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Rhizosphere effect on survival of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Typhimurium in manure-amended soil during cabbage (*Brassica oleracea*) cultivation under tropical field conditions in Sub-Saharan Africa

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ABSTRACT

The effect of cabbage (*Brassica oleracea*) rhizosphere on survival of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in manure-amended soils under tropical field conditions was investigated in the Central Agro-Ecological Zone of Uganda. Three-week old cabbage seedlings were transplanted and cultivated for 120 days on manure-amended soil inoculated with 4 or 7 log CFU/g non-virulent *E. coli* O157:H7 and *S.* Typhimurium. Cabbage rhizosphere did not affect survival of the 4 log CFU/g inocula in manure-amended soil and the two enteric bacteria were not detected on/in cabbage leaves at harvest. The 7 log CFU/g *E. coli* O157:H7 and *S.* Typhimurium survived in bulk soil for a maximum of 80 and 96 days, respectively, but the organisms remained culturable in cabbage rhizosphere up to the time of harvest. At 7 log CFU/g inoculum, *E. coli* O157:H7 and *S.* Typhimurium contamination on cabbage leaves occurred throughout the cultivation period. Leaf surface sterilisation with 1% $AgNO_3$ indicated that the organisms were present superficially and in protected locations on the leaves. These results demonstrate that under tropical field conditions, cabbage rhizosphere enhances the persistence of *E. coli* O157:H7 and *S.* Typhimurium in manure-amended soil at high inoculum density and is associated with long-term contamination of the leaves.

1. Introduction

Microbiological safety concerns attributed to the contamination of fresh produce with potential human pathogens such as *E. coli* O157:H7 and *Salmonella* spp. is well recognised (Barak et al., 2005; Doyle and Erickson, 2008; Nguyen-the and Carlin, 2000). Earlier studies aimed at finding solutions for microbiological safety problems of fresh vegetables suggest that sanitation of fresh produce can be executed at post-harvest level. Sanitation methods used are mostly based on a chemical decontamination step expected to ensure microbiological safety without loss of produce quality (Gopal et al., 2010; López-Gálvez et al., 2010; Vandekinderen et al., 2009; Weissinger et al., 2000). However, frequent outbreaks of *E. coli* O157:H7, *Salmonella* spp. and other microbial infections associated with fresh-cut vegetables raised concerns about the efficacy of such post-harvest sanitation approaches in guaranteeing the safety of fresh produce (Ölmez and Kretzschmar, 2009). Internalisation of human pathogenic microorganisms in plant tissues is probably one of the factors that limit the efficacy of sanitizers since such cells are completely protected from the biocides (Dong et al., 2003; Itoh et al., 1998; Solomon et al., 2002; Takeuchi and Frank, 2000; Takeuchi and Frank, 2001). Moreover, currently consumers have a preference for food that is free of additives. This shift in consumer behaviour discourages the use of post-harvest sanitizers for fresh produce decontamination. In addition, some chemical sanitation agents can result in the generation of potential hazardous by-products (Gil et al., 2009). For example, the reaction of chlorine with natural organic matter has been shown to generate by-products such as chloroform, haloacetic acids and trihalomethanes, which can elicit carcinogenic or mutagenic effects (Hua and Reckhow, 2007; Nieuwenhuijsen et al., 2000).

The inadequacy of post-harvest sanitizers to inactivate pathogenic organisms on fresh produce coupled with potential human health risks associated with in-situ-generated by-products strongly underscores the need to produce safe raw materials at pre-harvest level. Design of strategies to control pre-harvest vegetable contamination in the field requires thorough understanding of the factors that affect the survival of potential pathogens in the soil–plant ecosystem during cultivation. One such factor is the rhizosphere. The rhizosphere is a

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nutrient-rich ecological niche where the soil is under direct influence of the plant roots and where competition within the microbial community has been shown to be fiercely high (Berg et al., 2002). Since soil is not the primary habitat of *E. coli* O157:H7 and *Salmonella* spp., the rhizosphere of cultivated vegetable crops could either enhance their survival in soil increasing the risk of contamination of edible plant parts at harvest or reduce their survival and thus prevent contamination. Previous research conducted with cereals to elucidate the effect of the rhizosphere on survival of enteric pathogenic bacteria provided contradictory results. Gagliardi and Karns (2002) demonstrated enhanced persistence of *E. coli* O157:H7 in soils in the rhizosphere of maize compared to bulk soil. Williams et al. (2007) showed that the survival of *E. coli* O157:H7 in the rhizosphere of maize grown in waste-amended soil was affected by waste type but unaffected by the rhizosphere. Recently, Semenov et al. (2009) reported a significant positive effect of the rhizosphere of lettuce plants on average cell density of *E. coli* O157:H7 and *S.* Typhimurium in soil under controlled green house conditions (16 °C and 50% RH). However, we are not aware of any previous reports on the effect of rhizosphere of vegetable plants on survival of enteric food-borne pathogens in manure-amended soil under tropical field conditions as found in Sub-Saharan Africa. In previous research, we observed that *E. coli* O157:H7 and *S.* Typhimurium appear to survive longer in manure-amended soil on which cabbages were cultivated (Ongeng et al., 2011a) than in manure-amended bulk soil (Ongeng et al., 2011b) under field conditions in the Central Agro-Ecological Zone (CAEZ) of Uganda (Wortman and Eledu, 1999). The longer survival observed in the presence of plant roots suggested the possibility of a rhizosphere effect. However, no conclusions could be drawn about the rhizosphere effect since survival in bulk soil and in cultivated soil were determined in different experiments. The objective of this study was to determine whether under tropical field conditions encountered in the CAEZ of Uganda, the rhizosphere of cabbage plants influences survival of *E. coli* O157:H7 and *S.* Typhimurium in manure-amended soil, and to assess transfer of the pathogens to the foliage during cultivation.

