



**CPS 2011 RFP
FINAL PROJECT REPORT**

Project Title

Glucosinolate-derived compounds as a green manure for controlling *Escherichia coli* O157:H7 and *Salmonella* in soil

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Objectives

1. *Evaluate the antimicrobial activity of GSL-derived compounds in vitro against E. coli O157:H7 and Salmonella*
2. *Determine the glucosinolate content of different cultivars of broccoli grown in green houses*
3. *Investigate the persistence of enteric pathogens (surrogate strains) in soil tilled over with green manure (remnant crop after broccoli harvest) containing GSL-derived compounds.*

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Abstract

Cover crops planted after the harvest of a primary crop in the fall or intercropping a plow down/green manure could combine antimicrobial benefits derived from exudates secreted during the plant growth periods and in soil following residual incorporation (till over). This project investigated the role of broccoli remnants tilled over after harvest as antimicrobial in reducing enteric pathogens in soil. First, we investigated the antimicrobial effect of glucosinolate-derived compounds (GDC) for controlling enteric pathogens *in vitro* (**Objective 1**). The GDC and their enzymatic derivatives obtained commercially were evaluated for antibacterial activity using a disc diffusion assay on tryptic soy agar (TSA). *Salmonella* were more sensitive to these compounds than *E. coli* O157:H7 or non-pathogenic *E. coli*. Benzyl isothiocyanate exhibited a significantly higher zone of inhibition than other chemicals or Gentamicin (positive control) against *Salmonella* strains. The antibacterial effects of benzyl- and phenethyl isothiocyanate against *E. coli* O157:H7 were comparable to Gentamicin. Three broccoli cultivars: Arcadia, Belstar, and Diplomat were grown in a greenhouse to determine their glucosinolate content (**Objective 2**). Broccoli was harvested at maturity into 3 fractions (leaves and stems, roots, and florets). These broccoli fractions were freeze-dried, and then analyzed by high performance liquid chromatography - tandem mass spectrometry (HPLC- MS) for qualitative extraction of intact glucosinolate content. The glucosinolate (GSL) content of broccoli varied with cultivar and its fraction. glucotrapaeolin content was up to 10x higher in roots of the Diplomat cultivar than in roots of Arcadia or Belstar. In general, leaves and stems were rich in sinigrin content and roots were rich in glucotrapaeolin. A field study was conducted to evaluate the survival of surrogate strain O157:H12 in soil tilled over with broccoli (**Objective 3**). For field study, six-week old broccoli seedlings (Packman cultivar) were planted at Beltsville Agricultural Research Center (BARC) farm. After reaching full maturity, broccoli florets were harvested and remaining plants were tilled over with a tiller set at 4" depth. Tilled area were spray inoculated with *E. coli* O157:H12 strain (7 log CFU/ml) followed by additional treatment of glucosinolate or plant antimicrobials. Soil samples (100 g) were collected for up to 14 weeks from each subplot and analyzed for surviving populations of *E. coli* O157:H12 by direct plating on selective media and 8-block MPN assay. In general, populations of *E. coli* O157:H12 decreased in soil with time irrespective of treatment. *E. coli* O157:H12 populations in BIT-treated soil were not detected by direct plating after 7 days, whereas in other treatments populations ranged from 0.9 to 2.0 Log CFU/g of soil. The antimicrobial effect of broccoli plant remnants as well as that of cinnamaldehyde was more pronounced after 28 day from inoculation, Rapid inactivation of *E. coli* O157:H12 in soil could be attributed to glucosinolate derived compounds released from broccoli and additional antimicrobials sprayed in soil. The results reveal that the tilling over of the broccoli remnants as a green manure after harvest as well as GDC and natural plant volatiles has the potential in reducing *E. coli* O157:H12 populations in soil. Additionally, five broccoli cultivars (Arcadia, Diplomat, Green Magic, Belstar, and Imperial) grown in high tunnel were harvested at maturity. Following harvest, soil was tilled over with broccoli remnants and then spray inoculated with nonpathogenic *E. coli* O157:H12 strain as described earlier. Soil plug samples (Top and bottom layer of soil) were collected weekly/biweekly from each subplot using a soil sampler and then analyzed for surviving populations of *E. coli* O157:12 using MPN assay. Initial *E. coli* O157:H12 populations were 4-5 log and 2-4 log CFU/g soil for top and bottom layer samples, respectively. The *E. coli* O157:H7 persistence varied with type of broccoli cultivars tilled over in soil. *E. coli* O157:H12 were undetectable after 12 weeks in soil tilled over with Green magic and Imperial broccoli cultivars. However, they were still recovered after 16 weeks

in soil tilled over with Arcadia or Belstar broccoli. *E. coli* O157:H12 were reduced by up to 5 log CFU/g in soil within 28 days when soil was tilled-over with Marathon cultivar of broccoli.

Background

Overall incidence of illness linked to contaminated produce in the U.S. is quite low relative to the total number of produce (no. of bags) consumed. However, there has been increase in the number of produce outbreaks in the last 20 years. At least 10 of the 20-plus leafy greens-associated outbreaks occurred between 1995 and 2008 were linked to produce grown in or near Salinas Valley, and the produce was contaminated before harvest. Outbreak investigations of farms and ranches nearby leafy green production have identified new sources and information about the fate and transport of enteric pathogens (Cooley et al., 2007). The results suggest that some pathogenic strains may have evolved fitness to persist in different environments and become predominant. Soil or soil amendments may be a source of fresh produce contamination at the farm level. *E. coli* O157:H7 has been reported to survive for weeks or months depending upon soil type and soil amendments. *E. coli* O157:H7 has survived > 28 days in clay soil (Patel et al., 2010), 69 days in manure-amended soil (Mukherjee et al., 2006), and > 600 days in sheep manure (Kudva et al., 1998). Attenuated strains of *E. coli* O157:H7 survived for up to 217 days in manure-amended soil (Islam et al., 2004). Similarly, *Salmonella* Newport and *Salmonella* Enteritidis survived for 332 and 240 days in soil, respectively (Davies and Breslin, 2003; You et al., 2006). These enteric pathogens, which persist in soil for longer duration, may transfer to foliar or fruit surfaces of fresh produce during the splashing of irrigation water or rain, and may survive and grow under the appropriate environmental conditions.

Natural antimicrobials in some plants play key roles in their defense against fungal and bacterial phytopathogens. Glucosinolate (GSL), sulfur-containing organic anions with a β -D-thioglucose moiety and various differentiating side groups, are characteristic natural products of the *Resedaceae*, *Capparidaceae*, and *Brassicaceae* families implicated in plant defense responses (Brown and Morra, 1995). All plants which produce GSL also produce the enzyme myrosinase, which is physically separated from the GSL in the intact plant tissue. Upon tissue disruption the GSL and the myrosinase come into contact and myrosinase hydrolyzes the GSL into a number of bioactive hydrolysis products such as isothiocyanates, nitrile, and thiocyanate (Brown and Morra, 1997). These isothiocyanates are very toxic to a wide range of organisms due to a non-specific and irreversible reaction focusing on sulfur-containing groups in target proteins (Brown and Morra, 1997). The antimicrobial activity of GSL-derived products such as isothiocyanates has been demonstrated against a wide range of phytopathogens including *Verticillium dahliae*, *Fusarium oxysporum*, *Pseudomonas marginalis*, *Erwinia carotovora*, and *Alternaria brassicicola* (Njoroge et al., 2011; Smolinska et al., 2003; Tierens et al., 2001), and GSLs also play a role in disease resistance (Clay et al., 2009; Pawel et al., 2009; Rask et al., 2000). Likewise, mustard seeds (*Brassica* plants) produce isothiocyanates, and other metabolites with antimicrobial properties such as phenolic acid and phytin. In a recent study, Lucino et al (2011) reported that autoclaved mustard powder reduced > 5 log CFU/g *E. coli* O157:H7 during dry sausage ripening. They attributed the *E. coli* O157:H7 inactivation in sausage due to the antimicrobial effect of phenolic acid and isothiocyanates.

The specific chemical structure of the GSL-derived compounds are specific to the respective GSL present in the tissue and conditions under which hydrolysis occurs (Bones and Rossiter, 2006). Overall, the GSL content of *Brassica* roots is 4.5 times higher than in shoot tissues. Further, roots have a greater diversity of GSL than shoots. In addition to the differences in total GSL levels, differences exist in GSL composition between roots and shoots. For example, the predominant indole GSL in shoots of *Brassica* plants is indole-3-methylglucosinolate whereas higher concentrations of 1- and 4-methoxyderivatives are found in roots. The indole GSL has not been studied in detail probably because myrosinase-induced hydrolysis does not yield stable isothiocyanates, but rather less toxic nitriles and ascorbigens (Dam et al., 2009).

Broccoli (*Brassica oleracea* var. *italica* subvar. *Cyamosa*) and cauliflower (*Brassica oleracea* var. *botrytis* L.) are closely related; however, *Verticillium dahliae* (phytopathogen associated with wilt) is found on cauliflower and not on Broccoli (Subbarao and Hubbard, 1999). Fresh broccoli residues possess more antimicrobial activity against phytopathogen than dry broccoli residues over the temperature range of 10 to 35°C, and phytopathogen population reduces within 15 days of incorporation in soil (Subbarao and Hubbard, 1996). The GSL in presence of myrosinase are cytotoxic to *Salmonella* Typhimurium, generic *E. coli*, *Staphylococcus aureus*, *Bacillus subtilis*, and some fungi (Brown and Morra, 1997).

