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Effect of Some Microorganisms on Controlling Fusarium Wilt of Roselle (*Hibiscus sabdariffa* L.)

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ABSTRACT

Roselle (*Hibiscus sabdariffa* L.) is one of the most crucial medicinal plants all over the world. The present study was carried out with the intention of solving some problems related to soil-borne diseases. *Fusarium oxysporum* is associated with wilt disease of roselle plant. The application of some microbiota *i.e.*, a mixture of *Pseudomonas fluorescens* (PSR-11), *Bacillus subtilis* (BSR-8), *Pleurotus ostreatus* and mycorrhizae® individually or in combination were investigated for controlling wilt disease, growth, and yield of roselle plant. The study was conducted in sandy textured soil under sprinkler irrigation of Ismailia Agricultural Research Station, Ismailia, Egypt. Under greenhouse conditions, all the tested bioagents reduced wilt incidence and increased the percentage of survived plants. The conglomeration of the bacterial mixture of *Pseudomonas fluorescens* and *Bacillus subtilis*, *Pleurotus ostreatus* and mycorrhizae® was more effective in controlling wilt disease than using any of them individually. The application of the investigated bioagents showed a significant increase in phenol, photosynthetic pigments, and dehydrogenase contents, as well as root colonization percent. The obtained results showed the ability of *B. subtilis* and *P. fluorescens* to produce lytic enzymes such as β -1-3 glucanase, β -1-4 glucanase, chitinase and protease, in addition to their production of siderophore and HCN. The consortium of all microbiota gave the best results for growth and yield parameters of roselle plant.

Keywords: Roselle, *Hibiscus sabdariffa*, wilt, *Fusarium oxysporum*, biological control, *Pseudomonas*, *Bacillus*, *Pleurotus*, mycorrhizae, defense-related enzymes.

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INTRODUCTION

Medicinal and aromatic plants are of great importance for their multiple uses in medicine, cosmetics, nutrition and refreshing beverages. As well, they are used in natural flavoring and aroma additives in food industries, besides their use as a source of insecticides. They are among the most crucial agricultural export commodities for hard currency gaining and for satisfying the demands of the European markets (Mohamed *et al.*, 2015, Al-Sayed *et al.*, 2020).

The value of roselle exports is about 6.6% of the total Egyptian agricultural exports (FAO 2018)

Roselle (*Hibiscus sabdariffa* L.) belongs to the family Malvaceae, also called “karkade”, is an important annual crop grown in tropical and sub-tropical regions. The commercially important part of the plant is the fleshy calyx (sepals) surrounding the fruit (capsules). The whole plant can be used as a beverage, or the dried calyces can be soaked in water to prepare a colorful cold drink or may be boiled in water and taken as a hot drink. It also has some medicinal properties. The two botanical types of roselle are *Hibiscus sabdariffa* var. *sabdariffa*, grown for its fleshy, shiny-red calyx, and *Hibiscus sabdariffa* var. *altissima* grown for its phloem fiber (Purseglove, 1974 and Mohamed *et al.*, 2012).

One of the most urgent obstacles that limits roselle production is wilt disease. The most frequent pathogenic soilborne fungi consorted with root rot and wilt diseases are *Fusarium oxysporum*, *Fusarium solani* and *Macrophomina phaseolina* (Ooi and Saleh, 1999; Sie *et al.*, 2011; Hassan *et al.*, 2014 and Hashem *et al.*, 2017).

Chemical treatments are still a major practice in agriculture and are widely used to control fungal soil-borne diseases. However, fungicides have two major consequences, (1) fungicide overuse threatens human health and causes

ecological concerns; and (2) this practice leads to the emergence of fungicide-resistant microorganisms in the environment (Lamichhane *et al.* 2017).

Biological control is a non-chemical potential method for plant disease configuration by reducing the injurious effects of a parasite or pathogen via the use of other living entities. The rhizosphere is a nutrient-rich habitat that harbors a vast variety of bacteria and fungi that each can have neutral, beneficial or deleterious effects on the plant. Some of these organisms can improve plant growth by various mechanisms (Sahu *et al.*, 2018).

Microbial community constitutes most extraordinary and ubiquity life on the earth, but the commonness of microorganisms is higher in nutrient-rich niches as the rhizosphere. Thus, the rhizosphere is considered the 'focal point' for microbial colonization and activity (Sindhu and Sharma, 2020).

Plant growth promoting rhizobacteria (PGPR) has a vital role in agriculture. These positive effects of PGPR have direct or indirect performance on plants, direct promotion of growth by production of metabolites that enhance plant growth, indirect growth promotion occurs via the removal of pathogens by the production of secondary metabolites. *Pseudomonas* spp. and *Bacillus* spp. are the most promising groups of rhizosphere inhabitants able to control pathogenic soilborne microorganisms (Sarhan and Shehata, 2014; Vejan *et al.*, 2016; Tsegaye *et al.*, 2017 and Gouda *et al.*, 2018).

Bacillus subtilis has been widely used in agriculture attributable to its environmental safety, good biocontrol efficacy and straightforward industrial production. *B. subtilis* attains biocontrol natures, which include plant colonization, plant growth promotion, pathogen inhibition and induced systemic resistance activation (Hashem *et al.* 2019). In *B. subtilis*, biofilm formation on the root surface is critical for plant roots colonization and for biocontrol efficacy against plant pathogens (Al-Ali *et al.* 2018). Active substances production is an important aspect for assessing the biocontrol effectiveness of a beneficial strain (Gao *et al.* 2016). *B. subtilis* produces numerous substances with broad-spectrum antibacterial activity, which includes bacteriocins, lipopeptide antibiotics and antibacterial proteins (Gu *et al.*, 2021). It has been suggested that various antibacterial substances act synergistically to hinder phytopathogen growth (Alanjary and Medema 2018). The production of siderophores,

phytohormones, volatile compounds, lipopeptides and phytases help *B. subtilis* to provoke plant growth and induce plant immune responses (Franco-Sierra *et al.* 2020).

Pseudomonas fluorescens was adapted to survive in soil and colonize plant roots. The microbial inoculants utilized in agriculture as biofertilizers, biocontrol agents and plant growth promoting rhizobacteria (Kiely *et al.*, 2006). Fluorescent *Pseudomonas* spp. were among the most effective rhizosphere bacteria in diminishing soil-borne diseases in infected soils (Weller, 1988), where disease incidence was low, despite the presence of pathogens and environmental conditions conducive to disease popularity. Strains of *Pseudomonas fluorescens* enhance seed germination, shoot and root development of different crops. Some strains have the ability to devastate the fungal cell wall via secreting lytic enzymes and suppress the growth of fungal pathogens via secreting hydrogen cyanide and antibiotics. Several modes of action for antagonistic PGPR had been reported, for instance, *Pseudomonas* spp. production of various antimicrobial compounds and induction of plant defense mechanisms (Sindhu *et al.*, 2016 and Zian and Aly, 2020).

Increased plant growth of arbuscular mycorrhizal fungi AM colonization is well documented. AMF can act as biological agents for alleviating the severity of disease caused by root pathogenic fungi, bacteria and nematodes (Bagyaraj, 2018).

The induction of plant defense mechanisms such as phenolic compounds production through the application of products like mushroom culture filtrate of *Pleurotus ostreatus* is a promising strategy to help the plant to complete its life cycle in the presence of pathogen without a marked reduction in yield and the application of such a strategy is reflected on whole plant health. (Elsakhawy *et al.*, 2020).

The main objective of this investigation is to study the effect of some microbiota on controlling wilt disease and their effect on growth and yield of roselle plant.

MATERIALS AND METHODS

1- Isolation, purification and identification of the pathogen.

Infected roselle plants displaying typical symptoms of wilt were collected from different localities of Ismailia Governorate. Infected stem parts were cut into small pieces (1 cm), surface sterilized with sodium hypochlorite (2 %) for 2 minutes then immersed in distilled water 3 times

for washing. The washed pieces were dried between sterilized filter paper till dehydration of moisture. The pieces were then transferred to sterilized potato dextrose agar medium (PDA) plates. The plates were incubated at 28°C for 4-5 days until the colonies developed. Isolated fungi were purified by single conidial spores' techniques (Sinclair and Dhingra; 1995) and identified based on morphological characters as described by (Leslie and Summerell, 2006).

