INTERNALISATION OF HUMAN PATHOGENS INTO SALAD VEGETABLES DURING GERMINATING AND CULTIVATION

[1]Khalilah Abdul Khalil, [2]Keith Warriner and [2]William.M.Waites
[1] Faculty of Applied Sciences, Universiti Teknologi MARA, 43400 Shah Alam, Selangor
[2] Division of Food Sciences, School of Biosciences, University of Nottingham
Sutton Bonington Campus, Loughborough, Leicestershire LE12 5RD, UK

Abstract: Most of the recent food borne outbreaks has linked to *Escherichia coli* O157:H7 contamination of salad vegetables including lettuce. Though recent studies have examined the application of *E.coli* O157:H7 and other human pathogens like *Listeria monocytogens* and *Salmonella* on mature lettuce, little is known about these attachments of these pathogens to growing plants. The potential of *E.coli* and *L.monocytogens* to become internalised into germinating and hydroponically grown lettuce and watercress has been studied. With inoculated lettuce and watercress seeds (10^7 cfu/g) with *E.coli* strains and *L.monocytogens* could be recovered initially from external and internal sites in 4 days old seedling. When 10^3 cfu/ml of *E.coli* and *L.monocytogens* were introduces into nutrient solution of hydroponically cultivated lettuce and watercress, no internalisation of these pathogens was detected. As in the nutrient solution, inoculated pathogens declined significantly ($P<0.01$) during the initial inoculation period and were undetected thereafter. The current study suggested that the pathogens were able to internalised into germinating lettuce and watercress, however when these pathogens were introduced at low cell densities, pathogens were unable to internalised into inner tissues of salad vegetables. This suggests that the internalisation of pathogens has relied on cell numbers of population.

Keywords: Internalisation, Human pathogens, Salad vegetables

INTRODUCTION

In recent years an increase in human food borne illness has been associated with high consumption of fresh produce. It has been reported that 17 food borne outbreaks have been linked to contaminated lettuce or salad and about 50% of these outbreaks were attributed to contamination with *Escherichia coli* O157: H7 (18). Because *E.coli* O157: H7 lettuce outbreaks have been recognised only recently, little is known about this bacterial-plant interaction. Studies have been shown that *E.coli* O157: H7 can survive on lettuce leaf surfaces for extended periods of time when it was applied in aqueous solution using bovine faeces as a carrier [3]. Other human pathogens like *Listeria monocytogenes* and *Salmonella* were also found to be able to internalised into inner tissues of lettuce plants when these pathogens were introduced at high cell densities [17]. These researchers believe that these organisms might enter plants through stomata pores and gain access to the interior tissues via cut leaf edges. The internalisation of human pathogens via natural opening has also been reported for apples inoculated with *E.coli* 0157:H7 [5,6] and tomatoes with *Salmonella* [19]. Previous reports have suggested that human pathogens can also become internalised into the vascular system of growing plants [8,11,15] although the mechanisms by which the pathogen is introduced into the plant are not fully understood [14]. Devising successful intervention steps to reduce population of human pathogens on and in vegetables eaten raw should be aided by information concerning the source of contamination and the ecology of pathogens affected by agronomic and minimal processing practices[2,4].

The objectives of this study reported here were to investigate the possibility of association of relevant *E.coli* strains and *L.monocytogenes* with germinating lettuce and watercress seeds. Furthermore, the potential of these pathogens to become internalise into young lettuce and watercress plants grown in a hydroponics nutrient solution inoculated with low cell densities of *E.coli* and *L.monocytogenes* in the presence of an established competitive micro flora were investigated.

MATERIALS AND METHODS

Bacterial strains and preparation of suspensions: Suspensions of *E.coli* (JM109, P36 (*luxCDABE*), slaughterhouse and lettuce isolates) and *L. monocytogenes* (fla2 mutant) *luxAB* were prepared from overnight culture grown aerobically at 37°C in brain heart infusion (Oxoid, Hampshire, UK). The cells were harvested by centrifugation (4000 x g for 10 min at 4°C) and washed once in sterile maximum recovery diluent (MRD:Oxoid,Basingsstoke, UK). The cell pellet was finally prepared by resuspending the pellet in an appropriate volume of MRD to obtain suspension containing *ca* 10^7 CFU/ml (OD_{600} 0.2).

