Decontamination of Seeds for Seed Sprout Production by High Hydrostatic Pressure

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ABSTRACT

Garden cress, sesame, radish, and mustard seeds immersed in water were treated with high pressure (250, 300, 350, and 400 MPa) for 15 min at 20°C. After treatment, percentages of seeds germinating on water agar were recorded for up to 11 days. Of the seeds tested, radish seeds were found to be the most pressure sensitive, with seeds treated at 250 MPa reaching 100% germination 9 days later than untreated control seeds did. Garden cress seeds, on the other hand, were the most pressure resistant, with seeds treated at 250 MPa reaching 100% germination 1 day later than untreated control seeds did. Garden cress sprouts from seeds treated at 250 and 300 MPa also took about 1 day longer to reach average sprout length than sprouts from untreated control seeds did, indicating that sprout growth was not retarded once germination had occurred. Garden cress seeds were inoculated with suspensions of seven different bacteria (10^7 CFU/ml) and processed with high pressure. Treatment at 300 MPa (15 min, 20°C) resulted in 6-log reductions of *Salmonella Typhimurium*, *Escherichia coli* MG1655, and *Listeria innocua*, >4-log reductions of *Shigella flexneri* and pressure-resistant *E. coli* LMM1010, and a 2-log reduction of *Staphylococcus aureus*. Enterococcus faecalis was virtually not inactivated. For suspensions of the gram-positive bacteria, similar levels of inactivation in water in the absence of garden cress seeds were found, but the inactivation of *E. coli* LMM1010 and *S. flexneri* in water in the absence of garden cress seeds was significantly less extensive. These data suggest that garden cress seeds contain a component that acts synergistically with high hydrostatic pressure against gram-negative bacteria.

The consumption of seed sprouts is increasingly prevalent. Not only are seed sprouts rich sources of proteins, carbohydrates, minerals, and vitamins and thus nutritious foods (8), but epidemiological studies suggest that sprouts may also have a beneficial role in protection against a number of chronic diseases and conditions (6, 15, 16). However, over the past decade, the consumption of seed sprouts has been linked to multiple infectious outbreaks throughout the world (15, 34). The main disease-causing organisms are *Salmonella* and other enteric pathogens such as *Shigella* spp. and *Escherichia coli* O157:H7 (21). Sprouts can be contaminated with pathogens via different routes and at different stages of production, but contaminated seeds have been identified as the likely source of contamination in most sprout-associated outbreaks (21). Even a few cells of the pathogen on a seed present a potential health hazard because they can rapidly proliferate during sprouting. Once present on the seeds, pathogens are likely to survive for extended periods. Studies have shown that *Salmonella* can survive for months under dry conditions (1, 21). It is therefore essential that steps be taken to prevent the initial contamination of seeds and/or to eliminate any pathogenic organisms present, preferably before sprouting.

Several studies have addressed the effectiveness of a wide range of chemicals in killing pathogenic bacteria on seed sprouts and seeds intended for sprout production. These chemicals include well-established disinfectants such as chlorine or hypochlorite (9, 14, 17, 27, 33, 38), hydrogen peroxide (33, 38), ozone (29), and ethanol (33) as well as more natural alternatives such as citric, lactic, acetic, and other organic acids (7, 38, 39). Although substantial reductions of specific pathogens were often achieved, none of the treatment methods were able to completely eliminate such organisms. It has been suggested that the bottleneck to the disinfection of seeds lies not in the bactericidal potency of the chemicals but in the inability of the chemicals to reach pathogens inside seeds (2, 21). In addition, chemical disinfectants leave residues on the product that, even if they are believed to be harmless, are difficult to reconcile with the “health food” image of seed sprouts. Therefore, there is an interest in physical decontamination methods that leave no residues.

Research carried out by the U.S. Department of Agriculture (36) indicated that treatment of alfalfa seeds and sprouts with a combination of chlorine and irradiation is effective in safeguarding against *E. coli* O157:H7 and *Salmonella* contamination. The report further stated that the doses used to eliminate these organisms did not affect the germination ability of the seeds. However, while irradiation allows a reduction in the amount of chlorine necessary for decontamination, irradiated foods are not well accepted by the public.

