EGYPTIAN JOURNAL OF BOTANY (EJBO)

Print ISSN: 0375-9237
Online ISSN: 2357-0350

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PUBLISHED BY
THE EGYPTIAN
BOTANICAL SOCIETY

Using of *Ulva fasciata, Enteromorpha flexuosa*, *Pseudomonas fluorescens*, and *Pseudomonas putida* for managing cercospora leaf spot disease on sugar beet

Neveen F. Ali[1], Effat A. Zaher[2], Abd Allah A. El-Naggar[1], Marwa A. Zayton[2]
[1]*Maize and Sugar Crops Diseases Research Section, Plant Pathology Research Institute, Agricultural Research Centre, Egypt*
[2]*Plant Pathology Department, Faculty of Agriculture, Cairo University, Egypt*

REVIEW ARTICLE

This study was carried out to evaluate the potential effect of water-soluble (WE) and ethanol (EE) extracts obtained from macroalgae *Ulva fasciata* (UF) and *Enteromorpha flexuosa* (EF) on *Cercospora beticola* growth, conidial germination, disease severity, and sugar beet yield and its impact on the activity of resistance related enzymes (peroxidase and chitinase). *Pseudomonas fluorescens* (Ps-20) and *P. putida* (Ps-15) were also tested and compared with the recommended opus fungicide as well as the unsprayed treatment. All treatments noticeably inhibited the fungal growth (47.7-85.7% efficacy) and reduced the percentage of germinated spores (90% in control and 5.0-18.3% in bioagent treatments) *in vitro*. With foliar application in Damietta and Kafr El-Sheikh, the range of *C. beticola* spots number (SN) per 5 leaves (5L), spot area (SA), and disease severity (DS) was remarkably reduced (53.2-194.8 SN/5L, 1.0-2.7 mm^2, and 1.3-9.3%, respectively) compared with the untreated control (1399.7-1773.7 SN/5L, 7.5-11.1 mm^2, and 25.0-38.3%, respectively). Also, root weight and sucrose percentage (%) were significantly enhanced when the bioagents were sprayed on leaves in the two field trials. Additionally, foliar application with *E. flexuosa* crude extract (EE-EF), *P. fluorescens* (Ps-20), and *P. putida* (Ps-21) significantly increased chitinase but not peroxidase activity in leaves compared with the control in the two governorates. Accordingly, foliar application of these eco-friendly bioagents may be useful for decreasing both the disease and the use of environmentally polluting chemical fungicides.

Keywords: *Beta vulgaris*, *C. beticola*, *Ulva fasciata*, *Enteromorpha flexuosa*, ulvan, peroxidase, and chitinase

ARTICLE HISTORY
Submitted: May 30, 2024
Accepted: August 20, 2024

CORRESPONDANCE TO
Abd Allah A. El-Naggar,
Maize and Sugar Crops Diseases Research Section, Plant Pathology Research Institute, Agricultural Research Centre, Egypt
Emai: elnaggar06@gmail.com
DOI: 10.21608/ejbo.2024.293927.2866

EDITED BY: N. Khalil

INTRODUCTION

Sugar beet (*Beta vulgaris* L.) is one of the most important sugar crops in the world. In Egypt, it became the first source for sugar production followed by sugar cane. The total cultivated area of sugar beet in Egypt reached 0.6 million feddans that produced about 1.7 million tons of sugar (Sugar Crop Council, 2022). Most of these areas are cultivated at Kafr-El-Sheikh and Dakahlia Governorates. Sugar beet leaf spot caused by *Cercospora beticola* Sacc. is the most destructive foliar disease of sugar beet in warm and humid areas such as the Mediterranean basin (Mukhopadhyay and Rao, 1978; Rossi *et al.,* 1995; Weiland and Koch, 2004; Whiteny and Duffus, 1995; Gouda *et al*., 2022). In the absence of control measures, in areas with high disease severity, yield losses ranged from 25 to 50% (Shane and Teng, 1992; Byford, 1996). Crop losses due to cercospora leaf spot appeared as a reduction in root weight and reduced sugar content (Smith and Martin, 1978; Khan and Smith, 2005) which reflect on the final money return (Shane and Teng, 1992; Gouda *et al*., 2022). Cercospora leaf spot is managed by planting disease tolerant varieties, reducing inoculum by crop rotation and tillage, biological agents, and resistance inducing chemicals as well as fungicide applications (Miller *et al*., 1994; Hudec *et al*., 2023).

