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Evaluation of Different Mixtures of Bioagents and Antioxidants with Bioagents on Root-Rot in Strawberries

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Strawberry is one of the most important vegetable crops in Egypt for exportation. This is due to the good characteristic and early appearance in market. Black root rot is a common disease in strawberry caused by a group of soil borne pathogens include *Rhizoctonia solani, Fusarium solani* and *Macrophomina phaseolina*. This disease causes economic losses in strawberry and on a wide range of other cultivated plants. Biological control is used as environmental friend alternative method in plant protection. Bioagent alone, sometimes, cannot replace effective control achieve by chemical fungicides. Therefore, this research studied how to improve or increase the effect of biocontrol agents by mixing two different bioagents together (*Trichoderma harzianum* with *Bacillus subtilis*, *Trichoderma harzianum* with *P. fluorescens* and *Bacillus subtilis* with *P. fluorescens*) or by mixing these bioagents with different antioxidants instead of using single treatment. Two different antioxidants, *i.e.* potassium tartrate and salicylic acid+ ascorbic acid, were used under field conditions. Results showed that all treatments led to clear significant reduction in disease incidence compare with control treatment. Mixture of *T. harzianum* and *B. subtilis* gave the highest level of plant protection and led to increase the fruit yield. *T. harzianum* and *P. fluorescens* occupied the second rank, whereas mixture of *B. subtilis* and *P. fluorescens* was the least effective one. Combination between antioxidants and different bioagents resulted in significant reduction to the disease compared with single treatment. Synergistic effect was noticed when potassium tartrate was mixed with any of the used bioagents and reduction in disease incidence combined with yield increase were recorded. Mixture of potassium tartrate and *T. harzianum* was the best one, followed by mixture of potassium tartrate and *B. subtilis*. Potassium tartrate and *P. fluorescens* was the least effective mixture compare with control. Laboratory analysis for treated plants showed that disease control was positively correlated with amount of free and total phenols in treated plants. Amount of protein and chlorophyll positively correlated with fruit yield.

Keywords: Antioxidant, *Bacillus subtilis*, biological control, *Pseudomonas fluorescens, Trichoderma harzianum* and strawberry.

Several pathogenic fungi attack strawberry causing losses in quantity and quality of yield. Diseases caused by soil borne pathogens often more destructive compare with other diseases (Madkour and Aly, 1981). Although chemical control, sometime, achieves considerable results, but causes negative impact to environment and human health. This enforce operators to work hard to avoid these toxic chemicals and replace these chemicals with safe products. Biological control is one of the most promising and safe measure in this respect. Biocontrol agents work through different mode of actions. This help biocontrol agent to be more durable, more effective and without any chemical residues in human food chain (Abd El-Moity, 1981 and Choudhary and Johri, 2009).

Trichoderma harzianum can be considered as ideal biocontrol agent for its good characteristic. This antagonist is very easy to be isolated and grows rapidly on any organic staff. *Trichoderma harzianum* acts through different mode of actions, *i.e.* mycoparasitism (Hennis *et al*., 1983 and Abada *et al.*, 2002), production of antifungal substances (Lumsden *et al*., 1995 and Robinson *et al.*, 2009), also it owns enzymatic system causes destruction for the pathogens (Elad and Kapat, 1999 and Ziedan *et al*., 2005). In addition to these modes of action, Trichoderma also acts as inducer for resistance in treated plants against certain pathogens (Homer, 1993; Abd El-Moity, 2001 and Harman, 2006) and can grow within wide range of temperature and other environmental conditions (Bailey *et al*., 2008 and Singh *et al.*, 2010).

Bacillus subtilis and *Pseudomonas fluorescens* are known as effective antagonistic bacteria against many plant diseases. These antagonists act through antibiosis, secretion of volatile toxic metabolites, destructive enzymes and competition for space and nutrition (Nakkeeran *et al.*, 2002 and Michelsen and Stougaard, 2011). Mixing antagonists with each others may be lead to antagonistic effect consequently decrease efficacy of treatment (Robinson *et al.,* 2009) or lead to synergistic effect and increase the efficacy(Abd El-Moity,1985; Latha, *et al.*, 2009 and Yobo *et al.*, 2011). This increase or decrease is due to harmonic and compatible factors among bioagents. Elementary experiments must be carried out to study these points to decide exactly proper couples in mixture. Mixing bioagent with some antioxidants lead to synergistic effect (Saksirirat *et al.,* 2009; Houssien *et al.*, 2010 and Yousef *et al.*, 2010).

This work aims to study the effective of combination between bioagents and antioxidants in controlling the disease under study.