2- Pathogenic potential of the isolated pathogen:

The fungus recovered from the infected stems was tested for pathogenicity on roselle plants after 60 days of cultivation. Plant stems were punctured with a sterile needle, laden with a 5 mm disc of the isolated fungal growth (7 days old) which was confined to the stem.

3- Preparation of pathogen inoculum:

For soil infestation, 500 ml flasks, each containing 100 ml Potato Dextrose broth, were used for the preparation of *F. oxysporum* inoculum. Each flask was seeded with 5-mm disk of actively growing seven day's old *F. oxysporum* cultures, then incubated at 25 °C for 7 days. The contents of the flasks were homogenized in a blender for 1 min.

4- Preparation of biocontrol agents:

Bacillus subtilis (BSR-8), *Pseudomonas fluorescens* (PSR-11), *Pleurotus ostreatus* and mycorrhizal fungi were kindly obtained from Soil, Water and Environmental Institute, Cairo, Egypt.

4.1- Preparation of bacterial strains:

Pseudomonas fluorescens and *Bacillus subtilis* strains were cultured individually in King's broth medium in 250-ml flasks and placed on a rotary shaker at 120 rpm for 48 hrs at 28±1°C. Then, the cell suspension of each strain was adjusted to provide about 10⁷ CFUml⁻¹. A mixture of the two bacterial strains was applied before planting as seed treatment in addition to their application after 30 days of planting as soil treatment at a rate of 20 L fed⁻¹.

4.2 - Preparation of *Pleurotus ostreatus*:

Glass bottles containing sterilized PD broth were inoculated with *Pleurotus ostreatus*. The bottles were incubated on a shaker at 1200-1500 rpm for 7 days at 25°C. The contents of the bottles were homogenized in a blender for 1 min and applied before planting as seed treatment and applied after 30 days of planting as soil treatment at a rate of 20 L fed⁻¹.

4.3- Preparation of mycorrhizal fungi:

Mycorrhizeen® (mixture of mycorrhizal spores) was kindly obtained from Soil, Water and

Environmental Institute, Cairo, Egypt. Mycorrhizeen® was added as a recommended dose, 10 gm mycorrhizeen®/ seed.

5- Antagonistic test:

5.1- Antibiosis of biocontrol agents towards the fungal pathogen:

PDA medium amended with peptone, CaCO₃, and MgSO₄ (3.0, 0.2 and 0.2 gm/L, respectively) to examine the antagonism between the bioagents and the pathogen. The pathogenic fungi and each of the biocontrol agents were inoculated equidistantly from each other in Petri plates. Plates inoculated with the tested pathogen alone served as control. The plates were incubated at 25 -30 °C until the control plates reached full growth and inhibition zone diameters were measured. Five replicates were used for each treatment. Mycelial growth inhibition was calculated using the formula (Fokemma, 1973):

$$\text{Mycelial growth inhibition (\%)} = 100 (C-T/C)$$

Where:

C=growth in control

T=growth in treatment.

5.2- Antibiosis of biocontrol agents towards the fungal pathogen using the biocontrol agent filtrate:

The biocontrol agent filtrates were examined against *F. oxysporum* grown on PDA medium amended with peptone, CaCO₃, and MgSO₄ (3.0, 0.2 and 0.2 gm/L) to examine the antagonism between the bioagents and the pathogen. One ml of bioagent filtrate was transferred using sterile micropipette into the used media. The plates were centrally inoculated with the pathogenic fungi. Plates inoculated with the tested pathogen alone served as control. The plates were incubated at 25 -30 °C until the control plates reached full growth and inhibition zone diameters were measured. Five replicates were used for each treatment. Mycelial growth inhibition was calculated by using the formula (Fokemma, 1973):

$$\text{Mycelial growth inhibition (\%)} = 100 (C-T/C)$$

Where:

C=growth in control

T=growth in treatment.

6- Biochemical activities of bioagents:

6.1- Siderophores production:

Trypticase soy agar (TSA) medium amended with 8-hydroxyquinoline (50 mg/ liter) to chelate trace ferric iron (Fe³⁺) in a form that is unavailable to most microorganisms and thus 8-hydroxyquinoline proved to be an effective selective agent for siderophore-producing microorganisms (Alexander and Zuberer, 1991).

Plates of TSA medium were inoculated with filter paper disc saturated with freshly grown bioagent cultures and incubated at 28°C for 8 days. Organisms grown on TSA medium were considered positive for siderophoric compounds production.

6.2- Hydrogen cyanide production:

The cultures showing biocontrol potential against pathogens were streaked on Rose Bengal Agar (RBA) medium supplemented with glycine (4.4 g/L) for the quantitative determination of HCN from glycine by antagonistic bioagents. Inoculated plates were inverted and a strip of sterilized filter paper Whatman no. 1 saturated with 0.5% picric acid in 2% (w/v) sodium carbonate was placed in the upper lid. The plates were then sealed with parafilm in order to contain gaseous metabolites produced by the antagonists and to allow for chemical reaction with picric acid present in the filter paper adding and incubated at 28°C for 24-72 hr. A change in filter paper colors from yellow to orange-brown indicate the production of HCN. Yellow, limit cyanide production; orange, moderate cyanide production; light brown, relatively high cyanide production and brown, high cyanide production (Lorck, 2004).

6.3- β -1,3 glucanase (Laminarinase):

The enzyme-catalyzed reaction was measured with 2.5mg/ml soluble laminarin as a substrate in a 40mM potassium phosphate buffer, pH 7.0, at 30°C, for 15 min to obtain the reaction-time course. The reducing power caused by the enzymatic reaction from the substrate was determined by Somogyi-Nelson a method by which absorbance at 600 nm was measured (Somogyi, 1945). One unit of the enzyme was defined as the "amount of enzyme", which liberates 1 μ mol of reducing sugar equivalent to glucose per one min under the standard assay conditions.

6.4- β -1,4 glucanase (cellulase):

Cellulase activity was determined by incubation of 900 μ L of 1% (CMC) carboxy methyl cellulose in 20 mM phosphate buffer (pH 7.0) with 100 μ L of appropriate concentration of enzyme at 50°C. After 30 min reaction, 1 mL of dinitrosalicylic acid (DNS) was added and boiled in a water bath for 15 min to stop the reaction. The resulted samples were then cooled to room temperature and measured the absorbance at 540 nm (Miller, 1959). One unit of cellulase activity was defined as the amount of enzyme that could hydrolyze carboxymethylcellulose and release 1 μ mol of glucose within 1 min reaction time at 50°C.

6.5- Chitinase activity:

Colloidal chitin (1% in 50 mM acetate buffer) was used as the substrate for the measurement of chitinase activity. The mixture of enzyme solution (0.5 ml) and substrate (0.5 ml) was incubated at 40°C for 60 min. After centrifugation, the amount of reducing sugar produced in the supernatant was determined by the method of Monreal and Reese (1969) with N-acetylglucosamine as a reference compound. One unit of enzyme activity was defined as the amount of enzyme that produced 1 mole of N-acetylglucosamine per min.

6.6- Protease:

Protease activity (casein degradation) was determined from clearing zone in Skimmed Milk Agar (SMA) according to Nielsen *et al.* (1998).

7- Agricultural practices:

7.1- Seed treatments:

Two varieties of roselle seeds (*Hibiscus sabdariffa* L.) *i.e.*, bright (c.v. Light Red Sepals) and dark (c.v. Sabahia 17) cultivars were kindly obtained from Horticultural Research Institute, Agricultural Research Center, Giza, Egypt. Seeds were surface disinfected by immersing in sodium hypochlorite (1%) for 2 minutes, and washed several times with sterilized water, then soaked for one hour before planting in the tested treatments as following: (1)- Bacterial mixture of *Bacillus subtilis* + *Pseudomonas fluorescens* (BM), (2)- *Pleurotus ostreatus* (PO), (3)- Mycorrhizal fungi (M), (4)- BM+ PO, (5)- BM + M, (6)- PO + M, (7)- BM + (PO) + (M), (8)- Infected control, (9)- healthy control.

