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Antagonistic Rhizobacteria as Natural Foes Producing Antioxidants Against Onion Pathogens During Storage

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Three antagonists, *i.e. Pseudomonas fluorescens*, *Bacillus subtilis* and *Serratia* spp., were tested either alone or as a mixture for suppression of onion neck rot and black mould diseases under field conditions. Samples were taken for determination of total phenols, cartenoides and flavonoids. The average of disease reduction was 18.75 % for mixture compared to 47.0% in the non-treated control. In addition to disease suppression, treatment with a mixture of the antagonists promoted plant growth in terms of increased bulb yield. The mixture of the tested bioagents was more effective in reducing the disease and promoting both plant growth and bulb yield compared with using each of them alone. The result indicated the biocontrol efficacy and storage stabilities were not increased with the enhancement of the concentration of biocontrol agents. Treatment the onion bulb with the bioagents increased cartenoides in mixture treatment and the fungicide Folucolon compared with the control and another tested treatment. Treatment onion bulb with any of the tested bioagents or their mixture decreased total phenols compared with the fungicide Folucolon and control in the two concentrations (10^8 CFU/ml and10^9 CFU/ml). On the other hand, they also increased flavonoids compared with control and the fungicide Folucolon in the two concentrations.

Keywords: Antagonistic, antioxidant, onion, rhizobacteria and storage.

Onions are susceptible to Botrytis neck rot during storage. The disease is characterized by grey fungal growth, often watery in nature, at the neck area and on the outer scales. The infection usually spreads quickly through the whole plant (Chalutz and Wilson, 1990 and Jryandi *et al.*, 2009). Black mould, caused by *Aspergillus niger* is characterized by black discoloration at the necks of onions. The black discoloration can sometimes be found on outer scales (Shika and Doug, 2001). Stored onions are also susceptible to blue mould caused by genus *Penicillium*, which mould induces watery soft rot of onion tissue and/or blue–green discoloration at the neck or other tissue. Bacterial soft rots caused by *Erwinia* spp. often occur during storage of onions. Onions infected by bacterial soft rots often appear healthy on the outside but when cut open some of the inner scales seem brown-water-soaked and have a coo reed appearance. Bacterial rots caused by *Pseudomonas* spp. infect outer scales and are characterized by yellow slime which produces a sow dour

(Bailey *et al.*, 2006 and Rachel *et al.*, 2008). Biocontrol seems to be reliable alternative to synthetic chemicals, which have raised serious concerns of food contamination. Antifungal agents produced by microorganisms may be used as biocontrol agent. Antifungal metabolites produced by bacteria like *Pseudomonas* spp. and *Bacillus* spp. have been investigated for their antifungal properties (Motia *et al.*, 2005 and Nourozian *et al.*, 2006). The aim of this study was designed to evaluate the biocontrol potential of onion *Bacillus* spp., *Pseudomonas* spp. and *Serratia* spp. against food borne pathogenic fungi and the relation to defence of plants by determination cartenoides, total phenol and flavonoids.

Materials and Methods

Source of bioagents:

Pseudomonas fluorescens, *Bacillus subtilis* and *Serratia* sp. were taken from Bio-fertilizer-Unit; Agric. Res. Microbiol. Soils, Water and Environ, Res. Inst., Agric. Res. Centre, Giza, Egypt.

Preparation of the bioagents:

Two flasks containing 100 ml of Kings medium were inoculated with 1 and 2 ml respectively of either *P. fluorescens* and *B. subtilis* or *Serratia* sp. and a mixture of all and incubated at 27ºC for 48 hours.

In vitro antagonism of the bioagents:

The antagonism of the tested bacteria against *Aspergillus niger* (black mould) and *Botrytis allii* was *in vitro* studied on PDA medium. Tested bacteria were grown overnight on king's medium. Petri dishes containing PDA medium amended with gl^{-1}: peptone, 3.0; $CaCO_3$, 0.2 and $MgSO_4$, 0.2 were inoculated with pathogenic fungi in centralized sites. Three of the bioagents were inoculated onto agar plates. Plates were incubated at 28ºC for 3-5 days, and then inhibition zone diameters were measured.