Unfortunately, there is no breeding program now in Egypt due to the unfavorable conditions for seed production, and Egypt depends on importation as a source of varieties. At the same time, these varieties changed after a short period. Additionally, the use of chemical fungicides resulted in environmental pollution and poor health to the biotic community. So, looking for natural products' alternatives to synthetic fungicides is safer for sugar production. The potential for biological control of cercospora leaf spots, to supplement crop protection offered by resistant varieties and fungicides, is also being exploited (Collins, 1999; Georgieva *et al*., 2023; Azzam *et al*., 2023). On the other hand, biological agents like the systemic resistance inducing *Pseudomonas* spp. and green algae extract were used for foliar application (Wu *et al*., 2020) against numerous diseases (Umesha *et al*., 1998; Walsh *et al.*, 2001; Bolwerk *et al.*, 2003; Haas and Keel, 2003; Khan *et al*., 2009; Paulert *et al*., 2009, De Freitas and Stadnik., 2012; Mehamood *et al*., 2023) and already gave promising results for controlling and enhancing the productivity (Lemanceau, 1992; EL-Assiuty *et al*., 2009; Borsato *et al*., 2010; Craigie, 2011; Zodape *et al.*, 2011; Delgado *et al*., 2013; Singh *et al*., 2016).

The objectives of this study were to investigate the effect of water-soluble extracts named ulvan and

ethanol crude extracts isolated from each of *Ulva fasciata* and *Enteromorpha flexuosa* along with two *Pseudomonas* spp. and the recommended fungicide opus compared with the control on (1) *C. beticola* growth and conidial germination *in vitro*, (2) spots number, spot area, and disease severity *in vivo*, (3) sugar beet root weight and sucrose percentage, and (4) the activity of sugar beet defense enzymes, peroxidase and chitinase.

MATERIALS AND METHODS

Culture of *C. beticola*

Single spore *C. beticola* culture (k1 isolate), which was isolated from sugar beet field in Kafr EL-Sheikh Governorate (Ruppel, 1972) and identified at the Department of Fungal Classification and Identification, Plant Pathology Research Institute, Agricultural Research Centre, was used in this study.

Green Algae Collection and Water-soluble and Crude Polysaccharides Extraction

Green algae, *Ulva fasciata* Delile and *Enteromorpha flexuosa* Wulfen (Ahmed, 2018), were collected from Rockies in the Mediterranean Sea (El-Noras Village, Port Said). Cleaned and air- dried algae were grounded in fine powder by the aid of a mechanical grinder (Tornado Blender MX5200-2) and stored frozen until use (Aseer *et al.,* 2009). Two algal polysaccharides extraction methods were used. Water-soluble polysaccharides were isolated from each alga as described by Pengzhan *et al.* (2003), Cluzet *et al*. (2004), and Paulert *et al*. (2009). Fifty grams of each alga were added to a half liter of distilled water and autoclaved at 110°C for one hour. After that, aqueous solutions were filtered by filter paper (Double Rings, 150 mm). Then, 3 volumes of ethanol (95%) were added to the filtrated solution and stored at -20°C for 48 h. Afterwards, the precipitate was separated by filtration using filter paper (Double Rings, 150 mm), incubated at 40-45°C until dried and re-dissolved in distilled water and adjusted to 10 mg/ml as a final concentration (*U. fasciata* (WE-UF) and *E. flexuosa* (WE-EF)), kept at 4°C, and sterilized by filter syringe (Axiva, Syringe Filters Nylon, 0.2 µm). For ethanol extraction, 100 g of each alga were soaked in 300 ml of ethanol (95%) at room temperature (RT) for 24 h and subsequently incubated at 40°C until ethanol was fully evaporated. The obtained film was dissolved in water after incubation at 4°C for one day; then 10 mg/ml was adjusted for *U. fasciata* (EE-UF) and *E. flexuosa* (EE-EF). Finally, each extract was sterilized and preserved as mentioned above.

Antagonistic Bacteria

Pseudomonas fluorescens (Ps-20) and *Pseudomonas putida* (Ps-21) that have historical antifungal activity (Fatouh, 2018) were used in this study. The two bioagents were used *in vitro* (using the two concentrations, 10^6 and 10^8 cfu/ml) and in field (10^8 cfu/ml) after grown in King's medium B broth (King *et al.*, 1954) for two days at 25°C.

Opus Fungicide (A Positive Control)

The recommended fungicide for managing CLS (opus 12.5% at 1 ml/l, BASF Co.) was used in this study to compare the efficacy of the tested materials for CLS management.

Production of *C. beticola* Mycelia and Spores

For promoting fungal growth and spore production, tomato extract growth medium (TEGM) was used (Marcuzzo *et al*., 2015). A piece of 10-day (d) old *C. beticola* culture (2 x 2 cm) grown on PDA was scrubbed on TEGM by the aid of a sterilized needle and incubated at 25°C for 7 days under cool fluorescent light (12 h/d) and used as a source of spores.

In Vitro Trials

Evaluation of Tested Bioagents against *C. beticola* Growth: Adequate volumes of *U. fasciata* ulvan and *E. flexuosa* polysaccharides and ethanol crude extract were mixed separately with tomato extract growth medium in sterile Petri plates (9 mm) to obtain 1 and 5 mg/ml. Then, a 9 mm disc of 7 d old culture was inoculated in the center of the 7 cm Petri dish and incubated for 30 days at 25°C with 4 plates/ treatment. For *Pseudomonas* evaluation, one ml of the suspension of each concentrate was added to a 7 cm Petri dish containing 12 ml of TEGM and briefly shaken in invert direction to a quite mix. Fungal inoculation, incubation period, and incubation degree as well as replicates were as mentioned above. By the same way, opus fungicide (12.5%) was mixed with TEGM at the rate of 1 ml/l. On the other hand, sterile distilled water (SDW) was used as negative control. When the control treatment dishes were completed by the fungus (after 30 days of incubation), mean of the two perpendicular diameters fungal growth was recorded.

