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Original Paper

Preeliminary screening of endophytic bacteria associated with roots of potato plant grown in middle altitude as antagonist against bacterial wilt disease caused by *Ralstonia solanacearum*

Luqman Qurata Aini*, Abdul Latief Abadi, Mintarto Martosudiro, Muhammad Akhid Syib'li, Tita Widjayanti
University of Brawijaya, Faculty of Agriculture, Department of Plant Pests and Diseases, Jl. Veteran, Malang, Indonesia

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Bacterial wilt disease caused by *Ralstonia solanacearum* is a devastating plant disease on potato plant. This study aimed to screen endophytic bacteria isolated from potato roots planted in middle latitude areas and confirm their antagonistic potential against *R. solanacearum*. Endophytic bacteria were isolated from healthy potato roots (Granola cultivar) grown in the middle latitude area (500–700 m.a.s.l.) in Bumiaji Sub-District, Batu City, East Java, Indonesia. In this study, 130 endophytic bacteria were isolated. As a result, eight endophytic bacterial isolates were found as potential antagonists against potato wilt diseases (*R. solanacearum*) i.e., E1, E5, E6, E104, E117, E120, E121, and E129. Based on *in vivo* test, E1 isolate reduced the bacterial wilt disease in potato by 20.74% and significantly increased plant growth (plant height and plant dry weight). The ability of the endophytic bacteria to produce the antibiosis substance against the *R. solanacearum* and their ability to reduce bacterial wilt disease *in vivo* suggests that those bacterial isolates have the potency to be developed as the candidates for biocontrol agents against bacterial wilt disease caused by *R. solanacearum*. However, it is necessary to identify the molecular bacteria isolated in this study to determine which species can suppress wilt disease and increase the growth of potato plants.

Keywords: biological control, bacteria wilt disease, potato wilt disease, endophytic bacteria, middle altitude

1 Introduction

Bacterial wilt disease caused by *Ralstonia solanacearum* is a devastating plant disease on potato plant (Kurabachew and Ayana, 2017). The bacterial wilt disease on potatoes are widely spread in several countries in Asia such as Japan, China, India, Taiwan, and Indonesia (Tsuchiya et al., 2012) and have been reported to lead crop losses of about 33 to 90% (Souza et al., 2014). Bacterial wilt disease on potatoes found in Indonesia are commonly caused by *R. solanacearum* race 1 or race 3 (Karim et al., 2018). *R. solanacearum* race 1 is highly virulent, usually found in low land areas and has wide host range of about 200 plant species (Meng, 2013), whereas race 3 infected potato or sometimes tomato plants in highland areas (Scherf et al., 2010).

In Indonesia, potato plants are usually grown in the highlands (<1,000 m.a.s.l.), because in these areas the temperature is relatively lower and suitable for potato cultivation. However, due to the limited area of the highlands in Indonesia, the total potato production in Indonesia is still low (Swastika et al., 2021). Therefore, potato cultivars which can grow well in middle altitude areas (500–700 m.a.s.l.) and tolerance to higher temperatures were recently promoted to enhance the production of potato in Indonesia. However, based on our observation in the field, these potato cultivars have a major constraint particularly of their susceptibility against bacterial wilt disease caused by *R. solanacearum* that usually infect potato cultivars grown in warmer temperature. The use of bactericide showed not effective to control bacterial wilt disease on potato (Patil et al., 2012). Bactericide can harm the environment, and lead to the decrease of bacterial microflora in the soil as well as those on the phyllosphere which contribute on the plant health (Haruta and Kanno, 2015). Recently, the

***Corresponding author:** Luqman Qurata Aini, University of Brawijaya, Jl. Veteran, Faculty of Agriculture, Department of Plant Pests and Diseases, Malang, Indonesia; luqman.fp@ub.ac.id
ORCID https://orcid.org/0000-0003-2577-534X

global issues of food safety promote the environment and health friendly methods to control plant pest and disease (Rizzo et al., 2021). One of the methods is by using antagonistic microbes as biological control agent against plant pathogen (Lahlali et al., 2022).

