RHIZOSPHERIC *Bacillus spp*. AS BIOCONTROL AGENTS AGAINST MAIZE DOWNY MILDEW AND GROWTH PROMOTERS

BAKTERI RHIZOSFER *Bacillus spp.* SEBAGAI AGEN PENGENDALI HAYATI PENYAKIT BULAI DAN PEMACU PERTUMBUHAN PADA TANAMAN JAGUNG

Endang Mugiastuti[1], Abdul Manan[1], Loekas Soesanto[1], Deviana Primayuri[1], Dini Sundari[1*]

[1]Agrotechnology Study Program, Faculty of Agriculture, Jenderal Soedirman University, Jalan Dr. Soeparno No.63, Banyumas, Central Java, Indonesia

*Correspondence: dini.sundari@unsoed.ac.id

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ABSTRACT

Downy mildew is one of the major patogen limiting maize productivity in Indonesia. Effective mitigation strategies are essential due to the significant yield losses it causes. Biological control is an environmentally viable alternative method of disease management. *Bacillus* spp. are biological control agent capable of producing metabolic chemicals that can inhibit plant infections, hence holding potential for downy mildew management. This study aimed to evaluate the effectiveness of *Bacillus* spp. from the maize rhizosphere to manage downy mildew and promote maize plant growth. The research employed a completely randomized block design, consisting of four treatments and six replications. The treatments comprised *Bacillus amyloliquefaciens* BB.R3, *B. subtilis* BK.R5, *Bacillus* spp. BK.R9, fungicides treatment (metalaxyl), and control group for comparison. The observed variables included spore germination, incubation period, disease incidence, disease severity, Area Under Disease Progression Curve (AUDPC), number of leaves, plant height, fresh shoot weight, and fresh root weight. The findings revealed that *B. amyloliquefaciens* BB.R3, *B. subtilis* BK.R5, and *Bacillus* spp. BK.R9 effectively inhibited downy mildew by decreasing spore germination by 80.55-100%, prolonging the incubation period, and inhibiting disease incidence by 20.37-53.70%, disease severity by 25.64-62.56%, and AUDPC by 22.21-63.37%. *B. amyloliquefaciens* BB.R3 can enhance plant growth by augmenting root weight by 122.63% and maize plant weight by 80.26%.

Key words: *Bacillus,* Biological control, environmentally friendly, maize, *Peronosclerospora maydis*

ABSTRAK

Penyakit bulai merupakan salah satu penyakit utama yang menghambat produksi jagung di Indonesia. Upaya pengelolaan penyakit bulai perlu dilakukan mengingat besarnya kehilangan yang ditimbulkan. Pengendalian hayati merupakan salah satu alternatif pengendalian yang ramah lingkungan dan berkelanjutan. *Bacillus* spp. adalah bakteri yang mampu menghasilkan senyawa metabolik, dapat mengendalikan pathogen tanaman sehingga berpotensi sebagai pengendali penyakit bulai. Penelitian ini bertujuan untuk mengetahui kemampuan *Bacillus* spp. asal rizosfer untuk mengendalikan penyakit bulai dan mengoptimalkan pertumbuhan tanaman jagung. Penelitian menggunakan Rancangan Acak Kelompok Lengkap, dengan 4 perlakuan dan

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6 ulangan. Perlakuan meliputi *Bacillus amyloliquefaciens* BB.R3, *Bacillus subtilis* BK.R5, *Bacillus* spp.. BK.R9, serta fungisida (metalaksil) dan kontrol sebagai pembanding. Variabel yang diamati meliputi perkecambahan spora, masa inkubasi, kejadian penyakit, intensitas penyakit, AUDPC, jumlah daun, tinggi tanaman, bobot tanaman segar, dan bobot akar segar. Hasil penelitian menunjukkan *B. amyloliquefaciens* BB.R3, *B. subtilis* BK.R5, *Bacillus* spp.. BK.R9 mampu menekan penyakit bulai jagung, dengan menurunkan perkecambahan spora 80,55-100 %, menunda masa inkubasi, menurunkan kejadian penyakit sebesar 20,37-53,70 %, intensitas penyakit sebesar 25,64-62,56%, dan AUDPC sebesar 22,21-63,37%. *B. amyloliquefaciens* BB.R3 dapat memacu pertumbuhan tanaman, dengan meningkatkan bobot akar sebesar 122,63 % dan bobot tanaman jagung sebesar 80,26%.

