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Full Length Research Paper

Ozone to control *Rhyzopertha dominica* (Coleoptera: Bostrichidae) in stored wheat grains

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Alternatives for the control of insect pests in grain processing and storage units are necessary to maintain the characteristics of the product and decrease environmental impact. Although the toxic effects of ozone on some insect pests of stored products are already known, there is little information about their effects inside the grain column. This study aimed to evaluate *Rhyzopertha dominica* (Fabr.) (Coleoptera: Bostrichidae) control by ozone gas in wheat grains. Ozone was applied at a concentration of 1.61 mg L^{-1} and flow rate of 2.0 L min^{-1} in wheat grains. Inside the grain column, insects were placed in plastic cages distributed at three different points: on the plenum (basis), middle part and surface. The toxicity results indicated that the periods of ozone exposure that were necessary to cause mortalities of 50 and 95% in adult *R. dominica* ranged from 8.69 to 13.08 h and from 11.28 to 18.11 h, respectively. Shorter lethal times were observed when the insects were distributed on the basis of the grain column. A decreased instantaneous growth rate of *R. dominica* was observed with an increased exposure period. There was a significant correlation between the instantaneous growth rate and the physical and physiological characteristics of wheat grain.

Key words: Storage, insecticidal fumigant, toxicity, instantaneous growth rate, ozone, wheat grains.

INTRODUCTION

Rhyzopertha dominica (Fabr.) (Coleoptera: Bostrichidae) is known as the lesser grain borer. Because pesticides are effective, inexpensive and easy to handle, they are the most widely used form of control of stored-product pests (González et al., 2014). However, the continuous and indiscriminate use of pesticides over time has favored the development of insect populations that are resistant to these products (Guedes et al., 1994) and damaged the environment and human health (Stoppelli and Magalhães, 2005). Thus, it is necessary to develop research aimed at obtaining alternative methods for the control of insect pests of stored grain. Ozone gas (O_3)

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may be an alternative to pesticides used in stored grain, as it is highly toxic to fungi, bacteria, viruses, protozoa and insects (Isikber and Athanassiou, 2015; Kells et al., 2001; Mendez et al., 2003). Ozone affects living organisms by damaging cell membranes or triggering cell death by oxidative stress (Hollingsworth and Armstrong, 2005).

The use of ozone gas in the control of stored-grain insects is an attractive alternative for several reasons: It may be generated at the site of use; it self decomposes to molecular oxygen (McDonough et al., 2011; Mylona et al., 2014; Savi et al., 2015), which prevents the need to store and dispose of hazardous chemicals; there is no need for aeration to remove the gas after application (Isikber and Athanassiou, 2015); and it is classification as “GRAS” (Generally Recognized As Safe) by the United States Environmental Protection Agency (USEPA) (Isikber and Athanassiou, 2015).

Although there is knowledge of ozone’s potential as an insecticidal fumigant in the control of insect pests of stored-products, there is little information about its use for insect control within a column of grains or its effect on the growth of insect populations that are exposed to different lethal times. The present work evaluated ozone toxicity (including the effects on population growth rates) on R. dominica within a column of wheat, and determined the effect of population growth rates on the physical and physiological characteristics of wheat grain.

MATERIALS AND METHODS

Wheat samples were collected from an experimental field at Cooperativa Agropecuária do Alto Paranaíba (Coopadap), located in São Gotardo (MG), Brazil. We used wheat grains with a moisture content of 11.1%, hectoliter weight of 76.6 kg hL^{-1}, electrical conductivity of 21.4 μS cm^{-1} g^{-1} and initial germination percentage of 91.5%.

Insects

The insects were maintained in glass containers in rearing facilities under controlled conditions (30 ± 2°C and 70 ± 5% relative humidity, with a photoperiod of 14 h: 10 h dark:light [D:L]) and reared on insecticide-free wheat grains.

Ozone generation and system operation

Ozone gas was produced using an ozone generator (model O&L 10.0RM, Ozone & Life, São José dos Campos, SP, Brazil). The ozone generator had two outputs, one that released the generated ozone and the other that obtained atmospheric air by an air compressor coupled to the equipment. Ozone and atmospheric air (control) were distributed evenly to the cylindrical chambers where grains and cages containing grains and insects were placed. The residual ozone was treated in a column containing KI solution. The ozone concentration was quantified using the iodometric method, by indirect titration (APHA, 1981) as recommended by the International Ozone Association (IOA).

