Disinfection of Penicillium-infected Wheat Seed by Gaseous Chlorine Dioxide

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Seeds of wheat (Triticum aestivum L. cv. Olgeurumil) were infected with Penicillium sp. at mean infection rate of 83%. Penicillium sp. was detected in endosperm with bran but not in embryo. Gaseous chlorine dioxide (ClO₂) effectively inhibited growth of Penicillium sp. at concentration of 5 to 20 µg/ml. As treatment duration was extended from 1 to 3 h, growth of Penicillium sp. was completely suppressed even at 10 µg/ml. There was no significant reduction in the incidence of Penicillium sp. at 30% relative humidity (RH). However, the incidence of Penicillium sp. was 27.7% at 50% RH, further those were 3.5% and 0.2% at 70% and 80% RH, respectively.

Seed germination was not affected by ClO₂ treatment at all the RH conditions. Water-soaked seeds (30% seed moisture content) showed a drastic reduction in the incidence of Penicillium sp. when treated at more than 10 µg/ml of ClO₂. The incidences of Penicillium sp. were 3.3, 1.8 and 1.2% at 10, 15 and 20 µg/ml, respectively. The incidence of Penicillium sp. in dry seeds with 9.7% seed moisture content did not reduce when treated with 5 and 10 µg/ml at 50% RH although it tended to decrease as ClO₂ concentration increased to 20 µg/ml. Seed germination was not affected by ClO₂ treatment at the tested concentrations. These results indicated that gaseous ClO₂ was effective disinfectant to wheat seeds infected with Penicillium sp. and that the effectiveness of ClO₂ strongly increased when moisture content around or inside of the seed was increased.

Keywords: Chlorine dioxide, Disinfection, Penicillium sp., Relative humidity, Seed moisture content

Introduction

The storage of seeds as grain, other staple food and feed free of fungal contamination is of importance to consumers (Agarwal and Sinclair, 1996). Storage fungi of seeds comprising mainly species of Aspergillus and Penicillium normally do not play a role in disease development in the field but play a major role in seed deterioration in storage (Agarwal and Sinclair, 1996). Decrease in germinability, discoloration and shrinkage of the grains, heat damage by fungal respiration, and chemical breakdown of nutrients can be caused by interactions which occurred between fungi and the stored seeds. Moreover, they may produce mycotoxins that are injurious to human and animals.

Several chemicals have been used for disinfection of stored seeds, fruits and tubers to prevent insect damages which play an important role in making seeds vulnerable to fungal infection and distribution of fungal inocula. Phosphine, methyl bromide, ethylene and propylene oxides have been often used as fumigants to control insects (Sittisuang and Nakakita, 1985). Acetaldehyde, propanol, and butanal were tested on cherry fruit to control Penicillium expansum conidial germination (Matthesis and Burroughs, 1986). It is necessary to develop an effective seed disinfectant without toxicity to human and animals, affect on seed germinability and risk on abnormality of seedlings.

Chlorine has been used for surface sterilization of seeds (Sauer and Burroughs, 1986). It is easily available and very cheap,
however, it has disadvantages including extensive corrosion of metal equipment, sensitivity to organic load, and the formation of chlorinated byproducts (Hikada et al., 1992). For these reasons, new methods to disinfect storage fungi in seeds have been increasing. Chlorine dioxide (ClO₂) is less toxic than common chlorine, produces no byproduct, and has no particular odor or taste (Dietrich et al., 1992; Wondergem and van Dijk-Looijenga, 1991). Aqueous ClO₂ was reported as a highly efficient disinfectant against fungi including Alternaria sp. and Penicillium sp. (Errampalli et al., 2006), and Botrytis cinerea, P. expansum and Rhizopus stolonifer (Zoffoli et al., 2005). Gaseous ClO₂ was effective for controlling bacterial postharvest decays of apple (Lee et al., 2006), tomato (Mahovic et al., 2007) fruits. Trinetta et al. (2011) found that was effective for inactivation of Salmonella enterica and Escherichia coli O157:H7 on pre-inoculated seeds of tomato, lettuce and cantaloupe. Disinfection of seeds with gaseous ClO₂ may be useful for re-storage or transportation because it is a dry system.

The aims of this study were to reveal whether gaseous ClO₂ can inhibit growth of Penicillium sp. in wheat seeds and which condition is the best for disinfection of them. The effectiveness of gaseous ClO₂ was evaluated by investigating the incidence of Penicillium sp. in wheat seeds treated with various concentrations of ClO₂, relative humidity in treatment chamber and physical conditions of the seed. The toxicity of gaseous ClO₂ to the seed was evaluated by seed germination test.

Materials and Methods

Seed material. Seeds of wheat, Triticum aestivum L. cv. Olgeurumil, were harvested at Suwon, Republic of Korea and stored in a mesh bag at 4°C for 15 years. Mean germination rate was 42.9% as determined by between-paper method.

Incidence of Penicillium sp. in wheat seed. Fifty seeds were placed equidistantly on Komada’s medium in 90-mm Petri dishes (25 seeds per dish) and incubated at 25°C in the darkness for 7 days. The incidence of Penicillium sp. in seed was expressed as a percentage of the number of seeds with fungal growth to the number of seeds tested.

