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Genetic improvement of *Pseudomonas aeruginosa* and *Bacillus cereus* for controlling root knot nematode and two weeds under laboratory conditions.

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Abstract

Plant parasitic nematodes and weeds are among the many biotic stresses that crops production suffers from during their growing season. Their management relies mainly on chemical pesticides. To decrease the extent of environment degradation and hazards to human health and livestock due to the prolonged use of these chemicals, biological control using soil microorganisms is considered as a new ecofriendly and efficient control method. The main objective of this work was to improve the inhibition of two local bacterial strains, *Pseudomonas aeruginosa* and *Bacillus cereus*, against root knot nematode and weed seeds germination. To achieve this goal, protoplast fusion experiments were performed to gather all their properties in bacterial fusants and increase production of such toxic compounds. The results showed that *P. aeruginosa* was Rifampicin (Rif) resistance, but *B. cereus* was sensitive. In contrast to this *B. cereus* was Neomycin (Nm) resistance but *P. aeruginosa* was sensitive. A total of 40 fusants derived from the protoplast fusion experiments were selected by antibiotic resistance markers. SDS-PAGE analysis of the proteins confirmed that six recombinants acquired and expressed many specific protein bands from their parental strains. Three fusants, No. F7, F20 and F35, were selected and evaluated for their nematicidal potential in comparison with their parent against root knot nematode *Meloidogyne incognita* J_2 and *Echinochloa crus-galli* and *Portulaca oleracea* seeds germination during *in vitro* experiments. Data showed that the fusants exhibited more antagonistic effects than their parents. After 72hrs of exposure, the three fusants caused 80.6, 96.5 and 97.7% mortality as compared to control, while the % mortality after the same duration by *P. aeruginosa*, *B. cereus* singly and combined resulted in 52.2, 65.9 and 48.8%, respectively as compared to control. Furthermore, the three fusants completely inhibited the germination of *P. oleracea seeds* and resulted in small radicals *in E. crus-galli* seeds as compared to control. These fusants show great potential to be selected as possible potential biopesticide.

Keywords: Protoplast fusion, SDS-PAGE, Pseudomonas aeruginosa, Bacillus cereus, Biocontrol, Meloidogyne incognita, Echinochloa crus-galli, Portulaca oleracea.

1. Introduction

Plant parasitic nematodes are one of the most damaging and widespread pathogens that cause global losses to crop production with an estimated loss of $ 157 billion per year (Singh *et al*., 2015). Root-knot nematodes, *Meloidogyne* spp. especially *M. incognita*, has been found as the major limiting factor in vegetables production in tropical and subtropical regions. When the infective stage (J2) penetrates the roots and migrates directly to the vascular cylinder, it causes severe root galling, reduces nutrient and water utilisation efficiency, and affects photosynthetic products (Almaghrabi *et al*., 2013).

Throughout the world, weeds significantly contribute to reduce crop production, even more than all other pests combined (Adeux *et al*., 2019). They affect crop yield and quality by competing on space, nutrients, water, light and interfere with the distribution of fertilizers**.** Weed infestation resulted in yield losses of up to 45 % in wheat (Hussain *et al*., 2017). Moreover, weeds act as reservoir for plant pathogens like nematodes, thus facilitating the reinfesting of crops in the next seasons **(**Byron *et al.*, 2019).

Chemical pesticides have been used against plant parasitic nematodes and weeds with encouraging results since long, but many of these chemicals are proven to be carcinogens, infiltrate into ground water, build up residues in food plants and hazardous to the beneficial soil fauna and flora (Jabran *et al*., 2015). For more sustainable crop production, scientists are endorsing alternatives pesticides reduced toxicities. Using of non-pathogenic microbes with potential control activity against nematodes and herbicide resistant weeds has emerged as promising solution (Sayed *et al.*, 2014). One of the most promising microorganisms without negative effects on the users, consumers or the environment are Plant-Growth Promoting Rhizobacteria (PGPR) (Lee and Kim 2016**).** They affect nematodes and

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weeds directly through the production of toxic compounds, siderophores, hydrogen cyanide, antibiotic, competition for space and indirectly through promoting plant growth and induction of systemic resistance (Lakshmi *et al.,* 2015; Siddiqui *et al*., 2003).