Field experiment:

Field experiment was carried out in a sandy soil of Assiut Governorate during two growing seasons (2010-2011) and (2011-2012). A complete randomized block design with four replications was used in each site. The plot area was 2m wide by 1.5m long. At the end of the experiment, the onion was harvested and stored. The previously treated bulbs were kept in 40x40 cm Jute sacks each containing 400 bulbs and a piece of wetted cotton was kept at the jute sacks, to maintain suitable humidity around the bulb, and then incubated at room temperature (32ºC). Disease incidences were recorded after 30, 60 and 90 days.

Treatment of the seedlings by bioagents in the field:

Sixty days old plants of onion (cv. Giza 6) were soaked for 30 min in a suspension of either *P. fluorescens*, *B. subtilis*, *Serratia* sp. or in bioagent mixtures, at concentrations of 10^8 and 10^9 CFU/ml as well as in the fungicide Folucolon, at concentration of 187.5ml/100 l. Also, these concentrations were applied to the abovementioned plots (at rate of 20 l/fed) every two weeks until harvesting.

Production of antifungal metabolites:

Each plant growth promoting rhizobacteria (PGPR) strain was tested for production of hydrogen cyanide. Siderophore production and protease was semi quantified using chrome azurol (CAS) medium (Schwyn and Neilands, 1987). Glycerol (5% v/v) was used as the sole carbon source and the PGPR were selectively improved by the addition of 10 mg^{-1} fucidin and 50 mg^{-1} cephalceridine when the iron (III) is removed from the chrome azurol- complex by high affinity siderophore the colour changes from blue to orange. The diameter of orange halo around the colonies after incubation at 28ºC for 2 days was indicative of the relative level of siderophore production. Protease activity (casein degradation) was determined from clearing zones in skimmed milk agar (SMA) according to Neilsen *et al.* (1998). Production of hydrogen cyanide (HCN) was detected by growing bacteria for 16 h at 24ºC on plates containing nutrient agar with an indicator paper inoculated with 5 mg of copper (II) ethyl acetoacetate, 5 mg of methylene bis (n-n dimethyl aniline) and 2 ml chloroform as described by Castric and Castric (1983). Production of cyanide caused the indicator to turn blue.

Determination of cartenoides:

Total cartenoides were determined spectrophotometrically (in diethyl ether) at the wavelength of 440 nm as mentioned by Beadle and Zscheile (1942).

Total phenols:

Total phenols were determined as the method of Folin ciocalten (Heda *et al*., 2005).

Total flavonoids:

Total flavonoids were determined as the method of Folin ciocalten (Hung and Morita, 2008).

R e s u l t s

Frequency of pathogenic fungi and bacteria under storage conditions:

Data presented in Table (1) show that treating onion bulb by *Pseudomonas fluorescens* and *Bacillus subtilis* or *Serratia* sp. decreased the frequent occurrence of pathogenic fungi under storage conditions. *B. allii* was the most frequently isolated followed by *A. niger*, *P. digitatum* and *Pseudomonas* sp., while *Erwinia* spp. recorded the lowest frequency.

Table 1. Frequency of pathogenic fungi and bacteria under storage conditions

Fungus	Frequency (%)	Disease
P. digitatum	1.3	Blue mould
A. niger	10.0	Black mould
B. allii	12.0	Neck rot
Pseudomonas spp.	1.3	Soft root
Erwinia spp.	0.3	Soft root

Data in Table (2) indicate that tested bioagents inhibited the *in vitro* growth of *A. niger* and *B. allii*. *Pseudomonas fluorescens* showed the highest antifungal activity against *A. niger* and *B. allii*. Meanwhile, *Serratia* sp. recorded a moderate effect against *B. allii*.

Table 2. Effect of three bioagents on the inhibition zone of *A. niger* and *B. allii*

Tested bioagent	*A. niger* (mm)	*B. allii* (mm)
P. fluorescens	14.1	20
B. subtilis	12.0	10
Serratia sp.	11.0	7

Data in Tables (3 and 4) indicate that black mould of onion was significantly decreased when plants were treated with any of the tested bioagents was applied, either individually or in a mixture, in comparison with the control treatments.

