OPEN ACCESS

African Journal of Microbiology Research

14 March 2018
ISSN 1996-0808
DOI: 10.5897/AJMR
www.academicjournals.org

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Vol. 12(10), pp. 237-242, 14 March, 2018
DOI: 10.5897/AJMR2017.8770
Article Number: 753000D56328
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

The effect of *Thymus vulgaris* on growth and biofilm formation of uropathogenic *Escherichia coli*

Flores-Encarnación M.[1*], Nava-Nolazco R. M.[1], Aguilar-Gutiérrez G. R.[2], Carreño-López R.[3] and García-García S. C.[3]

[1]Laboratorio de Microbiología Molecular Celular, Facultad de Medicina, Benemérita Universidad Autónoma de Puebla, México.
[2]Centro de Investigación Sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Cuernavaca, Morelos, México.
[3]Centro de Investigaciones Microbiológicas, Benemérita Universidad Autónoma de Puebla, Puebla, México.

Recieved 23 November, 2017; Accepted 24 January, 2018

Currently, a problem related to public health is resistance to antibiotics because bacteria have been identified to be resistant. It has been reported that bacteria have developed resistance mechanisms to evade the effect of drugs, especially antibiotics. A resistance mechanism to antibiotics is biofilm formation. The biofilms are microbial communities embedded in an extracellular polymeric matrix and are ubiquitous in the microbial world. In recent years, there has been a special interest in studying new antimicrobial strategies to solve the great problem of resistance to antibiotics; thus, the use of essential oils could be an alternative to fight infections caused by biofilm forming bacteria. It has also been reported that essential oils have antiviral and antibacterial properties. In the present work, the effect of *Thymus vulgaris* on growth and biofilm formation of uropathogenic *Escherichia coli* was studied. This study demonstrated the strong effect of *T. vulgaris* essential oil on the growth and biofilm formation of uropathogenic *E. coli.*

Key words: *Thymus vulgaris*, biofilm, *Escherichia coli*, uropathogenic, growth.

INTRODUCTION

Urinary tract infections represent the most common bacterial infections. Over 150 million cases are estimated worldwide each year (Flores-Mireles et al., 2015). Another major problem has been resistance to antibiotics, since bacteria have been identified to be resistant to antibiotics. Resistance to antibiotics has been attributed to the excessive use and misuse of these drugs (Gould and Bal, 2013; Lushniak, 2014; Read and Woods, 2014; Ventola, 2015). It has been reported that bacteria have developed resistance mechanisms to evade the effect of drugs, especially antibiotics (Lin et al., 2015). So, bacteria that cause urinary tract infections have been found to form biofilm, which are mechanical barrier that protect them from the action of antibiotics (Delcaru et al., 2016). Biofilms are microbial communities embedded in an extracellular polymeric matrix; they are ubiquitous in

*Corresponding author. E-mail: mflores31@hotmail.com.

the microbial world (Costerton et al., 1995; Høiby, 2017). Reduced antibiotic susceptibility of biofilms contributes to the persistence of infections (Delcaru et al., 2016). It has been proposed that the poor antibiotic penetration, nutrients limitation, slow growth as an adaptive stress response, the formation of persister cells are essential components for the development of persistent and chronic infections (Delcaru et al., 2016). The above indicates that bacteria forming biofilm are associated with recurrent infections, which represent a major public health problem, and their economic impact is high because they cannot be treated with conventional therapies (Høiby, 2017). Therefore, in recent years, there has been a special interest in studying new antimicrobial strategies to solve the great problem of resistance to antibiotics and bacterial biofilm formation (Sharma et al., 2016). In this wise, the use of essential oils could be an alternative to fight infections caused by biofilm-forming bacteria (Sambyal et al., 2017).

Plant essential oils have been used as natural medicines to combat pathogens such as bacteria, fungi and viruses (Kon and Rai, 2012). The mechanism of action for some of them has been reported, for example: damaging the bacterial cell wall and membrane producing the cell destruction and leakage of the cell content (Sambyal et al., 2017). In the present study, the effect of *T. vulgaris* on growth and biofilm formation of uropathogenic *E. coli* was studied.

MATERIALS AND METHODS

Source of material

In this study, a commercial essential oil of *T. vulgaris* was used. It was obtained from a flavour and fragrance company at Puebla, México.

Bacterial strain

A strain of uropathogenic *E. coli* CFT073 was used. Bacterial strain was stored in cryovials at -40°C until analysis.

Culture conditions

The trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md) was used for bacterial culture. Test strains that had been cultured at 37°C for 18 to 24 h in trypticase soy broth were seeded crosswise in a Petri dish containing trypticase soy agar, and the plate was incubated at 37°C for 24 h.

Antimicrobial activity

The antimicrobial activity of essential oil was determined using the technique of disk diffusion in agar with some modifications and the antimicrobial susceptibility test discs. Briefly, tripticasein soy agar plates containing 20 mL of medium were prepared. Sterile Petri dishes (150 mm) were used. Plates were inoculated by cross-striation with uropathogenic *E. coli*. Each inoculum contained approximately 10^6 CFU mL^{-1}. Subsequently, 5 wells were made on the tripticasein soy agar plate with the aid of the mouthpiece of a sterile glass Pasteur pipette. Then, different concentrations (13.3 to 59.4 mg mL^{-1}) of the essential oil were placed in each well. The agar plates were allowed to stand for about 20 min at room temperature. Then, the plates were incubated at 37°C for 24 h. The effect of essential oil of *T. vulgaris* on uropathogenic *E. coli* growth was also tested using antimicrobial susceptibility test discs. For this, the plates were inoculated by cross-striation with uropathogenic *E. coli*. Each inoculum contained approximately 10^6 CFU mL^{-1}. Then, sterile filter paper disks (5 mm diameter) were placed on the surface of tripticasein soy agar plates. Different concentrations (0.66 to 13.2 mg mL^{-1}) of the essential oil were used. The agar plates were incubated at 37°C for 24 h. The diameters of the inhibition halos formed were measured using a caliper ruler. The analyses were conducted in triplicate. The bactericidal or bacteriostatic effect was determined by passing the bacteriological handle in the plate area without apparent bacterial growth and a fresh tripticasein soy agar plate was inoculated by cross-streak. The plate was incubated at 37°C for 24 h.

Determination of minimum inhibitory concentration

The minimal inhibitory concentration was determined spectrophotometrically using trypticase soy broth and different concentrations (2.64 to 79.2 µg mL^{-1}) of the essential oil. For this, cultures containing approximately 10^6 CFU mL^{-1} were prepared and the working volume was adjusted to 1 mL. The tubes containing trypticase soy broth were incubated at 37°C for 24 h; then, the absorbance at 560 nm was determined. The minimal inhibitory concentration was defined as the lowest concentration of compound at which no growth was evident.

Detection and measurement of biofilm

For the detection of biofilm, uropathogenic *E. coli* was pre cultured during 24 h in tripticasein soy broth. Then, *E. coli* was cultivated in tripticasein soy agar plates containing 0.2% calcofluor and incubated at 37°C for 24 h. Subsequently, the agar plates were irradiated with UV light. The bacterial biofilm was also measured using crystal violet staining. The bacteria were grown in four-fold on the 96-well sterile polystyrene plates and 250 µl of trypticase soy broth were used. A medium without bacteria incubated under the same conditions was treated as a negative control. The plate was incubated at 37°C for 48 to 72 h. After that time, the culture broth was removed and each well was washed with sterile phosphate buffer at pH 7.0; the biofilm was detected by staining with 0.1% violet crystal for 20 min at room temperature. The violet crystal was then solubilized using ethanol and measured spectrophotometrically at 595 nm.

The effect of essential oil on the formation of biofilm

In order to show the effect of *T. vulgaris* on biofilm formation, uropathogenic *E. coli* was inoculated on tripticasein soy agar plates containing 0.2% calcofluor dye. Different concentrations of *T. vulgaris* (1.32 to 13.2 mg mL^{-1}) were placed in wells made in the agar as described above. Plates were incubated at 37°C for 24 h. Later, the plates were irradiated with UV light. The effect of *T. vulgaris* on biofilm formation was also observed in 96 well plates using 0.1% crystal violet dye and different concentrations (0.66 to 52.8 mg mL^{-1}) of esterified essential oil. Thus, the plates were incubated at 37°C for 48 to 72 h and then the biofilm was quantified as indicated in the previous section.

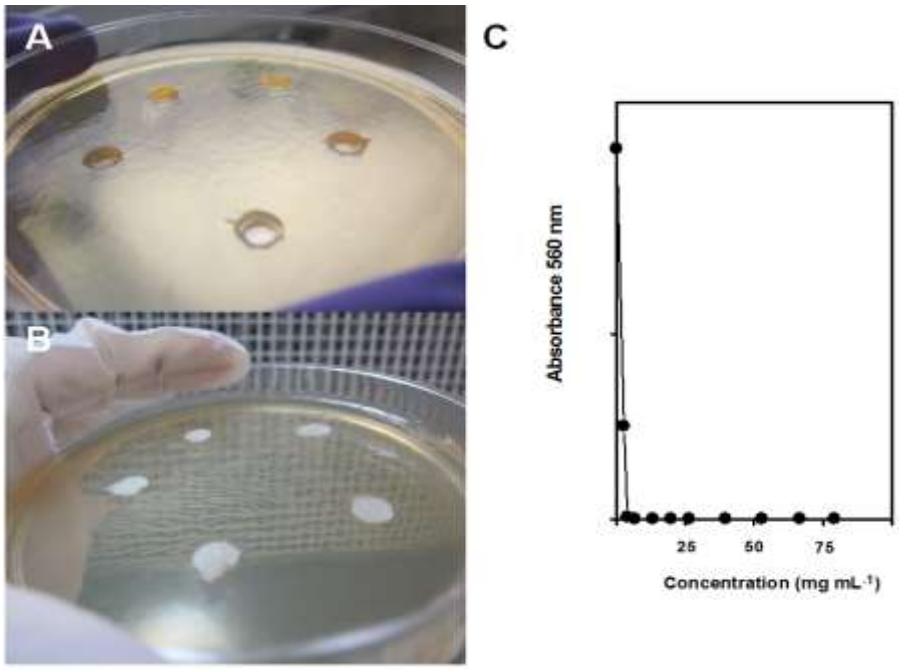

Figure 1. The inhibition of growth from uropathogenic *E. coli* using essential oil of *T. vulgaris*. **A.** The technique of disk diffusion in tripticasein soy agar plates; **B.** The antimicrobial susceptibility test discs; **C.** The minimum inhibitory concentration of *T. vulgaris* on growth of uropathogenic *E. coli*.

Obtaining the esterified essential oil

The esterification of essential oil was made in accordance with the official Mexican standard NMX-F-174-S-1981. 2 mL of *T. vulgaris* was placed in an Erlenmeyer flask containing 20 mL of potassium hydroxide in an alcoholic solution. A reflux condenser was added to the flask, which was placed in a boiling water bath for 15 min with constant stirring. When the saponification was completed, 400 µL of the 1.0% phenolphthalein indicator solution was added and it was titrated in the cold with 0.5 N HCl.

RESULTS

As described above, the antimicrobial activity was determined using different concentrations of the essential oil: 13.2, 19.8, 26.4, 39.6 and 59.4 mg mL^{-1}. The plates were incubated at 37°C for 24 h. The Figure 1A shows the surface of a tripticasein soy agar plate where uropathogenic *E. coli* was cultivated, observing that the growth was completely inhibited. On the other hand, the effect of essential oil of *T. vulgaris* on uropathogenic *E. coli* growth was also tested using antimicrobial susceptibility test discs. So the plates were inoculated by cross-striation with uropathogenic *E. coli* and sterile filter paper disks (containing different concentrations of the essential oil: 0.66, 1.32, 2.64, 6.6 and 13.2 mg mL^{-1}) were placed on the surface of tripticasein soy agar. The agar plates were incubated at 37°C for 24 h. The results obtained are shown in the Figure 1B. Although, lower concentrations of essential oil were used, the uropathogenic *E. coli* growth was completely inhibited as shown in Figure 1B. This test showed greater effectiveness because the essential oil of *T. vulgaris* diffused more easily in the agar and it was not diluted as it occurred in the test of wells on the agar. As mentioned earlier, the bactericidal or bacteriostatic effect was determined by passing the bacteriological handle in the plate area without apparent bacterial growth and a fresh tripticasein soy agar plate was inoculated by cross-streak and incubated at 37°C for 24 h. The results obtained indicated a bactericidal effect because the growth was not recorded (data not shown).

Once the inhibitory effect of the essential oil was determined, the minimum inhibitory concentration was calculated using spectrophotometer at absorbance of 560 nm with trypticase soy broth and different concentrations (0, 2.64, 3.96, 6.66, 13.2, 19.8, 26.4, 39.6, 52.8, 66.6 and 79.2 µg mL^{-1}) of the essential oil. The results obtained are shown in the Figure 1C. Figure 1C shows that low concentrations of essential oil of *T. vulgaris* produced growth inhibition of uropathogenic *E. coli*. From the results obtained, the minimum inhibitory concentration of *T. vulgaris* was calculated at approximately 4 µg mL^{-1}. This low concentration of the essential oil explains the strong inhibitory effect of the growth observed in the tripticasein soy agar shown earlier.

To determine the effect of *T. vulgaris* on the formation of biofilm, the essential oil was chemically esterified because previous biofilm measurements were not

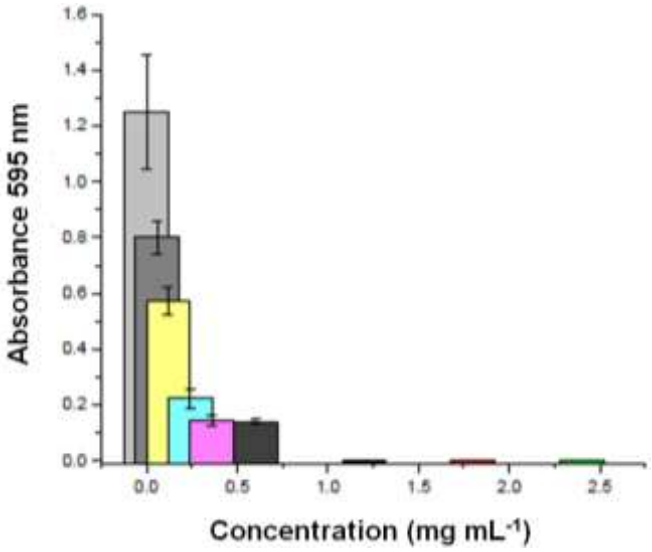

Figure 2. The inhibition of the biofilm formation of uropathogenic *E. coli* by esterified essential oil of *T. vulgaris*.

