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Enhancement of bacterial wilt resistance and rhizosphere health in tomato using bionanocomposites

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Abstract

Biological control agents are useful components in the enhancement of plant disease resistance and improvement of soil properties. Effect of biological control agents (BCAs) as a disease control method in plants is hampered by their vulnerability to environmental and edaphic conditions. This study entailed the use of chitosan-silica nanocomposites for delivery of BCAs. Effect of BCAs-nanocomposite complexes (bionanocomposites) on resistance of tomato plants to bacterial wilt, mycorrhizal root colonization and rhizosphere soil properties were investigated. Replacement of mesoporous silica nanoparticles (MSN) in the nanocomposite with nano synthesized clay was also assessed on disease resistance. Tomato seeds and seedlings were pre-treated using bionanocomposites and then inoculated by *Ralstonia solanacearum* isolated from infected tomato plants in a greenhouse. Bionanocomposites treatment of tomato plants caused a significant increase ($P \leq 0.05$) in the level of pathogenesis-related biochemicals such as chitinase and glucanase. Furthermore, beneficial microbial colonization was significantly ($P \leq 0.05$) induced in roots treated with the bionanocomposites. Wilting incidence and symptoms were reduced by over 50% when bionanocomposites were used. There was no significant effect ($P \leq 0.05$) on induced host plant resistance when mesoporous silica nanoparticles (MSN) were substituted with nanoclay particles. Therefore, due to ease of availability with no significant ($P \leq 0.05$) difference in efficacy between the nanoparticles, replacement of MSN with nanoclay in synthesis of the bionanocomposites is recommended. We argue that substitution of nanoclay with MSN makes the process of synthesizing the bionanocomposites sustainable.

Keywords: AMF colonization, host plant resistance, mycorrhiza-helper micro-organisms, nanoclay, resistance elicitors.

Introduction

Application of chemical pesticides against *Ralstonia solanacearum* is an infective control strategy mainly due to *R. solanacearum* variability (Agrios, 2005). Excessive use of pesticides causes loss of efficacy due to the pathogen variability. Excessive applications of pesticides also lead to residue toxicity and environmental pollution (Noor, 1999; Christos *et al*., 2011). Furthermore, application of pesticides after appearance of wilt symptoms is ineffective since the pathogen is highly fastidious which make it hard to control the pathogen after infection. In addition, most of the chemicals used for soil fumigation have been banned by the World Health Organization through

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various commitments such as the Kyoto protocol of 2005 (Christos *et al*., 2011; Karungi *et al*., 2011). The export markets have also introduced stringent conditions on minimum and maximum residue level of chemicals (KHDP, 2007; Karungi *et al*., 2011; KHCP, 2012). Therefore, an attempt to develop an effective and environmentally friendly method against pathogen is required. This could be a pre-infection package which can be applied before infection of plants by the pathogen (Jach *et al*., 1995; Maksimov *et al*., 2011).

Although using of biocontrol agents (BCAs) is environmentally friendly, increase plant productivity and improve soil structure, application of these agents is limited by i)harsh environmental conditions ii) reduced potency during storage and iii) inability to reach the target sites after application. Combination of biocontrol agents with other materials is required to increase efficacy, vitality and effective delivery within the plant system (Algam *et al*., 2010; Nguyen and Ranamukhaarachchi, 2010; Soad *et al*., 2013). Nanocomposites have the potential for delivery of BCAs due to their ease of fuctionalization, large surface area for adsorption and ability to penetrate in epithelial layers. Chitosan and silica nanoparticles are preferred because of their non-toxic property, capacity of enhancing host plant resistance and ease of assimilation by root hairs. These additives will increase the level of adsorbed materials and naturally deliver them to the host plant. The chitosan-silica nanocomposites also induce other effects in plants such as increased yield and elicitation of resistance (Helander *et al*., 2001). Previous work in this study showed compatibility and synergic effect when plants inoculated with microbes and applied together with chitosan-silica nanocomposites (Dennis *et al*., 2016). In this study we attempted to investigate the enhancement of bacterial wilt resistance in tomato plants by using of bionanocomposites.

Materials and Methods

Materials including mesoporous silica nanoparticles (MSN), acetic acid, NaOH pellets, tri-poly phosphate (TPP) obtained from Sigma Aldrich. Biocontrol agents; *Glomus mosseae* was obtained from Juanco Co. Ltd, effective micro-organisms were obtained EM Technologies Co. Ltd, *Bacillus subtilis* and *Trichoderma viridae* were sourced from Real IPM. *Ralstonia solanacearum*-phage and *Ralstonia solanacearum* were isolated from an infested greenhouse soil and tomato respectively. Chitin was obtained from Laborex and nutrient agar, potato dextrose agar, master mix PCR kit and primers were obtained from Bioneer Ltd.

Preparation of bionanocomposites

Chitosan immobilized silica nanocomposite were synthesized by use of physisorption process (Dennis *et al*., 2016). The nanocomposites were used for adsorbing biocontrol agents including: *Bacillus subtilis*, *Glomus mosseae*, *Trichoderma viride*, *R. solanacearum* phage and effective micro-organisms (EM). The microbes were cultured on the appropriate growth media namely nutrient and potato dextrose agar for bacteria and fungi respectively. A cellular suspension was prepared and standardized to 2.000 optical density (O.D) using Shimadzu Ultra violet visible (Uv-vis) spectrophotometer. The suspension was then adsorbed on 10% chitosan immobilized silica nanocomposite (CISNC) and chitosan immobilized nanoclay (CINC). The nanocomposites and bionanocomposites were characterized on Rigaku X-ray powder diffractometer (Christian *et al*., 2008). A suspension of 10% was prepared (1:10 for bionanocomposite to distilled water) for inoculation.

