

Antagonistic Potential of Some Bacterial Strains against *Xanthomonas campestris*, the Cause of Bacterial Blight in *Hordeum vulgare*

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Bacterial blight disease due to *Xanthomonas campestris* pv. *translucens* results in yield losses in barley, *Hordeum vulgare* L., especially in warm climates. Bio-based bactericides represent a safe alternative to harmful chemicals for controlling a wide range of phytopathogens. The bacterial strains (*Brevibacterium linens*, *Bacillus subtilis*, *B. thuringiensis*) were tested as antagonistic potential against *X. campestris* disease in barley seedlings. Antagonists were applied as seed biopriming and soil drench in *X. campestris* infested soil. Soil-drenching treatment was more efficient than the biopriming application. A significant increase in shoot length with a clear decrease in seed germination was recorded. Fresh and dry weights of shoot and root lengths of the treated plants were markedly improved. The remarkable antagonistic activity of *B. linens*, *B. subtilis*, and *B. thuringiensis* against *X. campestris* could be attributed to the capability to produce bioactive molecules that can trigger systemic resistance in the infected seedlings.

Keywords: Bacteria; *Hordeum vulgare* L.; Antagonistic; *X. campestris*; Antioxidant metabolites

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INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the principal cereal crops all over the world, and considered the fourth important cereal crop in terms of quantity produced and global area of cultivation (Fao-Stat 2009). For many years *Hordeum vulgare* L. has been of great economic importance, as approximately 57 million tons of barley were produced annually in the European Union, while global production reached 147 million tons annually (Edyta *et al.* 2019). In Egypt, barley is one of the most important seed crops. It is widely grown in the rain-fed areas of the north coastal region and in the newly reclaimed lands with saline soils. In addition, a little is grown irrigated in the Nile valley. It is mainly used as animal feed, though recently it has also been used as human food because of its nutritional and health properties (Hendawey *et al.* 2014). In Saudi Arabia *Hordeum vulgare* L is cultivated in specific regions suitable for growing this crop and used as human or animal food.

Bacterial blight is a disease of barley caused by the bacterial pathogen *X. campestris* pv. *translucens*. The disease affects many countries (Martens *et al.* 1984; Mathre 1997). The disease is characterized by small pale green spots or streaks on the leaves, which soon appear water-soaked. The lesions expand and then appear as dry dead spots (Harman 1991). The lesions elongate into linear streaks, which may eventually extend the full length of the leaf. In severe infections a milky gray exudate may ooze from the cut end of the leaf exhibiting symptoms.

Bacterial blight disease results in great economic losses. Control of bacterial blight disease is mainly by sowing healthy seeds; hence, it is a seed-borne disease. However, there are other control methods, such as rotations, use of resistant varieties, and applications of balanced levels of nitrogen. Moreover, the use of bactericides and fungicides as seed treatment can be effective in controlling the disease, but their toxic residues are considered a risk factor (Andersson *et al.* 2014).

Biological control is the purposeful utilization of introduced or resident living organisms to suppress the activities and populations of one or more plant pathogens (Meena *et al.* 2012). This approach can be an eco-friendly and effective component of an integrated disease management program (Mao *et al.* 1997). Although diverse bacterial genera are antagonistic to phytopathogens, maximum attention has been focused on the gram-positive genus *Bacillus* (Hiraoka *et al.* 1992). *Bacillus* spp. in particular are gaining recognition as safe biocontrol agents in a variety of crops, specifically as seed protectants and antifungal agents (Acea *et al.* 1988). Moreover, they are spore-formers, which imparts a natural formulation advantage over other microorganisms (Ahmad and Kibret 2004; Abuamsha *et al.* 2011).

Members of this genus have successfully controlled plant diseases in a wide variety of crops, including rice, wheat, and potato (Ashraf and Foolad 2005). Earlier works on biocontrol (Papavizas and Lewis 1989) reported the antagonistic potential of *Paenibacillus* in suppressing gray mold (caused by *B. cinerea*) in strawberry plants. The *Brevibacillus laterosporus* strain suppressed blast disease of rice caused by *Rhizoctonia oryzae* by 30-67% and decreased the weight loss by 35 to 56.5% in the greenhouse; maximum disease protection (67%) and weight loss (56.5%) were recorded when the bacterium was applied 2 days before the pathogen inoculation (Chang and Kommedahl 1968; Lumsden *et al.* 1995).

