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Original Research Article

Aqueous Garlic Extracts has Antibacterial Properties against {*Ralstonia solanacearum* (Smith) Yabuuchi} and Protected Tomato Plant from Bacterial Wilt Disease

Oluwayomi Emmanuel Ojo[1], Ayodele Martins Ajayi[1*], David Babatunde Olufolaji[1], Ganiyu Folusho Hassan[1]

[1]Department of Crop, Soil and Pest Management, Federal University of Technology, Akure, P M B 704, Ondo State, Nigeria

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Abstract: *In vitro* and *in vivo* studies were carried out to evaluate aqueous extracts from *Allium sativum* L. (Garlic) against *Ralstonia solanacearum* isolated from an infected tomato plant. Four concentrations, (10, 20, 30 and 40%) of aqueous garlic extracts were prepared using cool (35^o C) and hot (70^o C) water. Evaluation of the extract concentrations against the pathogen was done using the agar well diffusion method in a nutrient agar medium. Incubation was for 48 hours at 35^o C. In the *in vivo* studies, the most effective concentrations of garlic extracts were used as seed priming agents on tomato seeds sown in *R. solanacearum* infested soil in plastic pots. All data collected were subjected to statistical analysis using Minitab software (Version 17), while means were separated using Tukey's test. Results showed that cool garlic extracts at 40% had a 30.63 mm zone of inhibition against the pathogen. This was significantly higher than 10.90 mm recorded by 10% of both cool and hot extracts. Hot extract, 40%, treated seeds recorded the highest number of leaves (21.75), had no incidence of bacterial wilt, gave the best plant height (45.75 cm) and fruit weight (327.1 g), surpassed only by the standard check. There is a need for more work to be done to developing an antibacterial agent from garlic for the management of bacterial wilt disease of tomatoes.

Keywords: Tomato, *Ralstonia solanacearum*, bacterial wilt, *Allium sativum*, aqueous extracts, seed priming.

1. 0. INTRODUCTION

Tomato (*Solanum lycopersicum* L.) is a widely grown fruit vegetable globally, on account of its nutritional importance and value (Adepoju 2014). It is a member of the family Solanaceae, a classification it shares with many other important crops like potato, eggplant and chili pepper. Solanaceae is a complex family having about 1500-2000 species (Sato *et al*., 2012). Tomato can be grown under diverse environmental conditions in open fields or greenhouse (Tiwari *et al*., 2012), but diseases are a major limiting factor against its production in Sub-Saharan Africa (Kim *et al*., 2016). Bacterial wilt, also known as southern bacterial blight, is caused by a bacterium called {*Ralstonia solanacearum* (Smith) Yabuchi}. It is one of the most devastating diseases of tomatoes globally (Tahat and Sjam 2010; Kago *et al*., 2019). *Ralstonia solanacearum* is a member of Burkholderiaceae family. It is a gram-negative and non-spore-forming obligate aerobe with a single polar flagellum (Sneath *et al*., 1986). It grows best at 35^o C and the strain of the pathogen in Africa has been identified to be *R. solanacearum* Race 1 (Denny 2006).

The symptoms of bacterial wilt of tomato usually manifest shortly before or during flowering. It is characterized by sudden wilting of green foliage, especially the young leaves at the upper parts of tomato plants, during hot and sunny days. As the disease progress, the entire plant wilts and dies. A longitudinal section through the stem of an infected plant usually reveals a pale yellow coloured vascular bundle (Tahat *et al*., 2010), while the "Stem-streaming test" (Denny 2006; Garcia *et al*., 2019)] can be used as a tentative diagnostic test for the disease.