Inoculation of lettuce and watercress seeds: Batches of lettuce (*Saladin iceberg*) *ca* 70 seeds/g and watercress (*Spinacia spinosa*) (Nickerson-Zwaan, Lincoln, UK) *ca* 150 seeds/g were submerged for 20 min in suspensions of appropriate bacteria. The inoculated seeds were allowed to dry at room temperature for 3 to 4 hours on sterile filter paper. Batches (1g) of inoculated seeds were suspended in 9ml MRD and bacteria released by vortexing for 1 min. Aliquots of the diluted suspensions were then plated onto *E.coli* petri film (3M, MN, USA), Oxoid plate count agar, plate count agar with kanamycin (30μg/ml) and *Listeria* Oxford formula medium (Oxoid, Basingstoke, UK). Plate count agar and *Listeria* Oxford formula medium were incubated at 30°C for 48 hours. Plate count agar with kanamycin (to select for *E.coli* P36) and *E.coli* petri film were incubated at 37°C for 24 hours. The remaining seeds were germinated on the damp filter paper at 15°C in the dark for 7 days.

Plants cultivation: i).*Hydroponic cultivation of lettuce plants*:Germinated seeds were transferred to rockwool blocks (Growdan, Hedehusene, Denmark) pre-damped with a commercial hydroponics nutrient medium (Nutriculture, Suffolk, UK). Seedlings were allowed to develop in growth chambers maintained at 16°C with 12 hours per day illumination. After the seedlings had developed sufficiently (ca. 17 days) the lettuce plants were transferred to a nutrient film technique (NFT) hydroponic system. The hydroponic system consisted of three plastic channels 3 m long and 22 cm wide with 17 plants within each trough. The nutrient solution held in an 80 litre tank was continuously circulated in a closed loop via a centrifugal pump. The base of plants was covered with a polyethylene sheet layer to prevent contact of lettuce leaves with the underlying rockwool base substrate. The nutrient solution within the holding tank was inoculated with the appropriate bacteria to give final cell densities of 10^3 CFU/ml. ii) *Cultivation of watercress plants*. Germinated watercress germinated seeds were transferred into the gravel bedding within the plastic channels and nutrient solution was continuously circulated in a closed loop via a centrifugal pump. A hydroponic system containing no added human pathogen was examined in parallel as a control. Samples of lettuce and watercress plants and nutrient solutions were taken periodically throughout the cultivation period.

Microbiological analysis: i) *Lettuce and watercress plants*: The plant samples were placed in the appropriate volume of MRD and the loosely attached bacteria on the surface were released by vortex for 1 minute. The plants were then surface sterilised using 10% (v/v) sodium hypochlorite solution (in 50mM phosphate buffer at pH6) for 20 minutes and subsequently rinsed three times in sterile distilled water. The plants were macerated (extracted) using a sterile scalpel blade and suspended in MRD and plated onto the appropriate agar as previously described. *E.coli* P36 and *L.monocytogenes* were enumerated using plate count agar with kanamycin (incubated at 37°C for 24 hours) and Listeria Oxford formula (incubated at 30°C at 48 hours) respectively. *E.coli* JM109 was enumerated using chromogenic agar (Oxoid Basingstoke, UK) after incubation at 37°C for 24 hours. The luminosity of the colonies was determined using a Night Owl luminometer (Nightowl light imager, EG & G Berhold, Germany. ii) *Nutrient solutions*. Nutrient solutions from each tank were taken every 24 hours to determine the populations of the bacteria.

RESULTS AND DISCUSSIONS

Distribution of *E.coli* strains and *L.monocytogens* FLA2 mutant derived from inoculated seeds. The initial loading of *E.coli* strains and *L.monocytogenes* fla2 mutant recovered from inoculated seeds of lettuce and watercress were independent of bacterial type (Table 1). When the seeds were further germinated for 7 days at 15°C, the inoculated *E.coli* strains and *L.monocytogenes* fla2 mutant (*luxAB*)

were recovered from surface (wash) of seedlings (Table 1). When surface sterilised lettuce samples were screened, *E.coli* were recovered to varying degrees with *E.coli* P36 (*luxCDABE*) and *E.coli* JM109 reaching high cell densities compared to the other strain tested. *L.monocytogenes* also become internalised (Table 1). It was only *E.coli* P36 (*luxCDABE*), *E.coli* JM109 and *L.monocytogenes* (*luxAB*) were internalised into watercress (Table 1).

