Sodium Hypochlorite: Effect of Solution pH on Rice Seed Disinfestation and Its Direct Effect on Seedling Growth

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ABSTRACT

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Bacteria were completely eliminated from rice seeds following immersion in household bleach solutions (50% bleach and 2.6% NaOCl) adjusted to pH 7.0 in 0.5 M potassium phosphate, while fungi were eliminated at pH 5.0 and below. Mercuric chloride was used to disinfest nondormant rice seeds in order to test the direct effect of sodium hypochlorite on rice seedling growth apart from an indirect effect related to elimination of microbial contaminants. Seeds were immersed for 2 h in each solution or water in each of the following treatments: (i) sterile water followed by sterile water, (ii) HgCl₂ (1,000 µg/ml) followed by sterile water, (iii) HgCl₂ followed by KH₂PO₄ (0.3 M), (iv) HgCl₂ followed by NaOCl, and (v) NaOCl followed by sterile water. Sodium hypochlorite solutions were prepared in 0.3 M potassium phosphate, which gave a final pH of 7.3. Seedling growth in treatments in which seeds were treated with HgCl₂ followed by NaOCl, and NaOCl followed by sterile water, was significantly greater than those treatments in which seeds were treated with sterile water followed by sterile water, HgCl₂ followed by sterile water, and HgCl₂ followed by KH₂PO₄. Sodium hypochlorite stimulated rice seedling growth directly, as opposed to an indirect effect related to elimination of microbial contaminants or alleviation of dormancy.

Additional keywords: biological control, seed pathology, surface-sterilization

Household bleach, composed of 5.25% sodium hypochlorite (NaOCl) and 4% NaCl, pH about 11.4 (R. Tam, The Clorox Company, *personal communication*), is routinely used as a seed disinfestant in studies involving seedborne pathogens, biological control, endophytes, and seed germination, as well as in commercial applications (16,21–23). When using bleach in this manner, the implied assumption is that any effects subsequent to surface disinfestation are related to the experimental treatment, e.g., biological control agent, and there is no direct effect of hypochlorite on seedling growth.

The objective of this research was to determine the influence of sodium hypochlorite on rice (*Oryza sativa* L.) seedling growth apart from that attributed to disinfestation. Mercuric chloride and Vitavax 200FF (17.5% carboxin and 17.5% thiram; Gustafson, McKinney, TX) were included as additional seed disinfestation agents for comparative purposes in some experiments. A companion study was conducted to determine the relationship between pH of hy-

pochlorite solutions and efficacy of seed disinfestation. A preliminary report was published (4).

MATERIALS AND METHODS

The effect of pH of sodium hypochlorite solutions on disinfestation of rice seeds. A range of initial pH values of 50% household bleach (2.6% NaOCl) solutions (The Clorox Company, Oakland, CA) was established with various buffers including phosphoric acid (pH 2), potassium phosphate (pH 3 to 7), and Tris-HCl (pH 8 to 9). Buffered bleach solutions below pH 7.0 were prepared in an exhaust hood, because chlorine and HOCl are products of the chemical reactions (1). Final concentration of buffers was 0.5 M, which was required to prevent changes in solution pH during the experiments. Unbuffered bleach was diluted 1:1 (vol/vol) with distilled water, which gave a final pH of 11.6. Sterile buffered water (pH 7.1, 1.0 mM phosphate) was used as the control. Ten grams of rice seeds (cv. Lemont) was soaked in 100 ml of freshly prepared solutions contained in 150-ml beakers for 2 h at room temperature. The beakers were sealed with laboratory film (Parafilm 'M'; American National Can, Greenwich, CT) and agitated three times during the soak. The seeds were then rinsed, with agitation, in four changes of 150 ml of sterile distilled water. The seeds were removed from the final rinse and incubated on nutrient agar (NA)

at 32°C and on acidified (pH 3.0) potato dextrose agar (APDA) at 28°C for assessment of bacterial and fungal contamination, respectively. After 3 days of incubation, the numbers of seeds contaminated with bacteria and fungi and the number of germinated seeds on APDA were determined. In a preliminary experiment, the germinability of rice seeds was reduced on NA plates if there was a high rate of bacterial contamination. There were five replications (petri dishes) per treatment with 20 seeds per replication, and the experiment was conducted twice.

Efficacy of disinfestation with mercuric chloride and Vitavax 200FF as compared with household bleach. Ten grams of nondormant rice seeds was soaked for 2 h at room temperature in 100 ml of freshly prepared buffered (pH 7.3) bleach solution (2.6% NaOCl) as described above, by diluting household bleach with 0.6 M phosphate buffer (1:1, vol/vol) and in HgCl₂ solutions prepared in distilled water and adjusted to 500, 1,000, 2,000, 3,000, 4,000, and 5,000 µg/ml (1.85, 3.69, 7.38, 11.07, 14.76, and 18.45 mM, respectively). An unbuffered bleach solution prepared with distilled water and sterile distilled water treatments also were included. Seeds were then rinsed four times with sterile distilled water as described above. A separate treatment was included in which seeds were treated with Vitavax 200FF (2.5 ml/kg of seed) (25) as another means of reducing fungal contamination. Seeds were assessed for bacterial and fungal contamination as described above. There were five replications (petri dishes) per treatment with 20 seeds per replication, and the experiment was conducted twice.

