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Bio-Efficacy of Decomposed Crude Eucalyptus Leaves Extract and *Pseudomonas* Species against Faba Bean Brown Spot Disease

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ABSTRACT

Brown spot disease is one of the most common diseases in faba bean. Many studies have focused on finding natural compounds and biological control agents that can help combat these diseases. This study focused on the study of the effectiveness of decomposed crude eucalyptus leaf extract and *Pseudomonas* species against brown spot disease in faba bean caused by *Botrytis fabae* Sard. A field study was carried out at Toshka Research Station, Desert Research Center, Aswan Governorate, Egypt, during the 2022 winter growing season. In this respect, eleven *Pseudomonas* isolates were evaluated for their potential to suppress the phytopathogenic fungus *B. fabae*. The most effective isolates were identified using 16S rRNA nucleotide sequence. Molecular identification data revealed that selected isolates are blast identical to *P. stutzeri* with 95% and *P. fulva* with 100%. The decomposed extract of eucalyptus leaves reduced the mycelial growth of *B. fabae* in a concentration-dependent manner. The HPLC profile of phenolic compounds in the fermented extract revealed that both hydroxybenzoic acids and hydroxycinnamic acids are more abundant and pyrocatechol was the major phenolic compound in the fermented extract. Under field conditions, the least severity was recorded from treated plants with bioagents in combination with fermented eucalyptus leaf extract in the form of soil and spray applications. Treating faba bean plants with plant extracts and microbioagents improved most tested growth criteria, plant productivity and seed yield.

Keywords: *Vicia faba*, fermented eucalyptus extract, phytochemical qualitative screening, biocontrol, chocolate spot disease, suppressive soil

1. Introduction

Globally, faba bean (*Vicia faba* L.) is one of the most popular legume crops. In many nations, especially in the Middle East and Northern Africa, legume crop grains represent a significant source of dietary protein. Its great nutritional value due to its richness in vitamins and protein could explain the economic significance of bean farming throughout the world. Furthermore, beans also increased soil fertility in part because of their role in nitrogen fixation and soil biodiversity in crop rotations (Lee *et al.*, 2020). Consequently, one of the objectives in agriculture in many countries is to increase the production of this crop. The production of faba beans is influenced by fungi that include *Cercospora zonata, Ascochyta fabae,* and *Botrytis fabae* (Kimber *et al*., 2016). Brown spot is a destructive plant disease caused by *B. fabae* that is distinct from other foliar faba bean diseases (Tivoli *et al*., 2006).
Agriculture yields have long been supported and increased by plant protection agents, such as synthetic pesticides and herbicides. However, the chemical pesticide had several detrimental repercussions on the environment and human health (Maggi *et al*., 2020). The growth of resistant weeds and insects continues to highlight the pressing need for new and secure goods. Botanical pesticides pose less of a threat to the environment and human health than synthetic pesticides, so, they have long been regarded

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as preferred options. Although the exact date of human use of plants and their metabolites as pesticides against insects and bacteria has not been established, it is already associated with the advent of agriculture (Stevenson, 2017).

The advantages of using biopesticides are their low persistence and residuality, the prevention of environmental pollution, and the reduction of harmful impacts on life forms. They are less likely to develop pest resistance and can display high host specificity, which can delay knockdown (Hernandez-Fernandez *et al*., 2021). Additionally, the combined use of biopesticides and biofertilizers can enhance soil health and reduce environmental pollution, supporting sustainable agriculture. Crop yield can also be improved by using biopesticides in conjunction with natural enemies. Although the exact mechanisms of these combinations are less well understood, compatible interactions between different biocontrol agents that work in concert seem to be crucial factors to enhance plant protection in the future (Acheuk *et al*., 2022).

