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Efficacy of combinations of high pressure treatment, temperature and antimicrobial compounds to improve the microbiological quality of alfalfa seeds for sprout production

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Abstract

The effect of combined treatments of pressure, temperature and two disinfectant agents (hypochlorite and carvacrol) applied on alfalfa seeds, on their germination capability as well as on the reduction of the native microbial load of sprouts developed from treated seeds was evaluated by using response surface methodology (RSM). The germination percentage decreased as pressure and carvacrol concentration increased, while calcium hypochlorite concentration had no significant impact on seed viability. The counts of total aerobic mesophilic bacteria, total and faecal coliforms and moulds and yeast were reduced with increasing pressure and hypochlorite and carvacrol concentrations. The optimal conditions for improving the microbiological quality of alfalfa seeds (reductions between 4.5 and 5 log CFU/g) for sprouts production were 200 MPa and hypochlorite concentration of 18,000 ppm. On the contrary, the process parameters of the combined treatment HP/carvacrol that ensure the microbial safety of sprouts (250 MPa and 1500 ppm of carvacrol) reduced the germination percentage to unacceptable levels.

Keywords: Alfalfa seeds; High pressure; Carvacrol; Hypochlorite; Sprouts

1. Introduction

The use of germinated seeds as food originated in far east countries and has recently spread to the western world, where they are considered fashionable and healthy ingredients (Feng, 1997; Kuo, Rozan, Lambein, Frías, & Vidal-Valverde, 2004). A great variety of sprouts are easily available on the European markets, but the most popular are those from alfalfa, mung bean and radish. They are consumed often raw or slightly cooked in salads and sandwiches (Weiss & Hammes, 2003), or as decorative appetisers. It is well known that although the germination process improves the nutritional value of sprouts compared with unprocessed seeds (Ziegler, 1995), either by increasing digestibility (Sierra & Vidal-Valverde, 1999), by reducing antinutritional factors (VidalValverde et al., 2002), or by increasing compounds with antioxidant activity (Frias, Miranda, Doblado, & Vidal-Valverde, 2005; Doblado, Frías, & Vidal-Valverde, 2007), sprouting also provides suitable conditions for microbial proliferation. Seeds usually contain high microbial loads, ranging between 10^3 and 10^6 CFU/g (Prokopowich & Blank, 1991; Robertson, Johannessen, Gjerde, & Loncarevic, 2002), and these levels can increase during sprouting, reaching populations as high as 10^8–10^{11} CFU/g (Ghandi & Matthews, 2003; Peñas, Gómez, Frías, & Vidal-Valverde, 2008). These high microbial counts are the main reason for the short shelf-life of sprouts and increase the likelihood of infections as described by National Advisory Committee on Microbial Criteria for Foods (NACMCF) (1999) and Taormina, Beuchat, and Slutsker (1999), if seeds are contaminated with pathogens.

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US Food and Drug Administration (FDA) recommends the decontamination of seeds before sprouting. This is the most effective stage (Caetano-Anolles, Favelukes, & Bauer, 1990) due to lower levels of both microorganisms and organic material present on seeds than on sprouts and to the fact that internalisation of bacteria into sprout tissues during sprouting makes them physically inaccessible to sanitisers (Hara-Kudo, 1997; Itoh et al., 1998). Several methods have been evaluated for improving the safety of seeds, including heat treatment (Jaquette, Beuchat, & Mahon, 1996; Weiss & Hammes, 2003), exposure to ionizing radiation (Thayer, Rajkowski, Boyd, Cooke, & Soroka, 2003) and numerous chemical treatments such as chlorine or hypochlorite (Beuchat, Ward, & Pettigrew, 2001; Winthrop et al., 2003), hydrogen peroxide, ethanol (Piernas & Guiraud, 1997; Suzuki & Takizawa, 1997), ozone (Sharma, Demirci, Beuchat, & Fett, 2002), and commercial disinfectants. These treatments can only reach microorganisms on the seed surface, and there is still no guarantee that the contamination in the interior of seeds will be removed (Mundt & Hinckle, 1976; NACMCF, 1999). Besides, some of these methods affect the germination of seeds. Most of these treatments have been applied to seeds to prevent the proliferation of microbial pathogens. However, there is little information in the literature about treatments aimed at reducing both the high microbial load of seeds in order to increase shelf-life of sprouts and also reducing pathogens while preserving seed viability. Our group investigated the application of high pressure at different times and temperatures on mung bean and alfalfa seeds, to improve the safety of the resultant sprouts (Peñas et al., 2008). However, the optimal conditions found by us to maintain a high percentage of germination (90%), failed to produce a reduction of microbial levels high enough to ensure the microbial safety of sprouts. The combination of HP and antimicrobial agents could be an alternative treatment to reduce the microbial load of sprouts while maintaining a good germination capability. Calcium hypochlorite and carvacrol could be valuable tools to achieve these purposes, because of they have shown antimicrobial activity against many bacteria, yeast and fungi (Burt, 2004; Dusan, Marián, Katarína, & Dobroslaba, 2006; Jaquette et al., 1996). Until now, there are no literature data on the combined effect of HP and both disinfectant compounds on the germination capability of seeds and on the inactivation of microbial load of sprouts.

