CONTROL OF BACTERIAL WILT IN TOMATO USING CHITOSAN INTERCALATED WITH TEA EXTRACTS

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Abstract

In this study, tea extracts were intercalated in chitosan gel to enhance the inhibitory effect of the complex on bacterial wilt in tomato. The disease caused by *Ralstonia solanacearum* can result in 100% crop loss under severe infection. Chitin was ground into powder of 0.1 mm size, deacetylated using concentrated NaOH solution and tea extracts from green, purple and black tea adsorbed through rotary evaporation. Confirmatory tests on effective adsorption were done using FTIR and XRD, while bioassay experiments were performed to determine efficacy of the chitosan intercalated with crude tea extracts (CICTE) on the pathogen and tomato growth. *In vitro* and *In situ* tests were carried out in growth chambers and greenhouse respectively. The greenhouse trials were conducted for a period of 2 years in three sites i.e. Gatundu, JKUAT and Makuyu. The bioassays demonstrated significant ($p < 0.05$) reduction of *R. solanacearum* turbidity marked by change of optical densities (OD) from 3.55 to 1.04. In addition, there was significant ($p < 0.05$) inhibition of the cultured *R. solanacearum* and reduced wilt incidence in tomato plants treated with CICTE and later inoculated with the pathogen. Tomato plants treated with CICTE also recorded a significantly ($p < 0.05$) higher yield compared to the control. The study therefore recommends utilization of CICTE as an effective and environmentally safe biopesticide for the devastating bacterial wilt pathogen.

Keywords: polyphenols, bacterial inhibition, organic polymers, adsorption

Introduction

The solution to bacterial wilt problem lies in effectiveness of the control strategy (Kailash *et al*., 2012). Variability of *Ralstonia solanacearum* makes application of most control strategies infective. Some of the management strategies such as rotation and intercropping may not be applicable under greenhouse conditions due to persistence of the pathogen in the soil (Alvarez *et a*l., 2019). Use of chemicals is one of the most effective methods for managing pests and diseases. However, *R. solanacearum* mutates fast rendering most chemicals ineffective. Also, application of synthetic pesticides results in accumulation of chemical residues in the produce and cause environmental pollution (Christos *et al*., 2011). Further, some of the chemicals used for soil fumigation have been banned by the World Health Organization through various commitments such the Kyoto protocol of 2005 (Karungi *et al*., 2011). The chemical residues in harvested produce has led to introduction of, stringent measures on minimum and maximum residue levels by most export markets (KHDP, 2007; KHCP, 2012). Botanical extracts such as such as catechins, thymol, esters of chrysenthemic acid, natural oils and terpenes have the potential of managing the disease to below economic injury levels (Lee *et al*., 2012).

Tea contains high quantities of catechins and polyphenols. These secondary metabolites are produced by plants to protect themselves from pests and diseases. The phenolic compounds are known to have antimicrobial activities towards a number of pathogenic bacteria

(Almajano *et al*., 2005). Tea extracts have been associated with inhibition of various bacteria such as *Escherichia coli*, *Pseudomonas spp., Staphylococcus aureus* and *Xanthomonas spp.* (Williamson *et al*., 2005). The antimicrobial activities of these compounds such as the catechins and theaflavins have demonstrated inhibition of DNA and RNA synthesis in microbes (Koech *et al*., 2013). However, the antimicrobial effect of tea extracts is hampered by the instability of these antimicrobial compounds (Ikigai *et al*., 1993). Most natural plant extracts are rapidly photo degraded in the presence of ultra violet light. Entrapment and intercalation of botanical extracts including polyphenols by use of polymers such as cellulose, silica and clays have been shown to increase their efficacy over a prolonged period of time by protecting them from harsh environmental conditions and direct sunlight exposure (Tolaimate *et al*., 2003). Adsorption of these tea extracts into polymers that possess antimicrobial effect enhances their efficacy due to synergism (Williamson *et al*., 2005). The objective of this work was to synthesize stabilized chitosan intercalated with crude tea extracts (CICTE) through adsorption of tea extracts into chitosan for controlling bacterial wilt in tomato.