Table 3. Effect of the tested bioagents on disease incidence of *A. niger* on onion (cv. Giza 6) during storage season of 2010/2011

Tested bioagent	Concentration	Storage period (day)			
		30	60	90	Mean
P. fluorescens	1*	22.50	24.00	25.00	23.83
	2**	22.75	19.25	19.75	20.58
Mean		22.62	21.62	22.37	22.20
B. subtilis	1	31.50	32.75	33.25	32.50
	2	31.50	29.50	28.00	29.67
Mean		31.5	31.12	30.62	31.0 8
Serratia sp.	1	22.75	26.50	24.00	24.42
	2	23.50	21.50	22.25	22.42
Mean		23.12	24.00	23.12	23.42
Mixture	1	16.50	19.50	19.00	18.33
	2	18.00	18.25	20.25	18.83
Mean		17.25	18.87	19.62	18.58
Folucolon	187.5 ml/100 l	16.50	18.50	18.50	17.83
Control		44.56	45.25	46.75	45.50
Mean		25.00	25.50	25.68	

* 1: Concentration → 10^8 CFU/ml
** 2: Concentration → 10^9 CFU/ml

Data in Tables (5 and 6) show that neck rot caused by *B. allii* was significantly affected by bioagent treatment at two concentrations and storage for 30, 60, 90 days. The fungicide was more effective followed by the mixture for alleviating the disease severity and improving plant against *B. allii*.

Table 4. Effect of the tested bioagents on disease incidence of *A. niger* on onion (cv. Giza 6) during storage season of 2011 /2012

Tested bioagent	Concentration	Storage period (day)			
		30	60	90	Mean
P. fluorescens	1*	22.00	25.75	24.25	23.83
	2**	21.00	18.60	19.00	19.33
Mean		21.50	22.17	21.62	21.58
B. subtilis	1	32.00	33.50	33.75	34.42
	2	30.50	28.00	28.00	28.83
Mean		31.25	30.75	30.87	31.62
Serratia sp.	1	23.00	27.25	30.00	26.75
	2	22.50	21.75	22.25	22.17
Mean		22. 75	24.50	26.12	24.46
Mixture	1	17.25	19.75	20.50	19.17
	2	17.25	18.50	20.50	18.75
Mean		17.25	19.12	20.50	18.96
Folucolon	187.5 ml/100 l	18.00	17.50	19.50	18.33
Control		44.25	48.25	48.75	47.08
Mean		24.78	25.68	26.55	

* Concentration 1 and 2: As described in footnote of Table (3).

Table 5. Effect of the three bioagents on disease incidence of *B. allii* on onion (cv. Giza 6) during storage season of 2010/2011

Tested bioagent	Concentration	Storage period (days)			
		30	60	90	Mean
P. fluorescens	1*	22.00	25.75	24.25	23.83
	2**	21.00	18.60	19.00	19.33
Mean		21.50	22.17	21.62	21.58
B. subtilis	1	32.00	33.50	33.75	34.42
	2	30.50	28.00	28.00	28.83
Mean		31.25	30.75	30.87	31.62
Serratia sp.	1	23.00	27.25	30.00	26.75
	2	22.50	21.75	22.25	22.17
Mean		22. 75	24.50	26.12	24.46
Mixture	1	17.25	19.75	20.50	19.17
	2	17.25	18.50	20.50	18.75
Mean		17.25	19.12	20.50	18.96
Folucolon	187.5 ml/100 l	18.00	17.50	19.50	18.33
Control		44.25	48.25	48.75	47.08
Mean		23.95	26.68	25.58	

* Concentration 1 and 2: As described in footnote of Table (3).

Table 6. Effect of the three bioagents on disease incidence of *B. allii* on onion (cv. Giza 6) during storage season of 2011/2012

Tested bioagent	Concentration	Storage period (day)			
		30	60	90	Mean
P. fluorescens	1*	22.50	24.00	24.50	23.67
	2**	19.25	19.75	21.50	20.17
Mean		20.87	21.87	23.00	21.92
B. subtilis	1	28.00	29.50	31.50	29.67
	2	26.00	27.00	27.75	27.00
Mean		27.00	28.25	29.68	28.33
Serratia sp.	1	25.50	27.50	28.25	27.08
	2	23.50	24.50	25.00	24.00
Mean		24. 50	26	26.62	25.54
Mixture	1	18.00	19.75	20.75	19.50
	2	18.00	19.25	21.50	21.58
Mean		18.00	19.50	21.12	20.54
Folucolon	187.5 ml/100 l	14.00	17.25	18.75	16.33
Control		44.00	45.00	46.75	45.25
Mean		25.35	25.5 3	26.30	

* Concentration 1 and 2: As described in footnote of Table (3).

Illustrated data in Fig. (1) show that any of applied bioagents significantly increased yield production compared to untreated plants. The mixture of biocontrol agents increased the yield production in concentration 2 than in concentration 1.