Seed germination. One-hundred seeds were tested for germination using the between-paper method. Seeds were placed equidistantly between two layers of germination paper (Anchor paper Co., MN, USA) moistened with distilled water, after which the papers were loosely rolled up and placed in a plastic bag in an upright position to reduce surface evaporation. After incubation at 20°C in the darkness, the number of seeds that germinated normally after 4 and 8 days was determined and germination rate was expressed as a percentage of the number of normal seedlings to the number of seeds tested.

Seed moisture content. One-hundred seeds were ground and dried at 130°C for 2 hours in aluminum container, then they were allowed to cool in a desiccator for 40 min. The weights of empty containers (W1), the weights of seeds and containers before drying (W2) and the weights of seeds and containers after drying (W3) were measured to four decimal places. The seed moisture content (SMC) was calculated using the formula, \( \text{SMC} = \frac{(W2 - W1) - W1}{W1} \times 100 \), and expressed as percentage to one decimal place. SMC of 30% was accomplished by soaking dry sees in distilled water at 25°C for 24 hours.

ClO₂ treatment. Gaseous ClO₂ was generated by UV irradiation of Bactericide® gel (Oxy Therapy, Rep. of Korea) containing 2% (w/w) ClO₂. Within a treatment chamber, the ClO₂ concentration was measured by means of a portable detector (PortaSensII, Applied Technology Inc., USA). The relative humidity (RH) of the chamber was adjusted to 30, 50, 70 and 80% using a hand-made RH controller. Temperature within treatment chamber was kept around 23°C. To enable contact of seeds with ClO₂ during treatment, three hundred seeds were spread in a single layer without overlapping in a polypropylene mesh bag and placed on a tube rack. For treatment of Penicillium sp., fragment of 10-day-old fungal cultures grown on wheat seeds was suspended by sterile distilled water in an Eppendorf tube. The resulting suspension was manually counted with a haemocytometer and diluted in sterile water to 1 × 10³ conidia/ml. Two hundred microliters of the diluted suspension was spread on potato dextrose agar (PDA) medium in 90 mm-diameter Petri dish and then exposed to gaseous ClO₂ with half of the lid open.

Component plating test. Twenty-five seeds were used for detecting the Penicillium sp. associated with different components of wheat seeds. Seeds were soaked in sterile distilled water at room temperature for 24 hours. Seed components were separated into endosperm with bran and embryo using a sterile scalpel under a stereomicroscope. Each component was placed on PDA medium and incubated at 25°C for 7 days under alternating 12-h near UV and 12-h dark.

Statistical analysis. Each experiment was conducted three times except the measurement of SMC. An arc sine transformation was performed for percentage data. Analysis of variance and Duncan’s new multiple range tests with the package ‘agricolae’ (Mendiburu, 2014) was conducted using R version 3.1.0 (R Core Team, 2014). Significant differences were determined at the 95% confidence level (p ≤ 0.05).

Results and Discussion

Wheat seeds used in this study appeared to be relatively healthy without any shrinkage, discoloration and disease symptoms. There was, however, breakage of pericarp throughout the seeds (data not shown). Damage caused by breakage of the
protective outer seed layers can provide sites for fungal infection (Agarwal and Sinclair, 1996). Wheat seeds were infected with *Penicillium* sp. at mean infection rate of 83% as determined by incubation on Komada’s medium. *Penicillium* sp. was detected from endosperm with bran but not from embryo indicating that *Penicillium* sp. mainly existed near the seed surface (Fig. 1).

ClO\textsubscript{2} has received attention as a disinfectant for post-harvest fungal pathogens because of its stable efficacy and oxidizing power (Beuchat, 1998). Gaseous ClO\textsubscript{2} is expected to be more advantageous to disinfection of dry seeds because aqueous ClO\textsubscript{2} would increase the SMC, which may promote fungal growth and may reduce seed viability during storage. Gaseous ClO\textsubscript{2} inhibited growth of *Penicillium* sp. isolated from wheat seeds on PDA as the ClO\textsubscript{2} concentration increased from 5 to 20 μg/ml (Fig. 2). When treatment time was extended from 1 to 3 h, growth of *Penicillium* sp. was completely suppressed even at 10 μg/ml. Gaseous ClO\textsubscript{2} treatment was found to effectively suppress growth of *Penicillium* sp. *in vitro*. Extending treatment time was more effective for inhibition than increasing ClO\textsubscript{2} concentration.

Moisture content around seeds during ClO\textsubscript{2} treatment was controlled by adjusting RH in the treatment chamber. Dry seeds were exposed to 20 μg/ml for 10 h at 30, 50, 70 and 80% RH (Table 1). There was no significant reduction in the incidence of *Penicillium* sp. at 30% RH. However, reduction in the incidence of *Penicillium* sp. to 27.7% was observed at 50% RH, further reduction to 3.5% and 0.2% was accomplished at 70% and 80% RH, respectively. Seed germination was not affected by ClO\textsubscript{2} treatment at all the RH conditions. A high RH around the seed surface increased the effectiveness of ClO\textsubscript{2}. Based on the result, a RH higher than 70% is recommended. Moisture content in the dry seeds was increased to 30.0% by soaking in the water.

