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Soluble Concentrate Formulation of Oxalic Acid and N-Acetyl-L-Cysteine: Potential of Use in Controlling *Ralstonia solanacearum*

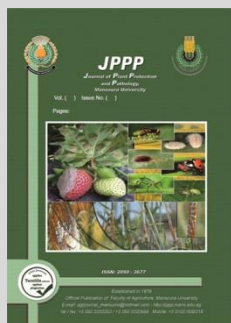
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ABSTRACT

Brown rot is a disease caused by the bacterium *Ralstonia solanacearum* and has the potential to cause significant losses due to tubers rotting of infected potato plants. The chemical control of *R. solanacearum* is ineffective and the biological control methods have not yet been developed against this organism. The antibacterial activities of a soluble concentrate (SL) formulations of oxalic acid (OXA), and N-Acetyl-L-cysteine (NAC) were tested against *R. solanacearum* race 3 biovar 2. Formulations of OXA SL 13%, NAC SL 10%, and OXA+NAC SL 23% showed inhibition zones against *R. solanacearum*. In the case of NAC SL 10%, and OXA+NAC SL 23%, the inhibition zone consisted of two regions, a clear inhibition zone, and a less clear inhibition zone. After 72 h. of incubation, the final total inhibition zone was 35.3, 33.3, and 60.0 mm for OXA SL 13%, NAC SL 10%, and OXA+NAC SL 23%, respectively. Both the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of OXA+NAC SL 23% against *R. solanacearum* were 62.5 µl/ml. Formulation of OXA+NAC SL 23% exhibited good stability at different storage conditions and no separation was observed during the stability test. In the pots experiment, the wilt index and the percentage of wilted plants were decreased significantly in OXA+NAC SL 23% treatments compared to the positive control (treated with *R. solanacearum* only) treatment. The number and weight of potato tubers were significantly increased using the concentrations 125 and 62.5 µl/ml of OXA+NAC SL 23% compared to the positive control. Field experiments are needed to confirm the results.

Keywords: Soluble concentrate, Formulations, Oxalic acid, N-acetyl-L-cysteine, Brown rot, Antibacterial activity, Control.

INTRODUCTION

The causative agent of bacterial wilt (brown rot) is *Ralstonia solanacearum*. This species is widespread in the world in tropical and subtropical regions. *R. solanacearum* causes significant yield losses and about 450 plant species have been recorded as hosts of this pathogen (Maji and Chakrabarty, 2014 and Wang *et al.*, 2019). Protection methods and chemical control of *R. solanacearum* are ineffective. Antibiotics show no effect and the biological control method has not yet been developed against this organism (Karim and Hossain, 2018).

Oxalic acid is a dicarboxylic acid that happens naturally in several plants and forms metal oxalates. Oxalic acid has the ability as an antioxidant. Oxalic acid stimulates disease systemic resistance and promoting plant growth (El-Shabrawi *et al.*, 2015). Oxalic acid showed good antibacterial activity against the brown rot bacterium *R. solanacearum* (Kwak *et al.*, 2016). In contrast, Wu *et al.* (2015) confirmed that the population of *R. solanacearum* was much higher in the rhizosphere soil of the susceptible tobacco cultivar (Hongda) compared to the rhizosphere soil of the resistant tobacco cultivar (K326), which led to a high disease index in the susceptible cultivar. The oxalic acid from Hongda root exudates was significantly higher compared to that from K326 root exudates. Oxalic acid significantly induces the chemotactic response and increases the biofilm biomass of *R. solanacearum*.

Clinical studies revealed that N-Acetylcysteine is an important modulator of antibiotic activity. The presence of N-Acetylcysteine can decrease the antibacterial activity of some

antibiotics (aminoglycosides, fluoroquinolone, and erythromycin) or enhance the effectiveness of others (β-lactam) against several bacterial strains. Besides, the presence of N-Acetylcysteine can be detrimental to some species of bacteria (Goswami and Jawali, 2010). In Agriculture, the minimal inhibitory concentration of NAC against *Xylella fastidiosa* was 6mg/ml. The plants absorb NAC at concentrations of 0.48 and 2.4mg/ml but not at 6mg/ml (Muranaka *et al.*, 2013).

The primary objectives of the formulation technology are to optimize the biological activity of the chemical and improve its storage, handling, safety, application, or effectiveness (Knowles, 2008). Solution concentrate is the simplest type of all formulations, in which the active ingredient is dissolved in water; all the components of the formulation SL are solubilized in the aqueous mixture to be sprayed, which is in the form of a clear solution or opalescent liquid, free from visible suspended matter and sediment, to be applied as a true solution of the active ingredient in water. The parameters necessary to have a good quality of the formulation are complete solubility of the active ingredient, chemical stability, and compatibility with most of the mixtures used (Knowles, 2008 and FAO/WHO, 2016).

This study aims to develop a stable soluble concentrate (SL) formulation of oxalic acid (OXA) and N-Acetyl-L-cysteine (NAC), individually or together, to evaluate the antibacterial activity of the SL formulations of OXA and NAC against *R. solanacearum in vitro*. Determination of the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of OXA+NAC SL 23% against *R. solanacearum* and

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study the characteristics of OXA+NAC SL 23% according to solution stability and physicochemical properties, to study the effect of OXA+NAC SL 23% on the development of wilt symptoms on potato plants and the yield of potato under conditions of brown rot stress.

MATERIALS AND METHODS

Source of *Ralstonia solanacearum*

Two virulent isolates of *R. solanacearum* Phylotype IIB sequevar 1 (PIIB-1), formerly referred to as race 3 biovar 2, were obtained from the collection of Bacterial Diseases Research Department, Plant Pathology Research Institute, ARC, Giza, Egypt. The afore-mentioned isolates were previously isolated, identified, and stored separately at room temperature in test tubes containing sterile water by Abd El-Rahman and Shaheen (2016). The isolates of *R. solanacearum* were propagated periodically and their pathogenicity was checked and stored in sterile water until use. A reference check isolate of *R. solanacearum* race 3 biovar 2 was kindly obtained from the potato brown rot project (PBRP), ARC.