Researchers have reported that mixtures of PGPR strains give better protection than one strain. But in certain cases, the establishment of more than one microorganism has no synergistic effect due to their different nutritional and environmental requirements. Furthermore, the combinations considered to exhibit improved efficacy under one set of conditions or one host may not produce equally favorable results under other set of conditions (Schisler *et al*., 1997). These open the door for utilizing some biotechnological approaches like protoplast fusion to gathering more than one mechanism of controlling in one individual and increasing the production of such toxic substances or enzymes (Abdel Salam *et al*., 2018; Soliman *et al*., 2018; Soliman *et al*., 2020).

The objectives of this study are to improve the antagonistic potential of such isolated bacteria using the protoplast fusion technique and to analyze the fusants products through SDS –PAGE technique. Under in vitro conditions, the antagonistic potential of fusants against root knot nematodes *M. incognita* J2 and *E. crus-galli* and *P. oleracea* weed seeds was evaluated in comparison to the parental types.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Two bacterial strains isolated from the Egyptian soil had nematicidal activity against plant parasitic nematodes. The bacterial isolates were identified based on 16S rDNA sequence analysis in the GenBank database nucleotides, as *Bacillus cereus* GEs (Accession No. LC215052) and *Pseudomonas aeruginosa* (under accession number LC215048) (Soliman *et al.,* 2019). The aforementioned bacterial isolates were used as parent strains in the protoplast fusion experiment and bioassay tests. Bacterial strains were grown in Luria–Bertani (LB) medium (Davis *et al*., 1980) at 30 °C for 24 h with shaking at 120 r/min.

2.2. Nematode inoculum preparation

Cultrue of root-knot nematode *M. incognita* was established from single egg-mass of an adult female, previously identified by the morphological characteristics of the female perineal patterns (Taylor and Sasser, 1978) and reared on tomato plants (*Lycopersicon esculentum* Mill) cv. Alisa in the greenhouse of the Plant Pathology Department, National Research Centre. Nematode eggs were extracted from the infected tomato roots using NaOCl solution as described by (Hussey and Barker, 1973). To perform *in vitro* tested, eggs were allowed to hatch for 48 h at 30±2°C in an incubator to obtain the 2nd stage larvae (J_2) to perform the *in vitro* test.

2.3. Antimicrobial susceptibility

Eleven antibiotics were used with final concentrations as follows: rifampicin (Rif), 100 µg/mL; ampicillin (Amp), 100 µg/mL; amikacin (Amk), 30 µg/mL; streptomycin (Sm), 200 µg/mL; kanamycin (Km), 40 µg/mL; tetracycline (Tc), 15 µg/mL; chloramphenicol (Cm), 35 µg/mL; gentamicin (Gm), 15 µg/mL; polymyxin (Pmx), 50 µg/mL; neomycin (Nm), 40 µg/mL; and erythromycin (Erm), 20 µg/mL. The Kirby–Bauer disc diffusion method for antimicrobial susceptibility test was followed (NCCLS, 1999).

2.4. Growth conditions and protoplast formation

Cultivation of *Bacillus cereus* and *Pseudomonas aeruginosa* was carried out in 250 ml flasks containing 70 ml of LB medium. Flasks were incubated for 24 hr. at 30^{o}C with shaking at 120 rpm. Cells were harvested at the mid-point of the log phase by centrifugation at 5000 rpm for 10 min and washed once with 1% N-laurylsarcosine. This was followed by washing three times with osmotic stabilizer buffer. The bacterial cells were then pelleted by centrifugation. Lysozyme was dissolved in osmotic stabilizer buffer to a final concentration of 15 mg/ml, sterilized by 0.2µm millipore filter. Lysozyme was then, added to the cell pellets at final concentration of 1/10th the volume and mixed thoroughly to make the suspension. The resulting mixture was incubated at 37^{o}C for 4h. The viable protoplast were counted by spreading appropriate dilution onto LB medium solidified by adding 2% agar, where all inviable protoplasts were lysed and only the intact protoplast will grow after incubation according to (Soliman *et al*., 2020; Mohamed *et al*., 2016).