The population dynamics of *Xanthomonas campestris* pv. *vitiensis* was studied both externally and internally in lettuce, tomato, and pepper plants. The application of bactericides during transplant production period was studied for the management of bacterial leaf spot of lettuce under greenhouse conditions (Al-Saleh *et al.* 2011). Epiphytic populations of *Xanthomonas citri* were found on symptomless grapefruit and mandarin fruits for as long as six and five days of incubation under laboratory and orchard conditions in Saudi Arabia, respectively. No differences were noticed between populations of the bacteria in the susceptible cultivar of grapefruit and the moderately susceptible cultivar of mandarin under both conditions (Al-Saleh and Ibrahim 2010).

This study aimed to evaluate the antagonistic potentiality of the bacterial strains *Brevibacterium linens*, *Bacillus subtilis*, and *Bacillus thuringiensis* for the suppression of bacterial blight disease of barley caused by *Xanthomonas campestris*.

EXPERIMENTAL

The bacterial strain of *Xanthomonas campestris* was isolated from barley (*Hordeum vulgare* L.) leaves showing the bacterial blight disease in the Jazan region in the southwest of Saudi Arabia, where the humidity and temperature was suitable for disease development. The antagonistic bacterial strains were *Brevibacterium linens*, *Bacillus subtilis*, and *Bacillus thuringiensis* and were isolated from the soil of apparently healthy barley plant rhizosphere. A sample of 10 g of soil was suspended in 100 mL of sterile physiological water and shaken vigorously at 28 °C for 30 min. Serial dilutions were softened over the

feed nutrient agar medium, and each dilution was incubated at 30 °C until colonies were observed. Purified colonies were grown on nutrient agar plates and identified according to Sun *et al.* (2011). Cultures were routinely grown aerobically on nutrient broth medium using a shaking incubator at 30 °C for 24 h (Salaheddin 2002). Proper aseptic techniques and standard laboratory operation procedures were followed during the study with these microorganisms (Lumsden *et al.* 1995; Mathre *et al.* 1999).

Plant materials, treatment and growth parameters.

Greenhouse studies were conducted during the season of October 2018 under greenhouse conditions at the College of Science, Jazan University (KSA). Cultivated barley grains were surface-disinfected by immersion in sodium hypochlorite (2.5%) for 2 min, then rinsed three times with sterilized deionized water. Surface-disinfected seeds were cultivated in 15-cm-diameter pots with 10 seeds. Triplicates of each treatment were performed, and all pots were kept in the dark at 18 °C ± 20 °C for 9 d. The treatments of barley grains were applied according to the seed biopriming technique, which employs bioagents to give protection through induced resistance against biological stress (Murunde and Wainwright 2018) and soil drench treatments, to ensure the application of any compound in a liquid form near the roots in the soil to absorb by the root. Such as with other methods of injection, it must be soluble in water, as shown in Table 1. The percentage of germinated seeds, length of seedlings, and fresh and dry weight of seedlings were determined according to Radwan *et al.* (2016). Seed germination was calculated when the radicle was 2 mm long. The germination percentage was determined by counting the number of germinated seeds relative to ungerminated ones as affected by different seed biopriming and/or soil drenching treatments relative to control.

Table 1. Treatments of Barley Grains with Antagonistic Bacteria and Pathogen

C1-2	Control in absence and in presence of <i>X. campestris</i>
B1-3	Grain biopriming with <i>Brevibacterium linens</i> , <i>B. subtilis</i> , <i>B. thuringiensis</i>
S1-3	Soil drench with <i>B. linens</i> , <i>B. subtilis</i> , <i>B. thuringiensis</i>
B4-6	Seed biopriming with <i>B. linens</i> , <i>B. subtilis</i> , <i>B. thuringiensis</i> in presence of <i>X. campestris</i>
S4-6	Soil drench with <i>B. linens</i> , <i>B. subtilis</i> , <i>B. thuringiensis</i> in presence of <i>X. campestris</i>

