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Biosurfactant Activity of *Bacillus* sp. Strain LP04 Isolate and Its Antifungal Potency against *Ganoderma boninense* and *Fusarium* sp.

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ABSTRACT

Biosurfactants are a class of amphipathic molecules that various microorganisms can produce. Biosurfactants are used as biopesticides and biocontrol agents because they have antimicrobial activity, especially as antifungal agents in several species of fungal pathogens such as *Ganoderma boninense* and *Fusarium* sp. that attack crops. This study aims to detect the biosurfactant activity of *Bacillus* sp. and its potential as an antifungal agent against the fungi *Ganoderma boninense* and *Fusarium* sp. Biosurfactants were produced in mineral salt medium (MSM) by harvesting cell-free supernatants. Screening of biosurfactant-producing isolates was carried out using an oil-spreading assay, a hemolysis assay, and an emulsification index. The antifungal activity of the isolates was then tested using the agar diffusion method. The LP04 isolate was closely related to *Bacillus thuringiensis* with a 99% similarity level. It has the potential to have biosurfactant activity, which is characterized by a positive result on the oil spreading assay test and has an emulsification index of 48.33±2.87%. The cell-free supernatants of the bacterial isolate were able to inhibit the growth of *Ganoderma boninense* and *Fusarium* sp. with growth inhibition rates of 51.11% and 56.92%, respectively.

1. Introduction

Biosurfactants are a class of amphipathic molecules that consist of hydrophobic and hydrophilic groups. The hydrophobic groups of biosurfactants are in the form of long-chain fatty acids, hydroxy fatty acids, or α-alkyl-β-hydroxy fatty acids. In contrast, the hydrophilic groups can be carbohydrates, amino acids, cyclic peptides, phosphates, carboxylic acids, or alcohols (Katemai *et al.* 2008). The chemical structures of biosurfactants are very diverse, such as lipopeptides, glycolipids, phospholipids, fatty acids, neutral lipids, and lipopolysaccharides (Dalili *et al.* 2015). Various microorganisms can produce biosurfactants in the form of extracellular compounds or compounds associated with cell walls (Balan *et al.* 2016). *Bacillus* is a genus of Gram-positive bacteria consisting of several species that have the potential to produce secondary metabolites such as lipopeptides, including *B. amyloliquefaciens, B. subtilis*, and *B. atrophaeus* (Sarwar *et al.* 2018).

The study of biosurfactants has been developing, especially regarding their application in various fields. Commercially, biosurfactants have applications in laundry detergent formulations, in the food and cosmetic processing industries, as well as petroleum processing and recovery. In the medical field, biosurfactants are applied as antimicrobial agents, anticancer, antiviral, antiadhesive, immunological adjuvants, and so on. In agriculture, biosurfactants are used as biopesticides and biocontrol agents because they have antimicrobial activity, especially as antifungal agents on several species of pathogenic fungi, such as *Fusarium moniliforme, Botrytis cinerea, Sclerotonia sclerotiorum, Colletotrichum gloeosporioides*, and *Phoma* sp. (Md 2012; Plaza *et al.* 2013; Jiang *et al.* 2016). In addition, the amphipathic characteristics of biosurfactants cause less pathogen resistance compared to ordinary antibiotics or fungicides. Biosurfactants are more environmentally friendly because they have low toxicity and high biodegradability and have high stability against extreme temperatures, pH, and salinity (Wu *et al.* 2019).

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Ganoderma boninense is a pathogenic fungus of the genus *Ganoderma*, which is a threat to agriculture in Indonesia, especially in oil palm commodities. This fungus causes disease on oil palm roots, including basal stem rot (BSR) and upper stem rot (USR). Although the process of *Ganoderma* infection is relatively long, it is estimated that this fungus will cause great damage to oil palm plantations in Indonesia if early prevention is not carried out (Hushiarian *et al.* 2013). *Fusarium* is a cosmopolitan genus of the filamentous ascomycetes fungus (Sordariomycetes: Hypocreales: Nectriaceae) that includes many pathogens with toxins in important crops. Collectively, diseases by the genus *Fusarium* include wilt, blight, rot, and rust on many horticultural crops, field crops, ornamental plants, and forests in both agricultural and natural ecosystems (Ma *et al.* 2013). This study aims to examine the biosurfactant activity in the cell-free supernatant of the bacterial isolate LP04 and its potential as an antifungal agent against *Ganoderma boninense* and *Fusarium* sp.

