

Research Note

Studies on the Effects of Phosphine on *Salmonella enterica* Serotype Enteritidis in Culture Medium and in Black Pepper (*Piper nigrum*)

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ABSTRACT

The effect of phosphine on *Salmonella enterica* serotype Enteritidis inoculated in culture medium and in black pepper grains (*Piper nigrum*), as well as on the reduction of the microbial load of the dried and moisturized product, was verified. The postfumigation effect was verified in inoculated samples with 0.92 and 0.97 water activity (a_w) exposed to 6 g/m³ phosphine for 72 h, dried to 0.67 a_w , and stored for 24, 48, and 72 h. No decreases were observed in *Salmonella* Enteritidis populations in culture medium when fumigant concentrations up to 6 g/m³ were applied for 48 h at 35°C. However, the colonies showed reductions in size and atypical coloration as the phosphine concentration increased. No reduction in *Salmonella* counts occurred on the inoculated dried samples after fumigation. On the other hand, when phosphine at concentrations of 6 g/m³ was applied on moisturized black pepper for 72 h, decreases in *Salmonella* counts of around 80% were observed. The counts of total aerobic mesophilic bacterium populations of the dried and moisturized black pepper were not affected by the fumigant treatment. The results of the postfumigation studies indicated that *Salmonella* Enteritidis was absent in the fumigated grains after drying and storage for 72 h, indicating a promising application for this technique. It was concluded that for *Salmonella* Enteritidis control, phosphine fumigation could be applied to black pepper grains before drying and the producers should rigidly follow good agricultural practices, mainly during the drying process, in order to avoid product recontamination. Additional work is needed to confirm the findings with more *Salmonella* serotypes and strains.

Black pepper is considered the most important spice consumed in the world, and Brazil is one of the largest producers and exporters of this product. In its producing areas, the high temperatures and humidity may favor the development of microorganisms. Research carried out in Brazil and abroad has revealed high levels of contamination of black pepper with enteropathogenic bacteria, especially *Salmonella* spp. (5, 6, 16, 21, 23–25). Recently, the U.S. Food and Drug Administration announced a white and black pepper recall due to potential contamination with *Salmonella* (14). Imported ground black pepper used to coat salami was responsible for a *Salmonella* outbreak involving 252 people in at least 44 states in the United States (12).

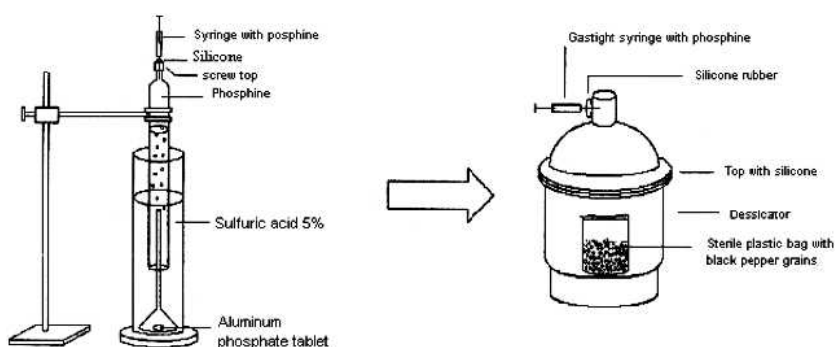
The presence of this pathogen in Brazilian black pepper has led to frequent rejections due to the detection of contaminated lots by sanitary authorities (26), mainly in the United States, the biggest Brazilian black pepper importer. The knowledge and adoption of good agricultural practices are fundamental for the control of this pathogen, but alternative methods for decontamination are also required. In this aspect, the available technologies mainly involve the

use of ethylene oxide and irradiation, approved in some countries but with legal and market restrictions in others. Currently in Brazil, the use of ethylene oxide for the sterilization or reduction of the microbiological load of foods is not allowed (1), and many importing countries do not accept irradiated black pepper. These facts emphasize the need to search for alternative decontamination methods that are easy to apply and inexpensive in order to either eliminate or reduce the level of contamination of black pepper.