MATERIALS AND METHODS

Materials

Green, purple and black teas were obtained from Kenya Tea Development Agency (KTDA) Kangaita Tea Factory Ltd (0^{o} 24.48 N, 37 ^{o} 15.2 E), while diseased tomato plants were obtained from a greenhouse infested with *R. solanacearum* in Kiambu County, Kenya. The area is an endemic zone of *R. solanacearum* pathogen with reported wilt incidences of over 70% (KHCP, 2012; KHDP, 2007). All materials were at least analytical grade and included; acetic acid and NaOH pellets obtained from Sigma Aldrich. Chitin was obtained from Laborex while selective TZC agar was obtained from Bioneer Ltd.

Preparation of crude tea extracts

The experiments were carried out in Jomo Kenyatta University of Agriculture and Technology (1.0901 ^{o}S, 37 0105 ^{o}E) at the Departments of Chemistry and Horticulture laboratories. The crude tea extracts were prepared by heating 60.0 g L^{-1} of the different tea types until boiling. After settling for 1.0 hr, the extracts were vacuum-filtered separately (Wan *et al*., 2009).

Preparation of Chitosan Intercalated with Crude Tea Extracts (CICTE)

Chitin (99%) was ground using a milling machine to obtain fine powder which was filtered through 0.1 mm mesh and autoclaved at 121 ^{o}C for 15 min. It was then treated with 80 % (w/v) NaOH solution and placed in an oven at 100 ^{o}C for 4 hr for deacetylation (removal of acetyl groups from the polymer) to take place. The product was then solubilized in dilute acetic acid. The acetic acid was then evaporated on a hot water bath leaving behind a whitish solid known as chitosan. The solid was ground and divided into three equal portions of 100 g, then placed in a rotary evaporator containing crude tea extracts (liquor) of green, black or purple tea. The samples were then characterized using a Fourier transform infra-red (FTIR, SHIMADZU) spectrophotometer and X-ray powder diffractometer (XRD, RIGAKU) (Roberts and Smith, 1961; Domszy and Roberts, 1985).

Isolation of Ralstonia solanacearum

The tomato plants were thoroughly washed to remove dirt. They were then dipped in 1% chlorox (Sigma Aldrich, UK) for sterilization. The basal part of the stem was cut into pieces of 5cm cross-sectionally and cut longitudinally then placed in a 1 L beaker containing distilled water to allow flow of bacterial exudates. The

obtained bacteria were cultured in a sterilized growth chamber for 48 hr at 32 ^{o}C on selective nutrient-tetrazolium chloride (TZC) agar contained in a petri dish (Jeong *et al*., 2007).

Isolation and Amplification of DNA

A suspension of the bacteria was used for extraction of DNA using the CTAB method and polymerase chain reaction (PCR) was done using touchdown procedures as described by Korbie and Mattick, 2008. The amplification reactions were performed in 25 µl volumes in thin-walled PCR tubes using *R. solanacearum* primers forward–GAA CGC CAA CGG TGC GAA CT-, and reverse –GGC GGC CTT CAG GGA GGT C-. The PCR (PTC-100) was programmed for an initial 5 cycles of 30 sec at 94 ^{o}C, 3 min at 48 ^{o}C, annealing at 58 ^{o}C for 1 min, extension for 1 min at 72 ^{o}C, followed by 10 and 15 cycles at the same timing and conditions. The samples were cooled up to 4 ^{o}C, subjected to electrophoresis on a 1.5% agarose gel in 1X TAE buffer (40 mM Tris acetate and 1.0 mM EDTA) (Wydra and Semrau, 2006). Touchdown PCR procedure was preferred as RT-PCR had limitations in determination of the optimal annealing temperature for clear display of the amplicons (Korbie and Mattick, 2008).

