Efficacy of Ozone and Hydrogen Peroxide on Controlling Crown Gall Bacterium and Root Knot Nematode Infected Guava Plants in Egypt

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ABSTRACT

Ozone gas (O_3), ozonated water (OW) and hydrogen peroxide (H_2O_2) were tested *in vitro* and *in vivo* in different concentrations against four isolates of crown gall bacterium and one isolate of root-knot nematode. The tested isolates were obtained from galled guava roots grown in Rosetta and Edkou farms, Beheira Governorate, Egypt. Biochemical and physiological characterizations, tumorgenicity and molecular features of the four isolates proved that these isolates belong to *Agrobacterium tumefaciens*. All concentration level of O_3 (50-450 ppm) gave bacteriostatic effect on bacterial cells as well as deactivated the tumor induction caused by *Agrobacterium* isolates. However, all the tested concentrations describe previously of O_3 showed nematicidal effect against the second stage juveniles (J_2) of *M. incognita* after 24 hrs of exposure. In addition, tested concentrations of OW (15-135 ppm) showed bacteriostatic and nematicidal effect on *A. tumefaciens* isolates and J_2 of *M. incognita*, respectively. On the other hand, all the tested concentrations (1, 3, 5, 7 and 9%) of H_2O_2 showed bactericidal and nematicidal effects against *A. tumefaciens* and J_2 of *M. incognita*, respectively. Scanning electron microscope photographs clearly showed the damage of *A. tumefaciens* cells and J_2 of *M. incognita* exposed to either O_3 or H_2O_2 at concentrations of 450 ppm and 9%, respectively.

Key words: *Agrobacterium tumefaciens*; *Meloidogyne incognita*; *Pisidum guajava*; Ozone; Ozonated water; Hydrogen peroxide; SEM.

INTRODUCTION

The causal agent of crown gall bacterium, *Agrobacterium tumefaciens*, is well-known in soil. An early comprehensive review on the host range of crown gall disease reported 643 susceptible hosts belong to 93 families, mainly gymnosperms and dicotyledons (De Cleene and De Ley, 1976). Crown gall bacterial pathogen infects the roots of dicotyledonous plants through lesions or wound injuries. These wounds may be caused by biological agents such as nematodes and insects or by mechanical tools (Kersters and Deley, 1984; Woese *et al*, 1984; Bouzar *et al*., 199; Setti and Bencheikh, 2013). The U.S. Environmental Protection Agency (EPA) classified methyl bromide as a restricted use pesticide (EPA, 1986) and currently being reduced because of its ability to deplete ozone layer (Kim *et al*., 1999). Many investigators are developing enviro-nmentally sound economically feasible alternatives that can be adopted once the phase out of methyl bromide is complete. Ozone (O_3) is highly reactive with many organic compounds (Razumovskii and Zaikov, 1984) and has been used in soils for environmental remediation of contaminated sites and degradation of pesticides (Somich *et al*., 1990; O'Mahony *et al*., 2006). The effective biocidal strength of O_3 due to its ability to oxidize organic molecules in living organisms (Qiu *et al*., 2009). Similarly, hydrogen peroxide (H_2O_2) may possess direct microbicidal activity at the sites of pathogen invasion (Alvarez *et al*., 1998). It is used for cell-wall reinforcing processes, lignification and oxidative cross-linking of hydroxyproline-rich proteins and other cell-wall polymers (Kuzniak and Urbanek, 2000).

The present investigation was undertaken to isolate and identify crown gall bacterium and root-knot nematode attacking guava plants grown in Rosetta and Edkou farms, Beheira Governorate, Egypt, using biochemical, physiological and molecular techniques as well as to study the effectiveness of O_3, ozonated water (OW) and hydrogen peroxide (H_2O_2)as alternative control compounds. Also, this study aimed to confirm the damage of *Agrobacterium* cells and root-Knot nematode J_2 as a result of O_3 or H_2O_2 treatment using scanning electron microscope.

MATERIALS AND METHODS

Samples collection and isolation

Guava (*Pisidum guajava* L.) plants showed crown gall and/or root-knot nematode disease symptoms were collected in April 2013 to September 2014 from Rosetta and Edkou farms, Beheira Governorate, Egypt. Symptomatic materials were placed in plastic bags and transported to the laboratory for further studies.

For bacterial isolation, Clark's selective medium designated as NASA, (Serfontein and Staphorst, 1994) was used. For nematode isolation, juveniles (J_2) of root-knot nematodes were extracted from 100 gm galled roots of Guava plants, which were cut into small pieces in a Petri-dish with 5 ml

distilled water. Under the magnification of a stereoscopic microscope, individual egg-masses which usually protruded from the root were observed. The eggs were passed through a tissue of filter and hatching to obtain J_2 as pure cultures for further studies.

Pathogenicity test

Pathogenicity test of bacterial isolates were done using squash fruits, *Cucurbita pepo* as recommended by Shoeib, (1997). The collected J_2 of nematode were used to inoculate roots of Guava seedling, 9 months in old, to confirm the pathogenicity, and then re-isolation was done. Roots of tomatoes seedlings (*Solanum esculentum*) cv. 'Super Marmand', 15 days in old, were inoculated with J_2 for preserving to further study.

