Effectiveness of Chlorine Dioxide as Influenced by Concentration, pH, and Exposure Time on Spore Germination of *Botrytis cinerea, Penicillium expansum and Rhizopus stolonifer*

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Abstract

J.P. Zoffoli, B.A. Latorre, N. Daire, and S. Viertel. Effectiveness of chlorine dioxide as influenced by concentration, pH, and exposure time on spore germination of *Botrytis cinerea, Penicillium expansum* and *Rhizopus stolonifer*. Chlorine dioxide (ClO_2) is an alternative to hypochlorites (NaOCl, $Ca(OCl)_2$) for fruit and vegetable sanitization to reduce postharvest decays caused, among other fungi, by *Botrytis cinerea*, *Penicillium expansum* and *Rhizopus stolonifer*. Due to economical reasons and because of its explosiveness, the use of chlorine dioxide has been relatively limited. However, the development of stabilized commercial formulations has allowed to reintroduce it. In this study, the efficacy of a new stabilized chlorine dioxide formulation (Tecsa®Clor, Protecsa S.A., Santiago,Chile) to inhibit conidial germination of *B. cinerea* and *P. expansum*, and sporangiospore germination of *R. stolonifer* was demonstrated. The efficacy was dependent primarily on concentration and exposure time but it was also affected by pH. Conidia germination of *B. cinerea* was inhibited over 98% at concentrations higher than 75 or at 25 $\mu g \cdot mL^{-1}$ after 1 or 30 min of contact, respectively. Similarly, over 90% inhibition of conidial germination of *P. expansum* was achieved with 100 and 25 $\mu g \cdot mL^{-1}$ after 1 and 30 min, respectively. Sporangiospores of *R. stolonifer* were inhibited by 90% at 100 $\mu g \cdot mL^{-1}$ for 30 min. The pH of the solution increased as chlorine dioxide concentration increased, and the biological activity decreased considerably at pH higher than 8, after 5 and 15 min of contact. No phytotoxic effects were obtained on pear cv. Packham's Triumph even at 1000 $\mu g \cdot mL^{-1}$ after 20-day-exposure at 20 ºC. Therefore the stabilized commercial formulation of chlorine dioxide is an alternative to chlorinate water to treat fruits by immersion in order to control *B. cinerea, P. expansum* and *R. stolonifer* during postharvest. For a complete control, concentrations higher than 75 $\mu g \cdot mL^{-1}$ should be used at pH between 7 and 8.

Key words: Apple, citrus, peach, pear, postharvest diseases, sweet cherry.

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INTRODUCTION

Postharvest diseases are very important for a successful commercialization of the Chilean fruit at the international markets. Prolonged transportation time to destination ports, as well as the need to storage the fruit for as long as possible,

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favor the development of diseases, allowing the development of soft rots on pome, stone fruit and other fruits caused by *Botrytis cinerea* Pers., *Penicillium expansum* Link and *Rhizopus stolonifer* (Ehrenb.) Vuill (Latorre, 2004; Ogawa and English, 1991).

The inoculum sources are commonly found in the orchard, and eventually it is carried on infected fruits, contaminating the water used in the hydrocooler, dumpers, washing area or along the fruit packaging line conveyors. Therefore, water sanitization is a required strategy of control. Consequently, treatment of the fruit by immersion in a chlorinated solution with hypochlorites (NaOCl, $Ca(OCl)_2$) has been widely used (Segall, 1968, Spotts and Peters, 1980, Zoffoli *et al*., 1996). Sodium and calcium hypochlorites dissociate in water, and depending on the pH of the solution hypochlorous acid (HOCl), hypochloric acid (HCl) and chlorine gas (Cl_2) are formed, but the hypochlorous acid is the biologically most active form. Additionally, the use of chlorine dioxide (ClO_2) has been suggested (EPA, 1999). Although this gas is soluble and stable in water is highly explosive under pressure. This forces its production *in-situ* mixing sodium hypochlorite and hydrochloric acid (HCl) or, sulfuric acid (H_2SO_4) under controlled conditions. Because of this and the relative high cost of production, chlorine dioxide has been difficult to market and it has limited the use for water sanitation treatment on fruits, and vegetables. However, the development of stabilized formulations have enabled the reintroduction of chlorine dioxide for this purpose (EPA, 1999).