7.2- Greenhouse experiment

A greenhouse experiment was carried out at Ismailia Agricultural Research Station, Agricultural Research Centre during the summer season of 2018/ 2019 to evaluate the previous treatments against roselle wilt and their effect on growth and yield of two roselle varieties. The experiment was conducted in a completely randomized design with 5 replicates. Pots (30 cm in diameter) with a bottom drainage hole filled with sandy disinfested soil (10 kg). Soil infestation was achieved by adding 100 ml of *F. oxysporum* (1.4 x 10⁶ spore/ml) pure culture grown on PD broth to each pot. The infested soil was irrigated before planting to insure its distribution in the soil. Five treated roselle seeds were sown in each pot just after irrigation. Pots were irrigated at field capacity whenever required. Before planting, all pots were received phosphorus as calcium super-phosphate (15.5 % P₂O₅) at a rate of 100 kg P₂O₅ fed⁻¹. Potassium was added in the form of potassium sulphate (48

% K₂SO₄) at a rate of 60 kg K₂SO₄ fed⁻¹ once before the 1st irrigation. Nitrogen was added as ammonium sulphate (20.5 % N) at the rate of 70 kg N fed⁻¹ in three equal doses i.e., 1/3 at cultivation time as based fertilization, 1/3 at stem initiation stage and the remaining was applied at bud initiation before flowering stage.

7.3- Impact of some biocontrol agents on Fusarium wilt incidence:

The disease incidence (DI) % was determined by recording percentages of wilted and survived plants according to the following formula:

Wilted plants % =

$$\frac{\text{Total No. of wilted plants}}{\text{Total No. of planted seeds}} \times 100$$

Survived plants % =

$$\frac{\text{Total No. of survived plants}}{\text{Total No. of planted seeds}} \times 100$$

8- Microbial activity after 60 days from planting:

Three replicate plants of each treatment were uprooted at 60 days after planting to determine the following parameters:

8.1- Dehydrogenase activity:

Dehydrogenase activity was determined according to Skujins (1976) based on the use of triphenyltetrazolium chloride (TTC) as an artificial electron acceptor. Nearly all microorganisms reduce TTC to triphenylformazan (TPF) which can be estimated after incubation at 30°C for 24 hrs. Because of the light sensitivity of TTC and TPF, all procedures should not be performed under diffused light; 2 g dry soil placed in test tubes (60 ml volume and 2.5 cm diameter) were mixed with 5 ml of TTC solution, and the tubes were sealed with rubber stoppers and incubated for 24 h at 30°C. The control contains only 5 ml tris buffer (without TTC). After the incubation, 40 ml acetone were added to each tube and the tubes were shaken at interval thoroughly and further incubated at room temperature for 2 hours in the dark. The soil suspension (15 ml) was filtrated, and the optical density of the clear supernatant was measured against the blank at 545 nm (red colour). The enzyme activity was determined using a standard curve of TPF according to the following equation:

$$\text{Dehydrogenase activity TPF } (\mu\text{g/D.Wt./g soil}) = \frac{\text{O.D/K}}{\text{D.Wt}}$$

Where:

O.D.= optical density

K = The obtained factor from the standard curve

D.Wt= dry weight of soil sample

8.2- Microbial root colonization:

Microbial root colonization was determined using the method described by Bilal *et al.* (1993). Ten centimeters of cleaned fresh root samples were chopped off and any soil particles were eliminated, then immersed with 2 ml of 2.3.5 chloride solution (TTC) in test tubes. Tubes were then incubated in dark at 30°C for 24 hours. After immersing roots in TTC, samples were left to dry in room temperature. Colonization was examined under a light microscope where plant tissues and tetrazolium reduction were visible as red color due to reduction of tetrazolium by bacterial cells. The percentage of colonization was calculated as follows:

Colonization length =

Root length – Non-colonized length

$$\text{Colonization \%} = \frac{\text{Colonization length}}{\text{Root length}} \times 100$$

8.3- Determination of total phenol contents:

Total phenol contents in plant leaves were estimated with the procedure given by Zieslin and Ben-Zaken (1993). One gram of tissue was homogenized in 10 ml of the methanol 80% and agitated for 15 min at 70°C. One ml of methanolic extract was added to 5 ml distilled water and 250µl of Folin reagent (1N), then the solution was kept at 25°C, after 3 min, 1ml of a saturated solution of Na₂CO₃ and 1ml of distilled water were added, and the mixture was incubated at 25°C for 1 h. The absorbance of the blue color developed was measured at 725nm. The content of total phenols was calculated according to the standard curve obtained from a Folin- reaction with a phenol solution and expected was phenol equivalent in µg g⁻¹ fresh tissue.

8.4- Determination of photosynthetic pigments:

The photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids) were determined quantitatively as described by Metzner *et al.* (1965). A known fresh weight of leaves (samples of the same fresh weight were dried at 50°C till constant weight to be used for final calculation), was homogenized in 85 % aqueous acetone for 5 minutes (the experiment should be carried out in the dark); the homogenate was centrifuged at 4000 rpm. The supernatant was made up to known volume with 85 % acetone. The absorbance was measured calorimetrically against a blank of pure acetone at wavelengths; 452.5, 644 and 663 nm, taking into consideration the dilution made. The concentrations of pigment fractions (chlorophyll a, chlorophyll b and

carotenoids) were calculated as $\mu\text{g/ml}$ by using the following equations:

$$\text{Chlorophyll a} = 10.3 E_{663} - 0.918 E_{644}$$

$$\text{Chlorophyll b} = 19.7 E_{664} - 3.87 E_{643}$$

$$\text{Carotenoids} = 42. E_{452.5} - (0.0264 \text{ chlorophyll a} + 0.426 \text{ chlorophyll b})$$

Where: E = absorbance

The results were expressed as mg/g dry weight.

9- Growth parameters:

Plant samples were collected at harvest stage after 160 days. Shoot length (cm plant^{-1}), fresh and dry shoot weight (gm plant^{-1}) were determined.

10- Yield parameters:

Yield parameters expressed as fruit fresh weight (gm pot^{-1}), fresh and dry calyx yield (gm plant^{-1}) and seed yield (gm pot^{-1}) were calculated.

11- Extraction of roselle root exudates:

Root secretions of two roselle varieties (bright and dark) seedlings were extracted and examined for their composition and influence on the growth of different microorganisms. The procedure of Vancura and Hanzlikova (1972) was adopted. Seeds were washed several times with water and then sterilized for 2 minutes with 2 % sodium hypochlorite. The sterilizing agent was removed thoroughly by washing with sterilized distilled water.

Seeds were then planted in sterilized coarse sand under greenhouse conditions. Fifteen days after planting, the above ground parts of seedlings were cut and sand with root system was repeatedly washed with sterilized distilled water to get 125 ml of the final fluid to obtain 100% root exudates solution. The solution enriched was kept in a freezer until analysis (Shehata, 1997).

Various concentrations *i.e.*, 10, 25, 50, 75 and 100 % of roselle root exudates were prepared. Ten ml of each concentration were transferred respiratory using sterile micropipette into 250 ml King's medium and PDA medium to investigate their effects on the growth of both *B. subtilis* and *P. fluorescens* and *P. ostreatus*, respectively. The appropriate media containing the previously mentioned quantities of root secretions were inoculated with 0.1 ml of 48 hours old cultures of the tested microorganisms, then incubated at 30 ° C. Colony counts of microbial groups were determined.

12- Statistical analysis:

Data were collected for statistical analysis according to Snedecor and Cochran (1980). Mean values were compared at a level of $P < 0.05$ by using Least Significance Difference (L.S.D) test. SPSS (v. 24, IBM Inc., Chicago, IL, USA)

and Costat (v. 6.400 Co Hort software., California, USA) were used for data statistical analysis.

RESULTS

1- Identification of the isolated pathogen:

The isolated fungus was identified according to its morphological characteristics and microscopic examination according to Leslie and Summerell (2006). The isolate was identified to its species by specialists in Plant Pathology. Res. Inst., Agric. Res. Center, Giza, Egypt.

2- Pathogenic potential of the isolated pathogen:

The pathogenic potentiality of the isolated fungus was determined. Disease symptoms observed on plants grown in artificially infested soil were almost similar to those noticed under field conditions, as illustrated in Figure (1). Symptoms started with leaves yellowing then turn to brown, and the entire plant became brown with dried leaves followed by death of the entire plant.