Identification of bacterial isolates

Classical and Molecular methods

Bacterial colonies suspected of being *Agrobacterium* sp. were initially identified using microscopically features included cell shape, Gram staining, motility and capsule formation, according to Moore *et al* (2001). Bacterial isolates were tested for their ability to produce acid from lactose, sorbitol, mannose, fructose, maltose and glucose. The bacterial growth at 37 °C and tolerance to grow on 2% and 5% NaCl were determined. The bacterial growth on potato dextrose agar medium (PDA) at pH 7.4 was also determined after 72 hrs at 25°C. The previous biochemical and physiological properties of the tested isolates were conducted according to Moore *et al*. (1988) and Brenner *et al*. (2005).

Polymers chain reaction (PCR) analysis was conducted using the two primer pairs; tms2A/tms2B and virD2A/virD2C primers (Table 1) designed by Haas *et al*. (1995) to identify the four tested *Agrobacterium* sp. isolates. These primers amplify the highly conserved DNA sequences in the *tms* and *virD2* gene on the Ti plasmid.

16S rRNA gene assays, full length (1550bp) of 16S rRNA gene was amplified for the four *A. tumefaciens* isolates using the two primers, P0 and P6 (Table 1).The amplified product of 16S rRNA gene (1550 bp) was sequenced using a Big Dye Terminator Cycle Sequencing Kit and resolved on an ABI PRISM model 310 Automated DNA Sequencer at the Sigma Scientific Services Company**.**

Identification of root-knot nematode isolates

Root-knot nematode isolate J_2 from Guava was used as pure culture to inoculate tomato seedling roots. Root-knot nematode females were picked up from galled tomatoes roots and identified based on the features of perineal pattern of the isolated adult females (Taylor et al., 1955).

Effect of ozone gas (O_3), ozonated water (OW) and hydrogen peroxide (H_2O_2) on the growth of *Agrobacterium* sp. and *Meloidogyne* sp. isolates

Effect of O_3 *in vitro* and *in vivo*

Bacterial isolates were grown in Nutrient Broth (NB) medium for 72 hrs at 25 °C. Bacterial suspension (100 ml) contains ca. 5×10^8 CFU/ml were exposed to O_3, using a Laboratory Corona Discharge Ozone Generator (Xetin Ozone Air & Water purifier, Model XT 301, Xetin Co. Ltd, Taiwan with an air flow rate of 2.5 L/min with output of 300 mg/h.

O_3 gas was passed through bacterial suspension for 0, 1, 3, 5, 7 and 9 min to give concentrations of 0, 50, 150, 250, 350 and 450 ppm, respectively. Before and after ozone treatment, a loopfull of bacterial suspension (10 µl) was streaked on PDA medium and incubated for 48 hrs at 25°C. The developed bacterial colonies were counted and used for testing its tumor induction on squash fruits using toothpicks.

At the same time, 10^4 of J_2/100 ml distilled water were taken in a flask and exposed to the same previously mentioned O_3 concentrations. Dead and alive J_2 were recorded after 24 hrs and mortality percentage was calculated. Three replicates for each treatment were used.

Table 1: Primers used for PCR and 16S rRNA test with *Agrobacterium tumefaciens* isolates

Primer name (forward/reverse)	Primer specificity	Nucleotide sequence (5` to 3`)	Length of amplified fragment (bp)
tms2A/ tms2B	*tms2* gene of the tumor inducing (Ti) on the Ti plasmid.	CGCCACACAGGGCTGGGGGTAGGC GGAGCAGTGCCGGGTGCCTCGGGA	220
virD2A/ virD2C	*virD2* gene of the virulence (vir) region on the Ti plasmid.	ATGCCCGATCGAGCTCAAGT TCGTCTGGCTGACTTTCGTCAT	224
P0 (F)/ P6 (R)	16S rRNA gene	GAAGAGTTTGATCCTGGCTCAG CTACGGCTACCTTGTTACGA	1550

Effect of OW and H_2O_2 *in vitro*

Ozonated water (OW) was prepared using O_3 flow rate of 300 mg O_3/hr for 30 min (150 mg/L) in distilled water. Dilutions of OW were prepared to give 0, 15, 45, 75, 105 and 135 ppm, respectively. A series of H_2O_2 (34%) concentrations were also prepared as 0, 1, 3, 5, 7 and 9 %.

The sensitivity pattern of tested *Agrobacterium* sp. isolates against OW or H_2O_2 were determined using disc diffusion method of Kirby-Bauer technique (Bauer *et al.,* 1966). In brief, filter paper discs, Whatmann No.1, with diameter 0.6 mm were saturated with 30 µl of different previously concentrations of OW or H_2O_2.The resulted inhibition zones on PDA medium around the developed colonies were measured.

For root-knot nematode *Meloidogyne* sp., one hundred J_2/ml distilled water in a test tube were carried out and exposed to previous dilutions of OW or H_2O_2. Numbers of dead and alive J_2 were recorded after 24 hrs and mortality percentages were calculated.

Bacteriostatic/bactericidal aspect after exposure to O_3, OW and H_2O_2

The resulting clear zone of inhibition was swabbed using a sterile cotton swab and used to inoculate new plates containing PDA medium to determine if O_3, OW and H_2O_2 were bacteriostatic (BS) or bactericidal (BC) in nature (Shoeib and Alkufeidy, 2014).