Research Methods and Results

Objective 1: Evaluate the antimicrobial activity of GSL-derived compounds in vitro against *E. coli* O157:H7 and *Salmonella*

Bacterial culture preparation

Five strains of *E. coli* O157:H7 isolated from fresh produce or environment samples (RM 4406, RM 1918, RM4407, RM4688, and RM5279); five *Salmonella enterica* serovars associated with produce commodities (*S. Thompson*, *S. Tennessee*, *S. Negev*, *S. Braenderup*, and *S. Newport*); five nonpathogenic *E. coli* strains; and three *E. coli* O157:H7 mutants lacking curli or cellulose appendages from EMFSL stock culture collection were used in disc diffusion assay to determine antimicrobial activity of GDC. These strains were streaked onto Tryptic Soy Agar (TSA), incubated 24 h at 37°C, and a colony of each strain was streaked on selective media (MacConkey Agar and XLT4 agar for *E. coli* and *Salmonella* strain, respectively) containing antibiotics as needed. Two successive transfers in TSBs were made before using for disc diffusion assay.

Disc diffusion assay

The glucosinolates and respective enzyme derivatives, mainly, isothiocyanates (benzyl-, butyl-, ethyl-, isopropyl-, methyl-, phenethyl-, and allyl-), indole methyl oxazolidinone, and methyl propyl pyrazole carboxylic acid were obtained from Sigma-Aldrich. These glucosinolates were dissolved in DMSO to obtain 10 mg/ml concentration. Actively growing bacterial strains described earlier were adjusted to 6 log CFU/ml concentrations and 100 µL were spread plated on TSA agar plates. Sterile filter discs (6 mm dia) impregnated with 10-20 µL of the test chemicals were placed on the agar plates seeded with the test bacteria and incubated at 37°C for 48 h (Figure 1). In all experiments, DMSO and Gentamicin were used as a negative and positive control, respectively. After incubation, the diameter in mm of the inhibitory or clear zones around the discs was recorded and antimicrobial activity was expressed as the mean inhibition zone.

Antibacterial effect of GDC was dose-dependent, increasing with the dose applied. *Salmonella* were more sensitive to these compounds than *E. coli* O157:H7 or non-pathogenic *E. coli*. Benzyl isothiocyanate (BIT) exhibited a significantly higher zone of inhibition than other compounds or Gentamicin (positive control) against *Salmonella* strains (Figure 2). The zone of inhibition was up to 64 mm for *S. Negev* serovar in presence of BIT. The antibacterial effects of benzyl- and phenethyl isothiocyanate against *E. coli* O157:H7 were comparable to that of Gentamicin (15-19 mm inhibitory zone). The antibacterial effect of 3-Methyl-1-propyl-1H-pyrazole-4-carboxylic acid and 1H-Indol-3-ylmethyl-2-oxazolidinone was not evident on test pathogens.

Objective 2: Determine the glucosinolate content of different cultivars of broccoli grown in greenhouses

Preparation of plant material for glucosinolate extraction

Greenhouse grown samples were harvested and separately bagged into 3 fractions (leaves and stems, roots, and florets). Following recordation of the fresh weights, the samples were stored frozen, then programmed-run freeze dried, and reweighed. Due to space limitations of the freeze drying equipment the leaf and stem samples were manually crushed, mixed and sub-sampled immediately prior to freeze drying. The dried material was then processed using a RobotCoupe RSI-6V spinning blade chopper until finely chopped.

Cultivar	Leaves and Stems		Roots		Florets	
	Fresh wt (g)	Dried wt(g)	Fresh wt (g)	Dried wt(g)	Fresh wt (g)	Dried wt(g)
Belstar	465.6	36.0	280.6	29.9	101.3	1.6
Arcadia	171.4	13.7	271.9	23.5	-----	-----
Diplomat	199.8	20.0	255.1	28.3	20.5	4.0

Extraction of intact glucosinolates

Qualitative extraction of intact glucosinolates in the various broccoli matrices was conducted by high-performance liquid chromatography – electrospray ionization – tandem mass spectrometry using the procedure described by Tian et al., (2005). A 40 mg aliquot of plant material was weighed into a 1.5 mL polypropylene snap-cap microcentrifuge tube to which was added 1 mL of 70% methanol (HPLC grade, EMD, MX0475-1). After sealing, the tubes were sonicated in a 70°C water bath for 180 min, removed and cooled in an ice bath, and then centrifuged at 12600 rpm for 15 minutes. A 200 µL aliquot was removed in a cryoprotective vial, dried under nitrogen gas stream, reconstituted with 1.2 mL nanopure water, and vortexed to mix. Samples were immediately analyzed for glucosinolate residues and maintained at 4°C throughout the analytical run.

HPLC analysis of broccoli residues

The broccoli extracts were analyzed for glucosinolate residues using high performance liquid chromatography – tandem mass spectrometry (HPLC – MS/MS) as per the published method. The inlet system was a Waters Alliance 2695 quaternary pumping separations module equipped with an automatic liquid sampler. Tandem mass spectrometry was performed using a Micromass Quattro Ultima ion-tunnel mass spectrometer operating in electrospray negative ionization mode, scanning for parents of m/z 97.0 DA, m/z 300-500, 0.50 second scan time, 0.1 second interscan delay. Instrument operation, data processing, and reporting was controlled using Waters MassLynx, version 4.1. The analytical parameters were as follows:

Column: Luna 5µ C18(2), 100Å, 250 x 4.6 mm (Phenomenex , 00G-4252-E0) with SecurityGuard® dual cartridge guard column – 4 x 3.0 mm C18 (Phenomenex, kit: KJO-4282 kit, cartridge: AJ0-4287), 40°C.

Column flow (1 mL/min) was split using a 3-way tee and restrictor lines so that the flow to the mass spectrometer was 200 µL/min.

Mobile Phase: 0.05% Trifluoroacetic acid (HPLC grade, Sigma-Aldrich 91707-10X1ML), prepared as 0.5 ml QS 1 L with Nanopure Water or Acetonitrile (HPLC grade, Fisher A998-4).

Gradient elution as follows:

Time (minutes)	% A (MeCN + 0.05% TFA)	% B (npH ₂ O + 0.05% TFA)	Curve
0	0	100	1
10	0	100	1
25	50	50	6

30	50	50	1
33	0	100	2
35	0	100	1

Mass Spectrometer: ES(-) ionization parameters were as follows:

<u>Source:</u>		<u>Analyser:</u>	
Capillary (kV)	2.00	LM 1 Resolution	12.0
Cone (V)	35	HM 1	12.0
RF Lens 1	20	Resolution	
Aperature (V)	0	Ion Energy 1	0.5
RF Lens 2	0	Entrance	-5
Temperature (°C)	120	Collision	20
Desolvation Temp. (°C)	450	Exit	1
Cone Gas Flow (L/Hr)	100	LM 2 Resolution	15.0
Desolvation Gas Flow (L/Hr)	400	HM 2	15.0
		Resolution	
		Ion Energy 2	2.0
		Multiplier (V)	650

Analytical standards

Analytical standards prepared in Nanopure water were injected simultaneously with the broccoli extracts. Combined standards of (-)-sinigrin and glucotropaeolin were prepared at 0.5, 1, 5, 15, 30 and 60 µg/mL for quantitative estimation of residues. Additionally, individual analytical standards of glucoiberin, glucoerucin, gluconapin, progoitrin, phenethyl glucosinolate, and glucocheirolin were prepared at approx. 80 µg/mL in Nanopure water and injected.

Several chromatograms (Figure 3) generated using the HPLC-MS were integrated to determine peak areas. Additionally, a combined spectrum was generated for each integrated peak to show the component parent ions.

Calibration:

Conc. (µg/mL)	(-)-Sinigrin		Glucotropaeolin	
	<u>Ret.Time</u> (min.)	<u>Peak Area</u>	<u>Ret.Time (min.)</u>	<u>Peak Area</u>
0.5	5.29	14,807	20.42	17,058
1	5.29	29,083	20.43	49,491
5	5.35	79,199	20.44	95,795
15	5.43	552,255	20.45	703,753
30	5.34	1,177,745	20.44	1,282,597
60	5.29	2,507,302	20.42	2,869,695
Linear	Regression: $y = 42,347x - 60, 215$ $R^2 = 0.997$		$y = 47,960x - 54,856$ $R^2 = 0.996$	

Based on elution time, the following observations were recorded for glucosinolate content.

GDC	Cultivar	Broccoli fraction		
		Roots	Leaves & stems	Florets
Sinigrin	Arcadia	459356	94173	NA
	Belstar	136940	429249	599423

	Diplomat	163967	590891	527932
Glucotropaeolin	Arcadia	100537	1030057	NA
	Belstar	729549	36373	26143
	Diplomat	1157011	49602	5357
Phenyl glucosinolate	Arcadia	NA	310246	NA
	Belstar	203195	0	0
	Diplomat	250732	0	0

Objective 3: Investigate the persistence of enteric pathogens (surrogate strains) in soil tilled over with green manure (remnant crop after broccoli harvest) containing GSL-derived compounds.