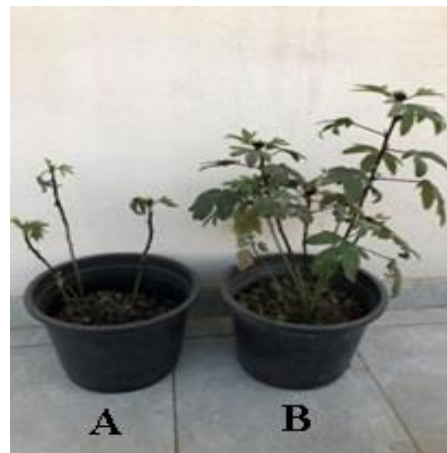


Figure (1): Wilt of roselle (*Hibiscus sabdariffa* L.) plants artificially infected with *F. oxysporum* (A) Infected plant (B) Control

3- Antagonistic tests:

3.1- Antibiosis of biocontrol agents towards the fungal pathogen:

Data in Table (1) reveal that *P. fluorescens* had significant antagonistic effect against *F. oxysporum* with a linear reduction of 79.63 %, followed by *B. subtilis* (70.93 %). While *P. ostreatus* showed the least ability to antagonize the pathogenic fungus with a reduction of 41.11 % only.

3.2- Antibiosis of biocontrol agents towards the fungal pathogen using the biocontrol agent filtrate:

The application of the microbial filtrate led to inferior effect comparing with the application of

the agent itself. This was adverted to in Table (2), where the bacterial mixture of both *B. subtilis* and *P. fluorescens* showed a significant linear

reduction of 75.37 %, however, *P. ostreauts* has no significant reduction 5.93 %.

Table (1): Effect of *B. subtilis* (BSR-8), *P. fluorescens* (PSR-11) and *P. ostreauts* on the linear growth in millimeter of *F. oxysporum* grown on potato dextrose agar medium:

Treatments	Linear growth (mm)	Reduction (%)
	Mean \pm S.E**	Mean \pm S.E**
<i>B. subtilis</i> (BSR-8)	26.2 \pm 1.856	70.93 \pm 2.062
<i>P. fluorescens</i> (PSR-11)	18.3 \pm 4.640	79.63 \pm 5.155
<i>Pleurotus ostreauts</i>	53 \pm 2.930	41.11 \pm 3.255
Control	90 \pm 0.000	0 \pm 0.000
L.S.D. (0.05)	11.483	12.759

Bacterial mixture (BM)*: Bacterial mixture of *Bacillus subtilis* (BSR-8) and *Pseudomonas fluorescens* (PSR-11). S.E**: Standard error

Table (2): Effect of bacterial mixture filtrate of both *B. subtilis* (BSR-8) and *P. fluorescens* (PSR-11) and of *P. ostreauts* filtrate on the linear growth in millimeter of *F. oxysporum* grown on potato dextrose agar medium:

Treatments	Linear growth (mm)	Reduction (%)
	Mean \pm S.E**	Mean \pm S.E**
Bacterial mixture (BM)*	22.17 \pm 22.167	75.37 \pm 5.215
<i>Pleurotus ostreauts</i>	84.67 \pm 0.333	5.93 \pm 0.370
Control	90 \pm 0	0 \pm 0
L.S.D. at 0.05	11.015	12.239

Bacterial mixture (BM)*: Bacterial mixture of *Bacillus subtilis* (BSR-8) and *Pseudomonas fluorescens* (PSR-11). S.E**: Standard error

4- Biochemical activities of the bioagents:

Data presented in Table (3) point to the production of hydrogen cyanide by both strains of *B. subtilis* and *P. fluorescens*. It was also clear from the displayed data, the variation among the two strains in the siderophore production, where it was higher with *P. fluorescens*. Protease production was detected in both *B. subtilis* and *P. fluorescens*. Biocontrol agents have the ability to

produce various enzymes such as β -1-3 glucanase, cellulase (β -1-4 glucanase) and chitinase which have the capability to degrade cell wall of pathogenic fungi. Data in Table (3) show that *P. fluorescens* surpass *B. subtilis* in the production of β -1-3 glucanase, β -1-4 glucanase and chitinase (22.2, 32.2 and 20.2 IU / ml, respectively).

Table (3): Plant growth promoting (PGRP) and lytic enzymes production by *Bacillus subtilis* (BSR-8) and *Pseudomonas fluorescens* (PSR-11).

Bioagent	Siderophore	HCN*	Protease	β -1,3 glucanase (IU/ml)	β -1,4 glucanase (IU/ml)	Chitinase (IU/ml)
<i>B. subtilis</i>	++	+	+	17.5	25.5	15.5
<i>P. fluorescens</i>	+++	+	+	22.2	32.2	20.2

HCN*: Hydrogen cyanide

5- Greenhouse experiment:

5.1- Impact of some biocontrol agents on

Fusarium wilt incidence:

The efficacy of bioagents as seed treatment and soil application against disease incidence of Bright and Dark roselle varieties was assessed under greenhouse conditions. Results in Figure (2) draw attention to the lack of significant

differences among varieties and among treatments in the disease incidence. It was clear that healthy control showed the highest level of plant survival in both Bright and Dark varieties, 88 and 84 %, respectively. Followed by the application of the mixture of *B. subtilis*, *P. fluorescens*, *P. ostreatus* and mychorrhizeen® in both Bright and Dark varieties, 80 and 64 %, respectively.

respectively. While the least plant survival percentage was recorded in the infected control, being 72 % in Bright variety and 56% in the Dark variety. The highest wilted plant percentage was

reported to the infected control grown in infested soil in Dark variety (44 %), and 36 % to the bacterial mixture treatment in Bright variety.