In more recent years, the application of gaseous chemical disinfectants for reducing microorganisms is gaining the interest of the food industry. Gases can penetrate into crevices and niches in foods and in facilities where entrapped microbes could be missed by conventional techniques of cleaning and sanitization. The gaseous antimicrobials already applied for black pepper were ethylene dioxide and ozone. Ozone reduced the microbial population of ground black pepper by 3 to 6 log, depending on the moisture content of the spice, but resulted in the oxidation of certain volatile oil constituents (27). Phosphine is one of the most used fumigants in the world. Because the previously popular fumigant methyl bromide has been banned under the Montreal Protocol, phosphine is the only

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FIGURE 1. Diagram of phosphine generator.



widely used, cost-effective, rapidly acting fumigant that does not leave residues on the stored product (residues in fumigated foods are 0.01 ppm or less and are negligible). Like other pesticides, it is highly toxic to human beings and animals (threshold limit value, 0.3 ppm as time-weighted average concentration and 1 ppm as short-term exposure limit (3)), so the prevention and protective measures listed on the International Chemical Safety Card should be followed (19). In Brazil, the application of phosphine in different crops during storage is established by the National Health Surveillance Agency (ANVISA) (2).

Previous research showed that phosphine, largely applied for insect control, can control mold growth (7, 8–11) and, thus, could also have some effects on other microorganisms. As this fumigant is an inhibitor of the respiratory enzymes (18), it may also have an effect on aerobic bacteria, but no published reports on this fumigant action in these microorganisms are available. The objective of the present research was to verify the effects of phosphine on *Salmonella enterica* serotype Enteritidis inoculated into culture medium and into black pepper grains. Studies of the survival period of this pathogen in dried black pepper were also carried out, as well as studies of the effects of phosphine in the reduction of the total microbial loads of dried and moisturized black pepper grains. The postfumigation effects were also verified after grain drying and storage.

MATERIALS AND METHODS

Raw material. Black pepper (*Piper nigrum*) (approximately 6 kg), freshly harvested and sun dried, kindly donated by the Association of Black Pepper Producers located in Belém, Pará, Brazil, was sent to the Instituto de Tecnologia de Alimentos (ITAL), Campinas, São Paulo, Brazil. The product was cleaned, manually homogenized, and sampled for analyses of water activity (a_w), moisture content, and aerobic plate counts. The moisture content was determined in a nonventilated air oven at 100 to 110°C for 2 h or until constant weight was obtained (17), the water activity was determined in a DECAGON apparatus model 2X-T, and the aerobic plating was done according to the methodology described by the American Public Health Association (APHA) (13). A subsample of 4 kg was taken and sent to the Companhia Brasileira de Esterilização for irradiation with 15 kGy. To evaluate the decontamination process, the irradiated samples were submitted to total aerobic plate counts. The remaining samples (2 kg) were kept in plastic bags at room temperature (22°C) for the nonirradiated studies.

Bacterial culture. A strain of *Salmonella* Enteritidis from the culture collection of the Laboratório de Higiene e Legislação,

FEA-UNICAMP, Brazil, was used in this study. This strain was isolated from poultry. The *Salmonella* Enteritidis culture was maintained in tryptone soy agar slants (Oxoid Ltd., Basingstoke, Hampshire, England) at 5°C. The identity of the strain was previously confirmed by biochemical and serological tests.

Inoculum preparation. An inoculum of *Salmonella* Enteritidis was transferred to a nutrient agar tube and incubated for 24 h at 35°C before the beginning of the experiments. Cells were collected from this medium and transferred to 5 ml of saline solution (0.85% NaCl) in order to adjust the suspension concentration to 10^8 CFU/ml according to the MacFarland turbidity scale, using Densimat equipment (bioMérieux, Inc., Marcy l'Etoile, France). The suspension was serially diluted (1:10) in 0.1% peptone water. Aliquots of 0.1 ml were spread onto the surface of XLD (xylose lysine deoxycholate) plates, followed by incubation at 35°C for 24 h in order to determine the viable cell concentration.

