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Disinfection with calcium hypochlorite and colloidal silver in the content of phenols of red and blue corn sprouts

Summary

The production of sprouts for human consumption represents a healthy option to combat the effects of oxidative stress, due to the bioactive compounds that are synthesized during germination. The safety of sprouts is an essential requirement. In this study, calcium hypochlorite and colloidal silver were used as options for not alter the nutraceutical content of corn sprouts and eliminate dangerous pathogens for human food. Treatments based on calcium hypochlorite in high concentrations (0.4%) were more efficient for food safety, but with the disadvantage of reducing the content of free phenols in blue corn only from 374.55 mg to 261.06 mg / 100 g. Colloidal silver at dose of 1.4 ppm, has a pathogen control similar to calcium hypochlorite without reducing the content of free phenols, even increasing it. The best treatment to treat corn seeds for sprout production is colloidal silver at 1.4 ppm.

Keywords: sprouts, oxidative stress, antioxidant, free phenols, pathogens

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Carreño Carrillo Carlos Vidal,[1] Villanueva Verduzco Clemente,[2] Villanueva Sánchez Evert,[3] Sahagún Castellanos Jaime,[2] Herbert Pucheta José Enrique[3]

[1]Horticulture Graduate Program, Universidad Autónoma Chapingo, Mexico
[2]Departamento de Fitotecnia Universidad Autónoma Chapingo, Mexico
[3]Laboratorio Nacional de Investigación y Servicio Agroalimentario y Forestal, Mexico

Correspondence: Clemente Villanueva-Verduzco, Departmento de Fitotecnia, Universidad Aut noma Chapingo, Carretera Mexico, Tel 01(595) 9521500, Email clemente.villanueva@gmail.com

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Introduction

At present there has been a trend in the increase of consumption of sprouts of different specie due to their nutraceutical properties; however, the vast majority of production systems do not have a really efficient patogen control or do not achieve a real disinfection of the seeds and sprouts. In recent years, throughout the world a lot of importance is given to food safety, due to the public health risks that they can generate mainly from a microbiological point of view, since there are bacteria such as Salmonella or *E. coli*, that can represent a severe problem. Reports have established that a large portion of foodborne outbreaks (*E. coli*) are attributed to the consumption of fresh fruits and vegetables. Almost half (46%) of foodborne illnesses between 1998 and 2008 in the United States, were attributed to fresh products.

Listeria monocytogenes, Salmonella typhimurium, and Escherichia coli are the main foodborne bacteria implicated in fresh products consumption.[1]

Khalil and Gomma[2] showed a maximum count of *E. coli* in carrot (4.96 log CFU / g), green pepper (4.57 log CFU/g), cucumber (4.38 log CFU/g) and zucchini (4.22 log CFU/g) in samples from street markets in Egypt. In countries as the United States, there are specific protocols by specialized agencies such as the Enviromental Protection Agency to deal with these types of problems, which include disinfection of food with the use of sodium hypochlorite or calcium hypochlorite.[3] The use of calcium hypochlorite has the advantage of being cheap if used on an industrial scale, it also presents a spectrum more comprehensive in pathogen control and the odor / taste it leaves on food is more acceptable and easier to remove compared to sodium hypochlorite, not to mention less is needed to have a similar effect.[4]

The use of calcium hypochlorite has the advantage over sodium hypochlorite of being cheap if used on an industrial scale, it also has a broader spectrum in the efficient control of pathogens and the odor / taste that it leaves in food is more acceptable and easy to eliminate, and it is mentioned that less is needed to have a similar effect.[4] However, there is only information about the optimal concentrations to disinfect vegetables and fruits, not for seeds in the production of sprouts.

The objective of this study is to find the optimal dose or an alternative to the use of calcium hypochlorite for the efficient disinfection of seeds used for the sprouts production, without compromising the concentration or composition of bioactive compounds present in sprouts.

Materials and methods

Experimental location and plant material

The experiments were carried out in the plant physiology laboratory (determination of phenols) and the seed laboratory (germination test) of Departamento de Fitotecnia of Universidad Autónoma Chapingo, located on the México-Texcoco highway km 38.5, Texcoco de Mora state of Mexico. The two creole varieties of blue and red seed corn were collected at municipal market of Chilchotla of Puebla State (19 ° 15′ 00″ NL; 97 ° 11′ 00″ WL) at an average altitude of 2,200 mosl, a total annual precipitation of 941.6 mm and an annual average temperature of 11.6°C.