2.4.1. The microscopic examination of the protoplasts

Aliquots (1.0 ml each) of the parental protoplasts were mixed in 25% PloyEthylene Glycol (PEG) 6000 and 100 mM $CaCl_2$ and incubated for 2 h at 30^{o}C. Aliquots of 100 µl from the mixture were prepared at 10 min intervals and diluted 10 times in protoplasting buffer. A total of six fusants were obtained after 2h of fusion time on selective medium containing antibiotics Neomycin (Nm) and Rifampicin (Rif).

2.4.2. Regeneration of protoplast

The protoplasts in the reaction mixture were collected by centrifugation at 3000 rpm for 10 min. The precipitate was washed with Tris-HCl buffer with an osmotic stabilizer and the resulting precipitate was re-suspended in the same buffer. Protoplast suspension was diluted and overlaid on the LB medium solidified by adding 2% agar. (Mohamed *et al*., 2016).

2.5. SDS–PAGE protein analysis

The parental and fusant strains were grown in suspensions following the method of (El-Kawokgy *et al*., 2015). SDS–PAGE 12.5 %, was done according to the method of (Laemmli, 1970) to compare the products secreted by the parental strains and those secreted by fusant strains. After size fractionation, the proteins were visualized by staining with Coomassie Blue R-250 dye. The gels were scanned using Gel Doc 2000 system, and molecular masses were determined using Total Lab version 1.10 software based on protein marker purchased from Biomatik Corporation (Wilmington, Delaware, USA).

2.6. Bioassay Tests

2.6.1. Efficiency of the nematicidal effect of three fusants in comparison with their parents P. aeruginosa and B. cereus against M. incognita J_2

To evaluate the nematicidal activity, Petri dishes 6cm in diameter were supplied separately with 4 ml cell

suspension of *P. aeruginosa* and *B. cereus* singly and combined and three fusants (No. F7, F20 and F35), then added one ml water containing 100 ± 5 freshly hatched *M. incognita* juveniles. Three concentrations were used and standard (S), S/2 and S/4 concentrations were prepared using distilled water. Each ml from standard containing (2×10^6cfu/ ml) Five ml distilled water containing 100 ± 5 freshly hatched *M. incognita* juveniles served as control. All preparations and the control were replicated in five times. All dishes were kept in incubator at 35°C and loosely covered to permit aeration and lessen evaporation. Number of survived and dead individuals was counted for three days. After the exposure periods, the nematodes in each treatment were transferred to distilled water and left for 24 hrs to see whether immobile nematodes resumed activity or not. The J_2 mortality (%) were assessed as compared to the control according to Mortality%= [C1-C2/C1] x100, where C1 is the number of live nematodes larvae in control treatment and C2 is the number of live nematodes larvae in other treatments.

2.6.2. Examine the inhibitory effects of P. aeruginosa and B. cereus as parental strains and their fusants on germination and growth of P. oleracea and E. crus-galli *weed seeds during in vitro analysis*

Seeds of targeted weeds were surface sterilized by immersing in 95% ethanol for a few seconds (3–4 s). After surface sterilization with 5% ethanol, seeds of targeted weeds were washed with sterilized distilled water several times to remove sterility. Filter paper was soaked with 3 ml of bacterial suspension (containing approximately (2×10^6 cfu/ ml) from *P. aeruginosa* and *B. cereus* as parental strains and the fusants No. F7, F20 and F35, were placed separately on Petri dish. Ten sterilized seeds from each weed species were placed separately in Petri dishes then incubated at 25°C. For control, ten seeds from the two weed species were placed separately on filter paper soaked with 3 ml of sterile water. Each treatment was replicated three times. Ten days later, germination of shoots and root lengths were observed.

