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Exploitation of Biostimulants as an Alternative Strategy to Control Bacterial Rot Diseases of Onion (*Allium cepa* L.)

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Bacterial soft rots, commonly caused by *Erwinia carotovora* subsp. *carotovora* or *Erwinia chrysanthemi*, and slippery skin, caused by *Burkholderia gladioli* subsp. *allicola* as well as sour skin, caused by *Burkholderia cepacia* of onion bulbs are among the most serious storage diseases of onion (*Allium cepa* L.) that lead to great loss in bulbs. In this study some biostimulants; chitosan, seaweed extract and humic acid were *in vivo* tested on onion plants, to determine their controlling potential against bacterial rot diseases. Application of biostimulants proved to be effective in reducing the bacterial rot in stored onion bulbs. All tested biostimulants increased photosynthetic pigments content in onion leaves and total phenols content in onion bulbs, meanwhile they declined lipid peroxidation and the electrolyte leakage percentage in onion bulbs. As a summary, chitosan, seaweed extract and humic acid could be applied to improve plant growth and stimulate plant defense against diseases in concern. These materials are cheap, available, easy to apply, hazardless and environmentally safe.

Keywords: Chitosan, humic acid, onion and seaweed.

Bacterial rot diseases of onion are among the most serious storage diseases of onion bulbs which may be recognized into at least three distinct forms; basically known as soft rot, caused by *Erwinia carotovora* subsp. *carotovora (Ecc)* or *E. chrysanthemi (Ec)*; slippery skin, caused by *Burkholderia gladioli* subsp. *allicola (Bga)* and sour skin, caused by *Burk. cepacia (Bc)* (McNab, 2004 and Chaput, 1995). Onion bacterial rots cause significant yield losses (Schwartz and Gent, 2004), thus the need for successful control measure is very utmost urgent.

In view of the hazardous impact of chemical pesticides and other agrochemicals on the ecosystems and human health, the biological or almost natural control of plant diseases as an alternative strategy has received increasing attention in recent years. Among the formulations that can be used in the biological control of plant diseases are the biostimulants which are defined as ''materials, other than fertilizers, that promote plant growth when applied in small quantities'' and are also referred to as ''metabolic enhancers'' (Zhang and Schmidt, 1997).

Among the most promising biostimulant oligosaccharides is chitosan (2-amino-2-deoxy-β-D-glucose), which has attracted tremendous attention because of its biocompatibility, non-toxicity and biodegradability. Inhibitory effect on the growth of various pathogens and stimulating plant growth was indicated (Farouk *et al.*, 2008 and 2012) and its ability to be potent elicitor of plant defense reactions was correlated (Sharathchandra *et al.*, 2004).

Chitosan also attracted attention by its great antioxidant activity (Park *et al.,* 2004), it can scavenge OH and O2 - radicals and has been shown to have DNA-protection properties (Harish Prashanth *et al.*, 2007). Recently, Sheikha and Al-Malki (2011) indicated that chitosan works as a positive factor in enhancing shoot and root length, fresh and dry weights of shoots and roots as well as leaves area. For some horticultural and ornamental commodities, chitosan increased harvested yield (Bautista-Baños *et al.*, 2006).

Application of seaweed extracts have been proven to have a wide range of beneficial effects on plants such as deeper root development, delay of fruit senescence (Zodape *et al.*, 2008 and 2011), early seed germination and improved crop performance and elevated resistance to biotic and abiotic stress, along with increasing shelf-life of perishable products were considered (Blunden, 1991 and Norrie and Keathley, 2006) as well as plant defense improvement against diseases (Farouk *et al*., 2012). Brown seaweeds are most commonly used in agriculture (Blunden and Gordon, 1986) especially, *Ascophyllum nodosum* L., commonly known as Norwegian kelp (Ugarte *et al.,* 2006). Seaweed contains all major and minor plant nutrients, all trace elements, alginic acid, vitamins, cytokinins, auxins, at least two gibberellins and antibiotics. Alginic acid is a soil conditioner, while the remainders are plant conditioners that affect cellular metabolism in treated plants leading to enhanced growth and crop yield (Khan *et al.,* 2009). The exact physiological mechanisms are still not known.