Source of materials and chemicals used in preparing the formulations

Oxalic acid (99% extra pure, solubility in water 1g/7ml), N-Acetyl-L-cysteine (99% white crystalline powder, solubility in water minimum 10 mg/ml and 100 mg/mL with heating), and triton X-100 (t-octylphenoxypolyethoxyethanol, polyethylene glycol tert-octylphenyl ether) were purchased from LOBA Chemie, Pvt. Ltd., India. Glycerol was purchased from BIOCHEM, Egypt. Propylene glycol was purchased from ADWIC, El Nasr Pharmaceutical Chemicals Co., Egypt. Deionized water was obtained through a Milli-Q system PROT. M PS LABCONCO Corporation, Kansas City, Missouri 64132-USA.

Confirmation of pathogen identity

Identification of *R. solanacearum* was confirmed by conventional PCR using two primers (Willowfort, Birmingham, UK), forward primer RS-1-F 5'- ACT AAC GAA GCA GAG ATG CAT TA -3' and reverse primer RS-1-R 5'- CCC AGT CAC GGC AGA GACT -3' (Pastrik *et al.*, 2002). Two bacterial colonies (2-mm diameter) were suspended in 100 µl of lysis solution (0.05 M NaOH, 0.25% sodium dodecyl sulfate [SDS]) and were incubated for 15min. at 100°C. The suspension was centrifuged for 1min. at 14,000xg and was diluted 20-fold in molecular grade water (pellet discarded). One microliter of the diluted suspension was used as a template for each 20µl of the PCR reaction mixture. The following amounts of reagents were used: 10µl of PCR reaction mixture (amaR OnePCR, Simply Biologics, Miaoli, Taiwan) + 7µl molecular grade water + 2µl primers (10 µM each) + 1µl DNA template. Amplifications were performed in a thermal cyclers (Applied Biosystems, 2720, Life Technologies Holdings Pte Ltd, Singapore). The PCR reaction conditions were as follows: initial denaturation at 94°C for 5 min, followed by 25 reaction cycles of 94°C for 30s, 55°C for 30s and 72°C for 30s; followed by a final extension at 72°C for 7 min. and 4°C (hold temperature). PCR products were separated on agarose gel electrophoresis using 1.5% agarose in 1x TBE buffer, stained with nucleic acid staining solution (RedSafe™, 20,000x, iNtRON Biotechnology, Inc.) and visualized on a UV-transilluminator.

Preparation of soluble concentrate (SL) formulation of oxalic acid (OXA) and N-Acetyl-L-cysteine (NAC)

SL formulation was prepared by direct mixing of 13% OXA and/or 10% NAC, 10% humectant (glycerol), 5%

antifreeze agents (propylene glycol), 10% wetting agent (triton x-100), and deionized water. Stirring was done (Magnetic stirrer with hot plate "Torry Pines Scientific", USA) at high speed for 10 min. until the formation of SL.

Preparation of bacterial suspensions

Isolates of *R. solanacearum* (two virulent isolates) were separately inoculated in Petri dishes containing King's B agar medium (proteose peptone 20.0g, K₂HPO₄ 1.5g, MgSO₄·7H₂O 1.5g, agar 20.0g, glycerol 15.0ml, distilled water to 1.0 liter and final pH7.2 ± 0.2). Inoculated Petri dishes were incubated at 28°C for 48h. Bacterial growth was harvested for each isolate separately, and the cell suspension was adjusted to 10⁹ CFU/ml using sterilized 10 mM phosphate buffer (2.7g Na₂HPO₄·12H₂O; 0.4g NaH₂PO₄·2H₂O; distilled water to 1.0 liter and final pH 7.2).

The antibacterial activity of different SL formulations of OXA and NAC

Antibacterial activity of SL formulations of OXA 13%, NAC 10%, and OXA +NAC 23% was tested against *R. solanacearum* using the well diffusion method (Mohammad and Abd El-Rahman, 2015). YPG agar medium (5g yeast extract; 5g peptone; 10g glucose; 20g agar; distilled water to 1.0 liter and final pH7.2 ± 0.2) was autoclaved for 15 min. at 121°C, allowed to cool in a water bath at 50°C and inoculated with *R. solanacearum*. The inoculum was 1ml of 10⁸ CFU/ml bacterial suspensions for flask containing 250 ml medium. Petri dishes (9 cm) were prepared by pouring 25 ml of inoculated medium. Once the agar was solidified, agar wells of 5-mm diameter were made with a sterilized stainless-steel cork borer, and were filled with 50 µl of the tested material. To ensure that antibacterial activity of the SL formulations of OXA and NAC were not due to low pH, the antibacterial activity of sterile distilled water adjusted to the same pH value (using 1M HCl) of OXA+NAC SL 23% was evaluated. Wells filled with 50µl of sterile distilled water were used as a control. The experiment was performed in three replicates. The Petri dishes were kept for 30 min to enhance diffusion. The Petri dishes were incubated at 28°C for 72h. Clear zones that formed around the wells were measured to the nearest millimeter after 24 and 72h of incubation.