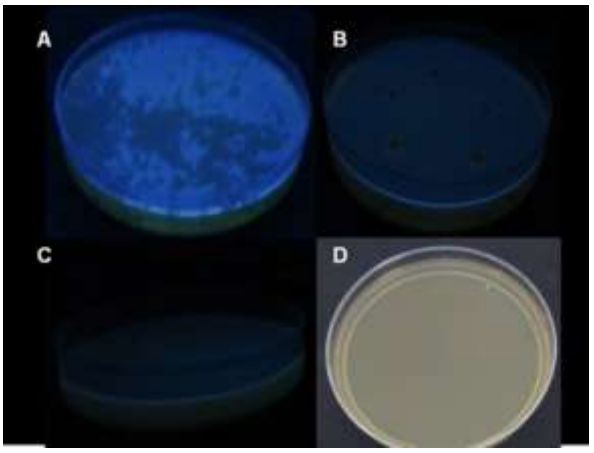

Figure 3. The production of exopolysaccharides by uropathogenic *E. coli* and the effect of essential oil of *T. vulgaris*. **A.** Presence of exopolysaccharides; **B.** Inhibition of the production of exopolysaccharides; **C.** The tripticasein soy agar plate containing 0.2% calcofluor dye and exposed to UV light; **D.** The tripticasein soy agar plate containing 0.2% calcofluor dye.

appropriate which was attributed to the insolubility of *T. vulgaris* in culture broths. The esterified form of the essential oil showed bactericidal activity similar to the non-esterified oil (data not shown). For the measurement of biofilm, *E. coli* was grown on the 96-well sterile polystyrene plates as indicated earlier. The biofilm was determined spectrophotometrically using 0.1% violet crystal. The concentrations of the essential oil used were: 0, 0.06, 0.12, 0.24, 0.36, 0.6, 1.2, 1.8 and 2.4 mg mL^{-1} (Figure 2). As shown in the figure, the formation of biofilm decreases with increasing concentration of esterified essential oil. In addition, at concentrations higher than 1 mg ml^{-1}, the biofilm formation is almost completely inhibited.

To verify that the biofilm of *E. coli* is inhibited by essential oil from *T. vulgaris*, the production of exopolysaccharides was detected using calcofluor. Figure 3A shows the presence of exopolysaccharides produced by uropathogenic *E. coli* on a tripticasein soy agar plate containing 0.2% calcofluor dye and exposed to

UV light. Figure 3B shows the results obtained when *E. coli* was cultured in tripticasein soy agar plate containing 0.2% calcofluor and different concentrations of *T. vulgaris* (in well); the plate was exposed to UV light. The concentrations of the essential oil used were: 1.32, 2.64, 6.6 and 13.2 mg mL^{-1}. As shown in Figure 3B, the essential oil inhibited the growth and the production of exopolysaccharides by uropathogenic *E. coli*. Figure 3C and D show the control conditions.

Given that a commercial essential oil was used in this study, its identity was verified using a spectrophotometric scanning in the UV region of 200 to 360 nm. The absorption spectra of essential oil showed characteristic signals corresponding to the monoterpenes (thymol and carvacrol) used as reference standards (data not shown).

DISCUSSION

Essential oils had been used since ancient times due to their medicinal properties. They also provide aroma and flavor to food (Shuaib et al., 2016). Essential oils are composed of natural terpenes in addition to some other non-terpene components. It has been reported that some essential oils have antioxidative and anticancer activities. In animal models, monoterpenes have been shown to act as chemopreventive or chemotherapeutic agents (Shuaib et al., 2016). It has also been reported that essential oils have antiviral and antibacterial properties. As antibacterial agents, the essential oils act against a wide range of pathogenic bacteria including *Staphylococcus aureus, Bacillus cereus, Shigella dysenteriae, Listeria monocytogenes, Salmonella typhimurium* and *E. coli* O157:H7 (Al-Shuneigat et al., 2014; Høiby, 2017; Hussein et al., 2014; Sambyal et al., 2017; Shuaib et al., 2016; Upadhyay et al., 2013).

In the present work, the effect of *T. vulgaris* on growth and biofilm formation of uropathogenic *E. coli* was studied. It has been reported that the *Thymus* species are considered as medicinal plants due to their pharmacological and biological properties (Kon and Rai, 2012; Shuaib et al., 2016). The flowering parts and leaves of *Thymus* species have been used for different purposes, for example as an antiseptic (Mohsenipour and Hassanshahian, 2015). Thus, in the present study, the high antimicrobial activity was determined using the technique of disk diffusion in agar and antimicrobial susceptibility test discs. As observed, the growth of uropathogenic *E. coli* was completely inhibited (bactericidal effect) at concentrations of the essential oil tested (using both methodologies); however, the antimicrobial susceptibility test was the best method because *T. vulgaris* diffused more easily in the agar. The high antimicrobial activity of *T. vulgaris* has been reported against Gram-negative bacteria such as *E. coli*, *Salmonella enteritidis*, *Salmonella choleraesuis, S. typhimurium, Vibrio cholerae*, *Proteus mirabilis*, *P. vulgaris*, *Pseudomonas aeruginosa* and Gram-positive bacteria as *S. aureus*, *S. epidermidis*, *Enterococcus faecalis* and *Bacillus cereus* (Al-Shuneigat et al., 2014; Hussein et al., 2014; Kon and Rai, 2012; Mohsenipour and Hassanshahian, 2015). Mohsenipour and Hassanshahian (2015) reported that the *T. vulgaris* extracts using the test of disc diffusion had high ability to inhibit the growth of *P. aeruginosa* and *S. aureus* and low inhibition efficiency on *E. coli* and *B. cereus*. These authors also reported that *T. vulgaris* extracts had low diffusion in solid media when compared with broth media and higher concentration of extract was necessary on solid media to observe the same inhibitory effect than in broth media (Mohsenipour and Hassanshahian, 2015). In the present work, similar results were observed when high concentration of essential oil was used for determination of biofilm in well polystyrene plates stained with violet crystal. The low solubility of the essential oil used at high concentration in broth media led to the esterification of the oil. As mentioned earlier, the minimum inhibitory concentration of *T. vulgaris* was calculated at approximately 4 µg mL^{-1} which was consistent with the inhibitory effect observed on agar plates with low concentration assayed. It has been reported that the hydrophobicity of the essential oils produce changes in bacterial membrane structure and wall structures. Alteration of the cell permeability, disturbance to respiration, modification of bacterial quorum sensing, potassium leakage from cells, effects on membrane potential (proton translocation), changes in pH gradient and ATP production of bacterial cell bacterial lipid membrane, lead to the lysis and death of bacteria (O'Bryan et al., 2015).

On the other hand, the effect of essential oil of *T. vulgaris* on biofilm formation of uropathogenic *E. coli* was determined. The results indicated that formation of biofilm decreased with increase in the concentration of esterified essential oil and at concentrations higher than 1 mg mL^{-1}, the biofilm formation was almost completely inhibited. Although, the minimum inhibitory concentration of *T. vulgaris* was calculated as 4 µg mL^{-1}, to inhibit the *E. coli* biofilm, a higher concentration was required because the essential oil was esterified. Microbial biofilms organized aggregations of cells attached to a substratum and surrounded by a self-produced extrapolymeric substance (EPS) matrix (Ta and Arnason, 2016).

In this work, the production of exopolysaccharides from uropathogenic *E. coli* was detected. The results indicated that uropathogenic *E. coli* exopolysaccharide production was inhibited by *T. vulgaris* using calcoflour dye. Al-Shuneigat et al., (2014) reported that *T. vulgaris* had an inhibitory effect on the formation of biofilm in several bacterial strains and that *E. coli* was the most sensitive, while *P. aeruginosa* was the most resistant for both planktonic and biofilm growth. Hussein et al. (2014) tested different plant extracts (including *T. vulgaris*) and they reported that the most efficient plant extract in inhibition of biofilm formation from *E. coli* was *T. vulgaris*;

Borago officinalis was the least efficient plant extract in inhibition of biofilm formation. It has been reported that thymol and carvacrol (components of the essential oil of *T. vulgaris*) produced the inhibition of biofilm formation in *S. aureus* and *S. epidermidis* against planktonic and biofilm strains (Ta and Arnason, 2015). The results indicated that uropathogenic *E. coli* exopolysaccharide production was inhibited by *T. vulgaris* using calcoflour dye, and also indicated that thymol and carvacrol (monoterpenes) had a strong inhibitory effect (data not shown) on the uropathogenic *E. coli* biofilm formation. These results are in agreement with previous results, which demonstrated that thymol and carvacrol are effective against biofilms of Gram-positive and negative bacteria (Ta and Arnason, 2016; Upadhyay et al., 2013). The thymol inhibited the formation of *L. monocytogenes* biofilms and genes critical to biofilm development were down regulated at concentration of 0.5 mM. Similar results were observed using 0.65 mM of carvacrol by inhibiting biofilm from *L. monocytogenes* (Upadhyay et al., 2013).

CONCLUSION

Antibiotic resistance remains a serious clinical problem; thus, it stimulates studies for search of new methods for treatment of infectious diseases using essential oils and plant extracts with antimicrobial activity. This study demonstrated the strong effect of *T. vulgaris* essential oil on the growth and biofilm formation of uropathogenic *E. coli*. This substance could be used as an alternative for the treatment of bacterial infections. However, the cytotoxic effect it has on humans, has not yet been determined

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank PRODEP and VIEP-BUAP for the facilities provided for the development of this work.

REFERENCES

Al-Shuneigat J, Al-Sarayreh S, Al-Saraireh Y, Al-Qudah M, Al-Tarawneh I (2014). Effects of wild *Thymus vulgaris* essential oil on clinical isolates biofilm-forming bacteria. J. Dental Med. Sci. 13:62-66.

Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM (1995). Microbial biofilms. Annu. Rev. Microbiol. 49:711-745.

Delcaru C, Alexandra I, Podgoreanu P, Grosu M, Stavropoulos E, Chifiriuc MC, Lazar V (2016). Microbial biofilms in urinary tract infections and prostatitis: Etiology, pathogenicity, and combating strategies. Pathogens 5:1-12.

Flores-Mireles AL, Walker JN, Caparon M, Hultgren SJ (2015). Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat. Rev. Microbiol. 13:269-284.

Gould IM, Bal AM (2013). New antibiotic agents in the pipeline and how they can overcome microbial resistance. Virulence 4:185-191.

Høiby NA (2017). Short history of microbial biofilms and biofilm infections. APMIS 125:272-275.

Hussein NH, Rasool KH, Hussein JD (2014). Effects of *Coriandrum sativum, Thymus vulgaris, Borago officinalis* and *Pimpinella anisum* on biofilm *Escherichia coli*. J. Gen. Environ. Res. Conserv. 2:219-230.

Kon K, Rai M (2012). Antibacterial activity of *Thymus vulgaris* essential oil alone and in combination with other essential oils. Nusantara Biosci. 4:50-56.

Lin J, Nishino K, Roberts MC, Tolmasky M, Aminov RI, Zhang L (2015). Mechanisms of antibiotic resistance. Front Microbiol. 6:1-4.

Lushniak BD (2014). Antibiotic resistance: a public health crisis. Public Health Rep. 129:314-316.

Mohsenipour Z, Hassanshahian M (2015). The inhibitory effect of *Thymus vulgaris* extracts on the planktonic form and biofilm structures of six human pathogenic bacteria. 2015. Avicenna J. Phytomed. 5:309-318.

O'Bryan CA, Pendleton SJ, Crandall PG, Ricke SC (2015). Potential of plant essential oils and their components in animal agriculture- in vitro studies on antibacterial mode of action. Frontiers Vet. Sci. 2:1-8.

Read AF, Woods RJ (2014). Antibiotic resistance management. Evol. Med. Public Health. 2014:147.

Sambyal SS, Sharma P, Shrivastava D (2017). Anti-biofilm activity of selected plant essential oils against *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Int. J. Curr. Microbiol. App. Sci. 6:444-450.

Sharma G, Sharma S, Sharma P, Chandola D, Dang S, Gupta S, Gabrani R (2016). *Escherichia coli* biofilm: Development and therapeutic strategies. J. Appl. Microbiol. 121:309-319.

Shuaib A, Rohit A, Piyush M (2016). A review article on essential oils. J. Med. Plants Stud. 4:237-240.

Ta CAK, Arnason JT (2016). Mini review of phytochemicals and plant taxa with activity as microbial biofilm and quorum sensing inhibitors. Molecules 21:1-26.

Upadhyay A, Upadhyaya I, Kollanoor-Johny A, Venkitanarayanan K (2013). Antibiofilm effect of plant derived antimicrobials on *Listeria monocytogenes*. Food Microbiol. 36:79-89.

Ventola CL (2015). The antibiotic resistance crisis. Pharm. Ther. 40:277-283.

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Vol. 12(10), pp. 243-247, 14 March, 2018
DOI: 10.5897/AJMR2017.8664
Article Number: 6397B0A56331
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Efficacy of two probiotics in the control of *Escherichia coli* O157:H7 in experimentally infected lambs

Kingsley Chineto Anyika[1*], Solomon Olu Okaiyeto[2], Shehu Na'allah Saidu[3] and Gabriel Ogbaji Ijale[4]

[1]Livestock Investigation Division, National Veterinary Research Institute Vom, Nigeria.
[2]Veterinary Teaching Hospital, Ahmadu Bello University Zaria, Nigeria.
[3]Department of Veterinary Medicine, Ahmadu Bello University Zaria, Nigeria.
[4]Unity Veterinary Clinic, Jos, Plateau State, Nigeria.

Received 1 August 2017; Accepted 18 October, 2017

This study was designed to determine the efficacy of probiotics mixture in the control of Shiga-toxin *Escherichia coli* (STEC) O157:H7 in experimentally infected lambs. Fifteen Yankassa breed of lambs aged between 3-4 weeks old were used. The lambs were divided into three groups of five lambs each (n=5). Group A: Neither probiotics nor STEC O157:H7 were administered (control), Group B: lambs were administered viable STEC O157:H7 cells at 6 x 10^8CFU/ml together with daily administration of probiotics mixture at 4.5 x10^8 CFU/ml, Group C: lambs were administered only viable STEC O157:H7 cells at 6x10^8 CFU/ml without probiotics. Faecal samples from all the experimental lambs were screened for the presence of STEC O157:H7 before the commencement of this study using Tryptone soy broth (TSB) as an enrichment media and Cefixine-tellurite sorbitol MacConkey agar (CT-SMAC) as a selective media. Following oral inoculation of the lambs with STEC cells, faecal samples were collected once weekly for six weeks, for STEC O157:H7 isolation and enumeration. STEC O157:H7 was confirmed by its reaction with O157 and H7 anti-sera (Wellcomex^r). STEC O157:H7 was not shed by lambs in group A (control). However, Group B lambs administered a mixture of probiotics shed significantly lower (P<0.05) counts of STEC cells in the six weeks post-infection than Group C lambs that received only STEC cells without probiotics. In conclusion, the use of probiotics mixture significantly (P<0.05) reduced the faecal shedding of STEC O157:H7. It was therefore recommended that probiotics should be administered to lambs to help control these pathogens.