Humic acid can be applied successfully in many areas of plant production as a plant growth stimulant or soil conditioner for improving natural resistance of plant against diseases and pests (Farouk *et al.*, 2012), through stimulating plant growth of nutrients and water (El-Ghamry *et al.*, 2009). Humic acid preparations were reported to promote the root length (Canellas *et al.*, 2002) and increase the fresh and dry weight of crop plants (Chen *et al.*, 2004a and 2004b). Several reports indicated the efficiency of humic acid in reducing some plant diseases, as chocolate spot and rust diseases of faba bean (El-Ghamry *et al.*, 2009), and early blight of tomato (Farouk *et al*., 2012).

The objective of this study was to evaluate the efficacy of chitosan, seaweed extract and humic acid on controlling onion postharvest bacterial rot and studying some physiological responses of onion plants as a result of the use of these materials.

Materials and Methods

The pathogenic bacterial strains:

Two isolates of the onion bacterial rot pathogens; *Erwinia carotovora* subsp. *carotovora* (Ecc) and *Burkholderia cepacia* (Bc) were recovered and identified in a previous study by Mansour *et al.* (2011).

The tested biostimulants:

Three biostimulants were used in this study; chitosan (Chito), seaweed extract (SW) and humic acid (HA). The chitosan was produced by ROTH Company, Germany. Chitosan solution was prepared using a modified method of El-Hassni *et al.* (2004) through dissolving it in 0.25 N HCl, the pH was adjusted to 5.6 with 2 N NaOH. The seaweed extract is an extract from the brown seaweed *Ascophyllum nodosum*, in form of water soluble paste, produced by Acadian Seaplants Limited Company (Dartmouth, Nova Scotia, Canada). While humic acid was obtained from company of Humax Manitoba Ltd (Carberry, Canada), in liquid form.

Screening antibacterial activity of the tested biostimulants against Erwinia carotovora subsp. carotovora and Burkholderia cepacia:

The cup plate method was used to determine the antibacterial activity of Chito, SW and HA against Ecc and Bc. One hundred micro litter of each biostimulant solution was placed in wells centrally made in nutrient agar (NA) containing plates seeded with Ecc and Bc, separately. Plates were incubated at 28°C for 24 h, and the diameter of inhibition zone was determined.

In vivo experiments:

Pot experiment:

The pot experiment was conducted to study the effect of the tested biostimulants (Chito, SW and HA) on controlling onion bacterial rot diseases under artificial inoculation condition. Onion transplants of Giza red cultivar were divided into four groups and their root systems were soaked overnight in the different treatments; Chito at 0.1%, SW at 2000 ppm, HA at 1000 ppm (the candidate concentration of each material was selected according to previous preliminary experiments) and water treatment as a control separately, then air dried. Plastic pots of 17 cm diameter containing clay: sandy soil mixture (2: 1), were planted with the different seedlings of onion transplants, two transplants per pots. All the suitable cultural practices were carried out through the growing period. Two months after planting, a second spray of each biostimulant was made. One month before the harvest, onion groups were inoculated with liquid bacterial cultures of rot pathogens (100 ml of the bacterial culture, 10^6 cfu/ml were poured in the soil of each pot), each in subgroup as follow:1) inoculated with Ecc, 2) inoculated with Bc and 3) treated with water as control. Onion was harvested after four months from planting (when 50 % of the onion necks fallen down). Then onion was leaved in the open air for two weeks before being stored.

Field experiment:

This experiment was carried out at Dakahlyia Governorate, Egypt in a field naturally infected with the bacterial rot pathogens to investigate the role of Chito, SW and HA in controlling onion bacterial rot. The experimentation area contained 16 plots (6.5m x 5m^2 each). Red onion transplants roots (cv. Giza) were soaked in different treatments of Chito at 0.1%, SW at 2000 ppm, HA at 1000 ppm (concentration of each material was selected according to previous preliminary experiments) and water as control. Planting of different treatments was made in the field plots, to make complete randomized design, with four replicates for each treatment. Two months after planting, all the treatments were applied for a second time by spraying the onion plants, downward until the soil. All the suitable cultural practices were carried out through the growing period. Onion was harvested four months after planting (when 50 % of the onion necks collapsed). Then crop was kept in open air for two weeks before being stored in the same experimentation pattern.

