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Impact of Genetically Modified Bacteria and Natural Rocks on Root-Knot Nematode Bio-Control, Improving Productivity and Chemical Properties of Grapes

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Abstract

This work aimed to improve grapes productivity as well as chemical and physical properties of the berries using genetically modified bacteria as bio-nematicides against Meloidogyne incognita and as a bio-fertilizer by facilitating the solubilization of natural phosphate rocks and feldspar (as a potassium source), to achieve that, protoplast fusion experiment was performed between Peanibacillus polymyxa and Lysinibacillus sphaericus. Fourteen fusants were isolated, fusants F11 and F15 achieved 100% mortality in vitro. A field experiment was then conducted in Wady Elnatroon, El-Beheira governorate, Egypt, to evaluate the genetically modified bacteria as bio-nematicides and bio-fertilizer for Flame seedless in 2022 and 2023 at two doses of 100 and 200 mL/vine which were referred to as (A and B), and natural rocks at two rates (¼ kg rock phosphate + ½ kg feldspar) and (½ kg rock phosphate + 1 kg feldspar) were referred to as (1 and 2) The treatment F11B2 achieved the best decrease in M. incognita juveniles (J2) number, galls, and egg-masses compared to the control. Generally, all treatments improved the yield, physical and chemical characteristics of the berries, and leaf mineral content compared to untreated trees. The F11A1 increased all growth parameters, yield, quality, total phenols, and anthocyanins in berries, While, F11B2 recorded the best-improved nitrogen, phosphate, and potassium in both seasons.

Keywords: Genetically modified bacteria; *Meloidogyne incognita*; bio-control; bio-fertilizers; natural rocks; grapevine productivity.

1. Introduction

In Egypt, grapes is considered one of the most important export crops, as production in 2022 reached about 1,571,989 tons, and this was produced from an area of 79,092 hectares, according to statistics from the Food and Agriculture Organization (FAOSTAT) [1]. Flame seedless table grapes are a significant Egyptian variety due to their excellent quality, high price, early maturity, and strong export position in Europe and Arabia. Most grapevine areas across the world face a significant threat from plant-parasitic nematodes (PPNs), especially the highly damaging root-knot nematodes (*Meloidomyne* spp.) that infest grapevines [2,3]. It was recognized that *Meloidogyne incognita* could pose an enormous risk to grapevine productivity. Its feeding habit on grapevine roots causes reduced vine vigour and production losses because it impedes water and nutrient uptake as well as root and shoots growth [4]. Furthermore, PPNs might increase vines' vulnerability to other infections [5]. Controlling nematodes is more challenging than controlling other pests because of their broad host range and soil-dwelling behavior [6]. To increase the productivity of grapes, meet the demands of the local market, and increase their exports, farmers think that increasing the amount of chemical fertilizers and nematicides they use will increase grape production [7]. Due to the greater risk of the usage of chemical nematicides and fertilizers to the environment and human health and the rising cost [2,8,9]. As a result, the need for effective, long-term nematode management techniques is growing, and modern agricultural methods use alternative chemical nematicides and fertilizers by adding natural rocks to the soil, especially rock phosphate and feldspar [10,11]. Rock phosphate, a natural

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source of phosphorus, is a less expensive alternative to industrial chemical fertilizers, reducing environmental pollution in agricultural soil and maintaining soil balance compared to chemical fertilizers [10]. In addition, feldspar is a potassium source that improves root growth, drought resistance, cellulose formation, sugar, starch, protein synthesis, and stress reduction [12]. Applied rhizobacteria, which have a critical role in controlling plant nematodes and releasing nutrient elements in soil from natural rocks, can accelerate the rate of crystal dissolution, mostly through the production of organic and inorganic acids [13,14,15]. So, the greatest option for managing nematodes is biological control, which replaces chemical nematicides and fertilizers, which need to be the main focus now.

Among the many benefits of bio-fertilizers are their ability to substitute chemicals such as phosphate (P) and nitrogen (N) through their ability to secrete organic acids, engage in exchange processes, and chelate inorganic phosphates to transform them into soluble forms, which promotes plant development. Bacteria are crucial to the bio-geochemical cycle of many elements [16,17,18,19]. Plant Growth Promoting Rhizobacteria (PGPR) as a bio-fertilizer is an ideal substitute for chemical fertilizers because it is cost-effective and environmentally friendly. Bacillus genus of rhizobacteria exhibits several strategies of action against plant-parasitic nematodes (PPNs), direct mechanisms include phosphate solubilization, nitrogen fixation, exopolysaccharide production, and phytohormone, while indirect mechanisms include lytic enzymes production, antibiotic production, Hydrogen Cyanide (HCN) production, siderophore production, induced bioremediation, and systemic resistance [20].

Genetic tools provide simple and effective techniques to improve bacterial strain properties, one of those techniques is protoplast fusion which is the only means of combining two cytoplasmic hereditary traits in a single genotype [21]. Homologous recombination between several *Bacillus* species with varying genetic distances was achieved Successfully using protoplast fusion [22,23,24]. A previous study found that the protoplast fusion of *B. cereus* and *B. thuringiensis* produced ten stable bacterial fusants with increased nematicidal activity compared to the parent strains [23]. The fusant F7 demonstrated high juvenile mortality, galls, egg-masses reduction, and improving plant growth in pot trials. It was discovered that F7 could fix atmospheric nitrogen more effectively than its bacterial parents.

Because of this, the beneficial impacts of bacteria are essential to the bio-geochemical cycle of numerous elements, as well as to bio-fertilization, their ability to release essential elements from natural rocks, and their involvement in controlling plant nematodes. Our experiment aimed to study the effect of the use of new genetically modified bacterial strains on plant nematode control and their role in facility the use of rock phosphate and feldspar as alternative chemical fertilizers, in addition to improving the productivity of 'Flame' seedless grapevines, the physical characteristics of the bunches, and the chemical properties of the grape juice under sandy soil conditions in Egypt.

2. Materials and Methods

2.1. In vitro experiments

2.1.1. Bacterial strains and growth media

Lysinibacillus sphaericus (Ls) CH4GES strain, NCBI accession number (LC215050.1), and *Peanibacillus polymyxa* (*Pp*) CH6GES strain, NCBI accession number (LC215049.1). The strains were maintained in Luria–Bertani (LB) medium [25].

2.1.2. Screening for bio-fertilization activities

2.1.2.1. Nitrogen fixing

The bacterial strain's activity for nitrogen fixation was examined on glucose nitrogen-free mineral (GNFM) agar medium [26], Bromothymol blue (BTB) was used as an indicator, and 0.5 g of BTB was dissolved in 100 mL of distilled water before being filter-sterilized. After inoculating each test strain onto GNFM plates, the plates were incubated for three days at 37 °C, flooded with BTB solution, and checked for a change in agar color from green to dark blue or bluish-green, which is a sign of nitrogen-fixing activity.

2.1.2.2. Phosphate solubilization

The bacterial strains were screened for inorganic phosphate solubilization by culturing them separately on Pikovskaya's agar medium, which containing 0.5% of tricalcium phosphate (Ca_3PO_4) as a complex insoluble phosphate source. The cultured plates were incubated at 37 °C for three days, the appearance of clearing zones and/or yellow halos is considered an indication of phosphate solubilization [27]. The solubilization index (SI) is measured using the following formula [28]:

$$SI = \text{colony diameter} + \text{halo zone diameter/colony diameter}$$

2.1.2.3. Potassium solubilizing

The bacterial strains were screened for their ability to solubilize potassium using Alexandrov medium which contains per liter (5.0 g Glucose, 0.1g $CaCO_3$, 0.5 g $MgSO_4.7H_2O$, 2.0 g Ca_3PO_4, 0.006 g $FeCl_3$, 3.0 g

Feldspar (Al-Ahram Mining Company, Egypt) as an insoluble potassium source, and 20.0 g agar).

The pH of the medium was adjusted to 7.5 using dilute acid and/or alkali. The spread plate technique was used and incubated at 37 °C for 3 days [29].

2.1.3. Protoplast formation, fusion and regeneration

2.1.3.1. Protoplast formation

The bacterial strains were incubated overnight in LB medium at 30 °C with shaking at 120 r/min. The bacterial cells were precipitated by centrifugation at 5000 rpm for 10 min and washed with 1% N-lauryl sarcosine, then rinsed three times with osmotic stabilizer solution (30 mM Tris–HCl buffer, pH 7.5, and 0.6 M $MgSO_4$). For protoplast formation, Lysozyme (Thermo Scientific) was dissolved in the osmotic stabilizer buffer at a final concentration of 4 mg/mL. The cell pellets were suspended and incubated with lysozyme at 37 °C for four hours. The protoplast formation was observed under a phase contrast microscope [23].