The use of heat to kill pathogens on alfalfa seeds has also been investigated, and Jaquette et al. (14) found treatment at 57 or 60°C for 5 min to be effective in killing *Salmonella* Stanley without substantially decreasing the germination ability of the seeds. However, heat treatment...
has limited appeal because the range of temperatures with which bacteria can be killed without germination being destroyed is narrow (15).

High hydrostatic pressure (HHP) treatment has been studied as a new technique for food preservation because it inactivates vegetative microorganisms while allowing better retention of a food’s sensorial properties than can be achieved with a comparable heat treatment. The purpose of this study was to evaluate the potential of HHP treatment for seed decontamination by studying its effects on the germination of various seeds and its effectiveness in the inactivation of different gram-positive and gram-negative bacteria inoculated onto the seeds.

MATERIALS AND METHODS

Seeds and bacterial strains. Garden cress, mustard, radish, and sesame seeds (Lima, Maldegem, Belgium) were studied. The bacterial strains used were the following: *Staphylococcus aureus* LMM02037, *Enterococcus faecalis* LMM02039, and *Salmonella enterica* serovar *Typhimurium* LMMBM01 (all from our laboratory collection), *E. coli* K-12 strain MG1655 (11), *E. coli* LMM1010 (a pressure-resistant mutant of strain MG1655) (12), and *Listeria innocua* LMG11387 and *Shigella flexneri* LMG10472 (both from the Belgian Coordinated Culture Collection of Microorganisms, Ghent, Belgium). These bacteria were stored at 2°C in 20% glycerol. Fresh master plates were streaked every week on Nutrient Agar CM3 (Oxoid, Basingstoke, UK).

Determination of seed germination. One hundred seeds were spread on water agar (1.2%) plates and stored at 20°C for several days in the dark. Every day, the percentage of germination was determined by counting the number of germinated seeds. The average sprout size for germinated garden cress seeds was determined with a vernier caliper (Mitutoyo Corporation, Tokyo, Japan).

Inoculation of seeds with bacterial suspensions. Cultures (20 ml each) in 100-ml Erlenmeyer flasks were inoculated from a single colony and grown to the stationary phase by shaking (200 rpm) at 37°C for 21 h; they were then centrifuged at 4,000 g for 5 min, and the pellets were resuspended at a concentration of approximately 10^7 CFU/ml in sterile deionized water. Garden cress seeds (4 g) were inoculated by immersion in 36 ml of bacterial cell suspension (10^7 CFU/ml) for 10 min at room temperature. After inoculation, the seeds were air dried at room temperature under a laminar flow cabinet on a sterile paper filter for 15 min.

HHP treatment. Pressure treatment was carried out with the use of a system with eight parallel thermostatically controlled 8-ml vessels that could be simultaneously pressurized and individually decompressed at different times (Resato, Roden, The Netherlands). The compression rate was approximately 100 MPa/min, and decompression was immediate. The temperature of the vessels was controlled by a circulating water jacket and was set at 20°C. Pressure levels ranged from 250 to 400 MPa, and it was previously established that sample temperature could increase to a maximum of 35°C owing to adiabatic heating for pressures of up to 400 MPa under these working conditions (12). For the pressure treatment of seeds, 1 g of dried seeds in 250 ml of sterile deionized water was pressurized in a heat-sealed sterile polyethylene bag for 15 min. For the pressure treatment of bacteria, a 250-μl cell suspension, prepared in the same way as the inoculum for the seeds, was pressurized in a heat-sealed sterile polyethylene bag for 15 min.

Determination of bacterial inactivation. To assess the loss of bacterial viability resulting from pressure treatment, untreated and treated samples were serially diluted in sterile deionized water and plated on plate count agar (Oxoid). Inoculated seeds were homogenized prior to dilution. Plates were incubated for 24 h at 37°C and CFU counts were determined. Inactivation was expressed as a logarithmic viability reduction and was calculated as follows: \( \log_{10} \text{reduction} = \log_{10}(\text{number of CFU before treatment} / \text{number of CFU after treatment}) \).