Scanning electron microscope (SEM) study

Samples preparations

The selected *A. tumefaciens* (RAt_1) isolate was grown in NB media and exposed to O_3 for 450 ppm or H_2O_2 (9%), then fixed in formaldhyde: glutaraldehyde (4:1,v/v) in phosphate buffer solution pH 7.2 at 4°C for 3 hrs. Bacterial cells were then postfixed in 2% osmium tetroxide (OsO_4) in the same buffer at 4°C for 2 hrs, washed in the buffer and dehydrated at 4°C through a graded series of ethanol ranging from 30-100%. The bacterial cells were dried by means of the critical point method mounted using carbon paste on an Al-stub and coated with Gold up to a thickness of 400 Å in a sputter-coating unit "JFC-1100E" (Oli *et al*., 2012).

Nematode specimens were prepared from a population of *M. incognita* cultured on tomato seedlings, grown in a greenhouse at 22-28 °C. Newly hatched J_2 were obtained by incubating egg masses on wet tissue paper supported by a plastic screen in a moist chamber (Eisenback, 1986). Specimens were chilled in 0.5 ml (12 drops) of tap water in a refrigerator (4°C) for 30 min, and then fixed by adding 4 drops of 4% cold glutaraldehyde-2% formalin (buffered with 0.1 M sodium cacodylate pH 7.2) every 30 min until the final concentration of fixative reached 2% glutaraldehyde-l% formalin (12 drops of fixative) Fixation continued overnight (15-20 hrs). After fixation, all specimens were postfixed in 2% OsO_4 (buffered in 0.1 M sodium cacodylate pH 7.2) for 8 hrs at 4°C, dehydrated in a seven-step ethanol series and dried (Eisenback, 1985).

Scanning electron micrograph

Changes in cell walls of *A. tumefaciens* and *M. incognita* J_2 head region treated with either O_3 (450 ppm) or H_2O_2 (9%) compared to the control treatment, were photographed using scanning electron microscope **(**SEM) JEOL, model JSM-5300 operated between 15 and 35 KeV.

Statistical Analysis

Data of experiments, 3 replicates for each treatment, were analyzed using statistical analysis system software (SAS) (SAS Institute, 1997). Treatment means were compared using the revised LSD test at the 0.05 level of probability.

RESULTS

Samples collection and isolation

Attempts to isolate *Agrobacterium* sp. from one hundred guava galled root samples resulted in 4 different isolates coded RAt_1, RAt_2, RAt_3 isolated from Rosetta farms and EAt_4 isolated from Edkou farms. After 4-6 days from incubation at 25°C, bacterial colonies grown on NASA medium looked like the typical growth of *Agrobacterium* sp. The bacterial colonies appeared convex with red centers and white margins (Fig. 1A), while the growth on PDA medium appeared convex, glistening, white to beige in color and circulated with an entire edge after 2-3 days at 28°C (Fig. 1B). For root-knot nematode J_2, eggs and females were obtained from galled guava root.

Pathogenicity test

The Pathogenicity test illustrated that all bacterial isolates induced tumors on squash fruits after 7-10 days from artificial inoculation (Fig. 2).

Roots of natural infected Guava plants (100 galls/10 gm roots) were taken to isolate egg-masses and hatching to obtain second stage juveniles (J_2) to make artificial infected in healthy guava seedlings resulted 59 galls/root

Identification tests

Classical and Molecular methods

On the basis of microscopic examination, the four tested isolates were identified using Gram staining, motility and capsule formation. Data in Table (2) showed that all bacterial isolates did not produce acids (-) when grown on media containing mannose, fructose, maltose and glucose. On the other hand, all these isolates produced acid (+) when grown on either lactose or sorbitol except RAt_2 isolate didn't produce acid (-) on sorbitol. Also, the obtained data indicated that *Agrobacterium* isolates didn't grow on media containing NaCl, but they grew at 37°C and gave positive (+) results with catalase test

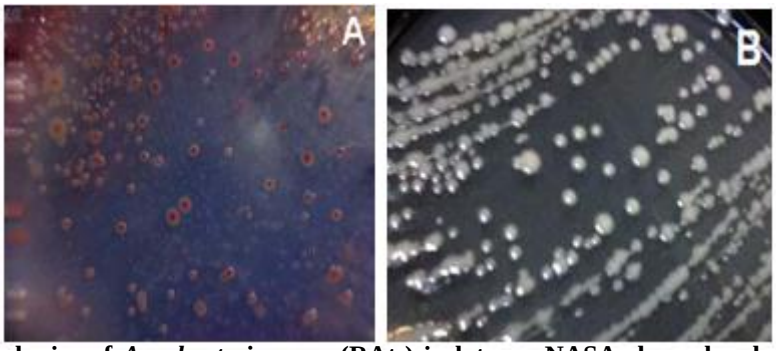

Fig.1: Bacterial colonies of *Agrobacterium* sp. (RAt_1) isolate on NASA showed red centers and white margins (A) and on PDA media showed circulated and white to beige in color (B).