Evaluation of Tested Bioagents against *C. beticola* Spore Germination: Bioagents' activity against spore germination was assessed as reported by Paulert *et al*. (2009). For WE-UF, WE-EF, EE-UF, and EE-EF assay, only 5 mg/ml was used due to the low performance

rate of 1 mg/ml for suppressing fungal growth. Then, 10 ml of each algal extract were added to sporulated *C. beticola* cultures (6 d old TEGM) and briefly agitated to spore lodging. Afterwards, 60 µl of the spore suspension were pipetted in a well of glass slide and incubated for 24 h at RT. Conidial germination was determined under stereomicroscope on twenty conidia per well. Three replicates were used for each treatment. For *Pseudomonas* spp., 10 ml (10^6 and 10^8 cfu/ml) of each of *P. fluorescens* (Ps-20) and *P. putida* (Ps-21) was added to the sporulated 6 d old TEGM cultures and briefly agitated to spore lodging. Thereafter, conidial germination was assessed in the well of glass slide as mentioned above. By the same way, opus fungicide (1 ml/l) was used. Additionally, the incubated fungal conidia in SDW for 24 h at RT served as a control. Generally, if the conidia had germ tubes, they were considered germinated. Also, the percentage of conidial germination (%) as well as efficacy of the tested materials was determined as reported by Gouda and El-Naggar (2014).

Field Trials

Evaluation of the Tested Bioagents against CLS: Randomized complete block field trials were conducted at Sakha (Kafr El-Sheikh) and Faraskur (Damietta) locations that were previously reported for the CLS epidemic (Khalil *et al*., 2007; EL- Fahhar and Abou El-Magd, 2009; Mohamed *et al*., 2019; Gouda *et al*., 2022). The susceptible cultivars Karam and Kawemira were sown at Sakha (September 10, 2018) and Faraskur (September 5, 2018), respectively, in three rows, 6 m long with 50 cm width, and three replicates for each treatment. Ninety days after sowing, each treatment was applied under natural infection. To avoid crossing over among treatments during the spraying process since the leaf canopy of adjacent rows was contacted, sugar beet middle row was used. Sugar beet leaves were sprayed with algal extracts (WE-UF, WE-EF, EE-UF, and EE-EF; 5 mg/ml), *Psuedomonas* (Ps-20 and Ps-21; 10^8 cfu/ml), and opus fungicide (1 ml/l) after the addition of tween 80 (0.05%) as a surfactant. However, distilled water amended with tween 80 was sprayed as negative control. The spray program was applied three times at intervals of 15 days with the aid of Volpi Originale atomizer (DEA, Italy, 2L). One week before harvest, disease severity (DS) (Shane and Teng, 1992) and mean of spots number (SN) in 5 leaves (5L) of three randomly selected plants per replicate were recorded. Also, mean of spot area (SA) was also recorded, after measuring the two perpendicular spot diameters mean, using the formula of area of circle (spot) = πr^2, where $\pi = 22/7$ and r = the half spot diameter.

Yield Component Assay

Roots Weight: Three roots of randomly selected plants per treatment with three replicates of each were weighted (scale of 10 kg capacity, Camry, China) and the mean was calculated.

Sucrose Percent Assay: The same weighted roots used above from each location were collected, labeled (three replicates of each treatment from both locations, Kafr El-Sheikh and Damietta), and sent to sugar manufactory (Delta Sugar Company, El-Hamoul, Kafr El-Sheikh) for sugar component analysis.

Assay of Enzymes Activity: To verify whether foliar application of bioagents was associated with changes of the activity of pathogenic related proteins, peroxidase (Po) and chitinase activities were determined.

- **Collection of Leaf Samples and Enzymes Extraction:** Prior to harvest, leaf samples were collected from three plants selected randomly for each treatment, labeled, maintained in ice box, transported to the laboratory, and then stored at -20°C immediately. Leaf samples were grounded with the aid of mortars and pestles using liquid nitrogen. As reported by Anand *et al.* (2007), one gram of the powder was transferred to a screw cap tube (5 ml capacity) followed by the addition of 2 ml of 0.1 M phosphate buffer (PH 7); then the mixture was homogenized using the vortex for 1 min. The mixture was centrifuged under cooling (4°C) at 4000 rpm for 20 min. After that, 500 µl of the supernatant (crude extract of enzyme (CEE)) were transferred in Eppendorf tubes and all tubes were maintained at -20°C until the assay of enzymes activity.
- **Peroxidase Assay:** According to Hartree (1955), 1.5 ml of pyrogallol solution (0.05M) were mixed with 0.5 ml of H_2O_2 (1%, prepared fresh) in cuvette (2.5 ml). After that, 8 µl of extracted crude enzyme were mixed with the components and the changes in absorbance at 420 nm (Unico 2000 Series spectrophotometer) were recorded at 30 s for 3 min. The blank contained the same component except for the crude enzyme extract (CEE). The enzyme activity was expressed as the change in the absorbance of the reaction mixture (min^{-1} g^{-1}) on a fresh weight as reported by Hammerschmidt *et al.* (1982) and Anand *et al.* (2007).