Key words: Shiga-toxin producing *Escherichia coli* O157:H7, probiotics mixture, cefixime-tellurite Sorbitol MacConkey Agar, tryptone soy broth, anti-sera.

INTRODUCTION

Shiga-toxin-producing *Escherichia coli* O157:H7 (STEC) is a food borne pathogen primarily transmitted to humans through the consumption of contaminated water or food (Caprioli et al., 2005). STEC represent the only

*Corresponding author. E-mail: chinetoanyika@gmail.com. Tel: +2347030564505.

pathogenic group of *E. coli* that has a definite zoonotic origin, although not all the STEC strains have been demonstrated to cause disease in humans. Six non-O157 groups have been identified by CDC (2008) to be responsible for over 70% of non-O157 STEC-associated illness (Bosilevac and Koohmaraie, 2011). The predominant STEC serotype associated with outbreaks and sporadic cases of serious STEC illnesses is STEC O157 (Karmali et al., 2010).

Ruminants such as cattle, sheep and goats are regarded as the main animal reservoirs of STEC (Nataro et al., 2011). Adult ruminants can be asymptomatic carriers of these pathogens, but it has been reported to cause mild to severe diarrhoea in lambs, calves and pigs (Cray and Moon, 1995). Similarly, Dean-Nystrom et al. (1997) also reported cases of mucoid diarrhoea and mortality in neonatal lambs and calves. Infected ruminants can also serve as source of infection to other susceptible animals such as pigs and rabbits. STEC O157:H7 also causes a wide range of human diseases, including mild to severe diarrhoea, haemorrhagic colitis (HC) and life threatening haemolytic uremic syndrome (HUS) (Caprioli et al., 2005). Both the European Food Safety Authority (EFSA) and the U.S. Department of Agriculture (USDA) have issued recommendations for laboratory testing for these pathogens (Eblen, 2010),

Lema et al. (2001) and Everlon et al. (2013) indicated that some strategies may be used in the rumen to decrease the number of viable STEC cells. One of such strategies is the use of probiotics supplemented in the ration. Probiotics are live organisms with the capacity to benefit the gastrointestinal tract microflora by promoting health or preventing diseases in the host (Papadimitriou et al., 2007; Hill et al., 2014). Several mechanisms have been proposed to explain the beneficial effects of probiotics. Among them are the production of organic acids by bacterial probiotics which can help decrease the gut pH, create more favourable ecological conditions for the resident microbiota and decrease the risk of pathogen colonization (Servin, 2004).

There is paucity of information on the use of probiotics in the control of STEC in Nigeria, Hence, this study will help explore alternative treatment regimen through the use of probiotics to improve small ruminant production in Nigeria.

MATERIALS AND METHODS

The study was carried out at the Livestock Investigation Division of the National Veterinary Research Institute, Vom, plateau State, Nigeria. The State is located in the North Central Geopolitical zone of Nigeria and has a land area of 26,899 sq km (NPC, 2006).

Experimental animals

Fifteen (15) Yankassa breed of lambs, aged between 3 and 4 weeks were used for this research. The lambs were sourced from the Small Ruminant Section of the Livestock Investigation Division of the National Veterinary Research Institute, Vom, Plateau State.

The lambs were tagged and allocated into pens. They were confined to their dams to enable them suckle. The dams were fed on concentrate and hay. Water was provided *ad libitum*

Escherichia coli strain used in the study

Shiga-toxin producing (STEC) *E. coli* O157:H7 strain, which is under the entero-haemorrhagic serogroup of *E. coli* was sourced from the National Veterinary Research Institute, Vom, Plateau State, Nigeria.

Cultures for use as inocula were produced in 10 ml of 0.9% saline solution after an overnight incubation on Sorbitol MacConkey agar (SMAC) supplemented with cefixime tellurite (Oxoid, UK) at 37°C for 24 h. Cell numbers were determined spectrophotometrically using the McFarland standard and were adjusted to contain 6 x 10^8 CFU/ml.

Probiotics used in the study

Sky-flo® Probiotics containing *Lactobacillus acidophilus* and Sanolife® PRO-F probiotics containing a balanced mixture of *Bacillus pumilus, Bacillus licheniformis* and *Bacillus subtilis* were used for this research. It was manufactured by Inve Aquaculture, Belgium.

Preparation of the daily doses of probiotics mixture

To prepare the daily doses of the probiotic strain used, individual tube containing the lyophilized bacteria, were inoculated into 9 ml of *Lactobacillus* selective broth de Mann, Ragosa and Sharpe (MRS) (Oxoid, UK) for selective enrichment and incubated at 37°C for 24 h aerobically. After the period of incubation, a loop full of the positive broth was then streaked on MRS agar (Oxoid, UK) plates and incubated at 37°C for 24 h aerobically. Colonies that grew were re-suspended in 10 ml of saline to generate a suspension containing *Lactobacillus acidophilus* 4.5 x 10^8 CFU/ml, *Bacillus subtilis* 4.5 x 10^8 CFU/ml, *Bacillus licheniformis* and *Bacillus pumilus* 4.5 x 10^8 CFU/ml using the McFarland standard.

Experimental design

Animal groupings

Lambs were divided into three (3) groups (A, B and C) of five (5) lambs each and confined to separate pens. The animals were all tagged for the purpose of identification.

Pre-experimental management of animals

The lambs were allowed to acclimatize to the environment for two weeks prior to the commencement of this study, during which the dams were de-wormed with albendazole (10 mg/kg) *per os* and administered ivermectin (0.2 mg/kg) sub-cutaneously to control ectoparasites. The lambs were weaned before the commencement of the study at four weeks of age, and fed concentrate and water during the study period.

Ethics

Ethical clearance was obtained from the Ahmadu Bello University Zaria Committee on Animal Use and Care, with approval number:

ABUCAUC/2016/Vet.Medicine/003.

Pre-infection data

Pre infection data collected from the lambs included: Faecal samples collected directly from the rectum of all the lambs into sterile polythene bags and immediately taken to the laboratory to screen for the presence of *E. coli* O157:H7 as described by Chapman et al. (1994). Briefly, 1 g of the faecal sample from each lamb was added to 10 ml of TSB and incubated at 37°C for 24 h aerobically for enrichment. Following incubation, a loop full of the overnight broth was streaked on CT-SMAC to selectively isolate *E. coli* O157:H7 colonies.

Animal groupings and treatment regimen

At the end of the two weeks acclimatization period and after the screening of all the lambs for the presence of *E. coli* O157:H7, they were subsequently subjected to different treatment regimens as follows: Group A: neither probiotic non *E. coli* (STEC O157:H7) was administered, as they served as control; Group B: Lambs were administered viable STEC O157:H7 ($6x10^8$CFU/ml) together with a mixture of probiotics (*L. acidophilus, B. pumilus, B. subtilis* and *B. licheniformis*) at 4.5 x 10^8 CFU/ml daily throughout the research period. Both were administered in a 1 ml normal saline, orally through the use of sterile syringe directly into the mouth; Group C: Lambs were administered 1 ml inoculum containing viable STEC O157:H7 (6 x 10^8CFU/ml) without probiotics, through the use of sterile syringe directly into the mouth.

Faecal sample collection, detection and enumeration of *E. coli* (STEC) O157:H7 post-infection

Faecal samples were collected from all the lambs on day three following the inoculation of *E. coli* O157:H7 to confirm the presence of the pathogen in the faeces. Subsequently, faecal samples were collected once weekly, directly from the rectum of each lamb every morning using sterile swab sticks, for a period of 6 weeks for detection and enumeration of *E. coli* O157:H7 colonies as described by Chapman et al. (1994). Briefly, 1 g of faeces from each lamb was aseptically added into 10 ml of normal saline and homogenized by shaking vigorously. Subsequently, 1 ml of the homogenate was then added to 9 ml of normal saline and a hundred (100) fold serial dilution was made after which 0.1 ml from each dilution tubes was spread plated onto CT-SMAC to selectively isolate *E. coli* 0157:H7 colonies for enumeration. Creamy white colonies that grew were regarded as presumptive isolates due to the inability of *E. coli* 0157:H7 to ferment Sorbitol and was further confirmed serologically using *E. coli* O157:H7 Monoclonal Antisera (Wellcolex *E. coli* O157:H7 kit, Oxoid). Presumptive *E. coli* colonies were also further verified by conventional biochemical methods as described by Harrigan (1998).

Presumptive colonies of *E. coli* O157:H7, were further confirmed by serotyping using commercial latex kit for *E. coli* O157:H7 (Wellcolex *E. coli* O157:H7 kit) which is a rapid latex agglutination test for confirming non-sorbitol fermenting colonies as possessing the O157 somatic antigen and H7 flagellar antigen.

Data analysis

The data obtained were expressed as mean ± standard error of mean (SEM) and presented in graphs. One way ANOVA with Tukey's post hoc test using SPSS version 20 for windows was used to determine significant difference in the shedding of STEC O157:H7 among the groups. Values of $P< 0.05$ were considered significant at 95% confidence interval.

RESULTS

Mean number of STEC O157:H7 isolates recovered

No STEC O157:H7 isolate was recovered from the faeces of all the lambs in group A throughout the six weeks of the study (Figure 1). However, the mean STEC O157:H7 isolates recovered from group B lambs increased significantly ($P<0.05$) from zero pre-infection to 2 x 10^4 CFU/g at the first week post infection and then decreased significantly ($P>0.05$) to 1.1 x 10^4 CFU/g during the second week, and progressively decreased to 8 x 10^3 CFU/g in the third week, 6 x 10^3 CFU/g in the fourth week, 4 x 10^3 CFU/g in the fifth week and 2 x 10^3 CFU/g in the sixth week post-infection (Figure 1). Group C lambs had a significant increase ($P<0.05$) in the mean STEC O157:H7 isolates over the six weeks post-infection: from no isolate pre-infection to 3.08 x 10^5 CFU/g in the first week post infection then decreased progressively to 2.3 x 10^4 CFU/g in the sixth week (Figure 1).

There were higher number of STEC O157:H7 isolates recovered from group C lambs that were challenged with viable STEC O157:H7 cells without the administration of a mixture of probiotics, than the group B lambs that received the viable STEC O157:H7 cells together with a mixture of probiotics over the six weeks period.

DISCUSSION

STECs are zoonotic pathogens that can cause food borne diseases in humans, ranging from diarrhoea to haemolytic colitis (HC) and the deadly haemolytic uremic syndrome (HUS) (WHO, 1998). There are several studies showing the prevalence of STEC in ruminants in different parts of the world. Therefore, the control of STEC is of great public health significance (Everlon et al., 2013; Martins et al., 2015). Several reports exist on the efficacy of probiotics in the control of STEC O157 (Avila et al., 2000; Lema et al., 2001; Everlon et al., 2016).

In this study, there were no clinical signs observed from all the lambs in the experimental groups throughout the six weeks period of research. This could be because ruminant such as cattle, sheep and goat are regarded as the main animal reservoirs and are usually asymptomatic carriers of these pathogens (La Ragione et al., 2009). It could also be due to the fact that the dose of the STEC O157:H7 inoculum used for the challenged was not high enough to produce clinical infection in the lambs. This finding agrees with the report by Lema et al. (2001), Avila et al. (2000) and Everlon et al., (2013) who also reported absence of clinical signs of STEC O157:H7 in experimentally infected ruminants. However, six weeks

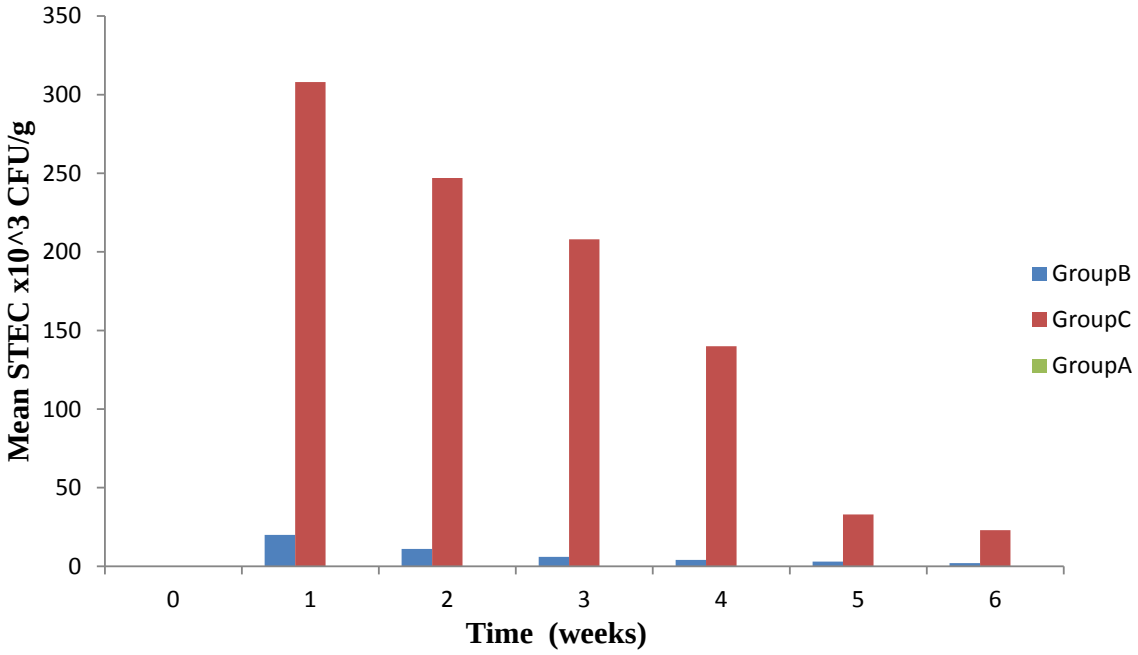

Figure 1. Mean *E. coli* (STEC) O157:H7 isolates recovered from the three experimental groups of lambs six weeks post-infection. Group A: Control; Group B: Lambs inoculated with STEC cells together with probiotics administered daily; Group C: lambs inoculated with STEC cells only without probiotics administration 0 week represents pre-infection period. 1 to 6 weeks represents post-infection period.

post-infection, it was observed that a lower count of *E. coli* (STEC) O157:H7 was recovered from lambs in Group B that received *E. coli* 0157:H7 inoculum together with the mixture of probiotics strains in comparison with Group C lambs that received only *E. coli* (STEC) O157:H7 inoculum without probiotics strains. The fewer counts of STEC O157:H7 recovered from the lambs in Group B could be as a result of the effect of probiotic strains administered. The mixture of probiotic strains perhaps, produced inhibitory substances which inhibited the adhesion and proliferation of the STEC cell, thereby leading to the death of these cells. Furthermore, the absence of a well-developed normal flora at this age of the lambs may also have facilitated the establishment of the probiotics strains in the gut and consequently, the establishment of the STEC O157:H7 was hampered. While the higher number of STEC strains recovered from group C lambs, could also be as a result of the absence of a well-developed normal flora in the young lambs; thereby, facilitating the adhesion and proliferation of the STEC O157:H7 cells in this group of lambs. It could also be as a result of the absence of any intervention (probiotics), so, the STEC O157:H7 cells did not suffer any competition with other micro-organism.