2.1.3.2. Protoplast fusion and regeneration

The obtained protoplasts were harvested by centrifugation at 2000 rpm for 10 min at 4 °C. The pellet was rinsed with the osmotic stabilizer, and the resulting pellet was resuspended in the same buffer. For protoplast fusion, aliquots (1.0 mL) of protoplast suspension of each parental strain were mixed together with 25% PEG 6000 (SRL, India) and 100 mmol/L $CaCl_2$ and incubated at room temperature for 20 min. For protoplast regeneration, 100 mL aliquots from the mixture were taken and added to a semisolid LB agar medium (2% agar) and overlaid on the solid agar LB medium and incubated at 30 °C for 2-5 days, then the selectable marker media were used for fusant selection [23].

2.1.4. Protein analysis by SDS–PAGE

The bacterial strains were grown overnight in LB medium at 30 °C and 150 rpm. The bacteria pellet was precipitated by centrifuging at 10000 rpm for 10 min at 4°C, and then the total proteins were extracted and separated by SDS–PAGE using 12.5% polyacrylamide gel according to Laemmli's method [30]. The protein profile was analyzed by GelAnalyzer 23.1.1 software based on the protein ladder (Thermo Scientific) and the phylogenetic tree was conducted by the NTSYSpc.v2.10e program.

2.1.5. Nematicidal effect of fusant against M. incognita juveniles

2.1.5.1. Nematode inoculum

The root-knot nematode *M. incognita* was reared on tomato plants in the Plant Pathology greenhouse of the National Research Centre using a single egg-mass of an adult female that had previously been identified based on the morphological features of the female perineal pattern [31]. To obtain second-stage larvae (J2) for the in vitro test, juveniles were extracted from the infected tomato roots by incubation with water for 48 hours at 30±2 °C in an incubator.

2.1.5.2. Nematicidal activity

The best six fusants (No. F1, F6, F9, F11, F15, and F17), which have a large number of bands by SDS-PAGE protein analysis against *M. incognita* compared to its parents, *L. sphaericus* and *P. polymyxa*, was selected to evaluate the nematicidal activity, 4 mL cell suspensions of each strain were put individually into test tubes. One ml of water containing 100 ± 5 freshly hatched *M. incognita* juveniles was then added. Four test tubes contain 5 ml of nematode suspension (100 ± 5) from a recently hatched *M. incognita* juvenile kept as a control treatment. All treatments and controls were replicated four times. Every treatment was maintained in an incubator at 35 °C with a loose cover to allow for evaporation to be reduced and aeration to occur.

The total number of deceased and alive individuals was counted. The J2 mortality (%) was assessed in comparison to the control after 24 h [23].

2.2. Field experiment

The experiment was performed on a grapevine to assess the ability of some modified bacteria as bio-nematicides on root-knot nematode control and their ability to act as bio-fertilizers on grapevines. The study was conducted in a private orchard in Wady Elnatroon City, El-Beheira governorate, Egypt, over the course of two seasons (2022 and 2023). Four-year-old Flame seedless cultivar grapevines were used for the investigation. The vines are irrigated with groundwater (EC 1032 micro mos and PH 7.68) under a drip irrigation system The grapevines are cultivated in sandy soil (Table 1), which displays the soil analyses based on the analysis conducted by Wilde et al. [32] and are spaced 2 x 3 meters. The vines are irrigated with groundwater (EC 1032 micro mos and PH 7.68) under a drip irrigation system. The selected trees had uniform forms and were treated with standard horticulture practices. One kilogram of rhizosphere soil per repeat was sampled, combined, and transported to a lab in order to create subsamples for nematode extraction. The initial *M. incognita* population densities were established prior to the administration of any treatment. The effectiveness of the treatment is evaluated using the reduction percentages.

Table 1 Some chemical properties of the orchard soil

	Soluble salts		Cations				anions			
Parameter	pH	EC	Ca^{+2}	Mg^{+2}	K^{+}	Na^{+}	HCO_3^{-}	CO_3^{-2}	Cl^{-}	SO_4^{-}
Unit		Ds/m	Meq/L	Meq/L	Meq/L	Meq/L	Meq/L	Meq/L	Meq/L	Meq/L
Sample	8.00	4.89	8.90	4.50	0.92	30.4	3.00	N. D	18.50	23.2

1.1.1. Experimental design and treatments

The design of the experiment is a simple experiment in the form of a randomized complete block design (RCBD). The experiment included 19 treatments, each treatment included four replicates, and each replicate was represented by three vines. The Ministry of Agriculture of Egypt recommends using potassium sulphate for K, phosphoric acid for P, and ammonium nitrate for N in a ratio of 60-30- 120 units for mineral fertilization. All grapevines were treated with the farm's horticultural program as the Ministry of Agriculture's recommendation, except for the trees under study, where potassium and phosphorus chemical fertilization were reduced by 25%. Rock phosphate (Al-Ahram Mining Company, Egypt) was added at a rate of 0.25 kg or 0.5 kg per vine/year, and feldspar was added at a rate of 0.5 kg or 1 kg per vine/year. Phosphate rock and feldspar were added once in mid-January. Bacterial strains were applied on three dates (mid-February, mid-March, and mid-April) at two doses (100 and 200 mL/vine) as soil drench application. The experiment included eighteen treatments (Table 2).

2.2.2. Measurements

2.2.2.1. Nematode parameters

Observations of *Meloidogyne incognita* parameters as numbers of juveniles (J2) in soil, galls, and egg-masses in roots of treated grapevine cv. Flame Seedless as well as untreated control were recorded after 6 months. The juveniles of nematode were extracted in soil samples of grapevine plants by sieving and decanting methods [33]. Numbers of egg-masses and galls were estimated in roots (5 g per plant) using a binocular microscope [34].

2.2.2.2.Leaf petioles mineral content

Leaf petiole samples were taken from mature leaves from the opposite side of the cluster and from all parts of the tree during the month of July. The leaf petioles were dried at a temperature of 70 °C in a drying oven. Then the samples were digested in hydrochloric acid and perchloric acid, and then the content of samples of mineral elements. The Kjeldahl method was used to assess the nitrogen content of leaf samples that had been digested in sulfuric acid [35]. Using spectrophotometric methods, the phosphorous element was estimated [36]. In addition, potassium is measured using the Flame photometric apparatus [37].

2.2.2.3.Fruit Yield:

At harvest time during the month of May (when the check treatment's berry juice's TSS reached 14– 16% brix). Yield/vine (kg): It was calculated by multiplying the average cluster weight by the average cluster number per vine.

2.2.2.4. Physical characteristics of grapes

Three clusters were taken at random from each vine and the following parameters were determined: Cluster weight (g) and berry weight (g), In addition, berry size (cm^3), length/diameter ratio of berry. In addition, the juice volume of 100g berries (mL).

2.2.2.5. Chemical characteristics of grapes

1- Soluble solids content percentage (SSC %) in fruit juice: It was determined by Hand refractometer apparatus.

Total acidity content (%): (as g tartaric acid/ 100 mL juice) by titration against 0.1 NaOH using phenolphthalein (PhTh) as an indicator [38].

2- Soluble solids content/acid ratio (SSC/acid ratio): calculated by dividing the percentage of SSC by total acidity %.

Total phenols (mg/100g fresh weight): the concentration of phenolic compounds was determined as described by Singleton and Rossi [39] and results were expressed as a tannic acid equivalent. The juices were dissolved in a mixture of methanol and The absorbance was then measured using a spectrophotometer at a wavelength of 535 nm, and the end result was displayed as mg/100 g fresh weight [40].