Preharvest estimations:

One month after planting the photosynthetic pigments were estimated for grown onion in the 3^{rd} upper leaf according to Lichtenthaler and Wellburn (1985). Furthermore, some foliar growth parameters as number and length of leaves were determined in both pots and field grown onions.

Postharvest determinations:

Total phenols content; was determined using Folin-Ciocalteau reagent according to the method of Singleton and Rossi (1965), anthocyanin was assessed following the method of Mancinelli *et al.* (1975). Total soluble solids; were determined using hand refractometer as described by Andersen (2000). Lipid peroxidation; was determined by the method of Shao *et al.* (2005) and electrolyte leakage; was estimated according to Goncalves *et al.* (2007) using an electrical conductivity meter.

Bacterial rot incidence was estimated for both pots and field grown onion after 3, 6, 8 and 10 months from the beginning of storage.

Statistical analysis:

Data were statistically analyzed with CoStat software (Anonymous, 2005). Data were first subjected to analysis of variance (ANOVA), then the multiple comparisons among means were made using new multiple range test at $P \leq 0.05$.

Results

Antibacterial activity of biostimulants against Erwinia carotovora subsp. carotovora and Burkholderia cepacia:

Chitosan (Chito), seaweed extract (SW) and humic acid (HA) had no antibacterial activity against Ecc and Bc where the diameter of inhibition zone was recorded as zero in all cases.

In vivo experiments:

Pot experiment:

Preharvest estimations:

Data in Table 1 showed that, Chito, SW and HA treatments increased the photosynthetic pigments concentration in onion plants compared to the untreated control plants. SW exhibited the best results in this regards hence it significantly increased chlorophyll A (Chl. A), chlorophyll B (Chl. B) and total carotenoids. Moreover, application of HA significantly increased Chl. A and Chl. B concentration in onion leaves as compared with untreated control plants. It was also observed that growth parameters, as leaves number per plant and leaves length, responded positively and significantly to the application of the biostimulants as compared with the control plants. Maximum leaves number per plant (9) was observed in the treatment of Chito and HA and maximum leaves length (51 cm) was observed in SW as compared with control plants.

Table 1. Photosynthetic pigments concentration in the 3rd upper onion leaves and plant growth as affected by biostimulants application under artificial inoculation condition in the pot experiment

Treatment*	Photosynthetic pigments content**			Foliar growth parameters	
	Chl. A (µg/gm)	Chl. B (µg/gm)	Total carotenoids (µg/gm)	No. of leaves	Length of leaves (cm)
Chito	687.6 b	410.4 b	494.1 b	9 a	45.5 b
SW	752.0 a	544.5 a	570.1 a	8 ab	51.0 a
HA	743.7 a	567.3 a	444.4 c	9 a	46.5 b
Control	654.7 c	355.5 b	460.8 bc	7 b	34.5 c

* Chito= chitosan, SW= seaweed extract and HA= humic acid.
** Chl. A= chlorophyll A and Chl. B= chlorophyll
\- Values within a column followed by the same letters are not significantly different according to CoStat software at $p < 0.05$. Values are means of six replicates.

Postharvest estimations:

Data in Table 2 showed that application of biostimulants decreased the disease incidence (DI) caused by Ecc and Bc three after storage of the pots grown onions. Chitosan exhibited promising results in this regard followed by SW and HA, thus the mean DI (DI mean of infected onion) was recorded as zero, 9.55 for Ecc and 19.09 % for Bc, respectively. Furthermore the disease incidence was zero when recorded after six, eight and ten months from storage.

Table 2. Bacterial rot incidence after three months from storage of pots grown onion bulbs as affected by the application of biostimulants

Treatment * (Biostimulant)	DI of onion ** infected with Ecc (%)	DI of onion infected with Bc (%)	Mean DI (%)
Chito	0	0	0.0
SW	9.1	10	9.5
HA	18.2	20	19.1
Control	20	33.3	26.7

* As described in footnote of Table (1).
** DI= Disease incidence.