This finding agrees with the report of Lema et al. (2001) who showed a protective effect of probiotics on STEC O157:H7 in lambs. Also, work done by Everlon et al. (2013) showed that probiotics was more efficient in reducing the faecal shedding of STEC cells in sheep that were younger than 45 days as compared to sheep that were older than 45 days. Similarly, Stanford et al. (2014) also reported a reduction of STEC O157:H7 in finishing feedlot cattle fed a mixture of probiotics.

The findings suggest the efficacy of probiotic mixture in the control of STEC O157:H7 in lambs through the reduction of faecal shedding of these pathogens.

Conclusion

Administration of a mixture of probiotics strains (*L. acidophilus, B. pumilus, B. subtilis* and *B. licheniformis*) at 4.5 x 10^8 CFU/ml was effective in the control of *E. coli* O157:H7 (STEC) in experimentally infected lambs. It was therefore recommended that probiotic mixture should be added to feed or water of lambs to help control these pathogens.

CONFLICT OF INTERESTS

The authors declare that there is no conflict of interest.

ACKNOWLEDGEMENT

Our profound gratitude goes to the director of the National Veterinary Research Institute Vom, for the

financial assistance to undertake this study and also the management and staff of Livestock Investigation Division, NVRI, Vom for their kind assistance during the course of this research.

REFRENCES

Avila F, Paulillo AC, Schocken-Iturrino RP, Lucas FA, Orgaz A, Quintana JL (2000). Evaluation of the efficiency of a probiotic in the control of diarrhea and weight gain in calves. Arq. Bras. Med. Vet. Zootec. 52(1):41-46.

Bosilevac JM, Koohmaraie M (2011). Prevalence and characterization of non-O157 Shiga toxin-producing *Escherichia coli* isolated from commercial ground beef in the United States. Appl. Environ. Microbiol. 77:2103-2112.

Caprioli A, Morabito S, Brugere H, Oswald E (2005). Enterohaemorrhagic *Escherichia coli*: emerging issues on virulence and modes of transmission. Vet. Res. 36:289-311.

Centers for Disease (CDC) (2008) Division of Foodborne, Bacterial and Mycotic Diseases (DFBMD). *Escherichia coli* (online). CDC DFBMD; 2008 Mar. Available at: http://www.cdc.gov/nczved/dfbmd/disease_listing/stec_gi.html. Accessed 3 Mar 2009.

Chapman PA, Siddons CA, Zadik PM, Jewes L (1994). A comparison of immunomagnetic separation and direct culture for the isolation of verocytotoxin producing *Escherichia coli* O157 from bovine faeces. J. Med. Microbiol. 40:424-427.

Cray WC (Jr.), Moon HW (1995). Experimental infection of calves and adult with *Escherichia coli* O157:H7. Appl. Environ. Microbiol. 61:1586-1590.

Dean-Nystrom EA, Bosworth BT, Cray WC Jr, Moon HW (1997). Pathogenecity of Escherichia coli O157:H7 in the intestine of neonatal calves. Infect. Immun. 65:1842-1848.

Eblen DR (2010). Public health importance of non-O157 Shiga toxin-producing *Escherichia coli* (non-O157 STEC) in the US food supply. (Last accessed May 28, 2010). Available from http://www.fsis.usda.gov/PDF/STEC_101207.pdf.

Everlon CR, Marita VC, Fernando AA, Patrick JB (2016). An evaluation of the use of probiotics and manure composting as strategies to reduce levels of Shiga toxin-producing *Escherichia coli* in sheep. Afr. J. Microbiol. Res. 10(26):1011-1017.

Everlon CR, Renato PM, Clariss AB, Livia GB (2013). Evaluation of the protective effect of probiotics in sheep against experimental infection by *Escherichia coli*. Afr. J. Microbiol. Res. 7(14):1262-1265.

Harrigan WF (1998). Detection and enumeration of pathogenic and toxigenic organism-pathogenic *Escherichia coli.* In W.F. Harrigan (3rd ed.), Laboratory Methods in Food Microbiology. London, Academic Press. pp. 186-189.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME (2014). Expert consensus document: The International Scientific Association for Probiotics and Prebiotics consensus statement on the scope and appropriate use of the term probiotic. Nat. Rev. Gastroenterol. Hepatol. 11(8):506-514.

Karmali MA, Gannon VP, Sargeant JM (2010). Verocytotoxin-producing *Escherichia coli* (VTEC). Vet. Microbiol. 140:360-370.

La Ragione RM, Best A, Woodward, MJ, Wales AD (2009). *Escherichia coli* O157:H7colonization in small domestic ruminants. FEMS Microbiol. Rev. 33:394-410.

Lema M, Williams L, Rao DR (2001). Reduction of faecal shedding of enterohemorrhagic *Escherichia coli* O157:H7 in lambs by feeding microbial feed supplement. Small Rumin. Res. 39:31-39.

Martins FH, Guth BE, Piazza RM, Leao SC, Ludovico A, Ludivico MS, Dahbi G, Marzoa J, Mora A, Blanco J, Pelayo JS (2015). Diversity of Shiga toxin producing *Escherichia coli* in sheep flocks of Parana state, southern Brazil. Vet. Microbiol. 30:150-156.

Nataro JP, Kaper JB, Robins-Browne R, Prado V, Vial P, Levine MM. (2011). Escherichia, Shigella and Salmonella. In. Manual of Clinical Microbiology; Versalovic J, Carroll KC, Funke G, Jorgensen J H, Landry ML, Warnock DW, Eds. ASM Press: Washington, DC, USA. pp. 603-626.

National Population Commission (NPC) (2006) Annual abstract of Statistic, 2007. Federal Government economic reform and governance project (ERGP). P 23.

Papadimitriou CG, Mastrojiannaki AV, Silva SV, Gomes AM, Malcata FX, Alichanidis E (2007). Identification of peptides in traditional and probiotic sheep milk yoghurt with angiotensin I-converting enzyme (ACE) inhibitory activity. Food Chem. 105:647-656.

Servin AI (2004). Antagonistic activities of lactobacilli and bifidobacteria against microbial pathogens. FEMS Microbiol. Rev. 28:405-440.

Stanford K, Bach S, Baah J, McAllister T (2014). A mixture of *Lactobacillus lactis* and *Paenibacillus polymyxa* reduces *Escherichia coli* O157:H7 in finishing feedlot cattle. J. Food Prot. 77:738-744.

World Health Organization (WHO) (1998). Zoonotic non-O157 Shiga toxin-producing *Escherichia coli* (STEC). Report of a WHO scientific working group meeting, Berlin, Germany, 23 to 26 June 1998. World Health Organization, Geneva, Switzerland.

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Vol. 12(10), pp. 248-253, 14 March, 2018
DOI: 10.5897/AJMR2018.8801
Article Number: 588318556334
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Potassium phosphite reduction of *Candidatus* Liberibacter spp. population on leaves of 'Ponkan' tangerines tree with huanglongbing

Rener Luciano de Souza Ferraz[1*], Marcelo de Andrade Barbosa[2], Maria José Araújo Wanderley[3], Patrícia da Silva Costa[4], Ivomberg Dourado Magalhães[5], Aldair de Souza Medeiros[5], Hugo Miranda Faria[2], Vinicius Marchioro[2], Alberto Soares de Melo[6] and Franklin Alves dos Anjos[7]

[1]Academic Unit of Agricultural Engineering, Federal University of Campina Grande, Paraiba, Brazil.
[2]Department of Soils and Fertilizers, São Paulo State University, São Paulo, Brazil.
[3]Department of Agriculture, Federal University of Paraiba, Paraíba, Brazil.
[4]Department of Animal Science, Federal University of Campina Grande, Paraíba, Brazil.
[5]Department of Plant Production, Federal University of Alagoas, CEP: 57072-900, Maceió, Alagoas, Brazil.
[6]Department of Agrarian Sciences, State University of Paraíba, Paraiba, Brazil.
[7]Federal Institute of Education, Science and Technology of Alagoas, Brazil.

Received 15 January, 2018; Accepted February 16, 2018

The objective of this work was to evaluate the population variation dynamics of *Candidatus* Liberibacter spp. under phosphite applications. The 'Ponkan' tangerines (*Citrus reticulata* Blanco cv. Ponkan) with symptoms and a high degree of huanglongbing (HLB) severity were used for the test. 'Ponkan' tangerine plants were treated with potassium phosphite leaf applications at a dose of 2.5 L ha^{-1}. Applications frequency was intervals of 25 days with a total of two applications. There was variation in bacterium *Candidatus* Liberibacter spp. population, depending on the application of phosphite. Phosphites have an indirect action on pathogens control, stimulating the formation of phytoalexins, a natural self-defense substance of plants. Indirect effects of these products were reported in citrus seedlings presenting symptoms of phosphorus deficiency, which when treated with foliar applications of potassium phosphite overcame these symptoms, restoring plant growth. *Candidatus* Liberibacter spp. population in 'Ponkan' (*Citrus reticulata* Blanco cv. Ponkan) leaves was reduced and the phosphorus and potassium contents increased with 2.5 L ha^{-1} of potassium phosphite by means of leaf application.

Key words: *Citrus reticulata*, leaf nutrition, huanglongbing, phosphorus, potassium.

INTRODUCTION

Citrus crops are an important activity worldwide with special relevance for Brazilian economy. Citrus were introduced

*Corresponding author. E-mail: ferraz340@gmail.com.

in Brazil by the first colonizing expeditions, probably in Brazilian State of Bahia. Citrus crop expansion is attributed to the emergence of new varieties
and to the development of cultivars more adapted to cultivated ecosystems (Lopes et al., 2011).

Brazil is the largest world producer of citrus plants and the largest exporter of sweet orange juice, which is the main product of the agro-industrial complex of Brazilian citrus growing sector.

Citrus crops are distributed in all regions of Brazil, but with a notable concentration in the Southeast region, especially in São Paulo State, whose orchards are mainly based on sweet orange trees, followed by tangerines and acid lime trees (Cunha Sobrinho et al., 2013). Based on the above, it is important to alert that citrus activity is threatened by the occurrence of the most destructive disease in the world, which is pointed out as the protagonist of the decline of the world citrus cultivation.

This disease is known as greening (huanglongbing, HLB), caused by the bacterial pathogen *Candidatus*.

The long asymptomatic phase of this disease consists of an obstacle in the decision-making process for strategies to control the pathogen (Aksenov et al., 2014).

Among the main damages caused by huanglongbing disease irregular leaf yellowing, fruit drop and deformity, as well seed abortion and significant reductions in fruits qualitative aspects, such as increased acidity and reduction of soluble solids content can be observed (Choi et al., 2013).

In this sense, Pourreza et al. (2014) reported that such problems can have a significant influence on the quality of the raw material supplied to the citrus industry, reflecting directly on the economic sustainability of the productive and industrial chain. This scenario denotes the importance of search for alternatives for controlling or reducing this disease by management of the vector *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) or reduction and eliminating the bacteria. In this perspective, the use of phosphite may be a viable alternative, especially because the action of phosphite has been reported many times against different pathogens of the most varied cultivated plants (Dianese and Blum, 2010).

These compounds are not phytotoxic and have high fungicidal activity, and can act directly by inhibiting the development of the pathogens, also indirectly activating the host plant defence system (Costa et al., 2014).

The objective of this work was to evaluate *Candidatus* Liberibacter spp. population dynamics under phosphite application via the leaves in 'Ponkan' (*Citrus reticulata* Blanco cv. Ponkan) to develop a strategy to reduce damages caused by huanglongbing on citrus plants.

MATERIALS AND METHODS

Experimental area

The experiment was carried out from April to May 2014, in an orchard, in the municipality of Pirangi (Sao Paulo State, Brazil) (21° 5' 28" S, 48° 39' 20" W), at 511 m altitude. Temperature, relative humidity, solar radiation and rainfall were obtained with the objective of characterizing climatic conditions during the period the research was conducted (Figure 1).'Ponkan' plants (*Citrus reticulata* Blanco cv. Ponkan) with symptoms and a high severity degree of huanglongbing disease were used for the experimental test. First, we found twelve plants with characteristics of disease; thereafter, leaves from symptomatic branches were collected to confirm the presence of the bacterium *Candidatus* Liberibacter spp. These branches were evaluated using a quantitative analysis of polymerase chain reaction (qPCR), and the branch was reserved for subsequent foliar biomass collections (Wang et al., 2006).

Treatments application

The plants were treated with foliar applications of potassium phosphite at a dose of 2.5 L ha^{-1}. Two applications occurred (each 25-day intervals) using a gasoline and oil (two-stroke) coastal farm sprayer, with a capacity of 10 L of mixture and horizontal of 14.5 m action radius. The application of mixture was on the lower part of the leaves with time of two minutes of application for each plant. This time was previously calculated for complete coverage of plant top.

Bacterial population

Twenty-five days after treatment and first collection of leaves, a new collection of plant material and new treatment was performed, and this new collection was performed after another 25 days. The collected material was sent to Sylvio Moreira Citrus Center in Cordeirópolis, State of São Paulo, to perform qPCR (Wang et al., 2006) to monitor bacterium population variation dynamics by means of Ct (Cycle Threshold) value, where Cts <29 denote positive reaction with expressive amount of nucleic acid (NA) of the target bacterium. Cts between 30 and 37 indicate moderate amounts of NA; Cts between 38 and 40 characterize minimal qPRC reactions denoting minimal NA amounts of the target bacterium in the sample.

Phosphorus and potassium contents

Ten leaves of the total collected in each evaluation were separated, conducted in a forced air circulation oven, and maintained at temperature of 65°C until reaching a constant mass. Subsequently, dried leaves were pre-processed manually in a sterile container and conducted to a grinding mill to obtain a homogeneous sample.

The samples were sent to Laboratory of the Department of Soils and Fertilizers of the Paulista State University, Campus of Jaboticabal, S.P., Brazil, to carry out the analyzes according to the method established by Bataglia et al. (1983). For determining macronutrients, such phosphorus (P) and potassium (K), nitroperchloric digestion was used. These nutrients are part of potassium phosphite nutritional composition and were evaluated to confirm their absorption by the plant and correlation with the bacterial population.