Experimental design

The experimental design used was completely randomized with a factorial treatment arrangement. The first factor was seed color, with two levels, red and blue and the second was disinfectant substance, with 5 levels: calcium hypochlorite solution Ca $(ClO)_2$ at 0.2%, Ca $(ClO)_2$ at 0.4%, colloidal silver 1.4 ppm, colloidal silver 2.8 ppm and control (sterile water); with a total of 10 treatments. The experimental unit was 100g of seed with 3 replications (Table 1).

Table 1 Treatments for corn seed disinfection for sprout producttion

Treatment	Maize seed colour	Disinfectant
1	Red	$Ca(ClO)_2$ 0.2%
2		$Ca(ClO)_2$ 0.4%
3		Colloidal silver 1.4 ppm
4		Colloidal silver 2.8 ppm
5		Control (sterile water)
6	Blue	$Ca(ClO)_2$ 0.2%
7		$Ca(ClO)_2$ 0.4%
8		Colloidal silver 1.4 ppm
9		Colloidal silver 2.8 ppm
10		Control (sterile water)

$Ca(ClO)_2$, Calcium hypochlorite

Seed washing and application of disinfection treatments

Before the application of treatments all the seed samples were washed three times with water and common detergent powder (Roma™) until the wash water coming clean. Then, three rinses were made with sterile water. The treatments witn calcium hypochlorite disinfectant base on Ca $(ClO)_2$ Meyer at 65% were prepared: 3.07 g and 6.15 g of calcium hypochlorite were weighed with an analytical balance and dissolved in one liter of distilled water each one, to obtain the treatments with 0.2 % and 0.4 % of calcium hypochlorite, respectively.

Treatments with colloidal silver were based on MICRODYNMR (with ionized colloidal silver at 0.35 %). Were used 8 and 16 drops.L-1 which are equivalent to 1.4 and 2.8 ppm of colloidal silver, respectively. A sample of 100 g of corn seed per repetition was weighed and placed in a beaker, immersed in 500 ml of the solution from each disinfectant treatment (Table 1) for 15 minutes. After disinfection time of 15 min in all the treatments, the 100 g of seed per replication were divided into 2 equal parts, 50 g were used to obtain flour and determine the phenol content after each disinfection treatment; and the remaining 50 g were subjected to a germination process to get sprouts where were determined the percentage of germination (PG) and pathogen incidence (PI).

Obtaining the flour

After disinfection time, seeds were rinsed 3 times with sterile water to eliminate residues of the disinfectant solution. Then, 50 g of seed were taken per repetition to place them in paper bags and put them in a drying oven for 12 h at 50°C[5] for drying and obtaining flour with a Hamilton Beach brand coffee grinder. The flour was stored in a desiccator until its analysis to determine phenols concentration.

Samples preparation for phenols extraction

The extraction method to determine the phenol content in corn seed meal was adapted from Bakan et al.,[6] with the following modifications: 1 g of corn flour was used, and 15 ml of methanol in water at 80% (v/v) were added. The sample was homogenized in a vortex and subjected to a water bath at 90°C for 20 min, the samples were left to rest for 24 h in the dark at 4-5°C, after that time the extracts were centrifuged for 5 min at 5000 rpm. Supernatants were recovered to quantify free phenols.

Quantification of free phenols

Quantification of phenols was done by the Folin-Ciocalteu method, adapted from Bakan et al.[6] The samples corresponding to the supernatants recovered from the centrifugation in the procedure of extraction of phenols from flours were subjected to a Folin reaction (Table 2) to quantify their content of free phenols by reading their absorbance in a spectrophotometer at 765 nm (Figure 1).

Table 2 Composition of the reactions to determine phenols in two varieties of corn

Maize variety	Sample	Distilled water	Folin reagent	Na_2CO_3 7.5%
	(µl)	(µl)	(µl)	(µl)
Blue	100	900	250	1,250
Redo	150	850	250	1,250

Figure 1 Good quality of roots and shoots in maize sprouts.

Glass optical cells were used to read the absorbances of each sample in the spectrophotometer. During the sample change and for reading of new data, the optical cell was washed with 80% ethanol in water (v/v) and rinsed with distilled water (Figure 2).

Figure 2 Incidence of patogens in maize sprouts.