Determination of Efficacy of the Chitosan Intercalated Crude Tea Extracts on Inhibition of *Ralstonia solanacearum*

Ralstonia solanacearum was standardized to 3.500 OD using Uv-vis spectrophotometer. Chitosan intercalated with crude tea extracts were placed in a beaker containing the bacterial suspension and the OD determined after 48 hr. Nutrient agar was weighed to 28 g, dissolved in 1L of water and sterilized in autoclave at 121 ^{o}C for 15 minutes after attaining a constant temperature of 121^{o}C. It was then cooled to 40 ^{o}C and dispensed into sterile petridishes for solidification. Each bacterial culture was pipetted and put in sterile TZC- nutrient agar plates and spread evenly using a sterile wire loop. The disks were infused with individual tea extracts and CICTE products, clearly labeled and incubated aerobically at 32 ^{o}C for 96 hr. The inhibition zones were measured (mm) and recorded (Koech *et al*., 2013).

Antimicrobial assays were carried out on a completely randomized design (CRD) with 8 treatments and 3 replications inside a growth chamber using the disk diffusion method. The inhibition experiments were repeated every week for a period of 1 month before the in situ experiments were conducted in greenhouses.

Experimental Sites and Design

The chitosan intercalated with crude tea extracts (CICTE) of green, black and purple were applied on tomato seeds prior to seeding by priming. Similar treatments were done on the planting media (cocopeat) and the primed seeds sown on matching treatments containing CICTE in trays. The seedlings were inoculated prior to transplanting, the plants were slightly injured using a scapel at the root zone then placed in CICTE gel before inoculation with a *R. solanacearum* suspension. Transplanting was done in greenhouses at 3 sites that is; Gatundu-Theta Tea Factory (0.9621 ^{o} S, 36.7683 ^{o} E and altitude 2050 m ASL), Juja-JKUAT (1.0891^{o} S, 37.0105^{o} E and altitude 1400 ASL) and Makuyu (0.9392 ^{o} S, 37.1249 ^{o} E and altitude of 1255 m ASL) on plastic pots with well-prepared soil in the ratio of 3:0.5:1 for soil, sand and manure respectively. The experiments involved 8 treatments i.e. purple tea, black tea, green tea, chitosan, purple tea-CICTE, black tea-CICTE, green-CICTE and distilled water. The treatments had 3 replications and were laid on a CRD in each site.

Determination of Efficacy of the Chitosan Intercalated Crude Tea Extracts on Bacterial Wilt Incidence and Severity in Tomato

Bacterial Wilt Incidence

Wilt incidence was estimated based on wilting plants and browning/ streaming tests. Wilt symptoms were observed through the growing period. Most severely infected plants didn't survive beyond the 10^{th} week. Wilt incidence was estimated and recorded as number of wilting plants per treatment. It was calculated using the formula;

$$\frac{(5A + 4B + 3C + 2D + E)}{1.75N}$$

where, A = number of plants on scale 5; B = number of plants on scale 4; C= number of plants on scale 3; D = number of plants on scale 2; E = number of plants on scale 1; N = total number of plants. From the scale, the lower incidence level the better the control measure (Tim *et al*., 2008).

Tomato plant stems showing signs of wilting were then cut and scored for stem browning and bacterial streaming. A scoring scale of 0-3 was adopted where, 0 - no browning, 1- light tan colour at the base, 2 - light brown colour above the basal part and 3 - dark brown colour spread throughout the vascular system. The streaming test was conducted by suspending cut stems in distilled water in a beaker and the ooze rate score of 0-3 used to determine severity, where, 0 - no ooze, 1 - thin strands of bacteria oozing, 2 - continuous thin flow and 3 - heavy ooze turning the water turbid (Elphinstone *et al*., 1998). The bacterial stem browning and streaming were done by selecting and evaluating 3 plants per treatment collected 120 days after planting.

Yield Estimation

Mature fruits were harvested from the plants 3 months after planting for a period of 6 months. From each treatment and replicate, a single plant was randomly sampled and marked. The yield from each marked plant was obtained by picking only the ripe fruits, weighing on a balance and aggregating the yield obtained for the entire season separately.