2. Materials and methods

2.1. Bacterial strains and culture media

Rifampicin-resistant derivatives of non-virulent *E. coli* O157:H7 ATCC 43888 (*E. coli* O156:H7-Rifr) and *S.* Typhimurium LT2A (*S.* Typhimurium-Rifr) were used. *Escherichia coli* O157:H7 ATCC 43888 lacks the genes for shiga-like toxins 1 (*stx*1) and 2 (*stx*2). The *stx*1 and *stx*2 genes in *E. coli* O157:H7 did not influence bacterial survival in bovine manure and manure slurry (Kudva et al., 1998). *S.* Typhimurium LT2A is a virulence-attenuated laboratory strain due to a mutation in the *rpoS* gene. The strain LT2A is a spontaneous derivative of the virulent strain *S.* Typhimurium LT2V (Swords et al., 1997). Despite the altered *rpoS* allele in *S.* Typhimurium LT2A, the organism survived stationary-phase and oxidative stresses as well as strains carrying the wild type *rpoS* allele. In addition, the mutated *rpoS* allele in *S.* Typhimurium LT2A did not affect survival of the organism in J774 cells and bone marrow-derived macrophages (Wilmes-Riesenberg et al., 1997). Moreover, *S.* Typhimurium LT2A colonised barley roots to the same degree as virulent *S.* Typhimurium DT104 strain (Kutter et al., 2006). CT-SMAC-Rif100-Cy50-Ny50 (Cefixime-Tellurite-Sorbitol MacConkey agar containing 100 μg/ml rifampicin, 50 μg/ml nystatin and 50 μg/ml cyclohexamide) and XLT4-Rif100-Cy50-Ny50 (Xylose-Lysine-Tergitol-4 agar containing 100 μg/ml rifampicin, 50 μg/ml Nystatin and 50 μg/ml cycloheximide) was used for selective detection and enumeration of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr, respectively. The enumeration method based on the use of rifampicin-resistant derivatives and appropriate selective media was previously validated for assessing the survival of *E. coli* O157:H7 and *S.* Typhimurium in soil (Ongeng et al., 2011c). CT-SMAC and XLT4 were obtained from Merck (Darmstadt, Germany) while rifampicin, nystatin and cycloheximide were procured from Fluka Biochemika (Milan, Italy).

2.2. Experimental set-up

Fresh cattle manure (pH: 6.7; N: 5.8; P: 3.2; K: 2.2; Ca: 1.7; Mg: 1.6 g/kg) and soil (organic matter: 3.2%; pH: 6.7; sand: 53.4%; silt; 12.65%, clay: 17.50%) was respectively obtained from the Animal Production Unit and experimental crop fields of the National Crop Resources Research Institute, Namulonge, Uganda. The manure originated from grazing dairy cattle of the Nganda breed while soil originated from an experimental field previously used for cultivation of maize and bean. The animals fed on natural pasture consisting of fresh grass and browse legumes. Inocula were prepared by cultivating *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in Luria–Bertani broth (5 g/L yeast extract, 10 g/L tryptone, 10 g/L NaCl and 14 g/L bacteriological agar; all from Merck) supplemented with 100 μg/ml rifampicin for 18 h at 37 °C. The cells were harvested by centrifugation ($4000 \times g$ for 10 min), washed and re-suspended in 0.9% saline to achieve inocula concentrations of 9 log CFU/ml. Fresh cow manure was inoculated with *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr at a rate of 5 or 8 log CFU/g, following the procedure previously described by Ongeng et al. (2011a). The inoculated manure was mixed with soil (1 part manure to 9 part soil) to achieve approximately 4 or 7 log CFU/g of each organism in the manure-amended soil. The inoculated manure-amended soil was dispensed in 6 L plastic pots and used in two different experimental set-ups. In the first set-up, three-week old cabbage (*Brassica oleracea* var. *capitata* cv. Gloria) seedlings were transplanted into the pots. Each pot contained one plant. In the second set-up, no seedlings were transplanted to the pots. This set-up served as control for the rhizosphere effect. The pots were randomly placed as sets of three replicates in an open space in the field. Each replicate consisted of 60 pots which provided sufficient experimental units for destructive sampling and analysis. The pots were spaced 60 cm apart within a set and 2 m apart between sets. The plants were cultivated for 120 days. Data on precipitation and minimum and maximum temperature during the experimental period were obtained from a nearby meteorological unit and are shown in Fig. 1.

2.3. Microbiological analysis

The CFU numbers of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr on/in cabbage leaves, and in the rhizosphere and in bulk soil were determined immediately after manure amendment and subsequently after every 3 and 8 days in case of the 4 and 7 log CFU/g inoculum, respectively. The rhizosphere was sampled by carefully removing plants from two pots per replicate from the soil matrix. The roots together with tightly adhering soil were separated from the plant stem using a sterile scalpel. Rhizosphere and foliage samples were kept separately for determination of the CFU number. Rhizosphere samples derived from young plants were placed into sterile 50 ml Falcon tubes containing 0.9% saline and vortexed four times for 1 min to separate cells from the rhizosphere. Rhizosphere samples of mature plants were placed in sterile zip-lock bags and physically massaged four times for 1 min. Dilution series of the extracts were then prepared in 0.9% saline followed by plating in duplicate 100 μl of appropriate dilutions on CT-SMAC-Rif100-Cy50-Ny50 and XLT4-Rif100-Ny50-Cy50 for CFU counting of *E. coli* O157:H7-Rifr and S. Typhimurium-Rifr, respectively. The CFU were counted after 24 h of incubation at 37 °C. Bulk soil samples were drawn from pots without plants (two pots per replicate). Five-gramme samples were taken from the middle of the pots and suspended in 50 ml falcon tubes containing 45 ml of 0.9% saline. The samples were vortexed 4 times for 1 min, diluted in 0.9% saline and spread plated in duplicate for determination of CFU numbers as described above for the rhizosphere

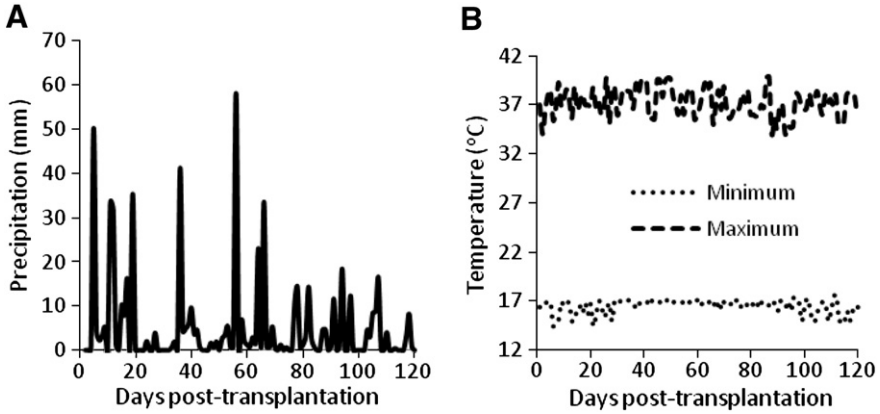

Fig. 1. Recorded daily precipitation (A) and temperature minima and maxima (B) during the experimental period.