2. Materials and Methods

2.1. Production of Biosurfactants

Biosurfactants were produced using bacterial isolates LP04 from the collection of the microbiology laboratory, IPB University, starting with rejuvenation of the isolate on TSA slanted agar medium for 24 hours. A full loop of the isolate was inoculated on 20 ml of preculture medium Luria Bertani (LB.) broth consisting of 0.5% yeast extract (Thermo Scientific), 1% NaCl (Merck), 1% tryptone (Merck), and incubated at room temperature (±25°C) for 24 hours using a shaker at 100 rpm (Jiang *et al.* 2016). After 24 hours, the preculture cultures were inoculated into mineral salt medium (MSM) culture medium consisting of 2% sucrose, 0.2% NH_4NO_3 (Merck), 0.3% KH_2PO_4 (Merck), 1% Na_2HPO_4 (Merck), 0.02% $MgSO_4 \cdot 7H_2O$ (Merck), 0.02% yeast extract, one µg/l $MnSO_4 \cdot 4H_2O$, as much as 10% of the culture volume and incubated at room temperature (± 25°C) for 48-72 hours using a shaker at 100 rpm. Cultures were harvested after the incubation period ended by centrifugation at 4032 g for 30 minutes. The supernatant was separated from the pellet and then used for the biosurfactant activity test (Setiani *et al.* 2020).

2.2. Screening of Biosurfactant Activity in Bacterial Isolates

2.2.1. Oil Spreading Assay

20 ml of Distilled Water was Poured Over the Surface of the Petri dish, then 5 ml of palm oil was added above the surface of the water to form a stable oil layer. As much as 100 µl supernatant was dripped onto the surface of the oil to see the surface activity. The supernatant of bacterial isolates that can produce biosurfactants will form a zone to replace the oil that spreads over the surface of the water (Gozan *et al.* 2014).

2.2.2. Hemolysis Assay

Bacterial isolates aged 24 hours were streaked on blood agar and incubated at room temperature (±25°C) for 24 hours. Hemolytic clear zones formed around the culture streaks were observed (Mahalingam and Sampath 2014).

2.2.3. Emulsification Index Test

2 ml of the cell-free supernatant and palm oil were added into a test tube. The mixture was shaken with a vortex for 1 minute and then incubated at room temperature (±25°C) for 24 hours. The emulsion layer formed is observed and measured, then the emulsification index is calculated by the following equation:

Emulsification index = ((emulsion layer height) / (total height of solution)) × 100% (Mahalingam and Sampath 2014).

2.3. Antifungal Activity Test

The ability of biosurfactants to inhibit the growth of fungi was tested using the agar diffusion method in Petri dishes containing potato dextrose agar (PDA; Merck). Fungus isolates were grown on one side of the media, while a well with a diameter of 1 cm was made on the opposite side using a sterile straw. For this test, 100 µl cell-free supernatant was put into the well. The media was incubated for 7-10 days, depending on the growth period of the tested fungus. MSM media was used as the negative control. The inhibition zone formed was observed, the growth diameter of the fungus was measured, and the antifungal index was calculated using the following equation: Inhibition percentage = (1 - A/B) × 100% (Mariastuti *et al.* 2018). A is the diameter of

the fungus on the treated plate, and B is the diameter of the fungus on the control plate.

2.4. Identification of Isolates Based on 16S rRNA Gene Sequences

The LP04 isolate was sequenced by P.T. Genetics Science Indonesia (GSI). Polymerase chain reaction (PCR) was carried out using universal 16S primers 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') for 35 cycles, with temperature and duration of each stage as follows: denaturation at 95°C for 15 seconds, annealing at 52°C for 15 seconds, and elongation at 72°C for 10 seconds. Sequence results were processed using the Seqtrace 0.9.0 application and then aligned using BLAST-N, which is available on the website https://www.ncbi.nlm.nih.gov/. The phylogenetic tree was created using the MEGA application version 11.0.13 using the Neighbour-Joining Tree method with a bootstrap value of 1,000x. The phylogenetic tree reference isolates used refer to publications containing the keywords "*Bacillus*" and "*surfactant*."