Endophytic bacteria (bacterial endophytes) are bacteria that reside in the plant tissues without causing harmful effects on host plants but sometimes contributes to the plant growth (Fadiji and Babalola, 2020). Endophytic bacteria can be isolated from different plant parts such as stems, roots and leaves (Kandel et al., 2017). Endophytic bacteria also contributes to the plant health by enhancing plant resistance by inducing systemic resistance or function as antagonist against plant pathogen (Morales-Cedeño et al., 2021). Bahmani et al. (2021) reported that 11 endophytic bacteria isolated from potato plant roots simultaneously reduced disease by 27–55% and also significantly increased plant growth. Several endophytic bacteria were antagonistic against vascular pathogens such as *Fusarium* sp., *Verticillium* sp., and *R. solanacearum* (Nandhini et al., 2012; Amaresan et al., 2012; Hasan et al., 2020). This study aimed to screen endophytic bacteria isolated from potato plant roots grown in middle latitude area and confirm their antagonistic potential against bacterial wilt disease caused by *R. solanacearum* on potato plant.

2 Material and methods

2.1 Isolation and characterization of R. solanacearum

R. solanacearum was isolated from roots and tubers of bacterial wilt diseased potato plant which grown in middle latitude area located in Bumiaji Sub-District, Batu City, East Java, Indonesia. Bacterial pathogen was isolated by small cutting (1 cm) of the infected the roots, stems and tubers. The infected roots, stems and tubers were then subjected to surface sterilization by using 3% of sodium hypochlorite solution for three minutes, rinsed three times with sterile aquadest and air dried. The materials were then macerated in sterile aquadest and set for 30 minutes. The suspension was then streaked on the Triphenyl tetrazolium chloride (TZC) medium in Petri dish and incubated for two days at room temperature (27–28 °C). The single white, fluidal, red centered colonies were the subjected to purification by sub-cultured in a new TZC medium. The presumed bacterial pathogen was then characterized by several physiological and biochemical assay including pathogenicity test, hypersensitive reaction test on tobacco, Gram reaction, catalase, oxidative/fermentative assay, fluorescent pigment on Kings B medium, soft rot assay on potato and production of acid in glucose, sucrose, lactose, maltose, galactose, mannitol and sorbitol.

2.2 Isolation of endophytic bacteria from potato roots

Endophytic bacteria were isolated from healthy potato roots (Granola cultivar) grown in the middle latitude area (500–700 m.a.s.l.) in Bumiaji Sub-District, Batu City, East Java, Indonesia. Endophytic bacteria were isolated from roots of potato by scrubbing the roots with aliquots of commercial detergent, washed in running tap water, rinsed in 95% ethanol, and air dried in sterile paper towels. The roots were then sterilized using 2% sodium hypochlorite solution for three minutes, rinsed three times with sterile aquadest and air dried. To confirm the biological contamination, the surface of potato roots was slide onto Nutrient Agar (NA) medium surface. When the contaminant colonies were grown in the medium, the samples were discarded. The potato roots were then weighed and macerated using sterile mortar and pestle. The macerated tissues were then resuspended with sterile aquadest, vortex mixed, and incubated for 15 minutes. Dilution series was made and the diluent was plated on to NA plates. The culture plates were incubated at room temperature (27–28 °C) for two days.