The objective of this work was to develop a set of optimum conditions of pressure, temperature and concentration of calcium hypochlorite or carvacrol for the treatment of alfalfa seed with an aim towards improving the safety of the sprouts, maintaining a high germination capability of seeds. Response Surface Methodology (RSM) was employed for these purposes.

Table 1
Coded and uncoded values of the experimental design

Independent variables	Levels					
	HP/Calcium hypochlorite treatment			HP/Carvacrol treatment		
	−1	0	1	−1	0	1
Pressure level (MPa)	100	200	300	100	200	300
Temperature (°C)	25	32.5	40	25	32.5	40
Disinfectant concentration (ppm)	1800	9900	18,000	250	875	1500

2. Materials and methods

2.1. Plant material

Alfalfa (*Medicago sativa*) seeds were provided by Man Fong Pacific Trading, S.A. (Spain) and stored at 4 °C until their treatment.

2.2. Experimental design

Response Surface Methodology (RMS) was used for investigating the effect of three independent variables (pressure, temperature and calcium hypochlorite or carvacrol concentration) on five response variables: (a) percentage of germination, reduction of: (b) total aerobic mesophilic bacteria, (c) total coliforms, (d) faecal coliforms and (e) mould and yeast counts on alfalfa sprouts. RSM is a statistical method based on the multivariate non-linear model that has been widely used model for optimisation of several processes (Box & Wilson, 1951). RSM evaluates the responses of several factors by varying them simultaneously with limited number of experiments (Mundra, Desai, & Lele, 2007). For this reason, it is less laborious and time-consuming than other approaches and today is one of the most popular optimisation techniques in the field of food science (Gao, Ju, & Jiang, 2006).

In the present work, the experiments were performed according to a central composite face-centered design. Three levels of each independent variable (pressure, temperature and calcium hypochlorite or carvacrol concentration) were chosen. The low, middle and high levels of each variable were designated as −1, 0, and +1, respectively, and are given in Table 1. Sixteen combinations of these three variables were performed following the design (Table 2). Two replications of each experimental condition were carried out.

2.3. Preparation of antimicrobial treatment solutions

The effect of two antimicrobial products for reducing the microbial load of alfalfa seeds was evaluated: Solutions of both calcium hypochlorite (1800, 9900 and 18,000 ppm) and carvacrol (Sigma–Aldrich Química, Spain) (250, 875 and 1500 ppm), separately, were prepared in distilled water, for treating (5 min at room temperature) the seeds

Table 2
Central composite design arrangement for combined HP, temperature and carvacrol/hypochlorite treatments