Chemical fungicides could be replaced with soil-borne, non-pathogenic microorganisms that have the capacity to combat fungal phytopathogens and so avoid plant disease. Since they work through a number of methods, including direct antibiosis, the stimulation of plant development, the induction of systemic resistance in the plant hosts, and competition for nutrients and space. *Pseudomonas* biocontrol strains and their bioactive secondary metabolites may be regarded as advantageous for the health of plants. These helpful bacteria have been shown to be particularly effective against nematodes, various insects, and bacterial and fungal phytopathogens (reviewed by Dimkić *et al*. 2022).

In comparison to synthetic chemicals, those derived from plant-based fungicides and pesticides are thought to be safer for the environment and human health because they are rich sources of antimicrobial agents (Suleiman, 2010) and are less persistent and hence easily biodegradable. Most studies on eucalyptus have mostly concentrated on the composition and biological effects of the essential oil extracted from the leaves, which have significant antibacterial activities (Luis *et al*., 2014). However, there are a few limited studies that focus primarily on the antibacterial qualities and chemical composition of fermented leaf extracts. Therefore, the purpose of this study was to determine the potential effect of natural metabolites obtained from fermented eucalyptus leaves in combination with *Pseudomonas* sp. against the brown spot disease of faba bean.

2. Methods:

Isolation of the Pathogen

The faba bean pathogen *B. fabae* was isolated from collected naturally infected leaves. The isolation of *B. fabae* and the subsequent antifungal assay was conducted using Faba bean dextrose agar medium (Haggag *et al*., 2006).

Isolation of antagonistic *Pseudomonas* bacteria

Pseudomonas bacteria were isolated from soil samples on King B medium using serial dilutions approach (Johnson *et al*., 1960).

Evaluation of the effect of antagonistic *Pseudomonas* bacteria on *B. fabae.*

Bacterial isolates were examined against *B. fabae* using dual culture technique according to Dennis and Webster (1971). The percentage of reduction in fungal growth was calculated according to Fokkema (1973) as follows:

$$\text{Reduction in linear growth (\%)} = \frac{R1-R2}{R1} \times 100$$

Whereas: R1 = the normal growth of the control
R2 = the inhibited growth

Identification of selected isolates

Cellular morphology and biochemical tests were carried out using the techniques outlined by Gordon *et al*. (1973). According to the modified approach of Ishikawa *et al*., (2000), isolates were identified by direct extraction of genomic DNA from the colonies grown on NA medium and used as a template for PCR using forward and reverse primers, F (5AGA GTTTGA TCC TGG CTC AG-3′) and R (5′ -GGT TAC CTT GTT ACG ACTT-3′). Using 16S rRNA sequencing, the chosen isolates

were sequenced and assembled using the BioEdit program (Hall 1999). The neighbor-joining NJ method was used to build the phylogenetic tree (Saitou and Nei 1987).

Plant growth promotion activities of selected isolates

Following the procedures outlined by Ahmad *et al*., (2008), bacterial isolates were tested for their plant growth-promoting traits, such as indoleacetic acid, hydrogen cyanide, siderophores, and phosphate solubilization.

Enzymatic activity of selected isolates.

The plate assay method, as designated by Seeley *et al*., (1981) was used to examine the ability of chosen isolates to produce cellulase, protease, and chitinase enzymes.

Solid-state fermentation of eucalyptus leaves

Fresh eucalyptus leaf samples were collected, washed, cut into small pieces and subjected to solid-state fermentation as described in a previous study (Yaseen, 2020). The obtained extract was concentrated and designated as the standard plant extract solution (100%).

Phytochemical screening

Phytochemical tests of total saponins, terpenoids, alkaloids and tannins were carried out for eucalyptus extract using chemical methods and high-performance liquid chromatography (HPLC) following standard methods (Biswas *et al*., 2013).

***In vitro* evaluation of the antifungal activity of eucalyptus extract**

The antifungal activity of eucalyptus extract was evaluated against *B. fabae* using the food poisoning technique (Babu Joseph & Kumar 2008). Different concentrations (5, 10, 15, 20, and 25 %) of crud extract were mixed with sterile potato dextrose agar medium and then poured into sterile Petri dishes. Discs of 7 mm diameter of *B. fabae* (6 days old cultures) were placed at the center of poured Petri dishes of treatment and incubated at 25 ± 2°C for 7 days. The percentage of reduction in fungal growth was calculated as mentioned before.