Experimental sites and design

The microbial and bionanocomposite complexes were applied on tomato seeds prior to seeding by priming. A similar

treatment was done on the growing medium (cocopeat) and the primed seeds were sown on a matching treatment in a tray. The seedlings were also treated with a similar complex prior to transplanting to the pots. Transplanting was done in greenhouses at two sites including; Gatundu-Theta Tea Factory (0.9621 ^{o}S, 36.7683 ^{o}E and altitude 2050 m ASL) and Juja-JKUAT (1.0891 ^{o}S, 37.0105 ^{o}E and altitude 1400 ASL) on plastic pots with well-prepared soil in the ratio of 3:0.5:1 for soil, sand and manure respectively. Another treatment was sown on cocopeat growing media with nutrients applied by fertigation system. The experiments involved 18 treatments and 3 replications based on a completely randomized design. Respective data was collected during the growth of plants.

Determination of Effective Concentration (EC) for the CISNC

The effective concentration (EC) of the CISNC was determined by a method so-called "up and down" or the "staircase method" using two hybrid tomato varieties (Choi, 1990). Twelve concentrations of CISNC and bionanocomposites were applied *in vitro* and *in vivo* for the determination of EC of the *R. solanacearum* and wilt reduction respectively. The *In vitro* tests were recorded five days after treatment while *in vivo* tests conducted in a period of six months. *R. solanacearum* inhibition experiment was performed *in vitro* while germination test, growth rate and wilting incidences were done under greenhouse conditions.

Assessment of mycorrhizal colonization

Root portions were sampled from all treatments. A sample of 50 g from each replicate was taken. The roots were carefully rinsed to avoid loss of fine roots and preserved in 70% ethanol. The preserved roots were then assessed for mycorrhizal colonization according to the procedures of Koske and Gemma, 1989. Estimation of percentage root mycorrhizal fungi colonization frequency and intensity was done using the subjective visual technique by Kormanik and McGraw, 1982, commonly referred to as the slide method. The roots were washed with 2.5% KOH (25g KOH in 1000 ml water) and subjected to70 ºC for 1 hr and then rinsed with tap water. To remove phenolic substances, alkaline hydrogen peroxide (60 ml of 28-30% NH_4OH, 90 ml of 30% H_2O_2 and 840 ml distilled water) was added to the samples. The roots were then placed in an oven at 70 ºC for 20 min. Oven dried roots were rinsed with tap water and acidified with 1% hydrochloric acid (HCl) for 30 min. The HCl was decanted and stained with 0.05% Trypan blue dissolved in acid glycerol (500 ml glycerol, 450 ml water, 50 ml of 1% HCl and 0.5g Trypan blue). The stained roots were placed in an oven at 70 ºC for 1 hr. The stain was decanted and a solution comprising of acid glycerol (500 ml glycerol, 450 ml distilled water, 50 ml of 1% HCl) was added to the samples. Proper stained root segments were cut into 1 cm-long pieces and 30 pieces randomly picked, mounted on slides and observed under the Nixon compound microscope to assess the frequency and intensity mycorrhizal colonization. Presence of arbuscules, vesicles, internal and external hyphae was examined. The frequency of mycorrhizal colonization was recorded as the number of root fragments infected with mycorrhizal fungi and expressed as a percentage of total number of root fragments observed. The intensity of mycorrhizal fungi colonization was also recorded as percentage cover of mycorrhizal fungi infective propagules in each 1cm root fragment.

Biochemical analysis (glucanase and chitinase)

The efficacy of resistance elicitation was carried out by determining the levels of chitinase and glucanase. A confirmatory test of the presence of the chitinase and

glucanase genes was done after amplification of DNA by use of polymerase chain reaction (PCR).

Foliage from treated tomato plants was ground to obtain a suspension for DNA isolation. DNA was extracted following the CTAB extraction method and then stored at -20 ^{o}C (Kumlachew, 2014). The polymerase chain reaction (PCR) was carried out using touchdown procedures as described by Khalil *et al*. (2003). The primers were a 21 mer forward primer –CGA ACC TAA TGG TGG TAG TGC-, and reverse –TCG CAA CTA AAT CAG GGT TG- for chitinase and22 mer forward primer –CGC CAT TGC TCG TGT TGA CAT G- and reverse -AAT TTC TCG CTC GGC GGT GGT G for glucanase. The samples were cooled at 4 ^{o}C and subjected to electrophoresis on a 1.5% agarose gel in 1X TAE buffer (40 mMTris acetate and 1.0 mM EDTA) and photographs taken under ultra-violet (Uv) light. The obtained ladders were interpreted using base pair amplicons of the enzymes (Chilvers, 2012 method). Amplified DNA (100 µL) was mixed with a binding buffer in a ratio of 1:1 mixed thoroughly by vortexing. Sodium acetate (10 µL of 3 M) was added and vortexed until a yellow colour appeared. A solution (800 µL) was transferred to the GeneJET purification column, centrifuged for 30-60 sec and the flow-through discarded. Wash buffer (700 µL) was added to the GeneJET purification column, centrifuged for 30-60 sec, flow-through discarded and the purification column placed back into the collection tube. The empty GeneJET purification column was centrifuged for 1 min. The GeneJET purification column was transferred to a clean 1.5 mL microcentrifuge tube and centrifuged for another 1 min. The GeneJET purification column was discarded and the purified DNA stored at -20 °C. Concentration of chitinase and glucanase was determined using Bioneer, nanodrop machine at 680/620 nm on purified samples (Korbie and Mattick, 2008).