The free phenol content of each sample was calculated based on the individual absorbance data of the samples and the formula of the calibration curve to transform the absorbance data to mg of gallic acid equivalents that serve as a reference for the phenol content of each sample. To prepare the calibration curve, a 100 ppm gallic acid stock solution was used. From this solution, eight Folin reactions were set up to determine the absorbance of each one with respect to their different known increasing concentrations 0, 30, 60, 90, 120, 150, 180, and 210 µl (Table 3).

Calibration curve of gallic acid over absorbance

Because gallic acid belongs to the family of polyphenols and to the group of hydrolyzable tannins, besides being easy to obtain, it can be used as a reference in quantitative methods to calculate the concentration of phenols in a solution.

Table 3 Gallic acid concentrations used to make the calibration curve

Reagents	Gallic acid standard curve concentration (ppm)							
	0	1.2	2.4	3.6	4.8	6.0	7.2	8.4
Gallic acid (µl)	0	30	60	90	120	150	180	210
Destilled water (µl)	1,000	970	940	910	880	850	820	790
Folin Reagent (µl)	250	250	250	250	250	250	250	250
Sodium carbonate 7.5% (µl)	1,250	1,250	1,250	1,250	1,250	1,250	1,250	1,250

Calculation of equivalents of free phenols. The concentration of free phenols (ppm) of each sample was calculated from its equivalence with gallic acid. The calculation was made by clearing x = gallic acid (ppm) from the equation of the line model from the calibration curve (Figure 3) and substituting the absorbance value (y = absorbance) given by the spectrophotometer for each sample; where

$$gallic\ acid\,(ppm) = \frac{(absorbance) + 0.1742}{0.1162}$$

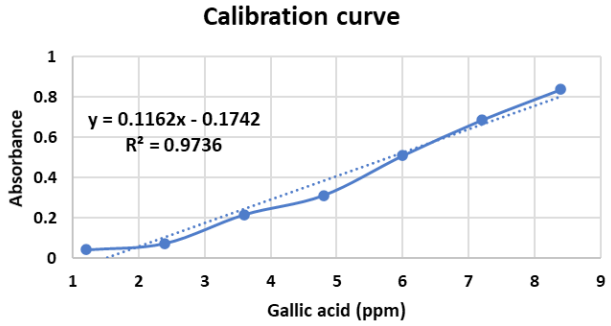

Figure 3 Calibration curve of gallic acid over absorbance.

Once the equivalence of free phenols with gallic acid (ppm) corresponding to each sample was obtained, the content of free phenols (FL) was reported in mg of gallic acid / 100 g of flour, using the following formula:

$$gallic\ acid\,(ppm) \times \frac{15\,ml\ methanol\ \ (solvent)}{1\,g\ of\ \ flour} \times 100 = \frac{mg\ of\ \ gallic\ acid}{100\,g\ of\ \ flour}$$

Germination (seeds) and patogen incidence (seeds and sprouts)

To determine the percentage of germination (PG) and pathogens incidence (PI), the remaining 50 g of seed per replication were taken of each treatment and germinated. Petri dishes 150 X 15 mm were used with a wet cotton towel at the bottom, in a germination chamber at 28°C for 72 h. In both varieties was calculated the germination percentage for determining if disinfection treatment affect the physiological quality of the seed. To determine the efficiency of the disinfection methods, the presence of any pathogenic agent (seeds and sprouts), was determined the percentage of incidence.

Results and discussion

Seeds: germination, microorganisms incidence and phenols content

The germination percentage (PG) in red variety was statiscaly higher (97.2%) than in blue (92.4%). Incidence of diseases in both varieties of corn (red and blue) was same (Table 4). Red corn presents a higher germination percentage (97.2%) than blue corn (92.4%) and both showed a statistically similar load of microorganisms. The content of free phenols in red grain corn was more than double (305,293 mg / 100g of maize seeds flour) than in blue grain corn (147.034 mg/100g of maize seeds flour), and vice versa in sprouts (Table 4). The good quality (Figure 2) in red maize, and Incidence of patogens in seed and sprouts in blue maize (Figure 3) are illustrated.