Survival of *Salmonella* Enteritidis on dried pepper. Aliquots of 10 μ l of the suspension previously described containing 10^8 CFU/ml were used for the inoculation of irradiated pepper samples at 0.67 a_w . The samples (11 g) were conditioned in sterile plastic bags and distributed in desiccators of 2,850 ml capacity (3 bags per desiccator) containing a KI saturated salt solution in order to create an environment of 67% relative humidity. After incubation for different periods (0, 3, 7, 14, and 21 days) at 25 and 35°C, sampling was carried out for *Salmonella* Enteritidis counts. Three repetitions were performed for each period and temperature.

Phosphine fumigation. Phosphine was obtained from Fer-toxin tablets in a generator, according to method no. 16 of the Food and Agriculture Organization of the United Nations (4) (Fig. 1). The gas concentration inside the generator was analyzed by gas chromatography using a flame photometric detector (Agilent Technologies, Palo Alto, CA) in phosphorus mode. The concentration of phosphine was assumed to be 86%, as previously determined by Oliveira et al. (20), and this value was used to calculate the required concentrations for the assays given that 1 ml of phosphine (99.9%) is equivalent to 1.39 mg of this gas. A phosphine analytical curve of concentration against peak area was obtained in the range of 0.2 to 6.0 g/m^3 and used to assess the experimental phosphine concentrations. A gas-tight syringe (Hamilton Company USA, Reno, NV) was used to take the phosphine from the generator through a septum and was introduced into the desiccators. The assays were carried out in triplicate, and the averages reported. Immediately after the phosphine fumigation periods (24, 48, and 72 h), the samples were subjected to the microbiological analyses.

Phosphine concentration. The phosphine concentration was measured inside the desiccators immediately before and at the end

of the exposure periods. An HP 6890 gas chromatograph with a flame photometric detector at a temperature of 150°C, a phosphorous filter (525 nm), hydrogen flow of 150 ml/min, and air flow of 110 ml/min was used. The capillary column was a 1909/j413 HP-5 with 5% phenyl methyl siloxane (30 m by 320 μm by 0.25 μm) with a constant nitrogen flow of 2.0 ml/min and a split injector (50:1) with make-up at 60 ml/min, both at 150°C. The sampling volume was 5 μl . The chromatography was linked to an HP Vectra XA computer (Chemstation software).

Evaluation of the phosphine effects on the bacterial cultures. Aliquots of 0.1 ml of a *Salmonella* Enteritidis suspension containing 10^3 CFU/ml were distributed on XLD plates and exposed to 0.0, 2.0, 4.0, and 6.0 g/m^3 phosphine for 48 h at 35°C. At the end of the exposure periods, the desiccators were opened in an exhaustion chemical chamber and the *Salmonella* Enteritidis colonies counted. Three repetitions for each treatment were carried out. Visual observations related to the colonies' morphology were also carried out. The plates containing the cultures that had been fumigated were reincubated in a B.O.D. (biochemical oxygen demand) incubator for an additional 24 h at 35°C in order to verify the colonies' recovery after the treatment.

