Research Article

Potential of Endophytic Bacteria in Controlling The Red Jabon Leaf Spot Pathogens In Vitro

Potensi Bakteri Endofit Dalam Mengendalikan Cendawan Patogen Bercak Daun Jabon Merah Secara In Vitro

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Abstract: Plant pathogens pose a significant challenge to the cultivation of red Jabon (*Anthocephalus macrophyllus*) seedlings. Notably, the fungi *Rhizoctonia* sp. and *Pestalotia* sp. are responsible for the destructive red leaf spot disease on Jabon leaves. To combat these fungal pathogens, the use of endophytic bacteria has emerged as a potential alternative. Endophytic bacteria can be isolated from various plant sources, although their effectiveness in controlling forest plant pathogens like Jabon has not been thoroughly explored. This study aimed to assess the potential of endophytic bacterial isolates obtained from *Pteris ensiformis* (Isolates APE15, APE22, APE33, and APE35) in controlling the growth of *Rhizoctonia* sp. and *Pestalotia* sp. through in vitro experiments. The antibiosis activity of the endophytic bacteria against the pathogenic fungi was evaluated using the dual culture method on PDA media. The results revealed that among the four endophytic bacterial isolates, APE35 exhibited the highest inhibitory effect on *Rhizoctonia* sp. (86.79%) and *Pestalotia* sp. (67.5%), while isolate APE22 only inhibited the growth of *Pestalotia* sp. (55%). In contrast, isolates APE15 and APE33 were unable to suppress either fungus. The antibiosis activity of APE35 and APE22 resulted in abnormal hyphal growth of the pathogenic fungi, characterized by shriveled, bent, dark-colored, and coiled hyphae. Physiological characterization of the endophytic bacteria revealed their ability to produce protease, cellulase, catalase, and phosphate-dissolving enzymes. Additionally, these bacteria exhibited a Gram-positive nature. This study provides valuable insights into the potential of endophytic bacterial isolates from *P. ensiformis* (APE35 and APE22) as biological control agents for *Rhizoctonia* sp. and *Pestalotia* sp., the causative agents of red Jabon leaf spot disease.

Keywords: antibiosis, physiological characterization, biological control, *Pestalotia* sp. *Rhizoctonia* sp.

Abstrak: Infeksi patogen menjadi faktor pembatas produksi bibit Jabon merah (*Anthocephalus macrophyllus*). Cendawan *Rhizoctonia* sp. dan *Pestalotia* sp. diketahui menjadi penyebab penyakit bercak pada daun Jabon merah yang merusak. Salah satu alternatif pengendalian cendawan patogen adalah pemanfaatan bakteri endofit. Bakteri endofit dapat diisolasi dari berbagai tumbuhan. Isolat bakteri endofit yang telah diketahui dapat mengendalikan patogen tanaman adalah bakteri endofit yang diisolasi dari akar tumbuhan paku pedang (*Pteris ensiformis*). Pemanfaatan bakteri endofit dalam mengendalikan patogen tanaman hutan seperti pada Jabon belum banyak dilakukan. Penelitian ini bertujuan untuk mengetahui potensi isolat bakteri endofit asal *P. ensiformis* (Isolat APE15, APE22, APE33, dan APE35) dalam mengendalikan *Rhizoctonia* sp. dan *Pestalotia* secara in vitro. Pengujian antibiosis bakteri endofit terhadap cendawan patogen dilakukan dengan metode dual culture pada media PDA. Selain itu juga dilakukan pengamatan abnormalitas hifa dan karakterisasi fisiologis bakteri endofit. Hasil uji antibiosis menunjukkan bahwa dari keempat isolat bakteri endofit, isolat APE35 menghambat pertumbuhan *Rhizoctonia* sp. (sebesar 86.79 %) dan *Pestalotia* sp. (sebesar 67.5 %), isolat APE22 hanya menghambat *Pestalotia* sp. (sebesar 55 %), sedangkan isolat APE15 dan APE33 tidak dapat menghambat kedua cendawan. Aktivitas antibiosis isolat APE35 dan

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APE22 menyebabkan abnormalitas pertumbuhan hifa cendawan patogen yaitu berupa hifa menjadi mengerut, membengkok, berwarna gelap, dan melilit, Karakterisasi fisiologis menunjukkan bakteri endofit mampu menghasilkan enzim protease, selulase, katalase, pelarutan fosfat, dan merupakan bakteri Gram positif. Penelitian ini memberikan informasi bahwa isolat bakteri endofit asal *P. ensiformis* (APE35 dan APE22) berpotensi sebagai agens pengendali hayati *Rhizoctonia* sp. dan *Pestalotia* sp. penyebab penyakit bercak daun jabon merah.