Antioxidant metabolites assay

Non-enzymatic antioxidant metabolites were analyzed in the barley seedlings exposed to the phytopathogens and plant oils. Total phenolic content of seed powder extracts was determined using Folin-Ciocalteu reagents (Singleton and Rossi 1965). Gallic acid standard solution (2.0 mg/mL) was prepared with distilled water. The solution was then diluted to working standard solutions of 1.5 mg/mL, 1.0 mg/mL, 0.5 mg/mL, 0.2 mg/mL, and 0.1 mg/mL. Forty µL of extract (in 80% methanol) or gallic acid standard was mixed with 1.8 mL of Folin–Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at room temperature for 5 min. Then, 1.2 mL of sodium bicarbonate (7.5% w/v) was added to the mixture. After standing 60 min at room temperature, absorbance was measured at 765 nm. Results are expressed as (µgCE / g DW). Total flavonoid content was measured in the methanolic extracts of powdered seeds according to the method of Dewanto *et al.* (2002). The methanolic extracts were diluted and mixed with 75 µL of NaNO₂ (5%). After 6 min, 150 µL of 10% AlCl₃ and 500 µL of

NaOH (1 M) were added to the mixture. Finally, the mixture was adjusted to 2.5 mL with distilled water. The absorbance *versus* prepared blank was read at 510 nm. Total flavonoid contents of seed extracts were expressed as μg of catechin equivalents per g of dry weight (mg CE/gDW) through the calibration curve with catechin. The levels of reduced glutathione (GSH) were estimated by the method proposed by Moron *et al.* (1979). Reduced glutathione was measured by its reaction with 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) to yield a yellow product that absorbs at 412 nm. The values are expressed as mol of GSH / g of tissue (mol GSH / g tissue).

Statistical analysis

The presented data are the mean values of at least three replicates. Standard errors were calculated for all values using M. Excel 2007 (Microsoft Corp. Redmond, WA, USA).

RESULTS AND DISCUSSION

Figure 1 shows the impact of different concentration on the germination percentage of barley grains. The bacterial strains *X. campestris* and *B. linens* distinctly inhibit grain germination, while *B. subtilis* and *B. thuringiensis* had either a positive or a neutral impact. In general, an increase in bacterial concentration led to a decrease in germination rate. The maximum percentages of germination (30.4% and 98.9%) were recorded at 5% bacterial concentration for *B. subtilis* (30.4%) and *B. thuringiensis* (98.9%), compared with the control treatment.

Figure 2 shows a clear increase of shoot length in barley plants exposed to the antagonistic bacteria as seed biopriming or soil drench treatments. Generally, the latter was more efficient than seed biopriming compared with the control. For the soil drench treatment, the maximum increase of shoot length was observed with *B. subtilis* and *B. thuringiensis* (26.9% and 18.6%) in the absence of *X. campestris*. Meanwhile, in the presence of the pathogen, the greatest values (23.0% and 15.2%) were recorded by the same strains. For the seed biopriming treatment, the maximum increases of shoot length were observed with *B. subtilis* (5.4%) in the absence of *X. campestris* and *B. thuringiensis* (6.9%) in the presence of the phytopathogen. Figure 2 also shows a clear increase of root length in the barley plants treated with bacteria. For the soil drench treatment, the maximum increases of root length were recorded with *B. subtilis*: 43.5 cm in the absence of *X. campestris* and 25.8 cm in the presence of the phytopathogen. For the seed biopriming treatment, the maximum increase of root length was observed with *B. subtilis*: 25.0% in the absence of *X. campestris*. Interestingly, the barley seedlings treated with *B. linens* demonstrated sharp decreases in root length, ranging from 16.1% to 54.0%, with all treatments.

Figure 2 shows distinct improvements of fresh and dry weights of the barley plants treated with bacteria as seed biopriming or soil drench. Generally, soil drench treatment was more efficient than the seed biopriming. Soil drenched with *B. subtilis* and *B. thuringiensis* showed increases of fresh weight in the absence of *X. campestris* (100.8% and 51.9%) and in the presence of the pathogen (54.9% and 35.1%). Similarly, seed biopriming with *B. subtilis* and *B. thuringiensis* showed clear improvements of fresh weight in the absence of *X. campestris* (48.2% and 23.8%) and in the presence of the phytopathogen (14.3% and 13.9%).