Chemical management of the disease is difficult and usually not effective. This is because the pathogen 'hides' within the xylem tissue where it is protected from reach by most chemical control agents. Additionally, *R. solanacearum* is a soil-born, and a quick build-up in population can result even if the plant

***Corresponding Author:** Ayodele Martins Ajayi

host is rid of the infection. Resistant cultivars are the best line of defence against the pathogen; unfortunately, however, such cultivars are usually not available or unaffordable for most peasant farmers in rural Africa. Research has shown that naturally derived plant products are sources of agrochemicals and have been employed in disease management. Furthermore, bioactive compounds from garlic have been reported to exhibit antimicrobial properties against bacterial pathogens of man (Wolde *et al*., 2018; Magrys *et al*., 2021), fungal/bacterial pathogens of plants (Abo-Elyousr and Asran 2009; Perello *et al*., 2013; Din *et al*., 2016; Abdel-Monaim *et al*., 2011). Phytochemicals from plants are safe, economical and biodegrade with ease (Okigbo and Ogbona 2006). It is on this premise that extracts from garlic were evaluated in this study as a possible antibacterial agent against *R. solanacearum* and the management of bacterial wilt disease of tomatoes. The aim is to develop an effective antimicrobial agent for the management of the disease, while the objectives were to;

i. Evaluate aqueous extract from garlic for antibacterial properties against *R. solanacearum in vitro* and *in vivo*
ii. Determine the effect of treatments on the growth and yield of tomato plants.

2.0. MATERIALS AND METHODS

2.1. Study location

The study was conducted at the Department of Crop, Soil and Pest Management, Federal University of Technology, Akure (FUTA), Ondo-State, Nigeria. Longitude 5°06′ E to 5°38′ E and between latitude 7°07′N to 7°37′N.

2.2. Preparation of culture medium and isolation of *R. solanacearum*

The culture medium employed was nutrient agar. It was prepared based on the manufacturers, *Biotek*, recommendation of 28 g in 1 litre of water. The Prepared medium was sterilized at 121^{o} C for 15 minutes in an autoclave. The Sterilized medium was amended with Nystatin and pour plated at 45^{o} C. *Ralstonia solanacearum* was isolated from the stem of an infected tomato plant showing symptoms of bacterial wilt disease. The method described by (Goszczynska *et al*., 2000) was adopted with slight modification. Segments of the infected tissue were obtained, washed in running tap water and surface sterilized in 70% alcohol. Sterilized tissues were then macerated in a sterile porcelain mortar with pestle. Inoculation was with the aid of a wire loop dipped into the macerated tissue and streaked on the prepared nutrient agar growth medium in Petri-dishes. Incubation was at 35^{o} C for 24 hours after which sub-culturing was done to obtain pure cultures of the bacterial colonies isolated.

2.3. Identification and characterization of *R. solanacearum*

Preliminary identification of *R. solanacearum* was based on the colony characteristic of the isolate, through visual and microscopic examination. Thereafter, biochemical tests were carried out for the characterization of the pathogen. A Pathogenicity test was also conducted on healthy tomato plants through soil infestation with the isolated pathogen.

2.3.1. Gram staining

The procedure described by Goszczynska *et al*., (2000) was adopted. A loopful of 24 hours old culture of *R. solanacearum* was used. A light smear of the isolate was made on a clean grease-free glass slide and air-dried. The sample was then heat-fixed by passing it several times over a Bunsen flame. Flooding of the smears with crystal violet was done for one minute, after which washing with distilled water, to remove excess stain, was done. Mordant (Gram's iodine) was added for one minute, rewash and slanted on the staining rack. Decolourization was done on the slanted slides by washing with 95% alcohol until no more violet colour runs from the slide. It was immediately washed and counterstained with 0.5% safranin for 30 seconds. The slides were washed for the last time with distilled water and blotted dry with Whatman's No. 1 filter paper. Microscopic examination was done using the oil immersion objective, X100, of a light microscope.

2.3.2. Catalase test

The slide method as described by Reiner (2010) was adopted with some modifications. One millilitre of 3% hydrogen peroxide was spotted on a clean slide and inoculum from 24 hours culture of *R. solanacearum* was emulsified on the spotted solution and examined for the formation of oxygen bubbles.