3. Results

3.1. Surface Properties of Biosurfactant on Isolate LP04

Biosurfactant activity can be observed from the surface properties of the supernatant harvested from the culture on MSM media. The cell-free supernatant from isolate LP04 could form zones on the oil surface, while the control treatment using MSM media did not form zones on the oil surface.

3.2. Hemolytic Character of Isolate LP04

A hemolysis test is performed to determine the ability of bacteria to lyse red blood cells. LP04 isolate was streaked and incubated on blood agar for 24 hours to form a clear zone around the growing colonies (Figure 1). The isolate can lyse red blood cells. This ability may be due to the surface activity of biosurfactants secreted by cells, and a higher concentration of biosurfactants can form a larger hemolytic zone on blood agar.

3.3. Emulsification Index of the Cell-free Supernatant

After 24 hours of incubation, the mixture of palm oil and supernatant, which has been shaken with a vortex for 1 minute, will form layers in the tube. The height of the emulsification layer and the total height of the solution are used to determine the emulsification index. The emulsification index recorded from the cell-free supernatant of isolate LP04 in the first, second, and third replicate was 46,67%, 48,33%, and 50%, respectively, so the average emulsification index of the cell-free supernatant was 48.33 ± 1.36% (Table 1).

Figure 1. The clear zone formed around the streaks of LP04 isolate after 24 hours of incubation on blood agar media

3.4. Antifungal Activity of LP04 Isolate against *Ganoderma boninense* and *Fusarium* sp.

The ability of LP04 isolate to inhibit the growth of *Ganoderma boninense* and *Fusarium* sp. was observed after 10 days of incubation. The diameter of the fungi in the treatment plate and the control plate were measured to calculate the inhibition percentage by the LP04 isolate culture. Hyphal growth of *Ganoderma boninense* and *Fusarium* sp. towards the wells on the treatment plate (Figures 2A and C) were 3.3 cm and 1.8 cm (radius), respectively. In contrast, on the control plate, the hyphae grew up in the wells (Figures 2B and D). The inhibition percentage of LP04 isolate in the culture broth was 51.11% against *Ganoderma boninense* and 56.92% against *Fusarium* sp. (Table 2).

3.5. LP04 Isolate Identification by 16S rRNA Analysis

The sequences of selected nitrogenous bases from the isolates were aligned for further analysis. Analysis of the phylogenetic tree shows that the LP04 isolate is closely related to *Bacillus thuringiensis* (Figure 3), with a 99.67% similarity level to the reference isolate and an E-value of 0.00 (Table 3).

4. Discussion

The results obtained at the end of the production stage were cell-free supernatant from LP04 isolate. The biosurfactant activity in the supernatant was tested using an oil-spreading assay to determine the surface-active properties of the biosurfactant. This method requires a small number of samples, and the process is easy and fast; it does not require special equipment (El-Sheshtawy and Doheim 2014). This method is commonly used in screening potential biosurfactants-producing isolates, both qualitatively and quantitatively. A positive test result is indicated by the presence of zones on the oil surface, which are replaced by supernatant droplets (Figure 4A).

The results in this study are similar to the oil-spreading assay carried out using bacterial isolates of *Bacillus* MG13 and MG20 (Moussa *et al.* 2017). The accumulation of biosurfactants triggers oil displacement by decreasing surface tension (between liquid and air) and interfacial tension (liquid water and liquid oil). This will reduce the repulsive forces between the two different phases and allow the two phases to mix and interact more easily; then, the oil moves and forms a clear zone on

Table 1. Emulsification index of LP04 isolate cell-free supernatant

Emulsion layer height (cm)	Total height of solution (cm)	Emulsification index (%)
1.45±0.04	3.00±0.00	48.33±1.36

Table 2. Growth inhibition percentage of *Ganoderma boninense* and *Fusarium* sp. by cell-free supernatant from LP04 isolate

Species	Diameter of fungus on treated plate (cm)	Diameter of fungus on control plate (cm)	Inhibition percentage (%)
Ganoderma boninense	6.6	13.5	51.11
Fusarium sp.	3.6	9.4	56.92