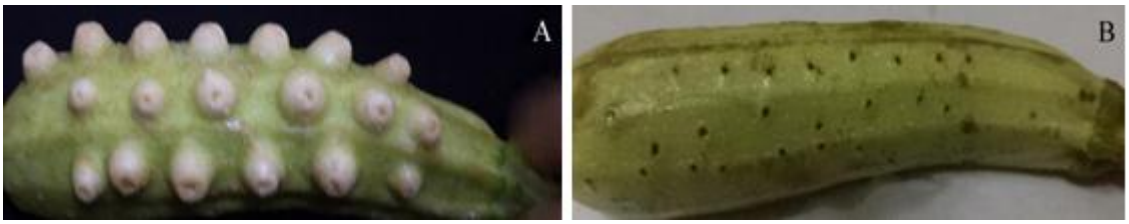

Fig. 2: Tumors symptoms on squash fruits inoculated(A); and non-inoculated(B) with *Agrobacterium* sp (RAt1).

Table 2: Physiological and biochemical reactions of tumorigenic *Agrobacterium* sp. isolates

Isolate code	Growth on NaCl		Acid production						Growth at 37°C	Catalase test
	2%	5%	Lactose	Sorbitol	Mannose	Fructose	Maltose	Glucose		
RAt_1	-	-	+	+	-	-	-	-	+	+
RAt_2	-	-	+	-	-	-	-	-	+	+
RAt_3	-	-	+	+	-	-	-	-	+	+
EAt_4	-	-	+	+	-	-	-	-	+	+

A specific band of 220 bp, amplified using primers tms2A/tms2B showed in the four tested *Agrobacterium*. isolates (Fig. 3A). Similarly, a specific band of 224 bp was appeared using virC/ virD primers tested (Fig. 3B).

PCR product of the 16S rRNA gene of the four *Agrobacterium* isolates resulted in a band of approximately 1550 bp (Fig. 4). This band was subjected to partial DNA sequencing. The obtained data revealed that the inferred 16S rRNA partial sequence of tested isolates were similar to *Agrobacterium tumefaciens*, one of them (RAt_1) was identified by GenBank as indigenous virulent *A. tumefaciens* isolate with accession No. LT630451.

Identification of root-knot nematode isolates

Root-knot nematode isolate obtained from Rosetta was identified based on the features of perineal pattern of females; all tested females were *Meloidogyne incognita*.

Effect of O_3 *in vitro* and *in vivo*

The results in Table (3) show that the main effect of lowest count of CFU/ml was observed with RAt_1 and RAt_2 isolates, which recorded 4.2×10^6 and 4.5×10^6 CFU/ml, respectively. The effect of ozone exposure in reducing CFU/ml (from 5.0×10^8 to 1.3×10^5) was significantly concentration-dependent (from 0 to 450 ppm, respectively). At all the tested ozone gas treatments, the tested bacterial isolates were still capable for inducing tumors on squash fruits (Fig. 5). On the other hand, all the tested ozone gas treatments applied on the root-knot nematode (*M. incognita*) induced 100 % J_2 mortality after 24 hrs.

Effect of OW and H_2O_2 *in vitro*

The results revealed no inhibition zones were appeared around the discs saturated with OW at different concentration on PDA medium inoculated with all tested *A. tumefaciens* isolates, for 48 hrs at 28 °C (Fig. 6). In case of *M. incognita* the recorded results after 24, 48 and 72 hrs of exposure to OW showed 50, 70 and 90% mortalities in J_2, respectively.

The efficacy H_2O_2 concentration for inhibiting bacterial growth was determined after 48 hrs of incubation (Table 4 and Fig. 7).

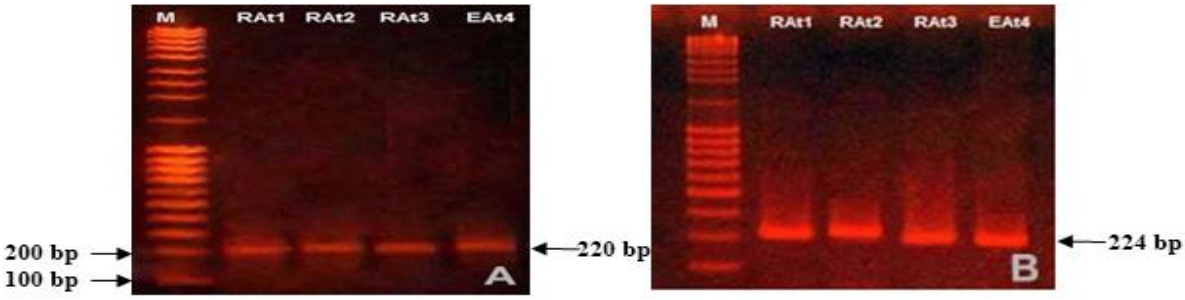

Fig.3: Amplified products resulted from the use of the two specific primers: tms2A/tms2B, (A); virD2A/virD2C (B); M: a 100 bp DNA marker ladder.

Table 3: Effect of different ozone gas treatments concentration/ time on number of CFU/ml of the four isolates of *A. tumefaciens* grown on nutrient broth medium.