Bacterial wilt incidence

The number of wilting plants per treatment was recorded as incidences of bacterial wilt symptoms. Wilting incidence was calculated using Equation (1).

$$\frac{(5A + 4B + 3\mathrm{C} + 2\mathrm{D} + \mathrm{E})}{1.75\ N} \quad (1)$$

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 indicates the better control measurement (Tim *et al*., 2008).

Bacterial wilt severity measurement

Tomato plant stems showing signs of wilting were cut and scored for browning and bacterial streaming. Scoring method was conducted based on 0-1, 2 and 3scaleswhere each scale indicates no browning, light brown color at the base, light brown color above the basal part and dark brown color spread through the vascular stem respectively. In addition, the streaming test was conducted based on suspending cut stems in distilled water in a beaker and the ooze rate score of 0, 1, 2 and 3 used to determine severity, where each score shows no ooze, thin strands of bacteria oozing, continuous thin flow and heavy ooze turning the water turbid respectively (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.

Determination of retention of biocontrolagents in soil/planting media

Biocontrol Agents stability in the soil was determined by using of available carbon percent (%) in the soil based on the Walkley-Black chromic acid wet oxidation

method. The amount of carbon was estimated as percentage using Equation (2).

$$= \frac{(B - T * 0.3 * V * 0.75)}{WB} \quad (2)$$

where C= Carbon percentage, B= amount of titrant consumed by blank, T= amount of titrant consumed by sample, W= weight of the sample, V= volume of $K_2Cr_2O_7$, 0.3= constant, 0.75= assumption that the sample had 75% carbon (Mylavarapu, 2009).

Determination of pH in the tomato rhizosphere

Soil pH was determined using a digital pH meter on all the treatments. The soil was dried at room temperature (25 ^{o}C) for 7 days then separated on the 6.3 mm sieve to obtain the proper soil sample for pH measurement. A sample of 30±0.1 g was weighed and placed in a glass beaker. Equivalent volume of distilled water was added to the soil sample and stirred thoroughly to obtain soil slurry and then cover with watch glass. The sample was allowed to stand for 1 hr with continued stirring every 10 to 15 min to allow the pH of the soil slurry to stabilize. The readings were taken after stabilization using electrodes of pH meter standardized with 7.0 buffer solution.

Data analysis

The data obtained from effective biochemical concentration wilt incidences, resistance associated enzymes and soil properties were subjected to analysis of variance (ANOVA) and means separated by Fischer's Least Significant Difference ($LSD_{0.05}$) to determine the significance level using Genstat statistical package version 12.

Results and Discussions

Determination of effective concentration of CISNC

Significant ($P \leq 0.05$) effects of different concentrations of the nanocomposite on inhibition of *R. solanacearum*, germination of tomato seeds, induction of chitinase, wilt incidence and tomato fruits shelf life were observed. However, the effect of concentration on most of the assessed parameters was not significant (Table 1).

Table 1. Effective concentration of CISNC in tomato development and wilt resistance

Treat/ conc. (%)	Inhibition (%)	Germination (%)	Wilt incidence (%)	Chitinase (%)	Shelf life (days)
0	15.33 a	72.11 a	30.00 a	1.543 a	12.01 a
0.5	18.67 a	77.89 b	31.11 a	2.029 b	20.11 b
5	45.00 b	82.56 c	33.78 b	2.081 b	21.67 b
10	75.33 c	82.89 c	34.00 b	2.096 b	21.67 b
20	77.33 cd				
30	81.00 de	85.11 cde	36.11 c	2.157 bc	21.78 b
40	84.33 ef				
50	87.67 fg	85.22 de	36.67 c	2.279 bc	22.22 b
60	88.67 fgh				
70	90.00 gh	86.11 ef	36.78 c	2.800 c	23.00 c
80	91.67 gh				
90	92.33 h	88.22 f	55.00 d	2.966 cd	23.56 c
100	92.33 h				

Means followed by the same letter are not significantly different. $LSD_{0.05}$

Validating effectiveness of CISNC on *R. solanacearum* inhibition, tomato seed germination, wilting of tomato plants, elicitation of resistance and tomato fruit shelf life was an important step towards synthesis of a bionanocomposite pesticide. The effective concentration intervals which caused significant ($P \leq 0.05$) bacterial inhibition, reduced tomato wilt, caused elicitation of chitinase and enhanced

postharvest shelf life of harvested tomato fruits were determined. Concentration of 0.5% reduced the *R. solanacearum* colony by 18.7%, germination was 77.9%, chitinase 2.029 nm, wilt incidence at 31.11 and shelf-life prolonged to 20.1 days. In comparison, a concentration of 10% resulted in 75.3% reduction in inhibition, seed germination of 82.9%, chitinase elicited to 2.081 nm, wilt incidence reduced by 33.8% and shelf-life enhanced to 21.7 days. The effect of 10% concentration of CISNC on *R. solanacearum* inhibition, seed germination, chitinase elicitation, wilt incidence and shelf life of treated fruits was not significant ($P \leq 0.05$) when compared to 20-50% concentration. Therefore, considering the concentration of 10% as the EC was done based on the five tested parameters, applying of high concentrations would be economically untenable and may imbalance other ecological aspects when additional amounts of reagents are added to the rhizosphere (Sharp, 2013).