Reproducibility of results. All experiments were replicated three times, and results were recorded as means ± standard deviations. Where appropriate, differences between treatments were analyzed for statistical significance by Student’s *t* test with Microsoft Excel.

RESULTS

Effect of HHP on germination of various seeds. The germination percentages for the four different types of seeds after high-pressure treatment are presented in Table 1. For each of the seed types treated at each pressure level used (250 to 400 MPa), the percentage of germination after 24 h was significantly lower than that for the untreated control seeds. However, the difference between treated and untreated seeds with respect to percentage of germination became smaller and eventually disappeared after longer germination times. Radish and mustard seeds were the most pressure sensitive, with seeds treated at 250 MPa reaching 95 to 100% germination 8 to 9 days later than the untreated control seeds did. At higher pressure levels, the germination percentages of both types of seeds remained very low for up to 11 days. Sesame seeds exhibited a germination delay of about 8 to 9 days when treated at 250 MPa but were able to recover almost completely when treated at 300 MPa. Garden cress seeds were the most pressure resistant, showing (almost) complete germination with a delay of <1 day when treated at 250 and 300 MPa and a delay of about 3 days when treated at 350 MPa. At a pressure of 400 MPa, the germination of garden cress seeds was more severely affected but still reached a level of >50% after 9 days. The results presented in Table 1 also show that the more germination is delayed, the more it is desynchronized.

For garden cress, the effect of high pressure on average sprout size over an 11-day germination period was also evaluated (Table 2). For garden cress seeds treated at 250 and 300 MPa, the attainment of average sprout length was delayed by about 1 day, the same delay as that for germination. This indicates that once germinated, sprouts do not suffer as a result of the high-pressure treatment. At higher pressures, the germination becomes strongly asynchronous (i.e., the inactivation of seed germination occurs over several days). Therefore, under these conditions, the average sprout size in Table 2 does not accurately reflect sprout growth but is an underestimation.

Inactivation of bacteria inoculated on garden cress seeds by HHP. Because they were the most pressure resistant of the tested seeds, we used garden cress seeds to study...
TABLE 1. Effects of high hydrostatic pressure (HHP) treatment (20°C, 15 min) on germination of seeds

<table>
<thead>
<tr>
<th>Seed type</th>
<th>Pressure level (MPa)</th>
<th>Germination % after HHP treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 1</td>
<td>Day 2</td>
</tr>
<tr>
<td>Garden cress</td>
<td>0</td>
<td>99.04</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>76.47</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>69.81</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>35.64</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>1.01</td>
</tr>
<tr>
<td>Radish</td>
<td>0</td>
<td>61.17</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>2.73</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>Mustard</td>
<td>0</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>8.55</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>0</td>
</tr>
<tr>
<td>Sesame</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>250</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>300</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>350</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>0</td>
</tr>
</tbody>
</table>

the decontamination of seeds by high pressure. The aerobic mesophilic plate count for the garden cress seeds was very low (3.9 × 10¹ CFU/g), and thus it was possible to conduct decontamination studies with well-chosen inoculant strains.

Garden cress seeds were artificially contaminated with a cell suspension of one of seven bacteria at a time and subjected to pressure treatment at 300 MPa. Inactivation levels were found to differ substantially between the different bacteria (Fig. 1). Inactivation levels of >6 log units were achieved for *Salmonella* Typhimurium, *E. coli* MG1655, and *L. innocua*. High levels of inactivation (>4 log units) were also achieved for *E. coli* LMM1010 and *S. flexneri*. *S. aureus* was inactivated by 2 log units, while *E. faecalis* was virtually not inactivated.

**Inactivation of bacterial cell suspensions by HHP.**

The same bacteria used in the inoculation experiments were subjected to high pressure in pure bacterial aqueous suspensions in the absence of garden cress seeds. Again, large differences in pressure sensitivity were observed (Fig. 2). However, some bacteria appeared to be less pressure sensitive in a pure suspension than they were when inoculated on garden cress seeds. For *E. coli* LMM1010, this difference (a <1-log reduction in pure suspension versus a >4-log reduction when inoculated on the seeds) was highly significant (*P* = 0.0001). For *S. flexneri*, the difference was smaller (ca. 1 log unit) but still statistically significant (*P* = 0.0488). For the other bacteria, the same results were obtained under both conditions. It should be noted, however, that the reduction levels for *E. coli* MG1655, *Salmonella* Typhimurium, and *L. innocua* exceed the detection limit of the experiment. Hence, it is not possible to state with certainty that the inactivation levels for these bacteria in the two experiments are not different.