samples. The CFU numbers on/in cabbage leaves were determined as follows. In the case of plants with less than 12 true leaves, all the leaves of the plant were processed while in the case of plants at the pre-cupping stage and beyond, all the outer (4–5) and 8–10 inner leaves were used. In each case, the leaves were divided into two sets by cutting each leaf longitudinally into two equal parts using a sterile scalpel. One set was surface-sterilised in 1% $AgNO_3$ in order to enumerate internalised cells as reported by Franz et al. (2007). Briefly; the leaves were washed twice for 15 seconds in distilled water in order to remove adhering soil. The leaves were then dipped in 1% $AgNO_3$, agitated by hand for 10 s and left in the solution for 1 min. The $AgNO_3$ treatment procedure was repeated once more followed by washing the surface-sterilised leaf (SSL) samples twice for 10 s in sterile distilled water to remove the remaining $AgNO_3$. The effectiveness of the method for leaf-surface sterilisation was examined first in a preliminary experiment with cabbage leaves artificially contaminated with *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr. In that experiment, cabbage leaves were cut into 3 cm discs (90 in total). The discs were divided into 3 sets and immersed in 7 log CFU/g inoculum suspension of *E. coli* O157:H7-Rifr, *S.* Typhimurium-Rifr or in sterile distilled water for 10 min followed by air drying for 5 min. In each case, half of the discs (15) were surface-sterilised as explained above and the other half (15) left as control. The discs were placed on XLT4-Rifr100-Cy50-Ny50 and CT-SMAC-Rif100-Cy50-Ny50 and incubated for 30 min at room temperature. The discs were removed and the plates incubated for 24 h at 37 °C. Results showed that all the prints made from $AgNO_3$-treated discs were negative for *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr while all the prints made from control discs were positive. The remaining set consisting of non-surface sterilised leaves (NSSL) was used to determine total CFU counts of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr present on and in the leaves. The SSL and NSSL samples were ground in a mortar containing 0.9% NaCl followed by quantification of CFU numbers of the test organisms as mentioned above. In case the detection limit of the plate count method (2 log CFU/g) was reached, an enrichment culture procedure was adopted. *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr was enriched in modified EC broth containing novobiocin and in selenite cystine broth (Merck), respectively, according to the instructions of the manufacturer. One hundred micro-litre aliquots of the enrichment broth was streaked on CT-SMAC-Rif100-Ny50-Cy50 and XLT4-Rif100-Cy50-Ny50 and incubated for 24 h at 37 °C for detection of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr, respectively.

2.4. Statistics and modelling bacterial survival in the rhizosphere of cabbage plants and in bulk soil

Survival curves of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr (log CFU versus time) for the soil ecosystem were fitted to the Double Weibull function described by Eq. 1 (Coroller et al., 2006) using GInaFiT, a Microsoft® Excel Add-In model fitting tool (Geeraerd et al., 2005). Each replicate was fitted separately.

$$N(t) = \frac{N_o}{1+10^{\alpha}}\left[10^{-\left(\frac{t}{\delta_1}\right)^{p}+\alpha} + 10^{-\left(\frac{t}{\delta_2}\right)^{p}}\right] \quad (1)$$

By Eq. 1, the overall population of the test organism is partitioned into two sub-populations 1 and 2 based on the assumption that sub-population 1 is more sensitive to the environmental stress than the second sub-population resulting into more rapid decay of sub-population 1. The parameters in Eq. 1 are: $N(t)$ = number of survivors (log CFU g^{-1}); No = initial inoculum size (log CFU g^{-1}); t = time (days); p = shape parameter (dimensionless); δ_1 = time needed for the first decimal reduction of sub-population 1 (days); δ_2 = time needed for first decimal reduction of sub-population 2 (days); α = ratio of the fraction of sub-population 1 to the fraction of subpopulation 2 at time zero (dimensionless). In addition to these parameters, the output of GInaFiT also provides the time to achieve a 4 log reduction (t_{4D}) in the overall population of the test organism (days). The goodness-of-fit of the survival curves was assessed using the root mean sum of squared error (RMSE). The *lsqnonlin* procedure of the MatLab Optimization Toolbox (The Mathworks Inc., Version 2007b; www.mathworks.com) was used to calculate the 95% confidence and prediction intervals for the fitted curves via the estimated parameter variance–covariance matrix. The parameter values of the fitted models were compared between curves obtained for the cabbage rhizosphere and curves obtained for the bulk soil using the independent 2-sample *t*-test at 5% level of significance. At each sampling point, the independent 2-sample *t*-test was used to compare mean CFU counts in the rhizosphere of cabbage plants with those in bulk soil at 5% level of significance. The models were used to derive the time for CFU counts of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the rhizosphere and in bulk soil to reach the detection limit (*ttd*) of the plate count method. In the case of plants cultivated on manure-amended soil contaminated with 4 log CFU/g inoculum, CFU of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr on the foliage were below or close to the detection limit of the plate count method, and were not subjected to statistical comparison. In the case of plants cultivated on manure-amended soil initially contaminated with 7 log CFU/g inoculum, counts of *E. coli* O157:H7-Rifr or *S.* Typhimurium-Rifr between SSL and NSSL samples at each sampling point were compared using the independent 2-sample *t*-test at 5% level of significance. GenStat Discovery Edition 3 (www.discovery.genstat.co.uk) was used for all statistical analyses.