2.3.3. Starch hydrolysis

The procedure described by Goszczynska *et al*., (2000) was followed. A basal medium having 1% soluble starch was employed for the test. Sterilization of the medium was done in an autoclave, after which pour plating was done in Petri-dishes and allowed to cool and set. Inoculation of *R. solanacearum* was through the streak method, once across the plate. Incubation was at 35^{o} C for 5 days, after which the culture plate was flooded with iodine solution. Motility and sugar fermentation tests were also carried out as described by Goszczynska *et al*., (2000).

2.4. Collection and preparation of garlic extract

Fresh garlic bulbs were purchased, cleaned and surface sterilized in 70% ethanol. Exactly 50 g was weighed out and crushed in a blender to obtain a paste. Fifty (50) ml of sterile distilled water at 35^{o} C was added to the paste and allowed to stand for 24 hours. Filtration was done with a sterile muslin clothe to obtain cool garlic extracts at 100% concentration. The

filtrate was reconstituted to obtain four concentrations of 10, 20, 30 and 40% respectively. The same procedure was repeated for hot (70^o C) water extraction.

2.5. *In-vitro* evaluation of garlic extracts against *R. solanacearum*

The antibacterial activity of garlic's aqueous extracts (cool and hot) against *R. solanacearum* was evaluated using the agar well diffusion method. A nutrient agar growth medium was prepared as described previously. Twenty-five (25) ml (at 40^o C) each of the medium was dispensed into Petri-dishes and 0.5ml of *R. solanacearum*, from a 24 hours old liquid broth, was added to each plate. Gentle swerving was done to facilitate the uniform mixing of broth and nutrient agar. A cork borer, of 7.5 mm, was used to punch wells in each nutrient agar/broth medium in Petri-dishes. Five wells, representing the concentrations of garlic extracts and the standard check, were punched in each medium for the evaluation of cool and hot aqueous extracts. Thereafter, 1.5 ml of each extract concentration was dispensed gently into a well as appropriate. The standard check was Mancozeb, reported to have antibacterial properties by Mikicinski *et al*., (2012), and is often used prophylactically for the management of tomato bacterial wilt disease. Each treatment was replicated thrice and incubation was at 35^o C.

2.6. Collection, sterilization and infestation of soil with *R. solanacearum*

Sandy loam soil, rich in humus, was collected from the Teaching and Research farm of FUTA. The soil was sterilized using the steam heat method, after which it was filled into plastic pots having a diameter of 41cm and a depth of 15cm each. The weight of soil in each pot was 15 kg. Plastic pots filled with sterilized soil were inoculated with 150mls of harvested cells of *R. solanacearum* at 10^6 cfu/ml. Planting of tomato seedlings occurred 48hrs after soil infestation.

2.7. Collection of test crop and extraction of seeds

A local variety of tomato, Beske, with a history of susceptibility to bacterial wilt was purchased from the open market. The fruits were cut and all the seeds were scooped out, washed in sterile distilled water and surface sterilized with 70% ethanol for 30 seconds.

2.8. Priming of tomato seeds with aqueous garlic extracts

Cool and hot garlic extract at 40% concentrations had the best inhibition on the growth of the pathogen. They were consequently evaluated in the in vitro study. Tomato seeds were drenched with the two concentrations separately and allowed to stand for 24 hours. The standard check consisted of tomato seeds primed with Mancozeb fungicide at the manufacturer's recommended rate, while the control was distilled water. Five primed seeds, from each treatment, were sown directly into *R. solanacearum* infested soil in plastic pots. Tomato seedlings were thinned down to two stands per pot at three weeks after germination. The treatments evaluated are listed below;

i. Cool garlic extract (40%)
ii. Hot garlic extract (40%)
iii. Standard check (Mancozeb)
iv. Control (Sterile distilled water)

Each treatment was replicated thrice and the experiment was laid out in the open field in a completely randomized design (CRD).