Table 1: Bacterial counts (log_{10} cfu/g) of initial loading on lettuce and watercress seeds and day 7 seedlings derived from inoculated seeds.

	Lettuce			Watercress		
Strains	Initial loading	Wash	Extract	Initial loading	Wash	Extract
P36	6.74 ± 0.70	5.04 ± 0.01	3.25 ± 0.02	7.18 ± 0.01	4.96 ± 0.02	2.49 ± 0.50
JM109	6.71 ± 0.73	4.34 ± 0.01	2.62 ± 0.01	6.69 ± 0.01	4.29 ± 0.03	2.15 ± 0.03
Let.iso.	6.19 ± 0.66	4.09 ± 0.07	1.59 ± 0.16	6.82 ± 0.11	4.13 ± 0.03	2.13 ± 0.31
I	6.22 ± 0.02	3.68 ± 0.02	1.30 ± 0.01	6.13 ± 0.11	3.67 ± 0.13	ND
II	6.39 ± 0.10	3.99 ± 0.03	1.39 ± 0.12	6.30 ± 0.10	3.93 ± 0.01	ND
L.mono	5.93 ± 0.05	3.27 ± 0.01	1.04 ± 0.19	5.91 ± 0.05	3.26 ± 0.01	ND

JM109: *Escherichia coli* JM109
P36: *Escherichia coli* P36 (bioluminescence)
I : *Escherichia coli* strain from slaughterhouse I
ND: Not Detected
*Bacterial counts >300 colonies
Let. iso : *Escherichia coli* strain from lettuce isolate
L.mono: *Listeria monocytogenes*
II : *Escherichia coli* strain from slaughterhouse II

Internalisation of *E.coli* and *L.monocytogenes* into hydroponically cultivated lettuce and watercress. Lettuce and watercress seedlings (14 day old plants) derived from non-inoculated seeds were introduced into a hydroponic growth medium containing a *E.coli* P36 (bioluminescence), *E.coli* JM109 and *L.monocytogenes* fla2 mutant at 10^3 cfu/ml. It was observed that *E.coli* P36 was recovered from surface on lettuce (n=2) at day 4 (log_{10} 2.56 ± 0.02) of the cultivation period but declined at day 8 (log_{10} 1.96 ± 0.17) and day 11 (log_{10} 1.65 ± 0.07) to undetectable levels thereafter (Figure1). *E.coli* P36 interact with watercress (n=2) to a lesser extent compare to lettuce and was recovered on the surface of the plants at start of cultivation period. *L.monocytogenes* was detected on lettuce surface at day 4 (log_{10} 2.51 ± 0.03) and day 8 (log_{10} 2.07 ± 0.17) of the cultivation period (Figure 1) but only at day 4 (log_{10} 2.10 ± 0.14) from watercress surface. Neither *E.coli* P36 nor *L.monocytogenes* was recovered from extracts of lettuce and watercress throughout the cultivation period of 39 days. *E.coli* JM109 under the same condition was recovered from the surface of the plants during initial 3 days (log_{10} 3.8 ± 0.08) of the growing period and slightly increased on day 6 (log_{10} 4.72 ± 0.03) before declining until day 13 (log_{10} 2.07 ± 0.1) of cultivation period. No internalisation of the pathogens was observed during the growing process.The counts of *E.coli* P36, *E.coli* JM109 and *L.monocytogenes* within the hydroponic solution progressively declined during the cultivation period of 39 days. *E.coli* P36 from the lettuce and watercress hydroponic solution reach undetectable levels after day 10 and day 3 respectively (Figure3). Whereas *L.monocytogenes* disappeared from lettuce hydroponic solution at day 3 and day 2 from watercress hydroponic solution (Figure 2). No *E.coli* JM109 was detected from lettuce nutrient solution 5 days after inoculation (Figure 2).