Effects of phosphine on *Salmonella* Enteritidis inoculated on black pepper grains with low and high moisture content. Samples of irradiated black pepper grains (11 g) with 0.67 a_w were conditioned in sterile plastic bags and inoculated with aliquots of 10 μl of the *Salmonella* Enteritidis suspension containing 10^8 CFU/ml. The bags containing the inoculated samples were distributed in desiccators with a 3,150-ml capacity (3 bags per desiccator) with approximately 300 ml of a KI saturated salt solution in order to create an atmosphere with a relative humidity equilibrium of 67% and a free space volume of 2,850 ml. The desiccators were kept in a chamber with a controlled temperature of $35 \pm 2^\circ\text{C}$ according to the previously established experimental design; the samples were subjected to the different phosphine treatments. Doses of 0.0, 3.0, and 6.0 g/m^3 for 72 h at 35°C were applied to the product with an initial a_w of 0.67 (dried product). At the end of the exposure period, the desiccators were opened in an exhaustion chemical chamber and the samples submitted to *Salmonella* Enteritidis colony counts. As no reduction in *Salmonella* Enteritidis populations was obtained in any of the phosphine treatments applied to dried black pepper, in a second step, the fumigant was applied to pepper grains with high moisture. Rehumidification of the product was performed by adding amounts of sterilized distilled water previously calculated in order to increase the initial water activities to 0.92 and 0.97 a_w (equivalent to moisture contents of 23 and 32%, wet basis). Desiccators containing saturated salt solutions of sodium tartarate and potassium sulfate were used in order to keep environments of 92 and 97.5% relative humidity. A complete factorial design with three variables—phosphine concentration (0.0, 3.0, and 6.0 g/m^3), exposure time (24, 48, and 72 h), and water activity (0.92 and 0.97)—was used. Treatments were performed in batches, and thus, inoculation procedures were carried out separately at the beginning of each assay. For this reason, reductions of log cycle CFU per gram in each trial were used to analyze the results. Three repetitions were performed for each treatment.

Effects of phosphine on total aerobic bacteria counts of pepper grains with low and high moisture content. Samples (11 g) of the nonirradiated pepper grains with water activities of 0.67, 0.92, and 0.97 were exposed to 0.0, 3.0 and 6.0 g/m^3 of phosphine for 24, 48, and 72 h. At the end of the exposure period,

the desiccators were opened in an exhaustion chemical chamber and the samples submitted to analyses of total plate counts.

Phosphine's postapplication effect. Black pepper grains with 0.92 and 0.97 a_w were fumigated with 6 g/m^3 phosphine for 72 h according to the same methodology previously described. Immediately after the treatment, the grains were dried in an oven at 40°C to 0.67 a_w and stored in desiccators at 67% relative humidity for 24, 48, and 72 h. *Salmonella* counts were performed before and after the fumigation and at the end of each storage time of the dried grains. Water activity content was monitored in the assays. Three repetitions were carried out for each treatment.

Enumeration of *Salmonella* Enteritidis on pepper. Appropriate dilutions of each black pepper sample were spread plated on XLD medium and incubated at 35°C. Black colonies were enumerated after 24 and 48 h of incubation for *Salmonella* counts, since no background colonies were found in preliminary tests with the irradiated samples before the inoculation procedures. When no *Salmonella* was detected on XLD medium, the treatment was repeated and samples were submitted to presence-or-absence *Salmonella* analyses following the methodology described by Downes and Ito (13).

Statistical analysis. A factorial design with three variables (a_w , exposure time, and phosphine concentration) was used. *Salmonella* Enteritidis counts were converted to logarithmic values for calculating means, standard deviations, and/or reductions. Logarithmic data were analyzed by analysis of variance and Tukey's test using ESTAT software, System for Statistical Analyses (version 2.0, Universidade Estadual Paulista, Jaboticabal, São Paulo, Brazil).

RESULTS AND DISCUSSION

Prior to the phosphine studies, the growth of *Salmonella* Enteritidis on dried pepper grains during storage time at 25 and 35°C was verified (Fig. 2). The mean a_w value for the dried pepper was 0.67. After 21 days, reductions of 43% (at 25°C) and 49% (at 35°C) in the *Salmonella* Enteritidis population were observed at both temperatures, but the numbers of viable cells were still over 100 CFU/g. Ristori et al. (22) verified the behavior of *Salmonella enterica* serotype Rubislaw in ground black pepper at different temperatures and water activities and observed that after 15 days at 0.67 a_w , the decreases were approximately 3.0 and 3.5 log cycles, respectively, at 25 and 35°C, while in our study, lower reductions (of around 2.0 log cycles) were observed after 14 days at both temperatures. Juven et al. (15), when studying the survival of *Salmonella* spp. in dry products, found that survival varied among different serotypes. The state of the product (whole or ground grains) may also contribute to extend or not extend the period of bacterial survival. Our study focused on whole pepper grains because this state represents most Brazilian exportation.