2.3 In vitro antibiosis assay of endophytic bacterial isolates

Antibiosis assay was performed using double layer or overlay method described by Wakimoto et al. (1986). Two days culture of presumed endophytic bacterial colonies were picked by sterile ose loop, resuspended in 1 ml sterile water and adjusted to approximately 10^9 CFU.ml^{-1}. The sterile 5 mm diameter of filter paper disk was dipped in the bacterial suspension for one minute and air dried for two hours in the clean bench. The filter paper disk was then put on the 9 cm diameter Petri dish plate containing NA or TZC medium and incubated for two days. One 1 ml of chloroform was then filled in the reversed Petri dish lid and incubated for two hours. Two loops full of *R. solanacearum* sp. colonies were resuspended in 14 ml ±45 °C medium of NA and overlaid on top of the medium. The cultures were incubated for 48 hours and the diameter of the clear zone produced by endophytic bacterial isolates which presumed as antagonist were then measured at one, two and three days after inoculations (DAI).

2.4 In vivo assay of endophytic bacterial isolates against R. solanacearum

In this study, eight isolates of endophytic bacteria recovered from potato roots were used for *in vitro* assay i.e., E1, E5, E6, E104, E117, E120, E121, and E129. The isolates of endophytic bacteria and *R. solanacearum* were sub cultured on NA and TZC medium, respectively, by streaking and were incubated for two days. The bacteria colonies were then used for propagation using 100 ml

of sterile Nutrient Broth (NB) medium enriched with yeast extract in a 500 ml sterilized Erlenmeyer tubes. Bacterial cultures were incubated for two days, then harvested using centrifugation at 5,000 rpm. Bacterial cells were resuspended in phosphate buffer and adjusted to a density of 10^9 CFU.ml^{-1}.

Potato seeds used in this study were the DTO cultivar (a cultivar commonly grown in the middle area) which had sprouted approximately 2 cm length and 30–50 g. tuber^{-1}. Potato seeds were surface sterilized with 70% ethanol for one minute then washed with aquadest. The seeds were then immersed in a suspension of endophytic bacteria for 24 hours. Inoculated seedlings were planted in 10 kg pots containing a mixture of sterilized soil and sand (1 : 1) and grown in the greenhouse under room temperature and irrigated as required. This study was conducted using a Randomized Block Design and five pots served as a single replication and all treatment were replicated tree times.

After 24 DAI, plants were collected and the effects of inoculation were evaluated i.e. the percentage of wilt disease (disease incidence rate), number of leaves, plant height, and fresh and dry weight of plant and tuber biomass. The disease's incidence rate (%) were calculated using the following formula (Gashaw et al., 2014):

$$DIR = (A/B) \times 100) \quad (1)$$

where: *DIR* – disease's incidence rate; *A* – number of the withered leaves in pot; *B* – total number of leaves in pot

2.5 Data analysis

Data of inhibition rate, percentage of disease incidence, number of leaves, plant height, and fresh and dry weight of plant and tuber biomass were analyzed by Analysis of Variance (ANOVA) with Duncan's test at P <0.05. All statistical analyses were performed using R Studio (R Core Team 2020) using Agricolae and ggplot2 packages.

3 Results and discussion

3.1 Isolation and characterization of R. solanacearum from infected potato planted in the middle latitude

The result showed that the pathogenic bacterium (UB-PRS1) was typical of *R. solanacearum* which had characteristics of Gram negative, showed hypersensitive reaction (HR) in tobacco leaves, oxidative, did not produce fluorescent pigment on Kings B agar, and accumulated Poly-β-hydroxibutirate in the cell (Table 1). Based on biochemical assays, the bacterial pathogen was indicated as *R. solanacearum* biovar 2. It has been widely reported that biovar 2 is consistently related to race 3 of *R. solanacearum* (Hayward, 1991; Gutarra et al., 2017).

