An assay on endophytic bacteria from corn and paddy to control damping-off of Rhizoctonia solani in corn seedling

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Abstract. In controlling Rhizoctonia solani a causal agent of damping-off to many important crops practical and chemical control are applied. However, these control are not successfully eliminate the disease. Furthermore, chemical application causes environmental problem, harm to human, and fungal resistance. Biological control of fungal disease has been developed. In this study four endophytic bacteria Bacillus sp. AJ02 and Xanthomonas sp. DJ01 isolated from healthy corn and Arthrobacter sp. AP01 and Bacillus sp. DP01 isolated from healthy paddy were employed to control corn seedling damping-off caused by R. solani. It was shown that all bacterial isolates inhibited R. solani growth with several hyphal decrements such as curved, petite, lytic, and swollen hyphae. Bacterial isolates were able to decrease corn seedling damping-off. One isolate Bacillus sp. DP01 reduced more ungrowth corn. Better performance including seedling height, seeding weight and dry weight, and leaf number was shown when the seedling was applied with bacterial isolates.

1. Introduction
Rhizoctonia solani is one important pathogenic fungus infecting many plants such as grasses, cereals, and potato affecting seed germination or seedling emergence and causing damping-off. It is found in most agricultural soils and survives between crops on plant residues and as microsclerotia [7, 24]. This pathogen usually attacks seedlings at or near the soil surface. Initial symptoms of the disease showed as stem lesions with brick red to brown color and sunken [4].

To reduce R. solani damping-off disease infection good seedbed preparation, culture practice such as shallow planting of seed and seedlings to promote rapid germination, and proper irrigation to enhance germination and growth and to avoid over watering have been applied [12]. Chemicals such as pentachloronitrobenzene, chlorothalonil, benomyl, thiophanate methyl, carboxin, mancozeb, vinclozolin, and iprodione have been used to control the disease [12; 24]. However, the chemicals are harms to environment and human, and increase pathogen resistance. Evaluation of commercial biofungicides to control R. solani has also been conducted [7]. Bacterial isolates such as Pseudomonas fragi [24] and Bacillus spp. [9, 24] and fungal isolates such as Trichoderma spp. [6, 16] have been reported to reduce damping-off disease caused by R. solani. Many were isolated from soil, rhizosphere, sewage, and fresh water. However, potential bacteria and fungi have also been found in many plant species and living asymptptomatically within plant tissues as endophytes root, stem, leaf, and seed [5, 10, 17, 20]. Using endophytic bacteria rather than rhizobacteria or from other sources was more promising since these bacteria interact more closely with the host plant, much less microbial
competition because of less diversity and lower populations of indigenous microorganisms inside plant tissues [8, 11] as protected site from environment stress [10, 17]. In this study, assay of endophytic bacterial isolates from healthy paddy and corn to reduce damping-off cause by R. solani has been conducted.

2. Materials and Methods

2.1. Endophytic Bacterial Isolates
Four endophytic bacterial isolates were used in this study. Bacillus sp. AJ02 and Xanthomonas sp. DJ01 were previously isolated from healthy corn, while Arthrobacter sp. AP01 and Bacillus sp. DP01 were previously isolated from healthy paddy. All bacterial isolates were kept in proper media and stored in refrigerator before used.

2.2. Isolation and Re-isolation of Rhizoctonia solani
Rotten corn trunk infected with potential pathogenic fungus was surface-disinfected, cut into small, and put with the cut part facing potato dextrose agar (PDA) added with chloramphenicol. Culture was incubated at ambient temperature for 2 days. Fungus colony appeared from inner part of cut part was sub-cultured in PDA until pure isolates obtained. Colony morphology and sclerotia were observed. Identification of potential pathogenic fungus was conducted using book of [2] R. solani was characterized as having 90°-branched hyphae.

Re-isolation of R. solani was conducted to infected corn seedling after infestation of the fungus. Infected trunk or root was disinfected with 2% of NaOCl ten seconds and washed with sterilized distilled water twice, and planted on PDA. Observation to colony morphology and sclerotia was done as previously described.

2.3. Assay of Bacterial Antagonism to Rhizoctonia solani
Assay of antagonism was conducted by dual culture method. Actively growth of R. solani hyphae was cut and put on the middle of PDA with 3% yeast extract in petridish. Blank discs (Oxoid) soaked in endophytic bacterial suspension of OD$_{560}$ = 0.5 were put symmetrically on both sides, 3 cm away from fungus growth. Culture was incubated at ambient temperature for 7 days. Bacterial isolate ability to inhibit fungal growth was shown as inhibition of hyphal growth surround bacterial colony.