Colonization of roots by biocontrol agents

Adsorption of *Glomus mosseae*, a type of arbuscular mycorrhizal fungi (AMF) onto chitosan immobilized silica nanocomposites, induced the highest and most significant ($P \leq 0.05$) root microbial colonization frequency (76.7%) and infection (81.7%). Interestingly, all microbes (BCAs), bionanocomposites, non-microbe adsorbed chitosan immobilized silica nanocomposites (CISNC) and chitosan immobilized nanoclay composites (CINC) treatments showed over 50% mycorrhizal infection rates compared to the controls (acetic acid (AA) and distilled water (DW)). There was significant ($P \leq 0.05$) difference in colonization when different soils and media were used. The montmorillonite soil showed the highest microbial colonization and infection, followed by the acidic nitrisol. Minimum microbial colonization and infection was observed in the inert media. The results are corroborated in Figure 1, Tables 2, 3 and Plate 1.

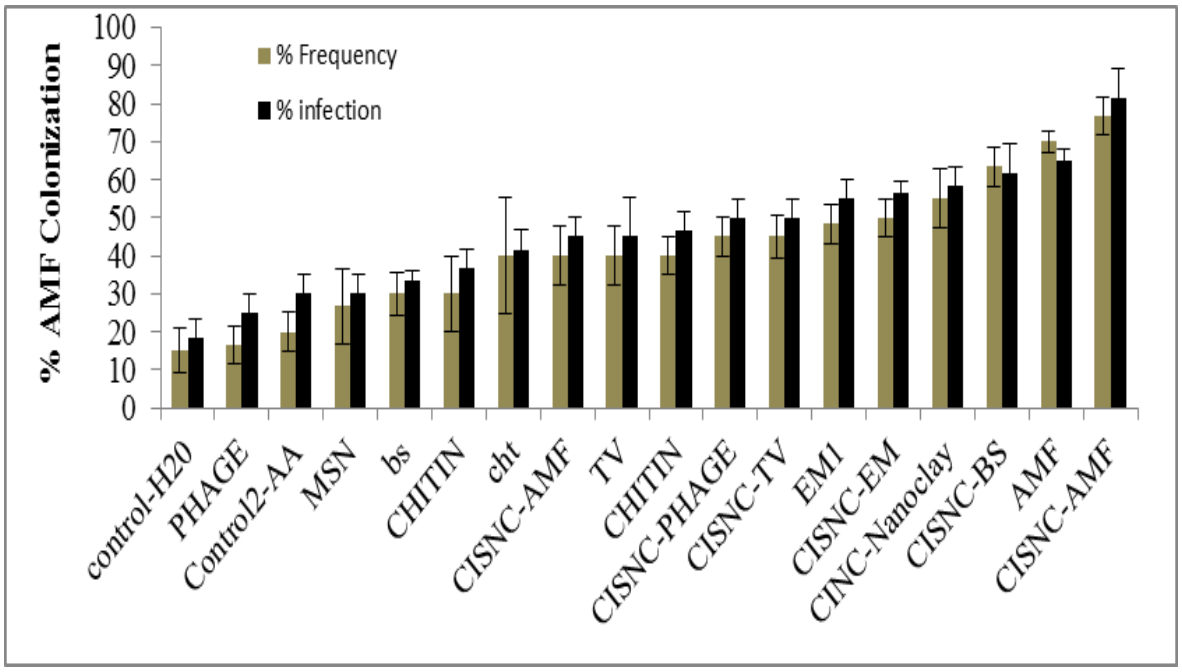

Fig. 1. Colonization and Frequency of Tomato Roots by Beneficial Microbes
Means significant at L.S.D $_{0.05}$ (F-test) [1]-LSD bar

Table 2. Microbial root colonization frequency in tomato roots (% AMF colonization)

Treat	Nitrisol (Gatundu)	Montmorillonite (Juja)	Cocopeat
Distilled water	15 a	19 a	0 a
Phage	17 ab	24 ab	5 a
Acetic acid	20 bc	27 b	0 a
Mesoporous silica nanoparticles	27 c	30 bc	0 a
B. subitilis (BS)	30 c	34 c	4 a
Chitin	30 c	35 c	0 a
Chitosan	40 d	40 cd	0 a
Chitosan immobilized silica nanocomposites (CISNC)	40 d	44 d	0 a
T. viridae	40 d	44 d	4 a
Chitosan nanoparticles	40 d	45 d	0 a
CISNC-Phage	42 de	48 e	0 a
CISNC-TV	45 de	50 e	6 ab
Effective micro-organisms (EM)	45 de	52 e	8 ab
CISNC-EM	48 de	54 e	10 b
Chitosan immobilized nanoclay	50 e	54 e	10 b
CISNC-BS	63 f	66 f	10 b
AMF	70 g	72 g	10 b
CISNC-AMF	77 g	74 g	20 c

Means followed by the same letter are not significantly different. $LSD_{0.05}$

Table 3. Microbial root colonization frequency in tomato roots (% AMF infection)

Treat	Nitrisol (Gatundu)	Montmorillonite (Juja)	Cocopeat
Phage	18 a	23 a	0 a
Distilled water	25 ab	28 ab	0 a
Acetic acid	30 bc	34 bc	0 a
Mesoporous silica nanoparticles	33 bc	37 bc	0 a
Chitin	37 cd	40 c	0 a
B. subtilis	45 de	44 c	5 a
Chitosan	45 de	44 c	0 a
T. viridae	47 def	45 c	5 a
Effective micro-organisms	50 efg	49 cd	8 b
CISNC-Phage	50 efg	49 cd	4 a
Chitosan nanoparticles	50 efg	49 e	0 a
CISNC	55 fg	54 e	0 a
CINC	55 fg	59 ef	4 a
CISNC-EM	57 gh	62 f	15 c
CISNC-TV	58 gh	62 f	15 c
G. mosseae (AMF)	62 h	68 g	40 d
CISNC-BS	65 h	69 gh	10 ab
CISNC-AMF	82 i	85 i	50 e