Kata kunci: antibiosis, karakterisasi fisiologis, pengendalian hayati, *Pestalotia* sp., *Rhizoctonia* sp.

INTRODUCTION

Pathogenic infection poses a significant obstacle to the cultivation of the red Jabon (*Anthocephalus macrophyllus*) seedlings. Notably, the fungi *Rhizoctonia* sp. and *Pestalotia* sp. are responsible for causing spot disease on red Jabon leaves (Herliana et al. 2020; Hidayah and Anggraeni 2015). Failure to control the spread of these pathogens can have detrimental effects, as observed in white Jabon (*A. cadamba*; Aisah et al. 2015; Herliana et al. 2022; Kobayashi and de Guszman 1988), as well as other tree species such as rubber (Damiri et al. 2022), Oak (Won et al. 2021), and Sengon (Istikorini and Sari 2020).

While various methods exist for controlling plant pathogens, including the use of antibiotics and chemicals, it is crucial to maintain a balanced planting ecosystem. Therefore, greater emphasis should be placed on biological control strategies (Pandit et al. 2022; He et al. 2021). Among these strategies, endophytic bacteria have emerged as a prominent option. Endophytic bacteria reside within plant tissues, exhibiting a neutral or beneficial relationship with their host plants. Their ability to produce growth hormones, facilitate nitrogen fixation, mobilize phosphates, and enhance plant resistance to pests and pathogens contributes to their role in promoting plant growth and development (Duan et al. 2013; Muthu Narayanan et al. 2022; Singh et al. 2020; Yadaf et al. 2021). In the case of red Jabon, the control of leaf spot disease has primarily relied on the application of fungicides, fertilizers, or a combination of both (Indhasari and Azisah 2021; Laode Mpapa and Romadhon 2015; Rustandi and Siti Fatimah 2015). However, there has been limited exploration of the use of endophytic bacteria as biological control agents for this purpose.

Extensive research has demonstrated the inhibitory effect of cultured endophytic bacteria on the development of pathogenic fungi, including *Rhizoctonia* sp. and *Pestalotia* sp, owing to their antibiosis ability. This effect is primarily achieved through the secretion of bioactive antimicrobial compounds or by mediating other systemic resistances in host plants (Digra and Nonzom 2023; Hasegawa et al. 2006; Shimizu et al. 2001a, 2001b). For instance, the addition of *Streptomyces* has been shown to suppress the growth of these fungi in tomato (Ebrahimi-Zarandi et al. 2021). Studies conducted by Bieber et al. (1998), Sasaki et al. (2001), and Shimizu et al. (2004) revealed that the antibiosis effects were triggered by various antibiotics secreted by *Streptomyces*, for example, actinomycin, fungichromin, cedarmycins, and alnumycin. Similarly, other studies have reported the successful control of these pathogens through the introduction of endophytic bacteria such as *Fraxinus* and *Bacillus* in plants like bayberry, clove, cowpea, rice, and tea (Ali et al. 2020; Siva et al. 2023; Wisanggeni et al. 2023; Xie et al. 2020; Zheng 2021). The secretion of antifungal enzymes by endophytic bacteria has been found to disrupt the integrity of the fungi's cell wall, resulting in osmotic imbalances, turgor pressure changes, and wall tension (Arlorio et al. 1992; Pusztahelyi 2018).

While numerous studies have emphasized on the potential efficacy of endophytic bacteria strains cultured from cultivated plants like rice, corn, oil palm, peach palm, sugar palm, and coconut (Eris et al. 2017; Suryanto et al. 2018), limited attention has been given to their wild isolates. In the subtropical region, Meguro et al. (2004) successfully extracted an endophytic bacterial strain, *Streptomyces padanus* AOK-30, from mountain laurel (*Kalmia latifolia* L), which demonstrated the ability to suppress the development of *Pestalotiopsis sydowiana* and *Rhizoctonia* sp. in cultured seedlings of similar plants. Furthermore, Asmoro and Munif (2019) isolated four wild endophytic bacteria (APE15, APE22, APE33, and APE35) from the root of the tropical sword fern (*Pteris ensiformis*), which exhibited the potential to control *Rhizoctonia solani* (Kuhn.), the pathogen responsible for causing sheath blight in rice.