Determination the MIC of OXA+NAC SL 23% by the well diffusion method

MIC of OXA+NAC SL 23% against *R. solanacearum* was determined using the well diffusion method (Bonev *et al.*, 2008). Seven serial two-fold dilutions of OXA+NAC SL 23% in sterile distilled water were prepared to obtain concentrations ranged from 500.0 to 7.81µl/ml. YPG agar medium was inoculated with *R. solanacearum*. Petri dishes (15cm) were prepared by pouring 60 ml of the inoculated medium. Once the agar was solidified, eight wells of 5-mm diameter were made around the middle of the plate and away from the edge of the plate at a reasonable distance. Each well was filled with 50µl of one of the tested concentrations. One well was filled with 50µl of sterilized water and used as a control. The experiment was performed in three replicates. The Petri dishes were kept for 30 min to enhance diffusion and were incubated at 28°C for 48h. Clear zones that formed around the wells were measured to the nearest millimeter. The lowest concentration forms a clear zone around the wells was considered the MIC.

Determination the MBC of OXA+NAC SL 23% by broth dilution method

Sterilized YPG broth medium was used to determine MBC using broth dilution method (Brumfitt *et al.*, 1984 and

Radaelli *et al.*, 2016). Serial doubling dilutions of OXA+NAC SL 23% in YPG were prepared to obtain concentrations ranged from 666.67 to 10.42 µl/ml. The OXA+NAC SL 23% concentrations were filtered through 0.45 µm filters. One millimeter of bacterial suspension of *R. solanacearum* (10⁶CFU/ml) was added to YPG medium (3ml) tubes containing different concentrations of OXA+NAC SL 23%. The final volume of each tube was 4ml containing 2.5x10⁵CFU/ml of *R. solanacearum*. Final OXA+NAC SL 23% concentrations were ranged from 500.0 to 7.81 µl/ml. Tubes of the YPG medium used as a negative control treatment. Tubes of the YPG medium containing 2.5x10⁵CFU/ml of *R. solanacearum* were used as a positive control treatment. The tubes were incubated at 28°C for 24h. MBC was determined by spreading 100 µl of each tube on the YPGA medium and incubated at 28°C for 48h. The experiment was replicated three times. The lowest concentration showed no growth, after this sub-culturing, was considered the MBC.

Characterization of the SL formulation of OXA +NAC 23%

The SL formulation of OXA +NAC 23% was observed for homogeneity, solution stability, or separation during storage.

Appearance

Prepared SL formulation was observed visually for clarity, homogeneity, or any sign of separation during storage (0±2°C for 7 days and 54±2°C for 14 days). The appearance of the formulation was determined by visual examination.

Dilution stability

The SL formulation, following dilution with CIPAC standard water D and standing at 30±2°C for 24h, should give a clear or opalescent solution. Any visible sediment or particles produced should pass through a 75 µm test sieve CIPAC MT 41.1 (CIPAC, 2017).

Stability Study

Different stress tests were conducted to obtain stable SL formulation. Centrifugation of the formulation was carried out at 3000rpm for 30min to confirm phase separation: if any. Freeze and thaw conditions were carried out alternatively at -21°C and 25°C test tubes (25ml) filled with the prepared formulation and hermetically closed were vertically stored for 12h in the freezer at -20°C, and then for 12h at room temperature (25°C±2). The formulation was observed for any changes. The formulation is considered "stable" if there is no substantial separation after four cycles. The formulation passed the thermodynamic stress tests, was processed for further characterization. Stability was checked at 0°C CIPAC MT 39.3. (CIPAC, 2000). After storage at 0±2°C for 7 days, the volume of the separated solid and/or liquid should not be more than 0.1ml. Stability was checked at elevated temperature CIPAC MT 46.3. (CIPAC, 2000). After storage at 54±2°C for 14 days, the volume of the separated solid and/or liquid should not be more than 0.3ml.

Physicochemical Properties

pH determination

The pH of a 1% solution of the prepared formulation was determined by a pH meter (Jenway model pH 3510), standard using pH 4 and 7 buffers before use. The measurements were carried out at 25°C by direct immersion of pH glass electrode into the prepared formulation samples CIPAC MT 75.3. (CIPAC, 2016).

Refractive index

The refractive index was determined by a simple Abbe's refractometer, ATAGO, Co., LTD, Japan by placing one drop of the prepared formulation on the slide at 25°C (ASTM, 2016).

Surface tension

The surface tension was determined by the Wilhelmy plate method using "Sigma 700". The instrument was recalibrated before testing. The surface tension of the samples was recorded (ASTM, 2014).

Density

The density was determined using a digital density meter model DDM 2910 with a touch screen, Rudolph Research Analytical, USA (ASTM, 2017).

Viscosity determination

The viscosity of the prepared SL formulation was determined without dilution using "Brookfield DV II+PRO" Digital Viscometer (Brookfield, USA) UL rotational adaptor. The temperature was kept at 25°C during the measurement by water bath (Model: TC-502 USA) and each reading was recorded after the equilibrium of the sample. Five replicates were conducted for the sample and the average was reported and expressed as milli Pascal-second (mPas) (ASTM, 2018).

Flash Point

Flash point is an important property of the formulation because it is used in various ways to control the conditions under which substances are stored and transported especially when they can be potentially dangerous and constitute a fire hazard. Determination of flash point of the prepared formulation was carried out by the tag open cup method (Koehler instrument company, INC, USA). The flash point was recorded as the temperature at the thermometer after appearance of the flash CIPAC MT 12. (CIPAC, 1995).