- **Chitinase Assay:** According to Miller (1959), 500 µl of crude enzyme extract (CEE) were mixed with 500 µl of colloidal chitin (0.5%, prepared fresh) in a glass tube (7.5 x1.2 cm) and incubated at 37°C for 30 minutes. After that, 1 ml of dinitrosalicylic acid solution (DNS) was added to each tube, incubated in boiling water (water bath, Kottermann) for 5 minutes, and cooled in running water, and subsequently 0.5 ml of Rochell salt (40%) was added. From each tube, 2 ml were transferred to a clean plastic cuvette to read the absorbance at 575 nm (Unico 2000 Series spectrophotometer). Three replicates were used for each sample and the average was calculated. Blank reaction mixture contained the same components of reaction mixture except for the CEE. Each treatment was triplicated. N-Acetyl glucosamine (GLcNAc) was used as a standard (Miller, 1959). The enzyme activity was expressed as µM GLcNAc/g fresh weight/min (Anand, 2007).

Statistical Analysis

All data of all experiments were subjected to the analysis of variance (ANOVA) and means were compared by Duncan's multiple range test (p=0.05) using COSTAT package version 6.311.

RESULTS AND DISCUSSION

***In Vitro* Trials**

Activity of Tested Materials against Fungal Growth: Application of the tested materials against *C. beticola* growth generally exhibited high potential activity since all materials means were highly significantly different (0.05%) compared with the control treatment even with the two rates used (Tables 1 and 2, and Figure 1). Water- and ethanol- soluble polysaccharide extracts from *Ulva fasciata* (WE-UF and EE-UF), *P. putida* (Ps-21), and opus fungicide suppressed the fungal growth (10-13 mm) with the same degree of significance and the efficacy ranged from 81.2 to 85.7% (Table 1). On the other hand, water- and ethanol-soluble polysaccharide extracts from *Enteromorpha flexuosa* (WE-EF and EE-EF) as well as *P. fluorescens* (Ps-20) exhibited moderate activity against the fungal growth (25.5-36.6 mm) and the efficacy ranged from 47.7 to 63.6%, respectively. Also, significant differences appeared among the two rates used (Table 2).

Bioagents' Activity against Fungal Spore Germination: Regarding spore germination in glass well-slide (Table 3 and Figure 2), all treatments significantly inhibited *C. beticola* conidial germination. However, the lowest degree of germination (from 5.0 to 8.3%) was recorded with *P. fluorescens* (Ps-20)*, P. putida* (Ps-21), and EE-EF without differences in significance between them and the opus fungicide (spore germination was 3.3%) reflecting the high efficacy for inhibiting this process (from 90.8 to 94.4%). Additionally, the remaining treatments WE-UF, EE-UF, and WE-EF exhibited a good degree for inhibition since spore germination was reduced from 90% (control (SDW)) to 18.3, 13.3, and 10%, respectively.

Field Trials

Effect of Tested Bioagents on Disease Parameters: In Damietta's trial, the disease severity of non-treated plants (control) was 38.3% under natural infection (Table 4). However, foliar application of WE-UF, EE-UF, WE-EF, EE-EF, *P. fluorescens* (Ps-20), P. *putida* (Ps-21), and opus fungicide reduced CLS severity on sugar beet leaves. It ranged from 5 to 9.3% compared with the fungicide and the untreated control (3 and 38.3%, respectively). On the other hand, no significant differences appeared among the activities of the tested bioagents against the disease. Also, the tested bioagents significantly decreased the number of spots (SN) that appeared on sugar beet leaves (which ranged from 57.6 to 130.7 SN/5L) compared with the fungicide and the untreated control (2 and 1773.7 SN/5L, respectively). Similarly, spot area (SA) was also significantly decreased by all tested bioagents since it ranged from 1.0 to 1.9 mm^2 with the tested materials; meanwhile it was 0.1 mm^2 with opus fungicide and reached 11.1 mm^2 with the control. In Kafr El-Sheikh's trial (Table 5), foliar application of WE-UF, EE-UF, WE-EF, EE-EF, *P. fluorescens* (Ps-20), and *P. putida* (Ps-21) significantly decreased SN, SA, and DS from 1399.7 SN/5L, 7.5 mm^2, and 25%, respectively, in untreated control to 53.2-194.8 SN/5L, 1.5-2.7 mm^2, and 1.3-6%, respectively. Meanwhile, these disease parameters were 4.1 SN/5L, 0.7 mm^2, and 0.8%, respectively, with opus fungicide (Table 5).