Study # 1: New Morning Farm, PA

An experiment was conducted at a farm in Pennsylvania to determine the GDC in soil. After tilling and raking the soil, soil was divided into 6 subplots of 25 sq ft each (Figure 4). A non-pathogenic *E. coli* O157:H12 strain was propagated in fecal slurry as described by Patel et al. (2010). One liter of inoculum containing 7 log CFU/ml nalidixic acid resistant *E. coli* O157:H12 was sprayed on each 25 sq ft area using a hand sprayer. After 30 min, these subplots were treated with (1) Benzyl isothiocyanate (BIT) at 0.039%; (2) 1% acetic acid; (3) 1% cinnamaldehyde; (4) 1% Sporan®; or (5) broccoli exudates obtained from grinding locally purchased broccoli. A plot without any treatment served as positive control. Soil samples collected from area without bacterial inoculation or antimicrobial treatment were used as negative control. Soil samples were collected weekly/biweekly for up to 14 weeks to determine the persistence of inoculated *E. coli* O157:H12 in soil. Soil (20 g) was added in a stomacher bag containing 80 ml mEHEC broth supplemented with 50 µg/ml nalidixic acid (mEHECN). The bag containing soil was sonicated for 1 min followed by pummeling for 1 min to dislodge bacteria attached to the soil. A 100 µl aliquot of appropriately diluted homogenate was spiral plated on MacConkey agar containing 100 µg/ml nalidixic acid (MACN) and Hetrotropic plate count agar (HPC) for *E. coli* O157:H12 and total bacterial populations, respectively. Following incubation for 24 h at 37°C, colonies were counted by Protocol automated colony counter. In addition, five-tube MPN procedure was employed to improve the sensitivity of the detection. For MPN; 10, 1, and 0.1 ml of the homogenate was transferred to each of five tubes containing 0, 9, and 9.9 ml mEHECN, respectively and incubated at 37°C for 24 h. After incubation, 10 µL from each tube was spot plated on MACN, incubated at 37°C for 24 h, and then positive samples were recorded.

The antimicrobial effect of BIT and other natural antimicrobials was not evident during the first week. *E. coli* O157:H12 populations were in the range of 5 - 6 log CFU/g during this period. Conversely, *E. coli* O157:H7 populations were higher in broccoli treated soil than in control soil during the 14 days period. *E. coli* O157:H12 were reduced in all treated soils after 21 days, the antimicrobial effect of BIT and broccoli was comparable to other natural antimicrobials during 21-42 days period. BIT and broccoli treatment reduced *E. coli* O157:H12 to undetectable level at 56 days. Occasional recovery of the target strain was observed at 79 days in other antimicrobial-treated soils. There was no adverse effect of these treatments on total bacterial populations in the soil (Figure 5). We observed the effect of soil moisture on total bacterial populations in soil, increase in populations was correlated with a rain event.

Study # 2: Beltsville Agricultural Research Center (BARC) Farm, Beltsville, MD

For field study, six-week old broccoli seedlings (Packman cultivar) were planted in a 1/2 acre field at BARC farm, Beltsville, MD. After reaching full maturity, broccoli florets were harvested and remaining plants were tilled over with a tiller set at 4" depth. The tilled area was subdivided as (1) uninoculated control broccoli tilled; (2) broccoli tilled + Benzyl isothiocyanate (BIT); (3) broccoli tilled + 2% cinnamaldehyde (CIN); (4) broccoli tilled + Isopropyl isothiocyanate (ITT); (5) inoculated control broccoli tilled, and (6) uninoculated control. A nonpathogenic *E. coli* O157:H12 strain enriched in bovine fecal slurry (7 log CFU/ml) as described above was sprayed on each plot (25 sq ft area), except negative control. One liter of 7 log CFU/ml bacterial suspension was uniformly sprayed per plot. Thirty minutes after application of bacterial suspension, plots that included volatile treatment were also sprayed with one liter of water suspension of a corresponding GDC or natural volatile. Soil samples were collected periodically for 14 weeks. At every sampling event, 100 g of soil was aseptically scooped in a whirl-pack bag from 3 separate locations in each subplot. Soil was collected from the surface to 2" depth in the area with soil sampler. Every sampling area was marked with a flag to avoid repeated samplings from the same spot during the experiment. Soil samples were analyzed for surviving populations of *E. coli* O157:H12 by direct plating and MPN assay.

In general, populations of *E. coli* O157:H12 decreased in soil with time irrespective of treatment. During first 3 days no significant differences among *E. coli* O157:H12 population in a control and broccoli/antimicrobial-amended soil were observed (Figure 6). However, *E. coli* O157:H12 populations in BIT-treated soil were not detected by direct plating at day 7, whereas in other treatments populations ranged from 0.9 to 2.0 Log CFU/g of soil. At 14 dpi (days post inoculation), *E. coli* O157:H12 populations were not detected by direct plating in control and treated broccoli/antimicrobial-treated soil. At this time point and at the consecutive sampling period (day 21) *E. coli* O157:H12 were detected by selective enrichment in all soil samples (Figure 6). The antimicrobial effect of broccoli plant remnants as well as that of CIN was more pronounced approximate after 28 dpi, *E. coli* O157:H12 being detected only in control untreated soil. The soil sampling continued for up to 14 weeks and did not detect *E. coli* O157:H12 by sensitive MPN assay in any but control samples. Collectively the field experiment data demonstrates that post-harvest tilling over of broccoli plants as well as GDC and natural plant volatiles has the potential in reducing populations of human enteric pathogens in soil. A glucosinolate-derived compound, BIT, had superior antimicrobial activity compared to cinnamaldehyde and broccoli plant remnants in reducing *E. coli* O157:H12 in soil.

Study # 3: High Tunnel at BARC, MD to evaluate the effects of broccoli cultivars

Additionally, the effect of broccoli cultivars on antimicrobial activity in soil was investigated. Five broccoli cultivars (Arcadia, Diplomat, Green Magic, Belstar, and Imperial) grown in high tunnel were harvested at maturity. Following harvest, soil was tilled over with broccoli remnants and then nonpathogenic *E. coli* O157:H12 strain enriched in bovine fecal slurry (7 log CFU/ml) was sprayed on each plot (1 L/25 sq ft area). Soil samples were collected weekly/biweekly for 18 weeks from three random locations of each subplot using soil sampler to obtain 4" soil plug. The top 2" and bottom 2" layer of soil plugs were collected separately and analyzed for *E. coli* O157:H12 populations using 8-block MPN assay. Every sampling area was marked with a flag to avoid repeated samplings from the same spot during the experiment.

Initial *E. coli* O157:H12 populations were 4-5 log and 2-4 log CFU/g soil for top and bottom layer samples, respectively (Table 7). The *E. coli* O157:H7 persisted at lower levels in bottom soil layers compared to those at top layers in most cases. Further, persistence varied with type of broccoli cultivars tilled over in soil. *E. coli* O157:H12 were undetectable after 12 weeks in soil tilled over with Green magic and Imperial broccoli cultivars. However, they were still recovered after 16 weeks in soil tilled over with Arcadia or Belstar broccoli. Recovery of *E. coli* O157:H12

also varied at certain sampling periods specifically with bottom soil layer; the strain was below detection limit in bottom layers of soil tilled with Arcadia, and Green magic broccoli at 35 days. A separate study was conducted with a Marathon cultivar of broccoli in high tunnel as harsh Winter climate in the mid-Atlantic area destroyed the field crop. In this study, up to 5 log reduction in *E. coli* O157:H12 populations was observed in soil after 4 weeks (Figure 8). A marginal increase in *E. coli* O157:H12 persistence was observed at 6 week sampling period.

PCR assay of soil samples

A total of 184 soil samples from high tunnel and field obtained during broccoli study were screened for the presence of *eae*, *stx1* and *stx2* gene of *E. coli*. The soil DNA was isolated using Power Soil DNA isolation kit (Mo Bio, USA) according to manufactures protocol. The primers used in this study for detection of *eae*, *stx1* and *stx2* genes are as follows:

eae Forward- GGCGATTACGCGAAAGATACC,
eae Reverse-CCAGTGAACCTACCGTCAAAGTTATTACC,
stx1 Forward- GTGTTGCAGGGATCAGTCGTACGG,
*stx1*Reverse- TGCCGGACACATAGAAGGAAACTC,
stx2 Forward- TTCCGGAATGCAAATCAGTCGTCA,
*stx1*Reverse- GCCGCCATTGCATTAACAAGAAAC.

Amplification reactions were carried out with 2 µl purified DNA, 800 µM deoxynucleoside triphosphate 4 mM MgCl₂, 1 µM of primers, and 2.5 unit of Taq polymerase. Molecular grade water was added to bring the final volume to 25 µl. PCR cycle included initial denaturation for 5 min at 94°C for 10 min and 35 cycles of denaturation for 20 sec at 94°C, annealing for 1 min at 57°C, and extension for 1 min at 72°C, with a final extension of 10 min at 72°C. PCR products were analyzed by gel electrophoresis through a 2% agarose gel, staining with Gel red (10 mg/ml), and then visualized using UV light. PCR results exhibited the presence of *stx1* and *stx2* genes in all the DNA isolated from soil samples by amplifying 434bp and 579bp products respectively, whereas, the *eae* gene was absent in *E. coli* O157: H12 strain used in this study (Figure 9).