***In vivo* Efficacy Test**

Field experiment

The study was conducted during the 2022 winter growing season at Toshka station, desert research center, Aswan government, Egypt. The soil texture was sandy with 59.5% nitrogen, 0.39 % organic matter, pH 7.95 and electrical conductivity of 1.82 $DS.m^{-1}$. The fertilizer used was NPK 19-19-19. A faba bean Maryout-2 cultivar obtained from the Plant Genetic Resources Department, Desert Research Center, Cairo, Egypt. The bio-control agent's crude extract of decomposed eucalyptus (50% dilution) and antagonistic *Pseudomonas* bacteria (10^8 cfu/ml) were added into the soil at 20 ml/plant during sowing. The experiment included eight treatments, which were a combination of four treatments of crude extract and two treatments of antagonistic bacteria. The statistical design of the experiment was split plot design with three replicates, whereas crude extract of decomposed eucalyptus treatments occupied the main plots and antagonistic bacteria were arranged in the sup main plots. Treatments of decomposed eucalyptus crude extract were as follows; T0: control (no application), T1: soil drench, T2: foliar spraying (sprayed 4 times at two-week intervals) and T3: soil drench + foliar spray, while the treatments of sub main were as follows; B0: control (no inoculation) and B1: bacterial inoculation.

Agronomic Data Recorded

Disease incidence: The disease incidence percentage (DI%) was calculated according to the following equation

$$\text{Disease incidence \%} = \frac{\text{Number of infected plants}}{\text{Total number of plants observed}} \times 100$$

Disease Severity (%)

Data of disease severity were recorded using the nine-category disease rating scale (Sahile *et al*. 2008). The disease severity (%) for each treatment was calculated using the equation:

$$DS\% = \frac{\sum n}{9N} \times 100$$

Where DS% = disease severity (%), ∑n= the sum of all scale ratings, 9 is the highest category on the rating scale and N= sample size).

Yield and yield components

At harvest, plant height (cm), number of branches, fresh weight (g), dry weight (g), number of pods/plants, (g) and 100 grains weight (g) were measured.

Statistical Analysis: The statistical test was performed using SAS statistical software. Means were sorted out by using Fisher's least significant difference (LSD) procedure.

Results

Pathogen isolation and pathogenicity test

Isolation trials carried out on faba bean leaves with chocolate spots collected from many locations in South Sinai governorate, New Valley governorate, and Moghra oasis, Matrouh Governorate, resulted in the isolation of different isolates of *B. fabae*. The virulence of the fungal isolates was tested in a pathogenicity test (unpublished data). The most virulent isolate was selected to use in this study.

Isolation of antagonistic *Pseudomonas* bacteria

Eleven bacterial isolates related to *Pseudomonas* genus were evaluated for their potential to suppress the phytopathogenic fungus *B. fabae*. The isolates that inhibited the growth of *B. fabae* to a high degree were selected. Table 1 showed that isolate Ps4 gave the highest reduction percentage (67.7%), followed by isolate Ps18 (54.4%) when compared to control.

Table 1: Antagonistic effect of bacterial isolates on *B. fabae* growth, *in vitro.*

Bacterial isolate	Ps4	Ps18	Control
Linear growth (Cm)	2.9±0.5	4.1±0.7	9.0±0.1
Growth reduction (%)	67.7	54.4	0

Identification of selected isolates

The morphological and biochemical features of selected isolates are presented in Table 2. Isolates Ps4 and Ps18 were Gram-negative, aerobic, short rod, motile and non-spore former. The evolutionary history was inferred using the Neighbor-Joining method. The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicated trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The 16S rRNA nucleotide sequence revealed that selected isolates are blast identical to *P. stutzeri* with 95% and *P. fulva* with 100% respectively. The phylogenetic tree of the bacterial strains and closely related strains is demonstrated in Fig 1.