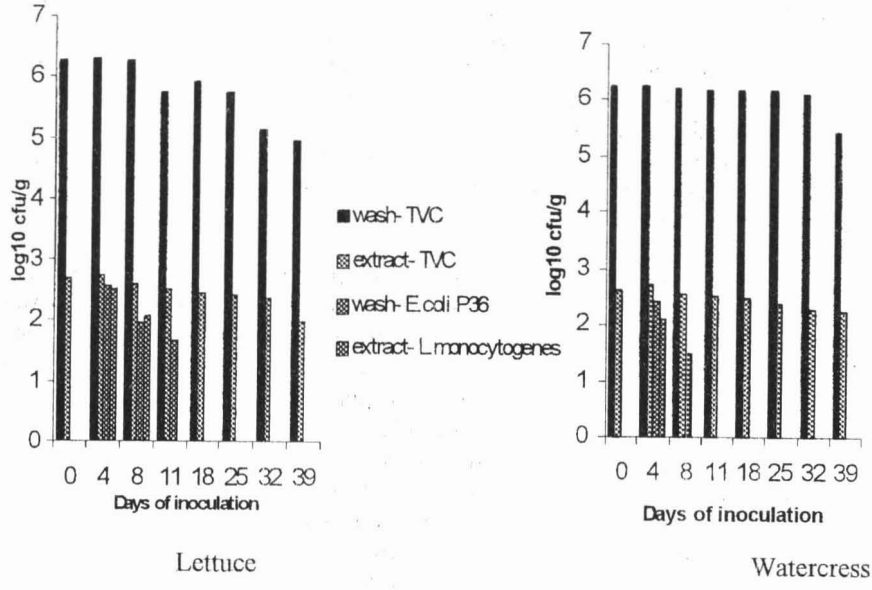

Figure 1: Bacterial counts (log_{10} cfu/g) of harvested lettuce and watercress plants cultivated in hydroponic solution inoculated with 10^3cfu/ml of *Escherichia coli* P36 (bioluminescence) and *Listeria monocytogenes*.

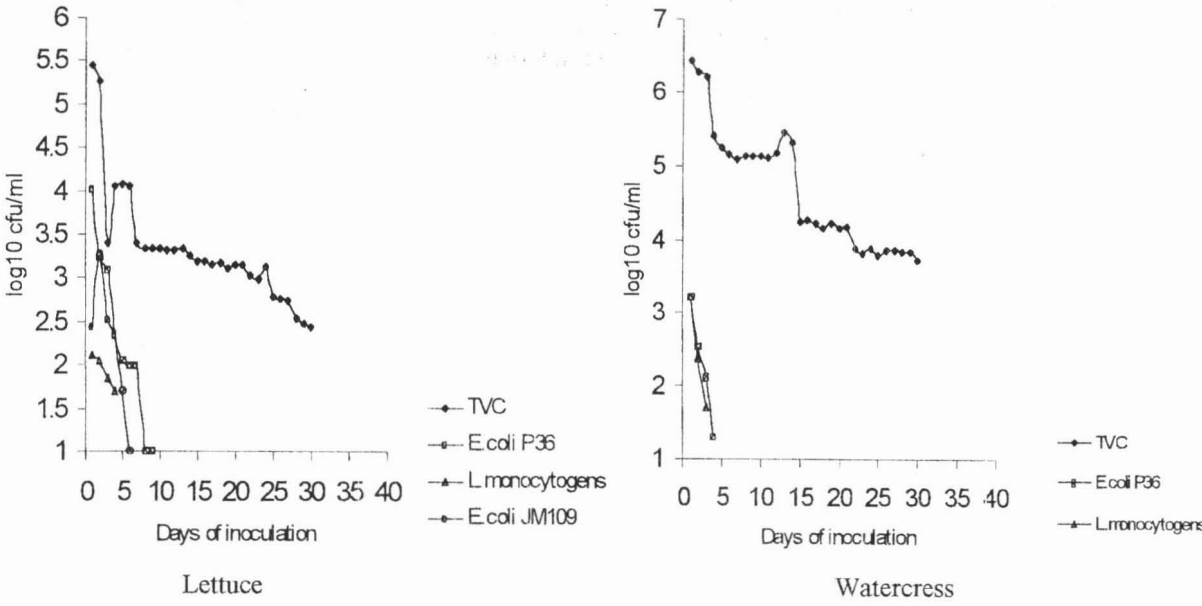

Figure 2: Total bacterial counts (log_{10} cfu/ml) of hydroponic solution of lettuce and watercress inoculated with *E.coli* P36 (bioluminescence) and *L.monocytogen* at inoculum level of 10^3 cfu/ml.