![Fig. 1. Detection of *Penicillium* sp. from whole wheat seed (left), endosperm and bran (middle) and embryo (right). Each component was placed on PDA medium and incubated at 25°C for 7 days under alternating 12-h near UV and 12-h dark.](image1)

| No treatment | Concentration of ClO\textsubscript{2} and treatment time | RH during ClO\textsubscript{2} treatment (%)* |
|-------------|------------------------------------------------|---------------------------------|
|             | 5 ppm, 1 h | 10 ppm, 1 h | 15 ppm, 1 h | 20 ppm, 1 h | 10 ppm, 3 h |
|             | 30 | 50 | 70 | 80 |
| Incidence of *Penicillium* sp. (%)\textsuperscript{a} | 47.0 a | 47.5 a | 27.7 b | 3.5 c | 0.2 c |
| Seed germination (%)\textsuperscript{a} | 42.2 a | 43.8 a | 42.0 a | 42.8 a | 43.7 a |

\textsuperscript{a} Each experiment was replicated three times and each value represents the mean. Means with the same letter did not differ significantly (p ≤ 0.05).

\textsuperscript{b} Dry seeds were treated with 20 μg/ml of ClO\textsubscript{2} for 10 h at each RH.
incidence of *Penicillium* sp. in dry seeds with 9.7% SMC did not show any reduction when treated at 5 and 10 μg/ml at 50% RH, however, it tended to decrease as ClO$_2$ concentration increased to 20 μg/ml (Table 2). Water-soaked seeds showed a drastic reduction in the incidence of *Penicillium* sp. when treated at more than 10 μg/ml. The incidences of *Penicillium* sp. were 3.3, 1.8 and 1.2% at 10, 15 and 20 μg/ml, respectively. It should be noted that the incidence of *Penicillium* sp. was reduced to 38.8% in water-soaked seed even without ClO$_2$ treatment, which was believed to be a result of secession of bran containing conidia or mycelia of *Penicillium* sp. during 24-h soaking. Seed germination was not affected by ClO$_2$ treatment, regardless of concentration. Increasing water content around or inside of the seeds during ClO$_2$ treatment strongly increased the effectiveness of disinfection. It is possible that water replaced the air between endosperm and bran of the seeds, which promoted diffusion of ClO$_2$, and activated sleeping pathogen, causing the pathogen to become more susceptible to ClO$_2$ (Agarwal and Sinclair, 1996). Above all, water might serve a medium for ClO$_2$ action in the seed, where the pathogens existed.

Wheat is the main cereal crop as it provides 20% of all calories consumed by people worldwide and also makes a significant contribution to animal feed. Contamination of wheat seed with storage fungi including *Penicillium* sp. may cause problems. Production of mycotoxins and loss of nutritive value by storage fungi are harmful or disadvantage for human and animal feed consumption. Deterioration of seeds by storage fungi becomes problematic for cultivation because deteriorated seeds may have the potential for low germinability and production of abnormal seedlings. The FDA has permitted the use of ClO$_2$ for disinfecting meat, poultry, fruit and vegetables for human consumption in an amount not to exceed 5 μg/ml residual ClO$_2$ for uncut fruits and vegetables. Our results indicated that there was a statistically significant effect of gaseous ClO$_2$ on disinfection of *Penicillium* sp. in wheat seeds at sites where moisture existed (30% SMC or > 70% RH). Gaseous ClO$_2$ treatment is expected to be effective and safe method for disinfection of *Penicillium*-infected wheat seeds.

Further investigations should be conducted to ascertain the best conditions for complete disinfection of *Penicillium* sp. in wheat seeds. We need to ascertain that ClO$_2$ treatment affect seed germination with highly germinable seed material. Once these problems are addressed, gaseous ClO$_2$ treatment can be applied to disinfection of *Penicillium*-infected wheat seeds as an effective but less toxic method.

### Table 2. Incidence of *Penicillium* sp. and seed germination in dry and soaked wheat seeds after ClO$_2$ treatment

| Seed | Concentration of ClO$_2$ (μg/ml) | 0 | 5 | 10 | 15 | 20 |
|------|---------------------------------|---|---|----|----|----|
| Incidence of *Penicillium* sp. (%) | Dry | 47.5 a | 42.5 b | 44.2 b | 34.8 d | 24.7 e |
| | Soaked | 38.8 c | 21.0 f | 3.3 g | 1.8 g | 1.2 g |
| Seed germination (%) | Dry | 43.7 a | 41.0 a | 43.0 a | 42.3 a | 42.8 a |
| | Soaked | 43.5 a | 41.5 a | 41.3 a | 42.2 a | 42.5 a |

*Each experiment was replicated three times and each value represents the mean. Means with the same letter did not differ significantly (p ≤ 0.05).*

*Seed moisture contents of dry and soaked seeds were 9.7% and 30%, respectively.*

*Seeds were treated with each concentration of ClO$_2$ for 6 h under 50 ± 2% RH.*

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