Piper canninum extract and...

by Ni Luh Suriani dkk

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Piper caninum extract and *Brevibacillus agri* mixture suppresses rice leaf spot pathogen; *Nigrospora oryzae* and improves the production of red rice (*Oryza sativa* L)

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Under the guise of enhancing productivity, using pesticides and artificial fertilizers in agriculture affects both the environment and living things. High chemical residues in food and the environment disrupt the health of consumers. One of the solutions that can bring about a reduction in the use of pesticides and chemicals is switching to organic fertilizers. The application of biopesticides originating from biological sources such as plant extracts and the use of microbes is gaining global acceptance. Therefore, this study aimed to obtain the best biopesticides and biostimulants that could suppress the leaf spot pathogen, *Nigrospora oryzae*, and increase the growth and yield of Bali red rice. The study contained four treatments, namely untreated control (F0), *Piper caninum* leaf extract (F1), *Brevibacillus agri* (F2), and fermented *P. caninum* leaf extract plus *B. agri* (F3). The treatments were arranged in a randomized complete block design, and each treatment was replicated three times. The parameters measured were the number of tillers per plant, number of leafs per plant, chlorophyll content, number of grains per panicle, grain weight, and grain yield. Furthermore, antimicrobial and antioxidants were assayed using SEM. GC-MS. At the end of the experiment, the disease index of the leaf spot was measured. The results showed that F3 significantly suppressed

leaf spots caused by *N. oryzae* compared to other treatments, including untreated control in red rice. Additionally, the F3 significantly increased the number of productive tillers, number of grains per panicle, and grain yield compared to all other treatments. The F3 enhanced the crop yield at 6.19 tons/ha, an increase of 50% compared to the untreated control. The SEM.GC-MS results showed the presence of 2.3 butanediol, tetra-decanoic acid, butanoic acid, ethyl ester, benzene propanal, 3-(1,1-dimethylethyl)-a-methyl, a-N-Normethadol in treated plants with *P. canicum* plus *B. agri*.

KEYWORDS

antimicrobial products, biopesticides, rice growth, green-house, GC-MS, IAA, piper caninum, SEM

Introduction

Rice is one of the staple food in Indonesia, and various varieties are being developed worldwide. It has been extensively studied due to the importance of production and consumption and its vast distribution and destructiveness worldwide (Mir et al., 2022). Several management practices are being followed to increase rice yields, including chemical fertilizers and pesticides (Sagar et al., 2020). However, these products are toxic to human health and the environment (Gh[illegible]k et al., 2013). Leaf spot of rice caused by *Nigrospora oryzae* is a severe fungal disease of rice (*Oryza sativa* L.), threatening global food security.

The most prevalent method for rice leaf spot disease is using chemical pesticides. For instance, chlorpyrifos, an organophosphate insecticide, can inhibit insects and pests and is found in various commercial pesticides (Shabbir et al., 2021). Agricultural scientists developed fertilizers from natural materials known as organic fertilizers and pesticides to overcome pesticides. Organic fertilizers and pesticides are very safe for the environment and humans due to their decomposition by microorganisms and not impacting non-target organisms (Moustafa et al., 2022).

Many organic fertilizers and biopesticides are developed from microbes and plant extracts (Reshma et al., 2018; Kumar et al., 2022). Biostimulants are one subcategory of rhizobacteria that can increase plant growth due to their ability to produce hormones (Khan et al., 2020) and other growth-promoting metabolites (Tembo et al., 2018; Kalam et al., 2020; Basu et al., 2021; Hamid et al., 2021; Kusale et al., 2021a,b; Lyu et al., 2022). Furthermore, they are an essential aspect of the integrated crop management (ICM) system to make agriculture more sustainable and resilient. When given to plants or the rhizosphere, these inoculants stimulate natural processes to improve nutrient uptake (Jabborova et al., 2020, 2021a,b; Moradzadeh et al., 2021; Nithyapriya et al., 2021; Saboor et al., 2021; [illegible]kar et al., 2021), usage efficiency, abiotic stress tolerance (Ilyas et al., 2020; Sagar et al., 2020; Khan et al., 2021), biocontrol (Sukmawati et al., 2021; Khumairah et al., 2022), and crop quality (Hamid et al., 2021; Kan[illegible]palli et al., 2021; Manasa et al., 2021). Plant extracts have been used to suppress diseases, such as spotting in rice caused by *Pyricularia oryzae*, *Nigrospora oryzae*, and other fungi.

Extracts from *Piper caninum* were reported earlier to suppress spotting disease caused by *P. oryzae* and contribute to increased rice growth and production (Suriani et al., 2020a). However, certain drawbacks exist in such studies either related to the over dosage of such extracts or shorter shelf-life (Suriani et al., 2020a). Therefore, there is a need for a new approach to overcoming the above problems by combining plant-based extract and selected bacterial isolate. The study combines *P. caninum* leaf extract with *Bacillus agri*, where *B. agri* is a fermented bacterium that uses *P. caninum* extract as a food source. New compounds from the fermentation in coordination with *B. agri*, which has integral antifungal properties, could serve as a more powerful biopesticide to effectively suppress spotting disease in rice. This approach aids to contribute for disease control, growth, and production of red Bali rice (*O. sativa* L).