Kata kunci: *Bacillus*, jagung, pengendalian hayati, *Peronosclerospora maydis,* ramah lingkungan

INTRODUCTION

Maize (*Zea mays* L.) is a primary food commodity that strategically strengthens food security and promotes economic growth in Indonesia. Indonesia's maize production from 2022 to 2024, respectively, was recorded at 16.53, 14.77, and 15.14 million tons per year (Badan Pusat Statistik, 2023). However, this production level has not been sufficient to fulfill the national demand, which has increased annually (Panikkai et al., 2017). In 2023, maize demand was estimated to reach 15.70 million tons, which was fulfilled through domestic production of 13.79 million tons and imports amounting to 1.19 million tons (Prasetyo, Sari, & Lestari, 2024)

The government continues to implement many initiatives to sustainably enhance maize production to achieve maize self-sufficiency, including through the Pajale (rice, maize, and soybean) program. However, these initiatives are confronted with several challenges, one of which is downy mildew disease that are caused by the fungus *Peronosclerospora maydis.* This disease is one of the major threats to maize and can result in yield losses of up to 100% (Adhi et al., 2024). *P. maydis* can infect maize from the time seeds are planted until they are 40 days after planting, with symptoms of chlorosis along the leaf veins, reduced growth, and absence of seed production (Semangun, 2004).

Management efforts for downy mildew must be implemented because of the considerable losses it inflicts. Nonetheless, downy mildew is exceedingly challenging to manage. Downy mildew disseminates swiftly via the air, and its oospores can persist in a dormant state in the soil for extended periods, with potential transmission through seeds. Downy mildew exhibits significant diversity and adaptation, rendering the efficacy of resistant types and fungicides short-lived (Ginting et al., 2020; Salcedo et al., 2021). Therefore, looking for safe, sustainable, and effective control alternatives is essential.

Biological control can safeguard plants during their entire life cycle, through the presence of poisonous compounds, resistance-inducing agents, and growth-promoting substances (Wulan et al., 2022). Biological control agents are live entities capable of proliferation, ensuring their efficacy in the field is enduring and durable (Correa-Galeote et al., 2018). The proliferation and advancement of biocontrol microbes are significantly affected by environmental factors. Thus, employing

microbes isolated from maize plants, whether endophytes or rhizosphere inhabitants, is anticipated to mitigate this concern.

Bacillus spp. bacteria are antagonistic microorganisms that act as biological control agents against various plant pathogens (Jatnika et al., 2013). Suriani & Muis (2016) assert that *Bacillus subtilis* can inhibit pathogen development via competition, antibiosis, and growth enhancement mechanisms. *Bacillus* spp. can produce antibiotic compounds, including chitinase enzymes that hydrolyze fungal cell walls and other antibiotics that impede pathogen development (Jatnika et al., 2013). Furthermore, *Bacillus* spp. bacteria can synthesize antifungal chemicals, including fengycin, bacillomycin, and other antibiotic peptides, which can inhibit the growth of phytopathogenic fungi (Daaboub et al., 2022). Mugiastuti et al. (2019) assert that this bacterium can induce plant defense-related chemicals, enhancing resistance to pathogens and is a group of beneficial bacteria that colonize plant roots and stimulate growth through various mechanisms. Previous research by Mugiastuti (2022) involved the isolation and characterization of several *Bacillus* spp. isolates from the rhizosphere of maize plants. Certain bacteria can control bacterial wilt and leaf blight diseases on maize while promoting maize growth through diverse antagonistic mechanisms, including production of antibiotics, cell lysis enzymes, hydrogen cyanide, phosphate-solubilizing enzymes, indole-3-acetic acid, and siderophores. It is anticipated that *Bacillus* spp. from the rhizosphere may be utilized to manage another significant maize disease, especially downy mildew. This study assesed the efficacy of *Bacillus* spp. in controlling downy mildew disease and enhancing growth in maize plants.

MATERIALS AND METHODS

The study was carried out from August to October 2022 at the Plant Protection and Greenhouse Laboratory, Faculty of Agriculture, Jenderal Soedirman University, Purwokerto.

Isolation of *Peronosclerospora maydis* and Propagation of *Bacillus* spp.