Impact of Bioagents Spray on Root Weight and Sucrose Percentage: All tested materials increased both root weight and sucrose % when sprayed on the surface of sugar beet leaves in Damietta and Kafr El-Sheikh compared with the control (Tables 4 and 5, respectively). In Damietta, the mean root weight and sucrose % of the plants sprayed with the tested bioagents increased (from 1705.7 to 2000.0 g/root and from 17.5 to 18.4%, respectively) more than those of the untreated control (1355.3 g/root and 14.6%, respectively). On the other hand, root weight

Table 1. Effect of tested bioagents on *C. beticola* growth after 30 days of incubation at 25°C with 12 h photoperiods.

Tested materials	Radial growth[1]	
	Mean (mm)	Efficacy (%)
1- WE-UF[2]	10.0 d	85.7 a
2- EE-UF	13.1 d	81.2 a
3- WE-EF[3]	34.4 b	50.8 c
4- EE- EF	25.5 c	63.6 b
5- P. fluorescens (Ps-20)	36.6 b	47.7 c
6- P. putida (Ps-21)	12.9 d	81.5 a
7- Opus fungicide 12.5%	10.0 d	85.7 a
8- Control (SDW)[4]	70 a	---

[1]Each value represents two concentrations with four replicates each; [2]WE-UF and EE-UF: water- and ethanol-soluble polysaccharides obtained from *U. fasciata*; [3]WE-EF and EE-EF: water- and ethanol-soluble polysaccharides obtained from *E. flexuosa*; [4]SDW: sterile distilled water; values with the same letter in the same column are insignificantly different.

Table 2. Effect of rate of use of tested bioagents on *C. beticola* growth and its efficacy.

Concentration	Radial growth[1]	
	Mean (mm)	Efficacy (%)
Rate-1[2]	30.22 a	64.9 b
Rate-2[3]	22.94 b	76.8 a

[1]Each value represents all materials with four replicates for each one; [2]rate-1 was 1 mg/ml for WE-UF, EE-UF, WE-EF, and EE-EF and 10^6 cfu/ml for Ps-20 and Ps-21; [3]rate-2 was 5 mg/ml for WE-UF, EE-UF, WE-EF, and EE-EF and 10^8 cfu/ml for Ps-20 and Ps-21; values with the same letter in the same column are insignificantly different. Also, significant differences appeared among the two rates used (Table 2).

Table 3. Effect of the tested bioagents on *C. beticola* spore germination and its efficacy.

Tested materials	Used rate	Spore germination[1]	
		(%)	Efficacy (%)
1- WE-UF[2]	5 mg/ml	18.3 b	79.6 d
2- EE-UF		13.3 bc	85.2 cd
3- WE-EF[3]		10.0 cd	89.0 bc
4- EE- EF		8.3 cde	90.8 abc
5-P. fluorescens (Ps-20)	10^8 cfu/ml	5.0 de	94.4 ab
6- P. putida (Ps-21)		6.7 de	92.6 ab
7- Opus fungicide 12.5%	1 ml/l	3.3 e	96.3 a
8- Control (SDW)[4]	---	90.0 a	---

[1]Spore germination was determined under stereomicroscope on twenty conidia per well-slide with three replicates; [2]WE-UF and EE-UF: water- and ethanol-soluble polysaccharides obtained from *U. fasciata*; [3]WE-EF and EE-EF: water- and ethanol-soluble polysaccharides obtained from *E. flexuosa*. [4]SDW: sterile distilled water; values with the same letter in the same column are insignificantly different.

and sucrose % of opus treatment were 1898.0 g/root and 18.6%, respectively. In Kafr El-Sheikh's field trial, the root weight and sucrose % ranged from 613.3 to 734.3 g/root and from 16.6 to 17.5%, respectively, with the bioagent treatments; meanwhile in the control treatment the root weight and sucrose % were 394.3 g/root and 15%, respectively. However, opus treatment exhibited 601.0 g/root and 18.4%, respectively, of these yield parameters (Table 5).

Impact of Bioagents Spray on the Activity of Sugar Beet Defense Enzymes, Peroxidase and Chitinase: Foliar application using EE-EF, *P. fluorescens* (Ps-20), and *P. putida* (Ps-21) significantly increased chitinase activity in sugar beet leaves compared with the control in the two governorates (Table 6). Meanwhile, it varied in significance from location to another when the remaining treatments were sprayed on leaves. In contrast, no significant differences were observed in PO activity between leaves that were sprayed by biagents and the control in the two experimental sites.

Developing any eco-friendly plant protection compound with no negative impacts on the environment and human health is strongly needed nowadays. In this study, foliar application of water-soluble polysaccharides (ulvan) and ethanol extracts obtained from *U. fasciata* and *E. flexuosa* as well as *P. fluorescens* (Ps-20) and *P. putida* (Ps-21) gave high potential activity for inhibiting *C. beticola* mycelial growth and its spore germination along with the recommended fungicide (opus 12.5%) compared with the control.