Field experiment:

Preharvest determinations:

Data in Table 3 indicated that the used biostimulants significantly increased the photosynthetic pigments Chl. A, Chl. B and carotenoids. HA in proven superior in this regard. It was also noticed an increase in the number of leaves, whereas the length of leaves was significantly increased by all treatments.

Table 3. Photosynthetic pigments concentration in the 3rd upper onion leaves and plant growth as affected by biostimulants application in the field experiment

Treatment * (Biostimulant)	Photosynthetic pigments **			Foliar growth parameters	
	Chl. A (µg/gm)	Chl. B (µg/gm)	Total carotenoids (µg/gm)	No. of leaves	Length of leaves (cm)
Chito	618.1 b	390.9 a	431.9 b	10 a	59.7 a
SW	684.8 a	399.5 a	480.5 a	10 a	61.0 a
HA	699.3 a	406.6 a	489.2 a	10 a	59.0 a
Control	547.4 c	279.5 b	397.8 b	10 a	48.7 b

*&** As described in footnote of Table (1).

- Values within a column followed by the same letters are not significantly different according to CoStat software at $p < 0.05$. Values are means of six replicates

Postharvest determinations:

Data in Table 4 pointed to the efficacy of the tested biostimulants total phenols, anthocyanin pigment, total soluble solids (TSS %), lipid peroxidation and electrolyte leakage percentage (ELP). It is evident that all treatments increased the total phenols significantly in onion bulbs. All similar effect was recognized of the treatments in concern on anthocyanin and it was slightly higher than that of the control. Moreover the TSS % was increased by Chito and HA treatments. All treatments significantly decreased the malondialdehye (MDA) concentration indicating significant decrease in lipid peroxidation. The ELP decreased by all treatments.

Table 4. Disease resistance indices in onion bulbs after harvest as affected by biostimulants application in the field experiment

Treatment * (Biostimulant)	Total phenols (mg/100 gm)	Anthocyanin (absorbance/ gm)	Total soluble solids (%)	Lipid** peroxidation (MDA= µ mol/gm)	Electrolyte leakage (%)
Chito	37.9 a	0.8 a	13.2 a	1.4 b	66 a
SW	35.6 b	0.8 a	12.3 a	1.6 b	62 a
HA	34.3 b	0.8 a	13.2 a	2.0 b	64 a
Control	31.4 c	0.8 a	12.5 a	3.8 a	69 a

* As described in footnote of Table (1).

** MDA= Malondialdehye.

- Values within a column followed by the same letters are not significantly different according to CoStat software at $p < 0.05$. Values are means of six replicates.

Data in Table 5 revealed that three months storage of the field grown onion decreased the DI incidence of the bacterial rot following treatment with Chito, SW and to a significant level HA in case of HA. Significant decrease in DI was detected after six months of storage, of onion bulbs by all treatments. Similar result was obtained eight and ten months after storage. In general; the reduction in DI values was more pronounced throughout storage durations in months.

Table 5. Bacterial rot disease incidence throughout storage of field grown onion bulbs as affected by the application of biostimulants

Treatment (Biostimulant)	DI after 3 months (%)	DI after 6 months (%)	DI after 8 months (%)	DI after 10 months (%)
Chito	12.2 ab	7.9 b	1.3 b	0.3 b
SW	9.7 ab	6.3 c	0.8 b	0.0 c
HA	7.5 b	6.0 c	1.9 b	0.1 bc
Control	16.3 a	11.6 a	3.9 a	0.8 a

* As described in footnote of Table (1).