Table 2 Scheme of the treatments and their abbreviations

Treatment full name	Dose	Treatment abbreviation
Untreated control	Untreated	Control
Natural rock fertilizers without bacteria	¼ kg rock phosphate + ½ kg feldspar without bacteria	1
Natural rock fertilizers and fusant bacterial strains	¼ kg rock phosphate + ½ kg feldspar + 100 mL of bacterial strain	F11A1
Natural rock fertilizers and fusant bacterial strains	¼ kg rock phosphate + ½ kg feldspar + 200 mL of strain	F11B1
Natural rock fertilizers and fusant bacterial strains	¼ kg rock phosphate + ½ kg feldspar + 100 mL of bacterial strain	F15A1
Natural rock fertilizers and fusant bacterial strains	¼ kg rock phosphate + ½ kg feldspar + 200 mL of bacterial strain	F15B1
Natural rock fertilizers and *P. polymyxa* as parental strains	¼ kg rock phosphate + ½ kg feldspar + 100 mL of bacterial strain	PpA1
Natural rock fertilizers and *P. polymyxa* as parental strains	¼ kg rock phosphate + ½ kg feldspar + 200 mL of bacterial strain	PpB1
Natural rock fertilizers and *L. sphaericus* as parental strains	¼ kg rock phosphate + ½ kg feldspar + 100 mL of bacterial strain	LsA1
Natural rock fertilizers and *L. sphaericus* as parental strains	¼ kg rock phosphate + ½ kg feldspar + 200 mL of bacterial strain	LsB1
Natural rock fertilizers without bacteria	½ kg rock phosphate + 1 kg feldspar without bacteria	2
Natural rock fertilizers and fusant bacterial strains	½ kg rock phosphate + 1 kg feldspar + 100 mL of bacterial strain	F11A2
Natural rock fertilizers and fusant bacterial strains	½ kg rock phosphate + 1 kg feldspar + 200 mL of bacterial strain	F11B2
Natural rock fertilizers and fusant bacterial strains	½ kg rock phosphate + 1 kg feldspar + 100 mL of bacterial strain	F15A2
Natural rock fertilizers and fusant bacterial strains	½ kg rock phosphate + 1 kg feldspar + 200 mL of bacterial strain	F15B2
Natural rock fertilizers and *P. polymyxa* parental strain	½ kg rock phosphate + 1 kg feldspar + 100 mL of of bacterial strain	PpA2
Natural rock fertilizers and *P. polymyxa* as parental strain	½ kg rock phosphate + 1 kg feldspar + 200 mL of bacterial strain	PpB2
Natural rock fertilizers and *L. sphaericus as* parental strains	½ kg rock phosphate + 1 kg feldspar + 100 mL of bacterial strain	LsA2
Natural rock fertilizers and *L. sphaericus* as parental strains	½ kg rock phosphate + 1 kg feldspar + 200 mL of bacterial strain	LsA2
A=100 mL of bacterial strain, B= 200 mL of bacterial strain, 1=¼ kg rock phosphate + ½ kg feldspar, 2= ½ kg rock phosphate + 1 kg feldspar,		

2.3. Statistical Analysis

Using the COSTAT program, User Manual, Version 3.03 (Barkley Co., United States of America) The data were tabulated and statistically evaluated in accordance with Gomez and Gomez [41], the data on nematode and plant-growth criteria were subjected to analysis of variance. Duncan's multiple-range test was used to compare the means [42].

3. Results

3.1. Protoplast formation and fusants selection

To select the fusants, the parental strains were examined to choose a selectable marker.

The selectable marker data of the *L. sphaericus* and *P. polymyxa* strains are presented in Table (3). *L. sphaericus* strain showed the ability for nitrogen fixing on glucose nitrogen-free mineral (N) agar medium, whereas *P. polymyxa* strain showed the ability to utilize insoluble phosphate and solubilize potassium by supplying Pikovskaya's (P) and Alexandrov (k) agar media.

Then, the protoplast formation of the two parental strains *L. sphaericus* and *P. polymyxa* was examined periodically by microscopic examination. The protoplasts were formed after 2 hours of incubation with lysozyme as shown in Fig. 1. After protoplast formation and regeneration, the obtained isolates were grown in selectable marker media (N, P, and K)

for fusants selection. Fourteen fusants were obtained and used in the next steps.

Table 3 The selectable marker data of the parental strains *L. sphaericus* and *P. polymyxa*

Strains	N	P	K
***L. sphaericus* (Ls)**	+	-	-
***P. polymyxa* (Pp)**	-	+	+

(+) refers to strain grow on the media
(-) refers to strain doesn't grow on the media

3.2. SDS analysis of the parental strains and their fusants

SDS-PAGE analysis of the total proteins of the two parental strains, *L. sphaericus* (Ls), *P. polymyxa* (Pp), and their fusants detected a total of 20 bands, their molecular weights ranged from 8 to 148 kDa Table (4). Each of the wild type stains revealed 10 bands, all the fusants acquired some bands from each parent as shown in Fig.2 and Table 5. The phylogenetic tree was conducted to determine the relationships between the parental strains and their fusants. The results showed that the fusants are more similar to the parental strain *L. sphaericus* than *P.* polymyxa fusants as illustrated in Fig. 3.

3.3. Effect of modified bacterial strains on *M. incognita* J2 mortality under laboratory conditions

All fusants had increased *M. incognita* J2 percentage mortality after 24 hours; the average was 73.15, 76.39, 70.43, 100, 100, and 79.51% for fusants F1, F6, F9, F11, F15, and F17 respectively. F11 and F15, in particular, both achieved 100% mortality when compared to the control mortality and were used in the field experiments.

3.4. Grapevine damage by nematode infection

The data presented in Figure 4 demonstrate that the use of fusants as a bio-control and bio-fertilizer in conjunction with natural rocks results in the greatest reduction of J2 in soil when compared to the parental strains. The reduction number of J2 in soil was found to be best treated with bacteria strain F11B2 when applied to vines. This resulted in reduction numbers of 64.26% and 71.47% in two seasons, respectively, compared to 5.04 and 5.91 in two seasons when natural rocks devoid of bacteria were used.

Data in Fig. 5 demonstrate how the addition of natural rocks to modified bacteria acting as a bio-control and bio-fertilizer can reduce the number of galls in all treatments. On the other hand, it was found that the application of the F11B2 treatment achieved the highest reduction of gall, which was recorded at 70 % and 74.56% in two seasons, respectively, as compared to 5.22 and 6.36 when natural rocks were treated in the same seasons without the presence of bacteria.

According to data in Fig. 6, and for the reduction number of egg-masses, it was found that treating vines with the bacterial strain F11B2 was the best treatment. This was recorded at 68.25% and 84.97% in two seasons, respectively, compared with 6.88 and 4.15 when using natural rocks devoid of bacteria in two seasons, respectively. Our current study's findings show that, in both of the experimental seasons (2022 and 2023), in comparison to the control (infected plants), all treatments used as a soil drench decreased different nematode parameters compared to parents and treatment with neutral rocks only. According to the results, the 2023 season shows the greatest decrease in all nematode parameters as compared to the 2022 season of grapevine root. A positive correlation was found between nematode reduction and the bacterial dose, rate of fertilizer used, and season.

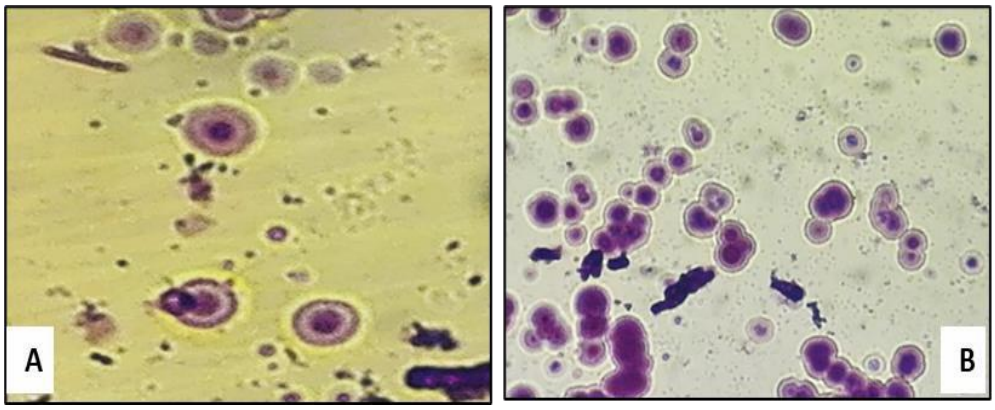


Fig. 1 Protoplast formation of the parental strains; A: *L. sphaericus*; B: *P. polymyxa*

Table 4 SDS-PAGE analysis of total proteins of the two parental strains *L. sphaericus, P. polymyxa* and their fusants

Band No.	MW KD	Parental strains		Fusant													
		Ls	Pp	F1	F6	F9	F11	F12	F13	F14	F15	F16	F17	F18	F19	F20	F
1	148	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	130	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	109		•	•	•	•	•	•	•	•	•		•	•	•	•	•
4	108		•				•				•	•					
5	102	+		+	+	+	+	+	+	+	+		+	+	+	+	+
6	100	+					+				+	+					
7	88	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	72		•	•	•	•			•	•	•	•					
9	70	+												+		+	
10	65		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
11	59		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
12	58	+		+	+	+	+	+		+	+	+	+	+	+	+	
13	52		•	•	•	•	•	•	•		•	•	•		•		•
14	46		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
15	35	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	30		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
17	22	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	19		•	•	•	•	•	•	•	•	•	•	•	•	•	•	•
19	11		•	•	•	•	•	•		•		•				•	•
20	8	+		+	+	+	+	+	+	+	+	+	+	+	+	+	+
Total no.of protein bands		**10**	**10**	**17**	**17**	**17**	**18**	**16**	**15**	**16**	**18**	**17**	**15**	**15**	**15**	**16**	**1**
No. of Ls bands expressed in fusants				**8**	**8**	**8**	**9**	**8**	**7**	**8**	**9**	**8**	**8**	**9**	**8**	**9**	**7**
No. of *Pp* bands expressed in fusants				**9**	**9**	**9**	**9**	**8**	**8**	**8**	**9**	**9**	**7**	**6**	**7**	**7**	**8**