Table 1 The characteristic of potato wilt bacterium, *R. solanacearum* strain UB-PRS1

No	Characters	Reactions
1	hypersensitive reactions (HR)	+
2	pathogenicity (wilt symptom on potato plant)	+
3	gram	-
4	catalase	+
5	oxidative/fermentative	O
6	nitrate reduction	+
7	gelatin hydrolysis	+
8	fluorescense pigment production	-
9	arginin hydrolysis	+
10	poly-β-hydroxibutirate accumulation	+
11	levan production	-
12	starch hydrolysis	-
13	soft rot on potato	-
14	acid production from:	
	a. glycerol	+
	b. sorbitol	-
	c. mannitol	-
	d. glucose	+
	e. galactose	+
	f. sucrose	+
	g. lactose	+
	h. maltose	+

+ indicates positive reaction, - indicates negative reaction

3.2 Endophytic bacteria isolated from roots of healthy potato plants and in vitro antibiosis assay against R. solanacearum

A total of 130 endophytic bacteria were isolated from inside part of healthy potato roots grown in middle altitude area in Bumiaji Sub-district, Batu City. These bacteria were included as endophytes since they can live in the internal part of plant tissue especially plant root tissue (Afzal et al., 2019). Our results are in accordance with previous reports which have been reported that several bacteria could be isolated from inside part of leaves, roots, stems, and nodules of pea, faba bean, rice, fenugreek, common bean and lupine (Zaghloul et al., 2016), leaves of tomato (Basumatary et al., 2021), potato tubers (Bahmani et al., 2021), and root of banana (Souza et al., 2014).

Based on *in vitro* antibiosis assay, eight of endophytic bacterial isolates had antagonistic activities against *R. solanacearum* i.e., E1, E5, E6, E104, E117, E120, E121, and E129 (Figure 1 and 3). Endophytic bacterial isolates of E1, E5 as well as E6 showed higher growth inhibition zones at 1 to 3 DAI (Figure 1). The inhibition zone was shown by the presence of a clear zone around the colony indicating they released antibiosis substances into agar medium on the area surrounding the colony which inhibit the growth of *R. solanacearum* (Figure 2). These results indicated that the endophytic bacteria isolated from potato roots had the ability to inhibit the growth of *R. solanacearum* under laboratory conditions. These results are in agreement with several studies that reported the endophytic bacteria isolated from potato plant had antagonistic activity against nematode, fungi and bacteria including *R. solanacearum* (Kheirandish and Harighi, 2015; Istifadah et al., 2018). For example, endophytic bacteria *Paenibacillus macerans* has been reported specifically inhibited *R. solanacearum* race 3 growth under *in vitro* conditions (Kheirandish and Harighi, 2015). Bahmani et al. (2021) also reported that endophytic bacterial strains i.e., *P. brassicacearum* Ps169, *P. brassicacearum* Psb101, *Bs. licheniformis* Bl17, *P. putida* Ps52, and *Pa. peoriae* Pa86 had the effective antagonistic activity against *R. solanacearum in vitro*.

The growth inhibition of *R. solanacearum* could be due to secretion of secondary metabolites from endophytic bacteria. Antibiosis substance is an organic substance produced by antagonistic microbes released in the agar media in low concentration and causing the inhibition on the growth of the cocultured opponent microbes (Wang et al., 2017). Secondary metabolites can be secreted from endophytic bacteria within plant parts (Vyas, 2021) and they can be in the form of volatile compounds that play an important role in the establishment of symbiotic relationship for intra- and interkingdom signaling (Soto et al., 2021). Ghadamgahi et al. (2022) also reported that the endophytic bacteria isolated from potato plant roots have the ability to produce metabolites such as proteases, siderophores, and hydrogen cyanide which have been shown to inhibit the growth of *R. solanacearum* under laboratory conditions.