Statistical analysis

The data obtained from response variables were submitted to analysis of variance ($p<0.5$). The means of each evaluation were compared by Tukey test (Barbosa and Maldonado Jr, 2015). The correlation between the bacterial population and phosphorus and potassium contents was investigated by means of Principal

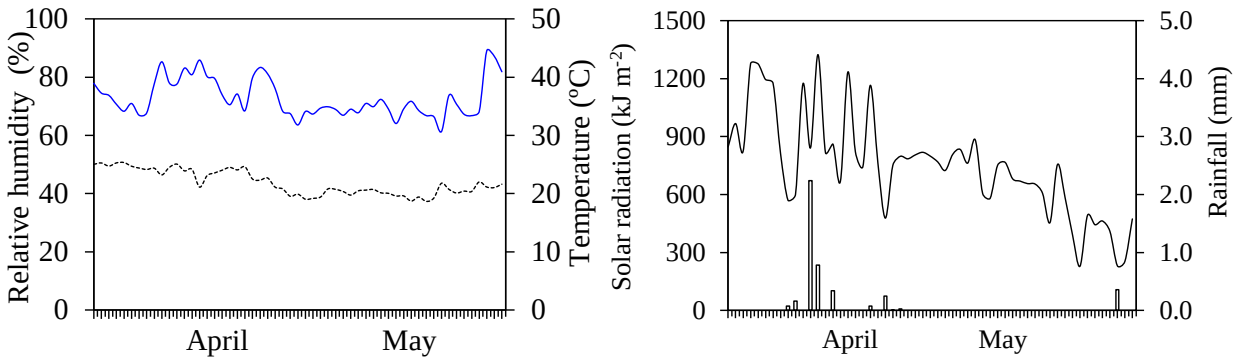

Figure 1. Graphical representation of climatic variables quantified from April to May. Pirangi, SP, Brazil. 2014. —— Relative humidity; **------** Average air temperature; □ Rainfall; and —— Solar radiation.

Table 1. Analysis of variance table for bacterial population (Ct), phosphorus content (Pg kg^{-1}) and potassium (kg kg^{-1}) in 'Ponkan' tangerine leaves under potassium phosphite applications in Pirangi, SP, Brazil during 2014.

Source of variance	Degrees of freedom	Mean squares		
		Ct	P	K
Apllications	2	14.34**	0.28**	2.97**
Residual error	9	1.51	0.01	0.16
CV (%)		6.33	6.39	4.24

**: Significant by test F ($p < 0.01$).

Component Analysis (PCA). For this, the data were standardized with means zero and unit variance (Hair et al., 2009).

RESULTS

A significant effect of the potassium phosphite applications on bacterial (Ct), phosphorus (P) and potassium (K) content in 'Ponkan' tangerine leaves is shown in Table 1. According to the results, there was variation in the population of the bacterium *Candidatus* Liberibacter spp. according to phosphite application. At the time of the first evaluation a Ct average value of 18.4±1.2 was found, indicating a high concentration of bacterium nucleic acids in the sample. In a new evaluation, after 25 days of treatment, the Ct mean value (18.3±07) remained stable. In the third evaluation, there was an increase of 14.8% in the value of Ct (21.6±2.2) when compared to the value observed in the first evaluation, and an increase of 15.3% in relation to the value of Ct verified in the second evaluation (Figure 2).

'Ponkan' tangerine leaves had a phosphorus content of 1.63 ± 0.10 g kg^{-1} in the first application of potassium phosphite on dose of 2.5 L ha^{-1}. Those value increased up 1.73±0.11 g kg^{-1} in the second application after 25 days, although the difference between the contents of the first and second application was not significant, varying in 5.8%. In the third application, there was a significant increase in P content which reached 2.13±0.10 g kg^{-1}, representing a percentage gain of 23.5 and 18.8% in relation to the first and second applications, respectively (Figure 3).

There was no significant difference in the potassium content of 'Ponkan' leaves in the first (8.8±0.78 g kg^{-1}) and second applications (9.4±0.20 g kg^{-1}), and percentage change of 6.4% was recorded. On the other hand, the contents of this nutrient increased by 10.5% with the third potassium phosphite application, reaching 10.5±0.15 g kg^{-1} (Figure 4).

By means of principal component analysis, it was verified that the three original variables (Ct, P and K) are in the same dimension (CP_1) with 83.15% of representativeness of total variance, which indicates the existence of an expressive correlation between these variables and the first main component. In fact, for the bacterial population, represented by Ct, a high correlation was verified (r = 0.86); and also to phosphorus (r = 0.94) and potassium contents, which had a correlation coefficient of r = 0.93 (Figure 5).

DISCUSSION

Phosphites have indirect action on pathogens control, stimulating phytoalexins production, a natural substance

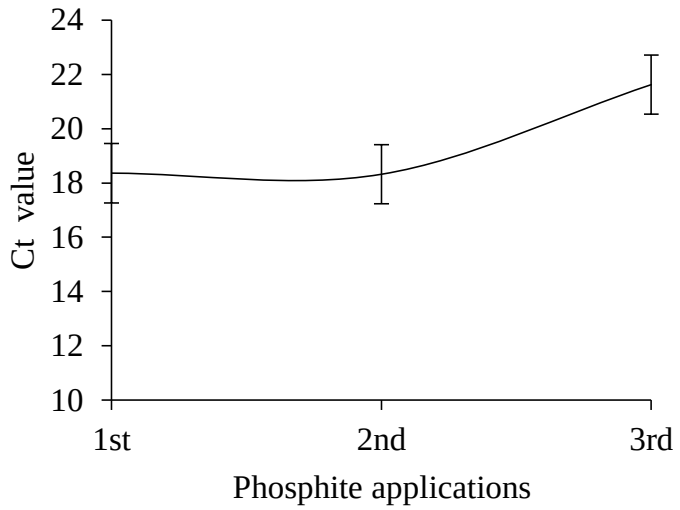

Figure 2. Ct values obtained from qPCR analyzes in 'Ponkan' tangerine leaves under applications of potassium phosphite in Pirangi, SP, Brazil during 2014.

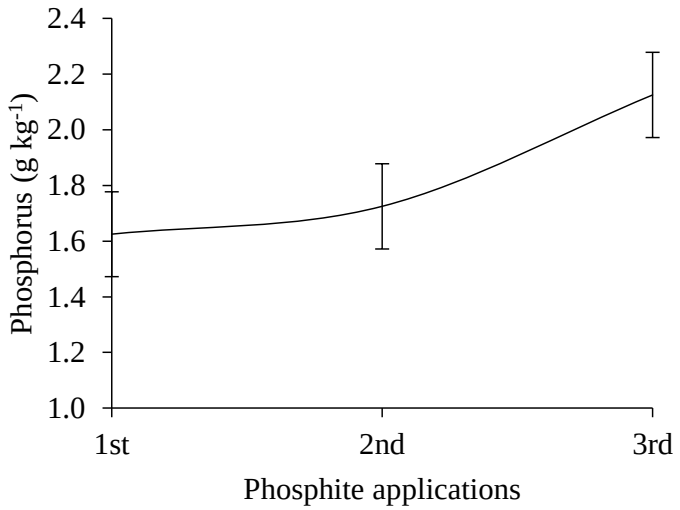

Figure 3. Phosphorus content in 'Ponkan' tangerine leaves under potassium phosphite applications in Pirangi, SP, Brazil during 2014.

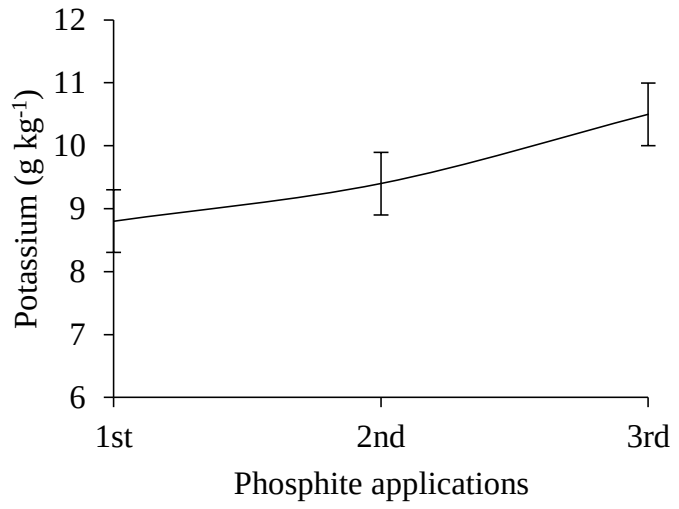

Figure 4. Potassium content in 'Ponkan' tangerine leaves under potassium phosphite applications in Pirangi, SP, Brazil during 2014.

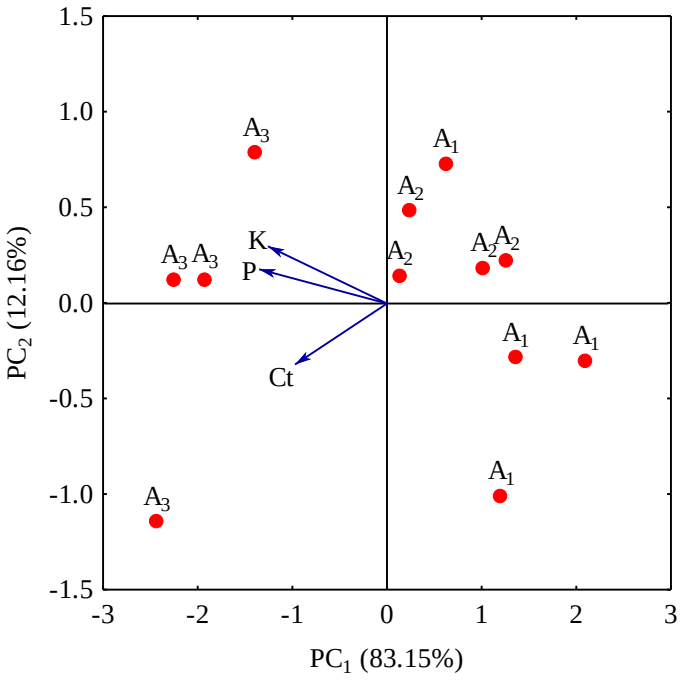

Figure 5. Two-dimensional projection of phosphorus and potassium contents and bacterial population in 'Ponkan' tangerine leaves under potassium phosphite applications in Pirangi, SP during 2014.

of self-defence of plants (Dercks and Creasy, 1989). The indirect effects of these products were reported by Lovatt (1990) who observed that citrus seedlings presenting symptoms of phosphorus deficiency, treated with foliar applications of potassium phosphite, exceeded these symptoms, restoring plant growth. Foliar applications of phosphite during pre-flourishing in 'Valencia' orange plants increased flower numbers, productivity and soluble solids content in fruits (Albrigo, 1997). Stansly et al. (2013) evaluated an orchard for four years with a significant contribution in the plant production when the foliar program was employed, which is most commonly adopted by farmers in Florida together with the intensive control of the vector.

Several studies show differences in the improvements provided by the phosphite application. In fact, Gottwald et al. (2012), working with nutritional treatments containing potassium phosphite, did not observe effect of the nutritional treatments on the bacterium *Candidatus*

Liberibacter spp. when compared to the control treatment in citrus plants. Ratifying this information, Johnson et al. (2013) also showed that the bacterial population in plants that received the nutritional management was similar to those that received the traditional nutrition.

It is important to note that in this research, there occurred population dynamics variation of *Candidatus* Liberibacter spp., Ct values that presented average of close to 20.0. Ct values that were close to this order are often found in leaves diagnosed with HLB in Florida (USA), and the range of 18.0 <Ct <30.0 is more common for more than 90% of the samples analyzed (McCollum et al., 2013). Therefore, we may infer that new researches with greater number of applications and evaluations are preponderant for better elucidation of the population dynamics of the bacterium in citrus plants.

In this study, the increases in P levels recorded are due to the supply of this nutrient via foliar and possible residual effect in the soil, as well as consequent root absorption, providing plants greater capacity in absorption and distribution of the nutrient, as far as new applications are applied (Boaretto et al., 2003). Increasing P is so important, because this nutrient is an essential cellular constituent for nucleic acids, phosphoproteins, phospholipids and ATP synthesis (Hammond and White, 2008). Increases of P contents in citrus were reported by Zhao et al. (2013) that verified the reestablishment of P levels in plants with severe HLB symptoms under foliar phosphorus applications. In a complementary sense, successive applications can be adopted by virtue of mobility and consequent translocation to plant organs where it will be used (Taiz and Zeiger, 2013).

Potassium acts as a regulator of the cellular osmotic potential, besides acting as an enzymatic activator on photosynthetic process and cellular respiration. Generally, due to its easy mobility in the plant, the nutrient is translocated to younger leaves, which justifies its foliar supplementation because part of this nutrient is compromised in older leaves as a result of disease severity. Increasing K content may also reflect improvements in the production quality, since this element influences the size, thickness of the bark, acidity and soluble solids content in fruits (Mattos Jr. et al., 2004; Marini et al., 2005; Taiz and Zeiger, 2013).

Potassium supplementation via foliar and its consequent increase in leaves is benefical for the plant, even under infection caused by *Candidatus* Liberibacter spp., because potassium is directly linked to stomatic movements, especially in the regulation and turgidity maintenance of guard cells, in a way that stoma opening depends on water absorption by these cells. This process is possible starting from K^+ ions accumulation; a fact that contribute to increased osmotic potential and to favour water entering in stomatal cells, facilitating stomatal orifice opening (Brodribb and Holbrook, 2003; Chaerle et al., 2005; Lemos et al., 2012; Morgan et al., 2016).

Conclusion

Candidatus Liberibacter spp. population in leaves of 'Ponkan' tangerine (*Citrus reticulata* Blanco cv. Ponkan) was reduced and phosphorus and potassium contents increased with application of 2.5 L ha^{-1} of potassium phosphite on leaves. Potassium phosphite is an alternative for reducing the bacterial population in orchards infected by huanglongbing. However, the Ministry of Agriculture, Livestock and Supply (Map) published in the Official Gazette of the Union, normative instruction obliging citrus farmers throughout the country to eradicate plants with symptoms of huanglongbing.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

Authors thank National Council of Scientific and Technological Development - CNPq and Coordination of Improvement of Higher Level Personnel - CAPES for granting a PhD scholarship to the first author; To the Graduate Program in Agronomy (Plant Production) of the Paulista State University - UNESP; and KeyPlex Company.

REFERENCES

Aksenov AA, Pasamontes A, Peirano DJ, Zhao W, Dandekar AM, Fiehn O, Ehsani R, Davis CE (2014). Detection of huanglongbing disease using differential mobility spectrometry. Anal. Chem. 86:2481-2488.

Albrigo LG (1997). Foliar application of major elements for flowering and fruit set. Indian River Citros Seminar, 50th Anniversary.

Barbosa JC, Maldonado JW (2015). Experimentação agronômica & agroestat - sistema para análises estatísticas de ensaios agronômicos. Fune P 396.

Bataglia OC, Furlani AMC, Teixeira JPF, Furlani PR, Gallo JR (1983). Método de análise química de plantas. Technical Bulletin, Agronomic Institute, Campinas, 78 p.

Boaretto AE, Muraoka T, Boaretto RM (2003). Absorption and translocation of micronutrients applied to orange leaves. Laranja 24:177-197.

Brodribb TJ, Holbrook NM (2003). Stomatal closure during leaf dehydration. correlation with other leaf physiological traits. Plant Physiol. 132:2166-2173.

Chaerle L, Saibo N, Straenten DVD (2005). Tuning the pores: towards engineering plants for improves water use efficiency. Trends in Biotechnol. 23:308-315.