Ozone toxicity

The toxicity of ozone to *R. dominica* was determined by estimating the lethal times of exposure for 50 and 95% of adult insects (LT_{50} and LT_{95}, respectively). The time-response curves were established by bioassays with increasing periods of exposure to ozone gas.

Wheat grain samples of 7.0 kg were ozonated inside cylindrical chambers of PVC (20 cm in diameter × 100 cm in height) with connections at the bottom and top for the input and output of ozone, respectively. Each chamber received a metal screen 10 cm from the bottom to support the grain, forming a plenum for the better distribution of the gas. Ozone was applied at a concentration of 1.61 mg L^{-1} and a continuous flow rate of 2 L min^{-1}. The tests were conducted under constant temperature (27 ± 2°C) and relative humidity (70 ± 5%).

Twenty non-sexed adult insects, aged from one to four weeks, were placed in plastic cages (10.5 cm in diameter × 9.0 cm in height) containing 400 g of wheat. The cages were distributed within the grain column at three distinct points: the bottom (on the plenum), in the middle and at the surface. The bottom and the cover of the cages were made of organza fabric to permit the free passage of gas through the grain column.

The experiments were carried out in three replicates for each period of exposure to ozone gas. The same system was used providing only atmospheric air to enable the estimation of natural mortality (control). Insect mortality was assessed 48 h after each exposure period.

Instantaneous growth rate (*r_i*)

The instantaneous growth rate (r_i) was determined using 50 non-sexed adult insects, aged from one to four weeks, that were kept in plastic cages containing 400 g of wheat. The cages were distributed inside the grain column at three different points (on the plenum, in the middle and at the surface). The grains and insects were exposed to ozone gas and atmospheric air (control) over five periods corresponding to exposure to the lethal-time (LT) that was obtained in bioassay toxicity (Table 1). Three replicates were conducted for each treatment and exposure period. After exposure to ozone and atmospheric air, the wheat grain column containing the insects was removed from the cages and transferred to glass vials of 0.8 L.

The bottles were placed in the climate chambers, under constant temperature (32 ± 2°C) and relative humidity (70 ± 5%) and 24 h scotophase. The adult progeny were recorded after 60 days of storage. The instantaneous rate of increase was calculated using Equation 1.

$$r_i=[\ln(Nf/ Ni)] /\Delta T \quad (1)$$

where Nf and Ni are the final and initial numbers of live (adult) insects, respectively, and ΔT is the duration of the experiment in days (Walthall and Stark, 1997).

Effect of ozone on wheat grain physical and physiological characteristics

Wheat grains (ozone and atmospheric air treatments) used for the instantaneous growth rate bioassays from the surface layer was used to evaluate the effect of ozone on the physical and physiological characteristics of the grains. Three replicates were used for each treatment (ozone and atmospheric air) and exposure period (10.11, 11.76, 13.05, 14.49 and 16.85 h).

The oven method was used to determine the wheat moisture content at 103 ± 1°C for 19 h, as recommended by the ASAE (2000). The hectoliter weight was determined using a balance (Hectoliter, Balanças Dalle Molle Ltda), according to the

Table 1. Periods corresponding to exposure to the lethal-time (LT) obtained in bioassay toxicity.

LT	Time (h)		
	Plenum	Middle layer	Surface layer
LT_{10}	7.10	7.67	10.11
LT_{30}	8.00	9.36	11.76
LT_{50}	8.69	10.35	13.05
LT_{70}	9.45	12.34	14.49
LT_{90}	10.65	15.06	16.85

Table 2. Relative toxicity of ozone to *rhyzopertha dominica*.