Materials and Methods

In all field experiments unless otherwise indicated, natural infested sandy soil belongs to PICO Company at Om Saber village, Behera Governorate was used. Strawberry cv. Festival seedlings were cultivated in plots and each plot was 1.2x5m as replicate. Each replicate contained 100 plants and three replicates were used for each treatment. Complete randomize plots design was used.

Isolation, purification and identification of biocontrol agents:

Three different biocontrol agents, *i.e. Trichoderma harzianum*, *Bacillus subtilis* and *Pseudomonas fluorescens,* were used in this study. The biocontrol agents were isolated from healthy strawberry plants grown in heavy infested soil. Isolated biocontrol agents were purified and identified according to Rifai (1969) for *T. harzianum* and Schleifer (2001) for *B. subtilis* and *P. fluorescens*. Purified isolates were used as bioagents in this research.

Propagation of bioagent:

Trichoderma harzianum isolate was grown on liquid gliotoxin fermentation medium (GFM) developed by Brain and Hemming (1945) for 11 days under complete darkness condition at 25ºC to stimulate toxin production (Abd El-Moity, 1981). *B. subtilis* isolate was grown in liquid nutrient glucose medium (NGM) developed by Dowson, (1957) for 2 days at 25ºC. *Pseudomonas fluorescens* isolate was grown on King's medium (King *et al.*, 1954) for 2 days at 25ºC.

Different bioagents were prepared as suspension at concentration of 30 x 10^6 /ml. Suspensions were mixed with 5% Arabic gum and 0.5% potassium soap to increase adhesive capacity and improve distribution of bioagent on the surface of treated seedlings. Strawberry seedlings cv. Festival were dipped in diluted (1:50) different bioagent preparation separately for 30 minutes, just before transplanting.

Preparation of antioxidants:

Two different antioxidants, *i.e.* 1 and 2, were formulated. Antioxidant 1: contains potassium tartrate combined with mixture of micronutrients (Yousef, 2007). Antioxidant 2: contains salicylic acid and ascorbic acid 1:1 (w. w). Solution of each antioxidant was prepared by solving 6 g/l water. Seedlings of strawberry cv. Festival were separately dipped in each of these solutions for 30 min.

Preparation of mixture of bioagents:

To increase the efficacy of the different single bioagents on disease control, three different combinations among the three used bioagents were prepared. Each mixture was prepared by mixing the two antagonists at the rate 1:1. The three different mixtures, *i.e. T. harzianum* + *B. subtilis*, *T. harzianum* + *P. fluorescens* and *B. subtilis* + *P. fluorescens,* were prepared.

Preparation of mixture of bioagents with antioxidants:

Different bioagents were mixed separately either with antioxidant 1 or antioxidant 2 at the rate 1:1 (v:v) to compare the effect of these mixtures with the effect of either single bioagent or single antioxidant and to determine their synergistic effect. Strawberry seedlings received different treatments were transplanted in highly infested soil at the rate of 100 transplants/ plot as described before. Strawberry seedlings cv. Festival without any treatment act as control.

Determine efficacy of different treatments:

Strawberry plants treated with different bioagents or antioxidants were received the same treatment, regarding irrigation or fertilization. After 45 days from transplanting all treatments were examined and percentage of dead plants was calculated as follows:

$$\text{Dead plants (\%)} = \frac{\text{Number of dead plants}}{\text{Total number of plants}} \times 100$$

Synergistic effect was calculated using the following formula:

$$\text{Synergism (\%)} = 100 - \frac{C \times 100}{(A+B)/2}$$

Whereas: C= Dead plants (%) in plants received mixture of (A+B)
(Mixture either two bioagents or bioagent with one of antioxidant).
(A+B) = Dead plants (%) in plants received single treatment A or B.

Laboratory experiment:

Healthy plant samples were collected from different treatments 45 days after transplanting. Healthy plants were extracted and total chlorophyll, protein, phenols and sugars were determined to figure out if there are any effect of the different treatments on plant chemical composition. Correlations between these chemical changes, disease incidence and yield were discussed.

Data were tabulated and statistically analyzed according to the standard procedures including general liner model (GLM) available in SAS (1996). Duncan's multiple- range test was used to illustrate means with significant differences.