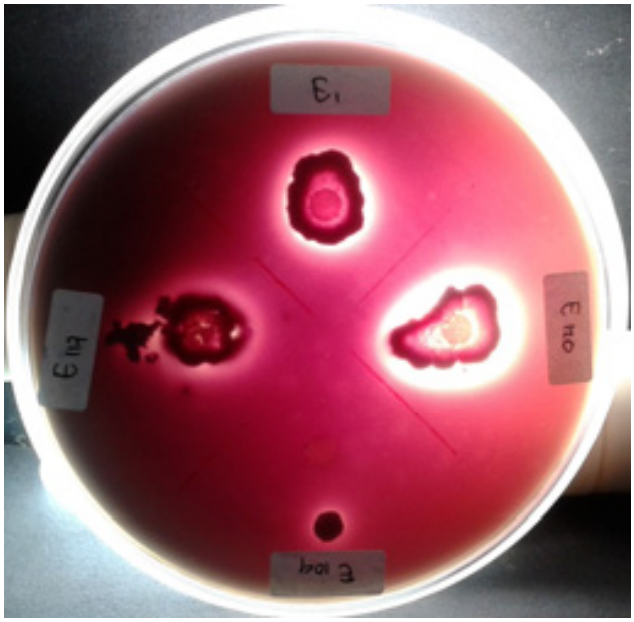

Figure 2 The clear zone produced by representative endophytic bacteria isolates

3.3 *Suppression of endophytic bacterial isolates against potato wilt disease caused by R. solanacearum*

In an *in vivo* experiment, we tested the suppression activity of endophytic bacterial isolates against bacterial wilt diseases on potato plants cultivar DTO. The results showed that bacterial wilt disease index on potato plants at 24 days after inoculation with endophytic bacteria were

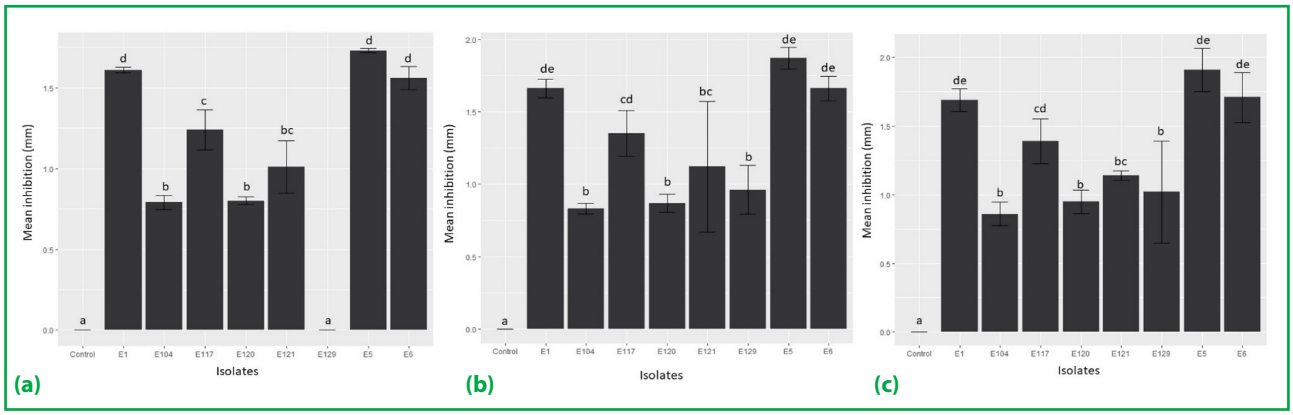

Figure 1 Inhibition zones of endophytic bacteria isolates against bacteria wilt disease (*R. solanacearum*) at (a) one (b) two and (c) three days after inoculation
Bar with different letters were significantly different at $p < 0.05$, according to Duncan's test