**DISCUSSION**

In the present study, we investigated the potential of high-pressure treatment for the decontamination of seeds to be used for sprout production. Compared with most other treatments, particularly chemical treatments, high pressure has the advantages that it does not leave any residues on

TABLE 2. Effects of high hydrostatic pressure (HHP) treatment (20°C, 15 min) on average sprout size for garden cress seeds

<table>
<thead>
<tr>
<th>Pressure level (MPa)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
<th>Day 7</th>
<th>Day 9</th>
<th>Day 10</th>
<th>Day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4.66</td>
<td>17.9</td>
<td>29.22</td>
<td>47.02</td>
<td>102</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>250</td>
<td>3.26</td>
<td>11.52</td>
<td>18.99</td>
<td>23.65</td>
<td>70.45</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>300</td>
<td>2.65</td>
<td>8.62</td>
<td>16.94</td>
<td>28.51</td>
<td>74.71</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>350</td>
<td>1.21</td>
<td>4.8</td>
<td>9.87</td>
<td>16.4</td>
<td>42.25</td>
<td>64.12</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>400</td>
<td>0.4</td>
<td>0.45</td>
<td>0.45</td>
<td>0.45</td>
<td>4.76</td>
<td>6.57</td>
<td>7.08</td>
<td>7.26</td>
</tr>
</tbody>
</table>

*Average sprout size was determined on the basis of germinated seeds only. ND, not done.*
the product and that homogeneous treatment conditions can be easily achieved with it because no spatial pressure gradients exist in the product during treatment. This homogeneity should ensure more efficient inactivation of bacteria in central parts of the seed that are difficult to reach with chemicals.

The first objective of this study was to investigate the effect of HHP on the germination of seeds, because the preservation of sufficient germinative capacity is a prerequisite for any new technique for seed decontamination. We used pressures of 250 to 400 MPa, a range within which several types of bacteria are inactivated. The germination of all four types of seeds was affected at 250 MPa, but garden cress seeds showed a delay in germination but retained 100% germination capacity when treated at up to 350 MPa for 15 min. This finding is remarkable because most experimental observations have led to the assumption that eukaryotes, particularly multicellular eukaryotes, are more pressure sensitive than prokaryotic cells owing to their more complex structure (3, 35). For example, pressure treatment for 10 min at 200 MPa was sufficient to completely kill *Anisakis simplex* larvae (20), and treatment for 30 min at 150 MPa killed Ehrlich ascites tumor cells (40). As far as we know, there are no reports in the literature on the sensitivity of plant seeds to high pressure, but experiments with cell cultures suggest that pressures exceeding 100 MPa result in plant cell inactivation (28, 32). One possible explanation for the observed resistance of plant seeds, particularly garden cress seeds, to pressure is their low water content. It is well documented that much higher pressures are needed to inactivate microorganisms at low water activity levels (23).

Although the seeds used in the present experiment were pressure treated in water, it is possible that they had not been fully hydrated at the time of pressurization. This possibility could be further investigated with the use of different hydration periods prior to pressure treatment. A delay in germination or a decrease in sprout growth is also commonly observed with other decontamination treatments, such as irradiation (26), organic acid rinses (38), and treatment with other disinfectant solutions (5). Weissinger et al. (39) on the other hand, found that seed germination was largely unaffected by treatment with acetic acid, allyl isothiocyanate, cinnamic acid, or thymol in the gaseous state. Garden cress seeds were used in further experiments to investigate bacterial inactivation because they were the most pressure resistant of all the seeds tested. Because the delay in germination for seeds treated at pressures of up to 350 MPa was limited to 1 day, a pressure level of 300 MPa was used in these experiments.