Therefore, this study aims to assess the potential of endophytic bacteria derived from sword ferns (*P. ensiformis*) in inhibiting the growth of *Rhizoctonia* sp. and *Pestalotia* sp., the causative agents of leaf spot disease on red Jabon leaves, through in vitro experimentation. Identifying endophytic bacterial isolates with the ability to effectively suppress the growth of these pathogenic fungi holds promise as an alternative approach for managing red spot disease on red Jabon leaves.

MATERIALS AND METHODS

This research was conducted at the Forest Pathology Laboratory, Silviculture Department, Faculty of Forestry, Bogor Agricultural Institute and Plant Nematology Laboratory, Plant Protection Department, Faculty of Agriculture, Bogor Agricultural University. The materials used were four endophytic bacterial isolates isolated from Asmoro and Munif (2019) (isolate codes APE15, APE122, APE33, and APE35), potato dextrose agar (PDA) media, the pathogenic fungus isolate used *Rhizoctonia* sp. and *Pestalotia* sp. from Sakbani (2017) isolation, tryptic Soy Agar (TSA) media, Skim milk agar (SMA) media, colloidal chitin media, Carboxyl methyl cellulose (CMC) media, Pikovskaya's media, 3% KOH, cyanide detection solution (CDS), crystal dye violet, iodine, safranin, alcohol, sterile distilled water. The tools used are petri dishes, test tubes, injection glasses, glass objects, cover glasses, compound microscopes, and laminar airflow.

Endophytic Bacteria Antibiosis Test against Pathogenic Fungi

The antibiosis test was carried out using the double culture test method on PDA media in 3 replications. Seven days old pathogenic fungi were taken 0.5 cm in diameter, then placed in the center of the petri dish. The endophytic bacterial isolates tested were streaked on both sides of the media at a distance of 2.25 cm from the tested fungus. The inhibition of endophytic bacteria was calculated using the modified formula (Kim et al. 2023):

$$I = \left(\frac{R1 - R2}{R1}\right) \times 100\%$$

I = percentage of inhibition, R1 = radius of fungus colonies growing to the edge of the petri dish, R2 = radius of fungi colonies growing towards the endophytic bacteria.

Observation of Hyphae Structure of Pathogenic Fungi After Antagonist Test

Observation of hyphae structure by observing the tips of the mycelium in the inhibition zone of *R. solani* using a microscope with magnifications of 10×10 times and 40×10 times. The tip of the *R. solani* mycelium growing on the surface of the media was cut, then placed on the object glass. Abnormal mycelium is characterized by bent, lysis, stunted, twisted, or broken ends of the mycelium.

Physiological Characterization of Endophytic Bacteria

Proteolytic Activity. This test was carried out by streaking the test bacteria on Skim Milk Agar (SMA) media. Bacteria capable of producing protease enzymes will form a clear zone around the colony (Baehaki & Budiman 2011).

Chitinolytic Activity. This test was carried out by streaking endophytic bacteria on colloidal chitin media. Bacteria capable of producing the chitinase enzyme will form a clear zone around the colony (Hariprasad et al. 2011).

Cellulolytic Activity. This test was carried out by scraping the test battery onto Carboxyl methyl cellulose (CMC) media and then dropping red congo dye after 24 hours of incubation. Bacteria capable of producing cellulase enzymes will form a clear zone around the colony (Ghose 1987).

Phosphate Dissolving Ability. This test was carried out by streaking the endophytic bacteria on Pikovskaya's agar media (Sarker et al. 2014). A clear zone around the bacteria indicates the ability to dissolve phosphate.

Properties of Grams. One loop of endophytic bacteria is dropped with 3% KOH; the mucus formation indicates the bacteria are Gram-negative, and if not, the bacteria are Gram-positive (Sarker et al. 2014).