All the *E.coli* strains and *L.monocytogenes* used in this study were able to attach to the surface of lettuce and watercress plants tissues during seedling growth. The ability of human pathogens like *E.coli* and *L.monocytogenes* to grow on seedlings for the first few weeks of growth has been reported by Hirano and Upper [13]. It has also found that *E.coli* 0157:H7 was able to proliferate $10^3 - 10^5$ fold at early stage of plant growth including germination [9]. Based on culture, 20 min. exposure of exterior surface on seedlings to 10% sodium hypoclorite eliminated most culturable bacteria, suggesting that *E.coli* P36 (bioluminescent tag), *E.coli* JM109 and *L.monocytogenes luxAB* (fla2 mutant) were able to

internalise into the plants whereas *E.coli* wild types strains grown at comparable levels and did not internalised to any greater extent except in the watercress (Table 1). A result which is in agreement with some of the researchers, who reported that several bacterial traits, including motility, chemotaxis, salt tolerance, binding to roots and the production of O-antigenic side chain of lipopolysaccharides have been correlated with the colonisation of seeds and roots [1,7]. However the capability of bacterial to establish on or within the plants varies with the plant types [12,18]. This could be due to different exudates constituent. The complex mixtures of carbohydrates, amino acids, organic acids, and other nutrients released from seeds and roots are thought to support the growth of bacteria. However it was proven by Robert *et al.* [16] that different plants released different constituent of compounds which produce an effect on bacterial growth during colonisation and the levels of seed exudates were believed to have an effect on bacterial growth.

The performance of *E.coli* P36 (*luxCDABE*), *E.coli* JM109 and *L.monocytogenes* (*luxAB*) were examined on hydroponics cultivated lettuce and watercress plants. From the result, it was suggested that introduction of these human pathogens at lower numbers (10^3 cfu/ml) into the nutrient solution in the presence of an established competitive micro flora did not result in internalisation. However the pathogens tended to attach to the outer surface of the plants at the initial stage of the cultivation process. This is supported by the fact that bacterial-plant association was generally dose-dependent, with the lowest inoculation dose resulting in the lower association levels [18]. It also believed that activation of plant defense mechanisms might play a role in suppressing saprophyte bacteria. Hawes *et al.*[10] found that the root border cells which play role in the defense mechanism are able to synthesize defensive structures and bind to the bacteria as well as control their growth. Another suggestion is that the inoculated bacteria being filtered by the gravel used as bedding for watercress. This might lead to lower numbers of pathogens present in the nutrient solution so that undetectable levels are present at the early stage of cultivation.

In conclusion, this study of the interaction of bacteria with lettuce and watercress seeds studies revealed that under high cell densities (10^7 cfu/ml), both *E.coli* and *L.monocytogenes* had the ability to colonise the surface and inner tissues of germinating plants. The result also revealed that the growth rate of pathogens was different with different types of seed or plants. However, under natural condition, introduction of these human pathogens in lower numbers (10^3 cfu/ml), with the presence of an established competitive microflora resulted no internalisation into both lettuce and watercress plants grown hydroponically in inoculated nutrient solution. From all the findings, it can be suggested that internalisation of *E.coli* and *L.monocytogenes* into growing lettuce and watercress are dependent on the cell numbers present.

ACKNOWLEDGEMENT

This research was supported by University Technology MARA and by University of Nottingham, UK. We would like to thanks Dr. P.Hill (Division of Food Sciences, Nottingham University) for donating the bioluminescence strains used in this study.