Table 2: Morphological and biochemical characters of selected bacterial isolates

Characteristic	Ps4	Ps18
Gram staining	-ve	-ve
Shape	Short rod	Short rod
Motility test	+	+
Spore forming	-	-
Methyl red test	+	++
Oxidase test	+	+
Catalase test	+	+
Arginie dihydrolysis	-	-
Starch hydrolysis	+	+
Gelatin liquefaction	+	+

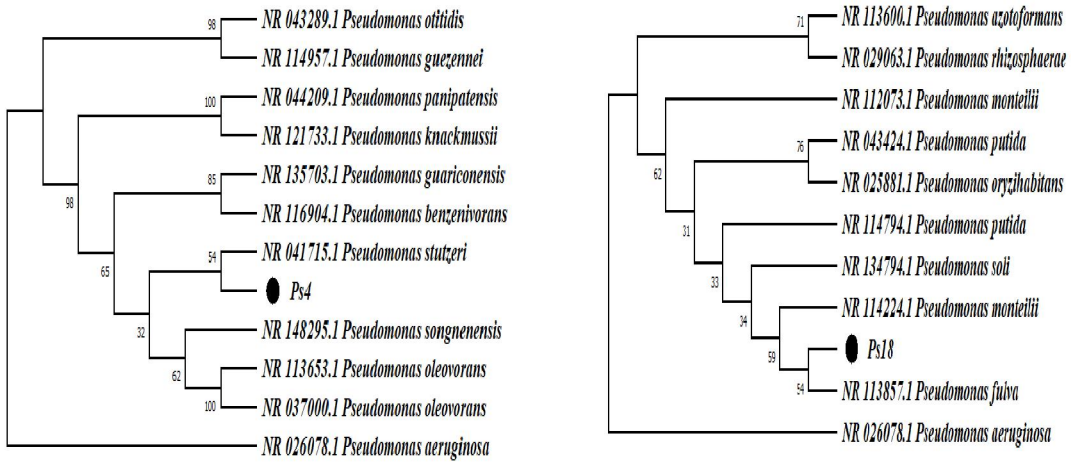

Fig. 1: Neighbor-Joining phylogenetic tree of partial 16S rRNA sequence of isolates Ps4 and Ps18. *Pseudomonas aeruginosa* was used as outgroup.

Enzymes and plant growth promotion activities of selected isolates

According to the results in Table 3, the bioagent strains could produce indol acetic acid as well as solubilize phosphate. They also showed siderophores, HCN, cellulase, protease, and pectinase production.

Table 3: Growth-promotion activities and enzyme production by bacterial strains.

Characteristic	*P. stutzeri*	*P. fulva*
Indole test	+	++
Phosphate dissolving	+++	++
HCN production	+	+
Siderophore production	+	+++
Production of enzymes		
Cellulase	+	++
Protease	+	++
Pectinase	+	++

Chemical analysis of the fermented eucalyptus extracts

Phytochemical screening

The preliminary phytochemical qualitative screening of the fermented eucalyptus leaf extract revealed the presence of tannins, terpenoids, alkaloids and flavonoids, while saponins were not detected in eucalyptus extract as presented in Table 4.

Table 4: Preliminary phytochemical screening of the decomposed eucalyptus leaves extract.

Phytochemicals constituents	Saponins	Tannins	terpenoids	Alkaloids	Flavonoids
decomposed eucalyptus extract	-	+	+	+	+

High-performance liquid chromatography (HPLC)

Different chemical classes of phenolic compounds were identified in the fermented extract of eucalyptus leaf by comparison of the retention times of standards and UV spectra under the HPLC conditions (Table 5). Both hydroxybenzoic acids and hydroxycinnamic acids are more abundant in the fermented leaf extract. As shown in Table 5, nineteen individual phenolic compounds were tested and quantified in the extract. Pyrocatechol was the major phenolic compound in the fermented extract of leaves (71.41 µg/ml), followed by ellagic acid (39.27 µg/ml) and gallic acid (23.33 µg/ml). Chlorogenic acid (7.13 µg/ml), ferulic acid (3.75 µg/ml), syringic acid (2.2 µg/ml), coumaric acid (1.03 µg/ml) and

coffeic acid (0.5 µg/ml) are other phenolic compounds identified in the crude extract. Minor amounts of ellagic acid, gallic acid, syringic acid, p-hydroxybenzoic acid and apigenin were also observed.