Data Analysis

The data on pathogen colony inhibition, optical densities, wilt incidence, bacterial streaming, browning effect and yield were subjected to analysis of variance (ANOVA) and means separated by protected Fischer's Least Significant Difference ($LSD_{0.05}$) at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Characterization of chitosan and chitosan-crude tea extracts (CICTE)

Fourier Transform Infrared Spectrometry Characterization

The absorption band at 1413 cm^{-1} characterized stretching vibration of amino group in chitosan (Figure 1). (Jolanta *et al*., 2010). Formation of CICTE attributed to the formation of new spectral peaks in the chitosan spectra namely 1180 and 2160 cm^{-1}. These peaks displayed stretching and vibration bonds attributed to the hydroxyl (3400 cm^{-1}), amine (2000cm^{-1}) and carbonyl (900-1600cm^{-1}) functional groups which depict chitosan and polyphenols numerous functional bonds (Giusti and Wrolstad, 2001; Ignat *et al*., 2011).

X-ray Diffractometry Characterization

The X-ray diffractograms showed characteristic peaks at 2-theta 8.25 and 9.05 but were shifted to 8.45, 9.25 and 21.89 suggesting formation of inter and intra-molecular hydrogen bonds in the complex. There was systematic increase in d- spacing (nm) when chitosan was intercalated with crude tea extracts confirming that chemical change had occurred between the compounds (Figure 2).

Detection of Isolated *Ralstonia solanacearum*

The isolated pathogen was cultured on semi selective TZC nutrient agar and detected by DNA amplification. The *R. solanacearum* was confirmed by observing the pink and whitish coloration on the TZC culturing media. In addition to PCR detection was employed as the ultimate confirmation test (Wydra and Semrau, 2006). The pathogen was detected at 600 bp (Plate 1)

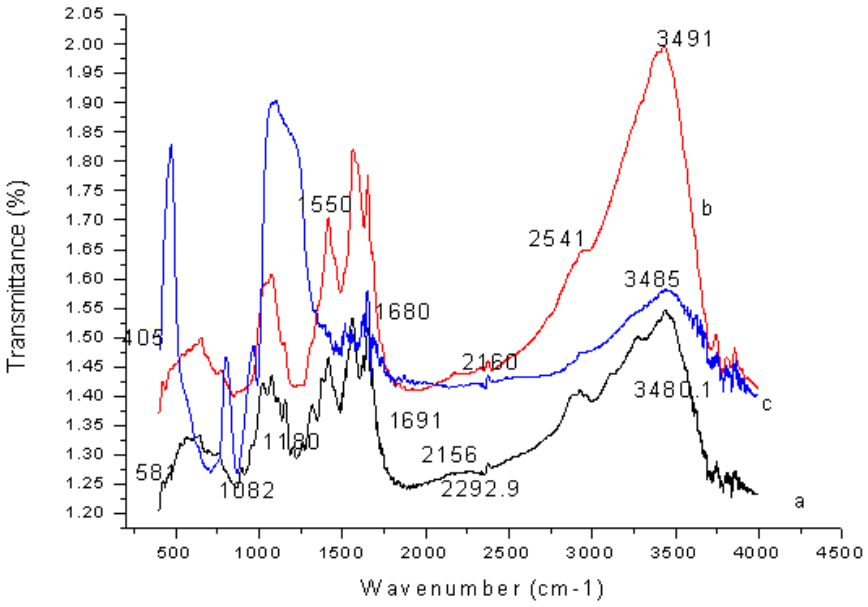

Figure 1: FTIR spectra for a) Chitosan b) Crude tea extracts and c) CICTE (chitosan intercalated crude tea extracts)