Choi D, Lee WS, Ehsani R (2013). Detecting and counting citrus fruit on the ground using machine vision. ASABE Paper No. 131591603. St. Joseph, Mich.: ASABE. 6 p.

Costa BHG, Resende MLV, Ribeiro JPM, Mathioni SM, Pádua MA, Silva JMB (2014). Suppression of rust and brown eye spot diseases on coffee by phosphites and by-products of coffee and citrus industries. J. Phytopathol. 1:1-8.

Cunha SAP, Magalhães AFJ, Silva SAS, Passos OS, Soares FWS (2013). Cultura dos citros, Brazilian Agricultural Research Corporation, Embrapa Mandioca and Fruticulture, Ministry of Agriculture, Livestock and Supply. Embrapa, Brasília, 1.

Dercks W, Creasy LL (1989). Influence of fosetyl – Al on phytoalexin accumulation in the Plasmopara viticola-grapevine interaction. Physiol. Mol. Plant Pathol. 34:203-213.

Dianese AC, Blum LEB (2010). Uso de fosfitos no manejo de doenças fúngicas de fruteiras e soja. Brasília: EMBRAPA, 29 p.

Gottwald TR, Graham JH, Irey MS, McCollum TG, Wood BW (2012). Inconsequential effect of nutritional treatments on huanglongbing control, fruit quality, bacterial titer and disease progress. Crop prot. 36:73-82.

Hair FJ, Black WC, Babin BJ, Anderson RE, Tatham, Ronald L (2009). Análise multivariada de dados. Tradução Adonai Schlup Sant'Anna. 6. ed., Porto Alegre, Bookman.

Hammond JP, White PJ (2008). Sucrose transport in the phloem: integrating root responses to phosphorus starvation. J. Exp. Bot. 59:93-109.

Johnson EG, Irey MS, Gast T, Bright DB, Graham JH (2013). Evaluation of enhanced nutritional programs for mitigating HLB damage. In: 3rd International Conference on Huanglongbing. 2013 Orlando. Proceedings... IRCHB III, 114 p.

Lemos LMC, Siqueira DL, Salomão LCC, Cecon PR, Lemos JP (2012). Características físico-químicas da laranja-pera em função da posição da copa. Braz. J. Frut. 34:1091-1097.

Lopes JMS, Déo TFG, Andrade BJM, Giroto M, Felipe ALS, Junior CEI, Bueno CEMS, Silva TF, Lima FCC (2011). Importância econômica dos citros no Brasil. Electron. J. Agron. 10:20.

Lovatt CJ (1990). A definitive test to determine whether phosphite fertilization can replace phosphate fertilization to supply P in the metabolism of 'Hass' on 'Duke 7'. Calif. Avoc. Soc. Yearb. 74:61-64.

Marini FS, Marinho CS, Muniz, RA, Carvalho AJC, Monnerat PHM (2005). Doses de sulfato de magnésio em substrato orgânico sobre o crescimento e nutrição de porta-enxertos cítricos. Laranja, 26:135-149.

Mattos JRD, Quaggio JA, Cantarella H, Carvalho SA (2004). Modelos de resposta do tangor Murcott à fertilização com N, P e K. Braz. J. Frut. 26:164-167.

McCollum G, Hilf M, Irey M (2013). Relationship between Ct values, HLB symptoms and CLas titer. In: 3rd International Conference on Huanglongbing. 2013 Orlando. Proceedings... IRCHB III, 18.

Morgan KT, Rouse RE, Ebel RC (2016). Foliar applications of essential nutrients on growth and yield of 'Valencia' sweet orange infected with huanglongbing. Hort. Sci. 51:1482-1493.

Pourreza A, Lee WS, Raveh E, Ehsani R, Etxeberria E (2014). Citrus huanglongbing detection using narrow-band imaging and polarized illumination. Amer. Soc. Agric. Biol. Eng. 57:259-272.

Stansly P, Arevalo A, Qureshi Q, Jones MM, Hendricks K, Roberts PD, Roka FM (2013). Vector control and foliar nutrition for management of huanglongbing in Florida Citrus. In: 3rd International Conference on Huanglongbing. 2013 Orlando. Proceedings... IRCHB III, 113.

Taiz L, Zeiger E (2013). Fisiologia vegetal. 4a ed. Trans. Eliane Romanato Santarém et al. Porto Alegre: Artmed, 848.

Wang Z, Yin Y, Hu H, Yuan Q, Peng G, Xia Y (2006). Development and application of molecular-based diagnosis for '*Candidatus* Liberibacter asiaticus', the causal pathogen of citrus huanglongbing. Plant Pathol. 55:630-638.

Zhao H, Sun R, Albrecht U, Padmanabhan C, Wang A, Coffey MD, Girke T, Wang Z, Close TJ, Roose M, Yokomi RK, Folimonova S, Vidalakis G, Rouse R, Bowman KD, Jin H (2013). Small RNA profiling reveals phosphorus deficiency as a contributing factor in symptom expression for citrus huanglongbing disease. Mol. Plant. 6:301-310.

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Vol. 12(10), pp. 254-262, 14 March, 2018
DOI: 10.5897/AJMR2017.8786
Article Number: F37BE5E56336
ISSN 1996-0808

http://www.academicjournals.org/AJMR

African Journal of Microbiology Research

Full Length Research Paper

Evidence of curtovirus competition and synergy in co-infected plant hosts

Stephen A. Peinado Jr.[1,3], Jorge Achata Böttger[1,4], Li-Fang Chen[2], Robert Gilbertson[2] and Rebecca Creamer[1*]

[1]Department of Entomology, Plant Pathology, and Weed Science, New Mexico State University, Las Cruces, NM, Mexico.
[2]Department of Plant Pathology, University of California, Davis, CA, USA.
[3]School of Veterinary Medicine, University of Wisconsin, Madison, WI, Canada.
[4]National Fund for the Development of Science, Technology and Technological Innovation, Lima, Peru.

Received 19 December, 2017; Accepted 24 February, 2018

Curtoviruses, members of the Geminiviridae, have wide host ranges, including weeds and crops and are often found in mixed infections of different strains. While other members of the Geminiviridae have been demonstrated to interact through competition and synergism in mixed infections in plants, either type of interaction has not been reported in curtoviruses. This research used qPCR to study the interactions between *Beet curly top virus*, pepper curly top strain, isolate BV3 (BCTV-PeCT-BV3) and *Beet curly top virus*, beet severe curly top strain (BCTV-Svr) in three plant hosts. A significant decrease in virus titer in both BCTV-PeCTV and BCTV-Svr in co-infected sugar beets was observed when compared to beets infected with either virus, indicating competition. Chile pepper showed a significant increase in BCTV-PeCT titer in co-infected plants, compared to singly infected plants, indicating synergism. BCTV-PeCT caused severe symptoms and yielded high virus titer in chile, compared to the lack of symptoms and extremely low titer of BCTV-Svr in that plant host. These results indicate that curtovirus symptoms and infection can be host specific and such host may influence mixed infections of virus. Curtoviruses can interact through both competition and synergism and the response may be dependent on the type of host plant.

Key words: *Beet curly top virus*, interspecific competition, host specificity, mixed infections

INTRODUCTION

Curly top disease is caused by members of the genus *Curtovirus*, within the family *Geminiviridae* (Bennett, 1971; Soto et al., 2005; Varsani et al., 2014). Curtoviruses are transmitted among dicot hosts by beet leafhopper [*Circulifer tenellus* (Baker)] in a circulative persistent manner (Soto and Gilbertson, 2002). Curtovirus host range is broad and includes crops as chile pepper [*Capsicum annuum* (L.)], tomato [*Solanum*

*Corresponding author. E-mail: creamer@nmsu.edu. Tel: 575-646-3068; 575-646-8087.

lycopersicum (L.)] and sugar beet [*Beta vulgaris* (L.)] as well as weeds such as London rocket [*Sisymbruim irio* (L.)], Russian thistle [*Salsola iberica* (Sennen and Pau)], and Kochia [*Kochia scoparia* (L.) Roth] (Creamer et al., 2005; Lam et al., 2009). In the field, infected weeds are usually asymptomatic while crops develop severe symptoms (Lam et al., 2009). Symptoms of curtovirus-infected chile pepper plants are stunting, chlorosis, thickened curled leaves, hyperplastic phloem growth, and reduced fruit set (Creamer et al., 2003), while curtovirus symptoms on sugar beets include stunting, chlorosis, severe leaf curling, and production of punctate growths on leaf veins (Bennett, 1971).

Co-infections with two or more viruses at the same time are commonly found in field samples (Roossinck, 2005) and can be beneficial to at least one of the viruses or antagonistic, where the co-infection is deleterious to one of the viruses. Co-infection is known to affect symptomology, host range, pathogen diversity, transmission rates, and infection prevalence (Lacroix et al., 2014). Since virus replication is an immediate indicator of competition for resources, changes in virus titer has been used to experimentally demonstrate competition between viruses (Roossinck, 2005; Hall and Little, 2013; Salvaudon et al., 2013).

Synergy between virus species is known to occur within the same genus and across virus families. Synergy has been reported between *Cucumber mosaic virus* (CMV) and potyviruses in cucurbits (Wang et al., 2002), potyviruses and *Potato virus X* (PVX) (Bowman-Bance 1991; Gonzalez-Jara et al., 2004), *Potato leafroll virus* and *Potato virus Y* (PVY) (Srinivasan and Alvarez, 2007) and among begomovirus species (Rentería-Canett et al., 2011).

Changes in virus transmission from co-infected hosts could also be an indicator of competition. Joint infection of two criniviruses, *Tomato infectious chlorosis virus* (TICV) and *Tomato chlorosis virus* resulted in the increased concentration and higher transmission efficiency of TICV (Wintermantel et al., 2008).

Order in which viruses are acquired can influence transmission, in that sequential transmission of two viruses, can favor transmission of one virus over another. For example, when whitefly vectors were allowed to acquire two strains of *Tomato yellow leaf curl virus* sequentially, 75% of the whiteflies transmitted both strains of the begomovirus and 25% only the first strain that they acquired (Ohnishi et al., 2011).

Host specificity also plays a role in curtovirus infection. *Beet curly top virus* spinach curly top strain (BCTV-Sp) infects a wide host range including spinach, sugar beet, bean and *Nicotiana benthamiana*, but not tomato or Samsun tobacco (Baliji et al., 2004). *Beet curly top virus* Worland strain (BCTV-Wor) causes mild symptoms on sugar beet, but severe symptoms on a variety of dicot species including tomato and pepper (Stenger et al., 1990). In contrast BCTV-Svr causes severe symptoms on sugar beet and mild symptoms on most other hosts (Bennett et al., 1971).

Mixed infections of curtovirus are common (Stenger and McMahon, 1997; Strausbugh et al., 2008; Lam et al., 2009; Chen et al., 2010). Mixed infections of curtoviruses from sugar beet nursery samples showed that, more than one strain or type was easily identified in mixed infections (Stenger and Ostrow, 1996). In New Mexico, *Beet curly top virus*, severe pepper strain (BCTV-SvrPep), BCTV-PeCT, *Beet curly top virus*, Worland strain (BCTV-Wor), and *Beet curly top virus*, pepper yellow dwarf strain (BCTV-PeYD) are prevalent in chile peppers, often in mixtures (Lam et al., 2009; Varsani et al., 2014). Preliminary research on serial acquisition of two of these viruses showed that once leafhoppers acquired BCTV-Svr, they could no longer acquire BCTV-PeCT. These results prompted a series of experiments designed to assess the potential for intrahost interaction as measured by changes in virus titer in crops and weed hosts.

MATERIALS AND METHODS

Plants, viruses, and leafhopper sources and maintenance

Plants used for greenhouse experiments were grown from seed. Plants used were Chile (*Capsicum annuum)* var. NM 6-4, which is susceptible to curtoviruses, Sugar beet [*Beta vulgaris* (L.)] highly susceptible to curtoviruses and a host of the beet leafhopper, grown from seed that was a gift of Robert Lewellen, USDA, Salinas, CA, USA and Kochia [*Kochia scoparia* (L.) Roth], a weed, known to be susceptible to curtoviruses and a host of the beet leafhopper, grown from seed collected near Las Cruces, NM, USA.

Beet leafhoppers were collected from Idaho (gift from Carl Strausbaugh, Kimberly, ID, USA) and maintained on BCTV-Svr-infected sugar beets and Kochia in a growth chamber at 29°C with 15 h of light.

BCTV-Svr(US-SVR-Cfh), abbreviated in this paper as BCTV-Svr was obtained from Idaho (gift of Carl Strausbaugh) and was maintained on sugar beets. BCTV-PeCT (US-NM-Pep-05) was collected from infected sugar beets near Las Cruces, NM, maintained on sugar beets, and used only for preliminary studies. BCTV-PeCT (US-CA- BV3) (GenBank JX487184.1) abbreviated in this paper as BCTV-PeCT-BV3, was originally isolated from tomato in California by Chen and Gilbertson (2009). This isolate was used for all but in the initial experiments. All strain designations were confirmed by PCR and sequencing prior to their use (Lam et al., 2009).

Agroinoculation of plants with BCTV-PeCT-BV3

Agro inoculation was used to infect plants with BCTV-PeCT-BV3 because leafhoppers are not able to acquire the virus from chile plants due to the toxicity of the plants to the insects (Sedano et al., 2012). An infectious clone (EHA105-BV3) of BCTV-PeCT isolate BV3 was produced by inserting a tandem dimeric construct into a binary vector pCGN1547 (Chen and Gilbertson, 2009). The clone was maintained in *Agrobacterium tumefaciens*. Cultures were incubated at 27°C for a minimum of 2 h prior to inoculation (Sedano et al., 2012).

Sugar beet, chile, and Kochia were planted in Metro-Mix 360 (Sun Gro Horticulture), maintained in an insect-free greenhouse and inoculated 10 to 15 days after germination by vascular puncture with 4 µl of culture containing the clone (Sedano et al.,

2012). After inoculation, plants were incubated in a growth chamber at 28°C with 15 h of light for 2 days, and then transferred to a greenhouse for 14 days.

Leafhopper transmission of BCTV-Svr

Plants were inoculated with BCTV-Svr by exposing them to feeding by, five adult leafhoppers that were reared on BCTV-Svr-infected sugar beets. Insects were inserted in leaf cages and allowed to feed for 16 to18 h. Plants were incubated 14 days in a growth chamber at 28°C with 15 h of light.

Plants previously inoculated with BCTV-PeCT-BV3 were inoculated with BCTV-Svr after 14-day recovery incubation in the greenhouse. Plants were not assessed for virus presence after the initial inoculation and prior to the second inoculation due to their small size.