3. Results

3.1. Survival of E. coli O157:H7-Rifr in cabbage rhizosphere and in bulk soil

In the case of an initial inoculum density of 4 log CFU/g, CFU of *E. coli* O157:H7-Rifr in the rhizosphere of cabbage plants and in bulk soil declined till below the detection limit of the plate count method at 21 and 24 days post-transplantation, respectively (Fig. 2A). At each sampling point, CFU number was not significantly different between the rhizosphere and bulk soil ($p>0.05$). Enrichment showed that the organism remained at low numbers in the rhizosphere and in bulk soil till day 27. Survival curves obtained with an inoculum density of 7 log CFU g^{-1} in cabbage plant rhizosphere and in bulk soil are shown in Fig. 2B. CFU counts of *E. coli* O157:H7-Rifr stabilised around 7 log CFU/g for at least 24 days both in the rhizosphere and in bulk soil before an appreciable decline in CFU number could be observed. Thereafter, CFU number of *E. coli* O157:H7-Rifr declined with time, but at each sampling point, mean CFU counts were significantly higher in the rhizosphere than in bulk soil ($p\leq0.05$). In bulk soil, CFU counts of *E. coli* O157:H7-Rifr reached the detection limit of the plate count method 72 days post-inoculation and could not be detected by enrichment beyond day 80. In the rhizosphere, CFU number of the organism remained above the detection limit of the plate count technique till crop harvest (120 days post-transplantation). Survival curves of *E. coli* O157:H7-Rifr were well fitted by the Double Weibull Model. Statistical measures of the fits and parameter values of the fitted curves are presented in Table 1. Graphical illustrations of the model fit, and the 95% confidence and prediction intervals determined by the model are shown in Fig. 3. In the case of 4 log CFU/g inoculum, the values of α, δ_1, δ_2 and p parameters were not statistically different between the rhizosphere and bulk soil ($p>0.05$). In the case of the 7 log CFU/g inoculum, the values of the δ_1, δ_2, and t_{4D} parameters were significantly higher for the rhizosphere than for bulk soil ($p\leq0.05$) while the values of α and p parameters were not significantly different between the rhizosphere and bulk soil ($p>0.05$). The times needed to reach the detection limit of the plate count method (*ttd*) according to the model are presented in Table 2. In the case of 4 log CFU/g inoculum, the model-derived *ttd* value was not significantly different between the rhizosphere and bulk soil ($P>0.05$) while in the case of 7 log CFU/g inoculum, the *ttd* value according to the model was significantly longer in the rhizosphere than in bulk soil ($P\leq0.05$).

3.2. Survival of S. Typhimurium-Rifr in cabbage rhizosphere and in bulk soil

Survival curves of *S.* Typhimurium-Rifr in the rhizosphere and in bulk soil obtained with an inoculum density of 4 log CFU/g are shown in Fig. 4A. CFU counts of *S.* Typhimurium-Rifr in the rhizosphere and in bulk soil dropped till below the detection limit of the plate count method 30 and 27 days post-inoculation, respectively. At each sampling point, CFU number in the rhizosphere was not significantly different from that observed in bulk soil ($p>0.05$). Using the enrichment method, *S.* Typhimurium-Rifr could not be detected both in the rhizosphere and in bulk soil beyond day 42. Survival patterns of *S.* Typhimurium-Rifr obtained with an inoculum density of 7 log CFU/g are shown in Fig. 4B. CFU number of *S.* Typhimurium-Rifr remained fairly stable for the first 24 days both in the rhizosphere and in bulk soil, after which decline became observable. During the decline phase, mean CFU counts of *S.* Typhimurium-Rifr in the rhizosphere were significantly higher than in bulk soil ($p\leq0.05$) except on days 32, 40, 64, 72 and 80 where CFU counts were not significantly different between the rhizosphere and bulk soil ($p>0.05$). In bulk soil, CFU counts of *S.* Typhimurium-Rifr reached the detection limit of the plate count technique 88 days post-transplantation and remained detectable by enrichment up to day 96. In the rhizosphere, CFU counts of *S.* Typhimurium-Rifr remained above the detection limit of the plate count method till harvest (120 days post-transplantation). Survival curves of *S.* Typhimurium-Rifr in the rhizosphere and in bulk soil were well described by the Double Weibull model. Table 1 presents data on statistical measures of the fits and parameter values of the fitted curves. The model fit, and the 95% confidence and prediction intervals are illustrated in Fig. 5. In the case of the 4 log CFU/g inoculum, none of the parameter values of the Double Weibull model describing survival in the rhizosphere and in bulk soil were significantly different ($p>0.05$). In the case of the 7 log CFU/g inoculum, the values of the δ_1 and p parameters describing survival *S.* Typhimurium-Rifr in the rhizosphere were not significantly different from those describing survival of the organism in bulk soil ($p>0.05$). In contrast, the values of α, δ_2 and t_{4D} parameters were significantly higher in the rhizosphere than those in bulk soil ($p\leq0.05$). The *ttd* values according to the Double Weibull model are presented in Table 2. The model-derived *ttd* value was not significantly different between the rhizosphere and bulk soil in the case of an inoculum concentration of 4 log CFU/g ($P>0.05$). However, when introduced into the soil at an inoculum density of 7 log CFU/g, the model-derived *ttd* value was significantly longer in the rhizosphere than in bulk soil ($P\leq0.05$).

3.3. E. coli O157:H7-Rifr and S. Typhimurium-Rifr contamination on and in cabbage foliage

For cabbage cultivated on manure-amended soil inoculated at an initial cell density of 4 log CFU/g, incidence of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr contamination on/in the foliage was especially

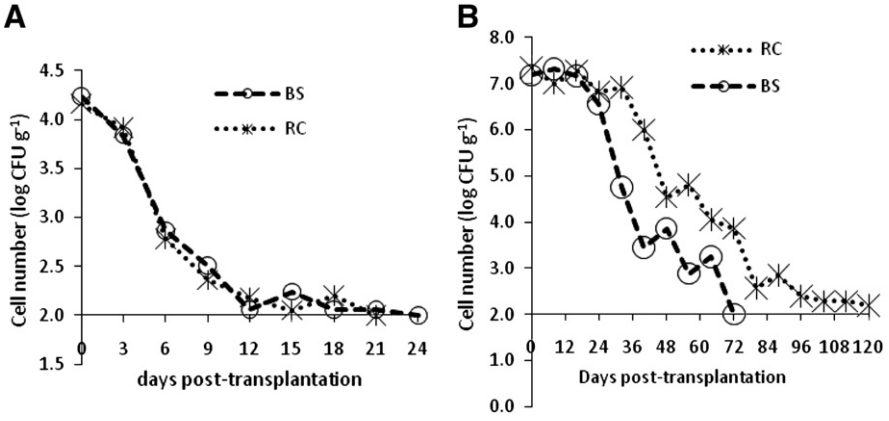

Fig. 2. Survival of *E. coli* O157:7-Rifr in the rhizosphere of cabbage plants (RC) and in bulk soil (BS). (A): 4 log CFU/g inoculum; (B): 7 log CFU/g inoculum. Data points are averages of three replicates (two pots per replicate, analysed in duplicates, n = 12). Standard errors are not shown for clarity of illustration.

Table 1
Statistical measures and parameter values of the fitted curves describing the survival of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in cabbage plant rhizosphere and in bulk soil according to the Double Weibull model.