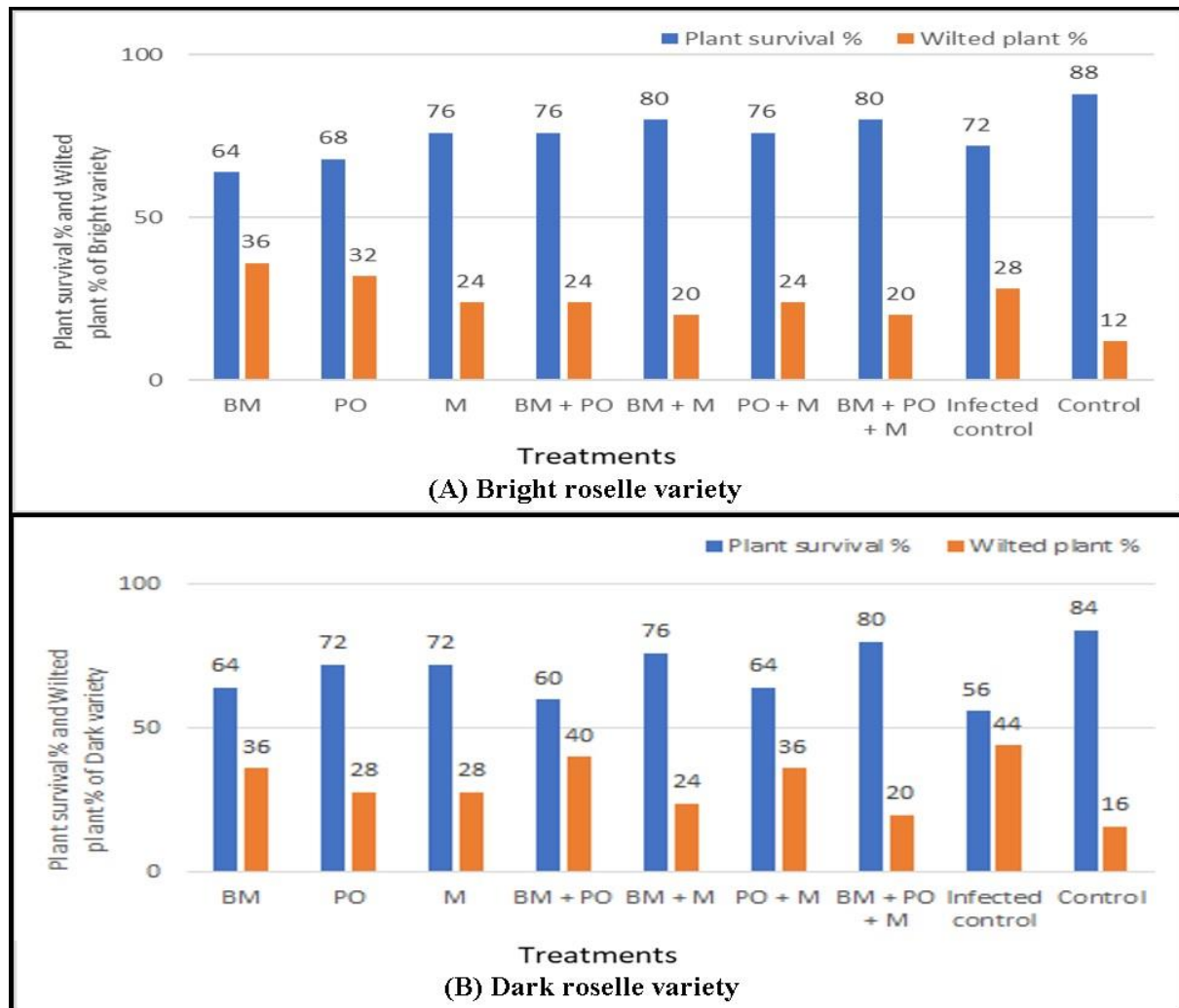


Figure (2): Percentages of wilted % and survived plants % as influenced by some bioagents on Bright (A) and Dark (B) roselle varieties grown in artificially infested soil by *F. oxysporum* under greenhouse conditions. Data are expressed as a mean \pm standard error, L.S.D. test (ANOVA). Statistical significance was considered as $P < 0.05$.

BM: Bacterial mixture, PO: *P. ostreatus*, M: Mycorrhiza.

5.2 - Dehydrogenase activity:

Data presented in Figure (3) show the ability of beneficial microorganisms to exist in a plenty population in the rhizospheric area and in soil. The existence of these microbes significantly increased the activity of dehydrogenase especially the application of the mixture of all microbes that led to the highest significant value which recorded 142 $\mu\text{g TPF/g}$ dry soil for the Bright variety and 134.9 $\mu\text{g TPF/g}$ dry soil for the Dark variety. The infected control recorded the least value of dehydrogenase, being 40.2 $\mu\text{g TPF/g}$ dry soil for the Bright variety and 32.1 $\mu\text{g TPF/g}$ dry soil for the Dark variety.

5.3-Root colonization

Referring to root colonization percent in response to inoculation with *B. subtilis*, *P. fluorescens*, *P. ostreatus* and mycorrhizeen® and their interaction with two roselle varieties, data in Figure (4) explicate the interaction effect between the varieties and the bioagents. Concerning the Bright variety, applying the bioagents mixture gave the highest colonization rate (90%), while applying *P. ostreatus* abandoned had no effect compared with the infected control (10%). Referring to the Dark variety, the application of *P. ostreatus* and mycorrhiza appeared to have the highest colonization rate (90 %).

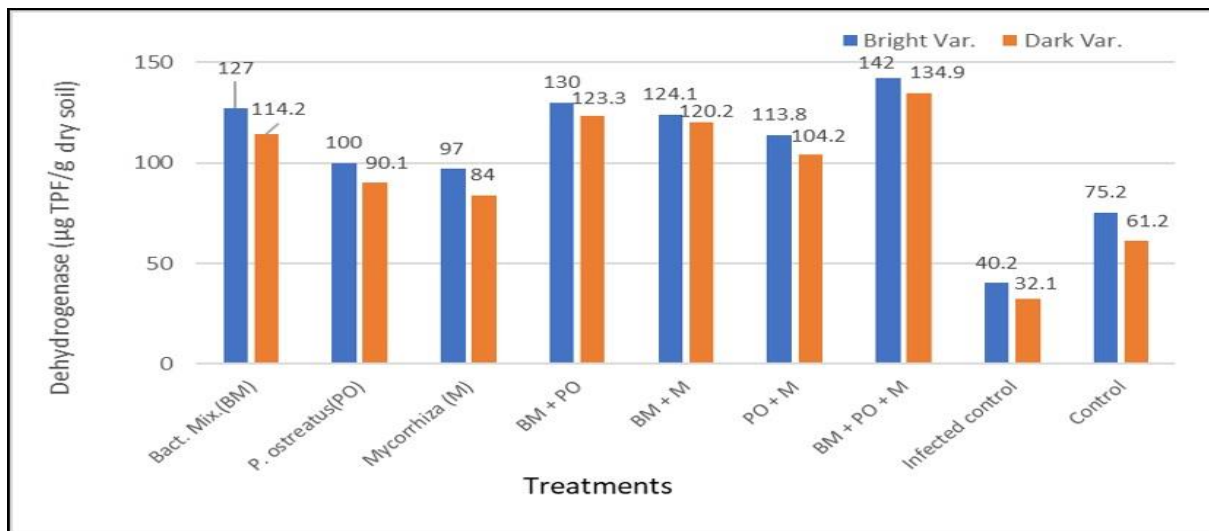


Figure (3): Biological influence on dehydrogenase content of Bright and Dark roselle varieties grown in artificially infested soil by *F. oxysporum* under greenhouse conditions. Data are expressed as a mean \pm standard error, L.S.D. test (ANOVA). Statistical significance was considered as $P < 0.05$.

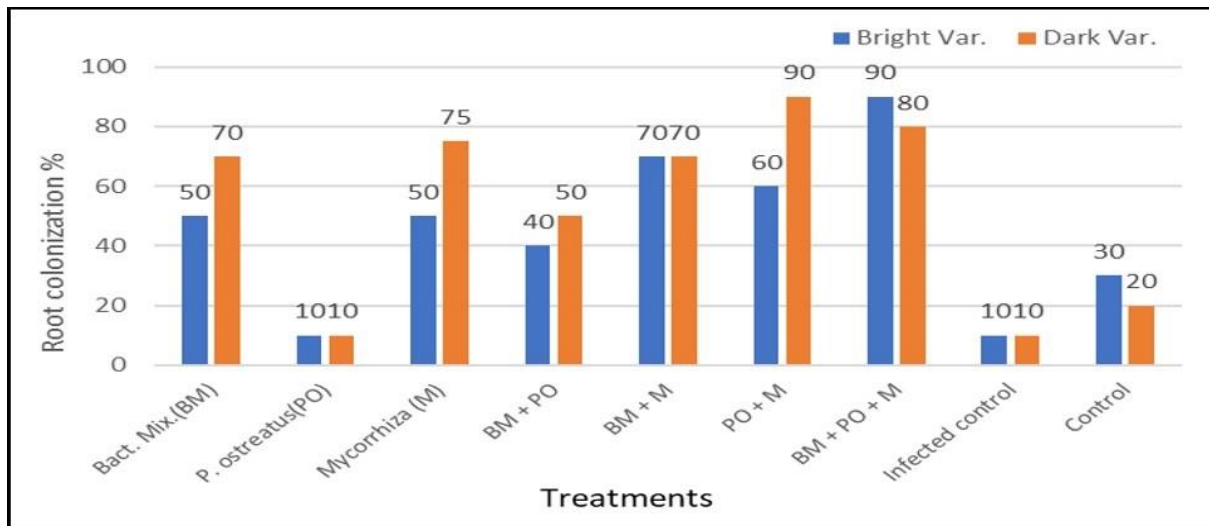


Figure (4): Root colonization % as affected by some microbiota on Bright and Dark roselle varieties grown in artificially infested soil by *F. oxysporum* under greenhouse conditions. Data are expressed as a mean \pm standard error, L.S.D. test (ANOVA). Statistical significance was considered as $P < 0.05$.

5.4- Determination of total phenols:

Determination of total phenols content in leaves of Bright and Dark roselle varieties demonstrated the significant difference among treatments, which is apparent in Figure (5). Data detected that in the Bright variety the mixture of *Bacillus subtilis*, *Pseudomonas fluorescens* and mychorrizeen® possess the highest value among treatments (4.98 mg/ g dry wt.), while the healthy control was the least value (0.94 mg/g dry wt.). Whereas, in the Dark variety the treatment using all the tested bioagents showed the highest value (3.95 mg/g dry wt.) and the control was the least (0.84 mg/g dry wt.).