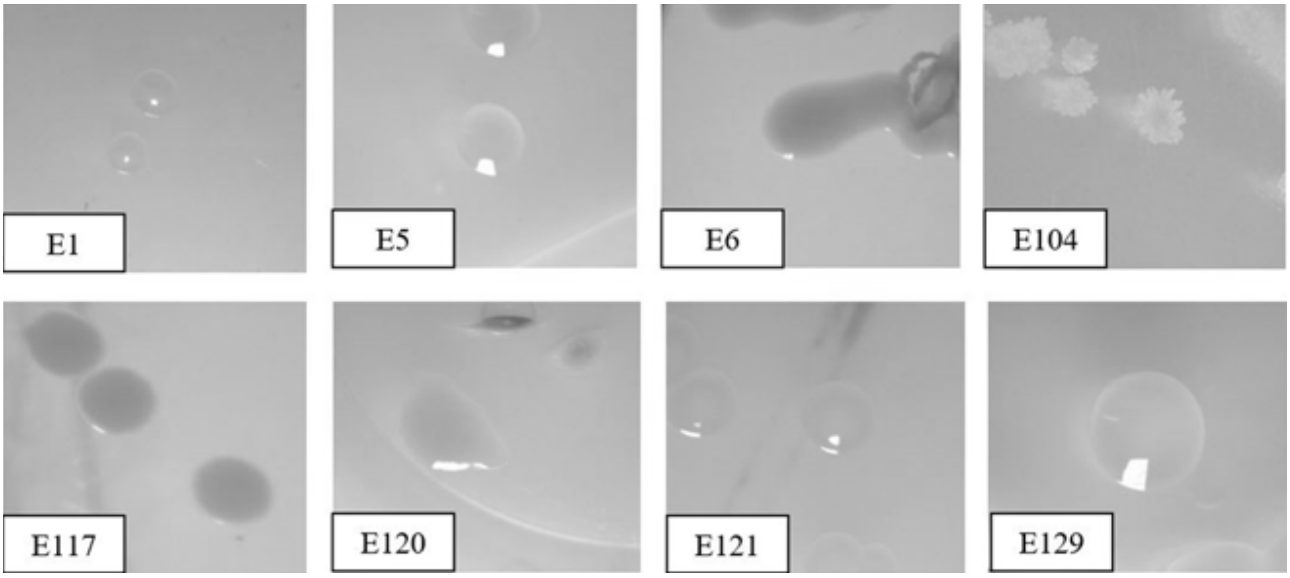

Figure 3 The endophytic bacterial colony morphology isolated from potato root in Bumiaji Sub-district, East Java

varied significantly (at $P < 0.05$). Disease index on potato plants treated with endophytic bacterial isolates E1, E104 and E6 was shown significantly lower than that of the untreated control (F = 3.857, P = 0.005) (Figure 4), indicated that those endophytic bacteria were able to suppress bacterial wilt disease on potato plants. Suppression of bacterial wilt disease on potato plant shown by isolates E1, E104 and E6 was similar to suppression by bactericide (Streptomycin Sulfate) (Figure 4). These results indicate that endophytic bacterial isolates E1, E104 and E6 have the high potential as biological control agent against bacterial wilt disease in potato plants

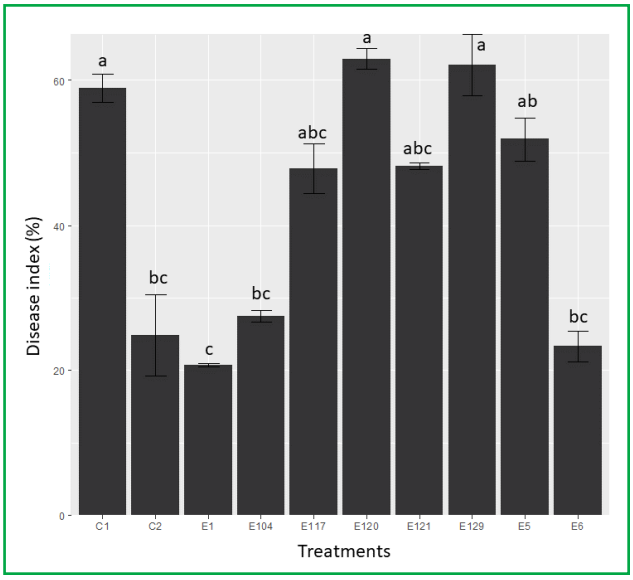

Figure 4 Effects of endophytic bacterial isolates recovered from potato root from middle altitude area on bacterial wilt disease severity
C1 – untreated; C2 – streptomycin sulfat. Bar with different letters were significantly different at $p < 0.05$, according to Duncan's test