P. maydis fungal spores were collected in the early morning (03:00-04:00 AM) by extracting spores from infected leaves with a fine brush and suspending them in sterile water. The propagation of *Bacillus* spp. was conducted utilizing Nutrient Broth (NB) media, with shaking performed on a Daiki Orbital Shaker at a velocity of 150 rpm for 2 days (Muis et al., 2015).

Spore Germination of *Peronosclerospora maydis*

The evaluation of *Bacillus* spp. efficacy in inhibiting spore germination was performed using a Randomized Block Design, incorporating treatments of *Bacillus amyloliquefaciens* BB.R3, *B. subtilis* BK.R5, *Bacillus* spp. BK.R9, alongside a control for comparative analysis. The experiment involved combining 25 µL of *Peronosclerospora* spp. suspension with a density of 10^4 spores mL^{-1} with 25 µL *Bacillus* spp. suspension on a glass slide, then covered with a coverslip (Mugiastuti et al., 2023). The sample was subsequently incubated in a sealed container with a moist tissue for 24 hours in the dark (Anugrah & Widiantini, 2018). Spore germination was determined using the formula:

$$P = \frac{A}{B} X\ 100\%$$

Definition:
P = represents spore germination, A = denotes germinated spores, and B = indicates the quantity of observed spores.

Application of *Bacillus* spp. and *Peronosclerospora maydis*

The evaluation of *Bacillus* spp. from the rhizosphere for downy mildew control was performed using a Randomized Block Design, incorporating control treatments (lacking *Bacillus* spp. and fungicide), *B. amyloliquefaciens* BB.R3, *B. subtilis* BK.R5, *Bacillus* spp. BK.R9, and metalaxyl fungicide. Each treatment unit had 4 plants, and each treatment was replicated 5 times. The maize seeds used were the sweet corn Bonanza variety, known to be susceptible to the fungus *P. maydis* (Wahyuni et al., 2019).

Maize seeds were treated by soaking in *Bacillus* spp. suspension with a concentration of 10^9 CFU mL^{-1} as described by Yasmin et al. (2017), for 12 h. Twenty seeds were immersed in 20 mL of bacterial suspension in a sterile container. To maintain a uniform bacterial distribution and prevent sedimentation during the soaking period, the suspension was gently agitated using an orbital shaker. After soaking, seeds were air-dried at room temperature for 30 minutes prior to planting. The fungicide was applied by seed coating. In the control treatment, the seeds were immersed in sterile water.

The application of *Bacillus* spp. was conducted by foliar spraying at 7 and 14 days after planting (DAP), using a bacterial suspension with a concentration of 10^9 CFU mL^{-1}. Similarly, the fungicide metalaxyl was applied by foliar spraying at a dosage of 1 g L^{-1}. Inoculation of *P. maydis* was conducted at approximately 3:00 AM, on seven days after planting maize plants. Inoculation was performed by applying 10 mL of a *P. maydis* spore suspension at a concentration of 10^6 spores mL^{-1} to all sections of the maize plants, including the growth point. The spore suspension was prepared by culturing *P. maydis* on susceptible maize plants under controlled conditions. After sporulation, spores were harvested and suspended in sterile distilled water.

Observation Variables and Data Analysis

The observed variables include the incubation duration, disease incidence, downy mildew severity, AUDPC (Area Under Disease Progression Curve), plant height, leaf count, fresh plant weight, and fresh root weight. The incubation time is defined as the duration from pathogen inoculation to the manifestation of initial symptoms. The disease incidence is determined using the formula proposed by Farida et al. (2022) as follows:

$$\mathrm{DI} = \frac{\mathrm{n}}{\mathrm{N}} \mathrm{X}\ 100\%$$

Definition:
DI = Disease Incidence (%), n = count of diseased plants, N = Total observed plants.

The assessment of disease severity was conducted using the methodology established by Farida et al. (2022) as outlined below:

$$\mathrm{DS} = \frac{\sum \mathrm{ni\ x\ vi}}{\mathrm{N\ X\ V}} \mathrm{x}\ 100\%$$

Explanation:
DS = represents disease severity, ni = denotes the number of plants exhibiting signs of downy mildew within a designated category, vi = signifies the scale, N represents the quantity of observed plants, while V is the maximum scale value of the

disease category. The scoring system for maize downy mildew disease is established according to (Farida et al., 2022): 0 = No visible symptoms; 1= chlorotic streaks affecting > 10% of the leaf area; 2= chlorosis and minor stunting covering 10-25% of the leaf area, with signs of leaf deformation; 3= stunting and extensive chlorosis involving 25-50% of the plant; 4= severe stunting, heavy chlorosis, and visible downy growth affecting >50% of the leaf area.