Materials and methods

Time and location of research

The present study was conducted from February 2021 to February 2022 at two locations, including the biopesticide laboratory of Udayana University, Bali, Indonesia, and the greenhouse at Munduk Paku Village, Senganan Penebel (Figure 1), Tabanan, Bali, Indonesia (8°22'49.3"S 115°09'43.2"E) and elevation 249 m above sea level).According to Schmidt & Ferguson's classification of climates, the climate in this area is a Type A climate. The average annual rainfall is between 2,000 and 2,800 mm, and there are typically 155.6 wet days. The average number of wet months is 4–10, and the average number of dry months is 0–5. The range of the

FIGURE 1
Map of research location (red color).

average air temperature is 25–28°C. Rice, horticultural crops (vegetables, fruits, and flowers), and flora kepodang birds, earthworms, and dragonflies (Sri Widari, 2021).

Isolation and identification of rice leaf spot pathogen and rhizobacteria

The leaf spot pathogen was discovered in a rice field in Bali, Indonesia's Senganan Village, Tabanan regency. After removing the diseased rice leaves (with the brown spot), they are thoroughly washed under running water, washed with 70% alcohol, and then rinsed under running water. They are then dried and cut into small pieces and placed in a petri dish with PDA medium, incubated for 2 days at room temperature, and the growth of pathogenic fungi is then observed under a microscope. Following its purification, the pathogen is spread and examined for the following step (Parwanayoni et al., 2021).

Sequencing of ITS regions and computer analysis of DNA sequences. The fungus pathogen was cultivated on PDA at ambient temperature (28°C) for 3 days. In Jakarta, PT Genetic Science, sequencing was carried out. Internal Transcriptional Spacer (ITS) 1 (5- TCCGTAGGTGAACCTGCGG-3) primer and ITS 4 were used in PCR to amplify the 18S rRNA gene (5-TCCTCCGCTTATTGATATGC-3). Takara PCR thermal cycler Personal (Takara Bio, Otsu, Japan) with Ex Tag (Takara Bio, Otsu, Japan) was used to perform the PCR in this study. The pre-denaturation step took 4 min, and then there were 35 cycles of denaturation at 94°C for 35 s, annealing at 52°C for 55 s, elongation at 72°C for 2 min, and post-elongation. The nucleotide sequences were determined using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) according to the guidelines of the tool and with the PE Applied Biosystems Automated DNA Sequencer (3130xl model, Applied Biosystems). Using Genetyx-ATSQ (version 4.0) software (Genetyx, Tokyo, Japan), compared with the same DNA sequence from DDBJ / EMBL / GenBank through the NCBI BLAST program (Thompson et al., 1997). Phylogeny analysis was carried out using the MEGA 6.0 program (Stecher et al., 2020), the Maximum Parsimony (MP) method with bootstrap1000x, with the following steps: (1). Search for similarities between sequences. Sequence data stored in the notes in the FASTA format is analyzed using the Blast-WU facility available online at www.ebi.ac.uk/Clustalw. (2). Making a tree of phylogeny with the MEGA program. Data from processing using the ClustalW facility will then be used as basic data to make phylogeny trees using MEGA data facilities (Darmadi et al., 2019).

The rhizobacteria were isolated from Senganan Village, Tabanan regency, Bali, Indonesia. For SRJL1 isolates, information was gathered from earlier research in which these isolates had IAA, protease, nitrogen. One strain—this isolate—was acquired from the 20 examined isolates in the earlier investigation. This isolate tested positive for all parameters. Therefore, this isolates and a squensing test were used in this study. Rhizobacteria were identified using an analysis of 16S rRNA gene sequences. First, the GeneJET Genomic DNA Purification Kit was used to extract and purify the DNA from rhizobacteria (Thermo Fisher Scientific). Using

the primer pairs 16S (63F 5′ - CAG GCC TAA CAC ATG CAA GTC-3′ and 1387R (5′ - GGG CGG WGT GTA CAA GGC-3′), the 16S rRNA gene was amplified by PCR with the help of 2 Kappa PCR ReadyMix (Kappa Biosystem) at 94°C for 5 min followed by 30 successive cycles at 94°C for 30 s, 55°C for nucleotide sequences were determined using the ABI PRISM 3100-Avant Genetic Analyzer. The DNA sequences were trimmed and assembled using the ChromasPro version 1.5 program. The assembled data was then processed for BLAST analysis with data registered in the National Center for Biotechnology Information (NCBI) through the website http://www.ncbi.nlm.nih.gov/BLAST. Several homologous sequence data from BLAST results, which are the closest species, were taken from GenBank data at the NCBI. The data were then analyzed again by aligning the sequences using the MEGA version 6.0 program. Furthermore, the data were analyzed using the PAUP 4.0b program with the maximum parsimony method with 1,000 replicates bootstrap (Vasseur-Coronado et al., 2021).

P. caninum extract

A total of 5 kg of mature *P. caninum* leaf (i.e., leaf number 3 from the top of the shoot of the plant were collected from Munduk Paku Village, Senganan, Penebel, and Tabanan Bali to make *P. caninum* leaf extract. The leafs were cleaned with running water, twisted, cut into small pieces, dried in a shaded place without sunlight until the weight was constant, and made into powder. Subsequently, the powder was macerated with methanol in a 1:10 (weight/volume) ratio for 48 h. The filtrate was filtered using Whatman filter paper (No. 2) then the obtained filtrate was evaporated using an evaporator (IWAKI, Japan) at a temperature of 40°C until the methanol evaporated, and the crude extract obtained was ready for use (Suriani, 2018).