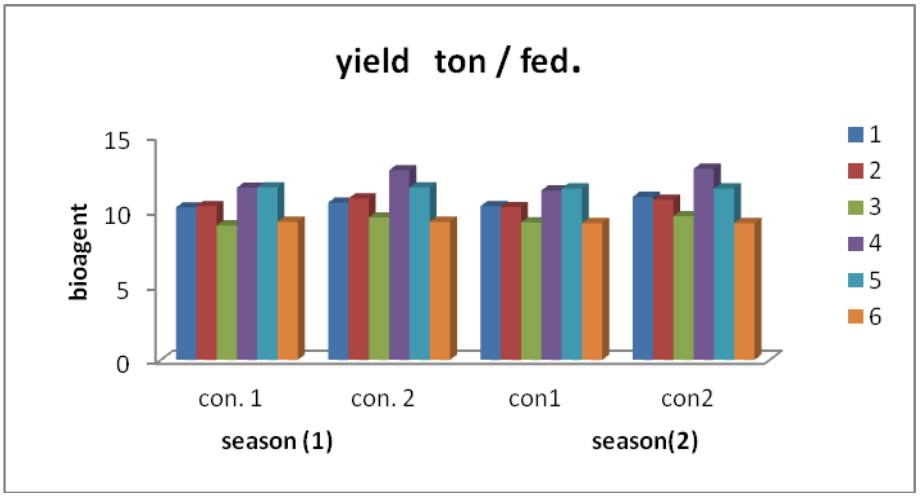

Fig 1. Effect of the tested bioagents on onion yield during 2010/2011 and 2011/2012 growing seasons

1- Control. 2- Folucolon. 3- *P. fluorescens*. 4- *B. subtilis*. 5- *Serratia* sp. 6- Mixture.

Illustrated data in Fig. (2) show that any of applied antioxidants increased the contents of endogenous non enzymatic antioxidants (flavonoids, cartenoides and phenols) in concentration 2 than in concentration 1. However, mixture of PGBR proved to be more effective in improving endogenous non enzymatic when compared with control and/or Folucolon.