3. Results

3.1. Bacterial strains and antibiotic susceptibility

Isolation of antibiotic genetic markers from *P. aeruginosa* and *B. cereus* was necessary for their manipulation. Data in Table 1 showed that *P. aeruginosa* was Rifampicin resistant and Neomycin sensitive in the contrary, *B. cereus* was Neomycin resistant and Rifampicin sensitive. Thus, Rifampicin and Neomycin were used as genetic markers for fusion products from the two strains.

Table 1. Antibiotics susceptibility of *P. aeruginosa* and *B. cereus.*

Bacterial strains	Antibiotic resistance	
	Nm	Rif
Pa (Rif ^{r})	---	+++
Bc (Nm^r)	+++	---

Pa = P. aeruginosa, Bc = B. cereus +++: = Very good growth ---: = no growth Rif = Rifampicin Nm = Neomycin

3.2. The microscopic examination of the protoplasts

The protoplast formation between the two selected strains, *P. aeruginosa* and *B. cereus* were tested periodically by microscopic examination. A total of six fusants were obtained after 2h of fusion time on selective medium containing the two antibiotics Nm and Rif as shown in (Figure 1).

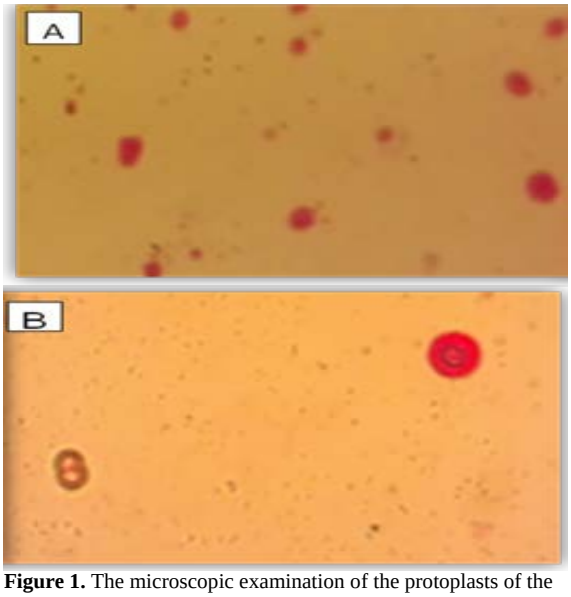

Figure 1. The microscopic examination of the protoplasts of the two parental strains *P. aeruginosa* and *B. cereus* (A) Protoplast formation .(B) Protoplast fusion

3.3. Fusants isolation

Neomycin (Nm) and Rifampicin (Rif) were added in LB medium as selective genetic marker, and only the fusants having combined the two selected isolates will grow in this media. Forty single colonies were randomly selected and retested for their ability to grow on the selective media. The fusants' growth was detected as follow: Six fusion products exhibited strong growth (F4, F5, F7, F15, F20 and F35), while forty fusion products showed weak growth and no growth in twenty single colonies refer to no fusion products.

3.4. Expression of the parental strains protein bands in their fusants

The SDS-PAGE protein patterns of the two parental strains *B. cereus* and *P. aeruginosa* and fusants are presented in (Figure 2). SDS-PAGE analysis of the total proteins of the two parental strains revealed a total of 11 and 12 protein bands, respectively. The molecular weights of parental strains ranged from 7 to 179 kDa. While the fusants showed a variable number of protein bands ranging from 11 in four fusants to 7 in one fusant (Table 2).