5.5- Determination of photosynthetic pigments:

Regarding the photosynthetic pigments in roselle plant, Figure (6) reveal that in both Bright and Dark varieties, the used microbiota insignificantly increased chlorophyll a and b compared with the control. It is obvious from the given results that carotenoid attained the highest significant contents in both Bright and Dark varieties when utilizing the mixture of *B. subtilis*, *P. fluorescens*, *P. ostreatus* and mychorrizeen® (0.82 and 0.6 mg/ g dry wt., respectively) in comparable with the infected control which attained the least value (0.33 and 0.4 mg/ g dry wt., respectively).

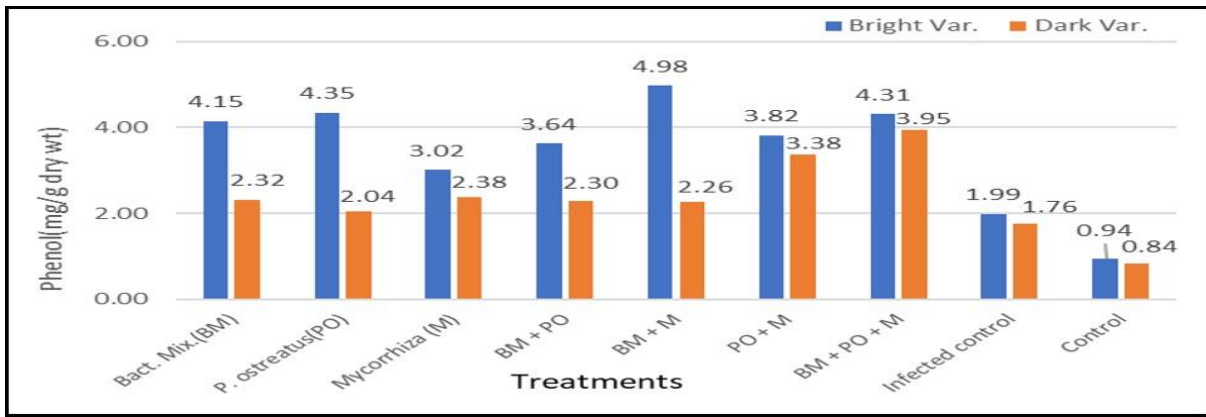


Figure (5): Biological influence on phenol content of Bright and Dark roselle varieties grown in artificially infested soil by *F. oxysporum* under greenhouse conditions. Data are expressed as a mean \pm standard error, L.S.D. test (ANOVA). Statistical significance was considered as $P < 0.05$.

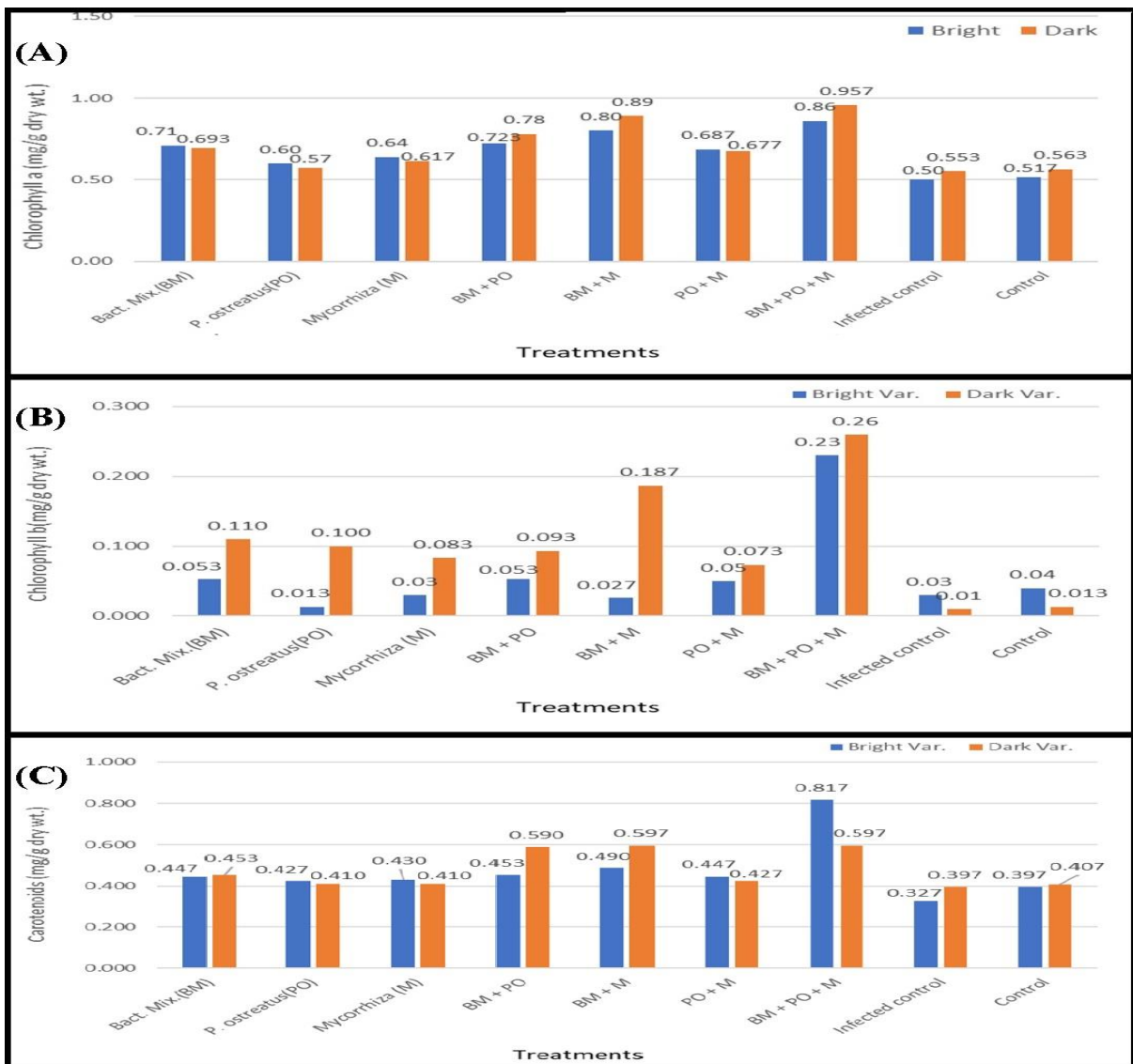


Figure (6): The impact of some microorganisms on photosynthetic pigment contents (chlorophyll a (A), chlorophyll b (B) and carotenoids (C)) of Bright and Dark roselle varieties grown in artificially infested soil by *F. oxysporum* under greenhouse conditions. Data are expressed as a mean \pm standard error, L.S.D. test (ANOVA). Statistical significance was considered as $P < 0.05$.

5.6- Growth parameters:

Growth parameters expressed as shoot height, fresh and dry weight were significantly affected by microbial inoculation. Data in Table (4) show that regarding the Bright variety, inoculation of the bacterial mixture together with mycorrhizae® was superior in shoot length which recorded 88.1 cm plant⁻¹ and shoot dry weight 20.8 gm plant⁻¹, while inoculation of *P. osteratus* and mycorrhizae® showed the highest shoot fresh weight with 79.5 gm plant⁻¹.

Regarding the Dark variety, implementation of *P. osteratus* showed the highest shoot height value which was 85.9 cm plant⁻¹, implementation of the mixture of all microbes showed the highest shoot fresh weight with 182.46 gm plant⁻¹, and implementation of the bacterial mixture in addition to *P. osteratus* showed the highest shoot dry weight with 31.2 gm plant⁻¹. In general, data indicated a markedly significant difference between the two varieties in morphological characterization.

Table (4): Impact of some bioagents on some shoot parameters of Bright and Dark roselle varieties grown in artificially infested soil by *F. oxysporum* under greenhouse conditions.