To study the phosphine effect on *Salmonella* Enteritidis inoculated on culture medium and in black pepper stored with low and high moisture content, a phosphine analytical curve was built. The areas were obtained after injections of 5 μl of phosphine concentrations of between 1 and 6 g/m^3 . The determination coefficient indicated an excellent adjustment of the data to the equation obtained (Fig. 3).

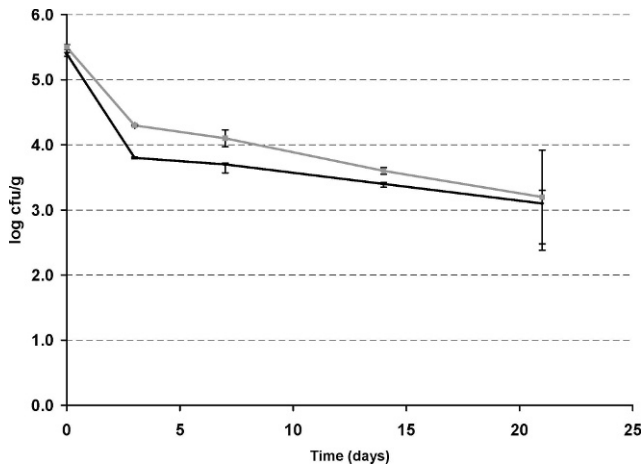


FIGURE 2. *Salmonella Enteritidis* (log CFU per gram) counts after different incubation periods at 25 and 35°C.

The results of the phosphine treatment of pure cultures of *Salmonella Enteritidis* inoculated onto XLD culture medium indicated that there were no decreases in the population of the tested pathogen when concentrations of up to 6 g/m³ for 48 h at 35°C were applied. However, the bacterial colonies showed a gradual reduction in colony sizes and atypical colorations as the phosphine concentration increased. After exposure to phosphine, the plates were reincubated for an additional 24 h at 35°C. It was observed that there was a gradual recovery of the colony size and coloration that was inversely proportional to the concentrations applied. Probably the growth rate was only reduced but not inhibited when phosphine was applied in pure cultures of *Salmonella Enteritidis* inoculated onto XLD medium for 48 h in concentrations up to 6 g/m³. Figure 4 shows the colony aspect immediately after the treatments

FIGURE 4. Pure cultures of *Salmonella Enteritidis* on XLD medium exposed for 48 h at 35°C at concentrations of 0 (A₁), 3 (B₁), and 6 (C₁) g/m³ and after reincubation for 24 h at 35°C (A₂, B₂, and C₂).