Crude extract test against *N. oryzae* (MIC test)

Petri dishes were filled with 200 µl of *N. oryzae* spores for this test and then added with 10 ml of diluted PDA, shaken horizontally to mix spores and PDA evenly. After solidification, diffusion wells were made with a *cork borer* (5 mm diameter) in each petri dish. Each diffusion well was filled with 20 ml of crude extract of *P. caninum* leaf. The plates were incubated in the dark at room temperature (Suriani et al., 2020a). The diameter of the resistance zone created around the diffusion well was measured. Meanwhile, the well method was also used to determine the minimum inhibitory concentration (MIC). There were five concentrations of crude extract of *P. caninum* leafs; 0% as a control, 0.5; 1; 1.5; and 2%. All tests were carried out in PDA, and each treatment was replicated five times. PDA was poured into a petri dish filled with extracts of 20 µl of each concentration and allowed to solidify, and then filled with discs of *N. oryzae*. Furthermore, the plates were incubated at ±25°C, and observations were recorded daily until the control fungal colony growth reached the Petri dish's edges. The plant's inhibitory effect of crude extract was calculated as control diameter- treatment diameter/ control diameter x 100% (Suriani et al., 2021).

Production of *B. agri*

B. agri was propagated on sterile potato dextrose agar, one liter of substrate is created from 200 grams of small-grained potatoes that have been cooked for 20 min with 1 liter of aquades, filtered, and then filled to capacity with 70 grams of dextrose and 20 grams of water. After that, the media is poured into each test tube, which is then compacted and autoclaved to make it sterile. Each test tube contains 10 ml of media. The bacterial isolates were transferred with an ose needle to the PDA media and incubated at 27 ± 2°C for 3 days. Fermentation (room temperature 28–30°C) of *P. caninum* leafs extract: a mixture of 20 mg of *P caninum* extract, 1 one liter of potato boiled water, and 1 ml suspension of *B. agri* was transferred into a glass bottle and incubated for 30 days at 27 ± 2°C) before application to rice plants (Suprapta, 2022).

Application of treatments to rice seeds

Bali red rice What is obtained from senganan village, penebel, Tabanan bali is local balinese rice was soaked for 48 h with *P. caninum* extract and *P. caninum* plus *B. agri*. There were four treatments, and each was replicated six times. The treatments were: F0 = untreated control; F1=*P. caninum* extract at 2%; F2 = *B. agri* at 2%; F3= Fermentation of *P. caninum* extract with *B. agri* at 2%. Approximately 20 g of each treatment was mixed with 100 ml for 30 min. It was then twisted, put in Petri disc dial tissue, and watered with soaking water daily. After 2 weeks of growth, the parameters recorded were root growth, wet weight, and dry weight of Bali red rice seedlings (Suriani et al., 2021).

Seed treatment

Rice seeds were immersed in water for 48 h. After soaking, the seeds were grown in a tray filled with soil, and periodic watering schedules were maintained. After germination, young seedlings were transferred to another tray filled with planting media. The planting medium used in the experiment was the top layer of soil collected from rice fields, precisely 20 cm from the surface. Furthermore, the seedlings were maintained in trays, ensuring a proper watering schedule for 2 weeks. After 15 days of growth, they were carefully removed and re-planted into pots (Suriani et al., 2020a).

Greenhouse trials

A randomized complete block design was used for the greenhouse trials. The treatments were as follows: F0 = untreated control; F1 = *P. caninum* extract at 2%; F2 = *B. agri* at 2%; F3 = Fermentation of *P. caninum* extract with *B. agri* at 2%. Each treatment was replicated six times. Therefore, there were 20 experimental units, each consisting of 10 clumps planted with two rice seedlings, totaling 200 trials. The implementation of the experiment includes sowing seeds, preparing planting media, planting seeds, maintaining plants, fertilizing, and harvesting (Suriani et al., 2019).

Rice is planted in a greenhouse using a 30-centimeter-diameter bucket as a container for the media. The media is created by mixing soil (Soil permeability calls for low to moderate bulk density, medium to outstanding porosity conditions, and moderately quick air or water movement through the soil. Typical prerequisites for soil infiltration include. Terrain type: clay loam) and compost (material from chicken waste and rice husk) (trade name- Surya compost, procured from the local market in Bali, Indonesia) to create a mud-like consistency. This planting is ready for use after receiving 1 component of compost (Suriani, 2019).

Planting

The pest and disease-free seedlings of uniform size (15 cm tall) and aged 15 days after planting. They were removed and planted in the morning, and a rice seed was sown in each pot. Planting is conducted perpendicular to the ground at a depth of 3 cm (IRRI, 2015).

Fungus inoculation

After planting rice for 30 days (HST), *N. oryzae* isolates are inoculated by spraying 20 ml of mold spore solution per clump with a hand sprayer, then covering it with plastic for 12 h to keep the moisture intact. A pure fungal culture is harvested with 10 ml of sterile water over an oatmeal medium. Subsequently, it is harvested with a nose needle and filtered with Whatman Filter Paper No. 2 to separate the mold spores from their hyphae. The collected fungal spores are diluted in sterile water till the volume is 20 ml. Meanwhile, the mold spores utilized have a density of 25×10^4 spores/ml (Hoesain et al., 2021).

Treatment application

Application of treatment is conducted by spraying on rice plants after 1-month-old. This application should be conducted the day after the inoculation of the fungus. Each rice plant is sprayed with 20 ml of treatment and repeated 4 times with an interval of 1 week (Suriani et al., 2021).