Effect of sodium hypochlorite on rice seedling growth. We attempted to separate the possible direct effect of hypochlorite on seedling growth from an indirect effect related to disinfestation. These two effects were separated by first disinfesting seeds with 1,000 µg/ml (3.69 mM) of HgCl₂ followed by treatment with NaOCl. In addition, Vitavax 200FF was used as another seed disinfestant. Seeds were immersed for 2 h in each of two solutions (or water) in the following treatments: (i) sterile distilled water followed by sterile distilled water, (ii) seeds treated with Vitavax 200FF (2.5 ml/kg of seeds) soaked in sterile distilled water for 4 h, (iii) HgCl₂ followed by sterile distilled water, (iv) HgCl₂ followed by KH₂PO₄ (0.3 M), (v) HgCl₂ followed

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by 2% NaCl, (vi) HgCl₂ followed by NaOCl (2.6% for bleach and 2 to 3% for reagent-grade NaOCl), and (vii) NaOCl (2.6% for bleach and 2 to 3% for reagent-grade NaOCl) followed by sterile distilled water. Mercuric chloride followed by 2% NaCl was included to test the effect of NaCl on seedling growth, because commercial bleach (Clorox) contains 4% NaCl. Sodium hypochlorite solutions were pre-

Table 1. Influence of pH of household bleach solutions on bacterial and fungal contamination of rice seeds and seed germination

pH ^w	Percent contamination with		Percent germination
	Bacteria	Fungi	
2	0 a ^x	3 a	89 c
3	0 a	0 a	42 a
4	0 a	0 a	78 b
5	0 a	0 a	82 b
6	0 a	9 a	89 c
7	0 a	22 b	95 cd
8	100 c	79 d	95 cd
9	100 c	39 c	96 cd
Unbuffered ^y	30 b	39 c	100 d
Control ^z	100 c	96 e	97 cd

^w The pH of household bleach solutions was adjusted with (1:1, vol/vol) 1.0 M phosphoric acid (pH 2), 1.0 M potassium phosphate (pH 3 to 7), and 1.0 M Tris-HCl (pH 8 to 9).

^x Means followed by the same letters within columns are not significantly different (Student-Newman-Keuls test, $P = 0.05$) (24). Statistical analyses were conducted following arcsine transformation of percent contamination (24).

^y pH of unbuffered bleach was about 11.6.

^z Sterile buffered water.

Table 2. Efficacy of surface-sterilization of rice seeds with different chemical treatments

Treatments	Percent of seeds contaminated with	
	Bacteria	Fungi
Control ^a	98 e ^v	100 f
Vitavax 200FF ^w	85 d	18 ab
HgCl ₂ (500) ^x	21 b	45 d
HgCl ₂ (1,000)	9 a	45 d
HgCl ₂ (2,000)	9 a	40 d
HgCl ₂ (3,000)	3 a	27 c
HgCl ₂ (4,000)	2 a	25 bc
HgCl ₂ (5,000)	2 a	13 a
Household bleach (pH 11.6) ^y	41 c	61 e
Household bleach (pH 7.3) ^z	3 a	43 d

^a Sterile distilled water.

^v Values followed by the same letters within columns are not significantly different (Student-Newman-Keuls test, $P = 0.05$). Statistical analyses were conducted following arcsine transformation of percent contamination (24).

^w Composed of 17.5% carboxin and 17.5% thiram (25). Treated seeds were soaked in sterile distilled water for 2 h.

^x Numbers in parentheses are the concentrations of mercuric chloride (μg/ml).

^y 50% household bleach (2.6% NaOCl) in distilled water.

^z pH of household bleach was adjusted (1:1, vol/vol) with 0.6 M potassium phosphate buffer.

pared by diluting (1:1, vol/vol) household bleach or reagent-grade NaOCl with 0.6 M potassium phosphate, which gave a final pH of 7.3. After treatment, seeds were planted in sterilized, covered glass storage dishes that contained 50 cm³ of sterile soil and 100 ml of sterile buffered water (pH 7.1, 1.0 mM phosphate). They were incubated in a growth chamber at 30/30°C (day/night) with 12-h photoperiods for 10 days, at which time the lengths and dry weights of shoots and roots were measured. There were four replications per treatment, 25 plants per replication, and the experiment was conducted twice.