Means followed by the same letter are not significantly different. $LSD_{0.05}$

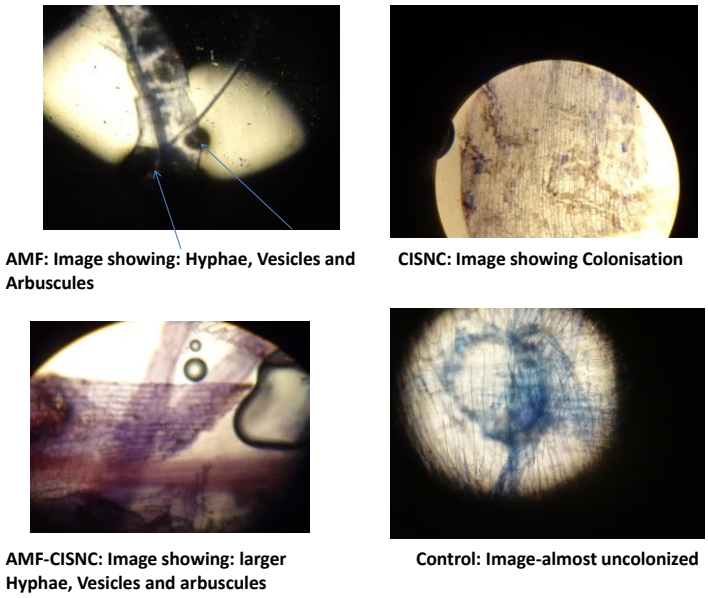

Plate 1. Microbial root colonization

The beneficial plant-microbe interaction results in antagonism of pathogens by enhancement of competition for space and nutrients in the root system. Tomato plant is a mycorrhizal "friendly plant" hence; *Glomus mosseae* readily colonized the root hairs. Mycorrhiza fungi enhances root establishment and by improving the root damages increase the nutrient uptake. This partnership helps to overcome soil borne pathogens and pests (Hodge, 2000; Glick, 2012), which eventually reduce tomato wilt incidences in treatments colonized by the BCAs. When diseased and mycorrhizal colonized roots were critically analyzed, there was evidence that growth of pathogens was only restricted to the epidermis and cortical tissues. Conversely, in diseased non-mycorrhizal roots, the pathogens infected through the stele. Mycorrhizal colonized roots also structurally disorganized and inhibited pathogen development (Barea *et al*., 2002; Park *et al*., 2007).

Resistance enhancement in tomato by bionananocomposites

Effect of treating tomato plants with BCA-nanocomposite complexes was observed byinducing pathogenesis related biochemicals in tomato plant system by increasing of chitinase and glucanase content. Expression of the biochemicals was confirmed by amplification of the DNA. BCAs, chitosan-silica nanocomposites and their complexes significantly ($P \leq 0.05$) increased the concentration of chitinase and glucanasewhen compared to the control plants. However, there was no significant difference ($P \leq 0.05$) in chitinase and glucanase elicitation when bionanocomposites were applied on Anna and Chonto F1 tomato varieties.

To ensure that microbial colonization and effect of bionanocomposites have occurred, plant materials for analysis were collected eight weeks post-transplanting. The enhanced resistance was monitored by measurement of elevated chitinase and glucanase concentrations (Tables 4, 5 and Plate 2). Hydrolytic enzymes related to plant resistance are regulated with two genes by which plant overcome over the pest attacks or when exposed to resistance eliciting agents (Jach *et al*., 1995). Our study showed a significant correlation between the induced chitinase and bacterial wilt incidences in two tomato varieties (Table 6).

Table 4. Concentration of chitinase in tomato varieties treated with bionanocomposites

Treatment	Anna F1	ChontoF1
Distilled water	1.06 a	1.10 a
Acetic acid	1.16 a	1.18 a
Mesoporous silica nanoparticles	1.23 b	1.26 b
T. viridae (TV)	1.26 b	1.29 b
Nanoclay	1.31 c	1.42 c
R. solanacearum	1.33 c	1.40 c
Phage	1.41 d	1.48 d
G. mosseae (AMF)	1.42 d	1.54 e
B. subtilis (BS)	1.42 d	1.54 e
Effective micro-organisms (EM)	1.56 e	1.55 e
Chitosan	1.60 e	1.59 ef
Chitosan nanoparticles	1.69 f	1.60 f
Chitosan immobilized nanoclay	1.70 f	1.61 f
Chitosan immobilized silica nanocomposites (CISNC)	1.76 g	1.61 f
CISNC-Phage	1.93 h	1.98 g
CISNC-TV	2.00 i	2.20 h
CISNC-AMF	2.03 i	2.37 i
CISNC-BS	2.24 j	2.45 j
CISNC-EM	2.61 k	2.74 k

Means followed by the same letter are not significantly different LSD $_{0.05}$.