** DI= Disease incidence.

- Values within a column followed by the same letters are not significantly different according to CoStat software at $p < 0.05$. Values are means of six replicates

Discussion

In the present study, three biostimulants were applied to control bacterial rot diseases of onion. Based on several studies, the biostimulants act through enhancing plant growth and inducing plant resistance to pathogens (Jayaraj *et al.*, 2008; El-Ghamry *et al.*, 2009 and Abdel-Mawgoud *et al.*, 2010). In the present study, number and length of onion leaves were responded positively upon the application of Chito, SW and HA as compared with the control plants, this is in agreement with Farouk *et al.*, 2012 who proved the enhancement of tomato plant growth by application of Chito, SW and HA under natural infection of early blight after one hundred days after transplanting. The enhancement of plant growth by the biostimulants may be attributed to their effect on some physiological processes in plant such as ion uptake, cell elongation, cell division, enzymatic activation and protein synthesis (Vick and Zimmerman, 1987). In this concern, Farouk *et al.*, 2012 found that application of Chito, SW and HA significantly increased ions percentage in tomato shoot represented as nitrogen, phosphorous and potassium which are necessary for plant growth and increase in cell permeability (Chen and Aviad, 1990).

The present work indicated that there was a significant increase in the photosynthetic pigments content in onion leaves after treatments with Chito, SW and HA, that may be due to enhancement in the efficacy of the photosynthetic apparatus and the decrease in photophosphorylation rate, usually occurring after infection (Amaresh and Bhatt, 1998). Biostimulants were found to increase potassium content (Farouk *et al.*, 2012), which may increase the number of chloroplasts per cell, number of cells per leaf and consequently leaf area (Taiz and Zeiger, 1991). The role of SW in increasing chlorophyll content was attributed to the presence of betaines in the SW which decrease chlorophyll degradation (Whapham *et al.*, 1993 and Blunden *et al.*, 1997). These results were confirmed in tomato plants by Zodape *et al.* (2011) and Farouk *et al.* (2012) and in faba bean by Al-Ghamry *et al.* (2009). Moreover, the positive effect of biostimulants on reducing disease incidence may be due to their efficacy as Alicitors since localized treatments of plants with biotic or abiotic defense Alicitors can result in localized or systemic responses. Such biostimulants has the ability to inhibit the devAlopment of the bacterial rot diseases in onion plants. Among natural Alicitor compounds, chitosan offers a great potential as a biodegradable material that have anti-microbial and alicitation activities (Benhamou, 1996). In addition to its direct antimicrobial activity, chitosan induces a series of defense reactions correlated with enzymatic activities. Chitosan has been shown to increase the production of glucanohydrolases, phenolic compounds and synthesis of specific phytoalexins with antifungal activity and also reduces macerating enzymes such as polygalacturonases and pectin methylestrase (Bautista-Baños *et al.*, 2006). In addition, chitosan induces structural barriers around pathogen penetration sites, for example inducing the synthesis of lignin-like material. Due to its ability to form a semipermeable coating, chitosan extends the shelf life of treated fruit and vegetables by minimizing the rate of respiration and reducing water loss (Bautista-Baños *et al.*, 2006). The antioxidant activity of chitosan has also attracted attention (Park *et al.*, 2004). Chitosan can scavenge OH and O2 - radicals and has been shown to have DNA-protective properties (Harish Prashanth *et al.*, 2007).

In the current study the preharvest application of chito on onion plants reduced the incidence of bulb rot throughout storage by inducing defense activities as shown by the significant increase in total phenols in onion bulbs upon chitosan application (Table 4), this is in agreement with the results of Liu *et al.* (2007) who used chitosan for controlling postharvest grey and blue mould diseases of tomato. The same results were confirmed by Al-Hassni *et al.* (2004) on the date palm after application of chitosan to control *Fusarium oxysporum* f.sp. *albedinis*, the causal agent of a major wilt in this crop. Chitosan application on onion was found to reduce the extent of lipid peroxidation and the Alectrolyte leakage (Table 4). This is in harmony with Yang *et al.* (2009) and Farouk *et al.* (2012) who reported that application of chitosan suppressed the increase in MDA concentration in plant tissue and the Alectrolyte leakage. The antioxidant properties of chitosan are primarily attributed to its abundant active hydroxyl and amino groups, which react with ROS to form stable and relatively nontoxic macromolecules radicals (Sun *et al.*, 2004 and 2008). The reduction of onion bacterial rot incidence by chitosan is also in accordance with the results of Abd-Al-Kareem *et al.* (2002) who reported that chitosan treatment induced resistance against late and early blight diseases of potato and Farouk *et al.* (2012) who used chitosan to control early blight disease in tomato.