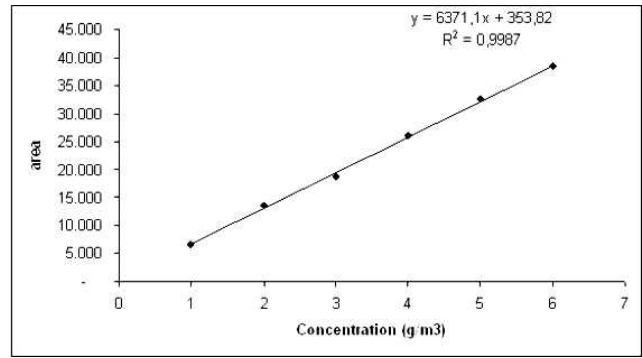
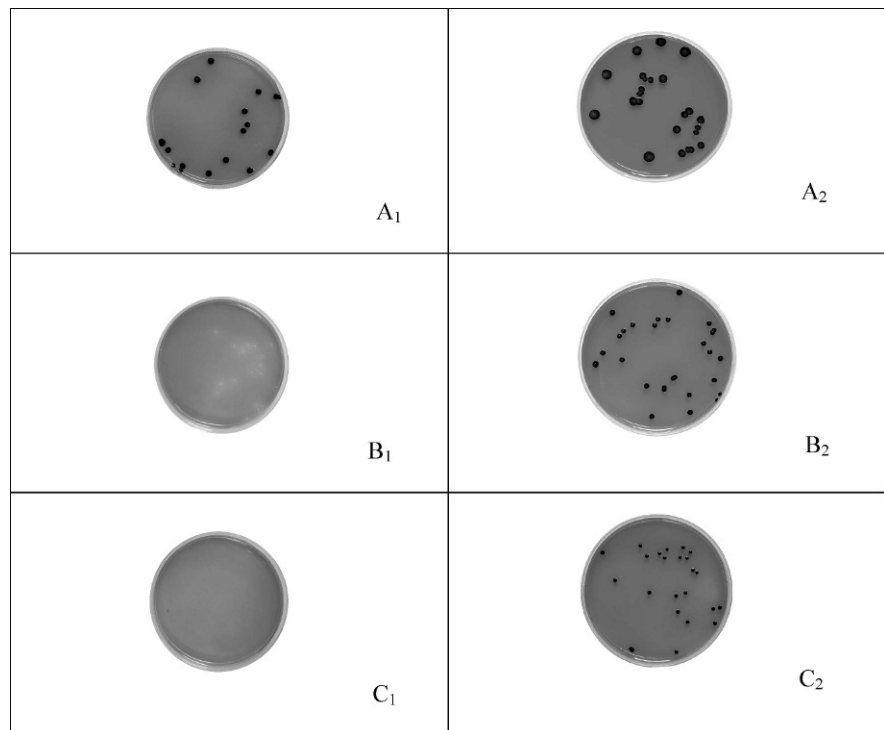


FIGURE 3. Phosphine analytical curve.

and after reincubation at 35°C for 24 h in the absence of phosphine.

The decreases in *Salmonella Enteritidis* populations inoculated into black pepper in grains with 0.92 and 0.97 a_w exposed to phosphine concentrations of 0.0, 3.0, and 6.0 g/m³ at 35°C for 24, 48, and 72 h are shown in Table 1. Ten-microliter amounts of the inoculum of *Salmonella Enteritidis* containing approximately 8 to 9 log CFU/ml were inoculated into 11-g samples, resulting in concentrations of 6 to 7 log CFU/g. Approximately 4 to 6 log CFU/g were recovered from pepper samples after drying. These values are shown in parentheses in Table 1 and correspond to time zero.

Significant differences in relation to the control were observed after exposure for 48 h when concentrations of 3 and 6 g/m³ were applied to the product with 0.92 a_w and when 6 g/m³ was applied to the product with 0.97 a_w. For the a_w of 0.92 after 48 h, reductions of 1.1 and 2.4 log cycles were observed for the populations exposed to concentrations of 3 and 6 g/m³, respectively, compared with the

TABLE 1. Decreases in *Salmonella Enteritidis* populations in irradiated black pepper grains with different water activities submitted to phosphine fumigation