The *Area Under Disease Progress Curve* (AUDPC) for maize downy mildew disease was calculated based on the method described by Habibullah et al. (2020), using the following formula:

$$\text{AUDPC} = \frac{\sum(Y_i + Y_{i+1})}{2} \left(t_{i+1} - t_i\right)$$

Defined as:

Y_i: Disease severity at the i-th assessment, t_i: Time (in days) at the i-th assessment.

In this study, disease severity was assessed six times at seven-day intervals. The assessment of plant height, leaf count, fresh and dry biomass of the plant, and the fresh and dry biomass of the roots, was performed 45 days post-planting, coinciding with the conclusion of the vegetative phase of the maize plants. The observation and measurement findings were analysed using the F-test, and if significant effects were detected, additional testing was performed using DMRT at a 5% error level. Data analysis was performed using DSAASTAT version 1.101.

RESULTS AND DISCUSSION

Evaluating the ability of *Bacillus* spp. to inhibit spore germination

The antagonistic bacteria's ability to inhibit spore germination is illustrated in Table 1. All investigated *Bacillus* spp. isolates demonstrated the ability to limit the spore germination of the fungus *P. maydis,* as indicated by the test findings. The proportion of *P. maydis* spore germination in the *Bacillus* spp. treatment was lower than that of the control. The suppression of spore germination in the *Bacillus* spp. treatment varied from 76.92% to 100%, with the most substantial inhibition noted in the *Bacillus* spp. BK R5 treatment, which did not substantially vary from the *Bacillus* spp. BK.R9 treatment. Figure 1 illustrates the spore germination of *P. maydis*.

The ability of *B. amyloliquefaciens* BB.R3, *B. subtilis* BK.R5, and *Bacillus* spp. BK.R9 to restrict the germination of *P. maydis* spores is presumed to be associated with the bacteria's production of chemicals that impede fungal proliferation via antibiosis and lysis mechanisms. Prior investigations on *Bacillus* spp. demonstrated their ability to produce protease, chitinase, and hydrogen cyanide (HCN) with differing efficiencies (Mugiastuti, 2022). Olanrewaju et al. (2017) state that protease enzymes can dismantle fungal cell wall proteins, while lipase enzymes can break down specific lipids linked to the cell wall. The combination of both can assist *Bacillus* spp. in lysing fungal cells. Lipids within the plasma membrane serve as crucial regulators of fungal pathogenicity. Numerous glycolipids have been demonstrated to impart virulence and resistance to multiple fungal species (Rella et al., 2016).

Table 1. Germination of spores of *Peronosclerospora maydis*

Treatment with antagonistic bacteria	Percentage of spore germination (%)
B. amyloliquefaciens BB.R3	8.19 b
B. subtilis BK.R5	0.00 a
Bacillus spp.. BK.R9	3.33 ab
Control	42.12 c

Note: Numbers accompanied by the identical letter within the same treatment and variable denote no significant difference according to the DMRT 0.05 test.

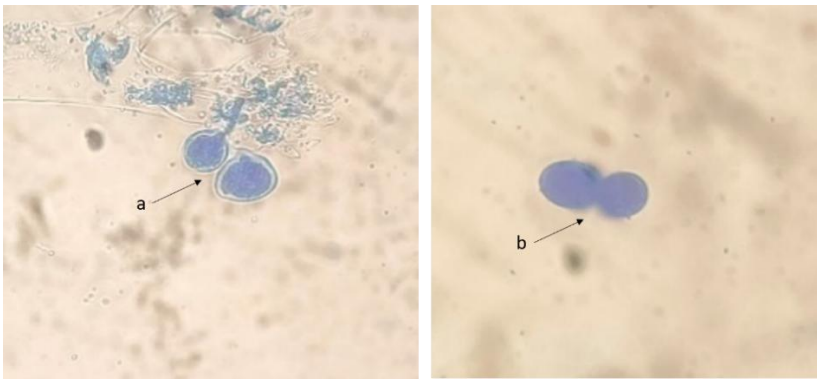

Figure 1. Spore *P. maydis*. (a) Germinating spore, (b) Non-germinating spore.