Matrix	δ_1	δ_2	α	p	Log N_o	RMSE	AdjR^2	t_{4D}
E. coli O157:H7-Rifr inoculated at 4 log CFU/g								
RC	5 ± 0.2^a	50 ± 9.0^a	1.93 ± 0.07^a	1.4 ± 0.12^a	4.3 ± 0.05^a	0.14	0.97	NA
BS	5 ± 0.2^a	34 ± 3.4^a	1.86 ± 0.05^a	1.7 ± 0.32^a	4.2 ± 0.05^a	0.16	0.96	NA
E. coli O157:H7-Rifr inoculated at 7 log CFU/g								
RC	32 ± 2.11^a	121.65 ± 4.2^a	4.26 ± 0.34^a	2.71 ± 0.73^a	7.39 ± 0.09^a	0.45	0.95	73 ± 0.52^a
BS	24 ± 0.99^b	72.60 ± 2.83^b	3.72 ± 0.11^a	3.43 ± 0.44^a	7.34 ± 0.09^a	0.44	0.96	50 ± 1.20^b
S. Typhimurium-Rifr inoculated at 4 log CFU/g								
RC	7.0 ± 0.3^a	36 ± 2.7^a	1.85 ± 0.06^a	3.48 ± 0.30^a	4.30 ± 0.05^a	0.20	0.95	NA
BS	8.0 ± 0.3^a	39 ± 2.6^a	1.75 ± 0.06^a	3.31 ± 0.50^a	4.30 ± 0.05^a	0.23	0.92	NA
S. Typhimurium-Rifr inoculated at 7 log CFU/g								
RC	27 ± 2.0^a	119 ± 9.2^a	4.38 ± 0.34^a	1.62 ± 0.16^a	7.71 ± 0.12^a	0.56	0.93	65 ± 0.72^a
BS	26 ± 2.0^a	70 ± 7.0^b	2.70 ± 0.26^b	1.99 ± 0.24^a	7.49 ± 0.12^a	0.57	0.93	55 ± 0.84^b

For each organism and at each inoculum density, means ($\pm$SE, n = 3) in the same column followed by the same superscripts are not significantly different ($p > 0.05$). RC: rhizosphere of cabbage plants; BS: bulk soil; δ_1: time for first decimal reduction of sub-population 1 (days); δ_2: time for first decimal reduction of sub-population 2 (days); α: ratio of the fraction of sub-population 1 to fraction of sub-population 2; p: shape parameter (dimensionless); No: original cell concentration (log CFU/g); RMSE: Root mean sum of squared error; AdjR^2: adjusted R^2; t_{4D}: time for 4 log reduction of overall population (days); and NA: not applicable.

observed in samples taken before 30 and 39 days post-transplantation, respectively. Contamination levels of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr on the foliage were always close or at the detection limit of the plate count method. Results are presented in terms of number of positive samples over the number of plants examined (Table 3). The organisms were mostly only detected on NSSL, and only rarely in SSL samples indicating that contamination was mostly on the leaf surface.

The evolution of *E. coli* O157:H7-Rifr CFU counts on/in NSSL and in SSL samples during cultivation on manure-amended soil contaminated with 7 log CFU/g inoculum is shown in Fig. 6A. Mean CFU counts of *E. coli* O157:H7-Rifr on/in NSSL samples increased to approximately 3.44 log CFU/g 8 days post-transplantation and stabilised around that level till day 40. Thereafter *E. coli* O157:H7-Rifr CFU number declined to the detection limit of the plate count method 80 days post-transplantation but remained detectable by enrichment till the time of harvest (120 days post-transplantation). In SSL samples, *E. coli* O157:H7-Rifr remained undetected for about two weeks, after which cell counts peaked at approximately 2.5 log CFU/g 24 days post-transplantation, stabilised at that level till day 32 and thereafter declined till below the detection limit of the plate count method 56 days post-transplantation. By enrichment, *E. coli* O157:H7-Rifr remained detectable in SSL samples up to the time of harvest. During cultivation, mean CFU counts of *E. coli* O157:H7-Rifr on/in NSSL samples was significantly higher than those in

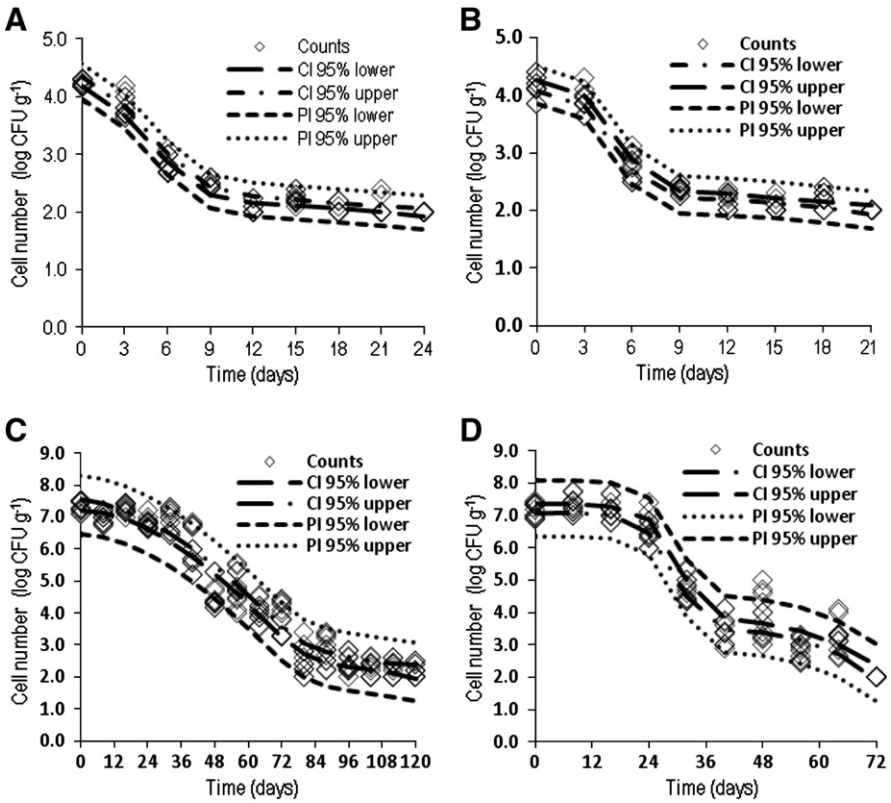

Fig. 3. Model fit, confidence interval (CI) and predicted interval (PI) of the survival curves of *E. coli* O157:H7-Rifr in the rhizosphere of cabbage plants and in bulk soil according to the Double Weibull model. A: 4 log CFU/g inoculum in rhizosphere of cabbage plants; B: 4 log CFU/g inoculum in bulk soil; C: 7 log CFU/g inoculum in the rhizosphere of cabbage plants; D: 7 log CFU/g inoculum in bulk soil. All data points are shown.

