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Efficacy of modified atmospheres on *Trogoderma granarium* and *Sitophilus zeamais*

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Abstract

We investigated the efficacy of two types of modified atmospheres (MA) against adults of *Trogoderma granarium* and *Sitophilus zeamais* under laboratory conditions. Adults of the above species were exposed at increased carbon dioxide (CO_2) of 70% or at low oxygen (O_2) of 0.1% for 0.67 (16 h), 1, 2, 4 and 6 d. After each exposure interval, mortality (immediate mortality) and knockdown was recorded and the surviving or knocked down individuals were transferred to normal conditions, where mortality was recorded again 7 d later (delayed mortality). Additionally, after the immediate and delayed mortality counts, all adults were removed from the treated substrate, and the number of progeny production was recorded 60 d later. Both MA condition totally controlled the adults of *T. granarium* and *S. zeamais* after 6 d of immediate exposure, or 4 d when delayed exposure was taken into account, revealing the post exposure effect of the MA. Moreover, high CO_2 was more effective than low O_2 for *S. zeamais*, while the reverse was true for *T. granarium*. The 4 d exposure period was crucial for the progeny production of both species, since in that period the transferred knocked down insects, after their exposure to MA, did not produce progeny. From the results of the present study, we can conclude that both MA can be used with success against the two target species.

Key words: modified atmospheres, khapra beetle, maize weevil, carbon dioxide, low oxygen*;* short exposures; post exposure.

Introduction

The khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) and the maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) are major stored product beetle species that infest a variety of stored grains and processed commodities, causing enormous quantitative and qualitative losses. *Trogoderma granarium* is a quarantine species in many parts of the world, has a remarkable tolerance to many of the currently used control methods, while its larvae can remain at diapause for extremely long intervals (EPPO 2017, Athanassiou et al. 2019). Moreover, *S. zeamais* is an important primary colonizer of grains, capable of a rapid population growth (Athanassiou et al. 2017). As in the case of all stored product insect species, chemical control is the main method used for the control of both species, such as the application of the fumigant phosphine, or the use of sprayable formulations of pyrethroids, organophosphates and other active ingredients. Nevertheless, both species have developed resistance to various traditional insecticides such as phosphine (Bell and Wilson 1995, Pimentel et al. 2009, Athanassiou et al. 2019) and some pyrethroids (Guedes at al. 1995, Kumar et al. 2010), while many novel active ingredients have been proved ineffective (Letellier et al. 1995, Haddi et al. 2015, Kavallieratos et al. 2016).

Modified atmospheres (MA) is an environmentally-friendly alternative control method, which can provide disinfestation of stored products from a wide range of pests without leaving toxic residues (Banks and Fields 1995). The application of MA results in the change of the concentration of gases like carbon dioxide (CO_2), oxygen (O_2) and nitrogen (N_2) in the stored product environment, which results on an atmosphere that is toxic for the target species (Jayas and Jeyamkondan 2002, Navarro et al. 2012). The toxic effect of MA can be achieved by adding CO_2 or N_2 in order to produce an atmosphere with high CO_2 or low O_2 concentrations or a combination of both. The toxic effect of the MA on insects depends on the concentration of the gases, the insect species, the development stage, the insect age and the exposure time (Fleurat-Lessard 1990, Navarro 2006). The reduction of exposure time without loss in efficacy levels is essential to enhance their use (Ruidavets 2014), although MA are considered as slow-acting control methods because exposed insects are able to gradually reduce their metabolic rate in order to overcome the toxic effect of changes in the ratio of gases in the atmosphere (Navarro 2006, Mitcham et al. 2006). The exposure of the insects to MA may result in their immobilization, which, after their removal from the treated substrate, may lead to either death or recovery. Important factors that will determine the outcome of this delayed effect after the return of the insects to normal conditions, are the exposure time and the toxicity of the MA environment, which increase with the decrease of the O_2 and the increase of the CO_2 to toxic levels (Fleurat-Lessard 1990, Navarro 2006, Mitcham et al. 2006).