Chitinolytic enzymes play a crucial role in the mechanism of biological control agents against pathogenic fungi because they break down fungal cell walls (Leoni et al., 2023). Veliz et al. (2017) assert that chitin is a crucial constituent of the cell walls of insects, fungi, nematode eggs, and certain protists. Chitinase enzymes will compromise and disintegrate the cell walls of several pests and diseases, consequently demonstrating antibacterial, antifungal, insecticidal, or nematicidal properties. HCN is a secondary metabolite, a toxic and volatile molecule synthesized by several bacteria, functioning as an antibiotic, insecticide, nematicide, and herbicide, while also exhibiting repellent properties (Flury et al., 2017; Gupta & Sinha, 2020).

Evaluating the efficacy of *Bacillus* spp. from the rhizosphere in managing downy mildew

The test results regarding the impact of *Bacillus* spp. from the rhizosphere on the elements of the downy mildew pathosystem, including incubation period, disease incidence, disease severity, and AUDPC, are presented in Table 2. The data analysis results indicate a substantial impact of *Bacillus* spp. treatment on the incubation period of downy mildew. The incubation period of downy mildew was noted from pathogen inoculation to the emergence of early symptoms.

The initial symptoms of the disease were characterized by chlorosis that manifests along the leaf veins. In the morning, a mass of fungal spores was observable beneath the leaf surface like a layer of white flour (Fig. 2). The control treatment exhibited the shortest incubation duration at 18.70 days post-inoculation (dpi), while the *Bacillus* spp. BK.R9 treatment demonstrated the longest at 32.30 dpi. These results indicated that the

Bacillus spp. BK.R9 treatment exhibited a significant difference compared to the control group. The finding demonstrates that *Bacillus* spp. BK.R9 bacteria can extend the disease incubation period by 13.6 days relative to the control treatment (untreated) (Table 2). While not statistically distinct from the control, the treatment with *B. amyloliquefaciens* BB.R3 and *B. subtilis* BK.R5 exhibited a propensity for an extended incubation period. This treatment did not significantly differ from the *Bacillus* spp. BK.R9 treatment.

Figure 2. The Disease Symptom of Downy Mildew.

The impact of *Bacillus* spp. significantly affects the variables of disease incidence, disease severity, and AUDPC (Table 2). The utilization of *Bacillus* spp. and fungicides effectively reduced disease incidence by 20.37-53.70%, disease severity by 30.42-81.25%, and AUDPC by 22.21-63.37% compared to the control treatment. Therefore, the effectiveness observed in this study was interpreted by comparing it with previous research findings. For instance, a *Bacillus* spp. consortium is reported to reduce downy mildew severity in maize by up to 82.71% and decrease AUDPC by 83.00% (Khoiri et al., 2021). Similarly, other studies have demonstrate that *Bacillus* spp. can suppress the disease incidence of downy mildew by 17-25% (Djaenuddin et al., 2021). These findings suggest that the reductions observed in the present study are within the biologically effective range reported in earlier biological control research. The ability of antagonistic bacteria to mitigate the prevalence and severity of downy mildew, along with AUDPC, yields results comparable to fungicide use. The treatment with *B. subtilis* BK.R9 showed superior efficacy compared to fungicides.

The ability of *Bacillus* spp. to prolong the incubation period and mitigate disease incidence, severity, and AUDPC is hypothesized to be associated with the competitive and inhibitory actions of *Bacillus* spp., thereby obstructing pathogens from infecting and colonizing maize plants. Besides the previously elucidated processes, the genus *Bacillus* is also documented to produce different antibiotics (Ashgar & Pessarakli., 2010), which state that various strains of *B. subtilis* can synthesize 68 antibiotics, whereas *B. brevis* can produce 23 kinds, predominantly from the peptide group, with additional contributions from the butirosin and protosin groups.

Table 2. Components of the downy mildew disease pathosystem

Treatments	IP (days)	DI (%)	DIS (%)	DS (%)	DSS (%)	AUDPC
Control	18.70 a	90.00 b	-	81.25 c	-	1,031.04 c
B. amyloliquefaciens BB.R3	25.80 ab	71.67 b	20.37 a	60.42 b	25.64 a	678.13 b
B. subtilis BK.R5	25.05 ab	68.33 b	24.07 a	53.33 b	34.36 a	802.08 b
Bacillus spp. BK.R9	32.30 b	41.67 a	53.70 a	30.42 a	62.56 a	377.71 a
Fungicide (Metalaxyl)	25.80 ab	66.67 b	25.92 a	62.50 b	23.08 a	764.16 b

Note: Numbers accompanied by the identical letter within the same treatment and variable denote no significant difference according to the DMRT 0.05 test. IP: Incubation period; DI: Disease Incidence; DIS: Disease Incidence Suppression; DS: Disease severity; DSS: Disease severity Suppression; AUDPC: Area Under Disease Progression Curve.