Run	HP/hypochlorite treatments					
	X_1	X_2	X_3	X_1	X_1	X_1
1	−1 (25)	−1 (100)	−1 (1800)	−1 (25)	−1 (100)	−1 (250)
2	1 (40)	1 (300)	1 (18,000)	1 (40)	1 (300)	1 (1500)
3	−1 (25)	1 (300)	−1 (1800)	−1 (25)	1 (300)	−1 (250)
4	−1 (25)	−1 (100)	1 (18,000)	−1 (25)	−1 (100)	1 (1500)
5	1 (40)	1 (300)	−1 (1800)	1 (40)	1 (300)	−1 (250)
6	0 (32.5)	0 (200)	−1 (1800)	0 (32.5)	0 (200)	−1 (250)
7	0 (32.5)	0 (200)	0 (9900)	0 (32.5)	0 (200)	0 (875)
8	0 (32.5)	0 (200)	1 (18,000)	0 (32.5)	0 (200)	1 (1500)
9	1 (40)	0 (200)	−1 (1800)	1 (40)	0 (200)	−1 (250)
10	0 (32.5)	−1 (100)	0 (9900)	0 (32.5)	−1 (100)	0 (875)
11	1 (40)	0 (200)	1 (18,000)	1 (40)	0 (200)	1 (1500)
12	0 (32.5)	0 (200)	0 (9900)	0 (32.5)	0 (200)	0 (875)
13	0 (32.5)	1 (300)	0 (9900)	0 (32.5)	1 (300)	0 (875)
14	−1 (25)	0 (200)	0 (9900)	−1 (25)	0 (200)	0 (875)
15	1 (40)	0 (200)	0 (9900)	1 (40)	0 (200)	0 (875)
16	−1 (25)	1 (300)	1 (1800)	−1 (25)	1 (300)	1 (1500)

Independent variables: Temperature (X_1), Pressure (X_2), Carvacrol/hypochlorite concentration (X_3).

previously soaked in distilled water for 3 h at room temperature.

2.4. High pressure treatment

A total of 800 seeds for each antimicrobial agent were packed in polyethylene bags under vacuum to be pressurised, after removing the antimicrobial solutions by decantation. The antimicrobial concentrations, as well as pressure and temperature conditions were chosen according to the experimental design using a Response Surface Methodology (RSM) described in Table 2. A total of 16 experiments were done for each antimicrobial agent, and the experiments were replicated two times.

A hydrostatic pump and a steel-vessel of 2.35 l capacity (100 mm in diameter and 300 mm in height) were used. The vessel was filled with water as fluid of low compressibility. The temperature inside the vessel and the quick thermal equilibration was controlled by a circulating-thermostatic bath. The come-up and decompression times were approximately 150 MPa min^{-1}. Unpressurised seeds not treated with the antimicrobial solutions were considered the control of the experiments.

2.5. Seed germination

Control seeds and those treated with combinations of the antimicrobial solutions and HP were washed twice with 10 ml of distilled water. Afterwards, the seeds corresponding to each treatment were germinated in a climatic cabinet (ASL Snijders Sci. International S.L., Tiburg, Holland) at 25 °C for 5 days in darkness. Seeds were sprinkled with distilled water every 12 h. Sprouts were obtained after 5 days and then the percentage of germination was determined by counting the number of germinating seeds. Fifty seeds were germinated for each treatment, and germination was performed in duplicate for each sample.

2.6. Microbiological analysis of sprouts

Microbiological analyses of sprouts obtained from control and treated seeds were performed. Microbial counts of sprouts from untreated seeds were used as initial values for calculating logarithmic reductions in microbial counts of sprouts from treated seeds. Alfalfa sprouts were added to Buffered Peptone Water (BPW) (Oxoid, Unipath Ltd., Basingstoke, UK) at a ratio of product to medium 1:9, and homogenised for 1 min on medium speed in a Stomacher Laboratory Blender Model 400 (Seward Medical, London, UK). One milliliter of each suspension was pour plated in triplicate on different media for the counting of the following microorganisms: Total aerobic mesophile populations on Tryptone Soya Agar (TSA), incubation at 30 °C for 72 h; total and faecal coliforms on Violet Red Bile Agar (VRBA), containing lactose as carbohydrate source, incubation at 37 and 44 °C, respectively, for 24 h; moulds and yeasts on Sabouraud Chloramphenicol Agar, incubation at 23 °C for 72 h.

2.7. Statistical treatment

Statgraphics Plus 5.1 (Statistical Graphics Corporation Inc., Rockwille, Md., USA) software was used for statistical analysis. Results were averages of three independent determinations.

The following quadratic polynomial equation was used to express responses as a function of independent variables:

$$Y = b_o + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j$$

where Y represents the dependent variable to be modeled; b_0, b_i, b_j and b_{ij} represent the coefficients of the model; and X_i and X_j represent the independent variables. The goodness-of-fit of the models obtained was evaluated by R^2 (multiple determination coefficient), the Fischer F-test

(and the derived *P*-values) and the standard errors of the estimate. Three-dimensional surface plots were drawn to illustrate the effects of the independent variables on the dependent ones.