Outcomes and Accomplishments

Glucosinolate are plant secondary metabolites commonly present in *Brassicaceae plants*, comprising more than 120 compounds with well-defined structures. The hydrolysis products of glucosinolate have been evaluated *in vitro* against plant pathogens. This is the first report on antimicrobial activity of GDC against enteric pathogens *in vitro* and in soil. Benzyl isothiocyanate was superior to other isothiocyanate in inhibiting *E. coli* O157:H7 and *Salmonella*. While antimicrobial activity of other isothiocyanates were also documented, their effects were marginal or lower than that of Gentamicin. *Salmonella* were most sensitive among the pathogens tested. Consequently, antimicrobial effects of these antimicrobials was not evident against generic *E. coli*. The antimicrobial activity of GDC was not influenced by surface appendages of *E. coli*.

Three broccoli cultivars were evaluated for their glucosinolate content using HPLC-MS procedure. There were significant differences in glucosinolate content among cultivars. Further, it varied with fractions (roots, stems and leaves, florets). Since broccoli florets would be harvested before tilling over the stems and roots, the beneficial effect of florets would not be observed at farm level. Nevertheless, florets were rich source of sinigrin but devoid of phenyl glucosinolate. The sinigrin and glucotropaeolin were 3x and 7x higher in roots of Arcadia and Diplomats cultivar, respectively. This is an important finding as GDC would be incorporated in soil during till over.

The *Brassica* family crops have been studied as a bio-fumigant to control soil pests and weeds by incorporating glucosinolate-derived compounds in soil. There are reports on control of

Verticillium wilt in cauliflower with broccoli residues in soil. This is the first investigation on role of GDC from *Brassica* plants in controlling *E. coli* O157:H12, a surrogate strain for enteric pathogen. Four studies were conducted either at a farm or in a high tunnel (to minimize the effect of cold winter in Maryland) to include more cultivars in field settings. Further, soil was incorporated with GDC along with broccoli till over to facilitate rapid *E. coli* O157:H12 reduction in soil. Low concentration of BIT (0.039%) as a spray was superior to 1% natural plant antimicrobials in reducing *E. coli* O157:H12 populations in soil. The organism was undetectable after 42 days whereas it survived in untreated soil during 14 weeks of study. Total bacterial populations in soil varied with rain event, increasing with an increase in soil moisture level. In a field study with Packman cultivar of broccoli, *E. coli* O157:H12 were non-detectable after 21 days when soil was treated with 0.039% BIT following till over of broccoli residues. *E. coli* O157:H12 was recovered from soil devoid of broccoli residues and GDC during 14 weeks of study. Differences in antimicrobial activity of broccoli residues due to cultivars could be attributed to differences in their GDC content. Green Magic and Imperial broccoli cultivars were more inhibitory towards *E. coli* O157:H12 in soil compared to that of Arcadia or Belstar. Up to 5 log reductions in *E. coli* O157:H12 were observed from soil tilled-over with Marathon broccoli when experiment was conducted in high tunnel to minimize the effect of adverse weather conditions.

Summary of Findings and Recommendations

Approximately 130,000 acres of broccoli are grown annually in the United States, with a value of \$742 million in 2009. California produces nearly 90 percent of the broccoli in the United States, and Arizona produces the rest. Demand for broccoli has steadily increased, both domestically and internationally, due to its health benefits. Production of broccoli is labor-intensive and net return on broccoli production may be negative (-\$248 per acre according to 2012 California Central Coast broccoli production, UC Davis publication). The GDC have been studied as a bio-herbicide to control brown rot on stonefruit, to suppress *Pseudomonas marginalis* responsible for soft rot, and to control *Verticillium* Wilt in cauliflower. This study reports microbial food safety benefit of broccoli production by controlling *E. coli* O157:H12 in soil.

Broccoli grown as a primary crop or intercropping is an excellent intervention strategy to control enteric pathogens in soil. *E. coli* O157:H12 populations as high as 6 log CFU/g soil are reduced below detectable limit (0.56 cfu/g) within 6 weeks. It is essential to increase the sensitivity of detection using MPN assay and confirm the results using molecular methodology. The rate of *E. coli* O157:H12 inactivation can be increased by spraying benzyl isothiocyanate at very low concentration (0.039%) in soil. Since the GDC content of broccoli cultivars varies, it is important to select cultivar based on its GDC content in addition to yield and disease resistance. We observed higher antimicrobial activity of Marathon, Green Magic, and Imperial cultivars compared to that of Arcadia and Diplomat. Additional studies to determine GDC content in other *Brassica* family crops will be helpful to compare the beneficial antimicrobial effect of these plants in soil. Plant breeding efforts should include glucosinolate levels as a factor in selecting broccoli for pathogen reduction in soil.

APPENDICES

Publications and Presentations (required)

Glucosinolate-derived compounds as a green manure for controlling *Escherichia coli* and *Salmonella* in soil (The 3rd Annual Produce Research Symposium, Center for Produce Safety, Robert and Margrit Mondavi Center for Performing Arts, Davis, CA; June 27, 2012)

Summary

Cover crops planted after the harvest of a primary crop in the fall or intercropping a plow down/green manure could combine antimicrobial benefits derived from exudates secreted during the plant growth periods (roots constantly releasing low concentrations of glucosinolate into the soil), with a sudden, more concentrated release at the time of residual incorporation (till over). The antimicrobial effect of Broccoli, a *Brassica* family crop, has been demonstrated against phytopathogen. In this study, we determined the antimicrobial effect of various glucosinolate-derived compounds (GDC) against enteric pathogens. Benzyl isothiocyanate (BIT) exhibited strong antimicrobial activity as observed by disc diffusion assay. *Salmonella* was more sensitive to these GDC than *E. coli* O157:H7. The antimicrobial effect of benzyl isothiocyanate was validated in field study using surrogate strain. The BIT was more efficient in reducing *E. coli* O157:H12 than acetic acid or Sporan[®] in the field study.

Glucosinolate-derived compounds as a green manure for controlling *Escherichia coli* and *Salmonella* in soil (The 4th Annual Produce Research Symposium, Center for Produce Safety, Wegman Conference Center, Rochester, NY).

Summary

Cover crops planted after the harvest of a primary crop in the fall or intercropping a plow down/green manure could combine antimicrobial benefits derived from exudates secreted during the plant growth periods (roots constantly releasing low concentrations of glucosinolate into the soil), with a sudden, more concentrated release at the time of residual incorporation (till over). The antimicrobial effects of green manure (broccoli remnants tilled over) and other glucosinolate derived compounds (GDC) were evaluated at the farm level using *E. coli* O157:H12 strain. The populations of *E. coli* O157:H12 decreased in soil with time irrespective of treatment. *E. coli* O157:H12 were undetectable in green manure- and GDC-treated soil after 28 days. Qualitative evaluation of intact glucosinolate in the various broccoli matrices was conducted using HPLC and mass spectrometry. The results revealed differences in GDC content of florets and root portions of different broccoli cultivars.

Budget Summary (required)

The fund provided by CPS was adequate for us to carry out the project. We spent much less in salaries due to (1) recruitment of research associate via University of Maryland at significantly lower salary than the salary of USDA research associate; and (2) USDA account setup delay resulting in failure to recuperate salaries of USDA personnel conducting research. The breakdown of the grant funds spent by category is:

Salaries:	\$88,171.72
Supplies:	\$29,496.08
Travel:	\$2233.00*
Registration:	\$555.00*

*includes expenses for attending 2014 CPS annual symposium at Newport Beach, CA.

Tables and Figures

Figure 1: Antimicrobial activity (zone of inhibition) of BIT using disc diffusion assay