Position	Slope ± s.e.	lt_{50} (95% ci) h	lt_{95} (95% ci) h	χ^2	p-value
Basis	14.56 ± 1.28	8.69 (8.46-8.93) [a]	11.28 (10.83-11.92) [a]	1.8	0.77
Middle	8.75 ± 1.25	10.75 (9.76-11.71) [b]	16.57 (14.48-22.04) [b]	8.85	0.65
Surface	11.56 ± 1.03	13.08 (12.57-13.51) [c]	18.11 (17.18-19.43) [b]	3.13	0.54

Lethal time ($lt_{50, 95}$) followed by the same letters in the same column are not significantly different based on the 95% confidence interval (ci) overlap (manonmani et al., 2011).

methodology described by Regras para Análise de Sementes (MAPA, 2009). The electrical conductivity of the solution containing wheat was measured using the conductivity mass and 50 grains that had been immersed in distilled water for 24 h (Vieira, 1994). The germination potential was determined by a germination test (MAPA, 2009), using 50 seeds per treatment.

Experimental design and statistical analysis

The r_i experiment and analysis of the physical and physiological characteristics of wheat grains were conducted in a split plot scheme, with the treatments as plots (atmospheric air and ozone) and the periods of exposure as subplots, arranged in a completely randomized design. The data were assessed by an analysis of variance and regression. For the qualitative factor, the means were compared using Tukey’s test at a 5% probability. For the quantitative factor, the models were chosen based on the significance of the regression coefficients using Student's t-test at a 5% probability, the coefficient of determination (R2) and biological phenomena. Regardless whether the higher degree interaction be or non-meaningful, we decided to investigate it further due to the interest of our study. The SAS software system (SAS Institute) was used for the analysis of variance. The SigmaPlot software system (SPSS version 7.0) was used to obtain the the regression equation and to plot the graphs.

Pearson's linear correlation coefficient was estimated between the instantaneous growth rate of insects and the moisture content, hectoliter weight, germination percentage and electrical conductivity of wheat grain. The correlation between these variables was tested by Student’s t-test at a 1% probability, using the SAEG software system (version 9.0). The toxicity data were subjected to probit analysis using the PROBIT procedure in the SAS software system (SAS Institute 1999), which generated the time-mortality curves.

RESULTS

Ozone toxicity

The results of the time-mortality bioassays for the three layers of the wheat grain column in which the insects were distributed (based on the plenum, middle and surface layer) showed low chi-square values ($\chi 2$) (<9.00) and high *P* values (>0.05), indicating the adequacy of the data probit model to estimate the time-mortality curves (Table 2). This table provides the estimates of the lethal times for the three layers of the wheat grain column in which the insects were distributed.

The ozone exposure times required to cause 50% mortality (LT_{50}) and 95% mortality (LT_{95}) in *R. dominica* adults, increased as the distance between the cages containing the insects and the ozone injection point increased. There was no overlap in the LT_{50} confidence intervals between the three layers in which the insects were distributed (Manonmani et al., 2011). For LT_{95}, there were overlapping confidence intervals between the middle layer and the top layer. The slope of the time-mortality curves ranged between layers; it was lower (8.75 ± 1.25) in the middle layer and higher (14.56 ± 1.28) in the basal layer.

Instantaneous growth rate (r_i)

The instantaneous growth rate of *R. dominica*, after 60 days of storage did not change significantly (*P*>0.05) between the layers of the wheat grain column in which the insects were distributed, regardless of the lethal-times for both wheat grains that were exposed to ozone gas and wheat grains that were exposed to the air (control) (Table 3). Given the above, the regression lines describing the growth rate behavior of *R. dominica* represent only the wheat grains of the surface layer (Figure 1).