Treatments	Shoot parameters					
	Height (cm/plant)		Fresh wt. (gm/plant)		Dry wt. (gm/plant)	
	Bright	Dark	Bright	Dark	Bright	Dark
	Mean ± S.E ***	Mean ± S.E ***	Mean ± S.E ***	Mean ± S.E ***	Mean ± S.E ***	Mean ± S.E ***
Bact. Mix. * (BM)	81.0 ± 1.46	79.5 ± 2.03	60.0 ± 2.89	65.0 ± 0.57	16.2 ± 0.50	12.7 ± 0.52
<i>P. osteratus</i> (PO)	64.4 ± 0.72	85.9 ± 1.45	42.5 ± 0.65	70.0 ± 0.63	11.2 ± 0.39	14.57 ± 0.34
Mycorrhizae (M)	73.5 ± 0.63	69.3 ± 0.96	60.0 ± 1.36	57.0 ± 0.47	11.9 ± 0.34	19.5 ± 0.66
BM + PO	87.5 ± 1.18	75.5 ± 1.29	50.0 ± 2.47	173.0 ± 0.72	16.5 ± 0.53	31.2 ± 0.49
BM + M	88.1 ± 1.15	73.3 ± 0.44	72.5 ± 0.77	57.5 ± 0.42	20.8 ± 0.35	14.01 ± 0.40
PO + M	71.7 ± 1.05	68.9 ± 0.32	79.5 ± 0.61	90.0 ± 0.45	17.8 ± 0.29	27.3 ± 0.58
BM + PO + M	77.4 ± 0.86	74.5 ± 1.62	76.3 ± 0.49	182.5 ± 1.15	18.8 ± 0.63	31.1 ± 0.72
Infected control	55.5 ± 0.42	53.3 ± 0.46	15.0 ± 0.50	15.5 ± 0.28	5.3 ± 0.31	6.5 ± 0.25
Control	56.5 ± 1.04	54.2 ± 1.39	25.0 ± 0.71	49.4 ± 0.56	5.0 ± 0.17	9.4 ± 0.31
L.S.D (0.05)	2.84	3.56	4.15	1.8	1.18	1.43
F. Test	**		**		**	
LSD at 0.05 V*B	3.194		3.151		1.269	

Bact. Mix. * (BM): Bacterial mixture of *Bacillus subtilis* (BSR-8) and *Pseudomonas fluorescens* (PSR-11), S.E***: Standard error, V= Variety, B= Bioagents

5.7- Yield parameters:

Biological influence on some yield parameters is expressed in Table (5). Data indicated that the impact of inoculation of the mixture of *Bacillus subtilis*, *P. fluorescens*, *P. osteratus* and mycorrhizae® of the two varieties was significantly reflected on the fruit fresh weight, the calyx fresh weight and calyx dry weight. The mixture of all bioagents was superior in both Bright and Dark varieties giving 178 and 146 gm/pot fruit fresh weight, respectively; 82 and 72 gm/ pot calyx fresh weight respectively; 7.3 and 7.4 gm/pot calyx dry weight, respectively; along with 14 and 12.9 gm/pot air dried fresh seed weight. The infected control reported the inferior yield in both Bright and Dark varieties.

6- Growth pattern of microbiota in culture media supplemented with roselle root exudes

Microbial candidates of inocula applied in the greenhouse experiments were separately examined for their response to root excretions of Bright and Dark roselle varieties when added to the culture medium at various concentrations. Presence of root exudates in the medium significantly increased growth of both *P. fluorescens* and *B. subtilis*. Such effect was more pronounced with a concentration 100 % in both varieties as shown in Figure (7). Higher numbers were scored with the Bright roselle variety. *P. fluorescens* multiplied better than *B. subtilis* when inoculated in a culture medium supplied with the same quantities of roselle root exudates.

Table (5): Some yield parameters as affected by some biological agents of Bright and Dark roselle varieties grown in artificially infested soil by *F. oxysporum* under greenhouse conditions.

Treatments	Fruit fresh wt. (gm /pot)		Calyx				Seeds fresh wt. (gm /pot) air dried	
			Fresh wt. (gm /pot)		Dry wt. (gm /pot)			
	Bright	Dark	Bright	Dark	Bright	Dark	Bright	Dark
	Mean ± S.E ***	Mean ± S.E ***	Mean ± S.E ***	Mean ± S.E ***	Mean ± S.E ***	Mean ± S.E ***	Mean ± S.E ***	Mean ± S.E ***
Bact. Mix. *(BM)	140.0 ± 3.39	124.0 ± 0.61	62.0 ± 0.36	60.0 ± 0.44	5.7 ± 0.21	6.9 ± 0.26	13.0 ± 0.51	11.3 ± 0.29
<i>P. ostreatus</i> (PO)	110.0 ± 3.24	86.6 ± 0.77	40.0 ± 0.71	22.8 ± 0.77	5.0 ± 0.25	4.7 ± 0.30	10.0 ± 0.44	9.6 ± 0.4
Mycorrhizae (M)	118.0 ± 3.31	98.0 ± 0.62	41.0 ± 0.79	38.8 ± 0.81	5.1 ± 0.24	5.2 ± 0.16	11.0 ± 0.5	8.2 ± 0.38
BM + PO	152.6 ± 1.68	146.0 ± 0.94	64.0 ± 0.42	62.0 ± 0.97	6.3 ± 0.22	7.0 ± 0.33	13.0 ± 0.45	12.1 ± 0.37
BM + M	156.0 ± 1.73	127.4 ± 0.79	68.0 ± 0.67	61.0 ± 0.96	6.4 ± 0.28	6.9 ± 0.32	14.0 ± 0.53	11.7 ± 0.41
PO + M	128.0 ± 1.12	118.0 ± 0.58	46.0 ± 0.67	58.0 ± 0.88	5.4 ± 0.23	5.9 ± 0.32	12.0 ± 0.53	10.2 ± 0.37
BM + PO + M	178.0 ± 3.76	146.0 ± 0.72	82.0 ± 0.75	72.0 ± 0.75	7.3 ± 0.45	7.4 ± 0.41	14.0 ± 0.56	12.9 ± 0.3
Infected control	80.3 ± 1.00	82.6 ± 0.77	27.0 ± 0.39	20.4 ± 0.68	4.6 ± 0.28	3.2 ± 0.27	7.2 ± 0.36	6.2 ± 0.17
Control	104.0 ± 1.82	86.2 ± 0.68	38.0 ± 1.30	21.0 ± 0.47	5.0 ± 0.26	4.1 ± 0.33	7.4 ± 0.32	7.4 ± 0.22
L.S.D (0.05)	7.31	2.09	2.08	2.2	0.79	0.88	1.35	1.13
F. Test	**		**		**		Ns	
LSD at 0.05 V*B	5.252		1.978		0.811		-	

Bacterial mixture * (BM): Bacterial mixture of *Bacillus subtilis* (BSR-8) and *Pseudomonas fluorescens* (PSR-11), S.E***: Standard error, V= Variety, B= Bioagents.