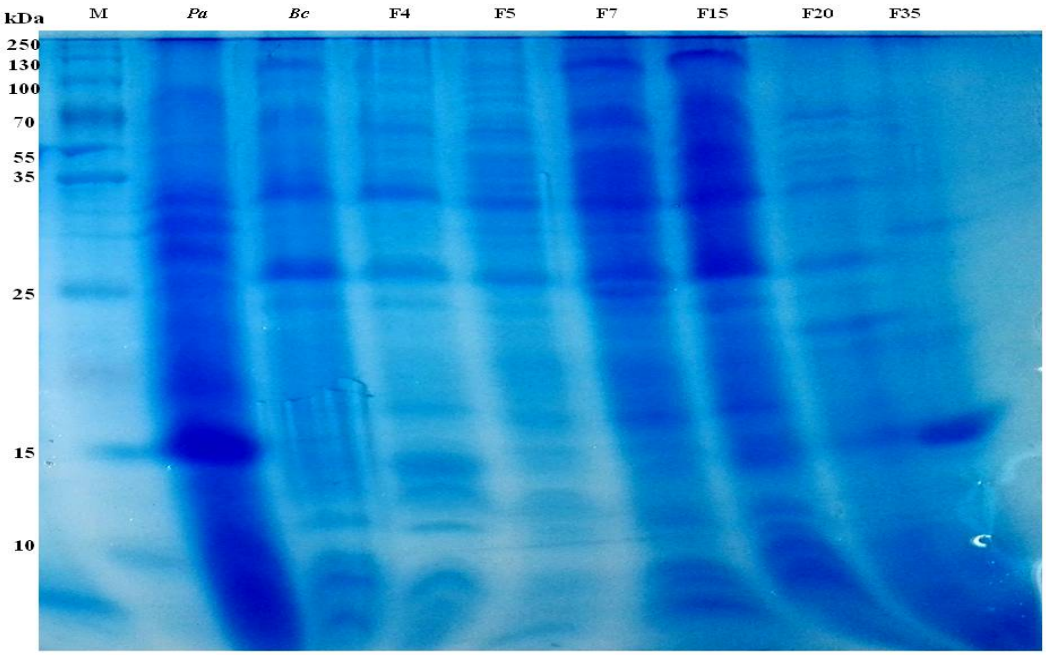

Figure 2. SDS-PAGE protein profiles of the two parental strains; *P. arugenosa* and *B. cereus* and their fusant (F). M is the protein marker (Biomatik Corp, (Wilmington, USA) with nine molecular weight bands (kDa).

Table 2. SDS-PAGE analysis of total proteins of the two parental strains *P. aeruginosa* and *B. cereus* and six of their fusants.

Band No.	MW KDa	Parental strains		*Pa* :: *Bc* fusants					
	MW	Pa	Bc	F4	F5	F7	F15	F20	F35
1	179	+		+	+	+	+	+	+
2	160	+		+	+		+	+	+
3	141		•	•	•	•	•	•	•
4	126	+		+	+	+	+	+	+
5	116	+			+	+	+	+	+
6	109		•	•	•	•	•	•	•
7	100		•	•	•	•	•	•	•
8	92	+			+	+	+	+	+
9	83	+		+	+	+	+	+	+
10	76	+				+	+	+	+
11	67		•	•	•	•	•	•	•
12	57		•		•	•	•	•	•
13	48		•	•		•	•	•	•
14	39	+		+	+	+	+	+	+
15	32	+			+	+	+	+	+
16	28		•	•	•	•	•	•	•
17	24		•	•		•	•	•	•
18	19		•	•		•	•	•	•
19	15		•	•	•	•	•	•	•
20	13		•	•	•	•	•	•	•
21	11	+		+		+	+	+	+
22	8		•	•	•	•	•	•	•
23	7	+		+	+	+	+	+	+
Total no. of protein bands		11	12	18	18	22	23	23	23
Numbers of *Pa* bands expressed in fusants				7	9	10	11	11	11
Pa bands (%)				38.8	50	45.5	47.8	47.8	47.8
Numbers of *Bc* bands expressed in fusants				11	9	12	12	12	12
Bc bands(%)				61.2	50	54.5	52,2	52.2	52.2

(+) Refers to presence of protein band of *Pa* . (•) Refers to presence of protein band of *Bc*