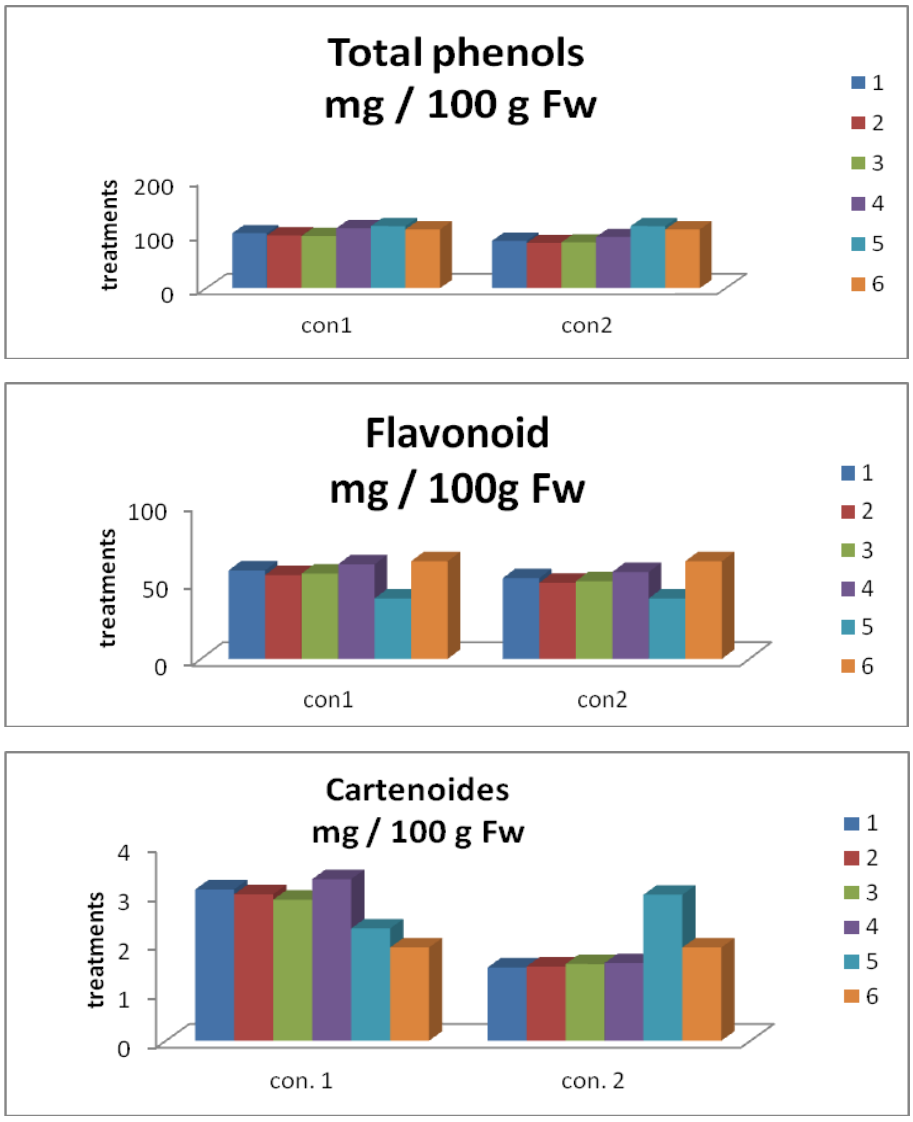

Fig 2. Effect of the tested bioagents on phenols, flavonoid and cartenoides production.

Discussion

During storage of onion in this study, the current status of post harvest losses of onion bulbs was examined during 2008/2009 and 2009/2010 growing seasons. Unexpectedly onion bulbs were found with severe infection during storage. These high losses could be attributed to the infection by storage fungi including *Aspergillus niger* and *Botrytis allii* (Schwartz and Mohan, 1995).

Among the fungi, the most frequent and damaging one was *B. allii* (Shika and Doug, 2001), which reported to infect bulbs surrounded wounds produce during harvesting leaf remove and marketing (Nunes *et al.*, 2002). On the other hand, black mould caused by *A. niger* was of severe infection during high temperature. Biological control using microorganisms is one of the most promising ways to reduce onion disease during storage. *Pseudomonas fluorescens*, *Bacillus subtilis* and *Serratia* spp. inhibited the *in vitro* growth of *A. niger* and *B. allii*. This result agrees with that obtained by Sobia *et al.* (2010).

Antibiotic production is now recognized as an important mechanism by which biocontrol agents, especially PGPR suppress plant pathogens. Interest in 2.4 diacetyl phloroglucinal–producing Pseudomonas has focused not only on its potential as introduced agents but also its activity in natural agroecosystems.

Pseudomonas that produce the antibiotic 2,4 diacetyl phloroglucinal (204 DAPG) inhibit a broad spectrum of plant pathogenic fungi and control a variety of root and seeding diseases (Masoud and Abbas, 2009). For antifungal activity, it was possible to obtain three points for high activity against each pathogen production of antimicrobial metabolites (siderophore, hydrogen cyanide, protease and 204 DAPG) among PGPR. *Pseudomonas fluorescens* occurs commonly in the rhizosphere of plants and represents an important functional group of beneficial bacteria of soil borne plant (Harish *et al.*, 2010).

According to Backman *et al.* (1997), the effectiveness of endophytes as biological control agents is dependent on many factors. Including, host specificity, the population dynamics, pattern of host colonization, the ability to move within host tissues and the ability to induce systemic resistance. For example, *Pseudomonas* spp. as onion endophyte inhibited *B. allii* and promoted vine growth in colonized. Demonstrating that divergent hosts could be colonized (Barka *et al.*, 2002 and Rachel *et al.*, 2008). Colonization of multiple hosts has been observed with other species of endophytes and plants, for example *Pseudomonas putida* and *Serratia mascescens* reduced Fusarium wilt in cucumber (Jetiyanon, 1994 and Liu and Tuzun, 1995).

Overall, *Bacillus* spp. was effective long-term colonists of leaf tissue and can suppress many diseases. Disease suppression was sustainable 80-70 days after a single application, but reapplication would likely be necessary in a planting due to its perennial non-deciduous mature that supports a typical leaf life of about one year. Based on previous experience, application of *Bacillus* spp. may have the advantage of longer time periods between application compared to chemical control methods (Adejumo, 2005 and Melentev *et al.*, 2006).

The application of endospore-forming bacteria native to the region could reduce the regulatory and environmental concerns associated with use of non-native microbes.

Plant phenolic compounds are secondary metabolites. The beneficial effects of those molecules are related to their antioxidant activity. They provide protection against pathogens (Sakr and El-Metwally, 2009). Total phenols in the plant tended to increase gradually with presents pathogens.