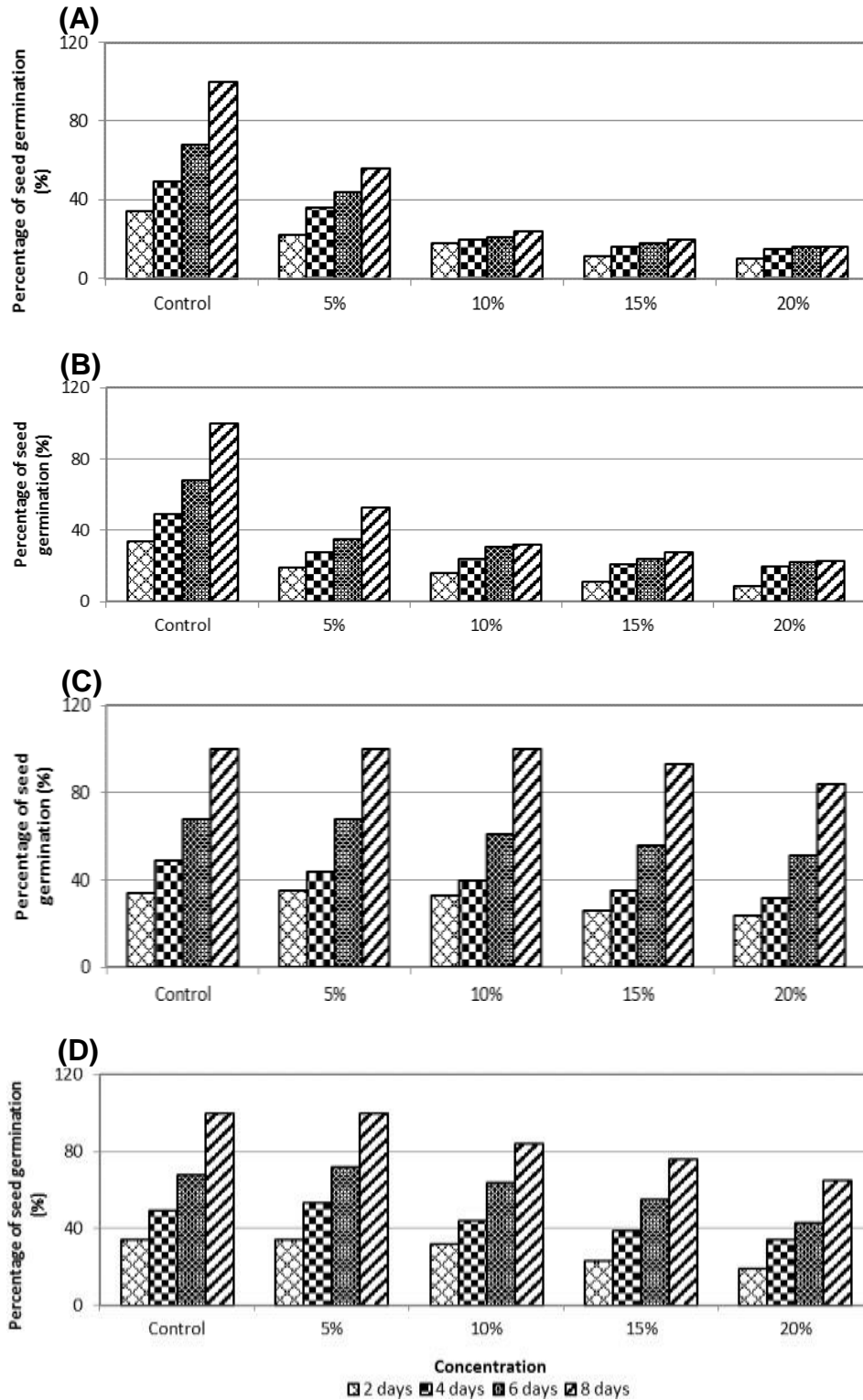


Fig. 1. Effect of different concentration on seed germination of *Hordeum vulgare* (A) *Xanthomonas campestris*, (B) *Brevibacterium linens*, (C) *Bacillus subtilis*, and (D) *Bacillus thuringiensis*