The efficacy of MA is well documented for several stored product pests, with a variety of O_2 or CO_2 contents (Krishnamurthy et al. 1986, Banks and Annis 1990, White et al. 1995, Riudavets et al. 2009, Navarro 2012, Iturralde-Garcia et al. 2016). For *T. granarium*, mostly in the case of its larvae, older works mainly examined different percentages of low O_2 or high CO_2 alone or in combination, at various temperatures, relative humidity and pressure levels (Navarro 2006, 2012). In contrast, to our knowledge, the data for the control of *S. zeamais* are mostly focused on the application of high levels of CO_2 (Banks and Annis 1990, Carvalho et al. 2012, Noomhorm et al. 2013), while the data for low O_2 are extremely few (Bailey and Banks 1975, Haojie et al. 2014).

Any application of MA should take into account the time that is needed for a satisfactory level of efficacy, as long intervals may allow insects to continue to cause damage on the commodity. Furthermore, delayed mortality may not deter progeny production, which will further increase the infestation after the termination of the application. This is particularly important, as, in many cases, eggs and larvae are less susceptible than adults in MA (Banks and Annis 1990, Riudavets 2009). In this context, we have tested the use of either low O_2 or high CO_2 for the control of *T. granarium* and *S. zeamais* adults, at various exposure intervals, by taking into account immediate and delayed mortality, as well as progeny production capacity.

Materials and Methods

Insects. *Trogoderma granarium* was reared on rice, while *S. zeamais* was reared on wheat at 28 ± 2 ºC, 70 ± 5% relative humidity (r.h.) and 16:8 light:dark photoperiod. All insects were obtained from cultures maintained in IRTA climatic chambers. Adults of mixed age and sex were used in the tests for both species.

Bioassays. Bioassays were carried out by exposing the adults of each species to two types of MA, i.e. either 70% CO_2, 6% O_2 and 24% N_2 or 99.9% N_2 and 0.1% O_2, for 16 h (0.67 d), 1, 2, 4 and 6 d. Plastic cups of 400 ml capacity with holes on their covers were used as experimental units. For each species and exposure interval one cup was prepared by adding 10 g of wheat and 10 adults (separate cups for each species). After that, the cups were placed into a 300 X 210 mm and 59 mm-thick plastic vacuum Cryovac Bag (Sealed Air, USA), in order to apply the desired type of MA.

The desirable MA conditions were prepared with a gas mixer (KM 100-3M, Witt, Germany) and were applied to the plastic vacuum bags by using a vacuum packing machine (EVT-10, Tecnotrip, Spain). Afterwards, a second sealing took place by using a portable sealer (Sealboy, Audion Elektro BV, The Netherlands) in order to avoid leakage of gasses. Then, the verification of the CO_2, O_2 and N_2 containment in the bags, was carried out by using a gas analyzer (Oxybaby, Witt, Germany). After the application of the gases, the bags containing the plastic cups were stored in climatic chamber set at 28 ± 2 ^oC, 70 ± 5% r. h. and 16:8 light:dark photoperiod for the tested exposure intervals.

Following each exposure interval, the bags were taken from the climatic chamber in order to measure the gas containment, through the gas analyzer. Subsequently, the bags and the cups were opened and dead, alive and knocked down adults (not having the ability to move, but showing a minimum movement of tarsi or antennae) were counted under stereoscope. Dead adults were removed, while the alive and knocked down adults were placed in new cups containing 10 g of new wheat and returned in the climatic chambers at the same conditions for 7 d more, in order to evaluate the post exposure effect. After the termination of the 7 d interval the cups were opened and dead and alive adults were counted and removed and then, the cups were returned to the climatic champers and remained for additional period of 60 d in order to measure the progeny production capacity. This was also done with the cups after the termination of the initial exposure period for measuring the progeny production. For each exposure interval there were separate bags and cups, while the whole procedure was repeated five times (5 replications).

Data Analysis. Immediate and delayed mortality (total mortality) levels were analyzed separately for each species by using the two-way Analysis of Variance (ANOVA) Procedure of JMP software (Sall et al. 2001), with exposure and MA treatment as main effects. Means were separated by the Tukey-Kramer HSD test at 0.05 (Sokal and Rohlf 1995). Additionally, the mean numbers of dead, alive and knocked down adults after the immediate exposure were calculated, in order to follow the "fate" of these adults after the termination of the 7 d post exposure period. Lethal Time (LT) of 50 and 99% was also calculated based on immediate mortality counts by using the same software. Means of progeny production counts were calculated, for the initial exposure and the 7 d post exposure periods, for the cups that were found to contain alive and knocked down adults.