Analysis of variance was performed for each response variable using the full models where *P* values indicated if the terms were significant. Lack of fit determined whether the selected model appeared adequate to describe the observed data or not. None of the predicted models had a significant lack of fit.

3. Results and discussion

3.1. Combined effects of temperature, HP and antimicrobial treatments on the germination capability of seeds

To evaluate the goodness-of-fit of the quadratic models obtained for the germination percentage of seeds treated by combinations of temperature, HP and hypochlorite or carvacrol to the experimental results, the coefficients of determination (R^2) were calculated (Tables 3 and 4). These values indicated that both fitted quadratic models accounted for more than 95% of the variations in the experimental data, which were highly significant. The adjusted values of R^2, that are more suited for comparing models with different numbers of independent variables, were 0.9853 and 0.9382, for the model of calcium hypochlorite and carvacrol, respectively (Tables 3 and 4). In general, a value of $R^2 > 0.75$ indicate the goodness of the model.

The regression coefficients of the quadratic polynomial equations were calculated for each model (Tables 3 and 4). These values can be used to examine the significance of each term relative to each other. The sign and magnitude of the coefficients indicate the effect of the variable on the response. Negative signs of the coefficient means decrease in response when the level of the variable is increased while positive sign indicates increase in the response. Significant interaction suggests that the level of one of the interactive variable can be increased while that of the other decreased for constant value of the response.

As it is shown in Table 3, for the calcium hypochlorite model, only the linear term of pressure had a significant negative effect ($P \leqslant 0.05$) on the germination percentage of alfalfa seeds. However, in the case of carvacrol, both linear terms of pressure and carvacrol concentration had adverse influence in germination capability of seeds (Table 4).

Response surface plots were generated to better visualise the combined effects of the three independent variables on the germination rate (Fig. 1). As can be observed from the Fig. 1a, which represents the response surface plot for calcium hypochlorite model, the germination percentage decreased as pressure increased. Alfalfa seeds treated at 100 MPa at 32.5 °C retained more than 70% germination capability, while the treatments at 200 MPa and 300 MPa reduced the percentage of germination to about 40% and 20%, respectively. The negative effect of pressure on the viability of alfalfa seeds has been previously observed by our group (Peñas et al., 2008). Pressures $\geqslant$250 MPa, applied for 5, 10 or 15 min, reduced the germination rate of alfalfa seeds to very low levels, and increases of temperature from 10 to 40 °C had a positive effect on seed viability. Wuytack, Diels, Meersseman, and Michels (2003) also observed that the percentage of germination after 24 h of garden cress, sesame, radish, and mustard seeds immersed in water and pressurised (250, 300, 350 and 400 MPa) at 20 °C for 15 min was significantly lower than that for untreated control seeds. On the other hand, Ariefdjohan et al. (2004) found that pressurised alfalfa seeds at 40 °C (275–575 MPa for 2 min or 475 MPa for 2–8 min) took longer to germinate, achieving germination rate of up to

Table 3
Coefficients of the second order polynomial equations and significance of each model and dependent response variables in alfalfa sprouts obtained from seeds treated by combinations of HP/hypochlorite/temperature

	% germination	Aerobic mesophilic bacteria counts reduction	Total coliform counts reduction	Faecal coliform counts reduction	Mould and yeast counts reduction
R^2	0.9941	0.9900	0.9863	0.9961	0.9875
R^2_{adj}	0.9853	0.9751	0.9657	0.9902	0.9688
Intercept b_0	81.9923	2.5733	0.1006	−3.0005	−3.0656
Linear b_1	3.2832	−0.0745*	0.0653	0.2913*	0.2361*
b_2	−0.6736**	0.0128**	−0.0002**	−0.0090**	0.0021**
b_3	−0.0015	0.00003**	0.00003*	0.00002*	−0.00001*
Quadratic b_{11}	−0.0540	0.0014	−0.0008	−0.0040	−0.0032
b_{22}	0.0008	−0.000002	0.00003	0.00003*	0.00003
b_{33}	2.993E−8	2.510E−9*	5.939E−10	1.014E−9	3.837E−10
Interaction b_{12}	−0.0056	−0.00004	0.00001	0.0002	0.000007
b_{13}	0.00002	−1.440E−7	−6.173E−8	−2.469E−7	0.000001
b_{23}	−6.327E−7	−5.093E−8	2.145E−7	2.407E−7*	3.837E−1

Independent variables: Temperature (X_1), Pressure (X_2) and Calcium hypochlorite concentration (X_3).
* $P \leqslant 0.05$.
** $P \leqslant 0.01$.