Maintenance

Feeding, watering, and weeding rice plants are part of the maintenance process. Weeding keeps plants from being disturbed and prevents weeds and rice plants from competing for nutrients. Fertilization is carried out with compost on the substrate's preparation. Furthermore, watering begins with the planting and continues once a day (morning or evening).

Harvest

Harvesting occurs after the rice has turned yellow after 4.5 months. Rice is dried entirely before being ground in the drain.

Growth and production parameters

The observed data was in the form of growth, including plant height, number of leafs, number of tillers and chlorophyll, number of grains per panicle, the weight of grain per clump, the percentage of empty grain, and yield. The formula for calculating the yield is ton/ha per treatment. Chlorophyll content was measured according to the method of Aron (1949) with the help of a chlorophyll meter (Konika-Minolta, Inc, Osaka, Japan-SPAD 502) (Zhang et al., 2022).

The intensity of leaf spot disease

The intensity of leaf spot disease was measured with the following formula:

$$IP = \frac{\sum_{i=0}^{i} (ni.vi)}{NV} x\ 100\%$$

IP = The intensity of leaf spot disease

Observations were recorded as follows (Suriani, 2019)-

0 - No attack,

1 - very mild attack (0–10% damage to leaf surface),

2 - mild attack (10–30% damage to leaf surface),

3- moderate attack (30–50% damage to leaf surface),

4- severe attacks (50–75% damage to the leafs' surface) and

5 -heavy attack (75–100% damage to the leafs' surface)

Gas chromatography–mass spectrophotometry (GC-MS) analysis

Analysis of GC-MS (QP2010SE, Shimadzu, Japan) was performed to identify active chemicals. The structure of the separated compounds was determined by comparing their molecular weight and fragmentation pattern by the GC-MS database library (Reshma et al., 2018). Additionally, this method was conducted on *P. caninum* extract fermented along with

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TABLE 1 The inhibitory *P. caninum* leaf extract against the diameter of the fungal colony.

N. oryzae on PDA media *in-vitro* no	The formula of *P. caninum* leaves extract (%)	Inhibitory (%)
1	0	0a* ± 0.0000
2	0.5	12.28b ± 5,276
3	1	22.20c ± 1.3140
4	1.5	30.69d ± 0.4329
5	2	34.90e ± 0.2658

*The same letter is not significantly different between treatments.

B. agri. This experiment was conducted at UNUD's Joint Mathematics and Natural Sciences Laboratory.

Data analysis

The data were analyzed by analysis of variance (ANOVA), and the treatment differences were separated by Duncans Multiple Range Test (DMRT) at a 5% significance level (Febrianna et al., 2018).

Results and discussion

Identification of bacterial isolate

The identification results showed that the SRJL1 isolate was a rhizobacteria *B. agri* shown in Figure 3 with an assessment number: OM510267. The synthesis of organic acids, phosphatase activity, and concentration of P-dissolved in Pikovskaya media indicated the phosphate solubilizing capacity of the isolates (Table 1). Analysis of the organic acids production revealed that both isolates, *Burkholderia* sp. and *Penicillium* sp. produced organic acids, including lactate acid, oxalate acid, citric acid, and acetate acid. Lactic acid was produced in more amounts compared to other organic acids. In comparison, glutamic acid was produced in the least amount. The capacity of these bacteria to dissolve phosphat will differ depending on their ability to produce organic acids (Sharma et al., 2013; Serna-Posso et al., 2017). Osmolovskaya et al. (2018) claimed that each organic acid has a different capacity to chelate metal ions. Two factors, including the stability constant of complex organic acids with metal ions and the structure of the hydroxyl and carboxyl molecules in the primary carbon chain, affect this variance. Yang et al. (2022) reported that the capacity of phosphate-solubilizing fungi to produce organic acids and a decrease in the pH of the medium is closely related to the ability of phosphate solubilizing to produce organic acids.

Identification of leafs spot pathogenic fungal isolates

Isolation of SUR fungus from rice plants in Senganan Village, Penebel Tabanan, with brown patches on rice leaf (Figure 2A). Spores of *N. oryzae* were black and round (Figure 2B). The 16S rDNA sequencing results matched with *Nigrospora oryzae* (Figure 3). Thus the fungus was identified as *N. oryzae*. The fungus's 16S rRNA nucleotide gene sequence was deposited in the NCBI gene bank under accession No. OP035911. *N. oryzae* can cause panicle branch rot disease in rice plants, cause decreased yields and decreased rice quality, is the first study in china (Liu et al., 2021).

The inhibition of crude extracts of *P. caninum* against *N. oryzae* under *in-vitro* condi-tions