Table 2
Time to reach the detection limit (*ttd*) of the plate count method (2 log CFU/g) for *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the rhizosphere of cabbage plants and in bulk soil according to the Double Weibull model.

Organism	Time to reach detection limit (*ttd*) of the direct plate count method (days)	
	Rhizosphere	Bulk soil
E. coli O157:H7-Rifr		
Inoculated at 4 log CFU/g	22 ± 0.52^{a}	24 ± 0.38^{a}
Inoculated at 7 log CFU/g	117 ± 1.70^{a}	85 ± 2.00^{b}
S. Typhimurium-Rifr		
Inoculated at 4 log CFU/g	30 ± 0.66^{a}	30 ± 0.83^{a}
Inoculated at 7 log CFU/g	119 ± 0.51^{a}	91 ± 1.50^{b}

For each organism and each inoculum density, means ($\pm$SE, n = 3) in the same row followed by the same superscripts are not significantly different ($p > 0.05$).

SSL samples from day 8 till day 72 ($p \leq 0.05$). However, from day 80 onwards, cell numbers of *E. coli* O157:H7-Rifr on/in NSSL and in SSL samples were statistically similar ($p > 0.05$). Indeed, all the 6 plants examined were positive for the organism at harvest irrespective of leaf-surface treatment with $AgNO_3$.

The evolution of *S.* Typhimurium-Rifr CFU numbers on/in NSSL and in SSL samples during cultivation on manure-amended soil contaminated with 7 log CFU/g inoculum is shown in Fig. 6B. With NSSL samples, mean CFU counts of *S.* Typhimurium-Rifr increased to approximately 3.3 log CFU/g 8 days post-transplantation, peaked at about 3.5 log CFU/g on day 32, and thereafter declined till below the detection limit of the plate count method 64 days post-transplantation. *S.* Typhimurium-Rifr remained detectable on/in NSSL samples by enrichment till the time of harvest. In SSL samples, CFU of *S.* Typhimurium-Rifr increased to about 2.5 log CFU/g 16 days post-transplantation, stabilised at that level till day 24 and thereafter declined till below the detection limit of the plate count method 48 days post-transplantation. However, *S.* Typhimurium-Rifr remained detectable in SSL samples by enrichment till harvest. From day 8 till day 56, CFU counts of *S.* Typhimurium-Rifr on/in NSSL samples was significantly higher than in SSL samples ($p \leq 0.05$). Beyond day 64, CFU number of *S.* Typhimurium-Rifr in SSL samples was the same as those observed on/in NSSL samples ($p > 0.05$). All the 6 plants examined were positive for the organism at harvest irrespective of leaf-surface treatment with $AgNO_3$.

4. Discussion

In this study, we examined whether under tropical field conditions, the rhizosphere of cabbage plants influences survival of *E. coli* O157:H7 and *S.* Typhimurium in manure-amended soil. Therefore, we compared persistence times of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the rhizosphere of cabbage plants with those in bulk soil. The results revealed that, both for *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr, the 7 log CFU/g inocula survived longer in the rhizosphere than in bulk soil whereas survival times of the 4 log CFU/g inocula in the rhizosphere and in bulk soil were identical. Different explanations can be provided to account for the difference, i.e., moisture availability and rhizosphere effect. Moisture is an important factor necessary for survival of pathogens in soil (Fenlon et al., 2000) and the canopy provided by cabbage leaves could have maintained moisture level better in the cultivated compared to the bulk soil. However, we showed in a previous study that the survival of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in manure-amended soil in the field was not affected by moisture condition in the matrix (Ongeng et al., 2011b), thus indicating that the longer survival of the 7 log CFU g^{-1} inocula in the rhizosphere was not due to a better moisture maintenance due to canopy provided by cabbage heads but rather due to the "rhizosphere effect". Rhizosphere effect refers to the phenomenon that in rhizospheres, in comparison to bulk soil, the biomass and activity of microorganisms is enhanced as a result of the exudation of growth beneficial compounds from the plant roots (Berg et al., 2005). The fact that the rhizosphere of cabbage plants enhanced survival of the 7 log CFU/g inocula but had no effect on survival of the 4 log CFU/g inocula in manure-amended soil suggests that high density inocula were probably more competent than low density inocula at withstanding pressures exerted by competitive and predatory microbial communities present in the rhizosphere. Rhizosphere competence has been linked to the ability of colonising bacteria to use root exudates as sources of carbon, and to synthesise compatible solutes to counter osmolarity dynamics in the rhizosphere (Lugtenberg and Dekkers, 1999; Miller and Wood, 1996; Sato and Jiang, 1996).

Curve fitting to a mathematical model can become a useful tool when quantification of biological phenomena can be achieved. Based on the results of this study, it is apparent that the Double Weibull model adequately determined the *ttd* for *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the rhizosphere of cabbage plants and in bulk soil. The *ttd* values according to the Double Weibull model identified in this research (Table 2) were proximate with the observed values (Figs. 2 and 4). This was also observed in previous studies (Ongeng et al., 2011a, 2011b), and therefore confirms that the Double Weibull model is a useful tool for describing the survival of *E. coli* O157:H7 and *S.* Typhimurium both in the bulk soil and in the rhizosphere under field conditions.

The observation that all the plants cultivated on manure-amended soil contaminated with 4 log CFU/g inoculum were negative for *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr at harvest is consistent with the results of the previous study (Ongeng et al., 2011a). This suggests that under tropical field conditions, cultivation of cabbage on soil amended with manure containing low cell densities of *E. coil* O157:H7

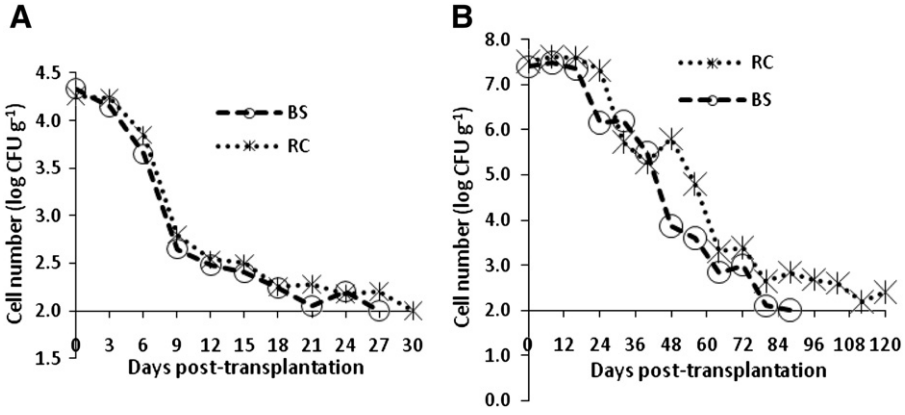

Fig. 4. Survival of *S.* Typhimurium-Rifr in the rhizosphere of cabbage plants (RC) and in bulk soil (BS). A: 4 log CFU/g inoculum; B: 7 log CFU/g inoculum. Data points are averages of three replicates (two pots per replicate, analysed in duplicates, n = 12). Standard errors are not shown for clarity of illustration.