HCN production. Tests were carried out using the Castric method (1975). The tested bacterial isolates were streaked on TSA media enriched with glycine. On the lid of the petri dish is placed sterile filter paper, which has been soaked in cyanide detection solution (CDS) and dried. A change indicated HCN production in the color of the filter paper from bright yellow to reddish-orange.

Catalase Test. The test was carried out by dripping 3% hydrogen peroxide (H_2O_2) on 1 loop of endophytic bacteria smeared on a glass object. The formation of air bubbles indicates a positive result (Reiner 2010).

RESULTS AND DISCUSSION

Antibiosis Activity of Endophytic Bacteria against Pathogenic Fungi

The assessment of antibiosis activity in endophytic bacteria against *Rhizoctonia* sp. and *Pestalotia* sp. revealed that not all tested endophytic bacterial isolates exhibited inhibitory effects on the growth of these pathogenic fungi (Figure 1). Specifically, when testing against *Rhizoctonia* sp., only the APE35 isolate demonstrated antibiosis activity, resulting in an 86.79% inhibition of growth by the fifth day. In the case of *Pestalotia* sp., two isolates, APE22 and APE35, displayed antibiosis activity, with inhibition rates of 55.00% and 62.75% respectively (Table 1).

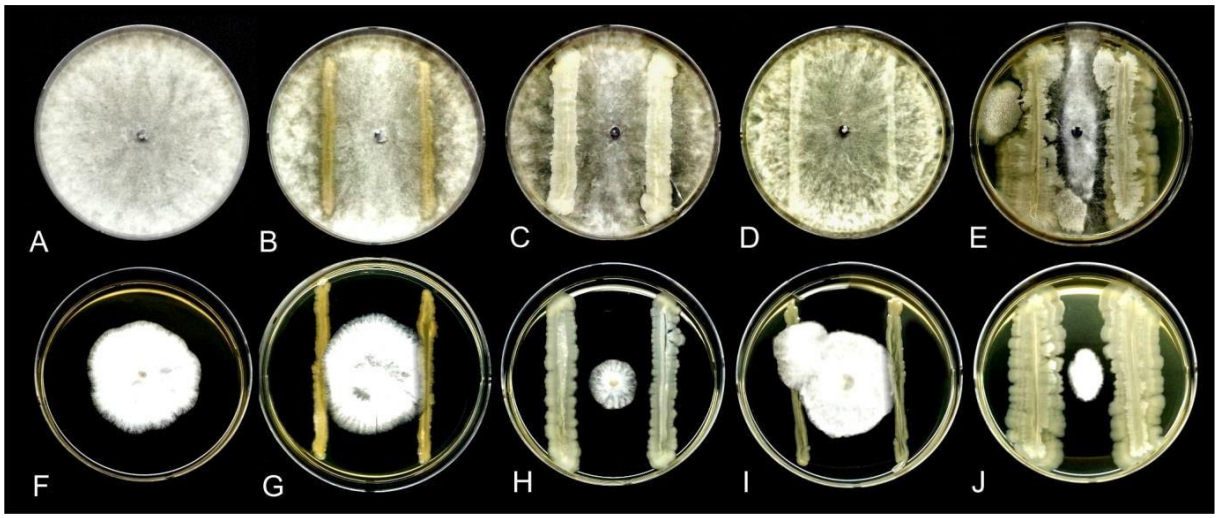

Figure 1. Results of the antibiosis test of endophytic bacteria against the pathogenic fungus Jabon red leaf spot. Colonies of *Rhizoctonia* sp. Control treatment (A), isolate APE15 – *Rhizoctonia* sp. (B), isolate APE22 – *Rhizoctonia* sp. (C), isolate APE33 – *Rhizoctonia* sp. (D), isolate APE35 – *Rhizoctonia* sp.(E). The *Pestalotia* sp. colony control treatment (F), isolate APE15 – Pestalotia sp.(G), isolate APE22 – *Pestalotia* sp. (H), isolate APE33 – *Pestalotia* sp. (I), APE35 isolate – *Pestalotia* sp. (J).

The inhibitory effect observed in fungal colonies can be attributed to the presence of endophytic bacteria, which exert control over the growth by means of their metabolites and by regulating nutrient availability and spatial constraints. It is noteworthy that endophytic bacterial isolates with potential as biological agents exhibit stability and a tendency to enhance the inhibition of fungal growth over time. Notably, the APE35 isolate demonstrated this trend against *Rhizoctonia* sp., with its inhibitory effect increasing from 62.75% on the 2nd day to 86.79% on the 5th day. Similarly, both APE35 and APE22 isolates exhibited an escalating inhibitory power against *Pestalotia* sp. from the second to the fifth day.