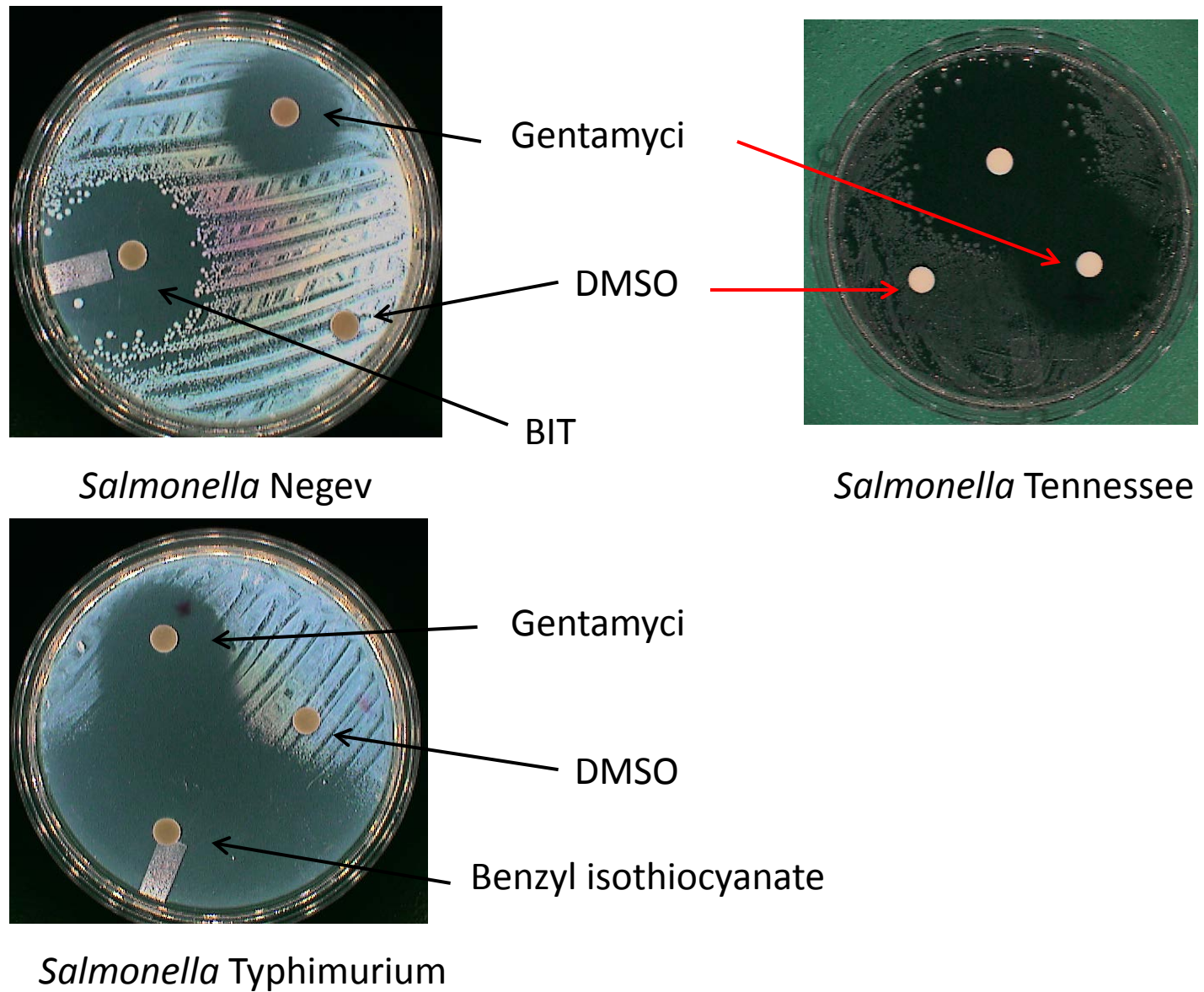
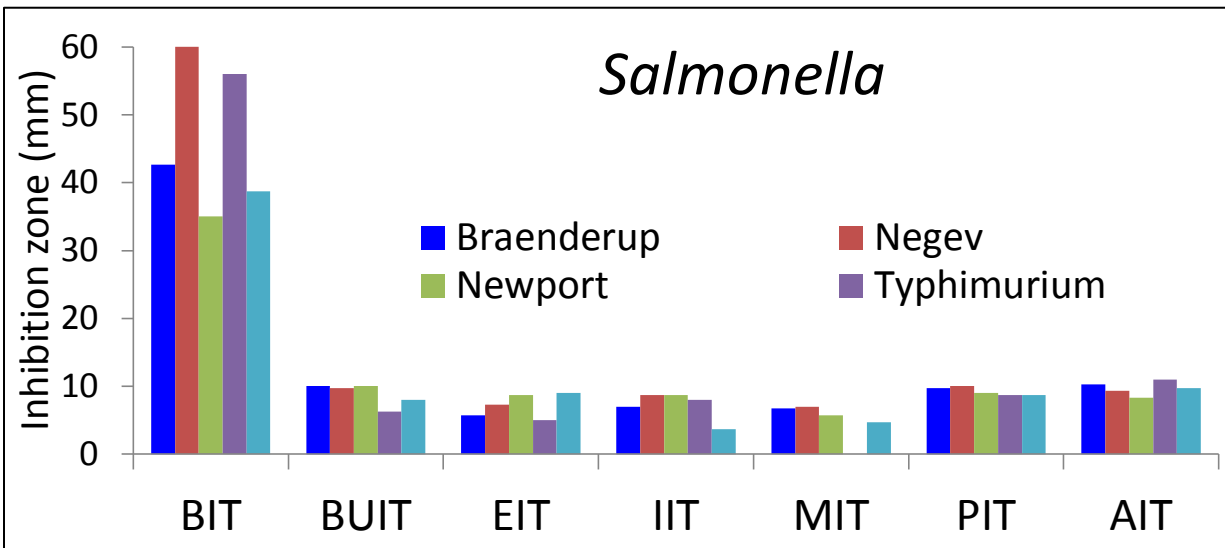
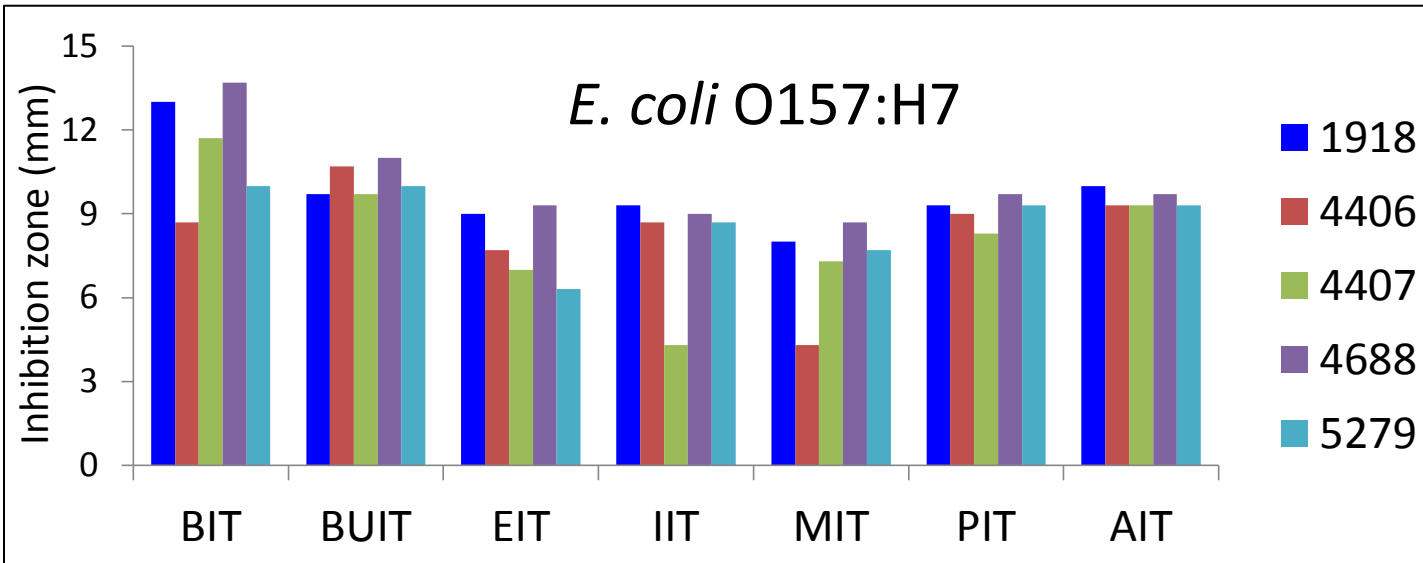


Figure 2: Antimicrobial activity of glucosinolate derived compounds against *E. coli* O157:H7 and *Salmonella* strains



Inhibition zone (mm) was measured using disc diffusion assay

BIT – benzyl isothiocyanate, BUIT- butyl isothiocyanate, EIT – ethyl isothiocyanate, IIT – isopropyl isothiocyanate, MIT - methyl isothiocyanate, PIT – phenethyl isothiocyanate, AIT – allyl isothiocyanate

Figure 3: Representative chromatogram for determination of glucosinolate-derived compounds in broccoli