(+) Refers to the presence of the protein band of *L. sphaericus*, (•) Refers to the presence of the protein band of *P. polymyxa*

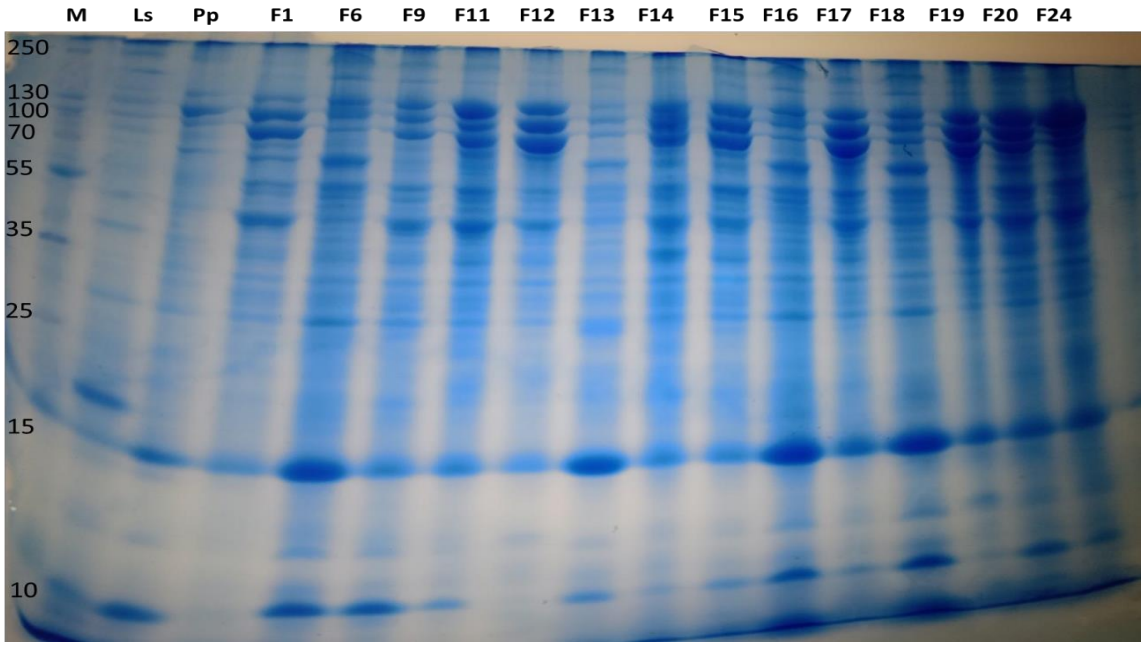


Fig. 2 SDS-PAGE for the total proteins from the paranal strains *L. sphaericus, P. polymyxa*, and their 14 fusants

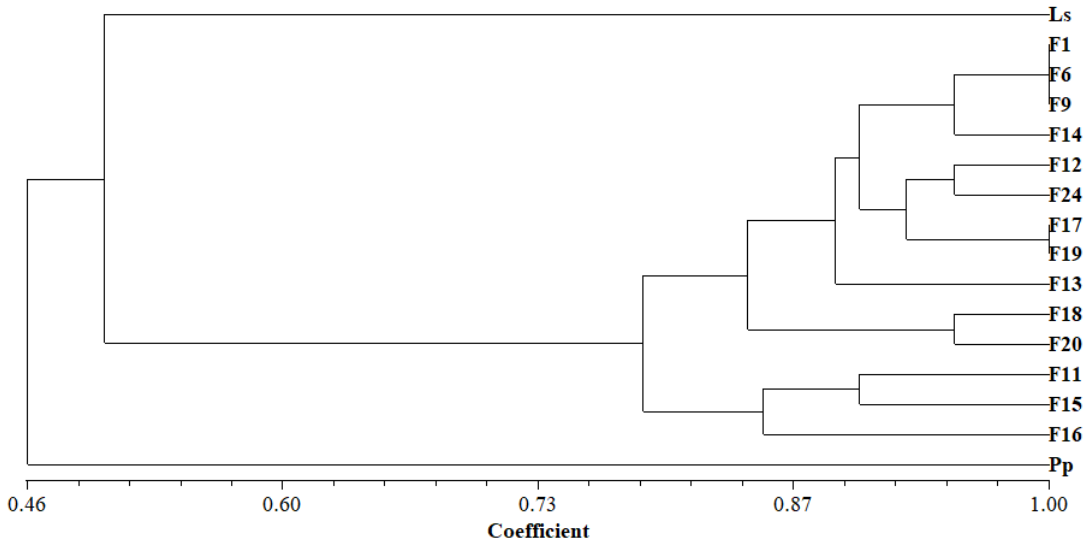
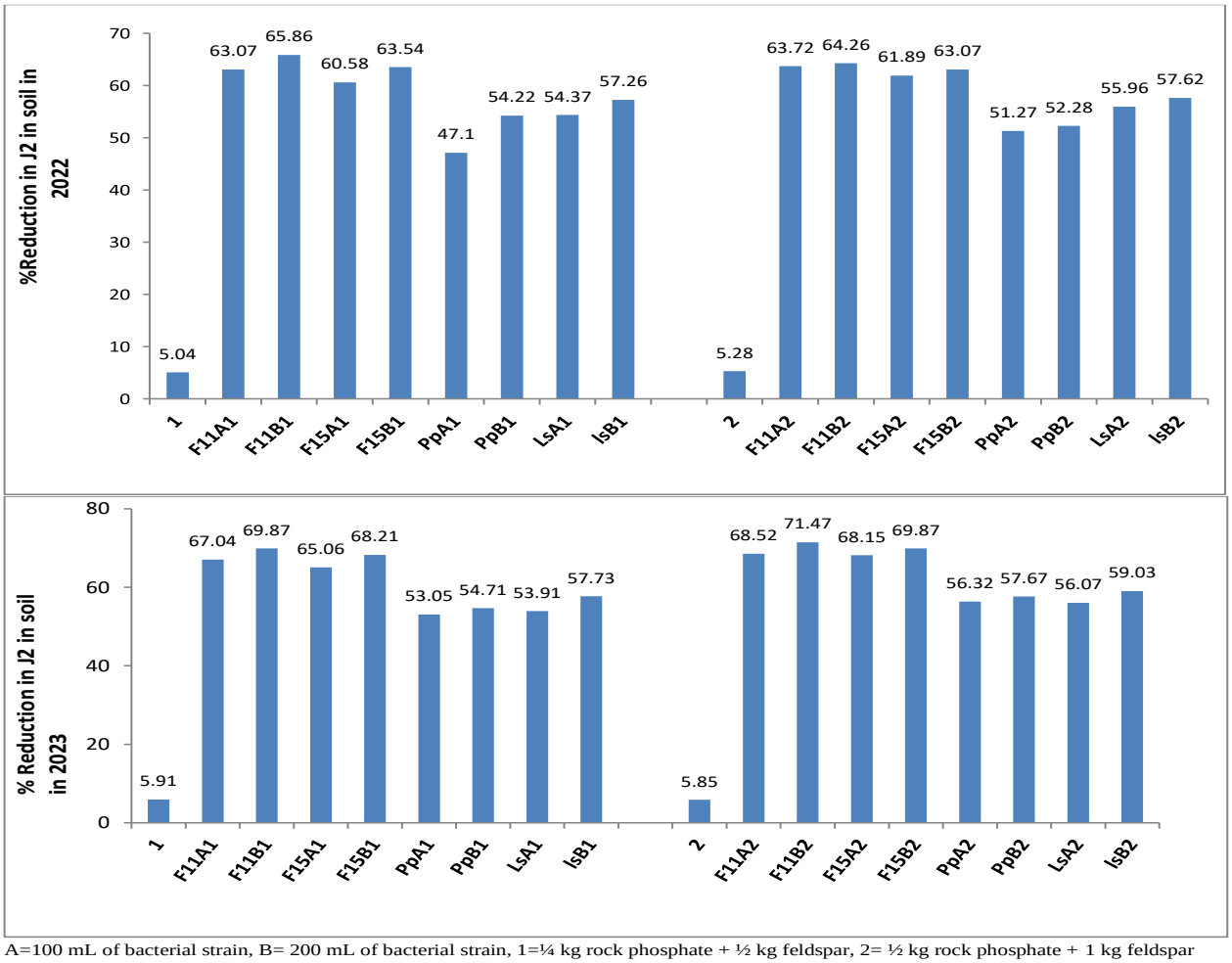
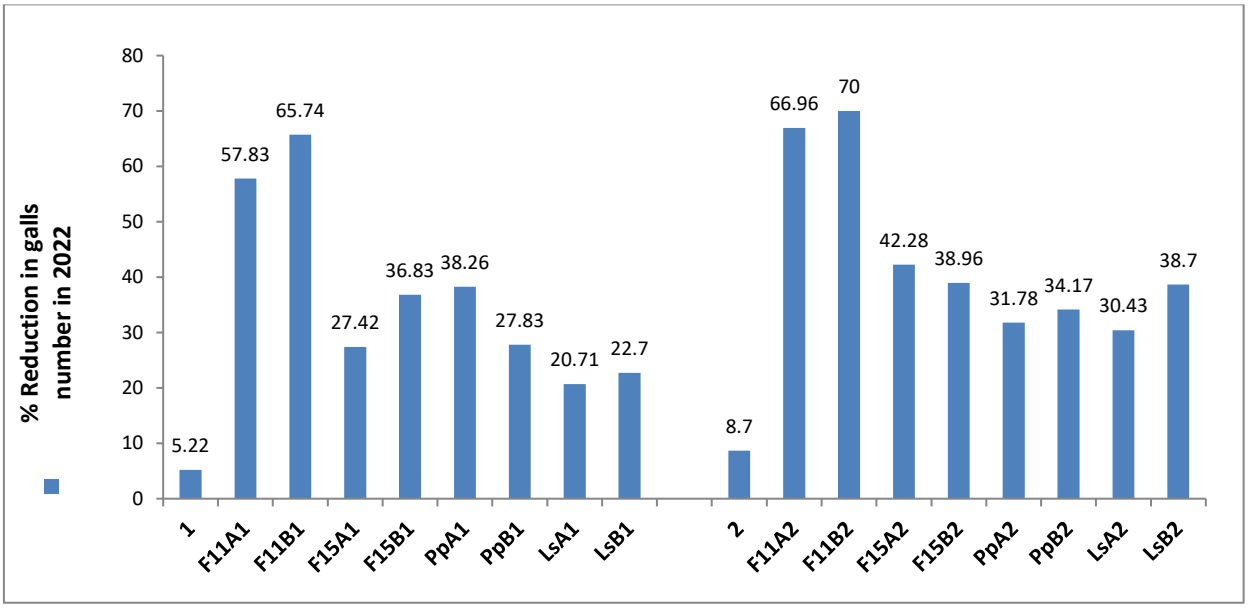