Results and Discussion

The aim of this work is to increase efficacy of the bioagent to increase yield component and eventually increase the final yield. To achieve this aim, the effect of mixing the bioagents with each other or in combination with antioxidants were studied. Data in Table (1) indicate that all treatments led to significant decrease to the percentages of disease incidence with considerable increase to the fruit yield and chemicals component (chlorophyll and protein) compared with control treatment. Data also indicate that, *T. harzianum* stand as the most effective one compared with the other two bioagents, *i.e. B. subtilis* and *P. fluorescens.*

Table 1. Effect of using single or mixture of bioagents on dead plants of strawberry in relation to fruit yield during period (15/Dec. – 15/Jan.) and chemical component of treated plants

Treatment	Dead plants (%)	Reduction (%)	Synergism (%)	Total Chlorophyll (Mg/g)	Protein (Mg/g)	Yield ton/ feddan
T. harzianum(T)	6.0 f*	84.2 b	0.0	28.6	1.7	3.9 c
B. subtilis (B)	6.7 e	82.4 c	0.0	30.8	2.2	4.1 b
P. fluorescens (P)	24.0 b	36.8 f	0.0	22.6	1.1	3.66 c
(T) & (B)	4.7 g	87.6 a	26.0	36.0	2.4	4.4 a
(T) & (P).	8.7 d	77.1 d	42.0	29.1	1.8	4.03bc
(B) & (P)	13.0 c	65.8 e	15.3	30.2	1.2	3.5 e
Control	38.0 a	00.0 g	0.0	20.0	0.9	2.5 f

* Values with the same letter are not significantly different.

B. subtilis followed *T. harzianum* in this regard and *P. fluorescens* occupied the last rank. Data also indicate that using any couple of bioagents as mixture led to synergistic effect in all cases. These results are in harmony with those results obtained by Saksirirat *et al.* (2009). Data also show that the most promising results were obtained when the mixture of *T. harzianum* and *B. subtilis* was used followed by the mixture of *B. subtilis* and *P. fluorescens*. Meanwhile, the mixture of *T. harzianum* and *P. fluorescens* occupied the last rank.

To study the mechanism of these treatments and their effect on yield increase, two chemical analyses, *i.e.* chlorophyll and protein contents in treated plants were carried out compare with control treatment. Obtained data in Table (1) indicate that as a result of reduction in disease incidence, chlorophyll and protein contents were increased in all treatments compare with control. The increase in chlorophyll and protein mean more photosynthesis and enzymes consequently more sugars (Prlak and kose, 2009). This will lead to increase in the yield of fruits. Data also indicate that the relation between reduction in disease incidence and increase of chlorophyll and protein contents was very clear when the most effective treatment (*T. harzianum* and *B. subtilis*) was compared with control. This relation cannot be detected in treatments with slight differences when compared with each other.

Data in Table (2) were tabulated to correlate disease reduction, in different treatments, with plant physiology. Chemical analysis of phenol and sugar contents were carried out on plants received different treatments compared with control plants. Obtained data indicate that in all treatments, phenolic content either free or total ones were higher than in plants of control treatment. The same situation was observed when total or non reduced sugars in treated plants were compared with plants in the control. On the light of these results we can explain the mechanism of disease control. This mechanism is due to the direct effect of the antagonist in addition to stimulation of plant resistance by producing active phenols (Cherif *et al*., 2007). Increase of yield also due to two factors, *i.e.* (1) healthy root system can absorb and supply adequate amount of raw nutrient substances and (2) syntheses of these raw nutrient materials effectively in presence of high amount of chlorophyll and protein, which lead to more fruit yield.

Table 2. Effect of using single or mixture of bioagents on dead plants of strawberry in relation to chemical component related to disease incidence

Treatment	Disease reduction (%)	Phenols(Mg/g)			Sugars(Mg/g)		
		free	Conjugated	Total	Reduced	Non-Reduced	Total
T. harzianum (T)	84.2 b*	31.5	25.4	56.9	2.0	1.7	3.7
B. subtilis (B)	82.4 c	24.6	25.8	50.4	2.4	2.6	5.0
P. fluorescens (P)	36.8 f	15.1	21.7	36.8	1.2	3.4	4.6
(T) & (B)	87.6 a	32.7	30.2	62.9	3.7	2.0	5.7
(T) & (P).	77.1 d	30.4	14.1	44.5	2.8	1.1	3.9
(B) & (P)	65.8 e	13.5	33.1	46.6	1.9	1.3	3.2
Control	0.0 g	12.3	23.9	36.2	2.3	0.3	2.6

* Values with the same letter are not significantly different.