RESULTS AND DISCUSSION

Bacteria were completely eliminated from rice seeds following immersion in bleach solutions (50% bleach and 2.6% NaOCl in 0.5 M potassium phosphate) at or below pH 7.0, while fungi were eliminated most effectively at pH 5.0 and below (Table 1). Buffer solutions without NaOCl did not affect levels of seed infestation (data not shown). Compared with higher pH values, seed germination was significantly lower at pH 3 to 5 (Table 1), but was not affected at pH 2. This may have been a response to a lower concentration of HOCl at pH 2.0 relative to higher pH values (1). Brazis et al. (2) reported that the bactericidal activity of HOCl is about 100-fold greater than OCl⁻, and this may explain why solution pH has such a marked effect. As pH declines from 10.0 to 5.0 in a solution with a given concentration of NaOCl, the concentration of HOCl increases, while that of OCl⁻ decreases. As pH declines from 5.0 to 1.0, the concentration of HOCl declines, while that of Cl₂ increases (1). Mercuric chloride at 1,000 μg/ml resulted in about the same level of disinfection as bleach at pH 7.3 (Table 2). The fungicide treatment significantly reduced fungal contamination, but bacterial contamination was relatively unaffected (Table 2).

The pH of 50% household bleach prepared in distilled water (2.6% NaOCl) was

about 11.6. Our results showed that buffering the pH of bleach solutions to 7.0 significantly increased the efficacy of disinfection. Efficacy of disinfection with bleach solutions is dependent upon NaOCl concentration, pH, water quality, storage condition, and age of the solution (1,10). These variables often are not considered or fully documented, which may account for inconsistent results in the literature (1,8).

The antimicrobial properties of hypochlorite solutions are affected by an equilibrium between the chlorine compounds present (1,2). When hypochlorite is dissolved in water, it produces HOCl, and HOCl dissociates reversibly into H⁺ and OCl⁻ with the final equilibrium dependent upon solution pH (1,2,10). Disinfection with hypochlorite is best achieved at about pH 5 and increases with higher temperature (1,22,23), but the stability of hypochlorite solutions decreases under these conditions (1,10). Recently, Robbs et al. (21) showed that initial oxidation-reduction potential of hypochlorite, which was higher at pH 6.0 than at pH 8.0, was correlated with the toxicity of chlorine solutions to *Erwinia carotovora* subsp. *carotovora* and conidia of *Geotrichum candidum*.

Because the efficacy of seed disinfection of HgCl₂ solutions equal to or greater than 1,000 μg/ml was similar to a 50% bleach solution at pH 7.3, and the solutions were not phytotoxic, HgCl₂ was used as a control for testing the effect of NaOCl on rice seedling growth apart from that attributed to seed disinfection. The only nonhypochlorite treatments that had statistically positive effects, relative to the water-water control, were HgCl₂-water and HgCl₂-NaCl on root dry weight (Table 3). It is possible that HgCl₂ eliminated seedborne contaminants that reduced root growth. These results suggest that roots and shoots responded differently to hypochlorite, with shoot growth being more responsive. We cannot rule out the possibility that NaCl had a beneficial effect on root dry weight in these experiments. However, when the experiment was

Table 3. Effect of household bleach and other sterilants on rice seedling growth

Treatments ^s	Shoot length (cm)	Root length (cm)	Shoot dry weight (mg)	Root dry weight (mg)
Water-water ^t	5.0 a ^u	3.5 a	2.3 a	1.2 a
Vitavax 200FF ^v -water	4.1 a	3.9 a	2.1 a	1.4 a
HgCl ₂ -water ^w	5.0 a	5.0 ab	3.0 a	2.8 b
HgCl ₂ -KH ₂ PO ₄ ^x	4.9 a	4.6 ab	2.8 a	1.8 a
HgCl ₂ -NaCl ^y	5.3 a	5.0 ab	2.9 a	3.1 c
HgCl ₂ -bleach ^z	6.8 b	5.7 b	4.1 b	3.2 c
Bleach-water ^z	7.0 b	7.1 c	4.2 b	3.2 c

^s Seeds were soaked for 2 h in the first solution and then 2 h in the second solution.

^t Control (sterile distilled water).

^u Values followed by the same letters within columns are not significantly different (Student-Newman-Keuls test, $P = 0.05$) (24).

^v Composed of 17.5% carboxin and 17.5% thiram (25). Treated seeds were soaked in sterile distilled water for 4 h.

^w Concentration of HgCl₂ was 1,000 μg/ml (3.69 mM).

^x Final concentration = 0.3 M KH₂PO₄.

^y 2% NaCl.

^z Household bleach was diluted (1:1, vol/vol) with 0.6 M potassium phosphate, final pH 7.3.

repeated with reagent-grade NaOCl, the direct stimulatory effect was unambiguous. Except for root length in the HgCl₂-water treatment, plant growth parameters were significantly greater in all reagent-grade NaOCl treatments (Table 4).