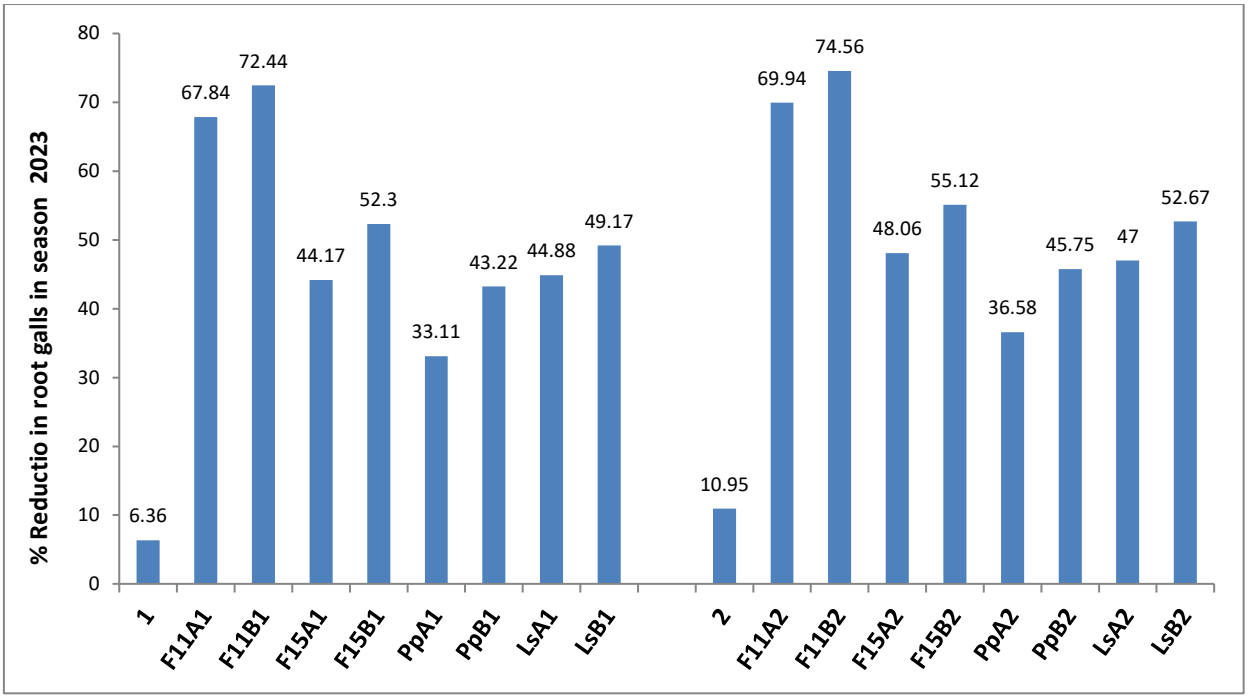
Fig. 3 Phylogenetic tree between the parental strains *L. sphaericus*, *P. polymyxa*, and their 14 fusants



A=100 mL of bacterial strain, B= 200 mL of bacterial strain, 1=¼ kg rock phosphate + ½ kg feldspar, 2= ½ kg rock phosphate + 1 kg feldspar

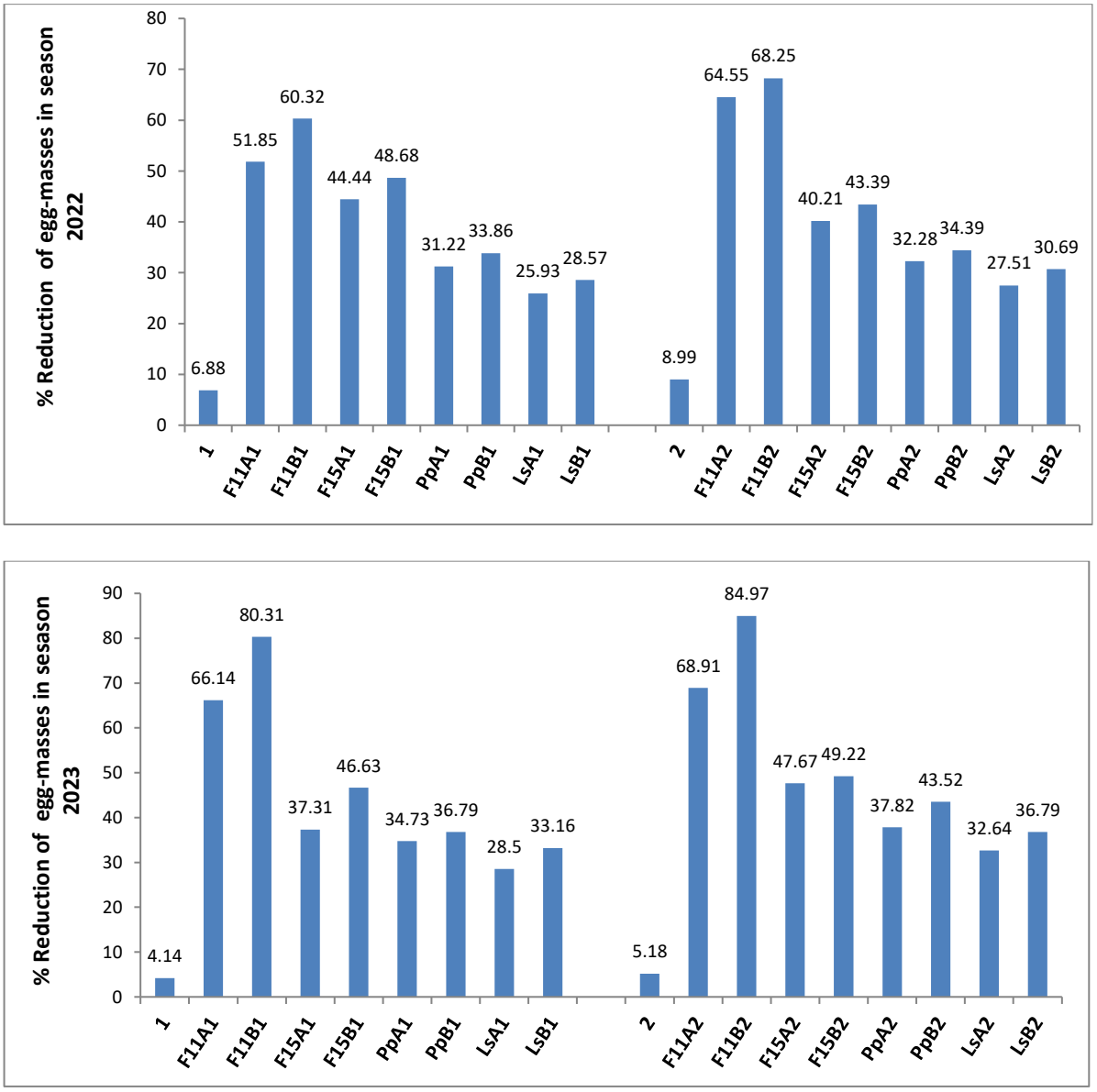
Fig 4. Effect of fusants and natural rocks on Reduction in J2 in soil in 2022-2023