Isolate code	Ozone concentration (ppm)						Isolate main effect
	0	50	150	250	350	450	
	CFU/ml						
RAt_1	$5.0\times10^{8\ a}$	$3.0\times10^{7\ bc}$	$3.3\times10^{7\ d}$	$3.3\times10^{6\ f}$	$2.3\times10^{5\ g}$	$2.0\times10^{4\ i}$	$4.2\times10^{6\ C}$
RAt_2	$5.0\times10^{8\ a}$	$4.6\times10^{7\ bc}$	$4.0\times10^{7\ b}$	$3.3\times10^{6\ f}$	$3.0\times10^{5\ g}$	$2.0\times10^{4\ i}$	$4.5\times10^{6\ BC}$
RAt_3	$5.0\times10^{8\ a}$	$4.3\times10^{7\ bc}$	$3.3\times10^{7\ cd}$	$3.0\times10^{6\ e}$	$3.0\times10^{5\ g}$	$2.6\times10^{4\ ih}$	$4.9\times10^{6\ B}$
EAt_4	$5.0\times10^{8\ a}$	$4.0\times10^{7\ bc}$	$3.6\times10^{7\ bc}$	$3.6\times10^{6\ e}$	$2.6\times10^{5\ g}$	$3.0\times10^{4\ h}$	$5.0\times10^{6\ B}$
Conc. main effect	5.0×10^{8A}	6.2×10^{7B}	5.2×10^{7C}	6.5×10^{6D}	9.5×10^{5E}	1.3×10^{5F}	

Data are means of 3 replicates. Means with the same letter(s) aren't significantly different at $P \leq 0.05$.

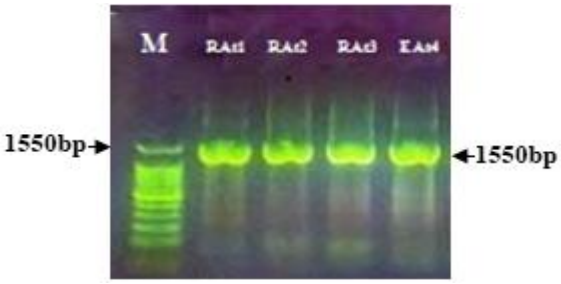

Fig. 4: Gel electrophoresis of 16S rRNA PCR product (1550 bp) for the four *A. tumefaciens* isolates, M: a 100 bp DNA marker ladder.

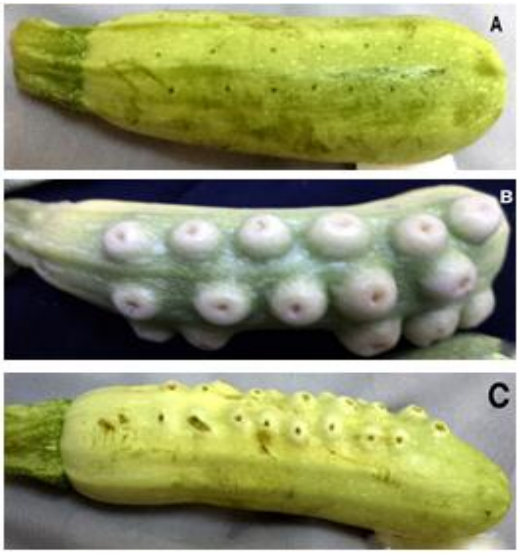

Fig. 5: Negative (-) control without bacterial inoculation (A); positive (+) control with bacterial inoculation (B); Tumor induced by *A. tumefaciens* inoculation which treated with ozone (450ppm) (C).

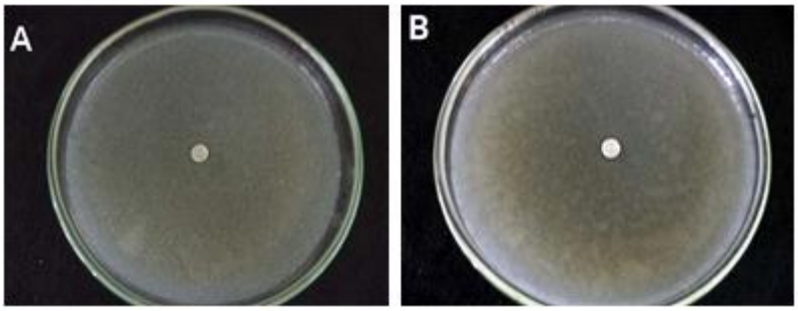

Fig. 6: *Agrobacterium tumefaciens* (RAt_1) isolate grow on PDA medium containing disc saturated with 30 µl sterilized water as a control treatment (A) and disc saturated with 30 µl (135 ppm) ozonated water (B).

Table 4: Effect of H_2O_2 concentrations on *Agrobacterium tumefaciens* isolates grown on PDA medium.

Isolate code	H_2O_2 concentration (%)						Isolate main effect
	0	1	3	5	7	9	
	Inhibition zone (mm)						
RAt_1	0.0^{m}	30.0^{d}	35.0^{c}	40.0^{b}	50.0^{a}	65.0^{a}	57.0^{A}
RAt_2	0.0^{m}	1.0^{k}	7.0^{i}	15.3^{g}	15.4^{g}	30.0^{e}	31.6^{D}
RAt_3	0.0^{m}	0.0^{m}	14.7^{g}	19.7^{f}	30.0^{e}	30.0^{e}	36.0^{B}
EAt_4	0.0^{m}	5.0^{j}	9.7^{hi}	11.0^{h}	22.3^{f}	30.0^{e}	34.2^{C}
H_2O_2 conc. main effect	1.0^{F}	2.2^{E}	3.2^{D}	3.7^{C}	4.7^{B}	5.3^{A}	

Means have the same letters aren't significantly different ($P \leq 0.05$).