Pots experiment

Pots experiment was carried out from February to early June at the time of summer potato planting in Egypt at the Plant Pathology Research Institute, ARC. Pots (25cm diameter) were filled with soil mixture (clay: sand at 1:1 ratio). Soil mixture was irrigated with water. After 24h, the soil was drenched with the suspension (10⁹cfu/ml) of a mixture of equal amounts of two virulent isolates (mixed immediately before use) of *R. solanacearum* race 3 biovar 2 at the rate of 100ml/pot. After 48h, potato tubers (cv. Spunta) kindly provided by the Potato Brown Rot Project (PBRP) were planted (one tuber/ pot) and then 100ml/pot of OXA+NAC SL 23% was added. The treatment was repeated with the OXA+NAC SL 23% (100ml/pot) 40 days after planting. Two OXA+NAC SL 23% treatments at concentrations 125.0 and 62.5 µl/ml were used. Plants treated with *R. solanacearum* only were used as a positive control treatment. The negative control treatment was kept without *R. solanacearum*, and OXA+NAC SL 23%. Five replicates (ten pots/ replicate) per treatment were used. The plants were watered regularly as required to keep the soil moisture. The development of wilt was recorded after 9 weeks of planting. This time was chosen because it is coincided with the greater vegetative activity of plants (Carvalho *et al.*, 2017). Disease severity was assessed using a scale of 0 to 5 where: 0= healthy; 1= wilting of one leaf; 2= wilting of two to three leaves; 3= wilting of most leaves; 4= wilting of all leaves; or 5= plant death. Wilt index was calculated using the following formula as described by (Hyakumachi *et al.*, 2013).

$$WI = \frac{5A+4B+3C+2D+1E+0F}{5N} \times 100$$

Where: A, B, C, D, E and F = number of plants on grades 5, 4, 3, 2, 1 and 0, respectively; N=total number of plants.

The percentage of wilted plants was determined. The plant was considered wilted when placed in grades 3,4 or

5(Carvalho *et al.*, 2017). After potato plants reached full maturity, the tuber yield of potato was collected. The average number and weight (g) of potato tubers per plant in each treatment were determined to study the effect of OXA+NAC SL 23% on the tuber yield of potato under conditions of brown rot stress. Plants were arranged in a completely randomized design. Means were compared by One-way ANOVA with Tukey test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Verification of the identity of *R. solanacearum* using PCR

The virulent isolates of *Ralstonia solanacearum* race 3 biovar 2 were subject to confirmation of identity using conventional PCR. A band at expected amplicon size (718bp) for *R. solanacearum* was generated by conventional PCR (Fig.,1) using two primers, forward primer RS-1-F 5'- ACT AAC GAA GCA GAG ATG CAT TA -3' and reverse primer RS-1-R 5'- CCC AGT CAC GGC AGA GAC T -3'.

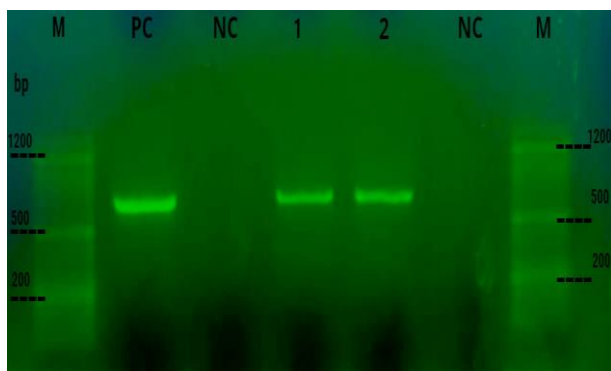


Fig. 1. PCR products based on RS-1-F and RS-1-R primers for *R. solanacearum* race 3 biovar 2. Lane M: 50bp DNA marker ladder. Lane NC: Negative (water) control. Lane PC: Reference isolate. Lanes 1-2: *R. solanacearum* (virulent isolates). A band at expected amplicon size (718bp) for *R. solanacearum* was generated.

Antibacterial activity of soluble concentrate (SL) formulations of oxalic acid (OXA) and N-Acetyl-L-cysteine (NAC)

Antibacterial activity of SL formulations of OXA and NAC was determined as the diameter of inhibition zone against *R. solanacearum* (Table, 1 and Fig.,2). After 24h, OXA+NAC SL 23% showed a total maximum inhibition zone (65.0mm) against *R. solanacearum* followed by NAC SL 10%

(61.0mm) and OXA SL 13% (35.3mm). In case of NAC SL 10% and OXA+NAC SL 23%, the inhibition zone consisted of two regions, a clear inhibition zone, and a less clear inhibition zone. The less clear inhibition zone was 39.7 and 20.3mm for NAC SL 10% and OXA + NAC SL 23%, respectively. After 72 h, the less clear inhibition zone decreased and stabilized at 12.0 and 15.3 mm for NAC SL 10% and OXA+NAC SL 23%, respectively. The final total inhibition zone was 35.3, 33.3, and 60.0 mm for OXA SL 13%, NAC SL 10%, and OXA+NAC SL 23%, respectively. Oxalic acid showed good antibacterial activity against the brown rot bacterium, *Ralstonia solanacearum*. Oxalic acid concentrations of 5000, 1000, and 500 mg/l were reported to show clear inhibition zones of 34, 27, and 12 mm, respectively against *R. solanacearum* (Kwak *et al.*, 2016). Concentrations of NAC over than 1 mg/ml decreased *Xylella fastidiosa* adhesion to glass surfaces, biofilm formation, and the amount of exopolysaccharides (Muranaka *et al.*, 2013). The presence of N-Acetylcysteine promotes the antibacterial activity of some antibiotics against several bacterial strains, and the presence of N-Acetylcysteine can be detrimental to some species of bacteria (Goswami and Jawali, 2010).