The inhibition of crude extract of *P. caninum* against *N. oryzae* was with an average diameter of 40 mm (Figure 4 and Table 1). The highest inhibition was found with *P. caninum* leaf extract at 2% (34.90%). The concentration of the extract is directly proportional to the inhibitory capacity against *N. oryzae*. Using *P. caninum* extract *in-vitro* can suppress leaf spotting disease in Bali red rice caused by *N. oryzae*. The test of the crude extract can inhibit *N. oryzae* with a diameter of 40 mm, classified as having powerful inhibitory properties. Tests of *P. caninum* leaf extract against *N. oryzae* fungal colonies showed the most significant inhibitory properties at a concentration of 2 at 34.90%. The higher the concentration of the extract, the greater the inhibition. The content of phytochemicals in *P. caninum* extracts, such as alkaloids, flavonoids, and phenols, can damage the fungus *N. oryzae*. The cell fluid flows out, and the cell becomes lysis. The extract can damage the cell wall of *P. oryzae*, causing blast disease, which can result in death. *P. caninum* also contains phytochemical substances such as Benzene, xylene, Tetradecane, dodecanoic acid, heptadecane, hexadecenoic acid, octadecamethyl cyclononasiloxa ne, Phthaic acid, and 8.11, 14-dococatrinic acid serves as fungicidal and bactericidal [33,34]. Essential oil from stems [Major compound: safrole (25.5%)] and leafs [Major compound: safrole (1[illegible]%)] have antimicrobial and antioxidant activities (Salleh et al., 2015; Najafi et al., 2021; Gowtham et al., 2022). Plant extracts for antifungal benefits (Arora et al., 2022) as they activate typical defense-related responses such as the production of H_2O_2, the up-regulation of genes encoding pathogenesis-related proteins, stilbene synthase, and the accumulation of resveratrol or its derivatives iceid. This is consistent with additional experiments on cell suspensions and plants (Krzyzaniak et al., 2018).

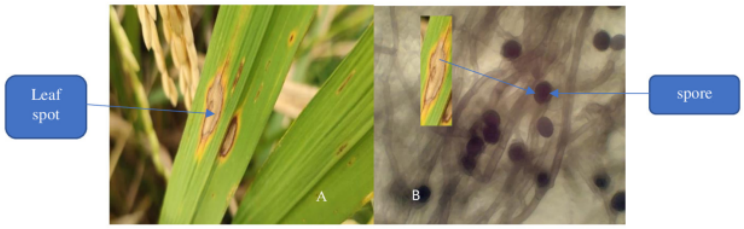

FIGURE 2
(A) Leaf spot caused by *N. oryzae*, **(B)** *N. oryzae* in rice.

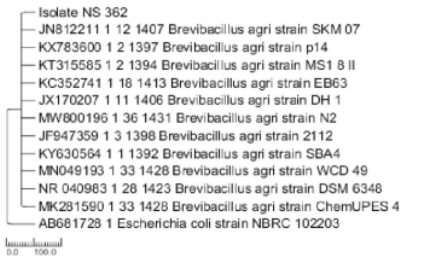

FIGURE 3
Phlogeny tree of *Brevibacillus agri* and *Nigrospora oryzae*.

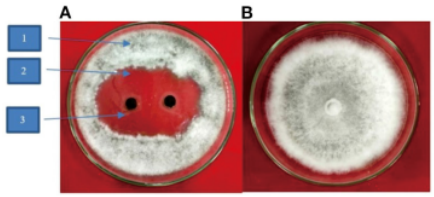

FIGURE 4
(A) Inhibitory of crude leaves extract of *P. caninum* against *N. oryzae*; 1-*N. oryzae*, 2-crude leaf extract, 3-clear zone **(B)** Control.

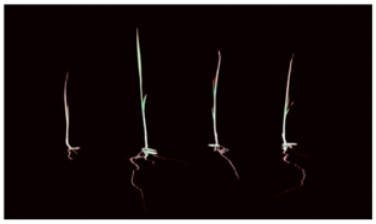

FIGURE 5
Root length from left to right F0, F3, F1, F2.

Effect of treatment in Bali red rice plant growth under *in-vitro* conditions

The study of the impact of treatment on the length of the roots grown on Petri dishes in the laboratory showed a difference in root length (Figure 5). The best treatment for long and compact development will be the F3 (fermentation of *P. caninum* leaf extract with *B. agri*), followed by F2 (*B. agri*) and F1 (*P. caninum* leaf extract), as described in Table 2. The use of *P. caninum* extract (F1), *B. agri* (F2), and fermentation extract of *P. caninum* with *B. agri* (F3) gave different results from the control. The *in-vitro* experiments prove they can increase the number of roots and compactness. Root length data show that control differs markedly from treatment, and the best roots are found in the F3 treatment (Table 2). Growth in rice plants is heavily influenced by the IAA hormone produced by rhizobacteria *B. agri*, where the qualitative test results in *B. agri* are positive. Additionally, there are growth hormones produced by *P. caninum* leaf extract in synergy with metabolites produced by rhizobacteria, which spurs the growth of rice plants. The results are supported by the research of *P. caninum* extract combined with rhizobacteria and compost. It

TABLE 2 Wet weight, dry weight, root length of seedling of Bali red rice.

Treatment	Root length (mm)	Wet weight (g)	Dry weight (g)
Fo	25a*	29a*	10.35a*
F1	45b	31a	10.45b
F2	80c	40b	10.90c
F3	90cd	44bc	11.10d

*The same letter shows a significant difference at the 5% level.

provides the best barrier to *Pyricularia oryzae,* causing spotting disease in rice. It also gives Balinese red rice the best growth and production response compared to control or without combination treatment (Suriani et al., 2020b). *B. agri* and *Brevibacillus formosus* produce various antagonistic metabolites, such as HCN, chitinase, and siderophore, and suppress *Salvia officinalis* wilt and root rot infections. IAA formation of *Brevibacillus* spp. has been postulated as a method for plant growth promotion. Some *Brevibacillus* spp. could synthesize 3.8 mgL^{-1} indole-3-acetic acid (IAA) *in vitro*, contributing to the favorable effects (Ahmed et al., 2018). Bacteria and smoke-water extract improve growth and induce the synthesis of volatile defense mechanisms in Vitis vinifera (Salomon et al., 2017). The combination of plant growth-promoting bacteria and botanical pesticides increases organic red rice yield and reduces the *Nephotocorisa acuta* population in Indonesia (Hoesain et al., 2021). Bacillus and Cyanobacteria isolates showed positive effects on rice seed growth compared to controls