Results

Immediate mortality. For immediate mortality all factors were significant (Table 1). For *T. granarium*, immediate mortality increased with the increase of exposure interval for both types of MA, while significant differences between the two MA treatments were noted only after the 2 d exposure (Fig. 1). At this exposure, the low O_2 caused higher mortality (86%) than the high CO_2 (40%). Moreover, mortality of *T. granarium* adults reached 100% only after 6 d and only in the case of low O_2, while for the same exposure at high CO_2 mortality reached 96%. For *S. zeamais* significant differences between the two MA treatments were noted only at the 2 and 4 d exposure intervals. After 4 d of exposure, mortality in high CO_2 was 100%, while in low O_2 reached merely 70%. For this species, mortality for both treatments was 100% after 6 d of exposure (Fig. 1). The calculated LT_{50} and LT_{99} values also indicate that *T. granarium* adults were more sensitive to low O_2 conditions while *S. zeamais* adults were more sensitive to high CO_2 conditions (Table 2).

Knockdown effect after exposure to high CO_2 was stronger to *S. zeamais* compered to *T. granarium* (Fig. 2A and 3). Similar results have been also recorded after exposure to low O_2, although the knockdown effect was weaker (Fig. 4A and 5A).

Delayed mortality. For delayed mortality all factors were significant with the exception of treatment and treatment X exposure for *T. granarium* (Table 1). For both species, delayed mortality was increased with the increase of the initial exposure in both treatments. For *T. granarium* mortality increased and reached 100% from the 4 d exposure in both MA treatments, while there were no significant differences among MA treatments in all exposure intervals (Fig, 6). In the case of *S. zeamais*, significant differences between treatments were noted only for the 1 and 2 d of exposure, while mortality was generally higher at high CO_2 compared to low O_2. For example for the 2 d exposure interval at low O_2 mortality was 14% while at high CO_2 100%. Moreover, mortality reached 100% at low O_2 after 4 d of exposure (Fig. 6).

The number of knocked down or alive adults after the initial exposure, did not affect their delayed response (7 d later) (Fig. 2, 3, 4 and 5) dead or alive condition. For the first three exposure intervals, all adults that had been initially recorded as knocked down, were either alive or dead 7 d later. Nevertheless, for longer intervals all adults that had been recorded as knocked down were dead at the post exposure period (Fig. 2, 3, 4 and 5).

Progeny Production. For both species and treatments, no progeny was produced after the initial exposure of the adults (Table 3). From the adults that had survived after the high CO_2 treatment, those that were classified as alive were able to produce progeny only at the 0.67 d exposure interval for *T. granarium*. Similarly, at the low O_2 treatment for both species, alive adults were able to produce progeny only at the 0.67 and 1 d exposure intervals. Moreover, for the adults of both species that had been classified as knocked down, no progeny production was recorded after 4 and 6 d of exposure in both MA treatments (Table 3).

Discussion

Our results indicate that both treatments were highly effective against the adults of *T. granarium* and *S. zeamais*, and totally controlled the adults of both species after 6 or 4 d of exposure, in terms of immediate and delayed mortality, respectively. For 60% CO_2 on *T. granarium*, Spratt et al. (1985) reported that all adults died within 5 or 6 days at 20 ^{o}C and in less than a 4 d at 30 ^{o}C, which is in accordance with our findings. Nevertheless, our data set shows that this species was more susceptible to reduced O_2 than to increased CO_2. Still, given that we only considered only two treatments, generalizations should be avoided, as more levels of either O_2 or CO_2 are needed to understand which technique is faster for the control of *T. granarium*. However, this difference in efficacy between high CO_2 and low O_2 treatments was also reported by Banks and Annis (1990) for *T.*

granarium pupae and diapausing larvae, where the authors calculated the LT_{95} for pupae in 4d at 0.0% O_2 and in 5.5d at 60% CO_2. Since both techniques are compatible with organic food production, they should be further examined, especially in the case of quarantine and pre-shipment treatments.