2.8. Data collection and statistical analysis

Data were collected on the following parameters.

i. Inhibition of *R. solanacearum* growth

The zone of inhibition on the growth of the pathogen by the treatments evaluated was measured 24 hours after incubation with a vernier calliper. The values obtained were recorded.

ii. Number of leaves

The number of leaves produced by each treatment was observed visually and counted from the 3^{rd} to the 9^{th} week after planting. The values obtained were recorded.

iii. Incidence of bacterial wilt disease

The incidence of bacterial wilt in each treatment was observed weekly. Disease incidence was obtained with the equation;

$$di = \frac{\text{nl}}{\text{tl}}\ x$$

Where;
di =disease incidence
nl = number of infected leaves
tl = total number of leaves on tomato stand

iv. Severity of bacterial wilt disease

The severity rating scale of Popola *et al*., (2012) was adopted. It consisted of a six-level rating as shown below;

0 = no symptoms of wilt 1= 1- 20% wilted leaves,
2 = 21 – 40% wilted leaves 3 = 41 -60% wilted leaves
4 = 61 – 80% wilted leaves 5 = 81 – 100% wilted leaves/death

vi. Plant height

The height of each tomato plant was measure and recorded, in cm, from the third to the 9^{th} week after planting

vii. Yield

The total number of fruits harvested from each treatment were counted and weighed. The weight of fruits was expressed in grams.

All collected data were subjected to statistical analysis using Minitab software. Means were separated using Tukey's test.

3. RESULTS

3.1. Identification of *R. solanacearum*

Results of the morphological and biochemical characteristics of the Isolated *R. solanacearum* is presented in (Table 1). Morphologically, the colour pigmentation observed for the isolated *R. solanacearum was* white/cream (Figure 1a). Microscopic observation after Gram staining showed several red coloured and rod-shaped cells. Biochemically, a negative result was obtained for Gram stain and starch hydrolysis. A positive result was however obtained for the catalase test, as well as the fermentation of lactose, manitol, dextrose, glucose and sucrose. The cells were also observed to be motile. Conclusively, results from the pathogenicity test confirmed the isolate to be virulent. Symptoms of bacterial wilt were observed on infected tomato plants shortly before flowering.

3.2. *In-vitro* evaluation of garlic extracts against *Ralstonia solanacearum*

Aqueous garlic extracts were found to have antibacterial properties against *R. solanacearum (Figure 1b and 1c). Statistically, significant differences in the zones of inhibition were observed among the treatments (Figure 2). Cool garlic extract at 40% significantly (*P≤0.05*) reduced the microbial growth of R. solanacearum*

Table 1: Morphological and biochemical characteristics of isolated *R. solanacearum*

S/N	Test/Activity	Results	
1	Gram stain		Negative
2	Starch hydrolysis		Negative
3	Catalase test	Positive	
4	Sugar fermentation		
	a. Lactose	Positive	
	b. Manitol	Positive	
	c. Glucose	Positive	
	d. Sucrose	Positive	
	e. Dextrose	Positive	
	f. Xylose	Positive	
5	Pathogenicity test	Positive	