Table 4
Coefficients of the second order polynomial equations and significance of each model and dependent response variables in alfalfa sprouts obtained from seeds treated by combinations of HP/carvacrol/temperature

	% germination	Aerobic mesophilic bacteria counts reduction	Total coliform counts reduction	Faecal coliform counts reduction	Moulds and yeast counts reduction
R^2	0.9753	0.9686	0.9880	0.9866	0.9780
R^2_{adj}	0.9382	0.9216	0.9700	0.9666	0.9451
Intercept b_0	139.928	1.7387	−0.0333	−4.4499	−1.4363
Linear b_1	−5.6087	−0.0721*	0.0160	0.2909*	0.2037**
b_2	−0.0332*	0.0136**	0.0123**	0.0090**	−0.0099**
b_3	0.0477*	0.0009**	8.276E−8*	0.00031*	−0.0004**
Quadratic b_{11}	0.1051	0.0012	0.0001	−0.0040	−0.0033*
b_{22}	0.00003	−0.00001*	0.000002	0.000007	0.00003**
b_{33}	0.000006	−1.456E−7	1.805E−7	2.503E−7	6.488E−8
Interaction b_{12}	−0.0056	0.00003	−0.00007	− 0.00001	0.0001*
b_{13}	0.00031	0.000008	0.000005	−0.000007	0.00001*
b_{23}	0.00008	−0.000001*	0.000001	9.400E−7	0.000004**

Independent variables: Temperature (X_1), Pressure (X_2) and Carvacrol concentration (X_3).
* $P \leqslant 0.05$.
** $P \leqslant 0.01$.

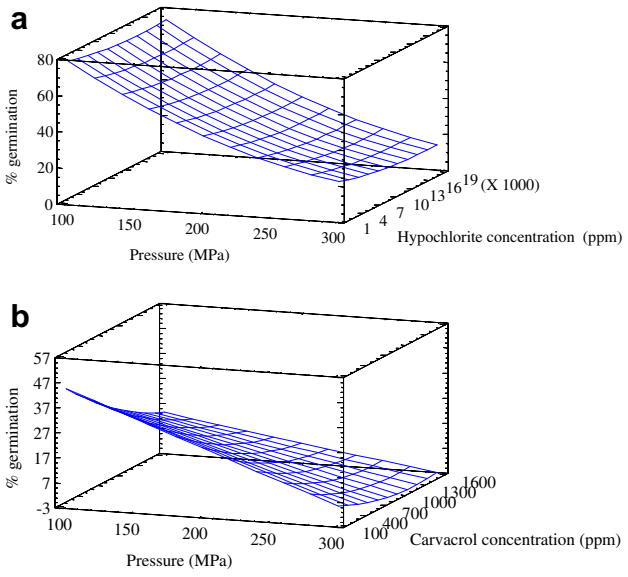

Fig. 1. Response surface plot of the percentage of alfalfa seed germination as a function of pressure and sanitiser concentration, while the temperature of treatment was kept constant (32.5 °C). (a) Hypochlorite treatment and (b) carvacrol treatment.

34%, while 95% of the control seed germinated. They also observed that coats of treated seeds were damaged. These germination rates were lower than those reported by Wuytack et al. (2003). The authors attributed this to the fact that the water used in the pressure treatment makes fluffy the seed coat, alleviating the damage and thus improving germination of seeds.

On the other hand, it can be noticed that calcium hypochlorite concentration had no significant impact on seed viability (Fig. 1a). This is a very positive result, because other treatments currently used to improve the safety of seeds adversely affect their germination rate. Our findings confirmed those obtained by Lang, Ingham, and Ingham (2000), who also observed no significant differences in the percentage of germination between control alfalfa seeds (seeds soaked in water; 95.7% of germination) and those soaked with calcium hypochlorite solutions containing between 2000 and 20.000 ppm active chlorine (germination percentage ranging between 90.0% and 96.5%).