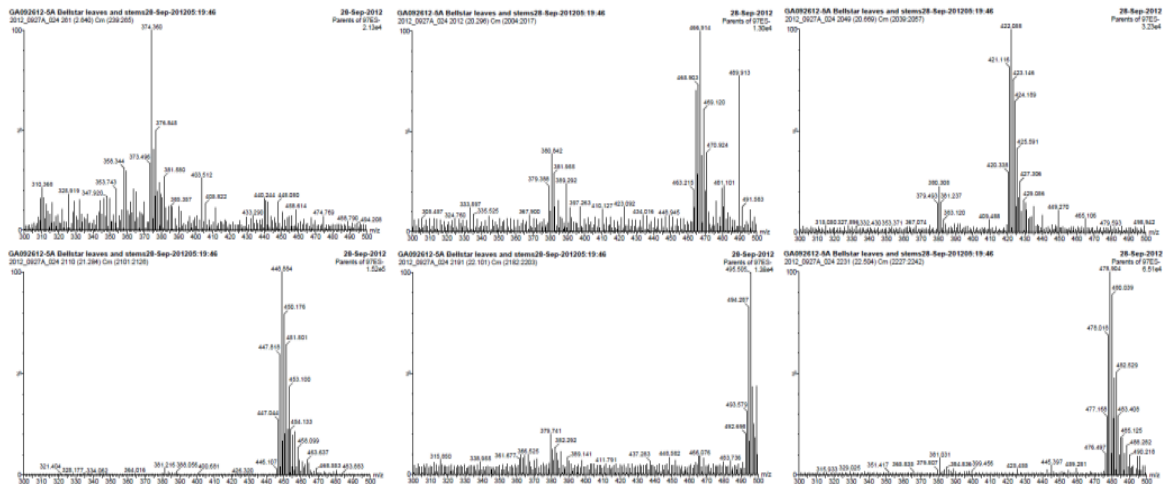
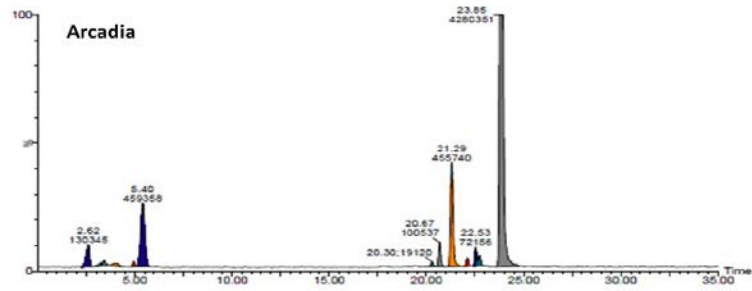
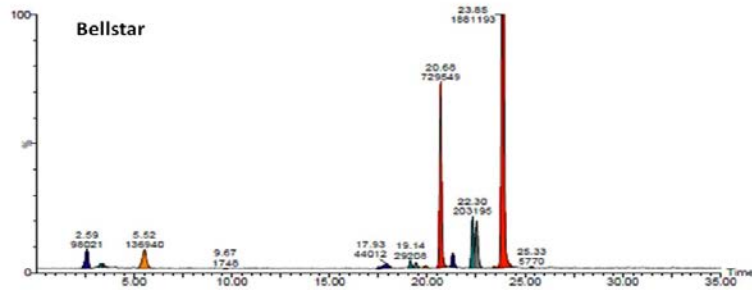
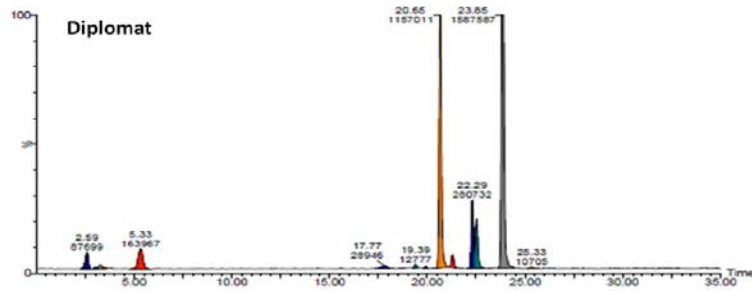
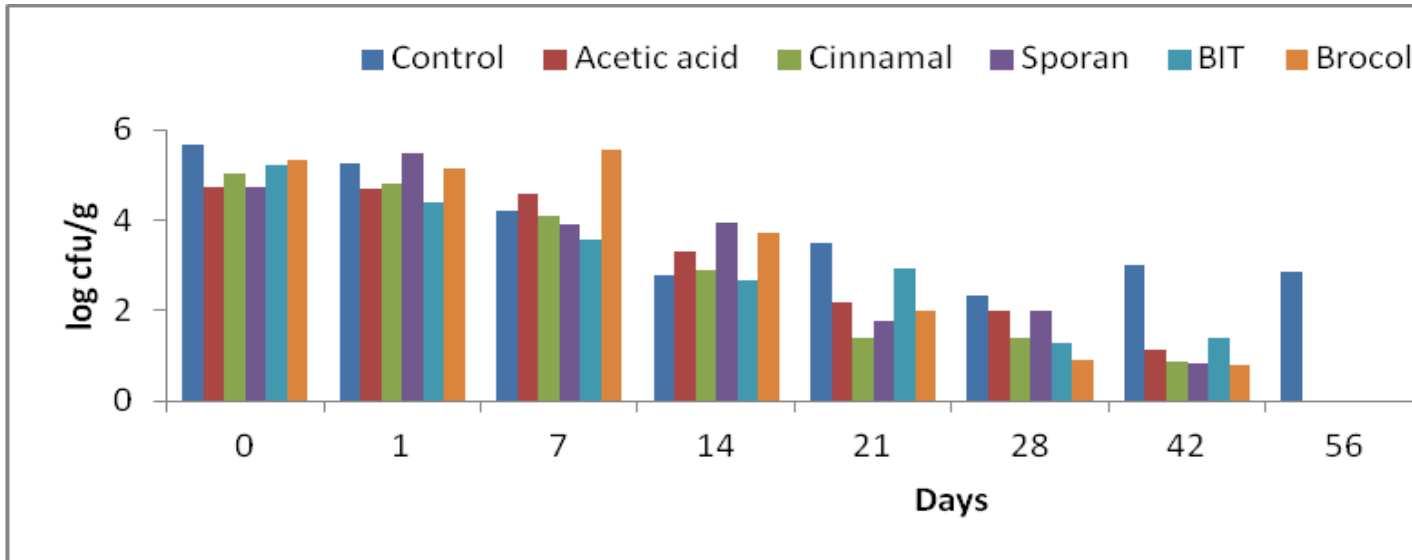


Figure 4: *Escherichia coli* O157:H12 inoculation and antimicrobial treatment of soil



Figure 5: Effect of glucosinolate-derived compounds on *E. coli* O157:H12 persistence in soil