The two methods have been also compared in the past for the granary weevil *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) by Adler (1994) as well as for the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) by Banks and Annis (1990). Both of these works reported that high CO_2 kills adults more rapidly compared to low O_2 (through the increase of N_2). This observation is in accordance with our findings, since after exposure to high CO_2, *S. zeamais* adults were totally controlled after 4 d of exposure, while the exposure time needed at low O_2 was 6 d. Our findings for the efficacy of low O_2 to *S. zeamais* is close to what was reported by Haojie et al. (2014), where the authors found that $LT_{99.9}$ for the adults of *S. zeamais* at 98-100% N_2 was 5.57 d. For the efficacy of high CO_2 to adults of *S. zeamais*, in large scale applications on rice, Carvalho et al. (2012) reported 100% mortality at an exposure interval of 10 d. Under laboratory conditions for *S. oryzae*, Riudavets et al. (2009) reported that for complete control of the adults, 4 and 8 d are needed, at 50 and 90% CO_2, respectively. Our results show that *S. zeamais* adults are more susceptible to high CO_2 compared to low O_2, but, as noted above, this conclusion is applicable only for the conditions tested here, and any data towards this direction are not transferable to other condition combinations. However, this observation is in agreement with previous findings reported by Spratt et al. (1985), where *Sitophilus* spp. are more susceptible to high CO_2 (60%) than to low O_2, in contrast with other major stored product insects, such as the rusty grain beetle, *Cryptolestes* *ferrugineus* (Stephens) (Coleoptera: Laemophloeidae) and the red flour weevil, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), which are more susceptible to low O_2.

Speed of kill is an essential factor for the selection of the appropriate control method of the stored grain pests, as the longer the insects remain active in the grains, the higher the losses are in the commodity. In addition, the need for longer exposures in controlled environments will increase the overall cost of the method, while keeping the insects alive for long intervals may pose certain risks for tolerance development (Boyer et al. 2011). In our study, we have found that for both species, all adults that had been survived after the 4 d of exposure eventually died, which clearly underlines the irreversible delayed effects of high CO_2 or low O_2. Both methods caused, in certain exposures, narcosis on *T. granarium* and *S. zeamais* adults, as a result of the metabolic changes that take place on the insects to overcome hypoxia or hypercarbia (Fleurat-Lessard 1990, Mitcham et al. 2006). Moreover, our results clearly suggest that this narcotic effect resulted in the elimination of progeny production, which can be considered more as a consequence of adult immobilization, thus inability for egg laying, rather than a direct effect of high CO_2 or low O_2 on immature life stages. The proportion of the adults that had been characterized as knocked down after the treatment was increased with the increase of the exposure interval, for both methods used. This state of the insects is critical, as, after their removal from the treated area and their return to normal atmosphere, knockdown may lead to either recovery or delayed mortality (Fleurat-Lessard 1990, Mitcham et al. 2006). In our study we found that knockdown is more likely to lead to delayed mortality than to recovery, even at exposures that were <4 d. Moreover, >2 d of exposure are needed to completely eliminate progeny production capacity of the insects that had survived both treatments. It is well established that sublethal exposure periods affect the reproduction potential of the insects (Fleurat-Lessard 1990, Dawson 1995, Navarro et al. 2012). In our experimental protocol we saw that both treatments worked well in progeny production suppression, either on wheat that had been treated along with the parental adults, or through the post-exposure effects on these adults. The scenario of post-exposure effects is considered as a reliable indicator for the application of other gases as well. For example, the basic protocol for the evaluation of resistance to phosphine, i.e. the Food and Agriculture Organization (FAO) protocol (FAO Plant Protection Bulletin 1975), is based on mortality levels that are recorded during a certain post-exposure period (Daglish 2004, Opit et al. 2012).

The overall results, provide data for the critical exposure intervals that are essential for the control of *T. granarium* and *S. zeamais*, after exposure to ether high CO_2 or low O_2. Based on our findings, this exposure is 4 d, for both treatments. Despite the fact it is generally regarded that controlled and/or modified atmospheres are slow-acting (Navarro 2012), the critical exposure proposed here is directly comparable with the application of phosphine fumigations, and thus, these levels of CO_2 and O_2 can be further evaluated as alternatives to conventional phosphine fumigations. Moreover, we found that low O_2 was preferable than low CO_2 for the control of *T. granarium*, while the reverse was true for *S. zeamais*. This fact should be taken into account in order to build a species-mediated application strategy. However, the experimental scenario tested here was based on adults and their progeny production capacity. Further testing with immatures is necessary to provide a complete “algorithm” on the critical exposures that are required to control the most difficult-to-kill life stages of each species.