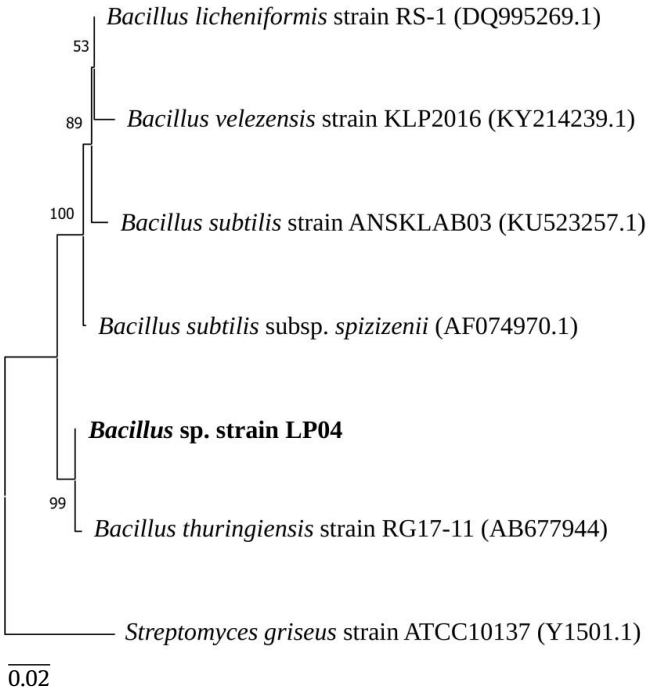

Figure 3. The phylogenetic relationship between LP04 and reference isolates based on 16S rRNA sequences.

Table 3. BLAST-n results of the LP04 isolate 16S rRNA sequence with reference bacteria

Isolate code	Reference accession number	Reference bacterial name	E-value	Similarity (%)
LP04	AB677944.1	*Bacillus thuringiensis* strain RG17-11	0.0	99.67

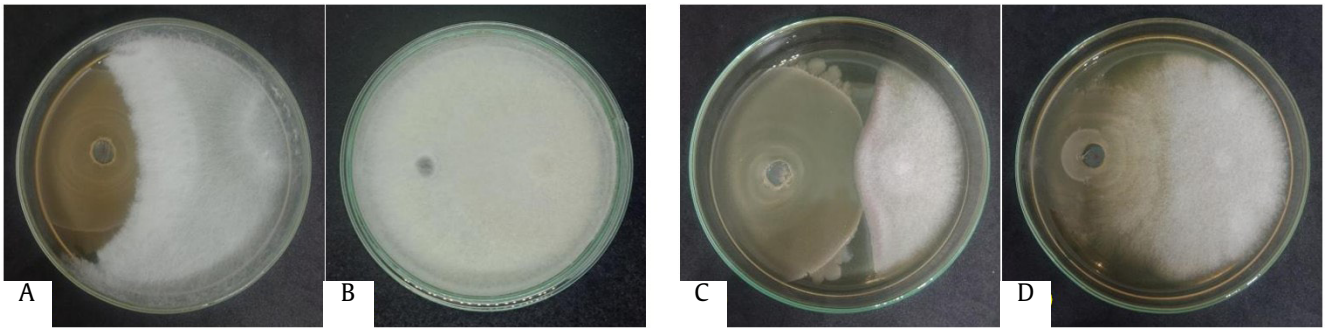

Figure 2. Antifungal activity of LP04 isolate against (A) *Ganoderma boninense* and (C) *Fusarium* sp., with (B) and (D) as negative controls, after 10 days of incubation