2.4. Observation on Abnormal Hyphae of R. solani
Observation of abnormal hyphae as a result of antagonism assay of endophytic bacterial isolates against R. solani was carried out using microscope. Abnormal hyphae might be indicated as curved, broken, lytic, petite, and swollen hyphae.

2.5. In vivo Examination of Endophytic Bacterial Isolate To Reduce Damping-off
A 120 ml of R. solani culture suspension was mixed with 1.5 kg of soil and sterilized compost (ratio 3:1) as media for corn growing. Growing media was put in a plastic tray of 30 cm x 22 cm x 7 cm. Corn seeds soaked in endophytic bacterial suspension of OD$_{560}$=0.5 were planted in growing media, twenty seeds each tray. Corn seeds with fungal infestation were used as (+) control and corn seeds without any microbial application were used as (-) control. To examine whether bacterial application harmed to corn seedlings, bacterial soaked corn seed were grown in growing media without fungus inoculation. Seedling growth was observed for 30 days. Percentage of seedling death or ungrowning corn of each bacterial treatment was counted.

2.6. Observation of Corn Seedling Performance
Corn seedling growth and development was measured as seedling height, weight and dry-weight, and leaf number.
3. Results and Discussions

3.1. Isolation and Re-isolation of Rhizoctonia solani

*Rhizoctonia solani* was isolated from rotten rot and trunk of infected corn and paddy plant. The fungus was characterized with its 90° branched brownish white hyphae. Colony was cotton or velvet-like with regular mycelia, grew fast in PDA. Black sclerotium was seen when colony getting old (Figure 1).

![Figure 1](image1.png)

**Figure 1.** (a). Colony of *R. solani* on PDA and (b). Normal branched hyphae (white arrow)

Re-isolation of pathogenic fungus from infected corn seedling showed that the seedling was infected by *R. solani*. *R. solani* causes seed rot and seedling blight on corn occurs before seedling grows. Seedling blight occurs in pre-grown phase which makes coleoptile and root brown, wet, and rotten. Late infection causes seed soft and brown. Seedling dies before growing to the soil surface. Post-grown infection happens when seedling grows which causes the plant yellowish, wither, and die [23].

3.2. In vitro Bacterial Antagonism Against *R. solani*

All bacterial isolates showed to inhibit *R. solani* growth to some extent indicated by the appearance of inhibition zone around the fungal colony as shown in Figure 2. Several bacteria were reported to have potential to reduce *R. solani* growth. [9] showed that endophytic bacteria such as *Bacillus pumilus* SQR-N43 were potential to control *R. solani*. *Pseudomonas fragi* and *Bacillus pumilus* isolated from sewages, soils and water samples inhibit *R. solani* growth by producing chitinase [24].

![Figure 2](image2.png)

**Figure 2.** *In vitro* antagonism assay of endophytic bacterial isolates against *R. solani* depicted as clear zone (arrow). (a). *Bacillus* sp. AJ02, (b). *Xanthomonas* sp. DJ01, (c). *Arthrobacter* sp. AP01, and (d). *Bacillus* sp. DP01
Endophytic bacterial antagonistic mechanism in inhibiting hyphal growth seemed to differ (Table 1). The antagonism works through antibiosis, competition, predation, or parasitism [1, 14, 18].

| Bacterial isolates  | Hyphal abnormality                                      |
|---------------------|--------------------------------------------------------|
| Bacillus sp. AJ02   | Curved hyphae with no normal angle as shown in normal hyphae |
| Xanthomonas sp. DJ01| Petite and broken hyphae; irregular septate            |
| Arthrobacter sp. AP01| Broken and lytic hyphae; petite hyphal tip             |
| Bacillus sp. DP01   | Swollen hyphae; irregular septate; lysis               |

Table 1. Hyphal abnormality after antagonism assay between endophytic isolates and R. solani

Many antagonistic microbes produce antimicrobial compound that released to the environment and inhibit other microbes of the same ecological niche [17]. Antifungal compound produce by endophytic bacterial isolates disturbs fungal cell wall showed as curved, petite, lytic, and swollen hyphae. [15] and [22] reported that broken, curled, twisted, and petite fungal hyphae showed as manifestation of antagonistic assay. Similar results were also shown by [9]. Bacillus pumilus SQR-N43 caused hyphal deformation, enlargement of cytoplasmic vacuoles and cytoplasmic leakage.