The instantaneous rate of increase of *R. dominica* was

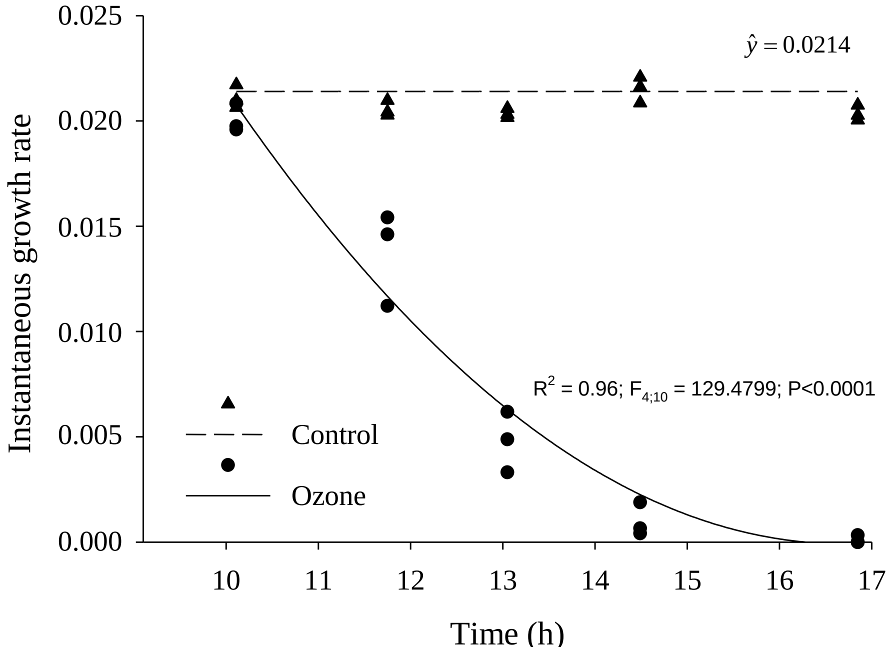

Figure 1. Instantaneous growth rate of *Rhyzopertha dominica* exposed to ozone gas and air (control) treatments, according to the lethal time, at a concentration of 1.61 mg L^{-1} after 60 days of storage.

significantly influenced (P<0.05) by the treatment with ozone gas in all periods of exposure and layers, except for LT_{10}, which was statistically equal to the control (Table 3). A reduced r_i of *R. dominica* treated with ozone gas was observed in the three layers after 60 days of storage. The instantaneous growth rate values of *R. dominica* located in the surface layer exposed to treatments with ozone and atmospheric air (control), at five lethal-times (LT_{10} = 10.11, LT_{30} = 11.76, LT_{50} = 13.05, LT_{70} = 14.49 and LT_{90} = 16.85 h), after 60 days of storage is illustrated in Figure 1. The r_i of *R. dominica* decreased as the exposure time of the wheat grain ozone gas increased. In contrast, the atmospheric air (control) did not affect r_i of *R. dominica* in any of the exposure periods.

Physical and physiological characteristics of wheat grain after instantaneous growth rate

After 60 days of storage, the moisture content of wheat grain changed significantly (P<0.05) between treatments with atmospheric air and ozone gas at all periods of exposure, except for the period of 10.11 h (Figure 2a).

In addition, ozonated wheat grains presented a higher average moisture content (12.80%) in the 10.11 h exposure period compared to that in the other ozone exposure periods after 60 days of storage. In addition, there was a significant positive correlation between the instantaneous growth rate and the moisture content of wheat grains that were exposed to ozone gas, that is, the moisture content of wheat grains increased with the increasing instantaneous growth rate of *R. dominica* (Table 3). However, no significant correlation was observed between the instantaneous growth rate and the moisture content of wheat grains that were exposed to atmospheric air (control).

The mean values obtained in the hectoliter weight of wheat grains that were exposed to the air (control) were significantly lower (*P*<0.05) than those of the wheat grains exposed to ozone gas, except for the wheat grains that were exposed for 10.11 h (Figure 2b). The correlation between r_i and the hectoliter weight of *R. dominica* was significant and negative, indicating that the hectoliter weight of wheat grains decreases when infestation by these insects increases (Table 4). Compared to the control (air), there was no significant correlation between the hectoliter weight of wheat grain and the instantaneous growth rate.

The electrical conductivity of the solution containing the wheat grains that were exposed to the air (control) showed significantly higher mean values (*P*<0.05) compared to those of the wheat grains that were exposed to ozone gas in all of the exposure periods after 60 days of storage (Figure 2c). An exception was observed only in the exposure period of 10.11 h, in which a positive and significant correlation was detected between the electric conductivity containing wheat grains that were exposed to ozone gas and the r_i of *R. dominica* (Table 4). In the control treatment (atmospheric air), the correlation between the electrical conductivity of the solution