Aside from the antimicrobial properties of hypochlorite, we present evidence that hypochlorite acts directly to stimulate rice seedling growth. Seedling growth was significantly greater after seed treatment with HgCl₂ followed by NaOCl (pH 7.3). This effect was not related to disinfestation, phosphate, or NaCl, in that the treatments that included HgCl₂-water, HgCl₂-KH₂PO₄, or HgCl₂-NaCl did not result in increased seedling growth. Our observations of the plants suggest that hypochlorite promoted growth by stimulating the development of primary leaves after emergence of the coleoptile. In related studies, Hall (9) reported that treatment of sweet potatoes (*Ipomoea batatas*) with calcium hypochlorite reduced the time interval from bedding to first plant harvest. Fieldhouse and Sasser (5) reported that residual sodium hypochlorite significantly increased the rate of emergence of pepper seedlings (*Capsicum annuum*) and dry weight of the above-ground portions of plants. When sodium hypochlorite-treated seeds were acid-rinsed, there were no significant differences between the acid-rinsed NaOCl treatment and nontreated control (5). However, Cantliffe and Watkins (3) reported that NaOCl reduced the rate of pepper seed germination, but total percent germination was not affected. These contrasting results may have been caused by procedural differences. The former and latter authors used the same concentrations of NaOCl (1% available chlorine), but soaked their seeds for 5 and 40 min, respectively.

Sodium hypochlorite has been used to break seed dormancy in several plant species including wild oat (*Avena fatua*), green needlegrass (*Stipa viridula*), wild buckwheat (*Polygonum convolvulus*), cow cockle (*Saponaria vaccaria*), *Bouteloua curtipendula*, and root parasites such as *Aeginetia indica* and *Alectra vogeli* (6,7,11-15,18). The promotion of rice seedling growth by

hypochlorite reported here is not related to the alleviation of seed dormancy in that seed germination in nonhypochlorite treatments was essentially 100% (Table 1). Furthermore, it is commonly known that rice seeds from short maturing varieties do not require a dormant period (17,19,20). Mikkelsen and Sinah (17) reported that dilute solutions of NaOCl enhanced seedling growth, and they suggested that a germination inhibitor was inactivated in the rice seed hull, although direct evidence was not presented. We saw no evidence for such an effect in an experiment in which rate and percent germination of rice seeds at 30°C were measured following treatments with NaOCl and water. Less than 2 days were required for 50% germination in both treatments, and final germination was 97%.

A precipitous decline in populations of biocontrol bacteria was observed when bacteria were inoculated onto rice seeds previously treated with bleach solutions adjusted to pH 7.0 and rinsed several times with distilled water (R. W. Schneider, unpublished data). This was not the case when unbuffered bleach solutions were used. Apparently, distilled water rinses do not remove all the hypochlorite from seeds treated with buffered bleach. Also, toxic concentrations of chlorinated amines may have been produced on the seeds (1). However, residual hypochlorite can be removed by soaking seeds in 0.01 N HCl without reducing germinability (1). Investigators conducting experiments in which seeds are surface-sterilized are cautioned to take into account the direct effect of sodium hypochlorite on seedling growth. Unless appropriate controls are included, spurious results may be obtained in studies involving plant growth regulators or biological control agents applied to seeds. Hypochlorite solutions should be acidified in a fume hood, because gaseous HOCl and Cl₂ are produced.

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Table 4. Effect of reagent-grade sodium hypochlorite and mercuric chloride on rice seedling growth

Treatments ^a	Shoot length (cm)	Root length (cm)	Shoot dry weight (mg)	Root dry weight (mg)
Water-water ^v	5.4 a ^w	4.0 a	2.2 a	1.4 a
HgCl ₂ -water ^x	5.7 a	4.9 bc	2.9 a	2.2 a
HgCl ₂ -HgCl ₂ ^x	5.5 a	4.2 a	2.8 a	2.2 a
HgCl ₂ -KH ₂ PO ₄ ^y	5.3 a	4.1 a	2.4 a	1.7 a
HgCl ₂ -NaOCl ^z	7.1 b	6.1 cd	3.8 b	3.2 b
NaOCl-water ^z	7.8 b	6.6 d	3.7 b	3.2 b

^a Seeds were soaked for 2 h in the first solution and then 2 h in the second solution.

^v Control (sterile distilled water).

^w Means followed by same letters within columns are not significantly different (Student-Newman-Keuls test, *P* = 0.05) (24).

^x Concentration of HgCl₂ was 1,000 µg/ml (3.69 mM).

^y Final concentration = 0.3 M KH₂PO₄.

^z 4 to 6% reagent-grade NaOCl was diluted (1:1, vol/vol) with 0.6 M potassium phosphate, final pH 7.3.

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