Effect of treatment on Bali red rice plant growth *in-vivo*

The results showed that the treatment's growth parameters, plant height, number of tillers, number of leafs, and chlorophyll content significantly differed from the controls. All treatments are significantly different from the controls for the high parameters of red rice plants. The best influence on the number of leafs, chlorophyll content, and several tillers is found in the treatment of *P. caninum* leafs extract fermented with *B. agri*, followed in a row by treatment *B. agri*, with *P. caninum* leafs extract (Figure 6). The highest chlorophyll content parameters are found in the treatment with the fermentation of *P. caninum* leaf extract and *B. agri.* However, using *P. caninum* leaf extract and fermentation treatment with *B. agri* is shorter. In the treatment using *B. agri*, when compared to control then, the height of the plant is higher. This is very profitable for red Bali rice, considering it has a plant height of up to 250 cm. The harvest obtained will also be reduced (Suriani, 2019) with a decrease in rice. The amount of chlorophyll influences rice yields as it regulates the photosynthetic rate, plant growth, and grain yield. Nitrogen-fixing bacteria in the rhizosphere are non-symbiotic microorganisms that can boost N availability for rice plants (Sagar et al., 2022a,b). The synthesis of growth hormones by these bacteria also aids growth (Bhat et al., 2022). Meanwhile, the environment and their relationship with plants significantly impact these skills (Dutta and Gachhui, 2006).

The parameters of the number of tillers and the highest number of leafs are also found in the treatment with the fermentation of *P. caninum* leaf extract with *B. agri*. A large number of tillers will increase the amount of dry grain in rice plants (Suriani et al., 2021). Furthermore, the control has an average number of tillers of 8.67, while in the treatment with extract of *P. caninum* fermented by *B. agri*, the number increased to 9.5 and 11. The highest number of tillers in the treatment using extracts of *P. caninum* leaf with B. *agri* is 12. The best treatment for the average growth of Balinese red rice uses the leaf extracts of *P. caninum* fermented with *B. agri.* Over the approved fertilizer dose, grain yield increased by 4.15–9.14%. Furthermore, grain yields improved significantly beyond the prescribed fertilizer dose when seaweed bio-stimulants were applied, ranging from 5.31 to 5.[illegible]t/ha (T7). As a result, the bio-stimulant LBS6 S can be used to improve the growth and yield of transplanted rice (Raklami et al., 2019). The use of PGPR *Bacillus megaterium* CCMMB583 and *Bacillus subtilis* KJB06 can increase plant height growth, dry weight of plants and also increase chlorophyll in rice plants (Abd El-Mageed et al., 2022).

Effect of treatment on Bali red rice crop production

The results showed that the data on the highest number of productive tillers was found in the fermentation treatment of *P. caninum* leaf extracts with *B. agri* (F3), followed by *B. agri* treatment (F2) and the treatment of *P. caninum* leafs extract (F1) with 10.33, 13.00, and 14.17, respectively. This indicates that there was a significant difference between the control and treatments. The most important number of threads are found in the F3 treatment, amounting to 242.83. Similarly, there was a significant difference in data on grain weight/clumps between treatments and controls, where the increased grain/clump yield is found in the treatment of F3 with 65.12 gr. The empty grain/clump parameter between treatment and control also significantly differed; the lowest weight was found in the treatment of F3 with 3.01 gr, as presented in Table 3. The yield of grain tons/ha and the addition of results between treatment and control were also significantly different (Table 3). The highest grain yield of tons/ha was found in F3 silverware at 6.19 tons/ha, with the highest crop yield of 50.08% (Table 4).

Combining 3 Piper extract with rhizobacteria and compost can increase the number of tillers, the number of leafs, and rice productivity. This is achieved by reducing the amount

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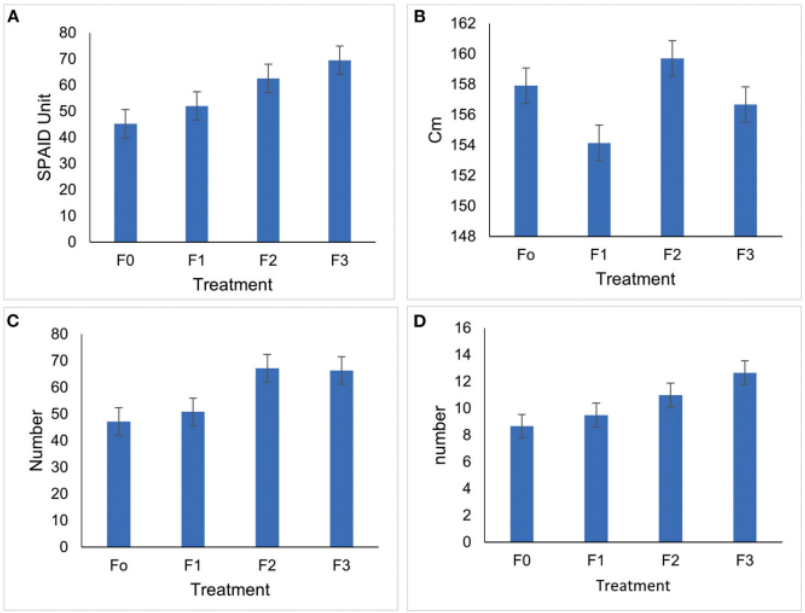

FIGURE 6
Average growth of Bali red rice plants **(A)** Chlorophyll content, **(B)** Height, **(C)** No. of leaves, **(D)** No. of tillers.