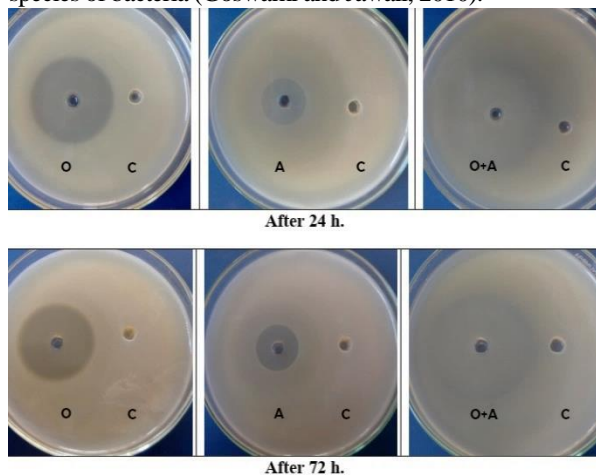


Fig. 2. Antibacterial activity of soluble concentrate (SL) formulations of oxalic acid (OXA) and N-Acetyl-L-cysteine (NAC) against *R. solanacearum*. O = OXA SL 13%, A = NAC SL 10%, O+A = OXA+NAC SL 23% and C (control) = sterile distilled water.

Table 1. Effect of SL formulations of OXA and NAC on the growth of *R. solanacearum* *in vitro*.

Treatment	Diameter of inhibition zone (mm)					
	After 24 h.			After 72 h.		
	Clear zone	Less clear zone	Total zone	Clear zone	Less clear zone	Total zone
OXA SL 13%	35.3±1.15	0.0±0.00	35.3±1.15	35.3±1.15	0.0±0.00	35.3±1.15
NAC SL 10%	21.3±1.53	39.7±2.52	61.0±3.06	21.3±1.53	12.0±2.00	33.3±3.21
OXA+NAC SL 23%	44.7±2.52	20.3±2.30	65.0±1.00	44.7±2.52	15.3±2.08	60.0±1.00
Sterile distilled water (pH = 2.1)	20.3±0.58	0.0±0.00	20.3±0.58	20.3±0.58	0.0	20.3±0.58
Control (sterile distilled water)	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00

Means of three replicates; values expressed as mean ± Standard Deviation (SD); SL =soluble concentrate; OXA = oxalic acid; NAC =N-Acetyl-L-cysteine.

MIC of OXA+NAC SL 23% against *R. solanacearum*

Oxalic acid exhibited antibacterial activities against *R. solanacearum*, *Agrobacterium tumefaciens* and *Xanthomonas oryzae* pv. *oryzae* at the minimum inhibitory concentration (MIC) of 250 mg/l (Kwak *et al.*, 2016). The minimal inhibitory concentration of NAC against *X. fastidiosa* was 6 mg/ml (Muranaka *et al.*, 2013). The MIC of OXA+ NAC SL 23% against *R. solanacearum* was determined. The inhibition zones

for 500, 250.0, 125.0, 62.5µl/ml of OXA+ NACSL 23% against *R. solanacearum* were 32.3, 20.0, 13.0, and 6.7mm, respectively. While the concentrations from 31.25 to 0.0µl/ml did not show any inhibition zone. The lowest concentration of OXA+ NACSL 23% that showed clear zones against *R. solanacearum* was 62.5µl/ml. The concentration of 62.5µl/ml of OXA+NAC SL 23% was considered the MIC value against *R. solanacearum* (Table 2 and Fig. 3).

Table 2. MIC of OXA+ NAC SL 23% against *R. solanacearum* using well diffusion method.

OXA+ NAC SL 23% concentration (µl/ml)	Inhibition zone(mm)*	MIC (µl/ml)
500.0	32.3±2.08	62.50
250.0	20.0±1.73	
125.0	13.0±1.00	
62.5	6.7±0.58	
31.25	0.0±0.00	
15.63	0.0±0.00	
7.81	0.0±0.00	
0.0	0.0±0.00	

* Means of three replicates, values expressed as mean ± Standard Deviation (SD); SL =soluble concentrate; OXA = oxalic acid; NAC =N-Acetyl-L-cysteine.

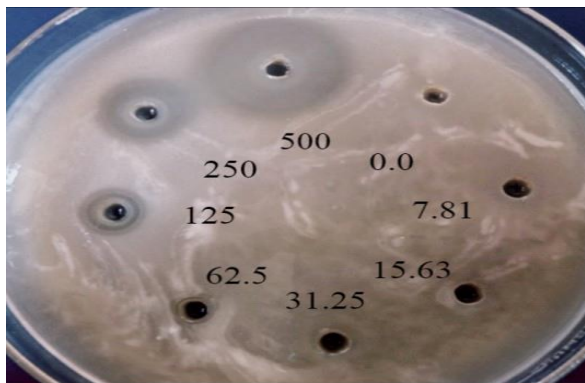


Fig. 3. MIC of soluble concentrate (SL) formulation of oxalic acid (OXA) + N-Acetyl-L-cysteine (NAC) 23% against *R. solanacearum*. Different concentrations of OXA+ NAC SL 23% (500, 250, 125, 62.5, 31.25, 15.63 and 7.81µl/ml) and control (0.0µl/ml i.e. sterile distilled water).

MBC of OXA+NAC SL 23% against *R. solanacearum*

The effect of different concentrations of OXA+NAC SL 23% was tested against the growth of *R. solanacearum* in the YPG broth medium to determine the MBC. The numbers of viable bacteria of *R. solanacearum*, after spreading 100µl of YPG broth medium on YPGA medium (sub-culturing), for 31.25, 15.63, 7.81 and 0.0µl/ml of OXA+NAC SL 23% were 5.4×10^2 , 2.6×10^3 , 1.8×10^4 and 19.7×10^8 CFU/ml, respectively (Table 3). The logs CFU/ml of *R. solanacearum* for 31.25, 15.63, 7.81 and 0.0µl/ml of OXA+NAC SL 23% were 2.73, 3.41, 4.27 and 9.18 CFU/ml, respectively (Fig. 4). The lowest concentration of OXA+ NAC SL 23% that showed no growth (viable bacteria) of *R. solanacearum* on the YPGA medium was 62.50µl/ml. The concentration of 62.5µl/ml of OXA+NAC SL 23% was considered the MBC against *R. solanacearum* (Table 3).