Primer design

Primers were developed that amplify the viral coat protein genes (CP) of BCTV-PeCT-NM (GenBank EF501977.1), BCTV-PeCT-BV3 (GenBank JX487184.1) and BCTV-Svr (Genbank U02311.1). The primer set PeCTVIIF (5'-GGAGTGCGTCGAGAGAACAAAC-3') and PeCTVIIR (5'-GCTTAGTAACGGTTATATTGTTGG-3') amplify a 400 bp fragment of the BCTV-PeCT CP that was used for detection by conventional PCR. CPIIF (5'-GTATCCATCAAGAGATAGAG-3') and CPIIR (5'-CGTCACAGTAACGTTCTTC-3') amplify a 402 bp CP fragment from BCTV-Svr which was used for both conventional and qPCR. PeCTVIIF and BV3R (5'-CCCTCGTGAGAGGACGT-3') amplify a 254 bp CP fragment from BCTV-PeCT-BV3 that was used for qPCR.

Virus detection and quantification by PCR

To determine if plants were infected with curtovirus, total DNA was extracted from young plant tissue, 14 days post infection (dpi) by alkaline lysis (Dellaporta et al., 1983) and quantified with Nanodrop ND-1000 (Thermo Scientific).

PCR reactions were carried out using GoTaq Flexi DNA Polymerase (Promega), following the manufacturer's guidelines. PCR parameters were as follows for CPIIF-CPIIR and PeCTVIIF-BV3R reactions: 94°C for 3 min, followed by 35 cycles consisting of 94°C for 30 s, 55/65°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min.

Virus titer was determined for all infected plants using quantitative PCR. Two independent groups of plants were incubated for 28 days post inoculation (dpi) and 56 dpi to allow symptom development before DNA extraction. BCTV-PeCT-BV3 and BCTV-Svr titers from plants infected with both viruses were quantified in independent qPCR reactions.

qPCR parameters were as follows: 94°C for 3 min, followed by 35 cycles consisting of 94°C for 30 s, 55°C (CPIIF/CPIIR) or 56.5°C (PeCTVIIF/BV3R) for 30 s, 30 s at 72°C for 30 s, followed by a final 72°C for 10 min. The temperature range for melting curve analyses was from 55 to 95°C rising 0.5 °C for 0.5 s for both primer pairs. Reactions were carried out in an IQ5 thermocycler (Bio Rad) using IQ SYBR green supermix (Bio Rad) at a final reaction volume of 20 µL with 2 µL of template and 0.5 µM primers.

The qPCR amplification products were used as reference amplicons for standard curves. After electrophoresis on an agarose gel, BCTV-Svr and BCTV-PeCT-BV3 PCR amplicons were extracted with a QIAquick gel extraction kit (Qiagen) and virus DNA concentration determined. Virus titer was calculated through the amplification of a five fold dilution series of viral DNA for BCTV-PeCT-BV3 from 1.027×10^7 ng/µL to 3.286×10^3 ng/µL) and BCTV-Svr from $8.020x10^6$ ng/µL to $2.566x10^3$ ng/µL.

BioRad IQ5 software automatically calculated the fluorescence threshold and a regression line was calculated with the threshold cycle ($C_{(t)}$) of serial dilution of standards. This equation used the molecular weight of curtovirus genomes to estimate the virus copy number per nanogram of total DNA in the samples.

Statistical analysis

SAS 9.2 (SAS Institute Inc., Cary, NC, USA 1989-2007) was used to conduct a regression analysis of qPCR standard curve amplification $C_{(t)}$ values and to compare the virus titer in infected plants. Simple linear regression was used by BioRad IQ5 software to produce a linear equation that relates RFU to $\Delta(C_t)$. The same data was used to produce a linear regression analysis in SAS and calculate the confidence intervals of the regression at α=0.05.

Normal distribution of virus titre data sets was tested by Kolmogorov-Smirnov goodness of fit test. Homogeneity of variance between data sets was tested with Levene's test. Correlation between BCTV-PeCT-BV3 and BCTV-Svr titers in co-infected beets and chile were tested, using the Spearman's non-parametric correlation analysis. A two-way factorial ANOVA was run to determine the relationship between the virus titer, virus species and infection status on sugar beets using JMP (SAS Institute Inc., Cary, NC, USA 1989-2007). Pairwise comparison between virus titers in co-infected chile plants was done using Kruskal-Wallis rank sum test. The significance level for all the analyses was set at α=0.05.

RESULTS

When initial leafhopper transmission tests were done by allowing leafhoppers carrying BCTV-Svr to acquire BCTV-PeCT-NM from infected sugar beets, and transmit to either sugar beets or chile, only BCTV-Svr was transmitted. The results were identical independent of the number of leafhoppers/plant (5 or 1) used for transmissions or source sugar beet plant (four attempted) used.

BCTV-PeCT-BV3 and BCTV-Svr were quantified using qPCR. Plants infected by a single virus were used to establish a titer baseline to determine if, co-infection influenced viral titer in sugar beet, chile, and kochia (Table 1). Melting curve analysis of the amplicon obtained from BCTV-PeCT-BV3 gave a primary peak at 84°C while that of BCTV-Svr gave a primary peak at 82°C (Figure 1). Neither amplicon produced secondary peaks indicating that, there was no primer dimer formation or nonspecific amplification. Quantification of BCTV-PeCT-BV3 was effective within the range of $1.027x10^7$ to $1.6432x10^4$ (r^2=0.995), while BCTV-Svr was quantified within the range of $8.020x10^7$ to $1.2832x10^4$ (r^2=0.994) (Figure 2).

Virus titer was significantly linked to both infection status and virus species ($p \leq 0.0001$), where the infection status is defined as a plant being infected by one virus alone or in combination of both (Supplementary Table 1S). While 22 out of 28 sugar beet plants pre-inoculated with BCTV-PeCT-BV3 were positive for both viruses, the remaining six did not become infected with BCTV-Svr. BCTV-PeCT-BV3 titer was significantly different in co-

Table 1. Range of viral titers based on infection type and host.

Virus	Infection	Host	Number infected plants/total	Titer log_{10} copies/ng	
				Range	Average ±SEM
BCTV-PeCT	Single	Beet	10/10	3.50 - 4.70	4.25±0.2
BCTV-Svr	Single	Beet	10/11	5.79 - 7.52	6.70±0.2
BCTV-PeCT	Mixed	Beet	22/28	1.72 – 4.00	3.28±0.1
BCTV-Svr	Mixed	Beet	22/28	3.37 – 6.74	5.47±0.1
BCTV-PeCT	Single	Chile	15/30	4.99 – 5.34	4.71±0.1
BCTV-Svr	Single	Chile	4/11	-	BT
BCTV-PeCT	Mixed	Chile	8/15	4.22 - 5.15	5.14±0.0
BCTV-Svr	Mixed	Chile	8/15	-	BT
BCTV-PeCT	Single	Kochia	8/384	-	BT
BCTV-Svr	Single	Kochia	3/10	-	BT
BCTV-PeCT	Mixed	Kochia	0/8	-	-

SEM=standard error of the mean, BT= below quantification threshold, BCTV-PeCT=BCTV-PeCT-BV3.

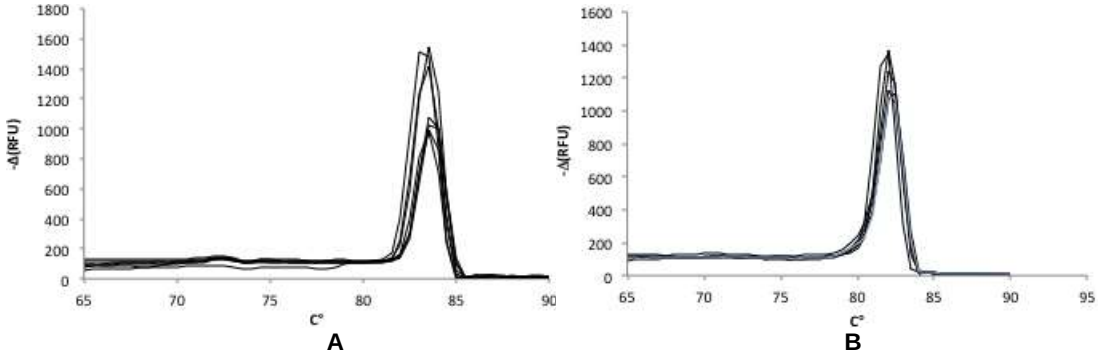

Figure 1. Melting curve analysis of (a) BCTV-PeCT-BV3 amplification indicating the melting point at 84°C (upper) and (b) BCTV-Svr amplification indicating the melting point at 82°C (lower).

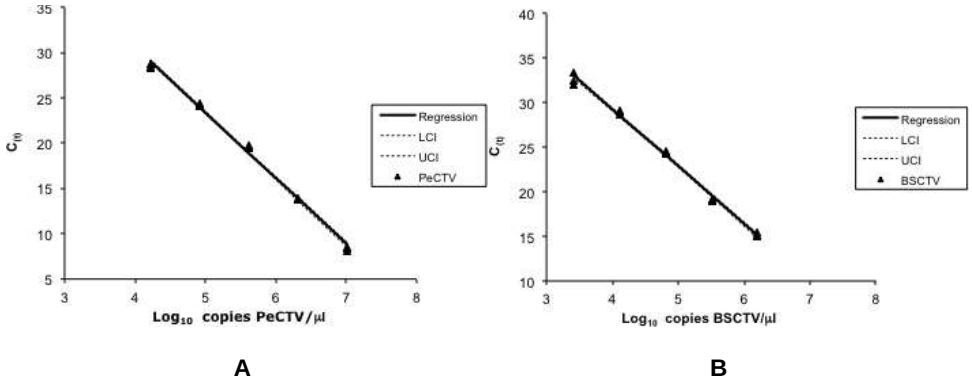

Figure 2. Representative qPCR standard for (a) BCTV-PeCT-BV3 (PeCTV) (upper) and (b) BCTV-Svr (BSCTV) (lower).

infected plants (2.8838×10^3 copies/ng) (*p*-value =0.0020) when compared to singly infected plants (2.6504×10^4 copies/ng) (*p*-value ≤0.0001). BCTV-Svr titer was significantly different in co-infected plants (1.1365×10^6

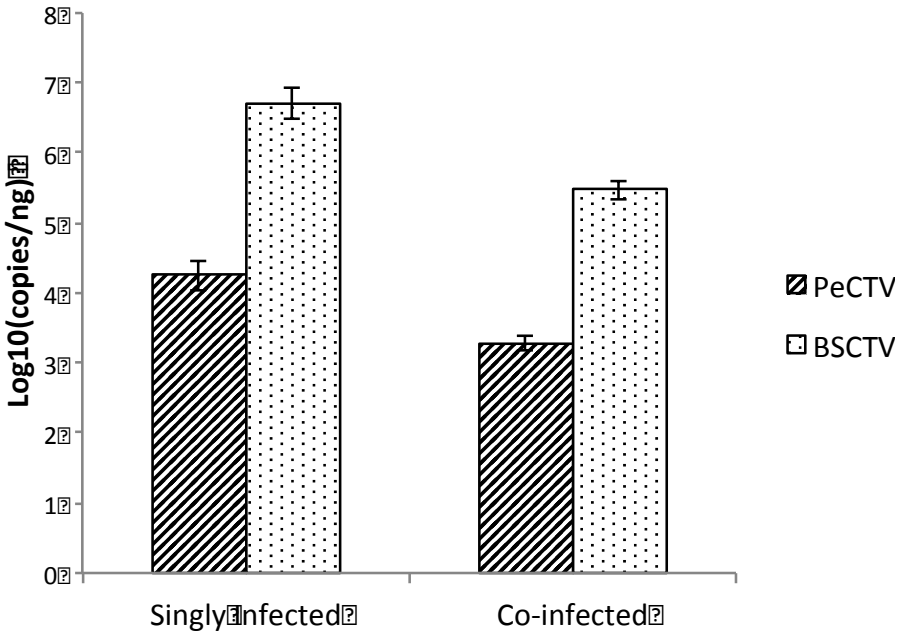

Figure 3. Comparison of average virus titer for BCTV-PeCT (PeCTV) and BCTV-Svr (BSCTV) in singly and co-infected beet plants α=0.05.

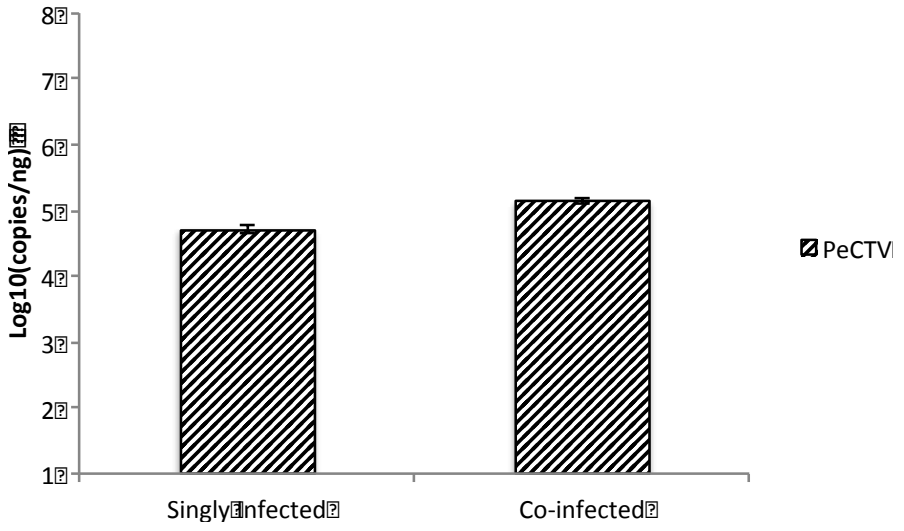

Figure 4. Comparison of average titer for BCTV-PeCT (PeCTV) in singly and co-infected chile plants. α=0.05.

copies/ng) when compared to singly infected plants (1.1806×10^7 copies/ng) (p-value ≤0.0001) (Figure 3).

In contrast, 8 out of 15 chile plants pre-inoculated with BCTV-Pect-BV3 were positive for both viruses. BCTV-PeCT-BV3 titer was lower in singly infected plants (5.8642×10^4 copies/ng) when compared to co-infected plants (1.4541×10^5 copies/ng) (p=0.0003, DF=1, χ^2=13.0538) (Figure 4). Chile plants infected with BCTV-Svr alone or with both BCTV-Svr and BCTV-PeCT-BV3 tested positive for BCTV-Svr with PCR but fell below the quantification threshold in qPCR. Chile plants infected with BCTV-PeCT-BV3 alone or in a mixed infection exhibited severe infection symptoms, and no change in symptoms was observed when infected with both viruses.

While over 380 Kochia seedlings were inoculated with BCTV-PeCT-BV3 infectious clone, only 8 plants tested positive for BCTV-PeCT-BV3, and none of these were infected with BCTV-Svr after leafhopper inoculation. The BCTV-PeCT-BV3 titer in these eight plants fell below the quantification threshold. Infection with BCTV-Svr alone

was determined in three Kochia plants by PCR, but the estimated virus titer was below the quantification threshold by qPCR.