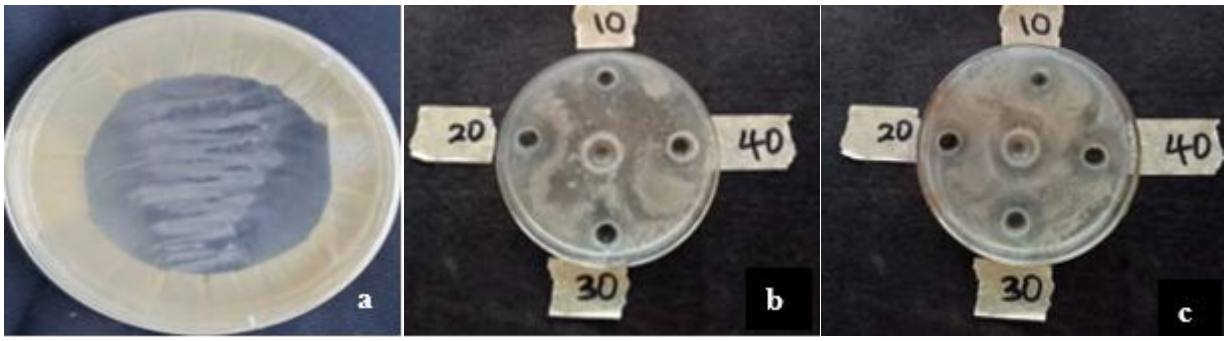

Figure 1: (a) Pure culture of R. solanacearum (b) Inhibition of *R. solanacearum* growth by cool garlic extracts (c). Inhibition of *R. solanacearum* growth by hot garlic extracts

Note. The values in figures 1a and b represent the concentrations of extracts. The central well is the standard check.

With the highest inhibition zone (30.63 mm) after 24 hours of incubation. However, the result also showed that cool garlic, 30%, and hot garlic, 40%, did not differ statistically in their zone of inhibition of the pathogen (20.90 mm and 30.00 mm respectively) after 24 hours of incubation. While cool and hot aqueous garlic extracts at 10%, recorded the lowest zone of inhibition with the values of 10.90 mm respectively which were not significantly different from one another. The standard check recorded a 20.67 mm zone of inhibition and was statistically similar to hot garlic at 30 and 40% concentrations (Figure 2).

3.3. Effect of treatments on leaf production

The effect of seed priming on leaf production is shown in Table 2. The number of leaves increased steadily across all treatments as the tomato plants increased in age. The highest number of leaves was 12.25, standard check, at the 6th week after planting (WAP). The value was not significantly different from the other treatments.

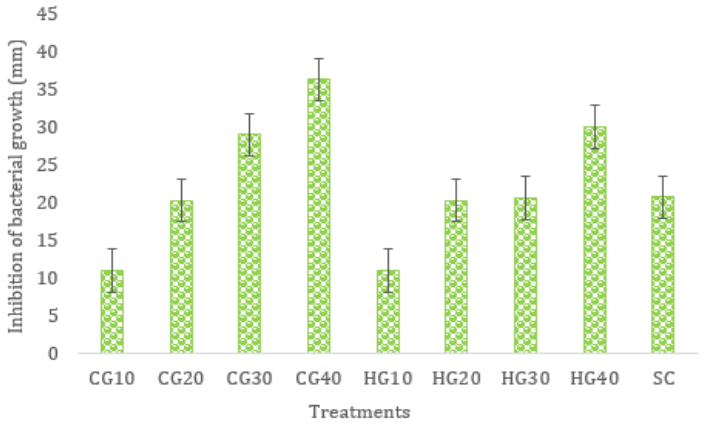

Fig 2: Effect of treatments on the growth of *R. solanacearum* in nutrient agar medium
Key: CG: Cool garlic extract, HG: Hot garlic extract, SC: Standard check

However, at the 9th WAP, hot garlic extracts recorded significantly (P≤0.05) highest number of leaves, 21.75.

Table 2: Effect of seed priming on the number of leaves produced by tomato plants

Treatments	Number of leaves/Weeks after planting		
	3	6	9
Cold garlic (40%)	3.33b	10.42a	13.00d
Hot garlic (40%)	3.67b	10.00a	21.75a
Standard check	3.51b	12.25a	20.50b
Control	4.17a	9.75a	16.17c

Means in a column with the same letter(s) are not significantly different by Tukey's test at P≤0.05.

3.4. Effect of seed priming on the incidence of bacterial wilt disease (%)

Disease incidence increased with increasing weeks after the first symptom was noticed (Figure 3a and b). It was significantly higher in control at 7, 10, and 12 WAP with the values of 79.73%, 83.33%, and 100% respectively (Table 3). Cool and hot garlic extracts and the standard check recorded no incidence of bacterial wilt across all the week for which data were collected.

