Growth of *Salmonella* on sprouting alfalfa seeds as affected by the inoculum size, native microbial load and *Pseudomonas fluorescens* 2–79

C.-H. Liao

Eastern Regional Research Center, Agricultural Research Service, U.S. Department of Agriculture, Wyndmoor, PA, USA

Abstract

**Aims:** To investigate the growth of salmonellae on sprouting alfalfa seeds as affected by the inoculum size, microbial load and *Pseudomonas fluorescens* 2–79.

**Methods and Results:** Alfalfa seeds pre-inoculated with $\leq 10^1$–$10^3$ CFU g$^{-1}$ of salmonellae and with or without *Ps. fluorescens* 2–79 were sprouted in glass jars and the population of salmonellae were determined daily for up to 6 days. The population of salmonellae on germinating seeds reached the maximum 2–3 days after sprouting when total bacterial count reached the maximum (10$^9$ CFU g$^{-1}$). The population of salmonellae on sprouting seeds not treated with *Ps. fluorescens* 2–79 showed a net increase of 3–4 log units. However, the population of salmonellae on alfalfa seeds treated with *Ps. fluorescens* 2–79 showed a net increase of only 1–2 log units. Disinfection of seeds with calcium hypochlorite enhanced the growth of salmonellae.

**Conclusions:** Treatment of seeds with *Ps. fluorescens* 2–79 reduced the growth of salmonellae by 2–3 log units.

**Significance and Impact of the Study:** The potential of *Ps. fluorescens* 2–79 as a biological agent for use in control of salmonellae on sprouting seeds was demonstrated and warrants further investigation.

Introduction

*Salmonella*-contaminated alfalfa sprouts have been implicated in more than 27 foodborne illness outbreaks between 1990 and 2005 (IFSN 2005). The source of pathogen associated with most of these outbreaks was thought to be originated from seeds used in sprouting (FDA/NAC-MCF 1999). As conventional washing and sanitization treatments currently available were unable to remove salmonellae from contaminated seeds to an acceptable level, more effective control strategies are needed to achieve the 5-log reduction target recommended (FDA/NACMCF 1999). Such strategies may include the use of biological intervention to suppress the growth of initial pathogens on seeds and to inhibit the outgrowth of survivors after chemical treatments.

Under the favourable conditions of the sprouting process, salmonellae can grow very rapidly on the surfaces of sprouting seeds. A number of studies (Charkowski et al. 2002) have shown that the population of salmonellae increased very rapidly during the first few days of sprouting. However, the maximum population of salmonellae detected in earlier studies varies very widely from $10^5$ CFU g$^{-1}$ (Stewart et al. 2001a), $10^6$ CFU g$^{-1}$ (Howard and Hutcheson 2003) to $10^7$ CFU g$^{-1}$ (Jaquette et al. 1996). It has not been investigated prior to this study if the growth pattern or the maximum population of salmonellae to be expected during sprout production may be affected by the initial pathogen number or the native microbial load on seeds destined for sprout production.

Fluorescent pseudomonads, especially *Pseudomonas fluorescens*, play an important role in the survival or growth of salmonellae on sprouts (Matos and Garland 2005). In earlier studies, certain strains of *Ps. fluorescens* isolated from raw foods of plant origins have been shown...
to inhibit the growth of a wide spectrum of spoilage and pathogenic bacteria (Liao 2006). One such strain identified as Ps. fluorescens 2–79 has been studied for many years as a biocontrol agent for a root pathogen Gaeumannomyces graminis var. tritici (Weller and Cook 1983). This strain has recently been found effective in inhibiting the growth of salmonellae and other pathogens on various agar media and sprouts (Fett 2006). The objective of this study was to investigate the growth dynamics of salmonellae on sprouting seeds as affected by the inoculum size, native microbial load and Ps. fluorescens 2–79.