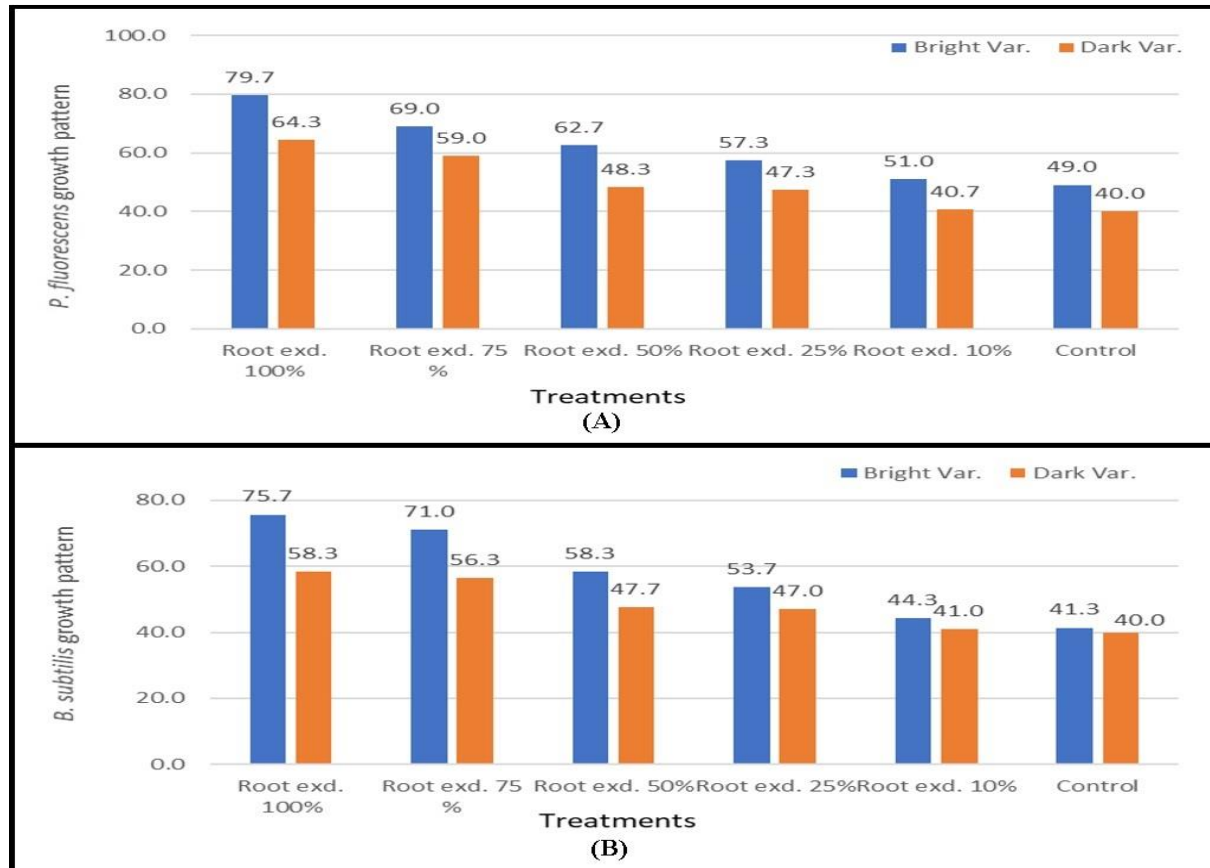


Figure (7): Effect of Bright and Dark roselle root exudates on growth pattern of (A) *P. fluorescens* and (B) *B. subtilis*. Data are expressed as a mean ± standard error, L.S.D. test (ANOVA). Statistical significance was considered as $P < 0.05$.

DISCUSSION

Roselle plant is considered being one of the crucial medicinal plants in Egypt. Its extracts became a basic material in medicine, cosmetic and food industries. Roselle cultivations concentrate in Upper Egypt. The cultivation area is increasing progressively attributable to the importance of the roselle plant (Ottai *et al.*, 2011).

Results of the present investigation revealed the ability of the bacterial isolates, *i.e.*, *B. subtilis* and *P. fluorescens* to antagonize the pathogenic fungus *F. oxysporum*. The inhibition in the growth could be attributed to antibiosis, hyperparasitism or production of chitinase, β -1,3 glucanase and β -1,4 glucanase which degrade the cell wall leading to lyses of mycelium of the pathogen. El-Gamal *et al.* (2016) reported a similar inhibitory result with *B. subtilis* and *P. fluorescens* against tomato root rot, where *B. subtilis* can cause 57.8 % reduction against *F. solani* while *P. fluorescens* led to 81.1 % reduction.

The production of various plant growth promoting and anti-fungal substances positively affected plant growth and resistance. Siderophore production was also noticed which enhances iron uptake by the plant which in turn enhances plant growth (Sarhan and Shehata 2014 and Gouda *et al.*, 2018). Voisard *et al.* (1989) found that HCN stimulated root hair formation.

In the present work, it was found that the mixture of bioagents decreased disease incidence than individual ones. Bagyaraj (2018) assumed that the protection mechanisms used by PGPR against the pathogen are: competition for space and nutrients, modification of Fe and Mn availability, liberation of antibiotics and HCN, plant growth promotion by modification of plant hormone balance, and stimulation of systemic and localized plant defense mechanisms. The protection mechanism used by *P. oostreatus* may be attributed to the production of secondary metabolites such as phenolic compounds. These phenolic compounds participate in the regulation of stages the development, and also contribute to the defense responses during exposure to pathogen infection, extreme sunlight, heavy metal stress and injuries. Such compounds often possess antioxidant activity, which is attributed to their unique chemical structure. Plant defense mechanisms based on phenolic compounds include physical changes, such as increasing lignification and suberization of the plant cell walls, as well as metabolic changes such as the de

novo synthesis of pathogenesis-related (PR) proteins and biosynthesis and accumulation of phenyl propanoid secondary metabolites. This is in agreement with the results of Elsakhawy *et al.* (2020). The current results are in agreement with Bagyaraj (2018) who stated that AMF cannot offer complete immunity against the soil-borne pathogens, they only impart a degree of resistance against these pathogens. Meanwhile, PGPR facilitates AMF formation, and the synergistic interactions between AMF and PGPR may enhance bioprotection against soil-borne plant diseases.

The contents of total phenols in both treated varieties (Bright and Dark) were higher than their control plants. The content in total phenols, which are involved in defense mechanisms against pathogens was higher in treated plants with *F. oxysporum* than the control ones in both varieties (Sie *et al.*, 2011). These compounds could be produced in response to the attacks of pathogens.

Photosynthetic pigments increased in response to microbiota inoculation may result from enhancing plant growth and biomass production. Bioagents may stimulate chlorophyll synthesis through encouraging pyridoxal enzymes formation, that play a role in α -amino levulinic synthetase as a primary compound in chlorophyll synthesis. These results are supported by other published papers (Khalil *et al.*, 2017 and Al-Sayed *et al.*, 2020).

Vegetative growth parameters *i.e.*, shoot length and number of branches recorded at harvest stage. Bio-fertilizer significantly increased growth parameters, which may be as a consequence of synthesis of phytohormones, N_2 fixation, synthesis of some enzymes that modulate the level of plant hormones, solubilization of inorganic phosphate and mineralization of organic phosphate which makes phosphorus available for the plant, the synthesis of growth promoting compounds like cytokinins, gibberellins and IAA. These results are similar to those obtained by Khalil *et al.* (2017) and Al-Sayed *et al.* (2020). Increase number of calyxes may be imputable to the increment in the number of branches.

Shoot fresh and dry yield, calyx fresh and dry yield and seed yield were significantly increased under inoculation by the combined bioagents comparing to individual ones and the control. This may be ascribed to the collective effect of bioagents *i.e.*, production of amino acids, vitamins and growth promoting substances like indole acetic and gibberellic acids secreted by

these introduced beneficial microorganisms which resulted in enhanced nutrient uptake, translocation and synthesis of photosynthate assimilates which resulted increased plant growth characters. These results are compatible with those of El Naim *et al.* (2017), Khalil *et al.* (2017) and Al-Sayed *et al.* (2020).

It was clear that AM yield production was not effective when used individually. This is in agreement with Sembok *et al.* (2015) who found that AM spores were not effective in enhancing the above-soil vegetative growth or the yield of roselle calyxes.

Results presented here demonstrate that the inocula (*P. fluorescens* and *B. subtilis*) were able to utilize a variety of compounds secreted by roselle, as a test plant, as carbon and energy sources where bacterial growth patterns were improved. As reported by Kipe-Nolt *et al.* (1985), bacteria utilize the exudates from a given genotype differentially. Many investigators (Kucey, 1988; Zaady and Okon, 1990) studied the root exudation phenomenon and concluded that, plants grown in soil under natural conditions are subjected to physical stresses which presumably bring about root-hair damage and the release of the organic zone.

The examination of the effect of root exudates on the growth of different PGPR strains revealed the higher ability of *P. fluorescens* to colonize roots than *B. subtilis*. In addition, different plant varieties affect the colonization of the same microbial strains. Root exudates are a mix of a wide variety of compounds, including primary and secondary metabolites. Primary metabolites, including carbohydrates, amino acids, and organic acids, are secreted in larger quantities than secondary metabolites, such as flavonoids, glucosinolates and auxins, etc. Root exudates mediate the interaction among plants of the same or different species, in a different manner depending on root exudate composition and the mechanisms of the relationship (Vives-Peris *et al.*, 2019). This behavior is in agreement with Bais *et al.* (2004), who stated that chemical components of root exudates may deter one organism while attracting another, or two very different organisms may be attracted with differing consequences to the plant.

CONFLICTS OF INTEREST

The authors declare no conflict of interest exists.

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