Table 5: HPLC analysis of phenolic compounds in fermented eucalyptus leaves extract.

No.	Compound	Chemical class	Conc. (µg/ml)
1	**Gallic acid**	Hydroxybenzoic acids	23.33
2	**Chlorogenic acid**	Hydroxycinnamic acids	7.13
3	**Catechin**	Flavan-3-ols	ND
4	**Methyl gallate**	galloyl esters	1.80
5	**Coffeic acid**	Hydroxycinnamic acids	0.50
6	**Syringic acid**	dimethoxybenzene	2.20
7	**Pyrocatechol**	Hydroxylated phenols	71.41
8	**Rutin**	Flavonoids	9.95
9	**Ellagic acid**	Hydroxybenzoic acids	39.27
10	**Coumaric acid**	Hydroxycinnamic acids	1.03
11	**Vanillin**	phenolic aldehyde	1.27
12	**Ferulic acid**	Hydroxycinnamic acids	3.75
13	**Naringenin**	Flavonoids	5.09
14	**Daidzein**	isoflavonoids	0.46
15	**Querectin**	Flavonoids	7.97
16	**Cinnamic acid**	carboxylic acid	0.05
17	**Apigenin**	flavanon-glycoside	ND
18	**Kaempferol**	Flavonoids	0.45
19	**Hesperetin**	flavanon-glycoside	ND

The *in vitro* antifungal efficacy of decomposed eucalyptus leaf extract (DELE)

Different concentrations of DELE (5- 25%) were tested for their inhibitory effect on thelinear growth of *B. fabae in vitro.* The results in Fig. 2 indicated that all tested concentrations of DELE reduced the mycelial growth of *B. fabae* in a concentration-dependent manner. Mycelial radial growth was highly significantly increased as theconcentration increased. The concentration of 25% provided the highest growth inhibitions (75%) whereas the lowest growth inhibition (6.25%) was observed at 5%.

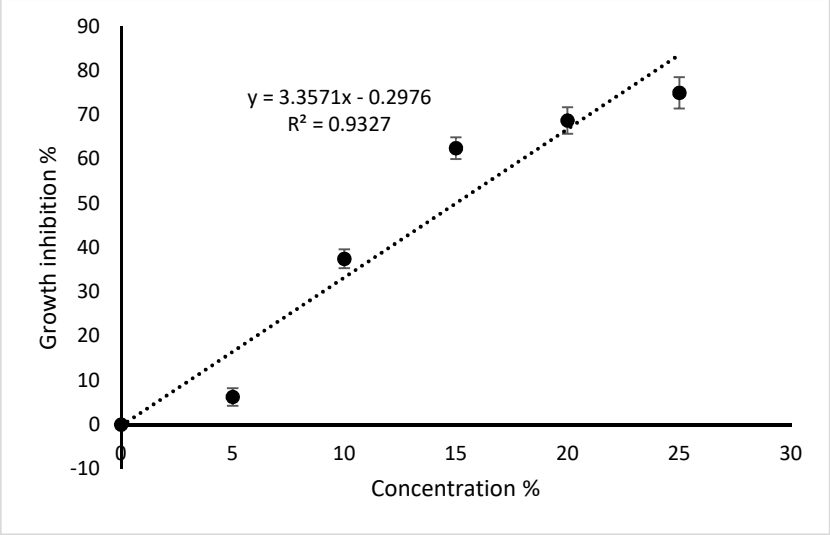

Fig. 2: Regression model predicting the relationship between in vitro mycelial Growth Inhibition of *Botrytis fabae* and crude extract concentrations of fermented eucalyptus leaves.