Table 4 Means comparisons of maize varieties for germination, contaminant microorganisms incidence, and concentration of free phenols in grain

Maize variety	PG (%)	PI (%)	CFP (mg/100g of maize seeds flour)
Red grain	97.2 a	13.8 a	305.293 a
Blue grain	92.4 b	18.0 a	147.034 b
MSD	2.82	5.4	9.83

CFP, concentration of free phenols, PG, percentage of germination, PI, percentage of incidence (seeds and sprouts), MSD, minimun significant difference. values with the same letter are statistically the same

Treatments: germination and microorgamisms incidence

The disinfectant treatments used, did not statistically affected negatively the germination percentage of any of the corn varieties with respect to the control, regardless of their nature and concentration (Table 5).

Germination: Given that most of the germination process in corn takes place in optimal pH ranges of 4.8-6.9[7] and that a solution of Ca $(ClO)_2$ at 1% in water (p/v) can reach pH ranges between 9.5-11.5 (Bernabel ES TESIS Yauri et al., 2013), it is expected that at this high pH, the germination process is affected due to the degradation of compounds or enzymatic inactivation. Therefore, some effect of the treatments with calcium hypochlorite at 0.2 and 0.4% on the PG could be also expected; however, it was not.

The percertage of germination (PG) of red and blue corn was not affected with the calcium hypochlorite solutions (same to control treatment) probably by containing anthocyanins, antioxidant bioactive compounds, and being exposed in a relatively short time (15 min) and at a relatively low concentrations (0.2 and 0.4%), the degradation by alkalinization was slow or it simply did not occur due to the protective action of bioactive compounds of maize seed or sprout, according with Pham-Huy (2008).

Microorganisms incidence. In the control treatment, the incidence (PI) of contaminant microorganisms after 72 h of germination was significantly higher (40% in red and 34% in blue corn) than in all the disinfection treatments. In both varieties all treatments were statistically the same to reduce the incidence of microorganisms, with a clear numerical trend in favor of calcium hypochlorite (0.4%), with 4% and 7% of incidence in red and blue variety, respectively (Table 5), probably due to the high oxidizing capacity of Ca $(ClO)_2$ that kills microorganisms compared to colloidal silver, which only prevents the development of the pathogen, unlike Ca $(ClO)_2$.

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The main contaminating microorganisms found in the sprouts of both varieties of corn (red and blue) were fungi of the genera Aspergillus and Fusarium, which numerically resisted better to colloidal silver than to Ca $(ClO)_2$. Most microorganisms that cause human disease (fungi or bacteria) have protective mechanisms and have the ability to form protective coatings. Bacteria, for the most part, form capsules of glycoproteins and polysaccharides, with which they adhere to surfaces or their host and protect themselves from dehydration, predation or antibacterial agents. Disinfectant compounds with oxidizing capacity can damage these natural protection mechanisms of pathogens and thus actually reduce harmful populations in seeds and their edible sprouts.

In Mexico since the cholera outbreak in 1993, the bactericidal effect of colloidal silver is widely known due to its oxidative action that prevents bacteria development, through a powerful action against enterobacteria such as *E. colli* and Salmonella spp.

Table 5 Comparison of means of treatments for germination and contaminant microorganisms incidence in seed of maize

	Red Maize		Blue Maize		Average	
Treatment	**PG (%)**	**PI (%)**	**PG (%)**	**PI (%)**	**PG (%)**	**PI (%)**
$Ca(ClO)_2$ 0.2%	99 a	5 b	95 a	12 bc	96.0 a	8.5 b
$Ca(ClO)_2$ 0.4%	96 a	4 b	89 a	7c	92.5 a	5.5 b
Colloidal silver 1.4 ppm	97 a	9 b	94 a	18 bc	92.5 a	13.5 b
Colloidal silver 2.8 ppm	98 a	11 b	95 a	19 b	97.0 a	15.0 b
Control (sterile water)	96 a	40 a	89 a	34 a	95.5 a	37.0 a
MSD	6.9	22.6	11.26	11.43	6.32	12.1

$Ca(ClO)_2$, Calcium hypochlorite, PG, Percentage of germination, PI, Percentage of incidence, MSD, Mínimum Significant Difference (Tukey, 0.05). Values with the same letter are statistically the same

Phenols content in seed and sprouts

The equivalence of free phenols with gallic acid (ppm) corresponding to each sample was obtained, the content of free phenols (FL) was reported in mg of gallic acid/100 g (Figure 3).