Materials and methods

Bacterial strains and culture conditions

Four Salmonella enterica serovars including Newport H1275, Anatum F4317, Stanley H0558 and Infantis F4319 were tested in the study. Antibiotic-resistant strains of these serovars were grown on tryptic soy agar (TSA) or in tryptic soy broth (TSB) supplemented with 100 µg ml⁻¹ of streptomycin and 100 µg ml⁻¹ of nalidixic acid. A rifampicin-resistant strain of Ps. fluorescens 2–79 was grown in TSB or on Pseudomonas agar F (Difco) supplemented with 100 µg ml⁻¹ of rifampicin. Antibiotic-resistant strains were isolated from the wild-type strains of Salmonella and Ps. fluorescens 2–29. Culture plates were incubated at 28°C for 2 days for enumeration of Ps. fluorescens 2–79 or at 37°C for 1 day for enumeration of Ps. fluorescens 2–79 and salmonellae, respectively. Total bacterial count was determined by plating the sample on TSA and then incubating culture plates at 28°C for 2 days. TSA, TSB and Pseudomonas agar F were obtained from Difco/BD Diagnostic Systems (Sparks, MD, USA).

Inoculation of alfalfa seeds with salmonellae and Ps. fluorescens 2–79

Inoculation of alfalfa seeds (Caudill Seed Company, Louisville, KY, USA) was carried out according to the procedure previously described (Fett 2006). Briefly, each of four Salmonella serovars was grown separately in 25 ml of TSB and incubated at 37°C for 18 h. An aliquot of 5-ml culture from each of four serovars was collected, combined and centrifuged, and the cell pellet re-suspended in 200 ml of 0·1% peptone water to make a cell density of approx. $7 \times 10^5$ CFU ml⁻¹. Alfalfa seeds (80 g) were inoculated by immersing in the suspension for 1 min. After removing the excess suspension, inoculated seeds were dried under a biological hood for 3 days and then stored at 4°C until use. The initial concentration of salmonellae on inoculated seeds was approx. $10^5$ CFU g⁻¹.

For preparation of seeds containing a series of 10-fold decreasing number of salmonellae (from $10^5$ to $\leq 10^3$ CFU g⁻¹), 10 g of seeds containing a given concentration of salmonellae was mixed with 90 g of noncontaminated seeds. Alfalfa seeds containing different concentrations of salmonellae (from $\leq 10^1$ to $10^3$ CFU g⁻¹) were also immersed in the Ps. fluorescens 2–79 suspension ($5 \times 10^5$ CFU ml⁻¹) for 2 min. Inoculated seeds were then placed in glass jars and subject to sprouting as described below. For preparation of Ps. fluorescens 2–79 suspension, this bacterium was grown in TSB supplemented with 100 µg ml⁻¹ of rifampicin at 28°C for 24 h. Bacterial cells from an aliquot of 5-ml culture were collected by centrifugation and the pellet was suspended in 250 ml of 0·1% peptone water to make the cell density of approx. $5 \times 10^5$ CFU ml⁻¹.

Recovery and propagation of native microflora from alfalfa seeds for inoculation studies

Alfalfa seeds (25 g) were placed in a stomacher bag containing 100 ml of 0·1% peptone water and allowed to soak at room temperature for 30 min. The bag was then pummelled at high speed for 2 min using a laboratory stomacher (Seaward Inc., London, UK). An aliquot of 0·2 ml of homogenate was used to inoculate 25 ml of TSB. After incubation at 28°C for 24 h, native bacteria were harvested by centrifugation and the pellet was rinsed once and re-suspended in the same volume of 50 µmol l⁻¹ of phosphate-buffered saline (PBS; pH 7·2; Gibco/Invitrogen, Carlsbad, CA, USA).