In contrast, the increase of carvacrol concentration caused a significant reduction in seed germination percentage, obtaining at each level of pressure, germination rates lower than those for calcium hypochlorite treatment. This effect was more pronounced at low pressures (100–150 MPa). Several reports showed that some essential oils act as inhibitors of germination and growth of dry seeds and plants (Muller, Muller, & Haines, 1964; Zhang, Yajima, Umezawa, Nakagawa, & Esahi, 1995). Moreover, Dudai, Poljakoff-Mayber, Mayer, Putievsky, and Lerner (1999) and Dudai et al. (2000), found that an essential oil from Oregano (*Origanum vulgare* L.), whose major component is carvacrol, inhibited the germination capability of wheat seeds.

3.2. Reduction of microbial load on alfalfa sprouts by combination of temperature, HP and calcium hypochlorite treatments applied to seeds

The magnitudes of regression coefficients in Table 3 show that the linear term of pressure had the most significant effect on the reduction of the microbial populations studied in the present work. The linear and quadratic term of hypochlorite concentration were also significant ($P \leqslant 0.01$ and $P \leqslant 0.05$, respectively) on the inactivation of mesophilic bacteria, while for total and faecal coliforms and moulds and yeast population, only the linear term had influence ($P \leqslant 0.05$). In addition, temperature had significant linear impact ($P \leqslant 0.05$) on the reduction of

mesophilic microorganisms, faecal coliforms and moulds and yeasts, and the interaction of pressure and hypochlorite concentration had a positive significant effect ($P \leqslant 0.05$) in killing faecal coliforms.

The combined effects of the three independent variables on the inactivation of the different microbial groups on alfalfa sprouts were also investigated by examining the response surface plots generated (Fig. 2a–d) by holding constant temperature at 32.5 °C. The counts of all microbial groups studied were reduced when pressure and hypochlorite concentration increased. The optimal conditions necessary to achieve an adequate margin of safety in sprouts were combinations of hypochlorite concentrations of 18,000 ppm and pressure of 200 MPa. In these conditions, reductions of 4.5–5 log CFU/g for all groups were reached, following the recommendations of the NACMCF (1999) for seeds intended for sprouts production. A previous work carried out in our laboratory demonstrated that the pressurisation of alfalfa seeds (250 MPa, 40 °C, 10 min) reduced the populations of total mesophilic microorganism, total and faecal coliforms and moulds and yeasts in alfalfa sprouts between 1.4 and 3.3 log CFU/g (Peñas et al., 2008). However, this level of pressure negatively affected the germination rate. Both microbial inactivation and impaired germination capability could be attributed to the combination of several factors: changes in cell morphology and cell membrane permeability, inhibition of genetic mechanisms, modifications of biochemical reactions and denaturation of proteins including key enzymes induced by HP (Linton & Patterson, 2002; McClements, Patterson, & Linton, 2001). On the other hand, the soaking of alfalfa seeds with 18,000 ppm calcium hypochlorite decreased these populations in alfalfa sprouts about 2–2.5 log CFU/g (data not shown). The results reported in the present work showed the synergistic effect of pressure and calcium hypochlorite combined at lower individual intensities to achieve higher reductions of microbial populations on alfalfa sprouts than both treatments alone, maintaining at the same time about 40% germination percentage. This germination rate can be considered acceptable, because of the viability of alfalfa seeds is impaired by the most of the treatments commonly used for their hygienisation.

3.3. Reduction of microbial load on alfalfa sprouts by combination of temperature, HP and carvacrol treatments applied to seeds

The coefficients of the regression equation describing the effect of pressure, temperature and carvacrol levels on the reduction of several microbial groups in alfalfa sprouts are given in Table 4. As can be observed, the microbial inactivation is primarily affected by the linear term of pressure ($P \leqslant 0.01$), as is reflected by the higher absolute value of this coefficient, followed by carvacrol concentration ($P \leqslant 0.01$ for aerobic mesophilic and moulds and yeasts; $P \leqslant 0.05$ for total and faecal coliforms). The quadratic term of pressure also showed a significant effect on mesophilic ($P \leqslant 0.05$) and moulds and yeast decrease ($P \leqslant 0.01$). The linear term of temperature had influence on aerobic mesophilic ($P \leqslant 0.05$), faecal coliforms ($P \leqslant 0.05$) and mould and yeast ($P \leqslant 0.01$) inactivation, while the quadratic term of temperature only was significant on mould and yeast reduction ($P \leqslant 0.05$). In addition,