DISCUSSION

A variety of factors influence the infection of a plant host by more than one virus. Mechanism of transmission, host range, order of infection, and viral interactions can affect, if a plant will be infected, the titer of the viruses, and the symptoms induced. This study views the effect of co-inoculation of curtoviruses onto different hosts. Presence of BCTV-PeCT-BV3 in chile pepper and beets had a significant impact on the likelihood of the host to be infected by BCTV-Svr. In beets infected with both viruses, the titer of both was lower when compared to plants infected by either one. The BCTV-Svr titers in plants infected with BCTV-Svr and BCTV-PeCT-BV3 were 1 to 2 orders of magnitude lower than those of plants infected with BCTV-Svr alone. This is similar to the preliminary results of Wintermantel (2011) which found BCTV-Svr titer alone in beets for at least 100 times higher than in plants infected with both BCTV-Svr and BCTV-Wor. The decrease in titer of both viruses in co-infected beets is suggestive of within-host competition, which had not been reported for BCTV-PeCT.

Viral interactions between species have been shown to change infection rates of each viral species compared with single species inoculations for closely related potyviruses and luteoviruses, but rarely for geminiviruses. Alves et al. (2009) found that *Tomato yellow spot virus* (ToYSV) established infection and accumulated to higher concentration earlier than *Tomato rugose mosaic virus* (ToRMV) in tomato and tobacco. Interestingly, ToRMV appears to interfere with ToYSV only during early infection. Mixed infections of potyviruses, *Watermelon mosaic virus* (WMV) and *Zucchini yellow mosaic virus* (ZYMV) were asymmetrical in that, while ZYMV replicated similarly in single and mixed infections, WMV accumulated to significantly lower levels in the presence of ZYMV than in single infections (Salvaudon et al., 2013). Studying mixed infections of luteoviruses, Lacroix et al. (2014) found that BYDV-PAV reduced the infection rate of CYDV-RPV, while CYDV-RPV had no effect of the infection rate of BYDV-PAV. Hall and Little (2013) found that BYDV-PAV dominated all mixed infections of wheat with BYDV-PAS, independent of the order of inoculation or length of time between inoculations. BYDV-PAV was also more likely to be aphid transmitted from a mixed infection. The authors speculated that the strength of the cross protections might be related to dose of the first virus or rate of movement through the phloem from site of inoculation.

Order of inoculation appeared to have an effect only in the preliminary experiments. For those experiments, allowing leafhoppers to acquire BCTV-Svr first appeared to interfere with acquisition of BCTV-PeCT-NM. However, for the balance of the experiments, order of inoculation did not appear to have had an affect on level of virus in our experiments, although it was not specifically tested. BCTV-PeCT-BV3 was inoculated first onto beets and was still dominated by BCTV-Svr in the mixed infection on this plant.

Inoculum delivery could have potentially been a factor in our results since BCTV-PeCT-BV3 was inoculated by agro inoculation and BCTV-Svr was inoculated using leafhoppers. However, Chen and Gilbertson (2009) showed that both inoculation methods, agroinoculation and leafhopper transmission, gave similar results when inoculating BCTV-Svr onto a range of plants.

Our study also shows evidence of viral synergy. In chile, BCTV-PeCT-BV3 titers were higher in the presence of BCTV-Svr compared with chile infected with BCTV-PeCT-BV3 alone. In contrast, in beets, both viruses showed decreases in virus titer in mixed infections. Synergism has been reported to occur with begomoviruses such that when *African cassava mosaic virus* (ACMV) and *East African cassava mosaic virus* (EACMV) co-infect a host, both symptom severity and EACMV titer increased due to trans-complementation by ACMV (Fondong et al., 2000). Rentería-Canett et al. (2011) found that co-infection of *Pepper huasteco yellow vein virus* and *Pepper golden mottle virus* had a synergistic effect that increased disease symptoms as well as the titer of both viruses. Similarly, Morilla et al. (2004) found that co-infection of begomoviruses *Tomato yellow leaf curl virus* and *Tomato yellow leaf curl Sardinia virus* (TYLCSV) increased symptom severity and infected even the same nuclei. BCTV has been shown to have a positive synergistic effect on virus titer (but not the symptom severity) of TYLCSV due to the replication enhancer of BCTV (Caracuel et al., 2012).

Quantification of both virus species in co-infected and singly infected plants showed clear indications that both BCTV-PeCT-BV3 and BCTV-Svr exhibit host specificity. BCTV-Svr titer was significantly higher than BCTV-PeCT-BV3 by three orders of magnitude in beets (Table 1). Chile plants infected with BCTV-Svr by leafhoppers in greenhouse and growth chambers routinely test positive for infection but fail to develop severe symptoms, if any at all (Sedano et al., 2012). In contrast, all chile plants that were infected with BCTV-PeCT-BV3 exhibited symptoms of infection. BCTV-PeCT-BV3 appears to be better adapted to peppers and replicates to high levels in this host. In our laboratory setting, BCTV-Svr was maintained in sugar beets and transmitted by leafhoppers. BCTV-Svr is known to be better adapted to infection of sugarbeets, and causes much more severe symptoms on sugar beets than on other plant hosts (Strausbaugh et al., 2008). BCTV-Svr had a low rate of infection in chile, and did not develop high titers.

Curtovirus species are known to accumulate differently depending on the host plant (Chen and Gilbertson, 2009;

Strausbaugh et al., 2008; Wintermantel, 2011). Chen and Gilbertson (2009) showed that while most cucurbits (cantaloupe, honeydew, watermelon) had low rates of infection and mild or symptomless phenotypes when inoculated with BCTV-Wor, tomatoes and shepherd's purse (a mustard weed) had high rates of infection and were highly symptomatic when inoculated with the same virus. In comparison, all of these plants are likely to be infected and show symptoms when inoculated with BCTV-Svr.

A significant difference in the accumulation of virus in agricultural hosts versus Kochia, a weed host was found in this work. This difference in virus titer between agricultural and weed hosts has been previously observed in a field survey of curtovirus (Creamer et al., 1996), and by Chen and Gilbertson (2009) which found that infected shepherd's purse had about half the titer of BCTV-Wor as the infected by sugar beets. Since Kochia serves as a reservoir host for BCTV, BCTV-Wor, BCTV-SvrPep, BCTV-PeCT, and BCTV-PeYD (Lam et al., 2009) in the wild, we expected it to be easily infected in the laboratory. Both viruses in Kochia could be detected, but well below the quantification threshold. Since beet leafhoppers prefer Kochia as a feeding and reproductive host (Hudson et al., 2010), feeding preference does not account for the very low titers found. The method of inoculation, vascular puncture is destructive to young seedlings. This may have led to the very low numbers of infected plants.

Evidence of synergism, competition, and host preference were reported here. This is the first reports for synergism and competition in curtoviruses, it presents additional evidence for host preference within the virus group. Further studies should focus on the molecular mechanisms underlying these relationships, and the implications for these on mixed infections in field settings.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENT

The authors thank the National Science Foundation funded by New Mexico Alliance for Minority Participation Bridge to the Doctorate program for funding for the senior author.

REFERENCES

Alves M, Alfenas-Zerbini P, Andrade EC, Esposto DA, Silva FN, da Cruz ACF, Ventrella MC, Ottoni WC, Zerbini FM (2009) Synergism and negative interference during co-infection of tomato and *Nicotiana benthemiana* with two bipartite begomoviruses. Virology 387:257-266.

Baliji S, Black MC, French R, Stenger DC, Sunter G (2004). Spinach curly top virus: A newly described curtovirus species form southwest Texas with incongruent gene phylogenies. Phytopathology 94: 772-779.

Bennett CW (1971). The curly top disease of sugar beet and other plants, Monograph 7, American Phytopathological Society, St. Paul, MN.

Bowman-Bance V (1991). Replication of potato virus X RNA is altered in coinfections with potato virus Y. Virology 182:486-494.

Caracuel Z, Lozano-Duran R, Huguet S, Arroyo-Mateo M, Rodriguez-Negre EA, Bejarano R (2012) C2 from *Beet curly top virus* promotes a cell environment suitable for efficient replication of geminiviruses, providing a novel mechanism of viral synergism. New Phytologist 194:846-858.

Chen L, Brannigan K, Clark R, Gilbertson RL (2010). Characterization of Curtoviruses associated with curly top disease of tomato in California and monitoring for these viruses in beet leafhoppers. Plant Dis. 94:99-108.

Chen LF, Gilbertson RL (2009). Curtovirus-cucurbit interaction: Acquisition host plays a role in leafhopper transmission in a host-dependent manner. Phytopathology 99:101-108.

Creamer R, Carpenter J, Rascon J (2003) Incidence of the beet leafhopper, *Circulifer tenellus* (Homoptera: Cicadellidae) in New Mexico chile. Southwestern Enotomol. 28: 177-182.

Creamer R, Hubble H, Lewis A (2005) Curtovirus infection of chile pepper in New Mexico. Plant Dis. 89:480-486.

Creamer R, Luque M, Howo M (1996) Epidemiology and incidence of beet curly top geminivirus in naturally infected weed hosts. Plant Dis. 80: 533-535.

Dellaporta SL, Wood J, Hicks JB (1983) A plant DNA minipreparation: version II, Plant Mol. Biol. Report. 1:19-21.

Fondong VN, Pita JS, Rey MEC, de Kochko A, Beachy RN, Fauquet CM (2000). Evidence of synergism between *Africa cassava mosaic virus* and a new double-recombinant geminivirus infecting cassava in Cameroon. J. Gen. Virol. 81:287-297.

Gonzalez-Jara P, Tenllado F, Martinez-Garcia B, Atencio FA, Barajas D, Vargas M, Diaz-Ruiz J, Diaz- Ruiz JR (2004) Host-dependent differences during synergistic infection by potyviruses with potato virus X. Mol. Plant Pathol. 5:29-35.

Hall GS, Little DP (2013) Within-host competition between barley yellow dwarf PAV and –PAS. Virus Res. 174:148-151.

Hudson A, Richman DB, Escobar I, Creamer R (2010) Comparison of the feeding behavior and genetics of beet leafhopper (*Circulifer tenellus*, Baker) populations from California and New Mexico. Southwestern Entomol. 35:241-250.

Lacroix C, Seabloom EW, Borer ET (2014) Environmental nutrient supply alters prevalence and weakens competitive interactions among coinfecting viruses. New Phytol., doi: 10.1111/nph.12909

Lam N, Creamer R, Rascon J (2009). Characterization of a new curtovirus, *pepper yellow dwarf virus*, from chile pepper and distribution in weed hosts in New Mexico. Arch. Virol. 154: 429-436.

Morilla, G, Krenz B, Jeske H, Bejarano ER, Wege C (2004). Tete a tete of *Tomato yellow leaf curl virus* and *Tomato yellow leaf curl Sardinia virus* in single nuclei. J. Virology 78: 10715-10723.

Ohnishi J, Kitamura T, Terami F, Honda K (2011) Co-transmission of *Tomato yellow leaf curl virus* (TYLCV)-Mld and TYLCV IL by the whitefly *Bemisia tabaci*. J. Gen. Plant Pathol. 77:54-59.

Rentería-Canett I, Xoconostle-Cazares B, Ruiz-Medano R, Rivera-Bustamante R (2011) Geminivirus mixed infection on pepper plants: Synergistic interaction between PHYVV and PepGMV. Virol. J. 104:1-13.

Roossinck MJ (2005) Symbiosis versus competition in plant virus evolution. Nat. Rev. 3: 917-923.

Salvaudon L, De Moraes CM, Mescher MC (2013). Outcomes of co-infection by two potyviruses: implications for the evolution of manipulative strategies. Proc. R. Soc. B 280:20122959.

Sedano MJ, Lam N, Escobar I, Cross T, Hanson SF, Creamer R (2012). Application of vascular puncture for evaluation of curtovirus resistance in chile pepper and tomato. J. Phytopathol. 160: 120-128.

Soto MJ, Cheng L, Seo Y, Gilbertson RL (2005). Identification of regions of the Beet mild curly top virus capsid protein involved in systemic infection, virion formation and leafhopper transmission. Virology 341: 257-270.

Soto MJ, Gilbertson RL (2002). Distribution and rate of movement of the curtovirus, Beet mild curly top virus in the beet leafhopper. Phytopathology 93:478-484.

Srinivasan R, Alvarez JM (2007). Effect of mixed viral infections (*Potato virus Y – Potato leafroll virus*) on biology and preference of vectors *Myzus persicae* and *Macrosiphum euphorbiae* (Hemiptera: Aphididae). J. Econ. Entomol. 100:646-655.

Stenger DC, Carbonaro D, Duffus JE (1990). Genomic characterization of phenotypic variants of beet curly top. J. Gen. Virol. 71: 2211-2215.

Stenger DC, McMahon CL (1997). Genotypic diversity of *Beet curly top virus* populations in the Western United States. Phytopathology 87:737-744.

Stenger DC, Ostrow KM (1996). Genetic complexity of a *Beet curly top virus* population used to assess sugar beet cultivar response to infection. Phytopathology 86:929-933.

Strausbaugh CA, Wintermantel WM, Gillen AM, Eujayl IA (2008). Curly top survey in the western United States. Phytopathology 98:1212-1217.

Varsani A, Martin DP, Navas-Castillo J, Moriones E, Hernandez-Zepeda C, Idris A, Zerbini FM, Brown JK (2014). Revisiting the classification of curtoviruses based on genome-wide pairwise identity. Arch. Virol. 153:1873-1882.

Wang YZ, Gaba V, Yang J, Palukaitis P, Gal-On A (2002). Characterization of synergy between cucumber mosaic virus and potyviruses in cucurbit hosts. Phytopathology 92:51-58.

Wintermantel WM (2011) Impact of curly top host plants on accumulation, competitiveness, and durability of curtovirus species. Am. Soc. Sugar Beet Technol., Orlando, FL.

Wintermantel WM, Cortez AA, Anchieta AG, Gulati-Sakhuja A, Hladky LL (2008). Co-infection by two criniviruses alters accumulation of each virus in a hot-specific manner and influences efficiency of virus transmission. Phytopathology 98:1340-1345.

Supplementary Table S1. Two-way factorial ANOVA: The effects of the factors virus species (BCTV-PeCT-BV3, BCTV-Svr) and infection status (singly infected, co-infected) (α=0.05).

Source	DF	Sum of squares	F Ratio	Pr>F
Virus	1	73.98	143.43	≤0.0001
Infection Source	1	16.61	32.21	≤0.0001
Virus * Infection Status	1	0.22	0.442	0.5087

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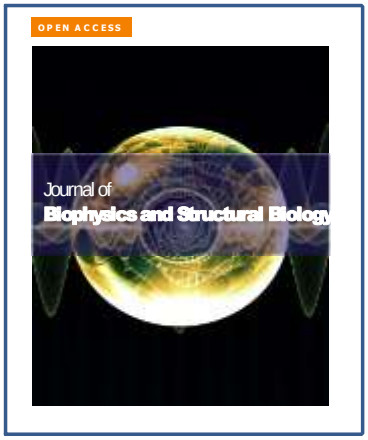

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