Surface sanitization of alfalfa seeds and inoculation of sanitized seeds with salmonellae, native microflora and Ps. fluorescens 2–79

Seeds were immersed at room temperature in a Ca(OCl)₂ solution containing 20 000 ppm of active chlorine for 10 min. The solution was decanted and PBS was added to remove chlorine residues from the seeds. Seeds treated with Ca(OCl)₂ or water were dried overnight and then used for inoculation of salmonellae, Ps. fluorescens 2–79 or native bacteria. To determine the change in the population of native bacteria, total bacterial count on seeds that had been treated with Ca(OCl)₂ or water was enumerated. Both sanitized and nonsanitized seeds were immersed in the suspension containing $7 \times 10^5$ CFU ml⁻¹ of salmonellae. Sanitized seeds were also immersed in the suspension containing a mixture of $7 \times 10^5$ CFU ml⁻¹ of salmonellae and $3 \times 10^8$ CFU ml⁻¹ of native bacteria or Ps. fluorescens 2–79.

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Sprouting in glass jars and recovery of salmonellae from seed sprouts

Sprouting was carried out at 20°C and in 20 200-ml glass jars, each containing 2 g of seeds pre-inoculated with approx. \( \leq 10^1 \), \( 10^1 \), \( 10^2 \) or \( 10^3 \) CFU g\(^{-1} \) of salmonellae. Alfalfa seeds inoculated with a given concentration of salmonellae were placed in five glass jars (2 g per jar) and one jar each was removed at day 0, 1, 2, 3 and 6 to determine the change in the population of salmonellae during sprouting. Seeds in glass jars were irrigated daily using 25 ml of sterile water for up to 6 days. For enumeration of salmonellae, sprouting seeds from a jar were transferred to a stomacher bag containing 20 ml of Dey/Engley (D/E) neutralizing broth (Difco/BD Diagnostic Systems) and the bag was then pummeled for 2 min using a laboratory stomacher. Appropriately diluted seed homogenates were plated onto TSA supplemented with streptomycin and nalidixic acid to recover and enumerate salmonellae.

Statistical analyses

The changes in the populations of salmonellae and total bacterial count on alfalfa seeds during sprouting in glass jars or on filter papers were analysed by performing analysis of variance to determine the effect of the treatments. Difference between treatments were performed using the Bonferroni least significant difference (LSD) mean separation procedure (Miller 1981) at the \( P = 0.05 \) significance level.

Results

Growth dynamics of salmonellae on sprouting seeds as affected by the inoculum size

Alfalfa seeds containing different concentrations of salmonellae were sprouted in glass jars and the change in the population of salmonellae was monitored for up to 6 days. The result (Fig. 1) shows that both salmonellae population and total bacterial count reached the maximum 2–3 days after sprouting and was not affected by the initial pathogen concentration (\( \leq 10^1 \), \( 10^1 \), \( 10^2 \) or \( 10^3 \) CFU g\(^{-1} \)) on seeds before sprouting. Salmonellae on seeds containing four different concentrations of pathogens grew at approximately the same rate during the first 2 days of sprouting. A net increase of 3–4 log units in the population of salmonellae on sprouting seeds was observed when total bacterial count reached the maximum (\( 10^9 \) CFU g\(^{-1} \)). The maximum population of salmonellae (3\( \times \)62–740 log CFU g\(^{-1} \)) detected during sprouting was directly correlated with the initial pathogen concentration present on seeds before sprouting.

Figure 1 Comparison of the growth patterns of Salmonella on sprouting alfalfa seeds pre-inoculated with different concentrations of salmonellae ranging from \( \leq 10^2 \) (–x–), \( 10^1 \) (– ), \( 10^2 \) (– – ), to \( 10^3 \) (– – – ) CFU g\(^{-1} \). Total bacterial count (–■–) is shown on top. The data are the means of three experiments and two duplicates in each experiment. The error bar represents the standard deviation (n = 6). The actual number of salmonella on seeds pre-inoculated with \( \leq 10^1 \) CFU g\(^{-1} \) of salmonellae was under the detection level by the culture method at day 0.