Chlorine dioxide exhibits a wide biocide action, being especially useful as a fungicide and bactericide (Benarde, *et al*., 1965; Du *et al*., 2003; EPA, 1999; Spotts and Peters, 1980). It acts by direct contact, as a superficial disinfectant, but it lacks a residual effect that allows fruit protection during transport and storage. The ClO_2 is a very reactive molecule, acts as an oxidant, and it is very effective at low concentrations in a range of pH 5 to 10. It has multiple sites of biochemical activity in microorganisms, interfering in the activity of some proteins, RNA and affecting the function of cell membranes. It is more stable than other chlorinated compounds in aqueous solution, slightly fixated by organic matter, scarcely corrosive and free of undesirable odors. The objective of this research was to study the effectiveness of a new stabilized formulation of chlorine dioxide, on the germination of spores of *B. cinerea*, *P. expansum*, and *R. stolonifer*; fungi commonly found as contaminants on fruit dumpers and other postharvest areas.

MATERIALS AND METHODS

Inoculum. Conidia of *B. cinerea* and *P. expansum* and sporangiospores of *R. stolonifer*, were obtained from 7- to 15 day old cultures, in potato dextrose agar, acidulated with 0.5 $mL \cdot L^{-1}$ (APDA), incubated at 20 ºC. Spores were suspended in sterile distilled water, filtered through fiberglass and agitated for 3 min before adjusting the final concentration to $10^6 \cdot mL^{-1}$ spores.

Chlorine dioxide. In this study chlorine dioxide (ClO_2) formulated as Tecsa®Clor (Protecsa S.A., Santiago, Chile) was used. It contains 5% (w/w) of stabilized ClO_2, free of chlorine or other chlorite compounds. Different concentrations of ClO_2 were obtained and used the same day by diluting the formulated product with distilled water, free of chlorine sources. The exact concentration of ClO_2 in the solution was determined by titration with

0.1% ferrous ammonium sulfate following the method proposed by the Environmental Protection Agency (EPA, USA),described by the APHA (1998), using N,N-diethyl-p-phenylendiamine (DPD) as indicator.

Effect of the concentration and exposure time. The effect of the exposure time and concentration of ClO_2 on spore germination were studied at 10 ºC, using 0, 10, 25, 50, 75 and 100 $\mu g \cdot mL^{-1}$ of ClO_2. After exposing the spores to each concentration for 1, 5, 10, 15, or 30 min, 100 µL of each spore suspension was plated in 2% water agar and incubated for 18 h at 20°C. Spore germination was determined in 100 spores per each of four replicates. Only those spores with a germinating tube equal or greater than the diameter of the spore were considered germinated. These results were expressed as the percentage of spores germinated in relation to the total number of spores of each replication. This study was repeated one time.

Effect of pH. The effectiveness of chlorine dioxide concentration (50 $\mu g \cdot mL^{-1}$), was studied at pH 7, 8 and 10 in aqueous solution of sodium phosphate buffer. The sodium phosphate buffer was prepared from different proportions of 0.1 M Na_2HPO_4 and 0.1 M NaH_2PO_4 stock solutions. Spores germination were determined after the ClO_2 application, considering a timing of 5 or 15 min following the germination period as was describe below.

Phytotoxicity. The possible side effects of chlorine dioxide on the fruit skin was studied on pears cv. Packham's Triumph, (firmness: 16.2 lb., soluble solids: 13.8% and titratable acidity: 0.21%) using 50, 150, 300, 500, 800 and 1000 $\mu g \cdot mL^{-1}$ of chlorine dioxide solutions. Fruits at 0°C were exposed for 10 min to each concentration and then they were stored for 7 days at 20°C before evaluating the external damages. Under any abnormal situation the evaluation was extended under the same conditions for 20 more days.

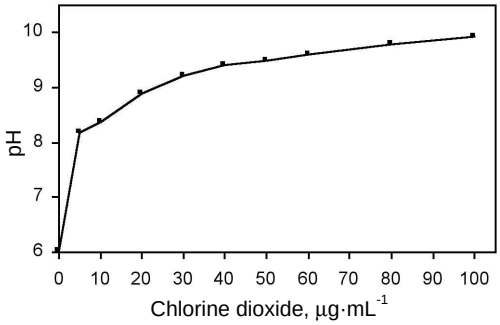

Figure 1. The effect concentration on pH of an aqueous solution of chlorine dioxide (Tecsa®Clor).