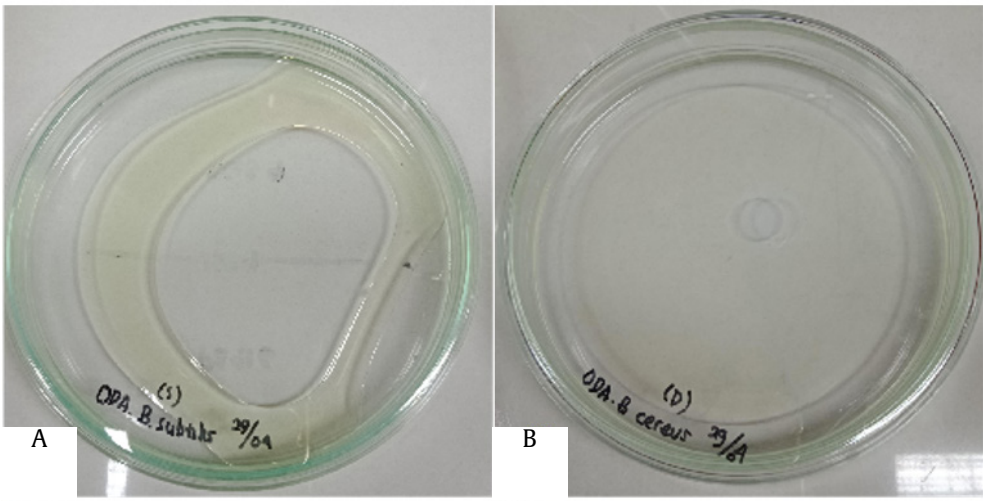

Figure 4. The zone formed by (A) cell-free supernatant of LP04 isolate and (B) MSM media as control

the surface of the water (Pandey and Anis 2013). The control treatment using MSM media did not show biosurfactant activity, as indicated by the absence of zones forming on the oil surface.

The biosurfactant activity of LP04 isolate was also tested on bacterial cells by hemolysis test on agar media containing fresh red blood cells from animals. Hemolysis is the most used initial toxicity assessment method (Greco *et al.* 2020). Hemolysis tests can be used to screen isolates that have the potential to produce biosurfactants qualitatively and to determine the presence of surfactin and rhamnolipids quantitatively (Youssef *et al.* 2004). Lipopeptide compounds from bacterial cells that have biosurfactant activity will lyse red blood cells in the agar so that a clear zone is formed around the streaks of the bacterial isolates (Walter *et al.* 2010). There are three types of hemolysis: α hemolysis, which forms a green-colored zone; β hemolysis, which forms a yellow-colored zone; and γ hemolysis, which indicates no hemolytic activity by cells. Bacteria that have the potential to produce biosurfactants can cause α and β hemolysis in red blood cells (Soltanighias *et al.* 2019).

The mechanism that causes hemolysis by biosurfactant compounds can be in the form of normal membrane dissolution at high biosurfactant concentrations or increased membrane permeability to small solutes at low biosurfactant concentrations, resulting in osmotic lysis (Setiani *et al.* 2020). Biosurfactants have the property of surfactants to lyse cells by inducing membrane reorganization and causing changes in cell morphology. The hydrophilic part modulates the penetration of the hydrophobic part, which disrupts the cell membrane (Manaargadoo-Catin *et al.* 2016). The hemolytic capacity of surfactants correlates with the concentration. It increases along with the increasing concentration (Dehghan-Noudeh *et al.* 2005; Zaragoza *et al.* 2010). Isolate LP04 showed a clear yellow zone (Figure 1), indicating that it could lyse red blood cells, and this strengthened the result of the biosurfactant activity of the isolate in the initial test.

The emulsification index test is a quantitative but indirect method to determine the stability of biosurfactants in cell-free supernatants. After 24 hours of incubation, the mixture of palm oil and supernatant, which has been shaken with a vortex, will form layers in the tube (Figure 5). The emulsification index is measured by the presence of about 50% by volume of the oil emulsion film, which persists after 24 hours of emulsion formation (Mahalingam and Sampath 2014; Nayarisseri *et al.* 2018). Biosurfactants that have an emulsification index value of >30% in the screening stage are considered positive for having significant emulsification activity (Shoeb *et al.* 2015). The interface emulsification ability of biosurfactants is influenced by their structure, components, and amphiphilic properties (Setiani *et al.* 2020; Durval *et al.* 2021).