Treatment of onion with SW significantly increased the total phenol contents, also significantly reduced the extent of lipid peroxidation and reduced Alectrolyte leakage (Table 4) confirming its Alicitation activity, these results in agreement with that of Jayaraj *et al.* (2008) who established the reduction of foliar fungal diseases on carrot upon the application of SW. They attributed the disease control in SW-treated plants to the accumulation of higher levels of defense-related gene transcripts and showed higher activities of defense-related enzymes. They involved as wall the effect of higher levels in the total phenolic content and phytoalexin. In this regard, tomato plants treated with SW showed resistance to leaf curl, bacterial wilt and fruit borer by Zodape *et al.* (2011) and the reduction in the early blight disease by Farouk *et al.* (2012). Seaweeds are a rich source of antioxidant polyphenols with bactericidal properties (Zhang *et al.*, 2006). The application of *Ascophyllum nodosum* extract to bentgrass (*Agrostis stolonifera*) increased SOD activity, which in turn significantly decreased dollar spot disease caused by *Sclerotinia homoeocarpa* (Khan *et al.*, 2009).

The current study demonstrated that HA at 1000 ppm decreased onion bacterial rot incidence throughout storage, the effect may be attributed is the significant increase in level of the total phenolic content and the significant decrease in the lipid peroxidation and Alectrolyte leakage in the HA-treated plants. (Table 4). These results confirm the role of HA in enhancing the natural resistance of plant against diseases and pests Abd-Al-Kareem *et al.* (2009) and Farouk *et al.* (2012) who proved the potentiality of HA in control early blight disease in potato and tomato, respectively. Furthermore, Abd-Al-Kareem (2007) reported that bean plants treated with humic acid induced resistance against root rot and Alternaria leaf spot in addition to increased bean yield under field conditions.

In conclusion, the tested biostimulants (chitosan, seaweed extract and humic acid) in this study proved to have a promising effect in reducing the onion bacterial rot diseases as shown by the potential to stimulate plant defence against diseases enhancement of plant growth. However, further studies are needed to evaluate in depth the protective role and impacts on diseases resistance.

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توظيف المستحثات الحيوية كوسائل بديلة لمكافحة أمراض الأعفان البكتيرية فى البصل

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الأعفان البكتيرية فى البصل مثل العفن الطرى المتسبب عن البكتيريا *Erwinia carotovora* تحت النوع *carotovora* أو النوع *E. chrysanthemi* والجلد المفكك المتسبب عن البكتيريا *Burkholderia gladioli* subsp. *allicola* أو الجلد النتن المتسبب عن البكتيريا *Burkholderia cepacia* تعتبر من أخطر الأمراض التى تصيب البصل أثناء التخزين حيث تؤدى إلى فقد كبيرفى الإنتاج .

فى هذه الدراسة تم إختبار قدرة بعض المستحثات الحيوية على مقاومة الأعفان البكتيرية فى البصل وهى الكيتوزان ومستخلص طحالب بحرية وحمض الهيومك ، لتقييم قدرتها على مقاومة الأعفان البكتيرية على نباتات البصل. وقد وجد أن إستخدام المستحثات الحيوية أدت إلى إختزال الأعفان البكتيرية أثناء التخزين. كما أدى إستخدام المستحثات الحيوية إلى زيادة كمية أصباغ البناء الضوئى فى أوراق البصل وكذلك زيادة المحتوى الكلى من الفينولات فى الأبصال ، بينما أدى إستخدام هذه المستحثات إلى إختزال تأكسد الدهون وكذلك إختزال نسبة تسرب الإلكتروليتات فى الإبصال المعاملة. وخلاصة القول فإن إستخدام الكيتوزان ومستخلص الطحالب البحرية وحمض الهيومك يؤدى إلى تحسين نمو النباتات وإستحثاث المقاومة ضد تلك الأمراض. علماً بأن هذه المواد رخيصة الثمن ومتاحة وسهلة الإستخدام وآمنة بيئيا.