E. coli O157:H12 populations in soil treated



Total bacterial populations in soil treated with anti

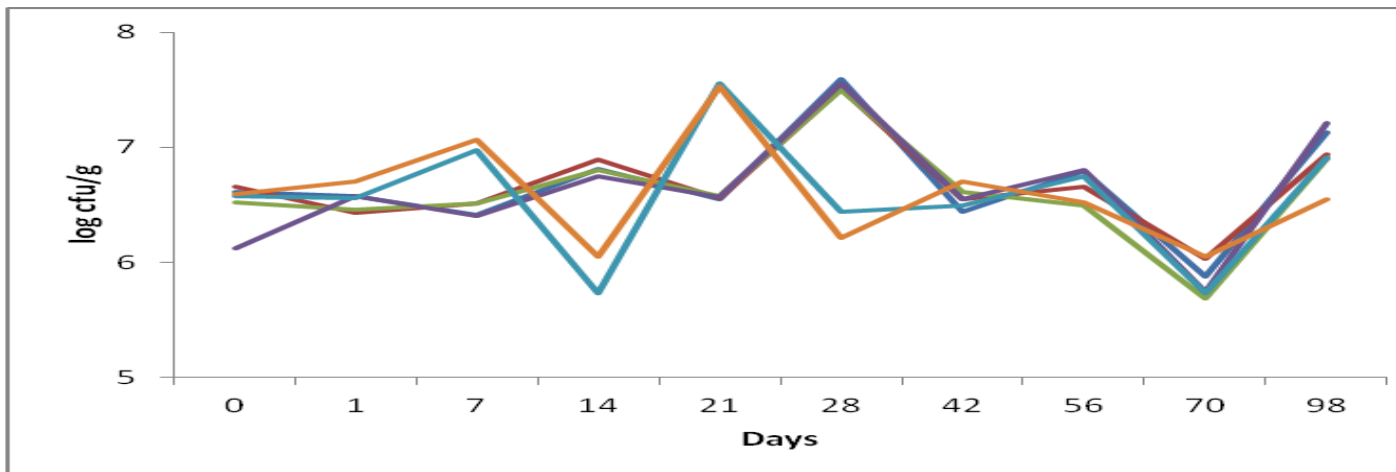
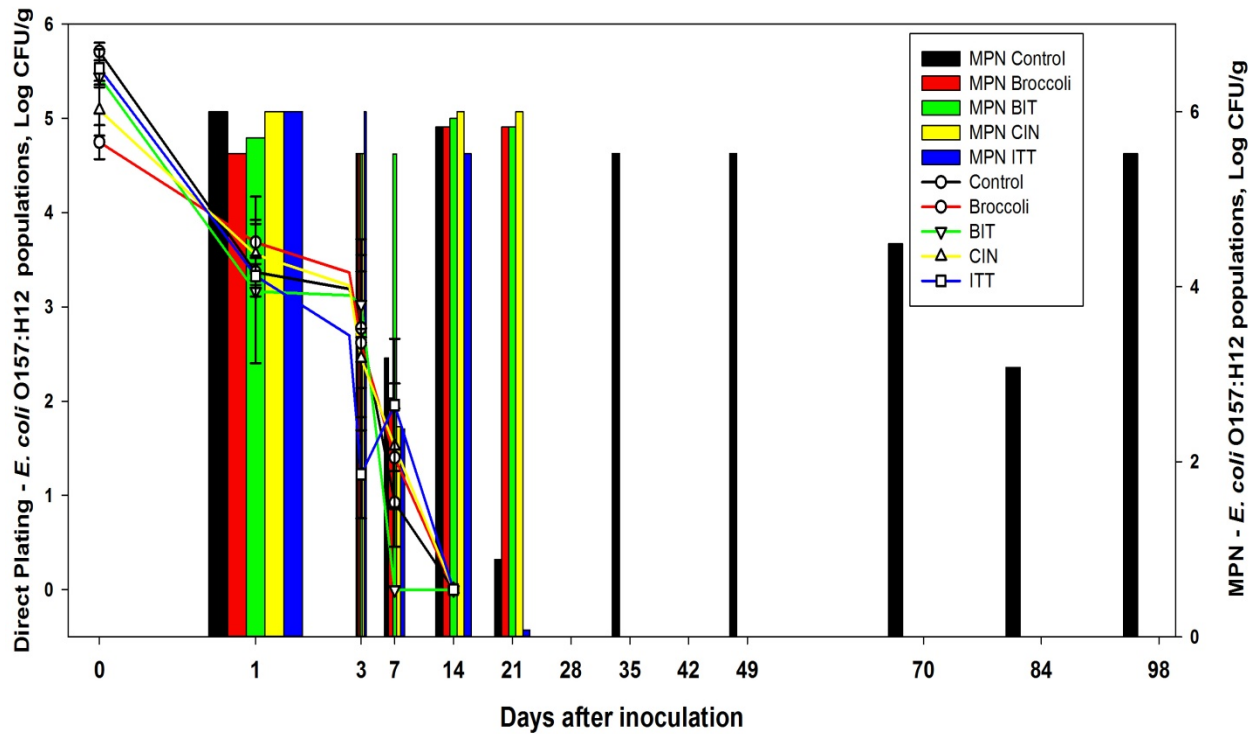
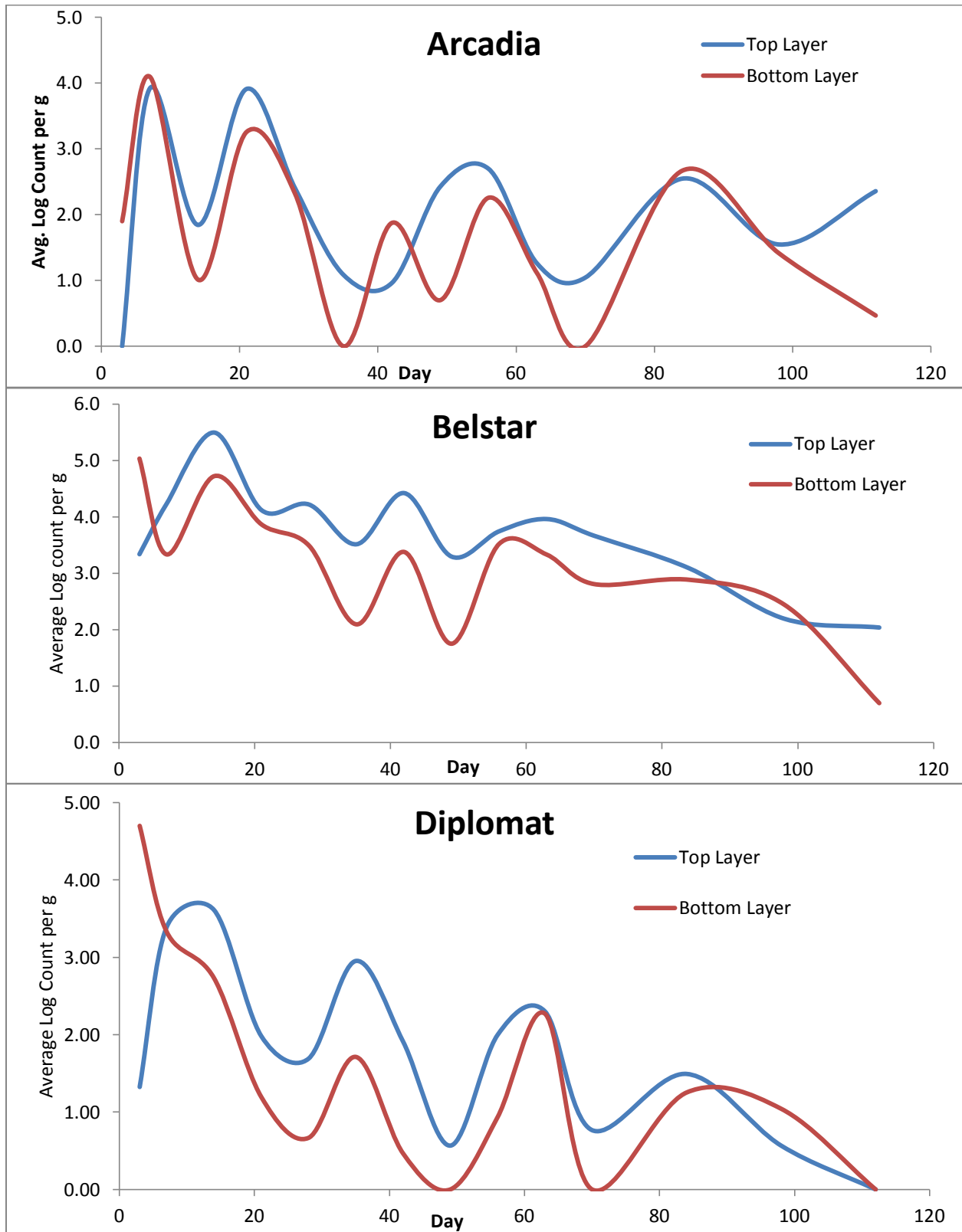


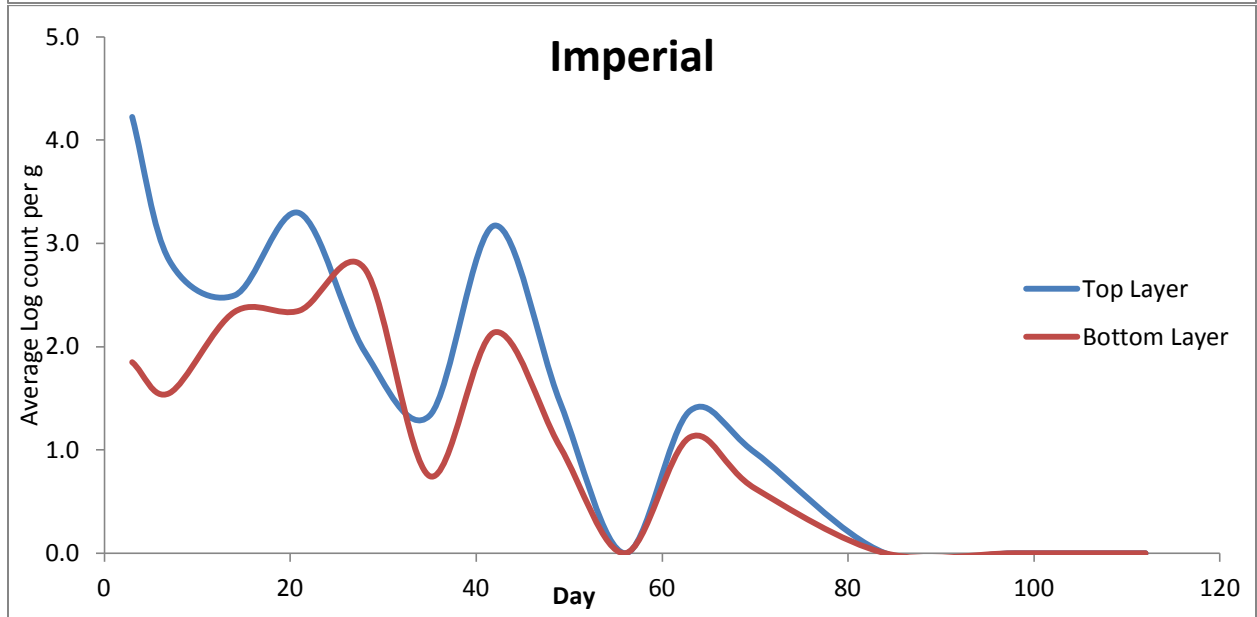
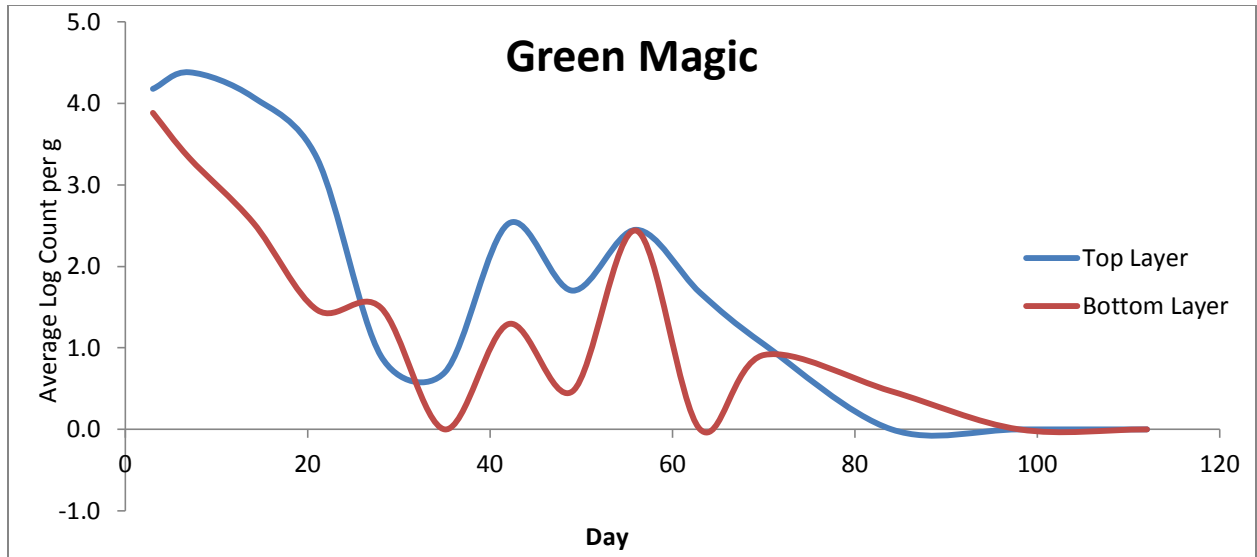
Figure 6: Survival of *E. coli* O157:H12 in soil tilled over with broccoli remnants