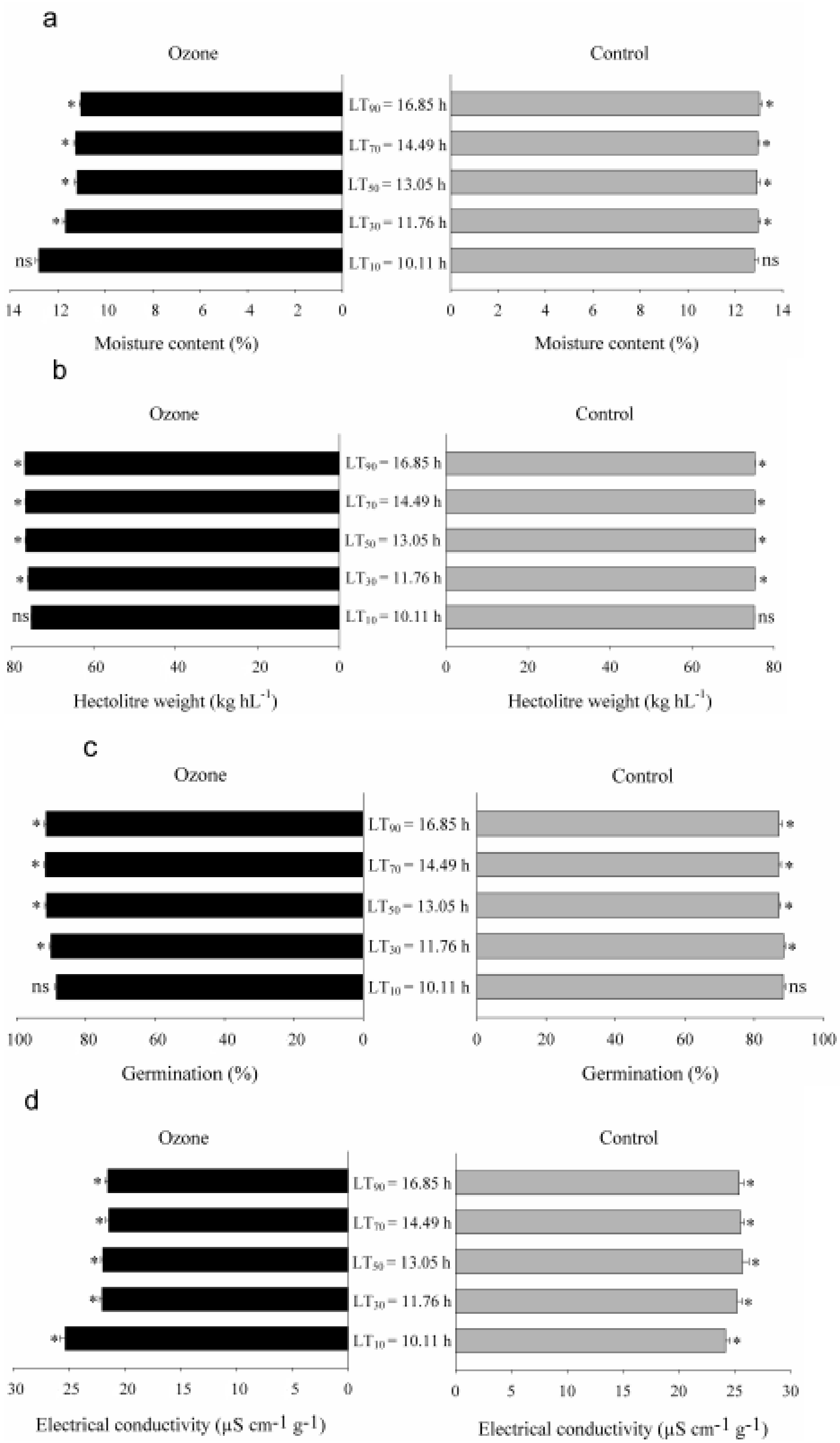

Figure 2. Moisture content (%) (a) hectoliter weight (kg hL^{-1}) (b), germination percentage (c) and electrical conductivity (µS cm^{-1} g^{-1}) (d) of wheat grain that were exposed to atmospheric air (control) and ozone gas (mean ± SE) at a concentration of 1.61 mg L^{-1} at different periods of exposure, after 60 days of storage. Means with asterisks indicate significant difference between treatments (ozone and control) by the F-test (P≤0.05).