A=100 mL of bacterial strain, B= 200 mL of bacterial strain, 1=¼ kg rock phosphate + ½ kg feldspar, 2= ½ kg rock phosphate + 1 kg feldspar

Fig 5. Effect of fusants and natural rocks on reduction in J2 in soil in 2022-2023



A=100 mL of bacterial strain, B= 200 mL of bacterial strain, 1=¼ kg rock phosphate + ½ kg feldspar, 2= ½ kg rock phosphate + 1 kg feldspar

Fig 6. Effect of fusants and natural rocks on reduction in egg-masses in 2022-2023

3.5. Yield and cluster weight

The results in Fig. 7 showed that fusants applied as a soil drench and natural rocks had a significant effect on the amount of yield (kg/vine) and cluster weight (g) compared to untreated vines in both seasons. When using bio-fertilization, natural rocks recorded the best improvement in yield and cluster weight compared to vines treated with only natural rocks. The treatment (F11A1) achieved the highest yield and cluster weight in both seasons as recorded (16.30 kg, 486.65 g, and 22.44 kg, 623 g), respectively, as vines treated with natural rocks (5.07 kg, 338.90 g, and 12.25 kg, 472, 7). While the control recorded (4.59 kg, 285.06 g, and 10.78 kg, 362 g), respectively, in yield and cluster weight

3.6. Physical characteristics of berries

From the results in Table 5, noticed that all treatments led to an increase in the berry weight, berry size, and juice volume of 100 g berries recorded increasing rates up to (25, 17.5, and 4%) and (34, 35, and 33%) in both seasons, respectively.

Vines treated with F11A1 were the optimum treatment for increasing the berry weight, berry size, and juice volume of 100 g of berries. For the length/diameter ratio of berries, there are some slight differences between all treatments.

3.7. Chemical characteristics of berries

Data in Table 6 show some chemical characteristics of flame seedless berries affected by different fusants and natural rocks. In the first season, it was observed that treating vines with the bacterial strains F15B1 and F15B2 were the best treatment for increasing SSC% for the berry. In the second season, all treatments recorded an increase in the percentage of SSC in the berry compared with the control, as treatment F11A2 achieved the maximum increase. Concerning juice acidity%, from the results for both seasons, it can seem that all treatments either added natural rocks with or without bacteria caused a significant decrease in juice acidity% compared with control. The minimum value for juice acidity was noticed when treated vines with (¼ kg rock phosphate + ½ kg feldspar) without bacteria. As for the SSC/acidity ratio, the data indicated that the highest ratio was observed when treated vines with F15B1 (31.11) in the first season. While in the second season, the highest ratio was F15A1 (43.55). Phenolic and anthocyanin compounds are considered to be health-promoting phytochemicals as antioxidants. From the results, it can be noticed that the vine treated with F11A1 in both seasons was the optimum treatment for increasing the values of the total phenols and anthocyanin.

3.8. Mineral content in leaves

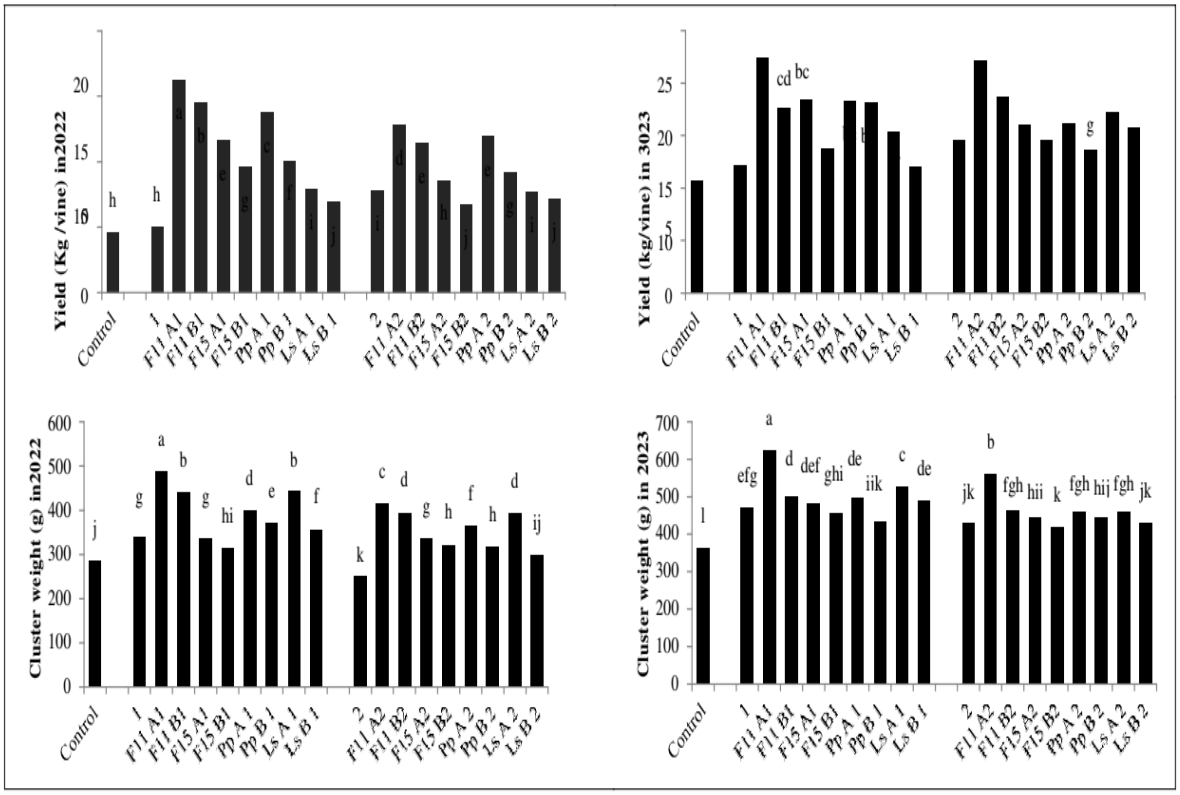
Data in Fig. 8 and 9 showed the impact of the application of fusants and natural rocks on the mineral content of grape leaves. As a result, the N% data indicated that adding the different bacterial strains for both levels (100 and 200 mL) to the natural rock caused a significant increase in the N% compared with the control and application for natural rock only.

In the second season, the same general trend was observed in N, P, and K%, where the maximum was when vines were treated with F11B2, which recorded 2.12%, 0.45%, and 1.90% in N, P, and K, respectively, in grape leaves compared with the application for natural rock only and the control.

4. Discussion

Plant parasitic nematodes (PPNs) cause significant damage and yield losses to agricultural, forestry, ornamental, and officinalis plants, resulting in annual losses of 12-25% of total production [43, 44]. Currently, the use of nematicides is restricted in agriculture according to EU legislation. This prompts a search for eco-friendly nematode control strategies, including bio-pesticides for sustainable agriculture [23.45,46]. It may be essential to apply bacteria as bio-control and bio-fertilizer agents in order to preserve an environment. We can reduce the use of nematicides by suppressing nematodes and replacing chemical fertilizers with one more safely [47].

Protoplast fusion is used to develop and improve bacterial strains and make an organism more capable of carrying out bioprocesses, and it is essential to the production of various chemicals in industry [23]. In order to achieve this, protoplast fusion was conducted between two strains: *L. sphaericus*, a nitrogen fixation strain that grows on free nitrogen media and *P. polymyxa*, which has the capacity to solubilize phosphate and potassium.



A= 100 mL of bacterial strain, B= 200 mL of bacterial strain, 1=¼ kg rock phosphate + ½ kg feldspar, 2= ½ kg rock phosphate + 1 kg feldspar

Fig.7 Impact of soil application of fusants and natural rocks on yield kg/vine and cluster weight (g) of Flame seedless grapevine during 2022-2023

Table 5 Impact of soil application of fusants and natural rocks on some physical characteristics of the berries of Flame seedless grapevine during 2022-2023.