Also, these bioagents reduced disease parameters (disease severity, spots number, and spot area) when sprayed three times upon sugar beet leaves. Successful protection of these bioagents to sugar beet plants against CLS disease was reflected on yield parameters (root weight and sucrose %); hence it was noticeably increased compared with the control (non-treated plants). As previously known in literature, ulvans are complex hetero polysaccharides which contain rhamnose, xylose, glucose, uronic acid, iduronic acid, and sulphate. These sugars are structurally grouped by two main repeating disaccharides, which are the ulvabiuronic acid types A and B, which serve as elicitors in controlling the disease by inducing tolerance in the plants (Paulert *et al*., 2007; Robic *et al*., 2009). On the other hand, the major polymeric units of the marine algal cell wall (sulfated hetero polysaccharides) act as antioxidant (Lahaye and Robic, 2007; Qi *et al.,* 2005, 2006) and enhance plant resistance against invading pathogens (Stadnik and De Freitas, 2014). Our result consistent with the previous result (El-Sheekh *et al*., 2021) demonstrated that all extracts from *U. fasciata* and *E. flexuosa* had the ability to inhibit *Fusarium solani* mycelial growth. Also, Paulert *et al.* (2009) found the

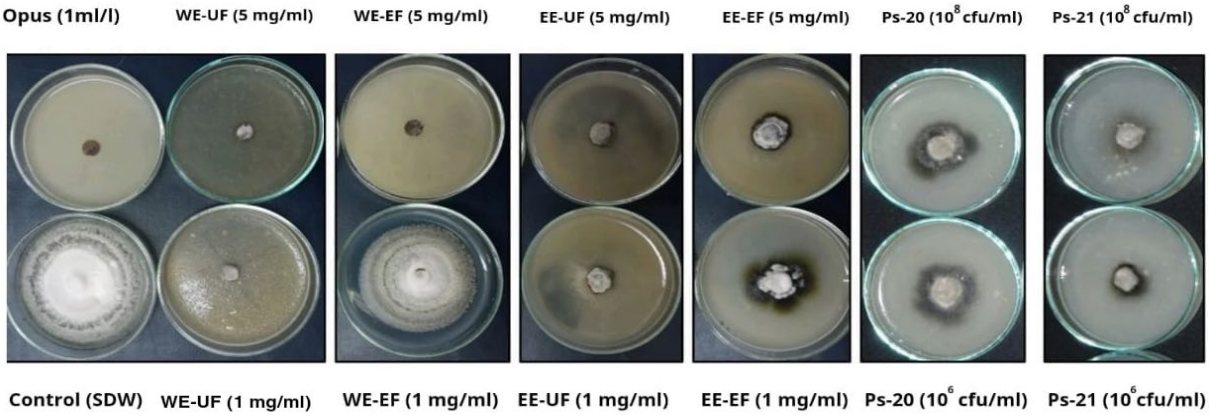

Figure 1. Antifungal activity of water-soluble polysaccharides and ethanol crude extract (1 and 5 mg/ml) obtained from *U. fasciata* (WE-UF and EE-UF), *E. flexuosa* (WE-EF and EE-EF), *P. fluorescens* (Ps-20; 10^6 and 10^8 cfu/ml), and *P. putida* (Ps-21; 10^6 and 10^8 cfu/ml) as well as opus fungicide against *C. beticola* growth compared with the control (SDW).

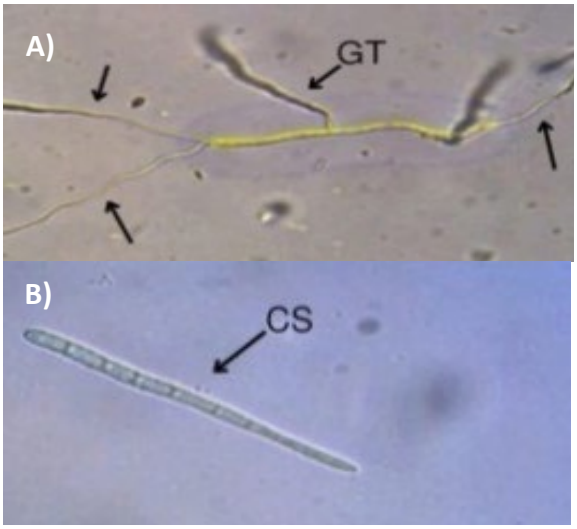

Figure 2. Germinated (A) and non-germinated (B) *C. beticola* spores in glass well-slide; CS: conidial spore and GT: germ tube.