In this study, several endophytic bacterial isolates had been shown to suppress bacterial wilt diseases caused by *R. solanacearum*. In accordance with our results, previous study reported that different kinds of endophytic bacteria have been shown to have antagonistic activity against pathogenic fungi and bacteria (Miliute et al., 2015). Until now, several endophytic bacteria have been isolated from potato plant and the number of genera found is <10 and is dominated by bacterial species in the genus *Agrobacterium*, *Enterobacter*, *Bacillus*, and *Pseudomonas* (Manter et al., 2010). The endophytic bacteria *Paenibacillus macerans*, *Pseudomonas fluorescens*, *Bacillus subtilis*, *Serratia marcescens*, and *Bacillus pumilis* were reported to be able to control *R. solanacearum* race 3 under *in vivo* conditions (Kurabachew and Wydra, 2013).

3.4 The effect of endophytic bacterial isolates on potato plant growth parameters

The endophytic bacterial treatments showed significant difference compared to control in all plant growth parameters (Figure 5). The highest effect of endophytic bacteria on plant height was found in E1 isolate (31.03 cm) (F = 3.241, P = 0.0137) (Figure 5a). The number of leaves were varied significantly in each treatment (F = 2.739, P = 0.029) (Figure 5b). The fresh and dry weight of plant and tuber were also showed varied significantly in each treatment (F = 6.412, $P < 0.001$), (F = 4.251, $P = 0.003$), (F = 2.588, P = 0.03), and (F = 4.978, P = 0.001), respectively (Figure 5c, 5d, 5e, and 5f). These results were probably due to the endophytic bacteria isolates had the ability to prevent the harmful effects of bacteria wilt diseases.