Data in Table (3) indicate that all treatments of single bioagent, single antioxidant and the combination between the bioagent and the antioxidant led to significant reduction in disease incidence compared with control treatment. Different single bioagents showed different effects regarding disease control. *Trichoderma harzianum* showed the highest effect followed by *B. subtilis*, being 6.0 and 6.7 % dead plants were recorded. Meanwhile, *P. fluorescens* showed the least effect, where 24% dead plants was recorded. On the other hand, using different antioxidants showed different effects. Antioxidant (1) was the most effective one, where 5.0% dead plants was noticed, compared with 10.0% in case of antioxidant (2). Combining antioxidant with antagonist in one treatment gave different results. When antioxidant (1) mixed with *T. harzianum* synergistic effect was noticed and percentage of reduction in disease was increased by 58.2% compared with the mean of the effect of antioxidant (2) and *T. harzianum*, each alone. On the contrary, when the same antagonist was mixed with antioxidant (2) slight synergistic effect (12.5%) was noticed compared with the mean of the two involved single treatments. When *B. subtilis* was mixed with antioxidant (1) or (2) nearly the same effect (about 43.0% increase in the efficacy of the treatment) was observed compared with the mean results of single treatments. When *P. fluorescens* was mixed with either antioxidant (1) or (2) a clear increase in the efficacy of the treatment was noticed and 63.4 and 47.0 % increase in the efficacy of *P. fluorescens* was observed.

Table 3 Effect of combination between two different antioxidant and bioagents on damping off in strawberry in relation to fruit yield during period (15Dec. – 15Jan.) and chemical component of treated plants

Treatment	Dead plants (%)	Reduction (%)	Synergistic (%)	Total Chlorophyll	Protein (Mg/g)	Yield ton/feddan
T. harzianum (T)	6.0 f*	84.2 e	0.0	28.6	1.7	3.9 e
B. subtilis (*B*)	6.7 e	82.4 f	0.0	30.8	2.2	4.1 abc
P. fluorescens (P)	24.0 b	36.8 i	0.0	22.6	1.1	3.66 f
Antioxidant (1)	5.0 gh	86.4 cd	0.0	34.1	3.1	4.03 cde
Antioxidant (2)	10.0 c	73.7 h	0.0	20.0	1.2	3. g
Ant. 1 & (T)	2.3 j	94.0 a	58.2	32.8	3.0	4.35 a
Ant. 2 & (T)	7.0 e	81.6 f	12.5	30.0	2.7	4.0 ed
Ant. 1 & (B)	3.3 i	91.3 b	43.6	38.5	2.6	4.23 ab
Ant. 2 & (B)	4.7 h	87.6 c	43.7	33.1	2.2	4.13 bcd
Ant. 1 & (P)	5.3 g	86.0 d	63.4	29.1	1.5	4.0 ed
Ant. 2 & (P)	8.0 d	79.0 g	47.0	26.5	1.8	3.7 f
Control	38.0 a	00.0 j	0.0	20.0	0.9	2.5 h

* Values with the same letter are not significantly different

The effect of the different treatments on plant physiology, specially chlorophyll and protein content was assessed, where these component directly affect on the yield. Data in Table (3) show that mixing any of bioagents with antioxidant (1) or (2) led to clear increase in chlorophyll content compared with the effect of the bioagent alone. When protein was determined, obtained data show that in all cases, except only one case (*B. subtilis* and antioxidant (2)), mixing the bioagents with the antioxidant (1) or (2) led to an increase in protein content compared with single bioagents treatment. In addition, the mixture of *B. subtilis* and antioxidant (1) resulted in nearly double amount of chlorophyll in treated plants compared with control treatment. These results are in agreement with those obtained by Yousef *et al.* (2010). No differ in the chlorophyll content of plants treated with the antioxidant (2) and the control was detected. Very slight difference in the chlorophyll of plants treated with *P. fluorescens* and control treatment was also noticed. The mixture of *B. subtilis* and antioxidant (2) occupied the second rank after the mixture of *B. subtilis* and antioxidant (1).Other treatments filled in-between. These synergistic effect could be explained on the light of the work of the authors (unpublished data), where these antioxidants positively affect on the rate of growth of *B. Subtilis* and *T. harzianum*.

Obtained data in Table (4) can explain the reason of different degrees of efficacy of different treatments. When *T. harzianum* and antioxidant (1) was used, the highest degree of disease control was observed compared with control treatment and 94.0% reduction in disease incidence was observed. The same treatment also showed the highest content in free and total phenols compared with any other treatment. This phenomenon is very clear at high differences in disease control. The result of this present study correlated with the report of Latha *et al*. (2009). In case of slight differences in efficacy among treatments, the relation between efficacy and phenols content can't be correlated. Regarding sugar content no clear relation between any sugar type content and degree of disease incidence was observed. The only clear relation was the total and non reducing sugars, where they were more in treated plants compared with the control.