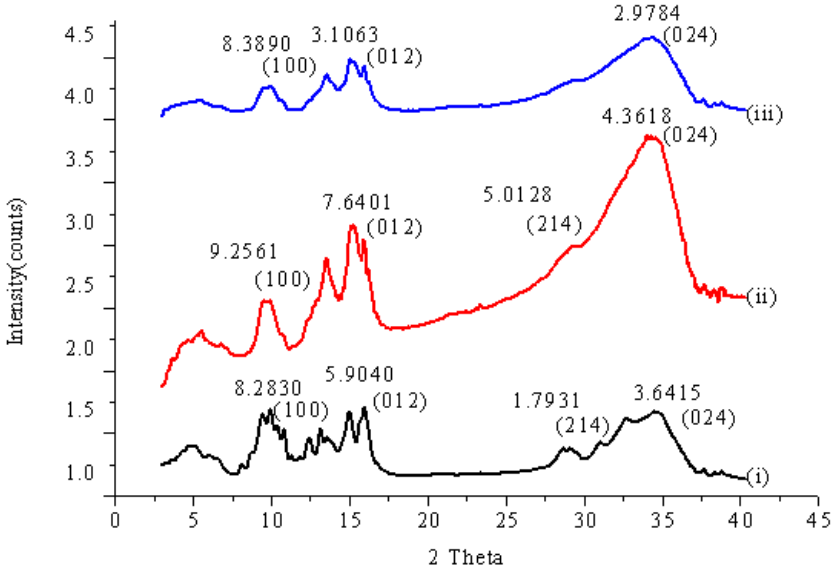

Figure 2: X-ray diffractograms of; (i) Chitosan, (ii) CICTE and (iii) Crude tea extracts

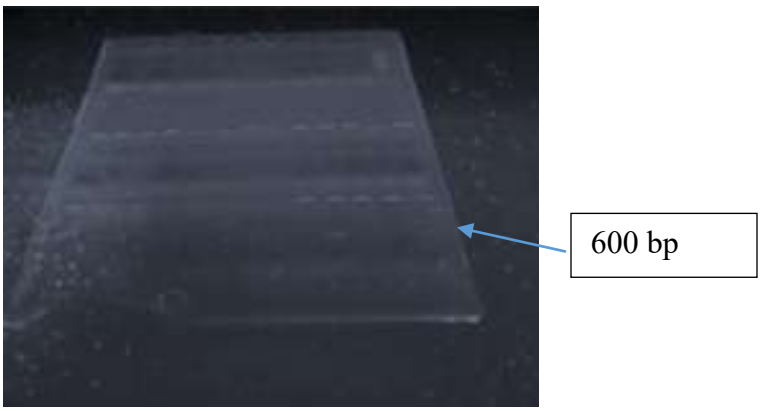

Plate 1: Detection of *R. solanacerum*

Table 1: In vitro Inhibition and Optical Density of *Ralstonia solanacearum* by Different Substances

Treatments	Inhibition (%)	Optical Density (600nm)
Distilled water	10.0a	3.55e
Purple Tea	34.0b	2.09d
Black Tea	52.0c	2.01d
Chitosan	55.0c	1.85cd
Green Tea	60.0c	1.18c
Purple CICTE	60.0c	1.65c
Black CICTE	69.0cd	1.28b
Green CICTE	86.0e	1.04a

[a]Means followed by similar letters are not significantly different at $\alpha = 0.05$.

Efficacy of the Chitosan Intercalated Crude Tea Extracts on Inhibition of *Ralstonia solanacearum*

Green and black CICTE had the highest significant ($p < 0.05$) pathogen inhibition effect (Table 1). Adsorption of crude tea extracts on chitosan significantly ($p < 0.05$) increased inhibition of *R. solanacearum* by 76 %. The antibacterial and antioxidant effects of tea extracts and chitosan derivatives have also been elaborated through the adsorption of tea extracts into chitosan. Their incorporation leads to an enhancement of the inhibitory effect of the biopolymer against gram-negative bacteria (Zhao *et al*., 2002; Ubonrat and Bruce, 2010). Reduction in the bacterial suspension OD after introduction of CICTE indicated destruction of the bacterial cells by interfacial interactions of the cells with the complex (Roccaro *et al*., 2005; Gupta, 2011). The interaction was attributed to the functional groups between the complex and the bacterial membrane which inhibited further growth and reduced the existing colonies (Zhao *et al*., 2002). The polymers-bacterial interactions also impeded cytoplasmic membrane function interfering with energy metabolisms of bacterial cells by green tea extracts. The biological activity of this polysaccharide that consists of glucosamine and N- acetyl glucosamine copolymers is generally attributed to the interaction between its ammonium cation and the negatively charged macromolecules of microbial cell (Williamson *et al*., 2005; Ubonrat and Bruce, 2010; Koech *et al*., 2014).