a _w	PH ₃ (g/m ³) ^a	Decrease in <i>Salmonella Enteritidis</i> population (log cycle CFU/g) during indicated treatment time (h) ^b		
		24	48	72
0.92	0.0	0.63 ± 0.06 A ^c a ^d (4.77, 4.14)	1.10 ± 0.01 B a (6.06, 4.96)	1.07 ± 0.12 B a (6.27, 5.20)
	3.0	0.42 ± 0.04 A a (4.19, 3.77)	2.12 ± 0.14 B b (4.96, 2.84)	3.07 ± 0.84 B b (5.10, 2.03)
	6.0	0.39 ± 0.28 A a (4.18, 3.79)	3.54 ± 0.31 B c (5.01, 1.47)	4.29 ± 0.03 c c (5.30, 1.01)
0.97	0.0	0.31 ± 0.05 A a (5.01, 4.70)	1.10 ± 0.00 B a (6.28, 5.18)	1.02 ± 0.01 B a (6.07, 5.06)
	3.0	0.45 ± 0.07 A a (5.01, 4.56)	1.39 ± 0.11 B a (5.18, 3.79)	2.17 ± 0.21 c b (5.06, 2.89)
	6.0	0.44 ± 0.07 A a (5.00, 4.56)	2.08 ± 0.21 B b (5.15, 3.07)	3.35 ± 0.06 c c (4.06, 1.45)

^a PH₃, phosphine.

^b Results are expressed as the averages of three repetitions ± standard deviations. Values in parentheses represent means of *Salmonella Enteritidis* populations (log CFU per gram) initially (time 0) and after phosphine treatments.

^c Results for concentration (rows) for the same water activity that are followed by different capital letters differed significantly at the 5% level.

^d Results for exposure time (columns) for the same water activity that are followed by different lowercase letters differed significantly at the 5% level.

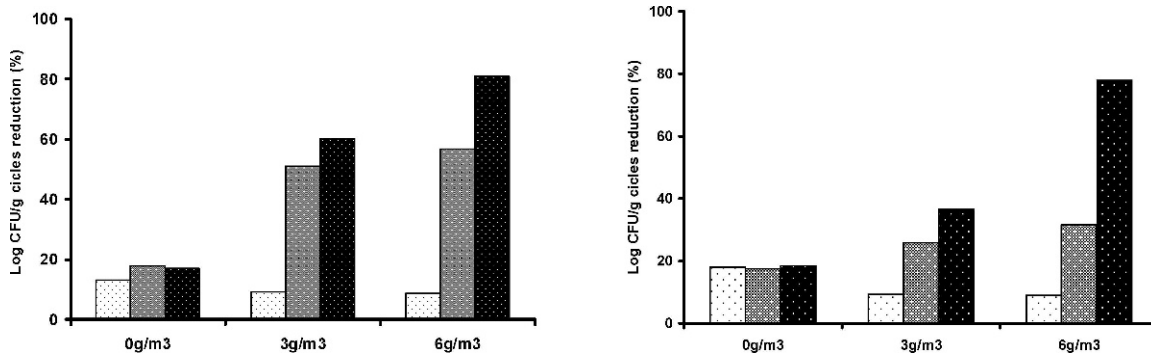


FIGURE 5. Reduction in *Salmonella Enteritidis* populations (log cycle CFU per gram) in black pepper grains with 0.92 (A) and 0.97(B) a_w submitted to phosphine fumigation.

nonfumigated samples (dose 0.0 mg/liter), as shown in Table 1.

In general, greater decreases in *Salmonella Enteritidis* were achieved at 0.92 a_w than at 0.97 a_w for the same phosphine concentration and exposure period. At 0.97 a_w, a significant decrease (1.0 log cycle) was observed only for the concentration of 6 g/m³. After 72 h, a decrease of 80% in relation to the initial population was observed for the concentration of 6 g/m³ for both water activities studied (Fig. 5).

The variance analyses showed that all factors studied (a_w, exposure time, and concentration) and their interactions were significant. The fumigant concentration was the main factor, followed by exposure time, a_w, and then the factors' interactions. Thus, at both a_w levels, the increase in fumigant concentration had more effect on the *Salmonella Enteritidis* population reduction than exposure time. The cost of phosphine is low, but to achieve high concentrations, such as 6 g/m³, hermetic storage structures are required. The maintenance of the desired levels becomes more critical with the extension of the exposure period. No decreases in the total aerobic mesophilic bacterium counts were observed in black pepper grains with 0.67, 0.92, and 0.97 a_w.