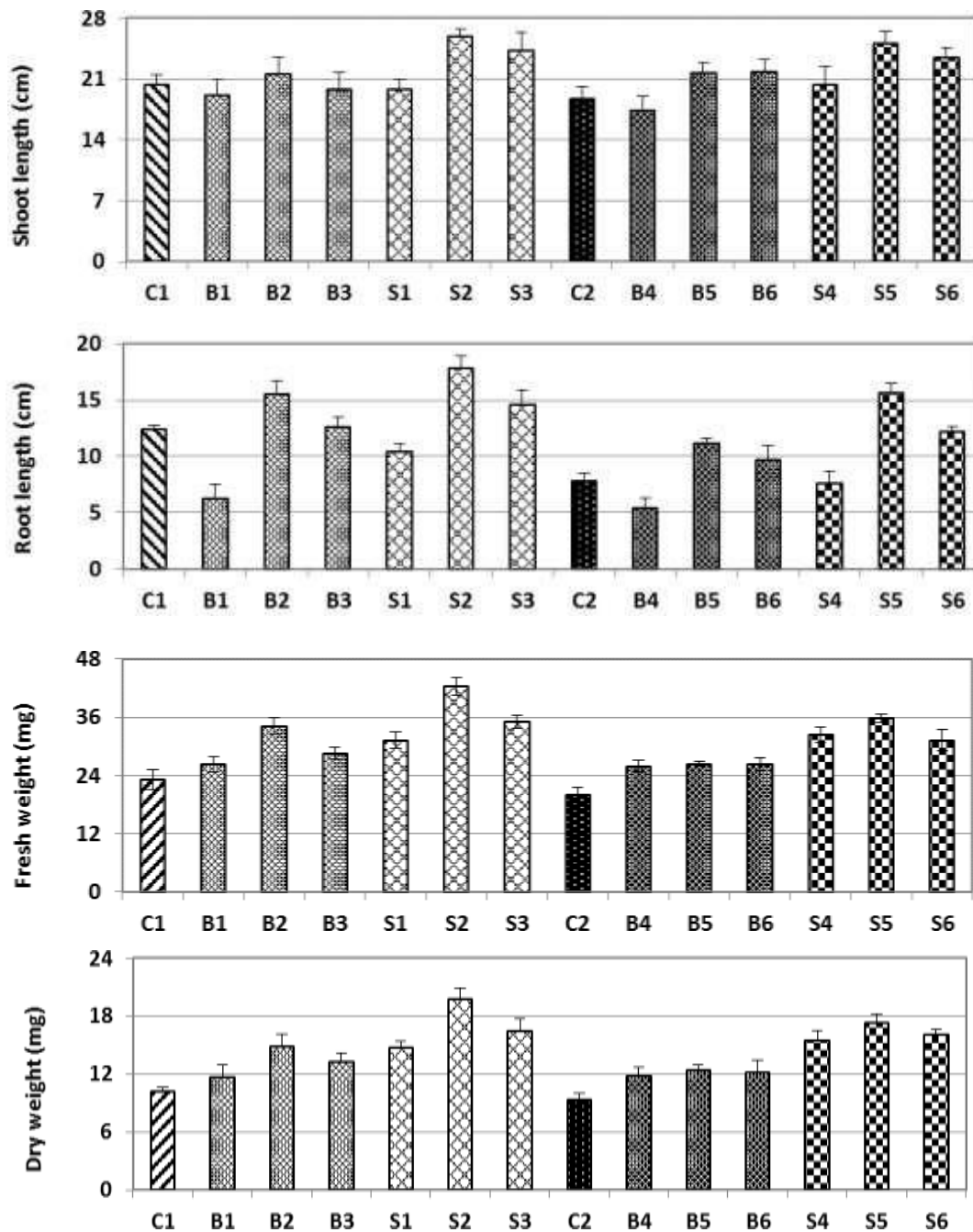


Fig. 2. Effect of seed biopriming and soil drench treatments on germination indices of *Hordeum vulgare*: (C1 - C2) control in absence and in presence of *Xanthomonas campestris*; (B1 -B3) seed biopriming with *Brevibacterium linens*, *Bacillus subtilis*, and *Bacillus thuringiensis*; (S1 - S3) soil drench with *B. linens*, *B. subtilis*, *B. thuringiensis*; (B4 - B6) seed biopriming with *B. linens*, *B. subtilis*, and *B. thuringiensis* in presence of *X. campestris*; (S4 - S6) soil drench with *B. linens*, *B. subtilis*, *B. thuringiensis* in presence of *X. campestris*