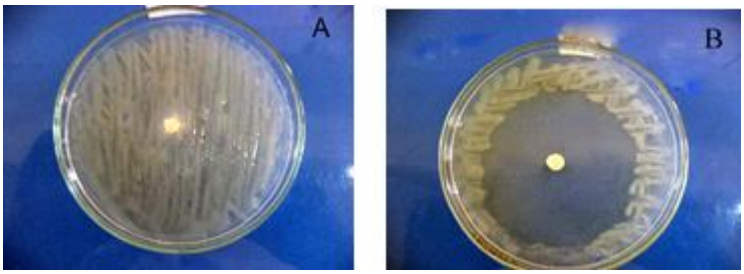

Fig. 7: *Agrobacterium tumefaciens* (RAt_1) isolate grow on PDA media containing disc saturated with sterilized water as a control treatment (A) and disc saturated with H_2O_2 (9%) (B).

Results revealed that the isolates markedly varied in their response to H_2O_2. The highest inhibition zone of H_2O_2 (57.0) was recorded with isolate RAt_1 followed by RAt_3, EAt_4 and then RAt_2, with 36.0, 34.2 and 31.6 mm, respectively (Table 4). Also, the highest inhibition zone was recorded with the highest concentration and vice versa.

In case of root knot-nematode, H_2O_2 resulted in a complete damage in J_2 of *M. incognita*.

Bacteriostatic/bactericidal aspect after exposure to O_3, OW and H_2O_2

Re-inoculated bacterial cells from inhibition zone on PDA were taken as criteria to detect bacteriostatic (BS) and bactericidal (BC) aspect of O_3, OW and H_2O_2. Results indicated that O_3 and OW gave bacteriostatic effect against all tested isolates which grow after re-inoculated on PDA medium, while isolates didn't grow after treatment with H_2O_2 after streaked on PDA medium which mean that H_2O_2 revealed BC effect against tested isolates. In case of RKN, J_2 were recorded mortality in all O_3, OW and H_2O_2 after 24 hrs, results mean that O_3, OW and H_2O_2 have nematicidal effect.

Scanning electronic microscope study

To detect the influence of O_3 gas or H_2O_2 in the change of ultrastructure bacterial cell walls and root-knot nematode J_2 head region were carried out using SEM. Cells wall of untreated *A. tumefaciens* isolate (RAt_1) showed straight rods arranged singly of cells (Fig. 8A), the cells after exposure to O_3 gas, shortness and some deformities in the external bacterial cell wall were observed (Fig.8B). In the case of H_2O_2 treatment, the micrograph of tested isolate, showed deformities and full lyses of bacterial cell walls (Fig. 8C). On the other hand, SEM photograph of *M. incognita* J_2 showed distorted in morphology of head region after treated with O_3 gas (Fig. 9B), while photograph clarified that nematode head region and entire bodies were disrupted after treated with H_2O_2 (Fig. 9C), in comparison with untreated J_2 where appear normal and free from distortion (Fig. 9A).