Table 5. Concentration of glucanase in tomato varieties treated with bionanocomposites

Treatment	Anna F1	Chonto F1
Distilled Water	0.12 a	0.13 a
Acetic acid	0.13 a	0.15 a
Mesoporous Silica Nanoparticles (MSN)	0.14 a	0.16 a
T. viridae(TV)	0.15 ab	0.18 b
Nanoclay	0.17 b	0.20 b
R. solanacearum(RS)	0.23 c	0.22 b
Phage	0.24 c	0.25 c
G. mosseae (AMF)	0.25 d	0.25 c
B. subtilis(BS)	0.26 d	0.25 c
Effective micro-organisms (EM)	0.26 d	0.25 c
Chitosan	0.26 d	0.26 c
Chitosan nanoparticles	0.26 d	0.26 c
Chitosan Immobilized Nanocomposites (CINC)	0.26 d	0.26 c
Chitosan Immobilized Silica Nanocomposites (CISNC)	0.27 e	0.27 d
CISNC-Phage	0.27 e	0.28 d
CISNC-TV	0.27 e	0.28 d
CISNC-AMF	0.27 e	0.28 d
CISNC-BS	0.27 e	0.28 d
CISNC-EM	0.27 e	0.28 d

Means followed by the same letter are not significantly different LSD $_{0.05}$.

Table 6. Pearson correlations for chitinase and glucanase in tomato treated with bionanocomposites

	N	Mean	SD
Chitinase	19	1.6212105263158	0.40112128658263
Glucanase	19	0.22962631578947	0.053181177143226

R^2= 0.71107331363471 (*P*= 6.422144212285), 2-tailed test of significance.

Plate 2. Gel image showing PCR product of chitinase and glucanase

Garcia-Garrido and Ocampo (2002) demonstrated that, in plants certain genes and biochemicals are associated with plant defense response. Mycorrhizal structures such as hyphae, vesicules and arbuscules induce expression of some pathogenesis related genes hence; plants colonized by mycorrhiza elevate defense related genes. Chitinase and glucanase biochemicals are synergistically induced during attack by pathogens and/or resistance elicitors. Total chitinase activity is higher in mycorrhizal host plants when colonized by the root-fungus complex when compared to non-mycorrhizal plants and their controls. Consistence with these observations, Sambrook *et al*. (1989) showed that constitutive activities of chitinase and glucanase were several times lower in wheat leaves before treatment with elicitors. The enzymes were significantly ($P \leq 0.05$) elevated upon treatment with *Stagono sporanodorum* isolates with high virulence.

However, Mandal *et al*. (2013) indicated that hydrolytic biochemicals are non-specific defense response in plants. In spite of this finding our study revealed a systematic reduction of disease symptoms in tomato plants with the elevated biochemicals. Therefore these biochemicals are suggested as bioprotector agents. The role of hydrolytic biochemicals; chitinase and glucanase in defense response of plants has also been described by Jongedijk *et al*. (1995). This has been attributed to the fact that, most pathogenic bacteria and fungi contain 1, 3 B-glucans, chitin and other substrates as cell wall components. These biochemicals effectively restrict growth of fungi and bacteria due to their lysozyme activity. Infection of healthy plants by pathogens is also associated with rapid activation of the corresponding gene containing chitinase and/or glucanase gene(s) which is expressed around the necrotic region in the leaf. Though chitinase and glucanase act synergistically in host plant defense responses and employ different mechanisms against pathogens (Soad *et al*., 2013).

For instance, while the chitinase enzyme catalyse the cleavage of site C_1-C_4 of two consequtive N-acetyl-D-glucosamine monomers of chitin, glucanase enzyme catalyse the cleavage of B,1-3 glucans. These compounds are ubiquitous in most pathogens (Neerja *et al*., 2010). Jongedijk *et al*. (1995) indicated that when chitinase is released, biosynthesis of chitinase, glucanase, catalyses and other defense related enzymes will be significantly induced. Also, co-transformation of plants with chitinase and glucanase related genes showed higher resistance to most pathogens when compared to plants transformed with these genes individually (Pratibha *et al*., 2012). This was consistent with the current study, where there was a strong correlation between the

concentration of both chitinase and glucanase biochemicals in tomato plants with less wilting incidences.

Bacterial wilt incidence assessment

Tomato seedlings treated with BCA-CISNC complex, particularly the effective micro-organisms and phages, showed minimum wilt incidences. Minimum wilt incidences occurred in BCAs-CISNC, CISNC and CINC composite treatments compared to plants treated with BCAs or nanocomposites (Table 7). This finding indicates the effect of elevated microbial root colonization in plant resistance enhancement (Tables 2 and 3). Control experiments including acetic acid and distilled water had significantly ($P \leq 0.05$) higher wilt incidences compared to all other treatments. Tomato varieties treated with bionanocomposites and the seedling inoculated with the pathogen showed the similar wilt incidences. However, in the control experiments, wilt incidence in Anna F1 was significantly ($P \leq 0.05$) higher than Chonto F1.Wilt incidences in tomato plants treated with BCA-nanocomposite is shown in Table 7.

Bacterial wilt severity assessment

There was significant ($P \leq 0.05$) difference in bacterial browning and streaming effect when different bionanocomposites were used to control bacterial wilt in the two tomato varieties. Comparatively, Chonto F1 had lower bacterial browning and streaming than Anna F1 variety (Table 8).

Our study revealed that combination of several resistance elicitor agents such as silica, nanoclay, chitosan and biocontrol agents known as co-inoculation resulted in maximum significant ($P \leq 0.05$) effects against wilt incidence (Tables 7 and 8). This resistance was caused by competition of colonization sites, carbon components and induction of systemically induced resistance as disease suppression (Algam *et al*., 2010). Use of chitosan in the nanocomposite carrier enhanced the biocontrol agents' efficacy against the pathogen. Thus, combination of chitosan nanocomposite and microbial antagonists, such as the *B. subtilis*, effective micro-organisms, T. *viride*, *G. mosseae* and *R. solanacearum*-phage, increase their efficacy. Chitosan acts as a propercarrier material due to high concentration of polysaccharides. Chitosan and its derivatives were also degrading produced pathogen repellents like ammonia which predisposed the *R. solanacearum* as a biological antagonists capable in controlling the pathogen as observed in this study.