REFERENCES

1. Anderson, T.A. Guthrie, E.A. and Walton, B.T. 1993. Bioremediation in the rhizosphere. Environmental Science and Technology 27:2630-2636

2. Beuchat, L.R. 1998. Surface decontamination of fruits and vegetables eaten raw: a review. Publication W.H.O/ FSF / FOS / 98.2. *World Health Organisation, Geneva, Switzerland.*

3. Beuchat, L.R. 1999. Survival of enterohemorrhagic *Escherichia coli* 0157:H7 in bovine faeces applied to lettuce and effectiveness of chlorinated water as a disinfectant. *Journal of Food Protection* 62: 845-849

4. Brackett, R.E. 1999. Incidence, contributing factors, and control of bacterial pathogens in produce. *Postharvest Biology of Technology* 15:305-311

5. Buchanan, R.L., Edelson, S.G., Miller, R.L., and Sapers, G.M. 1999. Contamination of intact apples after immersion in an aqueous environment containing *E.coli* 0157:H7. *Journal of Food Protection* 62:444-480

6. Burnett, S.L. Chen, J. and Beuchat, L.R. 2000. Attachment of *Escherichia coli* 0157:H7 to the surface and internal structures of apples as detected by confocal scanning laser microscopy. *Applied and Environmental Microbiology* 66:4679-4687

7. de Weger, L.A.Bakker, P.A.H.M.Schippers, B. Van Loosdrecht, M.C.M. and Lugtenberg, B.J.J. 1989. *Pseudomonas* species with mutational changes in the O-antigenic side chain of their lipopolysaccharides and affected their ability to colonise potato roots. *In B.J.J. Lugtenberg (ed.), NATO ASI series, Springer-Verlag, Berlin, Germany* 36:197-202

8. Guo, X.Van Iersel, M.W. Chen, J. Brackett, R.E. and Beuchat, L.R. 2002. Evidence of association of *Salmonella* with tomato plants grown hydroponically in inoculated nutrient solution. *Applied and Environmental Microbiology* 68: 197-202

9. Hallmann, J. Hallman, A.Q. Mahafee, W.F. and Kloepper, J.W. 1997. Bacterial endophytes in agricultural crops. *Canada Journal of Microbiology* 43: 895-914

10. Hawes, M.C. Gunawardena, U. Miyasaka. S. and Zhao, X. M. 2000. The role of root border cells in plant defence. *Trends in Plant Science*, vol. 5, No. 3: 128-132

11. Haywood, A.C. 1974. Latent infectious by bacteria. *Annual Review Phytopathology* 12:87-94

12. Huang, J.S. 1986. Unstructured of bacterial penetration in plants. *Annual Review Phytopathology* 24: 961-964

13. Hirano, S.S. and Upper, C.D. 2000. Bacteria in the leaf ecosystem with emphasis on *Pseudomonas syringae* – a pathogen, ice nucleus and endophyte. *Microbiology of Molecular Biology Review* 64: 624-653

14. Lund, B.M. 1992. Ecosystems in vegetable foods. *Journal of Applied Bacteriology* 73: 115S-126S

15. National Institute of Infectious Disease and Infectious disease control Division, Ministry of Health and Welfare of Japan. 1997. Verotoxins producing *E.coli* (enterohemorrhagic) infection Japan, 1996-Jun 1997. Infection Agents Surveillance Rep.18: 113-154

16. Roberts, D.P., Dery, P.D., Yucel, I. and Buyer, J.S. 2000. Importance of *pfkA* for rapid growth of *Enterobacter cloacae* during colonisation of crop seeds. *Applied and Environmental Microbiology* 66: 87-91

17. Takeuchi, K and Frank, J.F. 2000. Penetration of *Escherichia coli* 0157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. Journal of Food Protection, Vol.63,No.4.2000, pages 434-440

18. Wachtel, M.R., Whitehand, L.C and Mandrell, R.E.2002. Association of *Echerichia coli* 0157:H7 with preharvest leaf lettuce upon exposure to contaminated irrigation water. *Journal of Food Protection*. Vol 65, No.1, 2002, page 18-25

19. Zuang, R.Y.L.R, Beuchat., and F.J.Angulo.1995. Fate of *Salmonella Montevidoe* on and in raw tomatoes on effected by temperature and treatment with chlorine. *Application Environment Microbiology*. 61:2127-2131