Efficacy of Chitosan Intercalated Crude Tea Extracts on Bacterial Wilt in Tomato

Tomato seedlings treated with CICTE particularly the green CICTE and black CICTE had the least wilt incidences. Control

experiments; distilled water, purple, black and green teas had significantly ($p < 0.05$) higher wilt incidences compared to the treatments where CICTE had been applied. The wilt incidences were not significantly different ($p < 0.05$) between the tomato varieties. However, Anna F1 had a significantly ($p < 0.05$) higher wilt incidence than Chonto F1 (Table 2). There was significant ($p < 0.05$) difference in bacterial browning and streaming effect scores between the treatments. Comparatively, Chonto F1 had lower bacterial browning and streaming than Anna F1 variety (Table 3).

Table 2: Wilt Incidences in Tomato Varieties Treated with Chitosan Intercalated with Crude Tea Extracts

Treatments	Anna F1	Chonto F1
Distilled water (DW)	66.7e	64.8e
Purple Tea	54.5d	52.3d
Black Tea	46.7c	42.0b
Chitosan	37.8b	38.5b
Green Tea	37.5b	35.4b
Purple CICTE	35.8b	34.2c
Black CICTE	34.1b	32.5b
Green CICTE	20.5a	22.9a

[a]Means followed by similar letters are not significantly different at $\alpha = 0.05$.

Table 3: Bacterial Wilt Severity in Tomato Varieties Treated using Chitosan Intercalated with Crude Tea Extracts (CICTE)

Treatment	Anna F1	Chonto F1	Anna F1	Chonto F1
	Bacterial browning effect		Bacterial streaming effect	
Distilled water (DW)	2.3 a	2.0 a	1.8 a	1.6 a
Purple Tea	1.8 b	1.7 ab	1.4 b	1.3 b
BlackTea	1.4 b	1.3 c	0.8 c	0.7 c
Chitosan	1.4 b	1.2 c	0.7 c	0.7 c
Green Tea	1.2 c	1.2 c	0.6 c	0.6 c
Purple CICTE	1.0 cd	1.0 c	0.4 cd	0.3 d
Black CICTE	0.6 d	0.5 d	0.3 d	0.3 d
Green CICTE	0.3 e	0.3 e	0.1 e	0.1 e

[a]Means followed by similar letters are not significantly different at $\alpha = 0.05$. Score 0- no browning, 1- light browning at the basal stem 2 cm, 2- light brown colour spread in the vascular system and 3- dark brown colour widespread browning. Ooze rate score 0- no ooze, 1- thin strands of bacteria oozing, 2- continuous thin flow and 3- heavy ooze turning the water turbid (Elphinstone *et al*., 1998).

Chitosan in the Control of Bacterial Wilt

Chitosan reduced wilting in tomato plants significantly ($P < 0.05$). Adsorption of tea extracts on chitosan matrix known as co-inoculation resulted in highest significant ($p < 0.05$) effect against wilt in this study (Table 3). This inhibition was attributed to the synergy between the two compounds which are *R. solanacearum* inhibitors (Kusano *et al*., 2008). Use of chitosan in the composite enhanced the efficacy of tea extracts against the pathogen synergistically. Chitosan stimulates microbial degradation of pathogens in a way resembling the application of a hyper-parasite. Costa *et al*. (2013) reported that, chitosan is easily degraded producing pathogen repellents such

as ammonia which destroy pH sensitive micro-organisms. Alkaline conditions are not suitable for the bacteria.