Table 7. Wilt incidences in tomato varieties treated with bionanocomposites

Treatments	Anna F1	Chonto F1
CISNC-EM	17. 6 a	19.3 a
CISNC-BS	20.5 ab	22.9 ab
CISNC-Phage	26.4 bc	24.5 b
CISNC-AMF	26.3 bc	24.7 b
Chitosan immobilized nanoclay	28.6 c	26.1 c
Chitosan immobilized silica nanocomposites (CISNC)	28.1 c	26.8 c
CISNC-TV	28.0 c	29.2 cd
Effective micro-organisms (EM)	30.7 d	28.8 c
Mesoporous silica nanoparticles	34.1 e	32.5 d
Chitosan nanoparticles	35.8 e	34.2 de
G. mossea (AMF)	37.5 ef	35.4 e
Chitosan	37.8 ef	38.5 ef
Phage	39.7 f	38.4 ef
T. viridae	41.5 fg	39.3 f
B. subtilis	40.7 fg	39.8 f
Chitin	46.0 h	43.7 h
Acetic acid	54.5 i	52.0 i
Distilled water	55.8 ij	54.4 i

Means linked with a similar letter are not significantly different $LSD_{0.05}$.

Table 8. Bacterial wilt severity in tomato varieties treated with bionanocomposites

Treatment	Anna F1	Chonto F1	Anna F1	Chonto F1
	Bacterial browning effect		Bacterial streaming effect	
CISNC-EM	0.4 a	0.4 a	0.1 a	0.1 a
CISNC-BS	0.6 ab	. 5 a	0.4 b	0.3 b
CISNC-Phage	0.9 b	0.8 b	0.4 b	0.3 b
CISNC-AMF	0.5 a	0.4 a	0.1 a	0.1 a
Chitosan immobilized nanoclay	1.2 c	0.9 b	0.8 c	0.7 c
Chitosan immobilized silica nanocomposites (CISNC)	0.8 b	0.7 ab	0.6 bc	0.6 c
CISNC-TV	0.7 ab	0.8 b	0.6 bc	0.6 c
Effective micro-organisms (EM)	1.4 c	1.1 c	0.7 c	0.6 c
Mesoporous silica nanoparticles	1.8 d	1.5 cd	1.0 d	1.0 d
Chitosan nanoparticles	1.3 c	1.2 c	0.8 c	0.9 d
G. mossea (AMF)	0.8 b	0.7 ab	0.8 c	0.6 c
Chitosan	0.8 b	0.8 ab	0.8 c	0.7 c
Phage	1.4 c	1.2 c	1.0 d	0.9 d
T. viridae	2.2 e	1.6 cd	1.3 de	1.2 e
B. subtilis	1.6 cd	1.4 c	1.0 d	1.0 d
Chitin	1.0 b	0.9 b	1.0 d	1.0 d
Acetic acid	2.2 e	2.0 e	1.8 f	1.6 f
Distilled water	2.4 e	2.2 e	2.1 g	2.0 g

Means followed by the same letter are not significantly different. $LSD_{0.05}$,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).

However, according to Pal and Mc Spadden (2006), biocontrol agents are more likely to be rather preventive than therapeutic in disease control therefore their potential should be used in seed priming stage and/or in pre-treatment before transplanting. The biocontrol agents were found to be more effective in seed primed seedlings while chitosan and its derivatives showed better function as a soil drench (Prevost *et al*., 2006). Interestingly, substitution of mesoporous silica with nanoclay did not showed significant ($P \leq 0.05$) difference in tested parameters like wilt incidences. This was attributed to the fact that clay contains substantial quantities of silica in its composition (over 90% silica) (Saldajeno and Hyakumani, 2011; Pinto *et al*., 2012).

Total organic carbon accumulation in the soil

The duration of biocontrol agents, nanocoposites and microbial activity in the soil rhizosphere was monitored as a derivative of total organic carbon. Addition of BCAs, chitosan-silica composites and bio-nanocomposites in the rhizosphere, increased the carbon content significantly ($P \leq 0.05$) when compared to the controls. Application of bacteriophage did not increase the total organic carbon significantly ($P \leq 0.05$). The level of TOC was considerably ($P \leq 0.05$) higher in Juja clay soils than Gatundu's nitrisol, while cocopeat had the minimum carbon build up. The results of carbon content after treatment using the bionanocomposites complexes are shown in Table 9.

Table 9. Total Organic Carbon (TOC) in tomato rhizosphere

Treatment	Nitrisol (Gatundu)	Montmorillonite (Juja)	Cocopeat
Distilled water	2.4 a	3.8 a	30.6 a
Phage	2.7 a	3.9 a	31.4 ab
Acetic acid	2.8 a	4.3 b	30.8 a
Bacillus subtilis (BS)	3.2 b	4.8 bc	33.7 b
Trichodermaviridae (TV)	3.3 b	5.0 bc	34.3 b
Effective micro-organisms (EM)	3.5 b	5.3 c	35.8 c
Glomusmossea (AMF)	3.6 c	5.3 c	36.2 d
Mesoporous silica nanoparticles	3.6 c	4.4 b	30.7 a
CISNC-Phage	4.1 d	5.7 d	37.9 de
Chitosan nanoparticles	4.3 d	5.8 de	38.3 ef
CISNC-BS	4.3 d	5.9 e	38.6 ef
Chitosan	4.3 d	5.8 de	39.1 f
Chitosan immobilized nanocomposites (CISNC)	4.4 d	5.8 de	37.9 de
Chitosan immobilized nanoclay	4.4 d	5.9 e	38.3 ef
CISNC-AMF	4.5 e	5.7 d	38.6 ef
CISNC-EM	4.5 e	5.8 de	39.6 f
CISNC-TV	4.6 e	5.8 de	39.7 f
Chitin	5.6 f	6.1 f	40.3 g

Means followed by the same letter are not significantly different LSD $_{0.05}$.