Table 2 shows an increase of total phenolics in barley plants treated with the antagonistic bacterial strains at various degrees in non-infested or infested soil. The maximum total phenolic levels were recorded in plants treated with *B. subtilis* (161.4%) followed by *B. thuringiensis* (149.1%) as soil drench in the presence of the phytopathogen

X. campestris, compared with the corresponding control. Similarly, total flavonoids increased in plants under the stress of *X. campestris*. Moreover, there was a remarkable elevation of flavonoids with the antagonistic strains in barley plants in infested and non-infested soil. The level of flavonoids was recorded in plants treated with *B. subtilis* (296.9%) as soil drench compared with the corresponding control. Table 2 also shows the levels of reduced glutathione, which was markedly increased in barley plants exposed to *X. campestris*. Furthermore, in absence of the phytopathogen, plants treated with the antagonistic bacteria as soil drench or seed biopriming demonstrated highly significant increases of reduced glutathione, ranging from 11.1% to 77.6% compared with the control treatment. For plants treated with antagonistic bacteria in the presence of *X. campestris*, glutathione decreased in both treatments. The greatest increase (92.5%) was recorded in plants treated with *B. thuringiensis* as soil drenching.

Flavonoids are plant secondary compounds that have antioxidant activity dependent on the presence of free OH groups, especially 3-OH. Plant flavonoid that has antioxidant activity in the laboratory acts as an *in vivo* antioxidant (Geetha *et al.* 2003). The high phenolic and flavonoid content is responsible for the vital activity of these raw extracts. Flavonoids are very effective for most oxidizing molecules, including individual oxygen and various other free radicals involved in many diseases (Bravo *et al.* 1998). Flavonoids suppress the formation of reactive oxygen, the trace elements of flavonoids involved in the production of free radicals, scraping reactive species and regulating and protecting antioxidant defenses (Robene *et al.* 2006; Agati *et al.* 2012). Likewise, phenols give oxidative stress to plants. Raw extracts of fruits, herbs, vegetables, grains and other vegetable materials rich in flavonoids are increasingly being used in the food industry for their antioxidant properties and health benefits. These results are somewhat in agreement with the present results in applying seed biopriming and soil drench treatments.

Table 2. Effect of Seed Biopriming and Soil Drench Treatments with *B. linens*, *B. subtilis*, and *B. thuringiensis* on Total Phenolics, Flavonoids, and Reduced Glutathione of *Hordeum vulgare* in Absence and in Presence of *X. campestris*

Treatments		Total phenolic	Total flavonoids µg CE / g DW	Reduced glutathione
Seedlings without bacteria		0.57 ± 0.05	0.33 ± 0.06	3.61 ± 0.9
Non-infested soil	Biopriming	<i>B. linens</i>	0.93** ± 0.03	0.54 ± 0.07
		<i>B. subtilis</i>	1.01** ± 0.12	0.66** ± 0.04
		<i>B. thuringiensis</i>	1.07** ± 0.07	0.69** ± 0.11
	Soil drench	<i>B. linens</i>	1.02** ± 0.08	0.72* ± 0.06
		<i>B. subtilis</i>	1.26** ± 0.03	0.94** ± 0.09
		<i>B. thuringiensis</i>	1.32** ± 0.04	0.95** ± 0.09
Seedlings in presence of <i>X. campestris</i>		0.71 ± 0.09	0.48 ± 0.08	4.98* ± 0.4
Infested soil	Biopriming	<i>B. linens</i>	1.34** ± 0.09	0.79** ± 0.07
		<i>B. subtilis</i>	1.17** ± 0.11	0.81** ± 0.06
		<i>B. thuringiensis</i>	1.16** ± 0.09	0.78** ± 0.08
	Soil drench	<i>B. linens</i>	1.41** ± 0.08	1.25** ± 0.04
		<i>B. subtilis</i>	1.49** ± 0.05	1.31** ± 0.08
		<i>B. thuringiensis</i>	1.42** ± 0.12	1.29** ± 0.06