The wilt reduction could also be attributed to induction and accumulation phytoalexins in tomato plants by chitosan because it contains oligosaccharides which induce proteinase inhibitors that cause an increase in host plant resistance. Chitosan also acts as a resistance elicitor through induction of hydrolytic enzymes such as chitosanase, chitokanase and β-glucanase (Mandal *et al*., 2013). According to Taraskiewicz *et al.* (2013). Finally, chitosan induces increased lignification which makes pathogen penetration in plants difficult by fortifying the cell wall (Ambrorabe *et al*., 2008).

Tea Extracts in the Control of Bacterial Wilt

Tea extracts significantly ($p < 0.05$) reduced bacterial wilt in tomato. Green CICTE had the highest inhibition against *R. solanacearum* and wilt incidence. While, green tea extracts had higher bacterial inhibition and wilt reduction than black and purple tea extracts (Tables 1, 2 and 3). This can be attributed to high catechin content in green tea that has been shown to play an important role in the inhibition of bacterial growth and pathogenesis (Ko *et al*., 2015; Kerio *et al*., 2012). Koech *et al*. (2013; 2014) described green tea extracts as a strong oxidizing agent due to the high contents of catechins and polyphenols. Karori *et al*. (2007) observed that the oxidation process in black tea misnomally known as fermentation reduced the amount of catechins with formation of theaflavins and thearubigins a process that is not reversible. The resultant extracts from oxidized polyphenols have less microbial inhibitory effect than un-oxidized tea. In black tea manufacture, catechins are oxidized and dimerized during auto-oxidation to the yellow-orange pigments, theaflavins (TFs), or polymerized to the red pigments called thearubigins (TRs) (Wan *et al*., 2009; Owuor and Obanda, 2001). Finally, purple tea is rich in anthocyanins than the other types of teas. These anthocyanins have anti-microbial activity though not as strong as the catechins from green tea (Kerio *et al*., 2012). Anthocyanins inhibit bacterial growth and activity due to their ability to bind to adhesive sites on the bacterial cell walls (Ko *et al*., 2015).

Effect of CICTE on Tomato Yield

There was a significantly ($p < 0.05$) higher yield of harvested tomato fruits from plants treated with CICTE than in controls. However, Chonto F1 had a significantly ($p < 0.05$) higher yield than Anna (Table 4 and Plates 2 and 3). This can be attributed to the fact that, chitosan contains high carbon content which can stimulate growth of beneficial microbes resulting in higher microbial activity in the rhizosphere thus increased nutrients for plant uptake (Gaur *et al*., 2000). Cao et al. (2013) found out that, chitosan contains oligosaccharides that act on plants as phytohormones which increase plant growth and development. It also promotes availability and uptake of water and essential nutrients by adjusting osmotic pressure (Li *et al*., 2010).

Table 4: Yield of Tomato fruits obtained from plants treated with CICTE

Treatment	Anna F1 Yield (kg)	Chonto F1 Yield (kg)
Distilled Water	7.21a	8.24a
Purple Tea	8.47b	9.83b
Black Tea	8.64b	9.51b
Green Tea	8.28b	9.19b
Chitosan	10.13c	12.30c
CICTE-Green	10.28c	12.93c
CICTE-Purple	10.15c	12.64c
CICTE-Black	10.84c	12.07c

[a]Means followed by similar letters are not significantly different at α = 0.05.

Plate 2: Images of Chitosan and CICTE from a Compound Microscope

Plate 3: Images of Tomato Plants Treated with CICTE

Conclusion

The incorporation of crude tea extracts within the chitosan polymer matrix through adsorption modified the properties of chitosan. The complex is characterized by higher presence of functional groups such as hydroxyl and amino groups from the tea polyphenols and chitosan. These functional groups enhanced adsorption and efficacy of the resulting complex. These new elaborated assemblies were applied as a bacterial biopesticide. This product is more efficacious in bacterial wilt management, safe to the environment, reduces the dangers posed by the synthetic pesticides in the food chain.

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Conflict of Interest
The authors declare no conflicting interests.

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