Figure 3: (a). The onset of (b). Severe stage of bacterial wilt on tomato plant

Table 3: Effect of seed priming on the incidence of bacterial wilt disease of tomato (%)

Treatments	Disease incidence/Weeks after planting		
	7	10	12
Cold garlic (40%)	0.00b	0.00b	0.00b
Hot garlic (40%)	0.00b	0.00b	0.00b
Standard check	0.00b	0.00b	0.00b
Control	79.73a	83.33a	100.00a

Means in a column with the same letter(s) are not significantly different by Tukey's test at P≤0.05

Table 4: Effect of seed priming on the severity of bacterial wilt disease of tomato

Treatments	Disease severity/Weeks after planting		
	7	10	12
Cold garlic (40%)	0.00b	0.00b	0.00b
Hot garlic 40%)	0.00b	0.00b	0.00b
Standard check	0.00b	0.00b	0.00b
Control	3.00a	3.33a	5.00a

Means in a column with the same letter(s) are not significantly different by Tukey's test at P≤0.05.

3.5. Effect of treatments on disease severity

The severity of bacterial wilt increased with the increasing age of tomato plants (Figure 3a and b). The control showed a similar trend of disease severity as was observed for disease incidence across all treatments (Table 4). The control recorded the highest value for disease severity on the 7th, 9th and 12th WAP with the value of 3.00 and 3.33 and 5.00 respectively.

3.6. Effect of treatments on the height of tomato plants

The effect of seed priming on the height of tomato plants is shown in Table 5. An increase was recorded in the height of tomato plants in all treatments as the age of the plants increased. Significant differences ($P \leq 0.05$) were observed across the treatments. At 3 WAP, hot garlic extract recorded the highest plant height with the value of 7.00 cm while the lowest value of 6.00cm was recorded for the control. At 6 WAP, hot garlic was recorded 45.75 cm. This was significantly different from others. Whereas, at 9 WAP, a significantly higher value for plant height was recorded for the standard check, while the lowest value of 60.37 cm was recorded for the control.

Table 5: Effect of seed priming on the height of tomato plants (cm)

Treatments	Plant height/Weeks after planting		
	3	6	9
Cold garlic (40%)	6.33c	43.57c	61.37c
Hot garlic (40%)	7.00a	45.75a	65.50b
Standard check	6.75b	44.35b	73.62a
Control	6.00d	38.85d	60.37d

Means in a column with the same letter(s) are not significantly different by Tukey's test at P≤0.05.

3.7. Effect of treatments on fruit yield

The effects of seed priming on the yield parameters of tomatoes are presented in Figure 3. There were significant differences (P≤0.05) in the number and weight of fruits across treatments. Standard check recorded the highest number of fruits and fruit weight across the treatments with the values of 14.33 and 460.58 g respectively. Cool and hot garlic extracts were not significantly different from each other in terms of the number of fruits produced, 10.67, (Figure 3a) but differed significantly in terms of their weights. Hot garlic extract had 327.71 g fruit weight (Figure 3b).

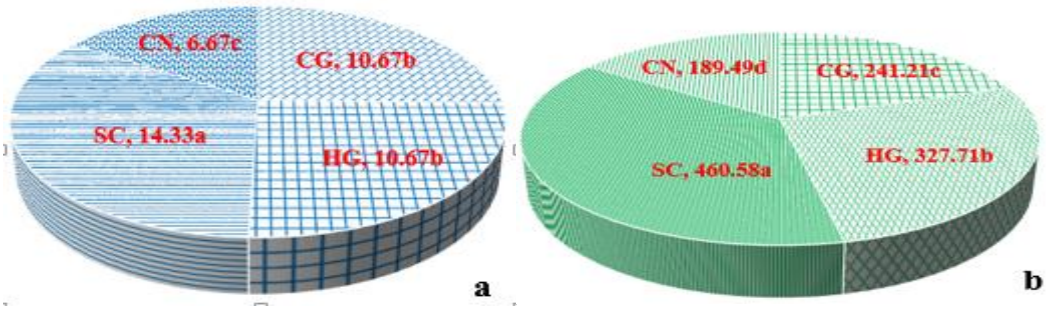

Figure 3: Effect of seed priming on a. number of fruits b. weight of fruits produced by tomato plants

Note: Pie segment values with the same subscript are not significantly different by Tukey's test at P≤0.05.