Table 4. Effect of combination between two different antioxidant and bioagents on dead plants disease incidence in strawberry in relation to chemical component of treated plants

Treatment	Disease reduction (%)	Phenols (Mg/g)			Sugars (Mg/g)		
		free	Conjugated	Total	Reduced	Non-Reduced	Total
T. harzianum (*T.h*)	84.2 e*	31.5	25.4	56.9	2.0	1.7	3.7
B. subtilis (*B.*s)	82.4 f	24.6	25.8	50.4	2.4	2.6	5.0
P. fluorescens (*P.f*)	36.8 i	15.1	21.7	36.8	1.2	3.4	4.6
Antioxidant (1)	86.4 cd	32.1	13.6	45.7	1.2	1.7	2.9
Antioxidant (2)	73.7 h	30.4	8.2	38.6	1.5	1.0	2.5
Ant. 1 & (*T.h*)	94.0 a	48.1	15.6	63.7	2.0	2.1	4.1
Ant. 2 & (*T.h*)	81.6 f	22.0	15.0	37.0	1.4	1.8	3.2
Ant. 1 & (*B.*s)	91.3 b	44.0	18.0	62.0	2.8	0.8	3.6
Ant. 2 & (*B.*s)	87.6 c	33.0	15.0	48.0	2.5	1.6	4.1
Ant. 1 & (*P.f*)	86.0 d	40.0	15.2	55.2	2.0	2.6	4.6
Ant. 2 & (*P.f*)	79.0 g	24.6	15.4	40.0	2.1	0.7	2.8
Control	00.0 j	12.3	23.9	36.2	2.3	0.3	2.2

* Values with the same letter are not significantly different.

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تقييم مخاليط مختلفة من كائنات المقاومة الحيوية ومضادات الأكسدة مع كائنات المقاومة الحيوية على أعفان الجذور فى الفراولة

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محصول الفراولة من أهم محاصيل الخضر التصديرية في مصر و ذلك لصفاتها الجيدة و الظهور المبكر في الأسواق. عفن الجذر الأسود من الأمراض الشائعة على نبات الفراولة و يسببه مجموعة من ممرضات أعفان الجذور وتشمل : *Rhizoctonia solani*, *Fusarium solani* and *Macrophomina phaseolina*. هذا المرض يسبب خسائر اقتصادية على نبات الفراولة و كذلك على عديد من النباتات الأخرى. تمثل المقاومة الحيوية طريقة بديله صديقة للبيئة لحماية النباتات من الممرضات. ولكن أحيانا لاتستطيع الكائنات المستخدمة فى المقاومة الحيوية بمفردها أن تعطى المقاومة الفعالة كما فى المبيدات الكيميائية. هذه الدراسة تناقش كيفية زيادة فعالية كائنات المقاومة الحيوية عن طريق استخدام خليط مكون من أكثر من كائن واحد أو بخلط هذه الكائنات مع مضادات أكسدة مختلفة بدلا من استخدام معاملة مفردة. فى تجربة حقلية تم استخدام مضاد الأكسدة (١) الذى يحتوى على طرطرات بوتاسيوم و مضاد الأكسدة (٢) الذي يحتوى على حامض السلسيليك. أظهرت النتائج أن جميع المعاملات أدت إلى خفض في حدوث المرض بدرجة معنوية مقارنة بالكنترول. أعطى الخلط بين *T. harzianum* and *B. subtilis* أعلى حماية للنباتات مما أدى الى زيادة المحصول. الخلط بين *T. harzianum* and *P. fluorescens* احتل الدرجة الثانية والخلط بين *B. subtilis* and *P. fluorescens* أعطى أقل تأثير. الخلط بين مضادات الاكسدة و الكائنات المختلفة أدى الى خفض فى حدوث المرض مقارنة بالمعاملات المفردة. لوحظ تأثير تكاملى عند خلط مضاد الأكسدة (١) مع الكائنات المختلفة وكذلك خفض فى حدوث المرض ملازما زيادة فى المحصول. نتيجة الخلط بين antioxidant (1) and *T. harzianum* كانت أفضل نتيجة يليها antioxidant (1) and *B. subtilis* ثم antioxidant (1) *P. fluorescens* مقارنة بالكنترول. التحاليل الكيميائية التى تمت على النباتات المعاملة اظهرت أن مقاومة المرض فى النباتات المعاملة ترتبط بشده بمحتوى هذه النباتات من الفينولات الحره والكليه. يوجد ارتباط ايجابى بين كميات الكلوروفيل و البروتين وزيادة المحصول.