Design and statistical analysis. Concentration and exposure time effect of ClO_2 were randomly distributed and analyzed for variance according to a 3x5x6 factorial model SigmaStat (Systat Software Inc., Richmond, California, USA). Pathogen was considered the main factor, and the time of exposure and concentration as sub-factors. All additional experiments were designed randomly with four replications, and means were separated according to the Duncan-Waller k ratio t-test. For the statistic analysis percentages were transformed to arcsine $\sqrt{x}$.

RESULTS

Effect of concentration and exposure time. Spore germination of *B. cinerea*, *R. stolonifer* and *P. expansum* varied between 0 and 100 % in function of the pathogen, concentration and exposure time (Table 1). There was a statistically significant effect ($p < 0.001$) for the pathogen factor (P), exposure time (Te) and concentration (C). Likewise, the interactions PxTe, PxC, TexC and PxTexC were highly significant ($p < 0.001$) (Table 2). Under these experimental conditions, the pH varied

from 8 to 10 when the concentrations increased from 10 to 100 $\mu g \cdot mL^{-1}$ (Figure 1). Concentrations equal or higher than 75 $\mu g \cdot mL^{-1}$ completely arrested the germination of conidia of *B. cinerea*. However, similar results were not achieved, even at 100 $\mu g \cdot mL^{-1}$ of chlorine dioxide, with conidia of *P. expansum* and sporangiospores of *R. stolonifer* in this case, the germination obtained was 9 and 2.4% respectively after 30 min of exposure (Table 1).

Table 1. Effect of concentration and exposure time on conidial germination of *Botrytis cinerea* and *Penicillium expansum* and sporangiospores of *Rhizopus stolonifer.*

Chlorine dioxide	Spore germination (%) after (min)[1]				
($\mu L \cdot mL^{-1}$)	1	5	10	15	30
			Botrytis cinerea		
0	100.0	100.0	100.0	100.0	100.0
10	100.0	98.0	91.0	98.6	99.5
25	100.0	100.0	90.4	87.8	0.9
50	79.1	55.5	47.7	55.1	0.9
75	2.0	0.9	1.4	nd	0.0
100	0.3	0.0	0.0	0.0	0.0
			Penicillium expansum		
0	100.0	100.0	100.0	100.0	100.0
10	nd	90.2	68.5	36.0	19.2
25	24.4	nd	24.0	8.2	3.1
50	37.9	45.9	10.7	15.1	7.1
75	43.7	45.4	18.8	6.8	0.5
100	6.9	12.3	5.1	7.8	9.0
			Rhizopus stolonifer		
0	100.0	100.0	100.0	100.0	100.0
10	100.0	100.0	99.4	98.1	nd
25	100.0	96.3	100.0	96.3	72.6
50	100.0	80.9	100.0	92.1	87.2
75	91.0	91.5	83.8	38.7	17.6
100	100.0	87.3	65.8	20.9	2.4

[1]Percentage of spore germination relative to untreated control. Spore germination without chlorine dioxide was 90, 85 and 65% for *B. cinerea*, *R. stolonifer* and *P. expansum* respectively. nd = not determined.

Effect of pH. The effectiveness of chlorine dioxide depended on the final pH of the solution, decreasing considerably as the pH increased between 7 and 10, maintaining a constant concentration of 50 $\mu g \cdot mL^{-1}$ (Figura 2). For example, in 5 min of exposure to 50 $\mu g \cdot mL^{-1}$ of chlorine dioxide at pH 7 and 8 there was 100% inhibition of conidial germination of *B. cinerea*, while under the same conditions at pH 10 only 38 % inhibition was obtained. Similarly, a complete inhibition of sporangiospores of *R. stolonifer* and conidia of *P. expansum* was obtained at pH 7 but spore germination was only partially arrested at pH 10, with 62.1 and 78.3 % germination inhibition, respectively, after 5 min of exposure to chlorine dioxide (Figura 2).