OXA+NAC SL 23% formulation characteristics

The agrochemical formulations cover a broad variety of systems that are prepared to suit specific applications. Any agrochemical formulations have the main function of making handling and application of the active ingredient as easy as possible. Biological effectiveness is an essential feature of the formulation. This is achieved in most cases by controlling the physical characteristics of the formulation. An important criterion for any agrochemical is its safety, both to the crop and to the agrochemical workers. We can predict the stability of soluble concentrate formulation by evaluating certain physical parameters before and after storage at 0°C and 54°C was used for the physical and chemical stability studies. It has been

generally accepted that two weeks at 54°C represent 2 years in normal conditions. No evidence indicates that a product has a satisfactory shelf life (of at least 2 years) in the different temperature zones. Therefore, the test thus offers a valuable output guide for performance after storage in warm or continental temperature climates. However, it is completely clear that the product that passes these testes would be sufficient in the field conditions (Gašić *et al.*, 2012).

Table 3. MBC of OXA+NAC SL 23% against *R. solanacearum* using the broth dilution method.

OXA+NAC SL 23% concentration (µl/ml)	Final concentration (µl/ml)*	Growth on YPGA medium**	Number of bacteria (CFU/ml)**	MBC (µl/ml)
666.67	500.0	-	0.0±0.00	62.5
333.33	250.0	-	0.0±0.00	
166.67	125.0	-	0.0±0.00	
83.33	62.5	-	0.0±0.00	
41.67	31.25	+	$5.4 \times 10^2 \pm 0.75 \times 10^2$	
20.83	15.63	+	$2.6 \times 10^3 \pm 0.12 \times 10^3$	
10.42	7.81	+	$1.8 \times 10^4 \pm 0.58 \times 10^3$	
0.000	0.0	+	$19.7 \times 10^8 \pm 17.0 \times 10^8$	

SL = soluble concentrate; OXA = oxalic acid; NAC =N-Acetyl-L-cysteine; * = in YPG broth medium; ** = after sub-culturing, means of three replicates and values expressed as mean ± Standard Deviation (SD).

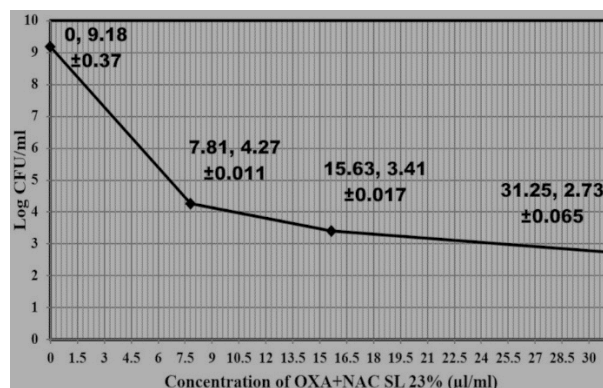


Fig. 4. Effect of OXA+NAC SL 23% on the viability of *R. solanacearum* in the YPG broth medium. SL = soluble concentrate; OXA = oxalic acid; NAC = N-Acetyl-L-cysteine; Means of three replicates; values expressed as mean ± Standard Deviation (SD).

The physical appearance of the prepared SL formulation of OXA+NAC SL 23% was readily dissolved and, when diluted at a ratio of 5: 95 (v/v) SL formulation: water with CIPAC standard waters A and D produced stable solution without precipitation and no change in color or appearance through the storage periods (7days at 0°C ± 2 and 14 days at 54 ± 2°C). The prepared formulation exhibited sustained stability after centrifugation and Freeze-thaw cycles without separation. Results of physical properties of the prepared OXA+NAC SL 23% formulation were shown in Table (4). pH is an important parameter for monitoring the stability of the prepared formulation, since a change in the value of pH indicates the occurrence of chemical reaction which may affect the quality of the final products (Seibert *et al.*, 2019) The pH values of the prepared formulation were in the range of 2.09 to 2.11, indicating that the prepared formulation under different storage condition has an acidic character which implies good biological activity (Molin and Hirase, 2004). The value of the refractive index was

in the range of 1.3806 to 1.3813, indicating that, the prepared formulation appears nearly transparent in the visible spectrum. The prepared formulation having the surface tension range of 31.22 to 31.53 mN/m. Lower surface tension is a beneficial function for most agricultural sprays because it promotes the spreading of droplets upon impact on leaves or other target surfaces, increases the surface-active area and enhances penetration and uptake of the product into the plants (Gaskin *et al.*, 2005; Giardino *et al.* 2006 and Damak *et al.* 2016). The prepared formulation has a high flash point (more than 60°C) makes it safer to transport and handle. The density value was in the range of 1.0803 to 1.0806 g/cm³ and the apparent viscosity was low, which in the range of 4.53 to 4.56 mpascal.

Table 4. Physicochemical properties of SL formulation of OXA+NAC SL 23% before and after storage.