Inhibition of salmonellae by \( Ps. fluorescens \) 2–79 on sprouting seeds containing different concentrations of salmonellae

Alfalfa seeds containing different concentrations of salmonellae (\( \leq 10^1 \), \( 10^1 \), \( 10^2 \) or \( 10^3 \) CFU g\(^{-1} \)) were inoculated with approximately the same concentration of \( Ps. fluorescens \) 2–79 (\( 10^5 \) CFU g\(^{-1} \)) and inoculated seeds were then sprouted in glass jars. The change in the population of salmonellae during sprouting was monitored daily for up to 6 days (Fig. 2). Similar to that described above, the population of salmonellae and total bacterial count reached the maximum approx. 2 days after sprouting when total bacterial count or the population of \( Ps. fluorescens \) 2–79 also reached the maximum (\( 10^9 \) CFU g\(^{-1} \)). The population of salmonellae increased 1–2–16 log units if seeds were treated with \( Ps. fluorescens \) 2–79. However, on seeds not treated with \( Ps. fluorescens \) 2–79, the population of salmonellae increased by 3–6–38 log units. These results consistently showed that treatment of seeds with \( Ps. fluorescens \) 2–79 could reduce the growth of salmonellae by at least 2 log units.

Growth of salmonellae on sanitized seeds supplemented with or without native microflora or \( Ps. fluorescens \) 2–79

Total bacterial count on alfalfa seeds was reduced from 4–38 to 2–48 CFU g\(^{-1} \) following the treatment with 20 000 ppm of Ca(OCl)\(_2\) for 10 min. To determine if
the reduction in native microbial load might affect the growth of salmonellae; growth of salmonellae on sanitized and nonsanitized seeds was determined. Results (Table 1) showed that, after 3 days of sprouting, the population of salmonellae on sanitized seeds was approx. 1 log higher than that on nonsanitized seeds which contained 2 logs higher number of native microflora. Sanitized seeds were later immersed in the suspension containing a mixture of \(7 \times 10^3\) CFU ml\(^{-1}\) of salmonellae and \(3 \times 10^6\) CFU ml\(^{-1}\) of native microflora or \(P.s.\) fluorescens 2–79 and then subjected to sprouting for 3 days. Results (Table 1) show that the population of salmonellae on sanitized seeds inoculated with native microflora or \(P.s.\) fluorescens 2–79 decreased from 6.41 to 4.82 and 3.68 log CFU g\(^{-1}\), respectively. These data showed that growth of salmonellae on sanitized seeds could be reduced by inoculating the seeds with native microflora or \(P.s.\) fluorescens 2–79.

**Discussion**

Data presented here (Fig. 1) showed that the population of salmonellae on alfalfa seeds reached the maximum after 2–3 days of spraying when total microbial density on the surface of seeds also reached the maximum (approx. \(10^9\) CFU g\(^{-1}\)). It has been reported before that the growth of \(L.s.\) monocytogenes (Palmai and Buchanan 2002) and Escherichia coli O157:H7 (Stewart et al. 2001b) on alfalfa seeds also reached the peak 2–3 days after spraying. However, the maximum number of salmonellae detected in earlier studies varies widely from \(10^3\) CFU g\(^{-1}\) (Stewart et al. 2001a) to \(10^7\) CFU g\(^{-1}\) (Jaquette et al. 1996). The data presented here showed a direct correlation between the initial salmonellae number and the maximal number of salmonellae to be expected during sprout production. A net increase of 3–4 log units was observed with seed lots containing different concentrations of salmonellae and after 2–3 days of spraying. These data are in agreement with those reported earlier (Fu et al. 2001; Charkowski et al. 2002; Howard and Hutcheson 2003) and also support the recommendation of testing pathogens at day 2 after spraying (FDA/NAC-MCF 1999).