TABLE 3 Effect of treatment on production in Week 18 on Bali red rice in greenhouse.

Treatment	Productive tillers produktif	Grain weight/clumps (gr)	Amount of grain/panicle	Empty grain/clump (gr)
F0	9.17a* ± 0.4083	43.32a* ± 1.5559	217.33a* ± 1.0328	12.58a* ± 07590
F1	10.33b ± 0.5164	50.96b ± 1.6384	233.83b ± 1.1691	9.26b ± 0.3305
F2	13.00c ± 0.8944	62.87c ± 1.2271	239.00c ± 1.8970	7.18c ± 0.4565
F3	14.17d ± 0.7528	65.12d ± 0.7768	242.83d ± 2.3166	3.01d ± 0.4628

*The same letter shows a significant difference at the 5% level.

TABLE 4 Grain conversion (tons/ha) and increased results over control in greenhouse.

Treatment	Grain conversion (tons/ha)	Increased results over control (%)
F0	4.82a* ± 0.1722	0a* ± 0.0000
F1	5.73b ± 0.1966	19.23b ± 6.1078
F2	6.98c ± 0.1472	43.89c ± 9.3043
F3	6.19d ± 0.0983	50.08c ± 4.4979

*The same letter shows a significant difference at the 5% level.

of empty grain, increasing the amount of grain/panicle, grain weight/clumps, increasing the yield of grain tons/ha, and also affecting the growth of tons/ha (Suriani et al., 2019). The increase in grain yield is due to the decrease in the intensity of spotting disease, where the intensity of spot leaf disease is inhibited by *P. caninum* leafs extract, and *B. agri.*, rhizobacteria *Pseudomonas* isolated from rice rooting in California can suppress spotting diseases caused by *Magnophore oryzae*, 90% of appressoria,

TABLE 5 The inhibition of treatment of *N. oryzae* on Bali red rice in greenhouse.

Treatment	Intensity	Inhibitory(%)
F0	75.48a* ± 0.5143	0a* ± 0.0000
F1	42.74b ± 1.2371	43.33b ± 1.3663
F2	23..73c ± 0.6683	68.00c ± 0.8944
F3	17.60d ± 0.8089	76.67d ± 1.2111

*The same letter shows a significant difference at the 5% level.

and 78% *M. oryzae* fungus because it contains antifungal phytochemical substances (Spence et al., 2014). *N. oryzae* causes panicle branch rot, and this disease reduces yields and lowers milling quality (Liu et al., 2021). It is also reported to cause spots on rice leafs in China (Organisms, 2018). Additionally, *N. oryzae* can also rod *Brassica juncea* in India (Sharma et al., 2013).

The colony of *M. oryzae* was inhibited by 68, 65, and 48% by *Pseudomonas* sp., *Burkholderia* sp., and *Bacillus* spp., respectively. The bacterial suspension filtrates inhibited leafs spotting by 81.0, 79.2, and 66.3%, respectively. They were reported to solubilize phosphate, generate siderophores and cellulose, form biofilms, and reduce spot leaf when tested as *M. oryzae* antagonists (Martins et al., 2020; Nithyapriya et al., 2021).

The F3 treatment gives the best results for growth, covering the number of tillers, number of leafs, and chlorophyll content as well as crop parameters comprising of a number of productive tillers, number of grains/panicles, grain weight/clumps, grain yield tons/ha, and addition of yields; while reducing the weight of empty grain/clumps. This is due to the low intensity of the disease in F3 treatment (Table 5). The intensity of the disease is inversely proportional to the inhibition of the leaf spot. The chlorophyll content in all treatments, which is highest in the F3 treatment, is directly proportional to photosynthesis, growth, and components of the yield. A consortium of *Bacillus* sp., *Pantoea agglomerans,* and mycorrhiza has been reported to increase chlorophyll, biomass amount, and proline of crops in saline soil (Diagne et al., 2020). PGPR in rice plants can increase plant height, the number of saplings, chlorophyll content of leafs, yield of tons/ha, and water use efficiency (Abd El-Mageed et al., 2022). PGPR isolates from aromatic local rice rhizosphere can be used as biostimulants, biofertilizers, and biocontrol agents to boost plant development (Ali et al., 2022). Furthermore, it can promote the high growth of plants, chlorophyll, and biomass. It also improved the yield and health of rice plant genotypes Jaya, PA6444, and Pusa basmati-1, in India (Sharma et al., 2013). It provides attractive alternatives to synthetic fertilizers that are both environmentally benign and financially effective (Cen et al., 2020). *Eucalyptus camaldulensis* leafs extract (LE), *Citrus sinensis* LE, *Ficus benghalensis* fruit extract (FE), and two microbial antagonists were used as plant extracts and bioagents. The botanical pesticides from *Azadirachta indica, Aglaia odorata*, and *Ageratum conyzoides* combined with rhizobacteria (*Bacillus* sp. and *Pseudomonas*) can increase the growth and production of rice and suppress *Leptocorisa acuta* disease in organic red rice in Jember, Indonesia (Hoesain et al., 2021).