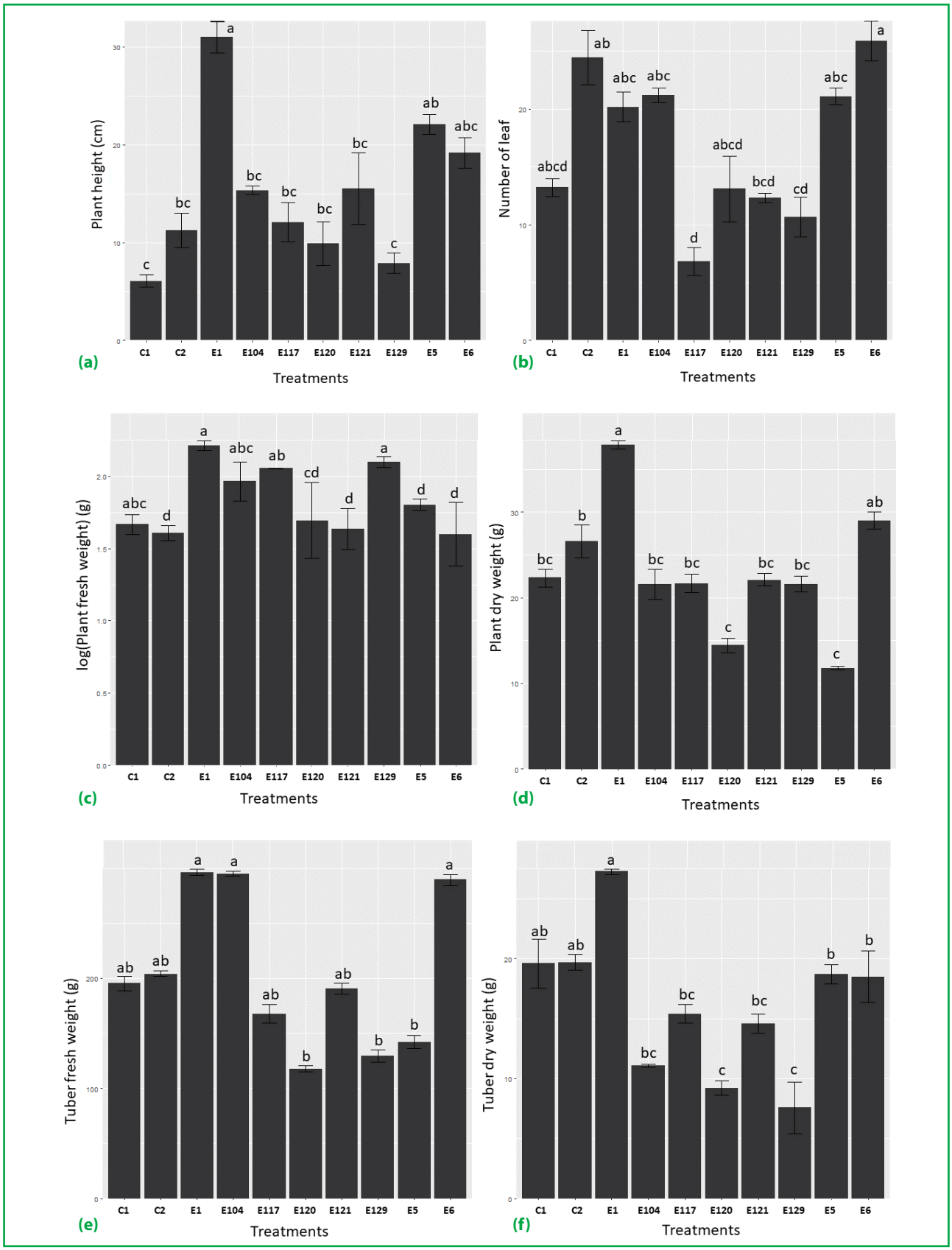

Figure 5 Effects of endophytic bacterial isolates recovered from potato root from middle altitude area against bacteria wilt disease (*R. solanacearum*) on potato growth parameters (a) plant height, (b) number of leaves, (c) plant fresh weight, (d) plant dry weight, (e) tuber fresh weight, and (f) tuber dry weight
C1 – untreated; C2 – streptomycin sulfat. Bar with different letters were significantly different at $p < 0.05$, according to Duncan's test

The beneficial effects of endophytic bacteria on host plants through different kind of mechanisms in rhizosphere associated bacteria (Pageni et al., 2014). In previous study, endophytic bacteria associated with root were found to be promising approach to control pathogens (soil-borne) and enhance plant growth (Lee et al., 2022). This is in agreement with our study which showed that EI endophytic bacterial isolate suppressed bacterial wilts diseases caused by *R. solanacearum* and significantly improved all parameters of plant growth. Bahmani et al. (2021) also reported that endophytic bacteria isolated from root of potato plant such as *P. putida* Ps52 and *P. brassicacearum* Psb101 increased the plant biomass. Endophytic bacteria associated with plant roots have been reported to play an important role in promoting plant growth, because they have direct or indirect mechanisms in facilitating and increasing plant growth by producing various secondary metabolites such as volatile compounds and phytohormones (Adeleke et al., 2021).

4 Conclusion

In conclusion, eight of endophytic bacteria isolates were isolated from potato root cultivated in middle altitude area in Bumiaji Sub-district, Batu City have promising antagonistic activities against bacteria wilt disease (*R. solanacearum*) i.e., E1, E5, E6, E104, E117, E120, E121, and E129 under laboratory conditions. Based on *in vivo* assay, the endophytic bacteria isolate especially EI was able to control the bacteria wilt disease (*R. solanacearum*) and had a significant effect on the growth (plant height and plant dry weight) of the potato plants. However, it is necessary to identify the molecular bacteria isolated in this study to determine which species can suppress wilt disease and increase the growth of potato plants.

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