Arios et al. (2014) reported that the size of the inhibition zone resulting from the interaction between endophytic bacteria and pathogenic fungi is dependent on various factors,

including the type, solubility, and stability of metabolites produced by the bacteria in the test medium, as well as the density and composition of the medium itself. Additionally, bacterial metabolite production is influenced not only by the presence of pathogenic fungi but also by environmental and nutritional stress. Notably, these metabolite compounds tend to be produced at their maximum levels in environments where nutrient availability for bacterial growth is limited (Brader et al. 2014).

Table 1. Inhibition of endophytic bacteria isolates against *Rhizoctonia* sp. and *Pestalotia* sp.

Treatments (isolate)	The average inhibition of *Rhizoctonia* sp. (%) on day-					The average inhibition of *Pestalotia* sp. (%) on day-				
	1	2	3	4	5	1	2	3	4	5
Control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
APE15	0.00	0.00	0.00	0.00	0.00	0.00	4.50	4.76	1.02	0.00
APE22	0.00	0.00	0.00	0.00	0.00	0.00	35.00	43.83	50.00	55.00
APE33	0.00	0.00	0.00	0.00	0.00	0.00	3.50	3.75	1.60	0.00
APE35	0.00	62.75	70.53	80.33	86.79	0.00	53.00	63.89	66.67	67.50

Abnormality of Pathogenic Fungal Hyphae after Antibiosis Testing

The growth of both *Rhizoctonia* sp. and *Pestalotia* sp. was inhibited as a result of the antibiosis activity exhibited by the endophytic bacteria. The ability of these four endophytic bacteria to produce metabolite compounds likely contributes to their antibiotic properties. These metabolites generally induce abnormal growth in the hyphae of the target fungi. The metabolic compounds produced by the endophytic bacteria have a significant impact on the growth of *Rhizoctonia* sp. and *Pestalotia* sp., leading to observed abnormalities and inhibition of hyphal growth (Figure 2). The abnormal growth of *Rhizoctonia* hyphae is characterized by swelling, bending, and blackening, while the hyphae of *Pestalotia* sp. display stunted growth (shorter than normal hyphae) and disjointed morphology.