Inhibition of chocolate spot disease of faba bean as treated with eucalyptus leaf extract and bioagents.

Brown spot disease incidence and severity highly significantly (at $p \leq 0.05$) varied due to different treatments. The highest disease incidence (DI, 86%) and disease severity index (DSI, 0.34) are observed in the untreated faba bean control plants as illustrated in Fig. 3. The least severity score of 14.2% was recorded from those treated with the bioagents in combination with fermented eucalyptus leaves extract in the form of soil and spray applications. Sett treated with bioagents combined with fermented extract spray application also had less DI (23.4%) and was the next best treatment. When the bioagent was added with fermented extract, the incidence of chocolate spots was reduced by 35.3%. However, a bean sett treated with a single bioagant was the least effective treatment in controlling chocolate spot disease.

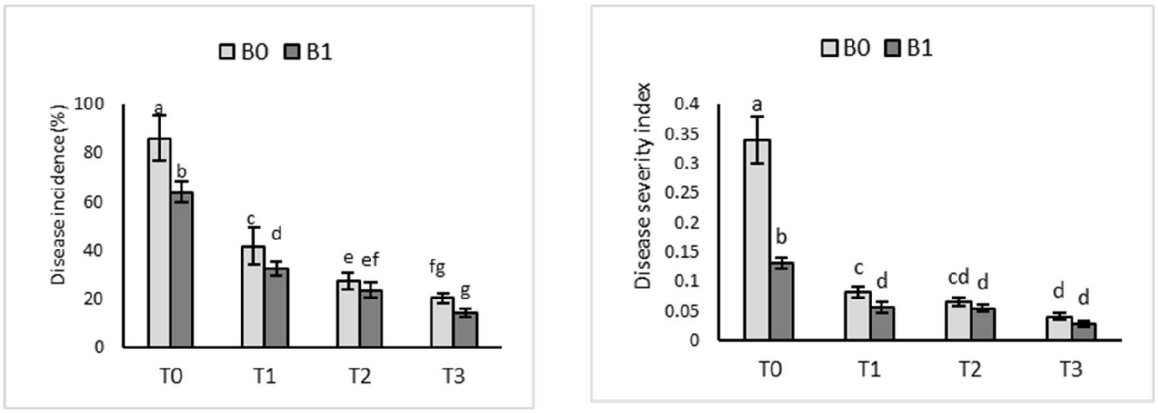

Fig. 3: Effect of fermented eucalyptus extract and inoculation with bioagents on the incidence and severity of *Botrytis Fabae.* T0: decomposed eucalyptus extract was not applied, T1: soil application, T2: foliar spraying and T3: soil application + foliar spray, B0: without bacterial inoculation and B1: bacterial inoculation.

Plant growth, productivity and yield as affected by decomposed eucalyptus leaves extract and bioagents treatment.

Plant height (PH) as well as the number of pods per plant (PN) were significantly increased by bio-treatment application compared with the control (Fig. 4). The lowest PH and PN were recorded in the infected treatment whereas the highest PH (131.4 cm) and PN (51 pods) were recorded from mixed bio-inoculations combined with fermented eucalyptus leaf extract (soil and spray applications). In contrast, it was also shown that plant branches were not significantly influenced by treatments. Plants treated with bioagents in combination with fermented extract produced the highest fresh and dry weight. The weight of 100 grains of faba bean was reduced due to *B. fabae* infection since infected plants gave 76 g while treated plants gave 103 g. The magnitude of the response was most pronounced in the case of T3, followed by T2, T1 and T0 in that order.