Table 2 shows the phosphine concentrations applied and those determined after different exposure periods. Although a slight decrease was observed in relation to the initial concentrations, these data did not show significant differences. When decreases in phosphine concentration occur during storage, it may be due to the fumigant sorption by the product. Castro and Leitão (7) observed phosphine sorption ranging from 0.07 to 0.7 g/m³ when corn at 0.92 a_w was fumigated with 4.0 g/m³ phosphine for 15 days.

TABLE 2. Phosphine concentrations determined initially and after 24, 48, and 72 h at 35 °C

a _w	PH ₃ concn (g/m ³) at indicated time (h) ^a			
	0	24	48	72
0.92	3.00 ± 0.00	2.92 ± 0.04	2.42 ± 0.12	2.56 ± 0.08
	5.99 ± 0.02	5.90 ± 0.04	5.51 ± 0.06	5.67 ± 0.03
0.97	2.97 ± 0.01	2.53 ± 0.01	2.57 ± 0.06	2.56 ± 0.04
	5.94 ± 0.01	5.71 ± 0.16	5.61 ± 0.32	4.52 ± 0.11

^a Results are expressed as the averages of three repetitions ± standard deviations. PH₃, phosphine.

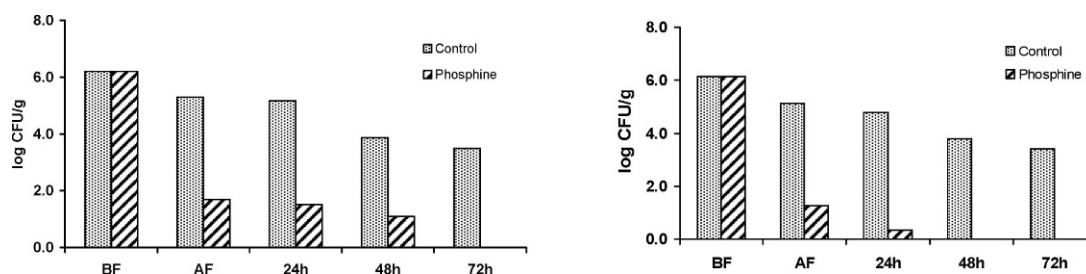


FIGURE 6. Phosphine postapplication effect in ground black pepper at 0.92 and 0.97 a_w . AF, after fumigation; BF, before fumigation.

Salmonella Enteritidis counts before and after phosphine treatment and 24, 48, and 72 h after drying of up to 0.67 a_w are shown in Figure 6. For the samples with 0.92 a_w , there was a reduction of 4.5 log cycles (73% of the *Salmonella* Enteritidis population) in the phosphine-treated grains immediately after fumigation, while in the control samples, only a slight reduction (0.9 log cycles, corresponding to 15%) was observed. After 72 h, *Salmonella* was absent, while 3.5 log CFU/g was still present in the control samples. For the samples at 0.97 a_w , a reduction of 4.9 log cycles (79%) of *Salmonella* Enteritidis in the grains was observed immediately after fumigation, while in the control samples, there was a reduction of 1.03 log cycles (17%). However, in contrast to the samples with 0.92 a_w , *Salmonella* Enteritidis was absent after 48 h, while in the control, 3.4 log CFU/g were still present in the grains after 72 h. These results indicated that phosphine treatment could be very useful for *Salmonella* Enteritidis decontamination in black pepper grains when treatment is applied before the drying process.

Phosphine acts in the cellular respiratory chain at the mitochondrial level, more specifically, in the Krebs cycle, so it is unlikely that serotypes or strains would differ in their responses to the fumigant treatment. For confirmation of our hypothesis, additional research is recommended.

The application of phosphine prior to the drying process showed very promising results and should be better evaluated in future research. Fumigation could be carried out in very well sealed fumigation containers before the product is dried, but after the treatment, producers should rigidly follow good agriculture practices in order to avoid product recontamination.

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