The second objective of this work was to investigate the inactivation of a number of important bacterial foodborne pathogens (*Salmonella Typhimurium*, *S. flexneri*, and *S. aureus*) and indicators of such pathogens (*E. coli*, *L. innocua*, and *E. faecalis*) that can occur on fresh sprouts. In addition, we compared the efficiency of this treatment for bacteria inoculated on seeds and for bacteria in pure suspensions, because bacteria are usually more resistant to high pressure in real (nonacidic) food products than in buffer systems or in water (24, 30, 31). It was found that the pressure sensitivity levels of the seven tested bacteria, both in pure suspension and on seeds, differ considerably. For three bacteria (*L. innocua*, *E. coli* MG1655, and *Salmonella Typhimurium*)...
Typhimurium), a reduction of $\geq 6$ log units could be achieved under both conditions. This level of reduction ensures sufficient safety. S. aureus and, particularly, E. faecalis were found to be highly resistant, with a reduction of 2 log units and no reduction at all, respectively, under both conditions. E. coli LMM1010 has been described as a highly pressure resistant mutant of strain MG1655 and, accordingly, exhibited a reduction of $< 1$ log unit when treated as a bacterial suspension in water. However, when inoculated on garden cress seeds, the inactivation of this bacterium increased by 3.6 log units. An $\sim 1$-log increase in the inactivation of S. flexneri on garden cress seeds was also observed. These results suggest that garden cress seeds may contain one or more antibacterial components that act synergistically with high pressure. These components alone do not exhibit bactericidal activity under our experimental conditions, because no reduction in bacterial numbers was observed in inoculated seeds that were not treated by high pressure (data not shown).

As a member of the Brassicaceae family, garden cress produces isothiocyanates that contribute to the typical aroma of these plants (7) but are also known for their antimicrobial properties (7, 13, 18, 19). The finding by Ogawa et al. (22) that E. coli can be sensitized to allyl isothiocyanate by high pressure lends credulity to the idea that these compounds could be the ones that are involved in the sensitization observed in our experiments. Zsolnai (41) proposed that the antimicrobial activity of isothiocyanates results from the inactivation of intracellular enzymes through the oxidation of sulfhydryl groups and disulfide bonds. Block (4) also ascribed these antimicrobial properties to the inhibition of metabolic enzymes and implicated the thiocyanate (SCN$^-$) ion, a potential degradation product of isothiocyanates with well-known antimicrobial properties (1). Thiocyanate can be converted to the even stronger antimicrobial hypothiocyanate (OSCN$^-$) by peroxidase enzymes, which are also present in several Brassicaceae organisms (25). The formation of OSCN$^-$ could be relevant, because we have previously shown that high pressure can indeed sensitize bacteria for OSCN$^-$ and perhaps for other reaction products generated by the lactoperoxidase enzyme (10). More importantly, we recently found (37) that this sensitization also occurs at reduced water activity levels, such as those that may exist inside seeds that are not fully hydrated (see above). Our finding that E. coli LMM1010 and S. flexneri are sensitized to the antibacterial compound in garden cress seeds and that E. faecalis and S. aureus are not (no determination could be made for the other bacteria because they were completely inactivated by a pressure of 300 MPa alone) further supports a role for the isothiocyanates because it is in agreement with the finding that gram-positive bacteria are more resistant to these compounds than gram-negative bacteria are (13).

In conclusion, we have demonstrated that HHP can be used to decrease the levels of some bacteria of concern on garden cress seeds. This treatment seems to be particularly efficient in inactivating enteric bacteria and Listeria, which are the bacteria of the greatest concern with regard to the safety of seed sprouts. An important challenge for future work will be to optimize the treatment to strike a more favorable balance between bactericidal efficacy and the preservation of seed germination capacity. The achievement of this goal will require a more detailed study of the effect of the process parameters (temperature, pressure, and treatment time) on bacterial inactivation and seed germination capacity. Another approach would be to use high pressure in combination with natural antimicrobial compounds such as organic acids, isothiocyanates, the lactoperoxidase system, and so forth. This hurdle approach may make efficient treatments at lower pressures possible and thus create opportunities for the sanitation of seeds that are more pressure sensitive.

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