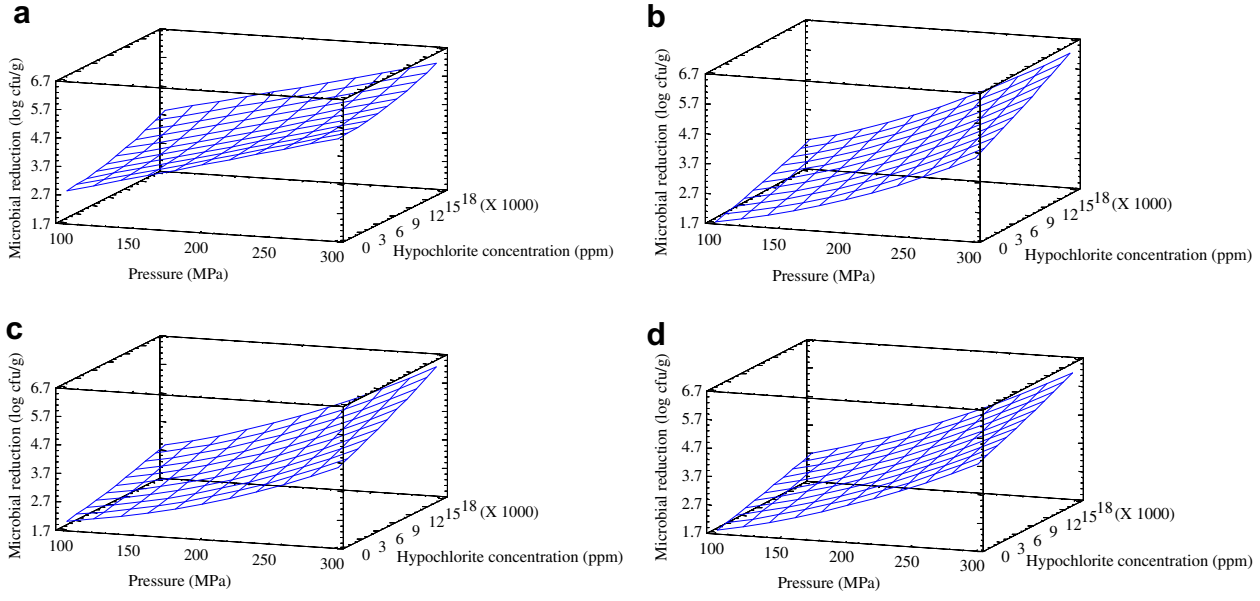

Fig. 2. Response surface plot for microbial counts reduction in alfalfa sprouts as a function of pressure and hypochlorite concentration, while the temperature of treatment was kept constant (32.5 °C). (a) Total aerobic mesophilic microorganisms; (b) total coliforms; (c) faecal coliforms; and (d) moulds and yeasts.

some significant interactive effects were found (Table 4). Thus, the interaction between pressure and carvacrol concentration was significant on aerobic mesophilic bacteria ($P \leqslant 0.05$) and moulds and yeast ($P \leqslant 0.01$) inactivation, while those interactions between temperature and pressure or carvacrol concentration were significant only on moulds and yeast reduction ($P \leqslant 0.05$).

All models presented satisfactory coefficients of determination between 0.969 and 0.987, indicating that the models are well adapted to the responses (Table 4).

The response surface plots obtained for the inactivation of the microbial groups studied in alfalfa sprouts as a function of the level of pressure and carvacrol concentration at constant temperature (32.5 °C) are shown in Fig. 3. The microbial counts in alfalfa sprouts were found to decrease with increase in the level of pressure and carvacrol concentration. The optimal processing conditions for obtaining about 5 log units reduction for all microbial groups, as indicated by NACMCF (1999), were 250–300 MPa and 1500 ppm of carvacrol. However, these conditions adversely affect seed viability, as we have described above. Only seeds treated at 100 MPa and 250 ppm of carvacrol maintained a satisfactory viability, reaching germination percentages > 50%. At these conditions, reductions between 2–2.5 log CFU/g for mesophilic bacteria and 1.5–2.5 CFU/g for total and faecal coliforms and moulds and yeasts were obtained. However, this decrease is not enough to ensure the safety of sprouts. It is evident from these results that the combination of high pressure and carvacrol is not feasible for industrial use as a alfalfa seed-decontamination treatment for sprout production, because the treatment conditions that ensure the microbial quality of sprouts reduced the germination percentage to unacceptable levels. On the contrary, this combined treatment was very adequate for the hygienisation of mung bean seeds, as we have previously observed (unpublished data), probably due to the different nature of both seeds.