inhibition effect of *Ulva fasciata* methanolic extract on the mycelial growth of *Colletotrichum lindemuthianum in vitro.* In contrast, De Borba *et al*. (2021) did not find any suppressive effect of *Ulva fasciata* ulvan on *Zymoseptoria tritici* spore germination and hyphal growth under *in vitro* and *in vivo* assays. Also, indirect antifungal effect of ulvan or green algae extract has been reported toward pathogens such as fungi, yeast, and bacteria (Paulert *et al*., 2007, 2009; De Freitas and Stadnik, 2012). In our study, three consecutive applications of ulvan and ethanol crude extract from the two macroalgae remarkably reduced CLS disease severity. This result was consistent with the previous result of Cluzet *et al.* (2004). They reported that two consecutive treatments of *U. armoricana* ulvan triggered a nearly complete protection of *Medicago truncatula* plants against *Colletotrichum trifolii*. In our study, *P. fluorescence* and *P. putida* also provided high efficacy for suppression of fungal growth and spore germination. On the other hand, foliar application by these bioagents approximately fully protected sugar beet leaves from CLS symptoms without significant differences between them and the opus fungicide and subsequently the yield was increased. As previously reported, fluorescent *Pseudomonas* spp. (non-pathogenic spp.) belong to the plant-growth-promoting rhizobacteria (Wu *et al*., 2020), are rapid root colonizers resulting in improving plant growth and yield, and in addition protect plant against pathogenic microorganisms (Lemanceau, 1992; Walsh *et al*., 2001; Bolwerk *et al.*, 2003; Haas and Keel, 2003; El Housni *et al*., 2024). Also, they act on plant pathogens by production of a various group of metabolites such as antibiotics and volatile compounds like hydrogen cyanide (Liu *et al.*, 1995; Thomashow and Weller, 1996; Raio, 2024), competition for nutrients specially iron by production of siderophore (Weller, 1998), and induction of systemic resistance (Chen *et al.*, 2000; Ryu *et al.*, 2004; Singh *et al*., 2013). On the other hand, *β*-1,3 glucanase, chitinase, cellulase, cyclic lipopeptide, and indole acetic acid were reported to be secreted by these bioagents (Kiewnick and Jacobsen, 1998; Gottschalk *et al.*, 1998; Karnwal, 2009; Sarhan and Shehata, 2014; El-Assiuty *et al*., 2016; Prasad *et al.*, 2017). One or more of these mechanisms may be the reason for successful activity of these biocontrol agents against CLS disease in our study. The obtained results corroborate previous reports in the fact that fluorescent *Pseudomonas* had potential activity against either *C. beticola* (Poornima *et al.,* 2011; Kiewnick and Jacobsen, 1998.

Table 4. Influence of foliar application of tested bioagents on spots number, spot area, and disease severity caused by *C. beticola* as well as root weight and sucrose % in Damietta, 2018-2019 Season.

Tested materials	Used rate	Disease severity %	Spots number/5 leaves	Spot area (mm^2)	Root weight (gm)	Sucrose %
1- WE-UF[1]	5 mg/ml	9.3 cb	111.8 b	1.9 b	1705.7 a	18.0 a
2- EE-UF		6.7 cb	104.4 b	1.8 b	1722.0 a	18.4 a
3- WE-EF[2]		9.3 b	130.7 b	1.3 b	1716.7 a	17.5 a
4- EE- EF		5.0 cb	58.1 b	1.2 b	2000.0 a	18.4 a
5- P. fluorescens (Ps-20)	10^8 cfu/ml	9.0 b	57.6 b	1.0 b	1700.0 a	17.9 a
6- P. putida (Ps-21)		8.0 cb	63.5 b	1.5 b	1778.0 a	17.1 a
7- Opus fungicide 12.5%	1 ml/l	3.0 c	2.0 b	0.1 b	1898.0 a	18.6 a
8- Control (SDW) [3]	---	38.3 a	1773.7 a	11.1 a	1355.3 b	14.6 b

[1]WE-UF and EE-UF: water- and ethanol-soluble polysaccharides obtained from *U. fasciata*; [2]WE-EF and EE-EF: water- and ethanol-soluble polysaccharides obtained from *E. flexuosa*. [3]SDW: sterile distilled water; values with the same letter in the same column are insignificantly different.

Table 5. Influence of foliar application of tested bioagents on spots number, spot area, and disease severity caused by *C. beticola* as well as root weight and sucrose% in Kafr-El-Sheikh, 2018-2019 Season.

Tested materials	Used rate	Disease severity %	Spots number/5 leaves	Spot area (mm^2)	Root weight (gm)	Sucrose %
1- WE-UF[1]	5 mg/ml	2. 7 bc	57.8 b	2.2 b	615.7 a	17. 5 b
2- EE-UF		4.0 bc	121.1 b	2.3 b	620.7 a	16.6 b
3- WE-EF[2]		4.3 bc	139.4 b	2. 7 b	613.3 a	17.1 b
4- EE-EF		6.0 b	194.8 b	2.1 b	734.3 a	17.2 b
5- P. fluorescens (Ps-20)	10^8 cfu/ml	1.3 bc	53.2 b	1.5 bc	703.3 a	17.1 b
6- P. putida (Ps-21)		4.3 bc	85.9 b	1.5 bc	713.3 a	17. 0 b
7- Opus fungicide 12.5%	1 ml/l	0.8 c	4.1 b	0.7 c	601.0 a	18.4 a
8- Control (SDW)[3]	---	25.0 a	1399.7 a	7.5 a	394.3 b	15.0 c

[1]WE-UF and EE-UF: water- and ethanol-soluble polysaccharides obtained from *U. fasciata*; [2]WE-EF and EE-EF: water- and ethanol-soluble polysaccharides obtained from *E. flexuosa*; [3]SDW: sterile distilled water; values with the same letter in the same column are insignificantly different.