Average values and standard errors as detected by direct plating on MACN are shown in lines. Columns represent average *E. coli* O157:H12 populations as detected by MPN enrichment procedure

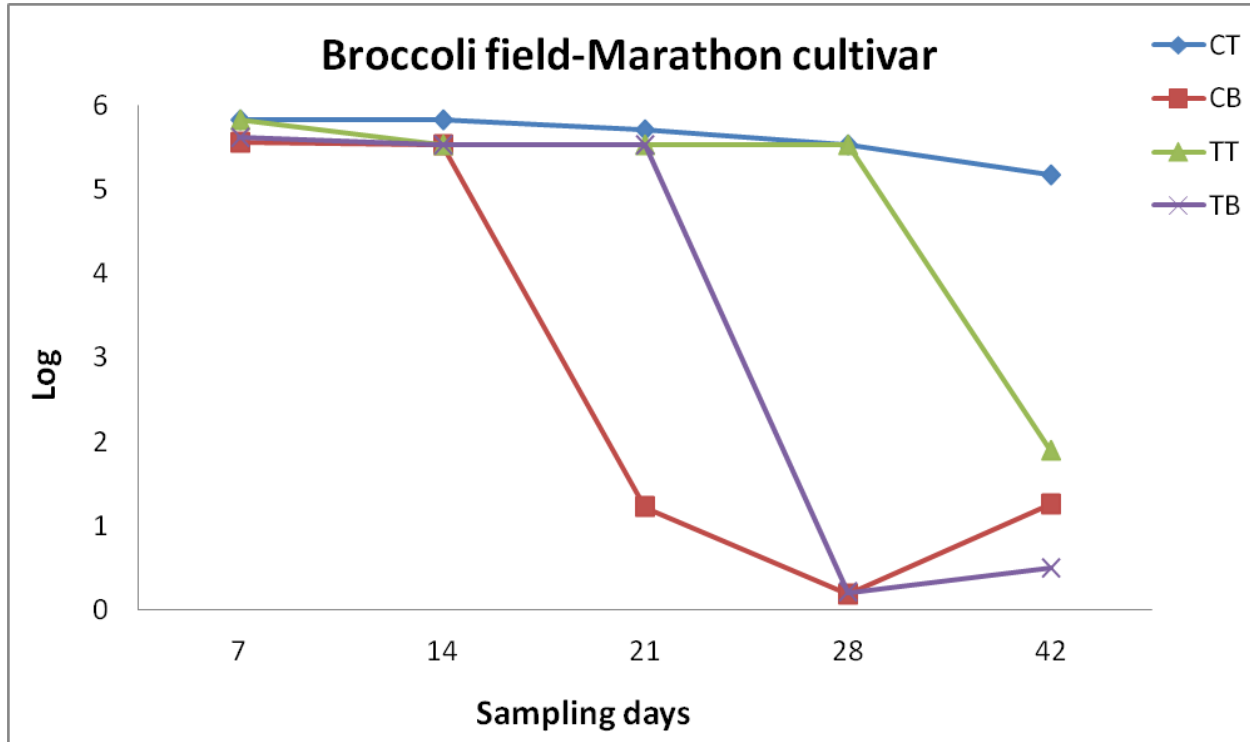
Figure 7: Effect of broccoli cultivar tilled over after maturity on *E. coli* O157:H12 survival in soil





Five cultivars were grown in high tunnel. At maturity stage, florets were harvested, broccoli remnants were tilled over, and then tilled over soil was inoculated with *E. coli* O157:H12. Soil samples (4" plug divided as top 2" layer and bottom 2" layer) were analyzed for *E. coli* O157:H12 for 112 days (16 weeks)

Figure 8: Reduction in *E. coli* O157:H12 populations in soil tilled-over with Marathon cultivar of broccoli

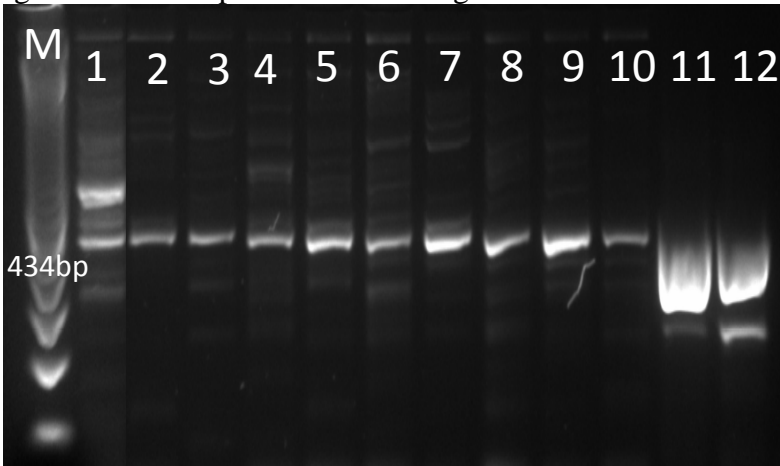


Log CFU/g soil, 4" layer of soil was sampled by soil sampler.

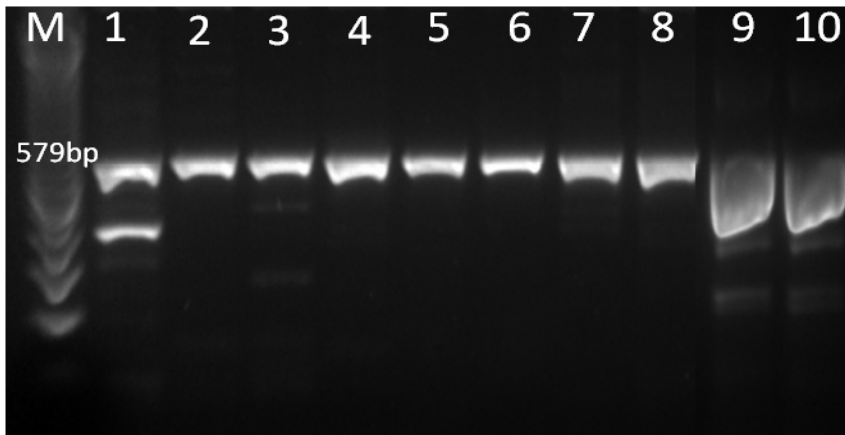
CT - control sample (top 2" layer of soil), CB - control sample (bottom 2" layer of soil), TT - soil tilled over with broccoli (top 2" layer of soil), TB - soil tilled over with broccoli (bottom 2" layer of soil)

Figure 9: Confirmation of *stx1*, *stx2*, and *eae* genes in soil samples by conventional PCR

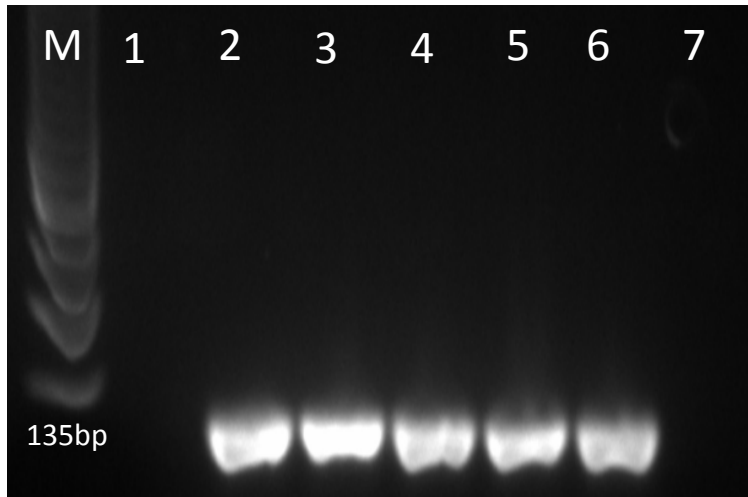
Figure 1. PCR amplification of *stx1* gene



Lane M: 100bp DNA marker; Lane 1 to 10: DNA of soil samples from broccoli study; Lane 11 and 12: Positive control for *stx1* gene



Lane M: 100bp DNA marker; Lane 1 to 8: DNA of soil samples from broccoli study; Lane 9 and 10: Positive control for *stx2* gene.



Lane M: 100bp DNA marker; Lane 1: *E. coli* O157:H12 strain; Lane 2 to 6: *E. coli* O157:H7 strains; Lane 7: Negative control

Suggestions to CPS (optional)

CPS leads in funding the research aimed at improving the microbial safety of fresh produce. The agency has done an excellent job in providing support to research interests significant to current problems with produce industry. We have enjoyed the close contact and professional relationship with CPS, attending research symposiums, and mutual understanding with industry leaders. It has also helped us in refining our research approaches in developing pre- and post-harvest intervention strategies for control of pathogens in fresh produce.