Table 3. Mean values of the instantaneous growth rate of *Rhyzopertha dominica*, distributed over the plenum and in the middle and superficial layers of the wheat grain column exposed to ozone gas and atmospheric air (control) at a concentration of 1.61 mg L^{-1} at different lethal times (LT) after 60 days of storage.

Treatment	LT (h)	Instantaneous growth rate		
		Basis	Middle	Surface
Ozone	LT_{10}	0.018^{aA}*	0.017^{aB}	0.020^{aA}
	LT_{30}	0.013^{aB}	0.012^{aC}	0.012^{aB}
	LT_{50}	0.003^{aC}	0.004^{aD}	0.004^{aC}
	LT_{70}	0.001^{aCD}	0.003^{aD}	0.002^{aC}
	LT_{90}	0.000^{aD}	0.000^{aD}	0.000^{aC}
Control	LT_{10}	0.021^{aA}	0.022^{aA}	0.021^{aA}
	LT_{30}	0.021^{aA}	0.022^{aAB}	0.020^{aA}
	LT_{50}	0.022^{aA}	0.020^{aAB}	0.020^{aA}
	LT_{70}	0.022^{aA}	0.021^{aAB}	0.021^{aA}
	LT_{90}	0.021^{aA}	0.021^{aAB}	0.020^{aA}

*Means followed by the same lower case letters in a line and capital letters on the columns do not differ significantly by Tukey's test ($P<0.05$).

Table 4. Estimation of Pearson's linear correlation coefficients between the instantaneous growth rate of *Rhyzopertha dominica* and the moisture content, hectoliter weight, germination percentage and electrical conductivity of wheat grains that were exposed to ozone gas and atmospheric air (control) after 60 days of storage.

Correlations	Atmospheric air (control)		Ozone	
	n	r	n	r
IGR × MC	15	0.0488	15	0.9168**
IGR × HW	15	-0.3094	15	-0.9451**
IGR × GP	15	0.1140	15	-0.8836**
IGR × EC	15	-0.3310	15	0.8607**

IGR = instantaneous growth rate; MC = moisture content; HW = hectoliter weight; GP = germination percentage; EC = electrical conductivity; n = sample number; r = correlation coefficient; ** significant at 1% probability.

containing the wheat grains and r_i was not significant.

The percentage of germination of wheat grains was significantly smaller ($P<0.05$) when exposed to atmospheric air (control) than in the grains that were exposed to ozone at all periods of exposure after 60 days of storage (Figure 2d). The correlation between the percentage of germination of wheat grains that were exposed to ozone and the r_i of *R. dominica* was significant and negative, indicating that the percentage of germination of wheat grains decreases with the increasing infestation of *R. dominica* (Table 4). In the control treatment, no significant correlation was observed between the germination percentage of wheat grains that were exposed to the air and the r_i.

DISCUSSION

LT_{50} and LT_{95} increased as the cages containing insects and wheat grains distanced from the injection point of the ozone gas (surface > middle > on the plenum), which can be explained by the variation in the gas concentration along the grain column. The concentration of ozone in the grain column depends on the resistance that the grain offers to the passage of fluid.

The closer the ozone gas injection point is, the more lethal are the lower times, which can cause the mortality of *R. dominica.* This pattern was also observed by Rozado et al. (2008), who studied the effectiveness of ozone at a concentration of 50 mg kg-1 (equivalent to 0.11 mg L-1) on the control of adult *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). These authors found time-response values when the removed insects were in an upper position compared to the injection point. These authors obtained LT95 values of 23.76 and 64.19 h when insects were distributed on the plenum, 118.61 and 140.83 h when the insects were in the middle layer and 240.75 and 390.18 h when the insects were on the surface for *S. zeamais* and *T.*

castaneum, respectively. According to Isikber and Athanassiou (2015), the ozone concentration and exposure time of the gas play important roles in the efficacy of the product in the control of insect pests of stored products. The effect of ozone at lower concentration (35 ppm) to control pests on stored grains was evaluated by Hansen et al. (2012) leading to a 6 days period of time for total control of the pests. We evaluated that the use of ozone at higher concentration reduced the exposure time necessary for an effective control of the pests.