Table 6. Influence of foliar application of tested bioagents on peroxidase and chitinase activity in sugar beet leaves at field in Damietta and Kafr El-Sheikh, 2018-2019 Season.

Tested materials	Used rate	Damietta		Kafr EL-Sheikh	
		Peroxidase	Chitinase	Peroxidase	Chitinase
1- WE-UF[1]	5 mg/ml	0.00082 a	0.0751 bc	0.0017 a	0.0915 ab
2- EE-UF		0.00062 a	0.1021 abc	0.0011 a	0.0819 ab
3- WE-EF[2]		0.00068 a	0.1288 a	0.0019 a	0.0905 ab
4- EE- EF		0.00116 a	0.1095 a	0.0014 a	0.0960 a
5- P. fluorescens (Ps-20)	10^8 cfu/ml	0.00048 a	0.1074 ab	0.0013 a	0.1024 a
6- P. putida (Ps-21)		0.00069 a	0.1108 a	0.0013 a	0.1007 a
7- Opus fungicide 12.5%	1 ml/l	0.00064 a	0.0961 abc	0.0011 a	0.0835 ab
8- Control (SDW) [3]	---	0.00052 a	0.0712 c	0.0015 a	0.0694 b

[1]WE-UF and EE-UF: water- and ethanol-soluble polysaccharides obtained from *U. fasciata*; [2]WE-EF and EE-EF: water- and ethanol-soluble polysaccharides obtained from *E. flexuosa*; [3]SDW: sterile distilled water; values with the same letter in the same column are insignificantly different.

Sarhan, 2018; El Housni *et al*., 2024) or other causal agents of diseases such as Fusarium wilt in cucumber (Liu *et al.*, 1995), Fusarium root rot of sugar beet (El-Assiuty *et al.,* 2016), damping-off in alfalfa (Sarhan and Shehata, 2014), downy mildew of pearl millet (Umesha *et al.,* 1998), or other fungi (Mawaddah *et al.,* 2023). Plant defense related enzymes, such as PO and chitinase, have been reported to play important roles in the defense mechanism against pathogens directly by inhibiting its development or indirectly by increasing antimicrobial activity that reduces the disease severity (Eckardt, 2004; Cao *et al.*, 2006; Lavania *et al.*, 2006; Ralph *et al.*, 2006; Tian *et al.*, 2006). In our study, we found that P. fluorescence, P. putida and the ethanol crude extract of green macroalga E. flexuosa (EE-EF) enhance the plant to secrete the defense enzyme chitinase. On the other hand, none of the tested bioagents increased the level

of peroxidase activity. Generally, these results indicate that foliar application by *P. fluorescence*, *P. putida*, and the crude ethanol extract (EE-EF) may elicit sugar beet plants to increase chitinase activity as a mechanism from multiples as reported above for enhancing plant resistance. On the other hand, the rest of the tested materials did not elicit that peroxidase enzyme may affect by a different mechanism for reducing the disease (Qi *et al.,* 2005, 2006; Lahaye and Robic, 2007; Robic *et al*., 2009). *P. fluorescens* was previously reported to afford resistance to plants due to increasing the levels of PO activity (Van Peer and Shipper, 1992; Saravanan *et al.,* 2004). In this study the insignificance in PO activity levels among all treatments and the control may be due to the long duration period between the application of treatments and the time of taking samples for enzyme assay (Van Peer and Shipper, 1992; Saravanan *et al.*, 2004).

CONCLUSION

Cercospora leaf spot disease caused by *C. beticola* is a serious disease that can cause huge losses. In Egypt, chemical fungicides remain a main method for controlling this disease since there is no breeding program for disease resistance. Unfortunately, excessive use of these fungicides could lead to many other problems such as fungal resistant development, environmental pollution, and harm to useful organisms and humans. So, looking for safe products' alternatives to CLS synthetic fungicides on sugar beet is needed for safer sugar production. In this study we used water and ethanol extracts obtained from *Ulva fasciata* (WE-UF and EE-UF, respectively) and *Enteromorpha flexuosa* (WE-EF and EE-EF, respectively) along with *P. fluorescens* (Ps-20) and *P. putida* (Ps-21) for managing CLS disease. Generally, these eco-friendly bioagents could protect sugar beet plants from CLS causal agent and to increase sugar beet yield (roots weight and sucrose %). Additionally, spraying sugar beet leaves by EE-EF, Ps-20 and Ps-21 enhanced the plants to increase their secretion from chitinase. Accordingly, foliar application of these bioagents is useful for decreasing both the disease and the use of environmentally polluting chemical fungicides. Additionally, to maximize the obtained benefit from these bioagents further studies including other diseases are needed.

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