Treatments	Berry weight (g)		Berry size (cm^3)		Juice volume of 100g berries (mL)		Length/diameter ratio of berry	
	2022	2023	2022	2023	2022	2023	2022	2023
Control	4.38 g	3.76 g	4.40 d	3.53 f	406.67ab	245.00gh	1.12 ab	1.03 a
NR only1	4.50 fg	3.83 fg	3.92 g	3.63 ef	371.67 efg	268.33de	1.07 bcd	0.99 a
F11A1	5.49 a	5.04 a	5.08 a	4.75 a	423.33 a	326.67 a	1.15 a	1.05 a
F11B1	5.12 b	4.78 ab	4.92 ab	4.43 b	375.00 ef	233.33 hi	1.02 d	1.01 a
F15A1	4.51 fg	3.80 fg	4.92 ab	3.58 ef	381.67c	196.67 j	1.04 c	0.99 a
F15B1	4.71 ef	3.92 efg	3.83 g	3.23 g	300.00 k	290.00bc	1.09 ab	0.99 a
PpA1	4.92 bc	4.57 bc	4.58 cd	4.23 bc	343.33 hi	300.00 b	1.09 ab	1.01 a
PpB1	4.58 e	4.52 cd	4.25 ef	4.13 cd	358.33 fgh	231.67 i	1.11 ab	1.01 a
LsA1	5.06 bc	4.46 cd	4.75 bc	4.17 cd	395.00 bcd	276.67cd	1.14 a	1.01 a
LsB1	5.03 bc	4.34 d	4.17 f	4.02 d	325.00 j	281.67cd	1.07 bcd	1.02 a
NR only2	4.78 de	3.85 efg	4.42 de	3.55 f	351.67 h	260.00 f	1.08 bc	1.03 a
F11A2	5.01 bc	4.61 bc	5.00 a	4.33 bc	343.33 hi	285.00 c	1.06 bcd	1.03 a
F11B2	5.00 bc	4.34 d	4.50 d	4.07 d	331.67 ij	280.00cd	1.07 bcd	1.00 a
F15A2	4.87 cd	4.46 cd	4.25 ef	4.17 cd	355.00 gh	246.67 g	1.09 abc	1.00 a
F15B2	4.64 ef	4.00 ef	4.25 ef	4.08 d	344.67 hi	270.00de	1.06 bcd	1.02 a
PpA2	5.43 a	3.56 h	5.08 a	3.33 g	291.67 l	230.61 i	1.10 ab	1.00 a
PpB2	4.82 cde	4.05 e	4.58 cd	3.75 e	378.33cde	285.00 c	1.06 bcd	1.02 a
LsA2	4.73 ef	3.93efg	4.75 bc	3.60 ef	343.33 hi	236.67gh	1.12 ab	1.03 a
LsA2	5.45 a	4.46 cd	5.17 a	4.20 cd	396.67bc	233.33 hi	1.06 bcd	1.03 a

A=100 mL of bacterial strain, B= 200 mL of bacterial strain, 1=¼ kg rock phosphate + ½ kg feldspar, 2= ½ kg rock phosphate + 1 kg feldspar

Table 6 Impact of soil application of fusants and natural rocks on some chemical characteristics of flame seedless grapevine during 2022-2023

Treatments	SSC%		Juice acid content %		SSC/acidity ratio		Total phenols (mg/ 100 g f.w)		Anthocieanine (mg/ 100 g f.w)	
	2022	2023	2022	2023	2022	2023	2022	2023	2022	2023
Control	15.60 b	13.83 k	0.76 a	0.80 a	20.53 i	17.40 i	33.00 ef	22.27 g	4.11 ef	4.61 fg
NR only1	14.20 fg	14.33 ijk	0.57 h	0.39 d	24.80 de	38.35 b	34.10 de	26.01 cd	4.90 c	5.25 d
F11A1	14.70 ef	15.33 gh	0.71 bc	0.40 d	20.67 i	38.37 b	40.97 a	31.11 a	7.97 a	8.28 a
F11B1	15.70 bc	18.43 ab	0.67 d	0.48 b	23.57 ef	39.06 b	29.62 g	26.65 c	7.25 a	7.52 b
F15A1	14.80 de	16.33 ef	0.68 cd	0.38 d	21.82 h	43.55 a	35.86 c	28.74 b	3.42 h	3.54 k
F15B1	18.20 a	17.63 bc	0.59 gh	0.43 c	31.11 a	41.70 a	35.23 cd	30.20 a	4.46 d	4.60 fg
PpA1	13.70 g	15.33 gh	0.73 ab	0.43 c	18.85 j	36.44 c	36.66 bc	21.91 gh	3.44 h	3.52 k
PpB1	13.70 g	17.50 cd	0.65 d	0.49 b	21.03 i	36.09 cd	23.66 j	22.68 g	4.54 d	4.99 e
LsA1	14.80 de	14.00 jk	0.72 b	0.43 c	20.79 i	33.04 ef	34.05 de	22.51 g	3.73 g	3.81 j
LsB1	15.50 cd	13.83 k	0.63 e	0.50 b	24.60 de	27.83 h	34.07 de	23.03 fg	4.59 d	4.76 ef
NR only2	14.50 ef	14.67 hij	0.65 de	0.43 c	22.25 gh	34.66 de	31.77 f	24.92 de	3.92 f	4.14 i
F11A2	15.50 cd	19.00 a	0.61fg	0.45 c	25.29 cd	42.81 a	33.31 ef	24.73 e	4.29 de	4.50 gh
F11B2	15.70 bc	16.67 de	0.68 cd	0.45 c	23.09 fg	37.50 bc	23.82 j	24.77 de	2.58 i	2.95 l
F15A2	14.50 ef	16.33 ef	0.52 i	0.43 c	28.17 b	38.81 b	28.18 h	21.00 hi	3.54 h	3.68 jk
F15B2	17.20 a	14.93 hi	0.66 de	0.48 b	26.14 c	31.49 f	37.78 b	21.06 hi	1.64 j	1.77 m
PpA2	15.70bc	14.00 jk	0.59 gh	0.48 b	26.78 b	29.74 g	23.58 j	20.39 i	4.49 d	4.88 e
PpB2	16.30 b	14.67 hi	0.67 cd	0.43 c	24.32 de	34.66 de	32.51 ef	21.96 g	5.76 b	6.07 c
LsA2	15.50cd	15.33 gh	0.73 ab	0.43 c	21.28 h	36.30 c	25.89 i	20.28 i	3.97 f	4.13 i
LsA2	14.80de	15.00 hi	0.62 ef	0.40 c	23.86 ef	37.63 bc	32.38 f	22.59 g	3.90 fg	4.29 hi

A=100 mL of bacterial strain, B= 200 mL of bacterial strain, 1=¼ kg rock phosphate + ½ kg feldspar, 2= ½ kg rock phosphate + 1 kg feldspar

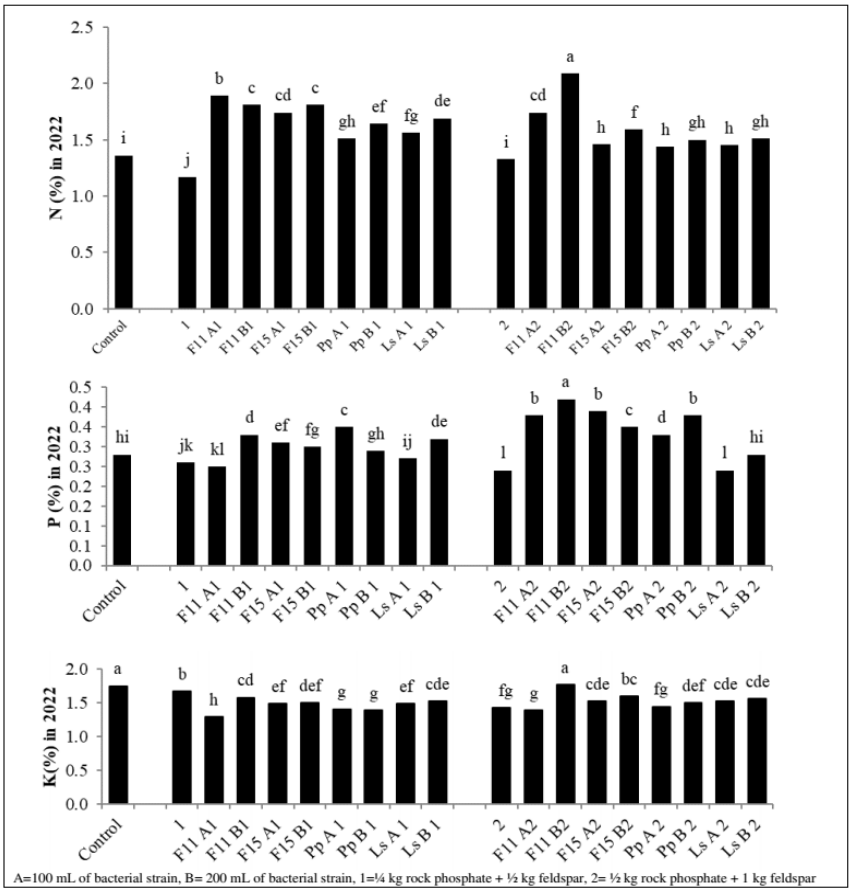


Fig. 8 Impact of soil application of fusants and natural rocks on nitrogen, phosphorus, and potassium percentages of Flame seedless grapevine during 2022