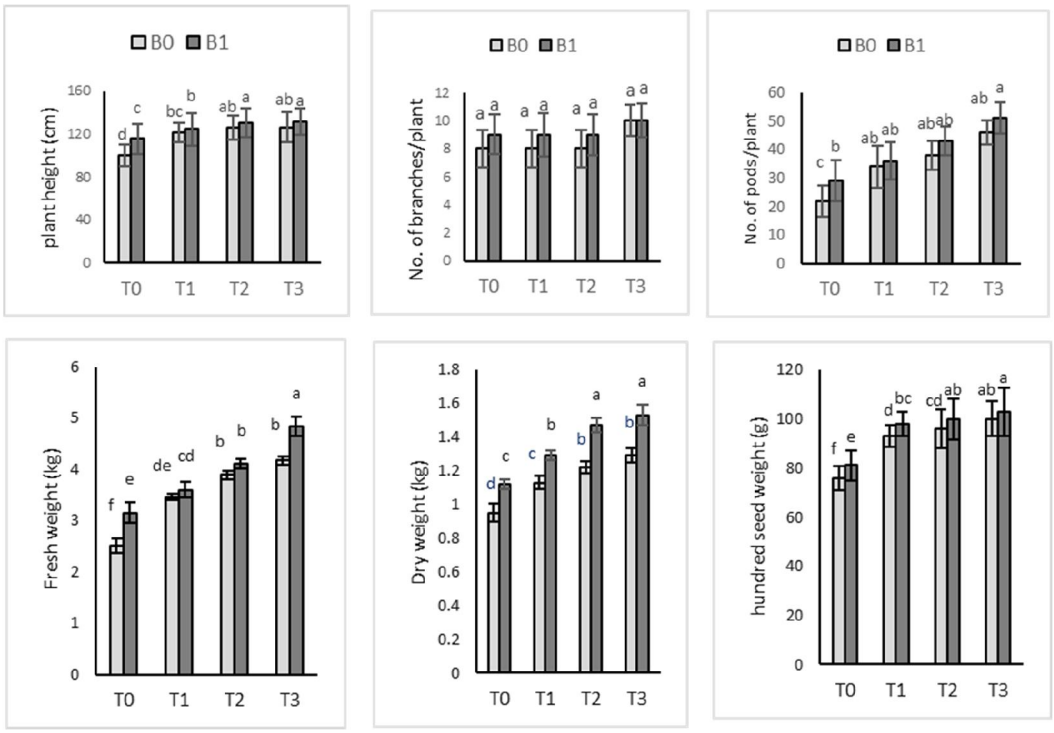

Fig. 4: Effect of fermented eucalyptus extract and inoculation with bioagents on the plant growth parameters. T0: decomposed eucalyptus extract was not applied, T1: soil application, T2: foliar spraying and T3: soil application + foliar spraying, B0: without bacterial inoculation and B1: bacterial inoculation.

4. Discussion

Brown spot disease caused by *B. fabae* is considered the most destructive plant disease of faba bean. In this study, *Pseudomonas* isolates as well as the fermented eucalyptus leaf extract exhibited a strong inhibitory effect on *B. fabae* spore germination. The presence of numerous antibacterial components with diverse spectra in the leaves of Eucalyptus spp. was emphasized by Takahashi *et al*. (2004). The bioactive components, such as flavonoids, terpenoids, alkaloids, and other phenolic compounds, are present in joint forms with other molecules, such as sugars, fatty acids, or amino acids, making it difficult to extract them using organic solvents, as highlighted by Adom and Liu (2002). enzymatic treatment may facilitate the extraction of natural bioactive chemicals from lignocellulosic tissues. Millet *et al*. (2012) highlighted that the fermented aqueous extract of onion bulb was more effective than both aqueous and methanolic extracts in terms of antibacterial, antigenotoxic, and antiproliferative activity. The antifungal action of the fermented eucalyptus extract may be attributed to the presence of phenolic compounds. In this case, the major or minor components of the extract, which are essential to the synergistic activity, may trigger the antifungal activity. A hydroxylated phenol known as pyrocatechol has been identified as a naturally occurring plant polyphenol with antibacterial properties (Alibi *et al*., 2021). The interactions occurring in these components may lead to antagonistic or additive impacts. The mechanism of phenolic compounds' toxicity may be due to reactions with sulfhydryl groups or more nonspecific interactions with the proteins and unavailability of substrates to microorganisms. Their possible consequence is leakage of cell membranes, therefore causing cell death (Endo *et al*., 2010). These results support previous reports on the antifungal effect of eucalyptus on mycelial growth of several plant pathogenic fungi, including *Botrytis cinerea, Rhizoctonia solani, Fusarium solani, F. oxysporum, Macrophomina phaseolina, Alternaria solani, Sclerotium rolfsii*, and *Phytophthora infestance* as reviewed by Barbosa *et al*. (2016). Leaves of eucalyptus were reported to have high amounts of oxalic acid, which is among the chemicals known to act as defense inducers in plants (El-Shafey *et al*., 2020). Similar observations were confirmed in the present study during the