Parameters	Before storage		After storage	
	Fresh formulation	7 days	14 days	
Temperature	Room temp.	0 °C	54 °C	
pH (1%)	2.12	2.11	2.09	
Refractive Index	1.3813	1.3806	1.3808	
Surface tension	31.22	31.53	31.26	
Density	1.0806	1.0805	1.0803	
Viscosity	4.54	4.56	4.53	
Flash point	Over 60°C	Over 60°C	Over 60°C	

The ability of OXA+NAC SL 23% to control brown rot

One of the most important factors limiting the cultivation of solanaceous crops in warm and humid climates is the occurrence of bacterial wilt, which is caused by *R. solanacearum* (Oliveira *et al.*, 2014). The ability of OXA+NAC SL 23% to control brown rot shown in (Fig., 5). Wilt index and the percentage of wilted plants were decreased significantly in OXA+NAC SL 23% treatments compared to the positive control (*R. solanacearum*) treatment. Wilt index was decreased from 25.2 % in *R. solanacearum* (positive control) treatment to 6.4 and 15.6 % using 125 and 62.5 µl/ml of OXA+NAC SL 23%, respectively. The percentage of wilted plants was 20.0% in the positive control (*R. solanacearum*) treatment, while the treatments 125 and 62.5 µl/ml of OXA+NAC SL 23% recorded 6.0 and 8.0 %, respectively. Wilt index was decreased significantly in treatment 125.0 µl/ml compared to treatment 62.5 µl/ml of OXA + NAC SL 23%. While no significant differences between the decrease in the percentage of wilted plants in treatment 125.0 µl/ml and treatment 62.5 µl/ml of OXA+NAC SL 23%. Percentage of plants with bacterial wilt symptoms of Brazilian potato cultivars was assessed by the relationship between the number of plants that had symptoms and the total number of plants, a plant was considered symptomatic when it showed more than 50% of wilted branches. ‘BRSIPR Bel’, ‘Agata’, and ‘BRS Camila’ showed susceptibility levels to bacterial wilt of 20, 30, and 80% of symptomatic plants at 59 days after planting, respectively (Carvalho *et al.*, 2017). Management of wilt disease includes methods adopted against *R. solanacearum* such as cultural practices, physical and chemical methods, and disease management through the use of antagonistic bacteria and fungi (Elazouni *et al.*, 2019). Cow manure decreased the wilt severity of potato by *R. solanacearum* from 47% to 20% in sandy soil (Messiha *et al.*, 2007). Treatment of tomato roots with *Bacillus thuringiensis* culture followed by challenge inoculation with *R. solanacearum* suppressed the development of wilt symptoms to less than one-third of the control (Hyakumachi *et al.*, 2013). Pesticides offered greater benefits than other control methods, but this has not always been the case. The combination of methyl bromide, 1,3-dichloropropene, or metam sodium with

chloropicrin significantly reduced bacterial wilt in the field from 72% to 100%. Infection by the bacterial wilt pathogen was prevented through bacteriostatic actions with a phosphoric acid solution (Yuliar *et al.*, 2015).

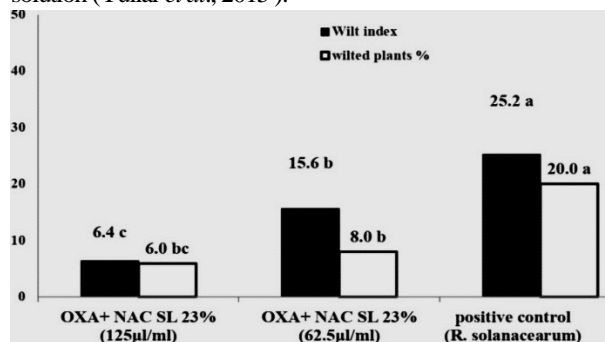


Fig. 5. Effect of OXA+NAC SL 23% on wilt index and percentage of wilted plant in potato. SL = soluble concentrate; OXA = oxalic acid; NAC = N-Acetyl-L-cysteine; Means of five replicates; Means that share a letter on the wilt index or wilted plant are not significantly different using Tukey test (p≤0.05).

Effect of OXA+NAC SL 23% on the tuber yield of potato under conditions of brown rot stress

The losses in weight of fruit yield for five eggplant genotypes due to the bacterial wilt disease were ranged from no significant loss to 99.53% (Oliveira *et al.*, 2014). The average weight of total potato tubers is equivalent very low values, but reasonable when considering an overall average incidence of plants with symptoms of bacterial wilt (Carvalho *et al.*, 2017). The yield of tomato (with bacterial wilt) treated with methyl bromide, 1,3-dichloropropene, or metam sodium with chloropicrin was 1.7- to 2.5-folds higher than that of the untreated control (Yuliar *et al.*, 2015). OXA+NAC SL 23% showed an increase in potato yield compared to positive control with *R. solanacearum*. The number of potato tubers was significantly increased from 4.1 in positive control (*R. solanacearum*) treatment to 6.1 and 5.3 using 125 and 62.5 µl/ml of OXA+NAC SL 23%, respectively. The weight of potato tubers was significantly increased from 105.5g in positive control to 148.5 and 130.9g using 125 and 62.5 µl/ml of OXA+NAC SL 23%, respectively. While the number and weight of potato tubers in the negative control treatment (free from *R. solanacearum* and OXA+NAC SL 23%) were 4.9 and 129.7g, respectively. No significant difference was found between the increase in the number of tubers in treatment 125.0 µl/ml and treatment 62.5 µl/ml of OXA+NAC SL 23%. While, the weight of potato tubers was significantly increased in treatment 125.0 µl/ml compared to treatment 62.5 µl/ml of OXA + NAC SL 23% (Fig., 6). The percentages of decrease in the number and weight of potato tubers (yield) due to bacterial wilt disease compared to the negative control (healthy plants) were 16.3 and 18.9%, respectively (Fig., 7). The percentages of increase in the number and weight of potato tubers (yield) due to treatment with 125 µl/ml of OXA+NAC SL 23% compared to the positive control (*R. solanacearum*) were 48.8 and 40.6%, respectively. The percentages of increase in the number and weight of tuber yield of potato due to treatment with 62.5 µl/ml of OXA+NAC SL 23% compared to the positive control (*R. solanacearum*) were 29.2 and 24.0%, respectively (Fig., 8).