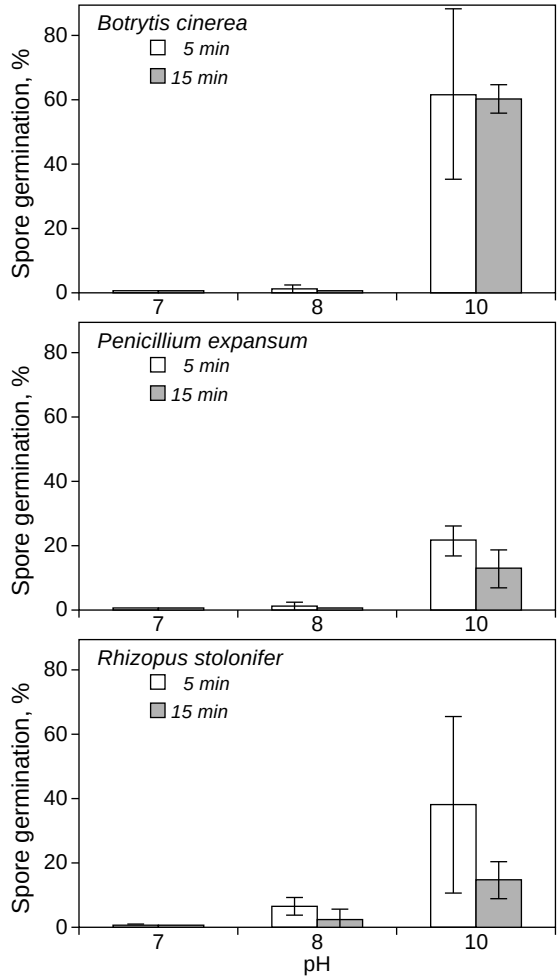

Figure 2. Effect of pH on the biological activity of an aqueous solution of chlorine dioxide (50 µL·mL^{-1}) determined at 10 ºC by the conidial germination of *Botrytis cinerea* and *Penicillium expansum*, and sporangiospores of *Rhizopus stolonifer*, after 5 and 15 min of contact. Means of 4 replication of 100 spores. Bars = standard deviation.

Table 2. Analysis of variance for the effect of pathogen, incubation time and concentration on the effectiveness of chlorine dioxide to inhibit conidial germination of *Botrytis cinerea* and *Penicillium expansum* and sporangiospores of *Rhizopus stolonifer.*

Source of variation	df	MS	F	*p*
Pathogen (P)	2	8,360	1,485,402	<0.001
Time of incubation (Te)	4	1,990	353,654	<0.001
Concentration (C)	5	11,166	1,083,993	<0.001
P x Te	8	0,238	42,212	<0.001
P x C	10	1.175	208,778	<0.001
Te x C	20	0.156	27,806	<0.001
P x Te x C	40	0.191	33,930	<0.001
Residual	245	0.00563		
Total				

[1] Data were transformed to arcsine√x before analysis.

Phytotoxicity. There were no signs of phytotoxicty associated to chlorine dioxide concentration between 50 and 1000 µg·mL^{-1}. However, deposits of salts were visible on the surface of the treated fruits without any direct damage even after 20 days at 20°C.

DISCUSSION

Sodium and calcium hypochlorite, and other compounds that dissociate into hypochlorous acid in water, have been extensively used for postharvest sanitization of fruits and vegetables. A concentration of 100 µg·mL^{-1} of free chlorine at pH 7 has been

recommended (Roberts and Reymond, 1994; Spotts and Peters, 1980; Zoffoli *et al*. 1996). However, its use has been questioned because of the collateral effect on irritation, corrosive action, loss of chlorine by volatilization and fixation with organic matter. Additionally, the biological activity decreases considerable above pH 7. Considering these aspects, numerous endeavors have been undertaken to find alternatives such as the chlorine dioxide (EPA, 1999; Robert and Reymond, 1994; Spotts and Peters, 1980).

The oxidating activity is the main property of the chlorine dioxide when it is reduced to chlorite ion (ClO_2^-), having a pka of 1.8, that it is considered relatively low, maintain its biological activity within a broad of pH range. However, according to our results the efficacy of the formulated and stabilized chlorine dioxide solution, on conidial germination of *B. cinerea* and *P. expansum* and sporangiospores germination of *R. stolonifer*, depended on the concentration, exposure time and pH of the solution.