Nor *et al.* (2023) reported that the Bacillus cereus KH1 cell-free supernatant emulsified with palm oil produced the largest emulsification index of 67% compared to other oils and hydrocarbons. Several other studies have also tested the emulsification activity using vegetable oils. The emulsification

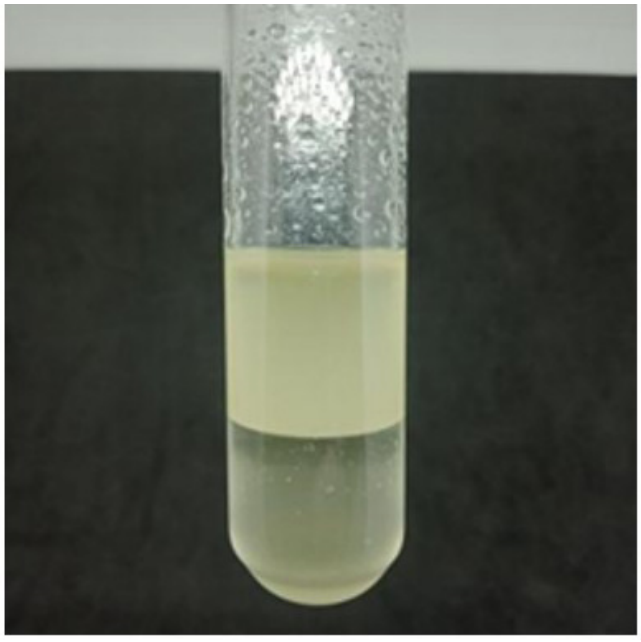

Figure 5. The emulsion formed on the mixture of palm oil and cell-free supernatant of LP04 isolate after 24 hours of incubation

index of biosurfactants produced by *B. subtilis* ICA56 bacteria is >50% in soybean oil (Lima de Franca *et al.* 2015). Another study was conducted on biosurfactants from the bacterium *Bacillus cereus* UCP1615 against several vegetable oils (canola, corn, peanut, and soybean) with different biosurfactant concentrations. Emulsification index values range from 36 to 68%, with the best index at a biosurfactant concentration of 10 mg/ml (Durval *et al.* 2021). The consistent screening test results supported each other; thus, the LP04 isolate has the potential to produce biosurfactant and secrete it extracellularly in growth media.

The percentage of inhibition was measured to determine extracellular biosurfactant antifungal activity. The inhibition percentage of LP04 isolates in liquid culture was 51.11% against *G. boninense* and 56.92% against *Fusarium* sp. (Table 2). Growth inhibition of *Ganoderma* sp. by the bacterial crude extract was also reported with an inhibition percentage of 65.25±3.78% by the bacteria *Bacillus siamensis* LDR (Santoso *et al.* 2022). Meanwhile, the value is higher than *Bacillus* sp. MA04 supernatant on *Fusarium stilboides*, which is 45.7% (Hernández-Morales *et al.* 2018).

The antagonistic activity of the *Bacillus* genus is widely known, arising from a variety of metabolites, one of which is lipopeptide. Surfactin, fengycin, and iturin act as a biocontrol against plant pathogens. One of the mechanisms of this antagonistic activity is direct antagonism against pathogenic fungi by inducing disruption of the plasma membrane. Another mechanism was reported in the lipopeptide iturin and fengycin by affecting the surface tension of the fungal cell membrane, causing the formation of micropores and ion leakage, which then led to cell death (Kumar *et al.* 2021). Antifungal activity is also influenced by the carbon source used in the biosurfactant production media. MSM media supplemented with sucrose as the carbon source was reported to form the largest zone of inhibition in the antagonist test against several fungus species (Singh *et al.* 2014).

Bacillus thuringiensis is a Gram-positive bacterium that is abundant in nature and can produce antifungal compounds such as lipopeptides (Kim *et al.* 2004). This species was reported to suppress the growth of *R. solanacearum* and the development of wilt symptoms in tomato plants (Hyakumachi *et al.* 2013). Isolates of *B. thuringiensis* strain AS17 japonensis and AS18 kurstaki were also reported to have antagonistic activity against *Fusarium oxysporum* f.sp. Lycopersici (FOL) race 2 (Qi *et al.* 2016). In addition, two isolates were reported with the ability to inhibit *G. boninense*, namely SW01-11 and SW02-08, which have a 100% similarity to *Bacillus thuringiensis* (Wibowo *et al.* 2017). Bacterial isolate LP04 has the potential to produce biosurfactants. The cell-free supernatant produced by *B. thuringiensis* LP04 has a surface activity that can degrade oil, emulsify oil, and lyse red blood cells. The isolate could inhibit the growth of the fungus *Ganoderma boninense* and *Fusarium* sp.

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