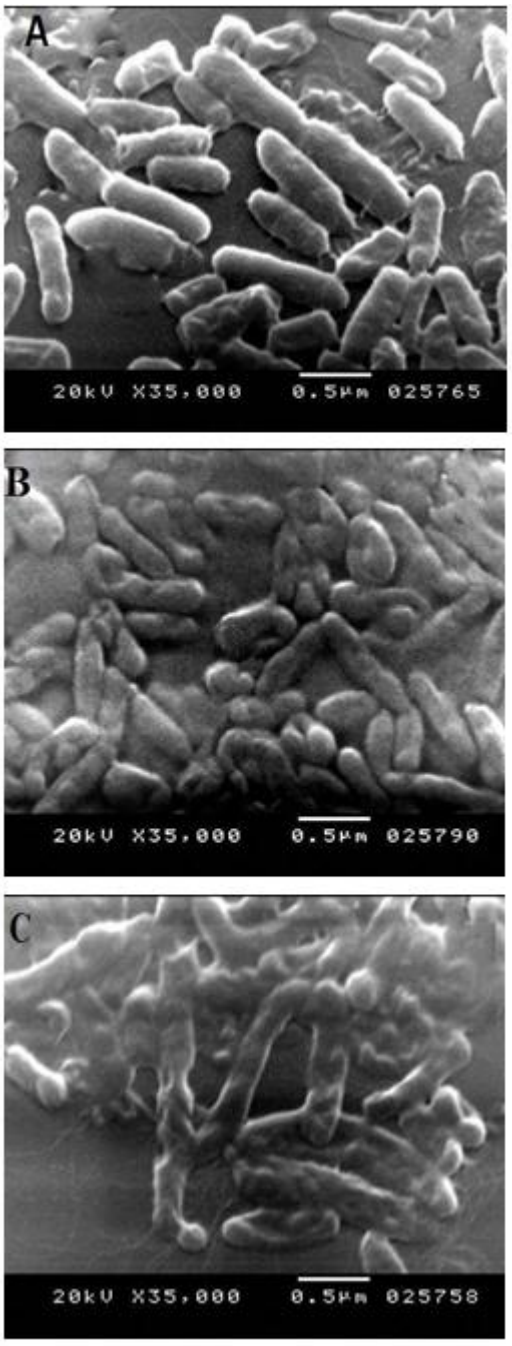

Fig. 8: Scanning electron microscope observation of *A. tumefaciens* (RAt_1) isolated from Guava plant. (A) Untreated bacterial cells showed straight rod occurring singly; (B) bacterial cells after exposure to O_3 (450 ppm) showed shortness and deformities; (C) bacterial cell after exposure to H_2O_2 (9%) showed deformities and full lyses.

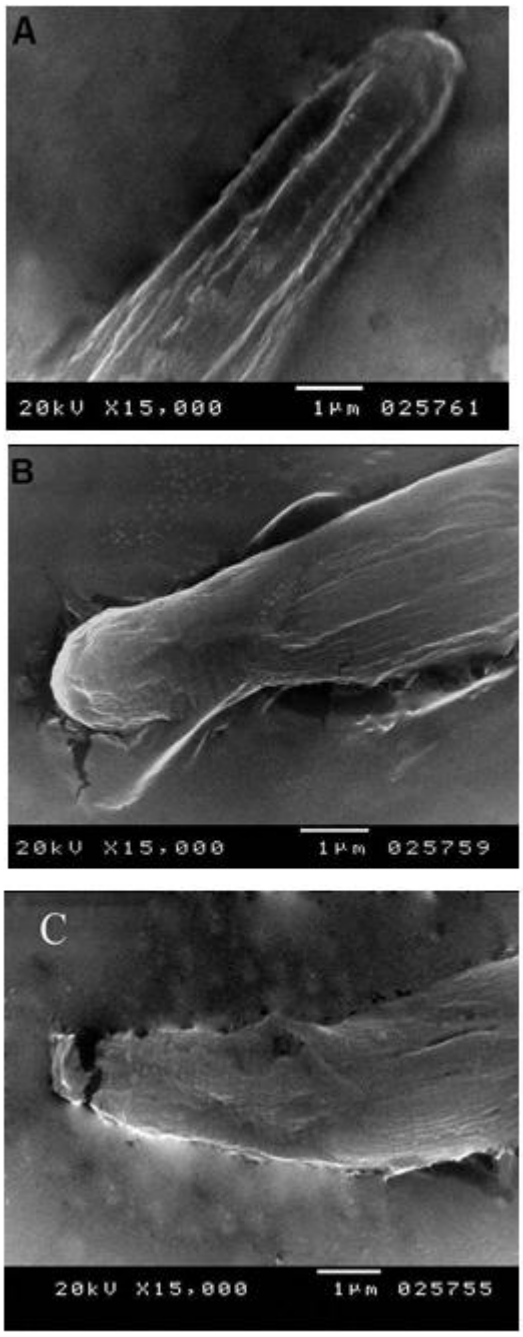

Fig. 9: Scanning electron microscope observation of *Meloidogyne incognita* Junveniles ($J_{2)}$, (A) Healthy control, (B) J_2 treated with O_3 (450 ppm) showed distorted in head region and (C) treated with H_2O_2 (9%) showed disrupted in head region.

DISCUSSION

In different parts of Beheira Governorate especially Rosetta and Edkou centers, 80% of guava plants are invased by bacterial pathogens, which cause crown gall disease. At the same time, this disease will be more unsafe when bacterial pathogens accompanied with parasitic nematodes, especially root-knot nematodes (M. A. Elsaedy, personal information's).

Isolation of tested *Agrobacterium. tumefaciens* was appeared smooth, round and bright transparent rings in the margins on the NASA selective medium, which supported by many authors (Holt *et al.*, 1994; Chen *et al.*, 1999; Koivunen *et al.*, 2004; Sarker *et al.*, 2011, Davoodi and Hajivand, 2013, Setti and Bencheikh, 2013). Tested of bacterial colonies were found that generally grow red colonies on NASA medium (Holt *et al.*, 1994 and Sarker *et al.*, 2011). The identity of each of the suspected *A.tumefaciens* isolates was determined by the appropriate biochemical and physiological tests. The results obtained were matched with Tiwary, et al. (2007) and Setti and Bencheikh (2013).

PCR has rapidly become a basic diagnostic and identification protocol in plant pathology. The PCR of the present results with tms2A/tms2B and virD2A-virD2C primers successfully proliferate the target sequences giving the specific bands expected for these primers with all tested isolates. These results are supporting further identity of these isolates as *A. tumefaciens*, and agree with the results of Tolba and Soliman (2014).

In the present study, the identification and description of root knot-nematode were based mainly on perineal pattern, where it was one of the most characteristic morphological features of the genus *Meloidogyne* to the tested adult females to describe *M. incognita* (Taylor *et al*., 1955).