3.3. Damping-off Reduction by Endophytic Bacterial Isolates

To know the effect of endophytic bacterial isolate infestation in reducing damping-off of corn, corn seeds were soaked in bacterial culture before planting. Seedling-death number as a result of damping-off disease was observed for 30 days of planting. Damping-off caused by R. solani was observed in (+) control a week after planting, and increased hereafter. It seemed that all isolates were able to reduce seedling-death to some extent (Figure 3). Seedling without bacterial application ((+) control)) was more susceptible to damping-off disease than those of seedling with bacterial application both with and without fungal infestation. Direct observation showed that no infected plant were observed in seed planted in soil without fungal infestation as shown in (-) control and sole bacterial application. Bacterial treatment of Bacillus sp. AJ02, Xanthomonas sp. DJ01, and Arthrobacter sp. AP01 slightly reduced seed viability, while Bacillus sp. DP01 seemed to increase seed viability. Endophytic bacterial isolates have been reported to control fungal diseases [25]. It was reported that endophytic bacteria controlled fungal disease on individual [25] and bacterial combination [17, 21]. Applications of a fermented organic fertilizer inoculated with B. pumilus SQR-N43 controlled R. solani by 68% of control efficiencies [9]. [16] used Trichoderma harzianum mutants to control Rhizoctonia solani in tomatoes cultivated under greenhouse and field conditions. Application of T. harzianum and B. subtilis increased the percentage of healthy corn seedlings and length, fresh and dry weight of corn seedling as well.
3.4. Seedling Development

In general, seedling development applied with endophytic bacterial isolates showed to have better performance as observed in increasing seedling height, leaf number, and dry-weight compare to that of (+) control as shown in Figure 4-6. It was interesting that both *Bacillus* isolate application with no fungal infestation contribute to higher seedling performance compared to that of both (-) control and (+) control (Figure 4.).
Although seedling fresh weight were higher in (-) control, endophytic bacterial isolates showed to increase seedling dry-weight in sole bacterial application and in fungal infestation (Figure 5.). This indicated that water content was lower in seedling of bacterial application. Direct observation showed that the seedlings were more vigor.

![Figure 5. Seedling fresh and dry-weight of corn treated with endophytic bacterial isolates](image)

All sole bacterial applications increased leaf number compared to that of both (+) control and of (-) control (Figure 6.). In fungal infested seedling the bacteria seemed to maintain leaf number as in (-) control.

It seems that two Bacillus isolates contributed more to control damping-off and to increase seedling performance. Instead of producing antifungal substances, these two isolates might excrete other secondary metabolites responsible for the increased plant growth. [13] reported that in the absence of pathogens, endophytes directly by promoting plant growth, or indirectly by protecting the plant against soilborne diseases. The growth promoting substances and plant growth hormones such as auxins, cytokinins and gibberellic acid etc., were produced by endophytes [10, 15]. [5] and [17] also showed that endophyte increases plant performance and protects plant again plant disease by producing plant growth hormon, chitinase and β-1,3-glucanase, peroxidase, phenylalanin ammonia-lyase, polyphenol oxidase, phenolat, jasmonic acid, and salicylic acid [3, 5, 19]. Unlike the Bacillus isolates, Xanthomonas sp. DJ01 and Arthobacter sp. AP01 seemed slightly harm to the seed. These bacteria was isolates from healthy plant with no symtoms appeared. Microorganism interaction in plant tissue might reduce of harmness of certain bacteria.
4. Conclusions
The result showed that all isolates were to show to inhibit the fungal growth. Abnormal hyphae such as curved, petite, lytic, and swollen hyphae were observed when *R. solani* was exposed to all bacterial isolates. In vivo examination showed that all bacterial isolates reduced corn seedling damping-off to 58-63% compared to 85% of (+) control, seed with only fungal infestation. Interestingly that one isolate *Bacillus* sp. DP01 decreased ungrowth corn seed to 17% compared to 34% of (-) control, seed without any microbial application. Moreover, all bacterial isolates showed to contribute to better plant performance including seedling height by 30.36-32.65 cm compared to 14.26 cm of (+) control, seeding weight and dry weight by 1.00-1.42 g and 0.19-0.24 g, compared to 0.36 g and 0.14 g of (+) control, and leaf number by 2.72-2.91 compared to 1.96 of (+) control, respectively.

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