Application of BCAs and nanocomposite carriers increases the microbial activity in the rhizosphere (Kubata *et al*., 2005). Use of organic carriers also increases the longevity of microbes in the soil and their efficiency in root hairs colonization. Microbial activity increases soil organic matter expressed as percent carbon, thereby affecting the soil physical and chemical properties. The microbial activity increases soil fertility by providing cation exchange sites and acts as a bypass for plant nutrients which are slowly released upon mineralization.

According to Gray and Smith (2005), there exists a strong correlation between soil organic matter and soil fertility. Addition of BCAs therefore, enhances mineralization due to increased microbial activity which ultimately causes nutrients availability and increased yield. The low carbon content in the control samples was attributed to continued cultivation of soil with addition of synthetic fertilizers which may reduce the microbial diversity and numbers. This will result in soil degradation that eventually increase the soil acidity and reduce the soil fertility (Vahjen *et al*., 1995). Addition of BCAs in the tomato rhizosphere therefore, caused restoration of the soil microbial activity. Adsorption of BCAs on chitin derivatives showed a positive effect of providing the microbes as substrates for consumption of energy and minerals before adapting to the rhizosphere. The polymer gradually increased the rhizosphere soil pH in this study, due to the released ammonia during breakdown of the nitrogen rich chitinous substrate (Rodrigo *et al*., 2006).

Effect of bionanocomposite son soil pH

Application of biocontrol agents and chitosan-silica nanocomposites affected the rhizosphere soil pH six months after application. However, there was no significant ($P \leq 0.05$) difference in soil pH when sole BCAs were applied. Chitosan immobilized silica or immobilized chitosan on nanoclay had significant ($P \leq 0.05$) effect on soil pH around the rhizosphere compared to the controls. Adsorption of BCAs on the nanocomposites showed significant ($P \leq 0.05$) increase on soil pH. There was also significant ($P \leq 0.05$) change in pH levels of entire growing medium i.e. nitrisol, montmorillonite and cocopeat (Table 10).

Table 10. Soil pH in rhizospherein different planting media, 6 months after application of the bionanocomposites

Treatment	Nitrisol (Gatundu)	Montmorillonite (Juja)	Cocopeat
Distilled water	5.2 a	6.7 c	6.5 a
Acetic acid	5.0 a	6.6 b	6.2 a
Effective micro-organisms (EM)	5.1 a	6.5 a	6.6 ab
Mesoporous silica nanoparticles	5.1 a	6.5 a	6.6 ab
Phage	5.1 a	6.5 a	6.6 ab
Bacillus subtilis (BS)	5.2 a	6.6 b	6.7 b
Glomusmosseae (AMF)	5.4 b	6.7 c	6.8 bc
Trichodermaviridae (TV)	5.3 ab	6.8 d	6.7 b
Chitin	5.6 c	6.8 d	6.9 c
Chitosan nanoparticles	5.7 cd	6.8 d	7.0 cd
CISNC-EM	5.8 d	6.8 d	7.1 d
Chitosan immobilized nanoclay	5.7 cd	6.8 d	6.9 c
Chitosan	5.7 cd	6.8 d	7.0 cd
CISNC-AMF	5.8 d	6.8 d	7.1 d
CISNC-Phage	5.7 cd	6.8 d	7.0 cd
Chitosan immobilized silica nanocomposites (CISNC)	5.7 cd	6.8 d	7.1 d
CISNC-BS	5.7 cd	6.8 d	7.2 de
CISNC-TV	5.8 d	6.9 e	7.3 e

Means followed by the same letter are not significantly different $LSD_{0.05}$.

Regulation of soil pH play critical role in optimal microbial colonization. For instance, an acidic soil inhibits the establishment of plant growth promoting fungi, while alkaline soils reduce colonization by the plant growth promoting rhizobacteria. A fairly neutral soil pH enhances development of both fungal and bacterial beneficial microbes. This promotes diversity of soil microbial communities and causes the desired property in soil fertility and crop productivity (Barea *et al*., 2002). Chitosan polysaccharides had a higher effect on the soil pH than chitin, attributed to the ease of polymer solubility. This can be due to the deacetylation of chitin into chitosan reduces the strength of bands and provide polar phase in polymer, which result in an easy cleavage of the chitosan (Prevost *et al*., 2006).

Conclusion

The attained complex after adsorption of biocontrol agents on the chitosan immobilized silica nanocomposite (CISNC) known as bionanocomposite showed considerable pathogen inhibitory effect and enhanced wilt resistance and rhizosphere health in tomato plants. Due to the diverse materials used in synthesizing the bionanocomposite, it functions as both biopesticide and biofertilizer. Our findings suggest that, the substitution of mesoporous silica nanoparticles (MSN) in the nanocomposite with nanoclay in the development of the bionanocomposite is desirable in sustainable production of the product.

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