In conclusion, OXA and NAC could be successfully formulated in the form of a stable soluble concentrate formulation. The prepared SL formulation of OXA+NAC SL 23% showed thermodynamic stability without presenting a

change in the physicochemical properties. The OXA+NAC SL 23% showed considerable inhibition zone against *R. solanacearum*. The OXA+NAC SL 23% showed promising results in controlling the disease and increasing the potato yield under conditions of brown rot stress in pots. Field experiments are needed to confirm the results.

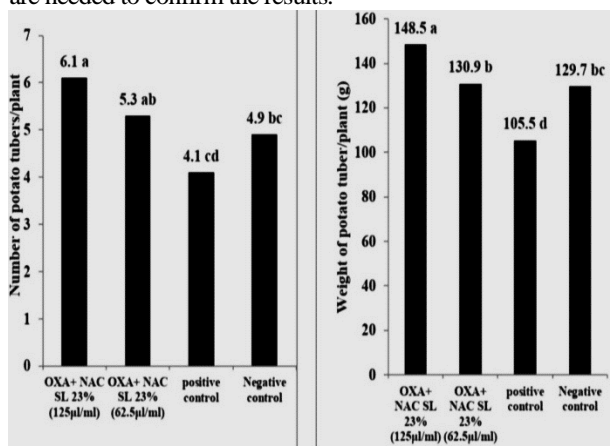


Fig. 6. Effect of OXA+NAC SL 23% on the number and weight of tuber yield of potato under conditions of brown rot stress. SL = soluble concentrate; OXA = oxalic acid; NAC = N-Acetyl-L-cysteine; Means of five replicates; Means that share a letter on the number of potato tubers or weight of potato tubers are not significantly different using Tukey test ($p \leq 0.05$).

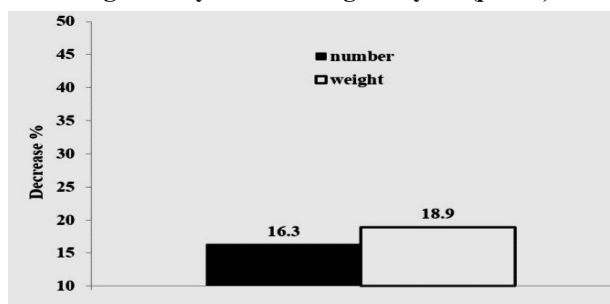


Fig. 7. The percentage of decrease in the number and weight of tuber yield of potato due to bacterial wilt disease compared to the negative control (healthy plants).

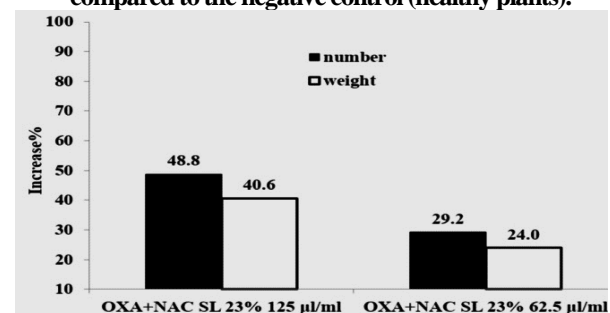


Fig. 8. The percentage of increase in the number and weight of tuber yield of potato due to treatment of OXA+NAC SL 23% compared to the positive control (*R. solanacearum*).

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تجهيز حمض الأكساليك وإن- أستيل-ال- سيستايين كمستحضر قابل للذوبان في الماء: إمكانية الاستخدام في مكافحة

رالستونيا سولاناسيرم *Ralstonia solanacearum*

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الغفن البني هو مرض تسببه بكتيريا الرستونيا سولاناسيرم *Ralstonia solanacearum* وله القدرة على التسبب في خسائر كبيرة بسبب تعفن الدرنات في نباتات البطاطس المصابة. مكافحة الكيمائية لـ الرستونيا سولاناسيرم *R. solanacearum* غير فعالة وطريق مكافحة البيولوجية لم يتم تطويرها بعد ضد هذا الكائن الحي. تم اختبار الأنشطة المضادة للبكتيريا للتجهيزات القابلة للذوبان في الماء (SL) لحمض الأكساليك (OXA) وإن- أستيل-ال- سيستايين (NAC) ضد الرستونيا سولاناسيرم *R. solanacearum*. أظهرت تركيبات OXA SL 13% و OXA NAC SL 23% و NAC SL 10% مناطق تثبيط ضد الرستونيا سولاناسيرم *R. solanacearum*. في حالة OXA NAC SL 23% و OXA NAC SL 23%، فإن منطقة التثبيط تتكون من منطقتين، منطقة تثبيط واضحة ومنطقة تثبيط أقل وضوحاً. بعد 72 ساعة من التحضين، كانت منطقة التثبيط الإجمالية النهائية 35.3 و 33.3 و 60.0 مم² لـ OXA SL 13% و OXA NAC SL 23% و NAC SL 10% على التوالي. كان الحد الأدنى للتركيز المثبط (MIC) والحد الأدنى للتركيز القاتل (MBC) لـ OXA NAC SL 23% ضد الرستونيا سولاناسيرم *R. solanacearum* 62.5 ميكروغرام / مل. أظهرت تركيبة OXA NAC SL 23% ثباتاً جيداً في ظروف التخزين المختلفة ولم يلاحظ أى فصل أثناء اختبار الثبات. في تجربة الأواني، انخفض مؤشر الذبول ونسبة النباتات الذابلة بشكل معنوي في معاملات OXA NAC SL 23% مقارنة بمعاملات المقارنة الإيجابية (المعاملة ببكتيريا *R. solanacearum* فقط). تم زيادة عدد ووزن درنات البطاطس بشكل معنوي باستخدام التركيزات 125 و 62.5 ميكروغرام / مل من OXA NAC SL 23% مقارنة بالمقارنة الإيجابية. هناك حاجة إلى التجارب الحقلية لتأكيد النتائج.