**Table 1** Comparison of the growth of Salmonella on sanitized and nonsanitized alfalfa seeds and also on sanitized seeds re-inoculated with native bacteria or \(P.s.\) fluorescens 2–79

<table>
<thead>
<tr>
<th>On alfalfa seeds</th>
<th>Total bacterial count (log CFU g(^{-1}))</th>
<th>No. Salmonella on seeds (log CFU g(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before sprouting</td>
<td>3 days after sprouting</td>
</tr>
<tr>
<td></td>
<td>658 ± 0.34 A</td>
<td>69 ± 0.34 A</td>
</tr>
<tr>
<td>Before sprouting</td>
<td>658 ± 0.34 A</td>
<td>69 ± 0.34 A</td>
</tr>
<tr>
<td>Sanitized with Ca(OCl)(_2)</td>
<td>2.48 ± 0.36 A</td>
<td>2.37 ± 0.37 A</td>
</tr>
<tr>
<td>Nonsanitized (Control)</td>
<td>2.43 ± 0.42 A</td>
<td>5.68 ± 0.33 A</td>
</tr>
<tr>
<td>Sanitized seed re-inoculated</td>
<td>6.71 ± 0.41 A</td>
<td>6.69 ± 0.39 A</td>
</tr>
<tr>
<td>with native microflora</td>
<td>6.71 ± 0.41 A</td>
<td>6.69 ± 0.39 A</td>
</tr>
<tr>
<td>Sanitized seed re-inoculated</td>
<td>7.13 ± 0.25 A</td>
<td>7.14 ± 0.25 A</td>
</tr>
<tr>
<td>with Ps. fluorescens 2–79</td>
<td>7.13 ± 0.25 A</td>
<td>7.14 ± 0.25 A</td>
</tr>
</tbody>
</table>

*Alfalfa seeds were sanitized using 20 000 ppm of Ca(OCl)\(_2\) and seeds treated with water as nonsanitized control. Seeds were then inoculated with \(10^2–10^3\) CFU g\(^{-1}\) of salmonellae and parts of sanitized seeds were re-inoculated with \(10^6–10^7\) CFU g\(^{-1}\) of native bacteria or \(P.s.\) fluorescens 2–79.

†Each value represents the mean of six determinations (n = 6) from three experiments and two duplicates ± standard deviation. Within a column, the numbers not followed by the same letter are significantly different (**P < 0.05**) by the Bonferroni least significant difference separation technique (Miller 1981).
Alfalfa seed has been shown to harbor a high level of native microfloral ranging from 10³ to 10⁷ CFU g⁻¹ (Liao and Fett 2001). Under the favourable conditions of the sprouting process, these indigenous bacteria can grow very rapidly and reach the maximum (10⁶ CFU g⁻¹) 2–3 days after sprouting (Splittstoesser et al. 1983). The data presented here showed that salmonellae ceased to grow when total bacterial count reached the limit usually 2–3 days after sprouting. Native microbial communities associated with alfalfa seeds have been shown to affect the growth of salmonellae during sprouting (Matos and Garland 2005). The data presented here (Table 1) also show that sanitization of alfalfa seeds can increase the growth of salmonellae presumably resulting from the removal of competing bacteria. Postharvest decontamination of fresh produce can reduce the spoilage and pathogenic microorganisms and also possibly reduce the microflora antagonistic to pathogens.

Result from earlier studies (Jaquette et al. 1996) have shown that washing and sanitization treatment can reduce the number of salmonellae by 2–3 log units. This study shows that that treatment of seeds with Ps. fluorescens 2–79 can reduce the growth of salmonellae by 2–3 log units. The extent of inhibition was not affected by the variation in the pathogen concentration on seeds. If contaminated seeds are first treated with a sanitizer to reduce the number of pathogens by 2–3 log units, a subsequent treatment of sanitized seeds with Ps. fluorescens 2–79 can reduce the number of pathogen by another 2–3 log units. A combination of chemical and biological intervention is therefore possible to reach the recommended 5-log reduction target (FDA/NACMCF 1999). The hurdle approach is especially useful for decontamination of naturally contaminated seeds which contain an extremely low level (≤1 MPN g⁻¹) of salmonellae (Stewart et al. 2001a).

References