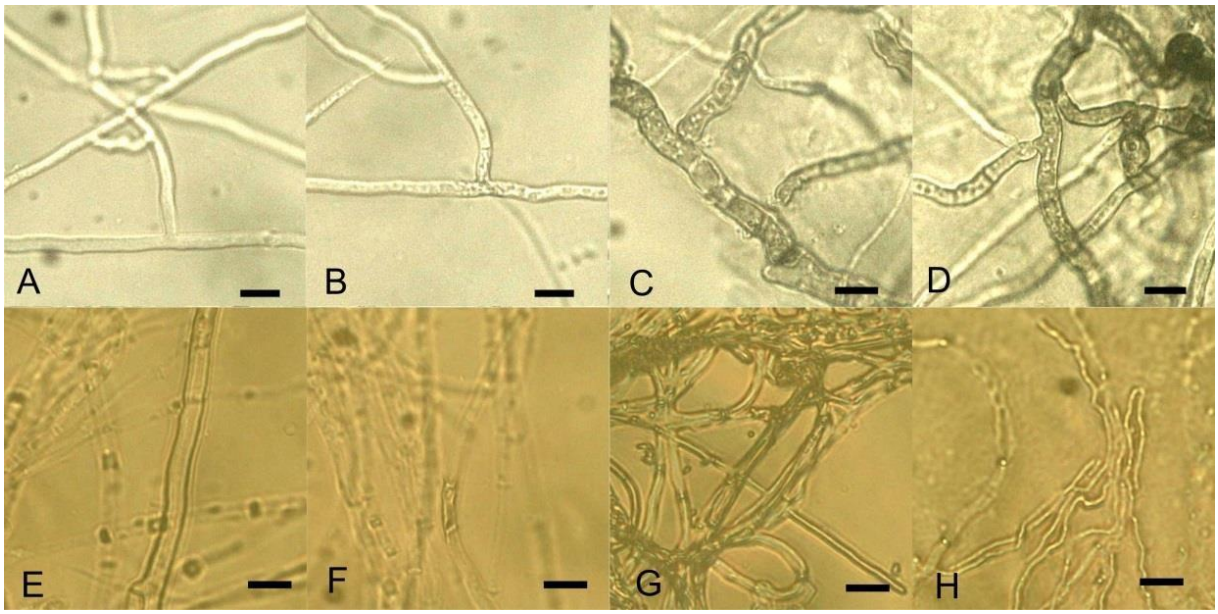

Figure 2. Abnormality of hyphae due to antibiosis activity of endophytic bacteria isolates APE35 and APE22. Hyphae *Rhizoctonia* sp. Normal (A-B), *Rhizoctonia* sp. Abnormal on testing of APE35 (C-D), the hyphae of *Pestalotia* sp. Normal (E-F), the hyphae of *Pestalotia* sp. Abnormal on testing of APE22 (G), the hyphae of *Pestalotia* sp. Abnormal on testing against APE35(H). Scale size in the figure: 10 µm.

Unlike Rangkuti et al. (2014), Arlorio et al. (1992), and Pusztahelyi (2018) studies, while the chitinolytic bacteria group is often associated with causing abnormalities in fungal hyphae, the two endophytic bacteria tested in this study did not exhibit chitinolytic activity based on physiological characterization tests. Therefore, it is believed that the hyphal abnormalities

observed in *Rhizoctonia* sp. and *Pestalotia* sp. are caused by other compounds, such as protease enzymes (Jalgaonwala and Mahajan 2011). The inhibition of fungal growth can be attributed to the antibiosis activity of the endophytic bacteria. The abnormality observed in fungal hyphae occurs because the antibiotic compounds produced by the bacteria can enter the fungal cells and cause protoplasmic dissolution (Compant et al. 2015).

Physiological Characteristics of Endophytic Bacteria

Biological agents employ diverse mechanisms to control pathogens, including the production of metabolite compounds that directly impact pathogen growth, stimulate plant growth, and enhance plant resistance to pathogens. The ability of a biological agent to produce metabolites is influenced by genetic and environmental factors. A favourable genetic background and suitable environmental conditions contribute to the expression of metabolites secretion (Caldwell 2005). The two tested endophytic bacterial isolates exhibited protein and cellulose hydrolysis capabilities, catalase enzyme activity, phosphate dissolution ability, while lacking chitin hydrolysis ability and HCN (cyanide acid) production. These physiological and biochemical characteristics are believed to play a role in suppressing the growth of *Rhizoctonia* sp. and *Pestalotia* sp. (Table 2).

Table 2. Physiological characteristics of endophytic bacteria APE15 and APE22

Physiological Characteristics	APE22	APE35
Gram	Positive	Positive
Protease	+	+
Cellulase	+	+
Chitinase	-	-
Phosphate Solvent	+	+
HCN	-	-
H_2O_2 (catalase test)	+	+