As a conclusion, the process parameters of the combined treatment of temperature, calcium hypochlorite/carvacrol and pressure can be effectively optimised using RSM, with a minimum number of experiments. We have demonstrated that HP enhances the effectiveness of calcium hypochlorite and carvacrol for inactivating the microbial populations in alfalfa sprouts. This hurdle approach exploits synergistic interactions between pressure and antimicrobial compounds, allowing use of lower pressures and antimicrobial concentration than when these treatments are used alone. Calcium hypochlorite is a more efficient decontamination treatment than carvacrol for alfalfa seeds, because carvacrol reduced the germination rate of seeds when it was used at concentrations that reduced the microbial load more than 5 log CFU/g. On the contrary, calcium hypochlorite showed a great potential, in combination with HP, as sanitiser treatment for alfalfa seeds intended for sprouts production, preserving the viability of seeds. The optimal process parameters that decrease the levels of microbial populations (total aerobic microorganism, total and faecal colifoms, moulds and yeasts) on alfalfa seeds more than 5 log CFU/g without affecting the germination percentage to unacceptable levels are 200 MPa and 18000 ppm calcium hypochlorite. These levels are in the margin of safety required by the FDA. These results indicate that calcium hypochlorite in combination with HP may be a feasible seed pretreatment for use it in the commercial production

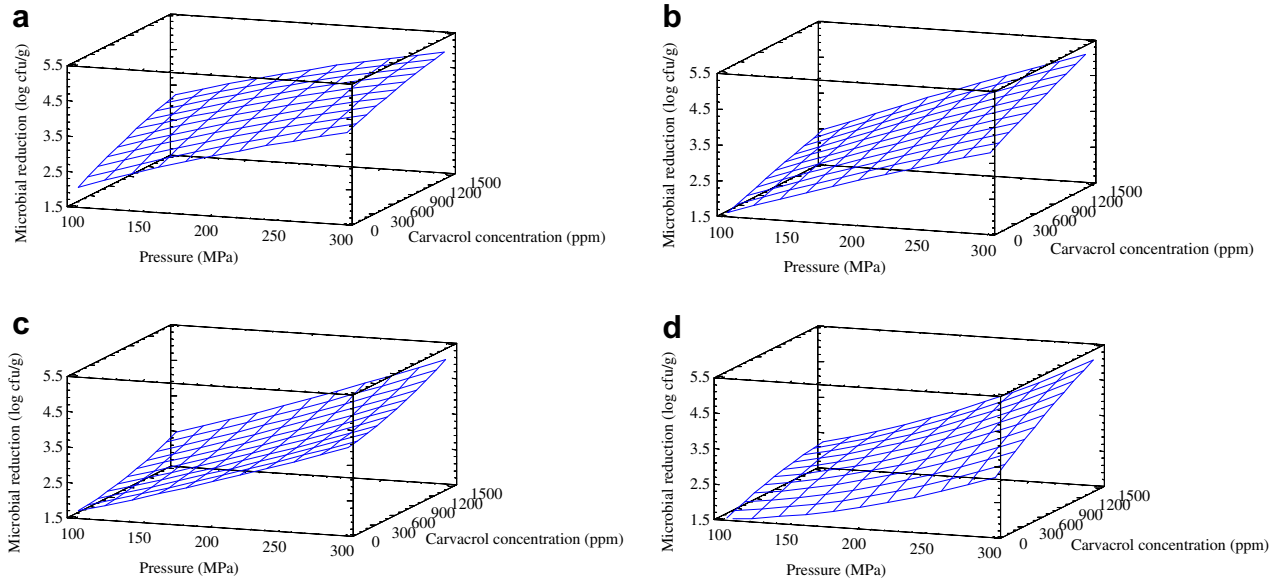

Fig. 3. Response surface plot for microbial counts reduction in alfalfa sprouts as a function of pressure and carvacrol concentration, while the temperature of treatment was kept constant (32.5 °C). (a) Total aerobic mesophilic microorganisms; (b) total coliforms; (c) faecal coliforms; and (d) moulds and yeasts.

of alfalfa sprouts. Moreover, it has the advantage that it is easy to implement by the industry.

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