The optimal relationship between concentration and exposure time for obtaining a complete inhibition of conidial germination of *B. cinerea* and *P. expansum* was achieved with 75 $\mu g \cdot mL^{-1}$ for 1 min and 30 min, respectively. Similarly, the sporangiospores of *R. stolonifer* required 100 $\mu g \cdot mL^{-1}$ for 30 min to completely arrest germination. These values differ from the 50 $\mu g \cdot mL^{-1}$ for 1 min reported for a complete control of conidia of *Monilia laxa* in nectarines (Mari *et al.*, 1999), or 10 $\mu g \cdot mL^{-1}$ for 0.5 min reported for a complete inhibition of spore germination on *B. cinerea*, *P. expansum,* and *Mucor piriformis* (Spotts and Peters, 1980). Robert and Reymond (1994) found 99% mortality in solutions of 3-5 $\mu g \cdot mL^{-1}$ with 1 min of exposure for these same pathogens.

The lower efficacy of the formulation of chlorine dioxide evaluated in this study, as compared with the efficacy of chlorine dioxide produced *in situ* (NaOCl and HCl) (Robert and Reymond, 1994, Spotts and Peters, 1994), was mainly attributed to the alkaline pH obtained with 50 $\mu g \cdot mL^{-1}$ of the commercial formulation of chlorine used in this study. Actually, the pH increased as the concentration of chlorine dioxide increased, whereas the effectiveness decreased (Tables 1 and 2). Thus requiring longer exposure time or higher concentrations to obtain a similar effect. Hence, the recommended use of the stabilized formulation of chlorine dioxide evaluated in this study should require the eventual adjustment of the pH in order to optimize the sanitization capability of this chlorine dioxide formulation.

According to the results of this study, chlorine dioxide is an alternative to hypochlorites for sanitization of pears and possibly for other fruits, enabling to reduce the inoculum potential by reducing spore germination of the main plant pathogenic fungi associated to postharvest decay.

RESUMEN

El dióxido de cloro (ClO_2) es una alternativa al uso de hipocloritos (NaOCl, $Ca(OCl)_2$) útil para la sanitización de la fruta en poscosecha y reducir el riesgo de pudriciones causadas entre otros por *Botrytis cinerea*, *Penicillium expansum* o *Rhizopus stolonifer*. Razones económicas y la explosividad de este gas al mantenerlo bajo presión han limitado su uso. Sin embargo, el desarrollo de formulaciones comerciales, estabilizadas, ha permitido reintroducir el dióxido de cloro con estos propósitos. En este trabajo se demostró la eficacia de este compuesto, formulado y estabilizado (Tecsa®Clor, Protecsa S.A., Santiago,Chile), en el control de la germinación de conidias de *B. cinerea* y *P. expansum* y esporangiosporas de *R.*

stolonifer. Sin embargo, la efectividad de este producto dependió primeramente de la concentración y del tiempo de exposición, pero también tuvo importancia en la mortalidad de las esporas el pH de la solución. Una concentración de dióxido de cloro > 75 o 25 $\mu g \cdot mL^{-1}$ 1 o 30 min de exposición, respectivamente, inhibió en más de 98% la germinación de conidias de *B. cinerea*. En forma similar, se obtuvo sobre un 90% de inhibición de la germinación de conidias de *P. expansum* con 100 y 25 $\mu g \cdot mL^{-1}$ por 1 y 30 min, respectivamente. La germinación de esporangiosporas de *R. stolonifer* se inhibió por sobre un 90% únicamente con 100 $\mu g \cdot mL^{-1}$ por 30 min. El pH de la solución acuosa de dióxido de cloro aumentó considerablemente con la concentración, disminuyendo la efectividad con pH superior a 8, tanto luego de 5 como 15 min de exposición. No hubo efectos fitotóxicos en peras cv. Packham's Triumph aun expuestas a concentración de 1000 $\mu g \cdot mL^{-1}$, luego de 20 días a 20 ºC. Por lo tanto, el dióxido de cloro formulado comercialmente es una alternativa para el control de *B. cinerea, P. expansum* y *R. stolonifer* en poscosecha. Sin embargo, se recomienda corregir la dosis en función de los patógenos predominantes y verificar el pH de la solución de modo que esta permanezca entre 7 y 8.

Palabras clave: Cerezo, cítricos, duraznero, enfermedades de poscosecha, manzano, peral.

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