Key: CG: Cool garlic extracts, HG: Hot garlic extracts, SC: Standard check, CN: Control.

4. DISCUSSION

Investigation on the anti-bacterial properties of *A. sativum* against *R. solanacearum in vitro* revealed that aqueous garlic extract has inhibitory properties against the pathogen. A significant reduction in the growth of the bacterium, as the concentration of the extracts increased, was observed. This finding is in agreement with results from other works on the antimicrobial properties of garlic against plant pathogenic bacterial and fungi (Popola *et al*., 2012; Abo-Elyousr and Asran 2009). These antimicrobial properties have been reported to be due to the presence

of some bioactive compounds, the most prominent of which is allicin, Diallylthiosulfinate (El-Arigi *et al*., 2005). A variety of alkaloids, terpenoids, flavonoids, tannins, and saponins have also been reported (Rahman 2007; Shang et al 2019)**.** The mechanism of inhibition of the active ingredients in garlic against microbial pathogens is well studied. They include competition, parasitism and antibiosis. Against bacterial pathogen, Wolde *et al*. (2018) reported that the primary mechanism of action of allicin is the inhibition of DNA, RNA and protein synthesis.

The difference in the antibacterial properties of cool and hot garlic extracts at the different percentages evaluated may be as a result of a difference in the concentration of the active ingredients at the different concentrations evaluated and the difference in the temperature of the extraction medium, water. It has been reported that the efficiency of any plant extract against pathogenic organisms depends on the nature and the quantity of the active ingredients it contains as well as the mode of extraction (Raghavendra *et al*., 2009*;* Senhaji *et al.*, 2013*)*. It can be concluded, therefore, that the increase in concentrations corresponds to an increase in the active ingredients present in the solution of garlic extracts. This may have had a corresponding increase in the disruption of metabolic activities in *R. solanacearum* cells. This may have also brought about a reduced rate of cell division and growth in the *in vitro* studies. This view is similar to the one held by Haris *et al*., (2001).

The priming of tomato seeds with garlic extracts prevented the symptomatic manifestation of bacterial wilt disease. It is not clear if the procedure prevented infection by *R. solanacearum* or simply enhanced the tolerance of tomato plants to infection. Reports from literature, however, suggests that seed priming with garlic extracts at low concentrations can promote rapid root development and seedling vigour, as well as the stimulation of defence response in plants (Perello *et al*., 2013; Perello *et al*., 2003; Hayat *et al.*, 2018). owing to the activity of Diallylthiosulfinate (Hayat *et al*., 2018). Additionally, garlic extracts have also been reported to protect tomato plants from *Pseudomonas syringae* pv. *tomato, Xanthomonas vesicatoria* and *Clav ibacter michiganensis* subsp. *Michiganensis* (Balestra *et al*., 2009), all of which are serious bacterial pathogens of the crop.

Infection from *R. solanacearum* is known to bring about reduced growth and yield of tomatoes. The increased growth and yield recorded in garlic extracts treated seeds may have resulted from the protection/induction of resistance in such tomato plants. A similar finding on an increase of yield in cowpea plants treated with hot garlic extracts was reported by Ajayi and Oyedele (2018).

5. CONCLUSION

This study has shown that aqueous garlic extracts have antibacterial properties against *R. solanacearum.* Priming tomato seeds with garlic extracts also protected tomato plants from bacterial wilt disease, bringing about increased growth and yield. Garlic is therefore recommended for further work in the development of a plant-based bactericide for the management of bacterial wilt disease of tomatoes.

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