Concentration of free phenols (CFP)

In seed: The concentration of free phenols (CFP) in blue corn seed was more than double (305,293 mg/100g of flour) than in red corn (147,034 mg/100g of flour) (Table 4).

In sprouts: In red corn, all disinfectant treatments were statistically equal to the control in content of free phenols (CFP); although numerically colloidal silver (2.8 ppm) exceeded by 17% (168.96 mg/100g of flour) the control (144,349 mg/100g of flour). This result is consistent with that of[8] who in adult L. gibba plants found that when applying a 1 mg/L (1 ppm) solution of colloidal silver, the concentration of total phenols increased by 156% with respect to the control. The two disinfectant treatments based on colloidal silver exceeded those of calcium hypochlorite free phenols (Table 6).

Table 6 Means comparisons of free phenols (CFP) in corn sprouts by treatment

	CFL (mg/100 g of flour)		
Treatment	**Red Maize**	**Blue maize**	**Average**
$Ca(ClO)_2$ 0.2%	138.439 b	275.37 c	214.54 b
$Ca(ClO)_2$ 0.4%	136.03 b	261.06 c	198.544 b
Colloidal silver 1.4 ppm	147.39 ab	290.64 b	246.905 a
Colloidal silver 2.8 ppm	168.962 a	324.85 b	211.378 b
Control (sterile water)	144.349 b	374.55 a	259.449 a
DMS	22.42	40.19	21.96

$Ca(ClO)_2$, calcium hypochlorite. CFP, concentration of free phenols. MSD, mínimum significant difference (Tukey, 0.05). values with the same letter are statistically the same

In contrast, in blue corn, the control significantly exceeded (374.55 mg/100g of flour) the four disinfectant treatments; and the colloidal silver treatments showed higher CFL (275.37 and 261.06 mg/100 g of flour; with 0.2 and 0.4%) than those of calcium hypochlorite (290.64 and 324.85 mg / 100g of flour; with 1.2 and 2.4 ppm), respectively (Table 6). However, the mechanism of action through which colloidal silver promotes this increase in CFL in the case of the red corn evaluated is not clear.

In blue corn, all the evaluated disinfectant treatments reduced the CFP with respect to the control; however, in the case of the treatments with calcium hypochlorite (0.2 % and 0.4 %) there was a significant ($p < 0.05$) greater reduction in CFL from 374.55 mg/100 g of flour in the control to 275.37 and 261.06 mg/100 g of flour, in the 0.2 % and 0.4% hypochlorite treatments, respectively (Table 6).

The pH has an effect on the structure, stability and coloration of anthocyanins, giving red pigmentations in acidic media (pH ± 2.0) and gradually changing to blue-purple tones as the pH levels increase (pH ± 7.0), beginning to degrade above pH = 7.[9]

In the case of blue corn, thanks to the compounds that give it its tonalities, its pH is close to neutrality and if we take into account that 1% calcium hypochlorite solutions can reach a pH between 9.5-11.5,[10] the degradation of phenols would be accelerated compared to the red maize variety. These results put into consideration the proposals established by the US EPA (Environmental Protection Agency), which recommend as a treatment to disinfect seeds for sprout production for human consumption. To reduce the risk of contracting viral, bacterial and / or fungal (mainly *Salmonella* spp. and *Escherichia coli*) diseases, they recommend a solution of Ca $(ClO)_2$ at 20,000 µg / ml.[3]). That is, a 2% solution of Ca $(ClO)_2$ which is equivalent to 5 - 10 times more concentrated than those evaluated in this study.

Fett[11] determined that to reduce populations of Escherichia coli to acceptable levels (4-5 log10 CFU.g-1) in mung beans, treatments with Ca $(ClO)_2$ solutions of 1,900 mg / L could be performed; that is, 0.19% for 15 min, without negatively affecting germination, which is more in line with what was found in this study.

Conclusion

Disinfection with sodium hypochlorite and colloidal silver significantly reduces the incidence of contaminating microorganisms in corn seed and sprouts for at least 72 hours, without affecting the physiological quality of the seed. In red grain corn, seed disinfection with calcium hypochlorite does not reduce the concentration of free phenols in seed or sprouts; but colloidal silver can increase it in some concentrations. In contrast, in blue grain corn both disinfection, with sodium hypochlorite and colloidal silver, reduce the content of free phenols in sprouts.

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Conflicts of interest

There are no financial conflicts of interest.

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