Mechanisms involvement two or more mechanism has been demonstrated in several systems, reported combinations include antibiosis with enzyme degradation of Botrytis cell wall; competition for nutrients followed by interference with pathogenicity enzymes of the pathogen or induced resistance and alteration of slant surface wetability combined with antibiosis. Since germination of Botrytis or Aspergillus conidia are depended on the presence of nutrient, competition for nutrient is regarded as important in systems where biocontrol is involved. Conidial viability and germination capacity are also potentially affected by the presence of antibiotics produced by biocontrol agents are present in the philosopher.

Biocontrol is established lesions and reduction of sporulation on necrotic slant tissue is a means to minimize the pathogen inoculums (Elad, 1996 and Rachel *et al.*, 2008).

Characteristics of phenols absent in healthy cells or present in very minute quantity produced in large quantity in response to weak pathogen or non pathogen than virulent one produced relatively quickly in cells after infection (Mazid and Mohammed, 2011).

Total phenols, flavonoids and cartenoides are known to play a role in plant deferens against abiotic stresses (Sakr and El-Metwally, 2009). Many flavonoid compounds can function as passive or inducible banes against microbial pathogen. In defiance of plants the role antioxidants to prevent disease.

The biological efficacies of three biocontrol agents had a positive trend with the increasing concentration. But when the concentration exceeds a certain level was higher than 10^8 CFU ml^{-1}. The biological efficacies of the biocontrol agents were weakened. This indicated the increasing of the three biocontrol agents concentration used were not always good for biological control.

Some studies showed that the high concentration of biocontrol agents has a better effect. But other reports detected that there was no positive effect of the concentration (Yizhan e*t al.*, 2011). It is possible that too many foreign agents in the micro-ecological environment in soil create unsuitable condition for indigenous bacteria and host plant, which might make it easier for the infection of pathogens. There are several mechanisms for different bio-control agents and the ultimate aim of their bacteria is to regulate ecological balance for controlling pathogen and promoting host plant growth (Yizhan *et al.*, 2011).

Obtained results exhibit the antifungal activity of bacterial species and indicated the possibility of using these bacterial genera as bioagents against these diseases.

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تم دراسة تأثير ثلاث أجناس من البكتريا وهى: *Pseudomonas fluorescens, Bacillus subtilis* and *Serratia* spp. منفردين أو خليط لمقاومة عفن الرقبة وأمراض التخزين فى نبات البصل تحت ظروف الحقل وتم تكرار الرش بعد شهر وفي نفس الوقت أخذت عينات لتقدير الفينول والكردينويدس والفلافينوديس. معدل تقليل الإصابة بخليط السلالات كان ١٨,٧٥% مقارنة بالكنترول الذي كان معدل الإصابة به ٤٧% بالإضافة إلى معدل تقليل الإصابة به كان تأثير الميكروبات المشجعة للنمو حيث زاد الوزن الجاف والمحصول. وأظهرت النتائج وجود تثبيطات للفطريات الممرضة المختلفة وكان أكثر التأثير للميكروبات المختلطة واستخدام تركيزين للبكتريات وهما 10^8 و 10^9 وحدة قياس للخلية لقياس قدرة أى تركيز منهم على تثبيط الفطريات وأظهرت النتائج أن تركيز 10^8 أفضل فى تثبيط الفطريات المسببة لعفن الرقبة والتخزين عن تركيز 10^9. معاملة الأبصال بخليط من البكتريا المضادة أحدثت زيادة فى الكردينويدس وأيضًا باستخدام المبيد بالمقارنة بمعاملات *P. fluorescens, B. subtilis and Serratia* spp. والكنترول باستخدام التركيز 10^8. معاملة الأبصال خليط من البكتريا المضادة *P. fluorescens, B. subtilis* and *Serratia* spp. أحدثت خفض فى الفينول بالمقارنة باستخدام المبيد والكنترول باستخدام تركيزين للبكتريات وهما 10^8 و 10^9 وحدة قياس للخلية ١ و٢. معاملة الأبصال بكل من *P. fluorescens, B. subtilis* and *Serratia* spp. أحدثت زيادة فى الفلافينوديس بالمقارنة باستخدام المبيد والكنترول باستخدام تركيزين للبكتريات وهما 10^8 و 10^9 وحدة قياس للخلية.