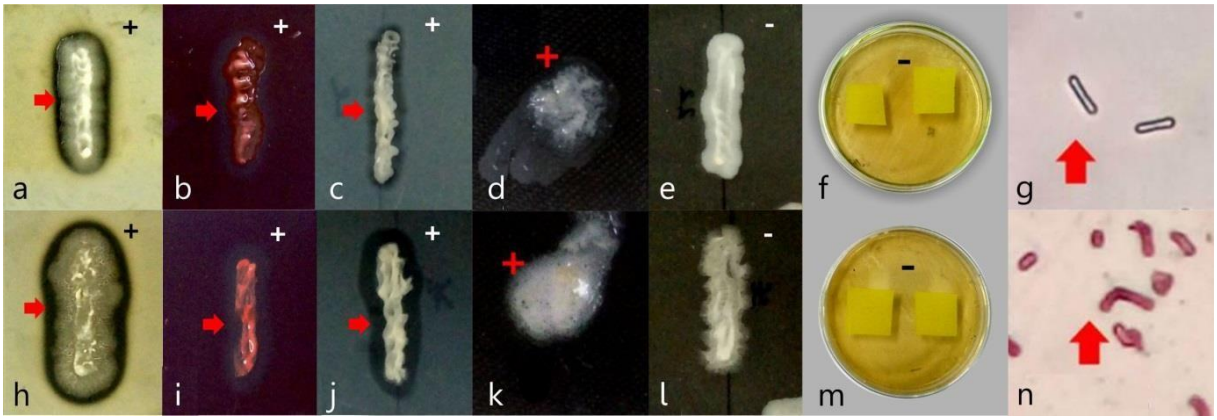

Figure 3. Characterization test results for isolates APE22 (A – G) and APE35 (H – N). Proteolytic test (A and H), cellulolytic test (B and I), phosphate solubility test (C and J), catalase test (D and K), chitinolytic test (E and L), HCN production test (F and M), Gram stain (G and N).

All tested endophytic bacteria demonstrated positive results in the proteolytic ability test, as evidenced by the formation of a clear zone surrounding their colonies on SMA media after 24 hours (Figure 3). The presence of this clear zone indicates the degradation of casein particles in the media due to the secretion of protease enzymes by the endophytic bacteria. These extracellular protease enzymes hydrolyze casein into peptides and amino acids. The proteolytic activity exhibited by the endophytic bacteria suggests their ability to produce protease enzymes capable of degrading the protein and chitin components of the *R. solani* cell wall. According to El-Deeb et al. (2013), Ali et al. (2020), and Abo-Elyousr et al. (2021), bacteria belonging to the *Bacillus*, *Paenibacillus*, and *Pseudomonas* groups are known to commonly produce protease

enzymes. In particular, *Bacillus* group bacteria tend to synthesize protease enzymes during the sporulation process (Contestini et al. 2017).

The cellulolytic activity test confirmed that both tested endophytic bacterial isolates possess the ability to produce cellulase enzymes, as indicated by the formation of clear zones around their colonies. Endophytic bacteria utilize cellulase enzymes to penetrate plant cell walls and colonize plant tissues (Tilak et al. 2005). The cellulolytic ability of these microbes plays a crucial role in biological control by facilitating the colonization of host plant tissues, thus inhibiting pathogen penetration. Moreover, according to Kuhad et al. (2011), the cellulolytic ability of endophytic bacteria can enhance plant quality and promote flowering.

The test results also revealed that the two endophytic bacterial isolates exhibited phosphate solubilization ability, as demonstrated by the formation of clear zones around their colonies. Phosphate is an essential nutrient for plant growth, and endophytic bacteria capable of solubilizing phosphate can contribute to the supply of this nutrient, thereby promoting improved plant growth (Vijayalakshmi et al. 2016). *Bacillus*, *Rhodococcus*, and *Serratia* group bacteria have been reported to possess phosphate solubilization ability (Wani et al. 2005). Both endophytic bacterial isolates were found to produce catalase enzymes, as evidenced by the formation of oxygen bubbles when hydrogen peroxide (H_2O_2) was added to the isolates. Catalase enzymes are crucial for aerobic bacterial cells and facilitate the breakdown of hydrogen peroxide into water and oxygen (Wilson 2014). However, the chitinolytic activity test indicated that the two bacterial isolates lacked the ability to produce chitinolytic enzymes, as there were no clear zones observed on chitin colloidal media around their colonies. Similarly, the ability to produce hydrogen cyanide was absent in both bacteria, as the filter paper remained unchanged from its original yellow to reddish-orange color. Gram trait tests and Gram staining revealed that isolates APE22 and APE35 belong to the group of Gram-positive bacteria, characterized by their purple color and rod-shaped morphology. These results were further confirmed by the 3% KOH test, which demonstrated mucus formation when reacted with isolates APE22 and APE35, indicating their Gram-positive nature.

CONCLUSIONS

The endophytic bacteria APE35 isolate exhibited inhibitory effects on the growth of *Rhizoctonia* sp., while isolates APE22 and APE35 demonstrated growth inhibition against *Pestalotia* sp. In vitro. The observed antibiosis activity of APE35 and APE22 isolates resulted in abnormal hyphal growth of the pathogenic fungi, characterized by shrivelled, bent, darkened, and twisted hyphae. Furthermore, physiological characterization tests indicated that these endophytic bacteria possess the ability to produce protease, cellulase, catalase, and phosphate-dissolving enzymes. Additionally, the Gram trait tests confirmed that the isolates belong to the Gram-positive bacterial group.

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