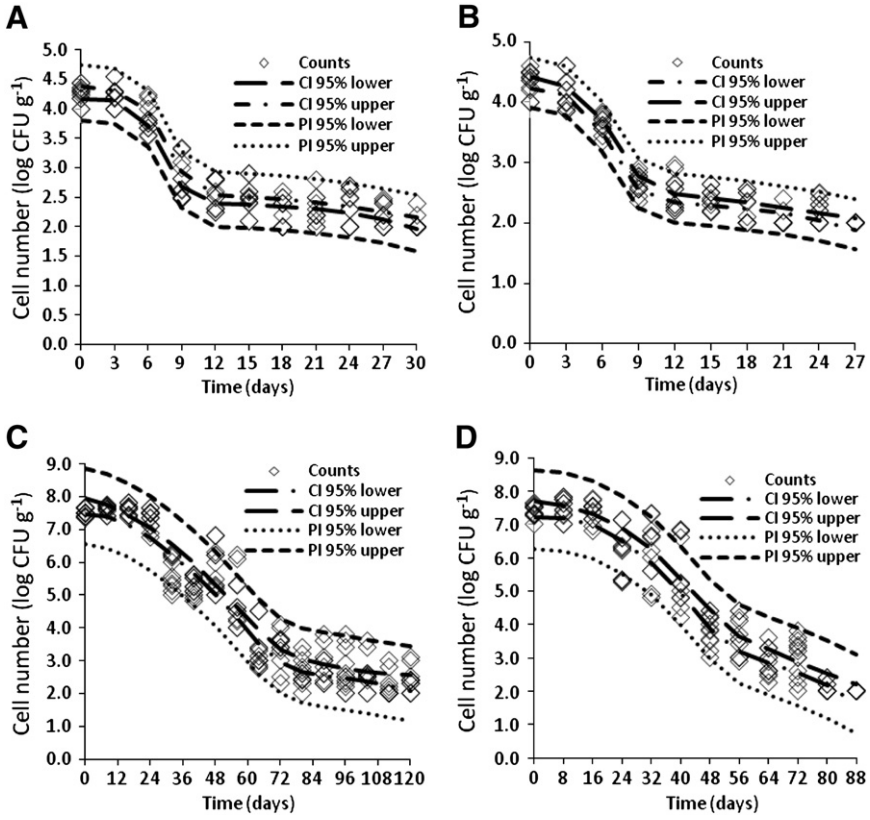

Fig. 5. Model fit, confidence interval (CI) and predicted interval (PI) of the survival curves of *S.* Typhimurium-Rifr in the rhizosphere of cabbage plants and in bulk soil according to the Double Weibull model. A: 4 log CFU/g inoculum in the rhizosphere of cabbage plants; B: 4 log CFU/g inoculum in bulk soil; C: 7 log CFU/g inoculum in the rhizosphere of cabbage plants; D: 7 log CFU/g inoculum in bulk soil. All data points are shown.

and/or *S.* Typhimurium (e.g., 4 log CFU/g) is not a priority risk factor for consumer health. It was evidently clear that at 7 log CFU/g inoculum, contamination of the leaves with *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr was both superficial and internal for most part of the cultivation period as demonstrated by analysing leaf samples before and after surface sterilisation with 1% $AgNO_3$. It took approximately two and three weeks, respectively before *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr initially introduced into the soil matrix at 7 log CFU/g could be detected in SSL samples (Fig. 6). This observation suggests the occurrence of a lag time between attachment and establishment of the organisms in protected areas of the leaf since the organisms were already detectable on NSSL samples one week post-transplantation. There is no apparent explanation for the observed lag. However, other researchers have shown that internalisation of food-borne bacterial pathogens in produce leaves depended on leaf age (Bernstein et al., 2007; Brandl and Amundson, 2008; Pu et al., 2009), presence of phytopathogens (Barak and Liang, 2008; Brandl, 2008) and on protozoal grazing (Gourabathini et al., 2008). Detection of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in SSL samples throughout the cultivation period for cabbage grown on manure-amended soil initially contaminated with 7 log CFU/g inoculum suggests that the organisms were present in internal locations within the leaves. We derive this assertion based on the findings of previous work which demonstrated entrapment of *E. coli* O157:H7 20 to 100 μm below the leaf surface in stomata and cut edges after immersion of lettuce leaves in approximately 7–8 log CFU/g inoculum suspension (Seo and Frank, 1998).

Table 3
Incidence of *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr contamination on/in the foliage of cabbage during cultivation on manure-amended soil contaminated at 4 log CFU/g.

Sampling time (DPT)	*E. coli* O157:H7-Rifr		*S.* Typhimurium-Rifr	
	NSSL	SSL	NSSL	SSL
0	0/6	0/6	0/6	0/6
3	6/6*	0/6	6/6*	0/6
6	6/6*	0/6	6/6*	0/6
9	6/6*	1/6**	6/6*	2/6**
12	6/6*	2/6**	6/6*	0/6
15	6/6*	0/6	6/6*	1/6**
18	4/6*	0/6	6/6*	0/6
24	2/6*	0/6	6/6*	0/6
27	3/6*	0/6	6/6*	0/6
30	0/6	0/6	4/6*	0/6
33	0/6	0/6	2/6*	0/6
36	0/6	0/6	1/6*	0/6
39	0/6	0/6	0/6	0/6
42	0/6	0/6	0/6	0/6
45	0/6	0/6	0/6	0/6
48	0/6	0/6	0/6	0/6
51	0/6	0/6	0/6	0/6
54	0/6	0/6	0/6	0/6

Values show number of positive samples per number of plants tested. DPT: days post-transplantation; SSL: surface-sterilised leaves; NSSL: non-surface-sterilised leaves.
* Observed with plating.
** Observed after enrichment.