In order to management these diseases on Guava trees, O_3; ozonated water (OW) and H_2O_2 applications were used. Although O_3 applications are more expensive than any control treatments such as soil fumigants, but the use of O_3 would become more feasible if the costs of perceived environmental and health effects were factored into the equation (Runia and Amsing 1996). In laboratory trials, the effect of O_3 and OW on bacterial isolates growth in decreasing CFU/ml was concentration-dependent, this finding agreement with Hussein *et al*., (2015). In addition the tested isolates were still able to induce tumors after exposure to O_3 and OW with bacteriostatic (BS) effect, these results in contradict with the resuls of Hussein *et al*. (2015) who found that O_3 was able to break cell membrane leading finally to kill the bacterial cells.

All tested concentration/time of O_3 and OW were induced 100% *M. incognita* J_2 mortality after 24 and 72 hrs, respectively. Similar results were obtained by Moens *et al*. (1991) who found that ozone treatment for 4 min totally inhibited the nematode infection potential on tomato plants, but the complete nematode kill was only achieved after a treatment time of 12 min.

The use of hydrogen peroxides for the control both *A. tumefaciens* and *M. incognita* was tested *in vitro*, results achieved bactericidal and nematicidal, respectively in 9% concentration of H_2O_2. Similarity results obtained by Hugo and Malan (2006), who found complete damage of *Radopholus similis* when treated with 400 ppm of H_2O_2.

Morphological changes of bacterial isolates *A. tumefaciens* and root-knot nematode *M. incognita* observed by SEM provided strong evidence that the effect of antibacterial agent act on the cell wall (Hara and Glenn 1994, Ritz et al., 2001; Neumann 2005 ; Oli et al., 2012). In the present study, SEM showed the modification of the external shape of cells of *A. tumefaciens* after treatment by O_3 and H_2O_2. These modification were deformities and decreasing of number cells in case of O_3 gas treatment, while in H_2O_2 treatment, the tested bacterial cells showed full lyses. Similar results were obtained by Shoeib and Alkufeidy (2014) who confirmed that Gram negative bacteria (G^-ve) appears deformities and lyses after treatment by aqueous clove's buds extract. Morover, The SEM results showed distorted and damage in the morphology shape of *an M. incognita* after treatment by O_3 and H_2O_2.

Public concern over the toxicity of nematicidal and their impact on the environment has focused research on alternative methods to control plant pathogen (Thomason, 1987).The high value crops have relied on the using of methyl bromide fumigation for controlling weeds, fungi and plant-parasitic nematodes. It is an effective disinfecting agent, and inhibits or kills bacteria, fungi, viruses and protozoa (Kim *et al*., 1999). Our conclusion have strengthened that O_3 and H_2O_2 have potential antibacterial and anti-nematode agents. Thus the efficacy of ozone gas and hydrogen peroxide may be used in future as alternative methods for controlling the double infection of crown gall bacterium and root – knot nematodes.

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الملخص العربى

دراسة تأثير الأوزون وفوق أكسيد الهيدروجين على بكتيرة التدرن التاجي ونيماتودا تعقد الجذور على نبات الجوافة في مصر

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تم اختبار غاز الأوزون، ماء الأوزون وفوق أكسيد الهيدروجين بتركيزات مختلفة على ٤ عزلات من بكتيرة التدرن التاجي وعزلة واحدة من نيماتودا تعقد الجذور. جُمعت العزلات البكتيرية والنيماتودية من جذور نبات الجوافة المصابة بالتدرنات والمنزرعة بمنطقتي إدكو ورشيد بمحافظة البحيرة، مصر. أثبتت كل من الإختبارات البيوكيميائية، الفسيولوجية، إختبار إحداث الورم، بالإضافة إلى استخدام طرق البيولوجيا الجزيئية للعزلات البكتيرية، أن الـ ٤ عزلات المدروسة تنتمي للنوع *Agrobacterium tumefaciens*. أظهرت نتائج تعرض كل من العزلات البكتيرية *A. tumefaciens* والطور اليرقي الثاني لنيماتودا تعقد الجذور *M. incognita* إلى تركيزات مختلفة من غاز الأوزون (٥٠ –٤٥٠ جزء في المليون)، أن جميع التركيزات كانت موقفة لنمو بكتيرة التدرن التاجي، كما أدت إلى تقليل حجم الأورام المتكونة بعد التعرض، بينما كانت التركيزات المستخدمة مميت للطور اليرقى الثانى للنيماتودا، وذلك بعد ٢٤ ساعة من التعرض. أيضا فقد أظهرت التركيزات المختبرة (١٥ – ١٣٥ جزء في المليون) من ماء الأوزون تأثيراً موقفاً لنمو العزلات البكتيرية المختبرة وتأثيراً مميتاً للطور اليرقي الثاني لنيماتودا تعقد الجذور. في حين أدى استخدام فوق أكسيد الهيدروجين بتركيزات ١، ٣، ٥، ٧ و٩%، إلى تأثير مميت لكل من العزلات البكتيرية والطور اليرقي الثاني لنيماتودا تعقد الجذور. أوضحت صور الميكروسكوب الإلكتروني الماسح تأثير غاز الأوزون (٤٥٠ جزء في المليون) وفوق أكسيد الهيدروجين (٩%)، على كل من خلايا بكتيرة التدرن التاجي والطور اليرقي الثاني لنيماتودا تعقد الجذور.