inactivation of the test organism at different extract concentrations. In the present study, the efficiency of tested plant extract and microbioagents (a mixture of *P. putida* and *P. stutzeri*) were evaluated for their efficiency to suppress brown spot disease caused by *B. fabae*. It was observed that the least severity score was recorded from plants treated with the bioagents in combination with fermented eucalyptus leaves extract in the form of soil and spray applications. Beneficial bacteria are efficient biocontrol agents against *B. fabea* through direct mechanisms, such as parasitism, antibiosis, and competition, or indirectly through the activation of systemic plant resistance. The interaction between plants and these microorganisms can lead to the development of defensive responses in distant plant organs, which is reflected in plant growth and productivity (Poveda *et al* 2020). Similar findings were also reported by Tola *et al*. (2016) who highlighted that the bioactive compounds in the leaves of *E. globulus* are active against mycelial growth and field severity of *B. fabae* of faba bean. Similarly, Jayakumar *et al*. (2007) suggested that the applicability of plant-passed extracts in combination with antagonistic bacteria for the suppression of sugarcane red rot disease in the field as an environment-friendly tool. Antimicrobial activity is attributed to diverse modes of action; mechanisms such as nutrient and space competition, induced resistance, antibiosis and parasitism operate alone or in concert with others and are involved in antagonistic interactions in the micro-organisms (Nxumalo *et al*., 2021). Different mechanisms of action through which phytochemicals can exert antimicrobial activities include (i) inhibition of the activity of enzymes and toxins; (ii) damage of the microbial membrane; (iii) suppression of virulence factors; (iv) inhibition of protein synthesis; and (v) quorum quenching. For example, the mode of action of tannins is based on their ability to bind proteins, thereby inhibiting cell protein synthesis, while phenolic compounds are known to alter the membrane functionality of pathogens (Nxumalo *et al.,* 2021). the disease in turn has a remarkable effect on the yield and yield components of the crop. Treating faba bean plants with plant extracts and microbioagents improved most tested growth criteria, plant productivity, and seed yield. The contrasting effects of *B. fabae* and the biocontrol agents on the growth, productivity and yield of faba bean may be due to the pathogenicity of *B. fabae*, the allelopathic effect of eucalyptus leaf extract, and/or the anti-*Botrytis* effect of both *Pseudomonas* species. The pronounced recovery of the growth, productivity and yield of infected plants that were treated with the bioagents in combination with fermented eucalyptus leaf extract could be ascribed to the additive effects of bio-stimulants produced by Pseudomonas isolates and present in eucalyptus leaf extract.

Conclusions

The crude metabolites present in fermented eucalyptus leaf extract and antagonistic *Pseudomonas* bacteria prevented disease symptoms remarkably when applied on bean seedlings before the *B. fabae* infection. It could be concluded that the fermented plant-based extract in conjunction with antagonists proved to be an ecologically friendly agent, preventing infection and promoting plant development at the same time. The approach may be improved to combat and prevent brown spot disease while also boosting faba bean yields in sustainable agriculture.

Abbreviations

HPLC: high-performance liquid chromatography, DNA: deoxyribonucleic acid, rRNA: ribosomal ribonucleic acid, DI: disease incidence, DS: disease severity, cfu: colony forming unit, HCN: hydrogen cyanide, DELE: decomposed eucalyptus leaves extract

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