Table 3. Components of the maize plant growth

Treatments	Plant height (cm)	Number of leaves	Plant weight (g)	Root weight (g)	Root length (g)
Control	89.75 a	7.10 a	51.77 a	3.80 a	57.96 a
B. amyloliquefaciens BB.R3	104.77 a	7.28 a	93.32 b	8.46 b	65.60 a
B. subtilis BK.R5	96.32 a	7.13 a	54.18 ab	4.04 a	48.10 a
Bacillus spp. BK.R9	100.65 a	6.30 a	64.52 ab	4.78 a	54.56 a
Fungicide (Metalaxyl)	101.23 a	7.23 a	73.52 ab	5.57 a	54.11 a

Note: Numbers accompanied by the identical letter within the same treatment and variable denote no significant difference according to the DMRT 0.05 test.

The low development of downy mildew in the fungicide treatment is related to the active ingredients in the fungicide that suppress the fungal growth. Antagonistic bacteria were effective in inhibiting the progression of downy mildew, showing comparable efficacy to the fungicide metalaxyl. Notably, treatment with *B. subtilis* BK.R9 was more effective than metalaxyl, suggesting that *Bacillus* spp. could serve as a potential alternative to this specific fungicide. These findings support the development of more sustainable disease management strategies, particularly in situations where metalaxyl efficacy has been compromised due to reduced sensitivity or resistance development in the pathogen population.

The impact of antagonistic bacteria treatment on plant growth parameters, such as leaf count, plant height, plant weight, fresh root weight, and root length, is illustrated in Table 3. The statistical analysis results indicate that treatment with antagonistic bacteria produced substantial variations in all components of root weight and plant weight. The treatment with *B. amyloliquefaciens* BB.R3 showed the best results compared to the control. *B. amyloliquefaciens* BB.R3 enhanced the fresh plant weight by 80.25% and the fresh root weight by 122.63% (Fig. 3).

Figure 3. The appearance of maize roots under different treatments. (a) control; (b) *B. amyloliquefaciens* BB.R3; (c) *B. subtilis* BK.R5; (d) *Bacillus* spp. BK.R9; (e) Fungicide (metalaxyl).

The ability of *Bacillus* spp. to promote plant growth has been suggested to be associated with the bacterium's ability to synthesize diverse chemicals or metabolites that induce plant growth. Mugiastuti (2022), states that the *Bacillus* spp. utilized in the experiments can solubilize phosphate, synthesize siderophores and IAA hormone, promote maize seed growth, and improve root development in maize plants. Phosphate-solubilizing bacteria transform insoluble organic and inorganic phosphates into absorbable forms for plants (Hassan, 2017). The IAA hormone can contribute to the augmentation of root surface area and enhance plant nutrient absorption (Shailendra, 2015; Olanrewaju et al., 2017).

The ability of *Bacillus* to enhance maize plant growth is suggested to be associated with the bacteria's proficiency in colonizing the root system. Weller (2007) states that the ability to colonize the root system involves colonization of the rhizosphere, rhizoplane, and/or inside the plant roots. The genus of *Bacillus* spp. exhibits rapid development by exploiting root and seed exudates, competing with other microorganisms, and adjusting to environmental conditions, thus efficiently colonizing the root system (Cavaglieri et al., 2005; Sundari et al., 2023; Weller, 2007). Subsequently, the bacteria will enhance the root surface, allowing the roots to assimilate the metabolites generated by the microbes, which will influence root development and physiology while protecting against pathogen attacks (Yulistiana et al., 2020).

CONCLUSION

B. amyloliquefaciens BB.R3, *B. subtilis* BK.R5, *Bacillus* spp. BK.R9 effectively control downy mildew in maize plants by suppressing spore germination (80.55-100%), prolonging the incubation period, reducing disease incidence (20.37-53.70%), disease severity (25.64-62.56%), and AUDPC (22.21-63.37%). *Bacillus amyloliquefaciens* BB.R3 also promotes plant growth by enhancing root weight (122.63%) and maize shoot weight (80.26%).

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