** Indicate levels of statistical significance between different treatments.

Glutathione tri-peptide (GSH) is the most abundant non-protein thiol within the cells, and it participates in many cellular functions including the temporary storage of

homogeneous reductase (Awasthi *et al.* 2009). Cellular radiation sensitivity has been shown to be inversely related to the internal level of GSH. On the other hand, controversy has arisen regarding its role in radiation protection because GSH has failed to provide consistent protection in many cases. Reports have been published that the DNA repair in cells is based on GSH. Subsequently, S-glutathionylation (disulfide formation mixed with protein-sulfidrel groups), provides an effective post-translation regulating mechanism for a variety of regulatory and metabolic proteins when there is a change in the state of cellular oxidation (Pineda-Molina *et al.* 2001; Sparaco *et al.* 2009).

Biological control of crop diseases can take advantage of conflicting microorganisms; this is one of the most effective alternatives to synthetic chemical pesticides for use in the future. Among the aggressive bacteria, several types of *B. linens*, *B. subtilis*, and *B. thuringiensis* have been successfully used for the biological control of plant diseases (Vidhyasekaran and Muthamilan 1999; Salaeddin 2002; Stoyanova *et al.* 2014). In general, the increase of bacterial concentration decreased the germination compared with the control treatment. Such results has been reported by many authors (Zhang *et al.* 2001). Salah eddin *et al.* (2005) reported that a simple inoculation technique for evaluation of cotton genotypes for resistance to bacterial blight caused by *X. axonopodis* pv. *malvacearum* can be employed by using biological control. Meanwhile, it must be examined which kinds of bacteria can produce any mycotoxins that affect the plants or are hazards to human health (Sanjay and Parashar 2002; Zohurul Islam *et al.* 2003; Zaidi *et al.* 2006; Mariya *et al.* 2014). This study demonstrated significant increases of total phenolics in barley plants treated with the antagonistic bacterial strains at various degrees in non-infested or infested soil. The maximum total of phenolic levels were recorded in plants treated with *B. subtilis* followed by *B. thuringiensis* as soil drench in the presence of the phytopathogen *X. campestris*, compared with the corresponding control. Similarly, the results indicated increases of total flavonoids in plants under the stress of *X. campestris*. Moreover, there was a remarkable elevation of flavonoids due to the antagonistic strains in barley plants in infested and non-infested soils, and these results agree with the results of Salaheddin (2002), Salaheddin *et al.* (2005), and Zhang *et al.* (2001). The effectiveness of biological control achieved by the biocontrol agents of the present study showed distinctive control mechanisms that may be due to additional or synergistic effects (Zohurul *et al.* 2003; Robe`ne-Soustrade *et al.* 2006; Salaheddin *et al.* 2010; Tolba 2017). The capability to produce bioactive molecules by this species of antagonistic microorganisms can trigger systemic resistance in the infected *Hordeum vulgare* seedlings and may be applicable than using the whole organism in future. Also, the identification for this kind of bioactive molecules by chemists in future research work (Sanjay *et al.* 2002; Abuamsha *et al.* 2011; Sun *et al.* 2011).

CONCLUSIONS

1. Application of bio-antagonistic organisms provides a safe way to protect a crop while avoiding risks to the environment and human beings all over the world.
2. Biological control of crop diseases by means of antagonistic microorganisms is one of the most effective and safe alternatives to toxic synthetic chemical pesticides.

3. The present study showed distinctive control mechanisms due to the additional or synergistic effects of microorganisms.
4. Application of beneficial bacteria to the soil or as seed biopriming can be regarded as safe to the environment and human health.
5. The present study indicates that biopriming can be used effectively. It gives enough numbers of bacteria in seeds and controls the phytopathogen (*X. campestris*).
6. Biocontrol can be considered as an eco-friendly and effective method of an integrated disease pest management program.
7. Unguided application of antagonistic biocontrol may lead to ecological problems.

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Conflicts of Interest

The authors declare no conflict of interest regarding the work of this manuscript.

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