The intensity of leafs spot disease in greenhouse

The leaf spot disease studied in this study is spotting caused by the fungus *N. oryzae*. The intensity of leafs spot disease between treatment and control showed a noticeable difference, and data between treatments also reported a significant difference. The lowest intensity was found in the treatment of F3, followed by F2 and F1, with data on the minor inhibitory disease in F3, followed by F2 and F1 (Table 5). Furthermore, data on the intensity of spotting disease with the resistance is inversely proportional. The higher the intensity of spotting disease, the smaller the inhibition. Phytochemical content in the fermentation of *P. caninum* extract with *B. agri* can suppress leafs spotting diseases caused by *N. oryzae* fungus because it is anti-fungicidal (Table 5). Growth hormones such as IAA produced by *B. agri* and found in the leaf extract of *P. caninum* can increase growth. Additionally, new phytochemical substances result from the fermentation of *P. caninum* extract with *B. agri* to produce 2.3 butanadiol, tetradecanoic acid, Butanoic acid, ethyl ester, Benzenepropanal, 3-(1,1-dimethylethyl)-a-methyl, a-N-Normethadol strengthens the durability of rice plant tillers and increase growth. These findings contribute to comprehending bacterial volatiles in the rhizosphere and their functions (Yi et al., 2016). The bioactive components of tetradecanoic acid have been employed as additives in insecticides, pest control, insect repellents, and insecticidal agents (Bharathithasan et al., 2021).

GC-MS results

Gc-MS results showed that fermentation of *P. caninum* leafs extract with *B. agri* produced seven peak compounds (Figure 7). However, it showed different results for the extract of *P. caninum* without fermentation with *B. agri*. It revealed the presence of benzene, xylene, tetradecane, dodecanoic acid, heptadecane, hexadecenoic acid, octadecamethyl cyclononasiloxane, phthaic acid, and 8.11, 14- dococatrinic acid (Suriani et al., 2021). After the *P. caninum* leafs extract is fermented using *B.agri*, 2.3 butanadiol, tetradecanoic acid, Butanoic acid, ethyl ester, benzenepropanal, 3-(1,1-dimethylethyl)-a-methyl, a-N-Normethadol will be obtained. These compounds function

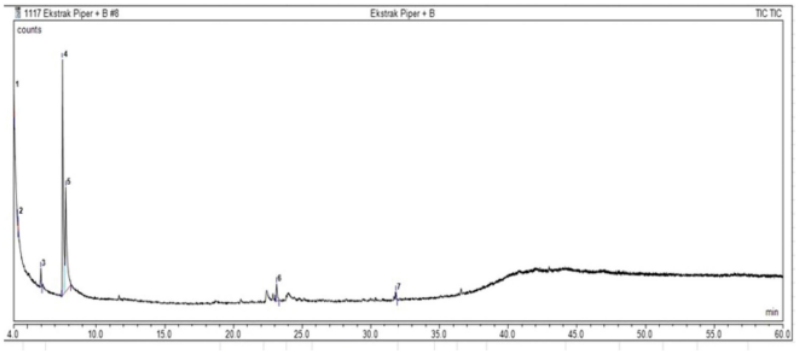

FIGURE 7
GC-MS chromatogram fermentation of *P. caninum* extract with *B. agri*.

TABLE 6 Compounds of GC-MS analysis of fermented *P. caninum* with *B. agri*.

Peak	Retention time	Name of the compound	Area (%)	Biology activities
1	4.01	2,3-Butanediol	0.32	Antifungal
2	4.29	2,3-Butanediol	0.89	Antifungal
3	5.97	Tetradecanoic acid	2.86	Larvicidal and repellent activity
4	7.55	Butanoic acid, ethyl ester	53.89	Antibacterial, antimicrobial
5	7.78	Butanoic acid, ethyl ester	36.64	Antibacterial, antimicrobial
6	23.13	Benzenepropanal, 3-(1,1-dimethylethyl)-a-methyl	4.40	Antifungal
7	31.83	a-N-Normethadol	1.00	Antibacterial and antioxidant

as anti-fungicidal, and some as antioxidants such as a-N-Normethadol (Table 6). Meanwhile, 2,3-butanediol causes the release of root exudates, which influence soil fungus and bacteria. *Tagetes erecta* plant extract with phytochemical combined with rhizobacteria (*Bacillus* sp.) more effectively suppresses pathogenic fungi such as *Monilinia laxa, Fusarium gramin earum, Aspergillus niger* (Perisoara et al., 2022).

Conclusions

The treatment of *P. caninum* leafs extract, *B. agri*, and the fermentation of *P. caninum* with *B. agri* affects the intensity of leaf spot disease caused by *N. oryzae*. The best influence on the growth and production of Bali red rice is the treatment of F3, which is the fermentation of *P. caninum* extract using *B. agri*. The new phytochemical substances produced possess antifungal, antimicrobial, and antioxidant properties. These compounds are 2,3 butanediol, tetradecanoic acid, Butanoic acid, ethyl ester, Benzenepropanal, 3-(1,1-dimethylethyl)-a-methyl, and a-N-Normethadol.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/~~OP035911.

Author contributions

Conceptualization, methodology, and writing original draft: NS. Data analysis: DS. Writing, review and editing: NR, EP, SG, MR, SA, MA, and MS. Formal analysis: IS, NS, EP, SG, and MR. Fund acquisition: SA and MS. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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