An important observation from the current study is that enhanced survival of the 7 log CFU/g inoculum in the rhizosphere was associated with long-term contamination of cabbage leaves. This was in contrast with the scenario observed when 4 log CFU/g inocula were used. Dong et al. (2003) observed a strong correlation between colonisation of the rhizosphere and endophytic colonisation of *Medicago sativa* seedlings by *S.* Typhimurium for plants cultivated in a growth chamber maintained at 22 °C with respective day and night cycle of 15 and 9 h. However, the results of Dong et al. (2003) cannot be directly compared with the results of this study since Dong et al. (2003) did not follow the fate of *S.* Typhimurium in the plant and in the

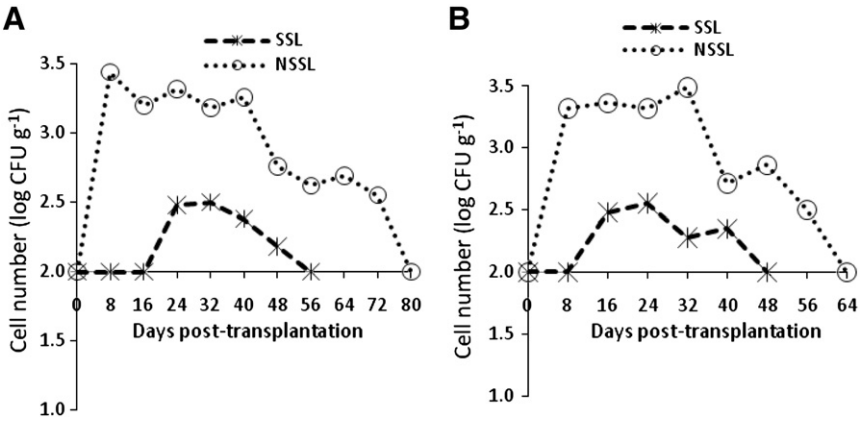

Fig. 6. Population size dynamics of *E. coli* O157:H7-Rifr (A) and *S.* Typhimurium-Rifr (B) on/in non-surface-sterilised (NSSL) and in surface-sterilised leaf (SSL) samples of cabbage during cultivation on manure-amended soil contaminated at a rate of 7 log CFU/g inoculum. Data points are averages of three replicates (2 plants per replicate, analysed in duplicates, n = 12). Standard errors are not shown for clarity of illustration.

rhizosphere till plant maturity. Based on the fact that the infectious doses of *E. coli* O157:H7 and *Salmonella* spp. are rather low (<100 cells) (Darwin and Miller, 1999; Paton and Paton, 1998), therefore, the low levels of the organisms detected on and in cabbage leaves by enrichment at harvest for plants cultivated on manure-amended soil with initial inoculum level of 7 log CFU/g would still compromise microbiological safety of the produce.

This study applied solely a culture-based methodology and did not include measurements of the viable but non-culturable (VBNC) fraction of the test organisms. The use of culture-based technique as a sole method to study environmental survival of bacterial pathogens as applied in this study has been criticised by proponents of the VBNC physiological state of bacteria (Ritchie et al., 2003). The basis for the criticisms have been that culture-based methods may fail to recover the fraction of the test population that has entered into a VBNC state as a result of environmental stress (Oliver, 2005; Ritchie et al., 2003), albeit controversies surrounding the occurrence and public health implications of pathogenic bacteria in the VBNC physiological state (Caro et al., 1999; Kell et al., 1998; Oliver, 2005; Sylvester et al., 2001). Quantitative polymerase chain reaction (qPCR) technique is believed to overcome the limitations of culture-based method since it is thought to quantify both the culturable and the VBNC fractions of the test strains under investigation (Garcìa et al., 2010; Ibekwe et al., 2002; Ibekwe and Grieve, 2003; Novinsack et al., 2007). However, constraints such as the requirement for an enrichment step (Klerks et al., 2006; Marsh et al., 1998), lack of an efficient method for extracting nucleic acids from environmental samples (Artz et al., 2006; Klerks et al., 2006; Martin-Laurent et al., 2001), the negative impact of PCR inhibitors on DNA amplification (Monterio, et al., 1997; Tebbe and Vahjen, 1993; van Wintzingerode et al., 1997; Wilson, 1997), the fact that DNA can persist longer after cell death (Artz et al., 2006; Ibekwe et al., 2002; Josephson et al., 1993) and the poor detection limit of qPCR for organisms from environmental samples (3 log CFU/g: Ibekwe et al., 2002; 5 log CFU/g: Artz et al., 2006; 4 log CFU/g: Jacobsen and Holben, 2007) hamper the suitability of the qPCR methodology in survival studies. Notwithstanding the controversies surrounding VBNC physiological state of bacteria, we still believe that a culture-based approach still stands useful in survival studies. In fact, up to date, the bulk of information on the survival of manure-borne bacterial pathogens in agricultural and other environmental matrices has been derived from culture-based studies (Ongeng et al., 2011b and references therein) while qPCR has not yet found a wide application in survival studies due to potential pitfalls mentioned above. In addition, qPCR methodology requires expensive equipment which is usually not available in most laboratories especially in developing countries such as Uganda where the current study was performed.

Various inoculum levels have been used to investigate the behaviour of enteric bacteria in real or simulated agricultural environment (Avery et al., 2004; Islam et al., 2004, 2005; Moontian et al., 2009; Pu et al., 2009; Solomon et al., 2002, Warriner et al., 2003). However, the use of high inoculum density to investigate the interaction of salad crops with enteric food borne pathogens as done in this study has been criticised by other researchers (Moontian et al., 2009; Pu et al., 2009). The critics believe that inoculum densities such as 7 log CFU/g are far much greater than what would be expected in the environment. However, Fukushima and Seki (2004) recovered *E. coli* O157:H7 up to a concentration of 8 log CFU/g from bovine faeces. We believe that such a finding provides a valid reason for challenge tests with high density inocula.

5. Conclusion

This study demonstrated that the effect of rhizosphere of cabbage plants on survival of *E. coli* O157:H7 and *S.* Typhimurium in manure-amended soil under tropical field conditions in Sub-Saharan Africa depended on inoculum density. The study indicated clearly that: i) the rhizosphere of cabbage plants enhanced the persistence of the 7 log CFU/g *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr but had no effect on survival of the 4 log CFU/g inoculum; and ii) enhanced persistence of the 7 log CFU/g *E. coli* O157:H7-Rifr and *S.* Typhimurium-Rifr in the rhizosphere of cabbage plants was associated with long-term contamination of edible plant parts. Therefore, future studies should determine the fate of the rhizosphere-persistent cells beyond the time of harvest.

Acknowledgements

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