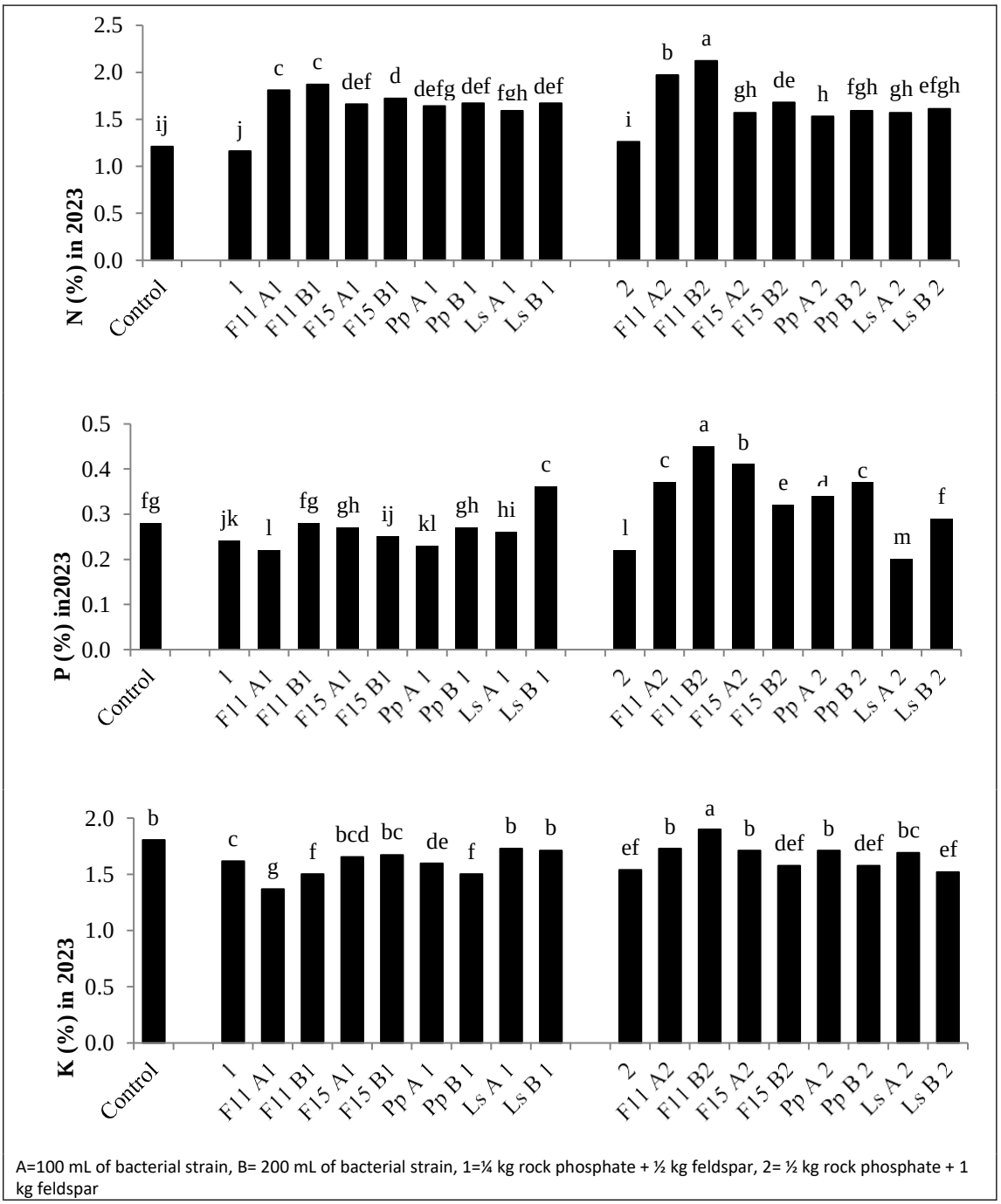


Fig. 9 Impact of soil application of fusants and natural rocks on nitrogen, phosphorus, and potassium percentages of Flame seedless grapevine during 2023

Fourteen fusants that were obtained from the protoplast fusion experiment were cultivated on N, P, and K media. The proteins' SDS-PAGE examination verified that all 14 recombinants had inherited and expressed a large number of distinct protein bands from their parent strains. By transferring and expressing genes that code for nitrogen fixation and the solubilization of potassium and phosphate elements, the two parental strains enhanced the fertility of the new recombinants F11 and F15 by transferring their genetic material to them. The findings presented in this study are in agreement with

Vasileva et al. [24] who reported that bacterium can recombine through protoplast fusion, which permits recombination between the parental chromosomes to create new strains with enhanced phenotypes for biotechnological and agricultural applications by fusing advantageous alleles from different species. The induction or inhibition of genes is shown by an increase or decrease in the number of protein bands, and this change was reflected in nematode control. The outcome was consistent with that of Mohamed et al. [23] and Soliman et al. [3] who detected an increasing number of bands in the fusants when compared with their parent and reflected on improvements in nematode bio-control and plant growth parameters.

The data related to nematode infection indicated that the use of fusant strains as a bio-control and bio-fertilizer in conjunction with natural rocks results in the greatest reduction of different nematode parameters when compared to the parental strains treated with neutral rocks only as control. These results are in agreement with those of Mohamed et al. [23] and Soliman et al. [3]. In the present study, we found that the use of the protoplast fusion technique enhances biological control of root-knot nematode *M. incognita* in grapevines, with fusants F11 and F15 achieving the best reduction in nematode reduction in flame seedlings. According to the results, the 2023 season shows the greatest decrease in all nematode parameters as compared to the 2022 season of grapevine root. There was a positive correlation found between nematode reduction and the bacterial dose, rate of fertilizer used, and season this result agrees with Ismail et al. [48].

It was generally observed that adding natural rocks and bacteria increased the amount of the yield, this is in agreement with Hagagg, et al [49] who found that rock phosphate enriched with P mobilizing bacteria gave higher yield and fruit weight and improved the physical and chemical properties of fruits 'Wonderful' pomegranate. Ganzour et al. [13] highlighted the role of phosphate solubilizing bacteria in providing phosphorus to plant roots, reducing soil acidity, increasing nutrient availability, and enhancing growth and productivity. Bio-fertilization is more environmentally beneficial than acidulation, biological solubilization of rock phosphate will release phosphorus for plant uptake and lessen pollution to the surrounding area, and the use of rock phosphate for crop productivity by inoculation with bacteria has been demonstrated to be an effective method [14]. Moreover, the usual method for releasing potassium from rock-feldspar is to employ B. circulans, a bacterium that dissolves silicate [50]. On the same side, El Sayed et al. [16] revealed that in addition to the chemical phosphorus and potassium that are readily available in the soil, as well as their content and absorption, the natural P and K rock fertilizers applied in conjunction with biofertilizers in sandy soil will provide high available and uptake nutrients, yield, and fruit quality comparable to those obtained by applying chemical phosphorus and potassium.

The F11A1 is the best treatment for an increase of yield and cluster weight or the majority of plant growth in both seasons 2022 and 2023, the explanation is that by increasing fertilization the concentration increased nutrients, which prompted grape vines to increase vegetative growth at the expense of bunches and yields, as grape yields are negatively affected when fertilization exceeds what is required, this result agree with Hagagg et al. [49] on pomegranate and Hagagg et al. [51] on olive where they found that the amount of the yield at the low level of rocks and bacteria was better than the high level. On the contrary, Dursun et al. [52] reported that the bacterial bio-fertilizers improve the yield and nutrition of the tomato. However, when the amount of fertilization and the amount of bacteria were doubled, it did not affect the amount of the yield.

While, in N, P, and K the F11B2 recorded the best increase in the leaf where when the amount of fertilization and the amount of bacteria were doubled increasing N, P, and K level, this data agrees with Mehata et al. [53] who reported that bio-fertilizers have the ability to solubilize phosphorus and are now an efficient technique to boost organic farming and agree with Rogiers et al., [54], illustrated that potassium the most prevalent cation in grape berries at all stages of growth is a crucial nutrient for grapevines. Given that the structure of feldspar is potassium combined with aluminium silicate (feldspar contains a potassium level of 10% to 13%), it is challenging to apply directly to soil without bio-fertilization. In the same context, Hosny and Bakr [15] explained that adding several different bacterial strains in a liquid form to the soil has a high ability to decompose phosphate rock, and this gave positive results in terms of productivity and quality characteristics of the crop.

5. Conclusions

Protoplast fusion is a promising approach that holds great potential for generating genetically modified bacterial strains with enhanced bio-control or bio-fertilizer properties leading to improving management of plant parasitic nematodes and increasing grapevine yield production. In general, the F11B2 recorded the best nematode reduction and fixing of nitrogen, solubilize phosphorus and potassium. While F11A1 recorded an increase in yield and the majority of physical and chemical characteristics of berries in both seasons 2022 and 2023.

6. Conflicts of interest

The authors declare that they have no conflicts of interest

7. Acknowledgment

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