The highest number of *P. aeruginosa* bands (11 bands) was displayed in 3 fusants, while the lowest number (7 bands) was shown by F4. The total *P. aeruginosa* expressed bands in the rest of the fusants were classified in ascending order as follows: 9 bands in F5, 10 bands in F7, 11 bands in F15, F20 and F35 fusants. On the other hand, variable number of protein bands of *B. cereus* was shown in the fusants. The highest number of *B. cereus* (12 bands) was displayed in 4 fusants, while the lowest number (9 bands) was revealed by F5 fusant. The 6 fusants were characterized based on the absence and presence of the 11 *P. aeruginosa* protein bands. A total of 11 *P. aeruginosa* protein bands with different molecular weights were expressed in all the 6 fusants. The presence of the

remaining 11 bands was distributed in descending order as follows: five bands with molecular weights of 179, 126, 83, 39 and7kDa, and five bands were molecular weights 160, 116, 92, 32 and 11kDa, and one band with molecular weights 76kDa existed in four fusants, respectively.

The 6 fusants were characterized based on the absence or presence of the expression of the 12 *B. cereus* protein bands. A total of 8 protein bands with different molecular weights were found in all fusants. The presence of the remaining 8 bands was distributed in descending order among the 6 fusants as follows: each of the eight bands with molecular weights of 141,109,100,67,28,15,13 and 8kDa was displayed in 6 fusants, while 4 bands with molecular weights of 57, 48, 24 and 19kDa were detected in 5 fusants.

3.5. Bioassay tests

3.5.1. The nematicidal potential of P. aeruginosa and B. cereus parental strains and their fusants against M. incognita J_2 under laboratory conditions

As illustrated in (Table 3), the bacterial strains under investigation had a lethal effect on *M. incognita* J_2 as detected by the percentage mortality when compared to control. The reduction in the movement was irreversible, and the mortality of the juveniles was confirmed when they were transferred to distilled water for 24 hrs. Nematode mortality was positively correlated with suspension concentration and times of exposure. Fusants were more effective than their parent singly or combined. The fusant F35 had shown a strong nematicidal activity against *M. incognita* J_2. The recorded percentages mortality as compared to control were 97.7%, 90.9% and 86.3% for S, S/2 and S/4 concentration respectively, after 72hrs of exposure (Table 3).

Table 3. The nematicidal potential of *P. aeruginosa* and *B. cereus* and their fusants on mortality of *M. incognita* juveniles under laboratory conditions.

Bacterial strains	Percentage mortality at different exposure periods								
	S			S/2			S/4		
	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs	24hrs	48hrs	72hrs
Pa	7c	28.4d	52.2d	2e	25.2c	35.2e	0 c	10.5c	14.7e
Bc	10c	46.3c	65.9c	5d	10.5d	43.1d	1c	5.2cd	20.4d
Pa+Bc	8c	44.2c	48.8d	2e	5.2e	28.4f	0c	4.2d	17.1de
F 7	29b	48.4c	80.6b	18c	36.8b	62.5c	10b	25.2b	48.8c
F 20	40a	70.5a	96.5a	27a	56.8a	79.5b	20a	31.5a	71.5b
F 35	30b	55.7b	97.7a	22b	37.8b	90.9a	10b	25.2b	86.3a
Control	---	---	---	-----	-----	-----	-----	-----	----

Pa=Pseudomonas aeruginosa Bc= Bacillus cereus F= fusant S= Standard concentration Averages followed by same letter(s) are not significantly ($P \leq 0.05$) different according to Duncan'sMultiple Range Test

3.5.2. Evaluation of the inhibitory effects of P. aeruginosa and B. cereus and three fusants on germination of P. oleracea and E. crus-galli *weed seeds under laboratory conditions*

All bacterial suspensions completely inhibited seeds germination of *P. oleracea* as compared to control. *P. oleracea* seeds were swollen in size but did not germinate in a response to all bacterial suspensions (parents and fusants). However, no radicle growth was observed in plates, while *E. crus-galli* seeds were less influenced than *P. oleracea* seeds. The seeds germinated with small radicle as compared to control. It is worthy to mention that both parents and fusants retard *E. crus-galli* germination. Moreover, fusants suspensions inhibited the weed germination more than parents suspension.