The instantaneous growth rate measures the ability of a population to increase exponentially in an environment. This rate can be considered a practical model, due to its fast results (Walthall and Stark, 1997).

As these authors increased the exposure period of the insects to ozone gas, the instantaneous growth rate in the population of *R. dominica* decreased. During the degradation of ozone to diatomic oxygen, free radicals may be formed from reactive oxygen species. In addition, O3 may cause the peroxidation of polyunsaturated fatty acids, resulting in the destruction of critical molecules, such as DNA and proteins (Holmstrup et al., 2011). Thus, these effects, either alone or together, may result in cell damage and the death of the insects that are exposed to ozone gas thus reducing the instantaneous growth rate of the *R. dominica* population.

According to Bodroža-Solarov et al. (2012), insect infestation in the grain mass can increase the moisture content of the grains. This increased grain moisture can be explained by insect metabolism. Thus, the high moisture content that was observed in non-ozonated wheat grains (control) and during the exposure period of 10.11 h to ozone may be explained by the presence of *R. dominica.*

According to Özkaya et al. (2009), *R. dominica* remains within the grain during its development, feeding on the endosperm, which reduces the dry matter and therefore the mass and/or volume of the grains. Thus, the lower values that were observed in the hectoliter weight of wheat grain are explained by the high *ri* of *R. dominica.* Bodroža-Solarov et al. (2012) found a decrease in 2.3 and 8.3% in wheat grain weight as a result of *Sitophilus oryzae* (Linn.) (Coleoptera: Curculionidae) attack.

The increased electrical conductivity of the solutions is related to the deterioration of the grains, which is one of the main causes of insect attack (Loeck, 2002). Thus, it is suggested that high ri, resulted in the increased electrical conductivity of the solution containing wheat grains after 60 days of storage. The behavior of the electrical conductivity of the solution containing wheat grain agrees with the study of Pereira et al. (2007), who evaluated the effects of fumigation with 50 ppm (0.11 mg L-1) of ozone gas for 168 h on stored maize. The increased electrical conductivity of the solutions containing corn grain was more significant in treatments with a high rate of insect infestation.

Storage usually reduces the grain germination percent, which can be enhanced by the action of biotic (fungi, insects and mites) and abiotic (high water content and mass temperature of grains) factors (Chen, 2000; Locher and Bucheli, 1998). The germination percentage of wheat grains was negatively correlated with the instantaneous growth rate of *R. dominica*. The insects feed on grain embryos, consequently reducing the percentage germination (Rocha Júnior and Usberti, 2007).

The results presented in this study corroborate those of Guenha et al. (2014), who found a negative correlation between an insect population and the germination percentage of rice seed stored for three and six months. Rocha-Júnior and Usberti (2007) also obtained similar results in their study on the physical and physiological characteristics of wheat seed purged with phosphine during storage. These authors observed a decreased germination percentage in the control treatments during the storage period, due to heavy insect attack.

The results of this study, combined with information available in the literature confirm that the use of high ozone gas concentrations can reduce the lethal time of exposure to insect pests of stored products. In the surface layer, the time to reach an ideal mortality was greater than in the middle and on the plenum. To solve this problem, ozone gas could be injected at different points along the grain column. This solution is also advocated for by Isikber and Athanassiou (2015), who proposed the application of ozone injection at multiple points along the grain column. Importantly, one of the alternatives to maintain the susceptibility of an insect population to ozone is the adoption of integrated management measures, including the use of the correct concentration and application of ozone either alternated or interspersed with other insecticides.

Conclusion

To obtain 50 and 95% mortalities of *R. dominica* adult insects in the surface layer of the grain column, 13.08 h and 18.11 h were necessary, respectively. The instantaneous growth rate of *R. dominica*, after the application of ozone and after 60 days of storage ranged from 0.000 (LT_{90}) to 0.020 (LT_{10}), regardless of the distribution of insects in the column of wheat. The physical and physiological characteristics of the wheat grains changed with the increased instantaneous growth rate of *R. dominica.*

Conflict of Interests

The authors have not declared any conflict of interest.

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