4. Discussion

Protoplast fusion of two parental *Pa* and *Bc* strains was expressed in the 6 selected fusants. SDS–PAGE proteins indicated that some parental protein bands expressed in all fusant strains this study agree with (Khan *et al.*, 1998), who studied the whole cell protein profiles of 42 strains of *P. aeruginosa*, isolated from clinical samples, using the SDS-PAGE method and reported the presence of protein bands, ranging from 340kDa to 14.3kDa. On the basis of Dice Index of similarity, the strains could be grouped into 20 types. Protoplast fusion has been glorified as the method adequate for a new type and good reproducibility.

The present study demonstrated that biocontrol of *M. incognita* J_2 could be effectively achieved using *P. aeruginosa*, *B. cereus* strains and their fusants. These rhizobacteria appear to suppress root knot nematode via different mechanisms. Oka *et al*., (1993) reported that ammonia was exited during protein degradation by *B. cereus* improving its nematicidal activity. Xioa *et al*., (2018) mentioned that the extracellular metabolites like protease, chitinase, and siderophore in *B. cereus* cell free supernatant significantly increase the mortality of *M. incognita* J_2 and decrease egg hatching. Chen *et al*., (2015) mentioned that *P. aeruginosa* can kill nematodes via the production of hydrolytic enzymes, like protease and diffusible toxins like cyanongen, phenazines, and pycoyanin which degrade nematode cuticle and inhibit metabolic pathways. In addition, *P. aeruginosa* has the ability to produce hydrogen cyanide which is responsible for killing nematode (Patil, 2014).

Moreover, the present study indicated that rhizobacterial strains remarkably reduced the germination of *P. oleracea* and *E. crus-galli* seeds. These are in accordance with the previous studies of (Sardar *et al*., 2020) who reported that bacteria inhabiting plant rhizospheres could be applied as a biocontrol agent to control weeds associated with rice plants. Also, (Carvalho *et al.*, 2007) found that *B. cereus* produced at least two phytotoxins which acted a vital role in the production of sodium vanillate and 2-aminobenzoic acid which inhibit lettuce seedling. (Patil, 2014) confirmed the ability of *P.*

aeruginosa to produce the toxic secondary metabolite hydrogen cyanide inhibiting the enzymes involved in plant respiration, carbohydrate metabolism, CO_2 and nitrate assimilation. Lakshmi *et al.*, (2015) mentioned that seed bacterization with *P. aeruginosa* caused reduction in shoot and root length of *P. oleracea* and *A. spinosus* weed seedlings.

Our results illustrate that fusant products were more efficient in killing *M. incognita* J_2 and inhibiting germination of seeds weed than their parents. Fusants produced more toxins, antibiotics, and lytic enzymes than the parents, according to (Zaied *et al*., 2009), who discovered that fusion between *Serratia* spp. and *Pseudomonas* spp. produced more chitinases and bacteriocin than the parents, resulting in high mortality levels in nematodes when compared to the parental strains.. Elkylany, (2017) mentioned that fusants from *Anoxybacillus flavithermus* and *B. pumilus* were more effective in killing *M. javanica* J_2 than their parent, and Abdel Salam *et al*., (2018) discovered that a higher level of chitinase production in the fusants between *B. amyloliquefaciens* subsp. plantarum SA5 and *Lysinibacillus sphaericus* Amira than the parents resulted in higher nematode mortality..

5. Conclusion

We are focusing here on genomics and genetic engineering techniques as helpful tools for developing more powerful biocontrol agents. The efficacy of nematode and weed seeds suppression by tested materials depends on the toxic compound released from these organisms. The obtained fusants from the two local bacterial *P. aeruginosa* and *B. cereus* showed high efficacy against *M. incognita* J_2 and *E. crus-galli* and *P. oleracea* seeds germination because they produced more toxins, antibiotic, lytic enzymes than the parent strains. These new strains show great potential to be formulated as an effective biopesticide.

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