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Tables and Figures

Table 1. ANOVA parameters for immediate and delayed mortality of the species tested (error df=40)

Immediate Mortality					
		T. granarium		*S. zeamais*	
	df	*F*	*P*	*F*	*P*
Treatment	1	22.17	<0.01	138.46	<0.01
Exposure	4	75.94	<0.01	396.60	<0.01
Treatment * exposure	4	5.46	<0.01	61.44	<0.01
Delayed Mortality					
		T. granarium		*S. zeamais*	
	df	*F*	*P*	*F*	*P*
Treatment	1	0.07	0.79	124.26	<0.01
Exposure	4	27.44	<0.01	253.28	<0.01
Treatment * exposure	4	0.54	0.71	50.51	<0.01

Table 2. Lethal Time (LT) estimates for 50 and 99% mortality of *T. granarium* and *S. zeamais* adults exposed to either 70% of CO_2 or 0.1% or O_2

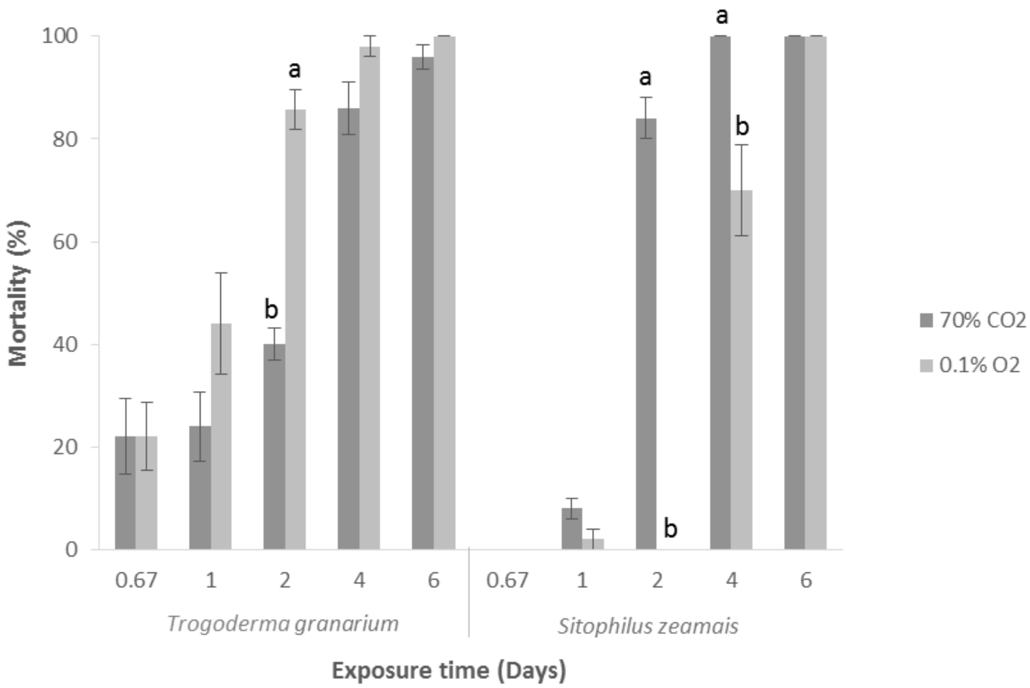
	T. granarium		*S. zeamais*	
	LT_{50} (days)	LT_{99} (days)	LT_{50} (days)	LT_{99} (days)
70% CO_2	1.6	7.2	1.9	3.7
Probit model	y=3.43log(x)+4,35		y=4.8log(x)+4,6	
0.1% O_2	1.2	3.7	3.5	6.6
Probit model	y=8.5log(x)+2,5		y=8.5log(x)+0,4	

y value can be expressed to % by using the Probit Transformation Table (Finney's table)

Table 3. Mean progeny production (adults per cup) of the tested species after immediate exposure for 0.67, 1, 2, 4, and 6 d to either 70% of CO_2 or 0.1% or O_2 and the corresponding 7-d post-exposure period for each exposure interval, i.e. 7 d after the removal of the alive and knocked down adults from the substrate

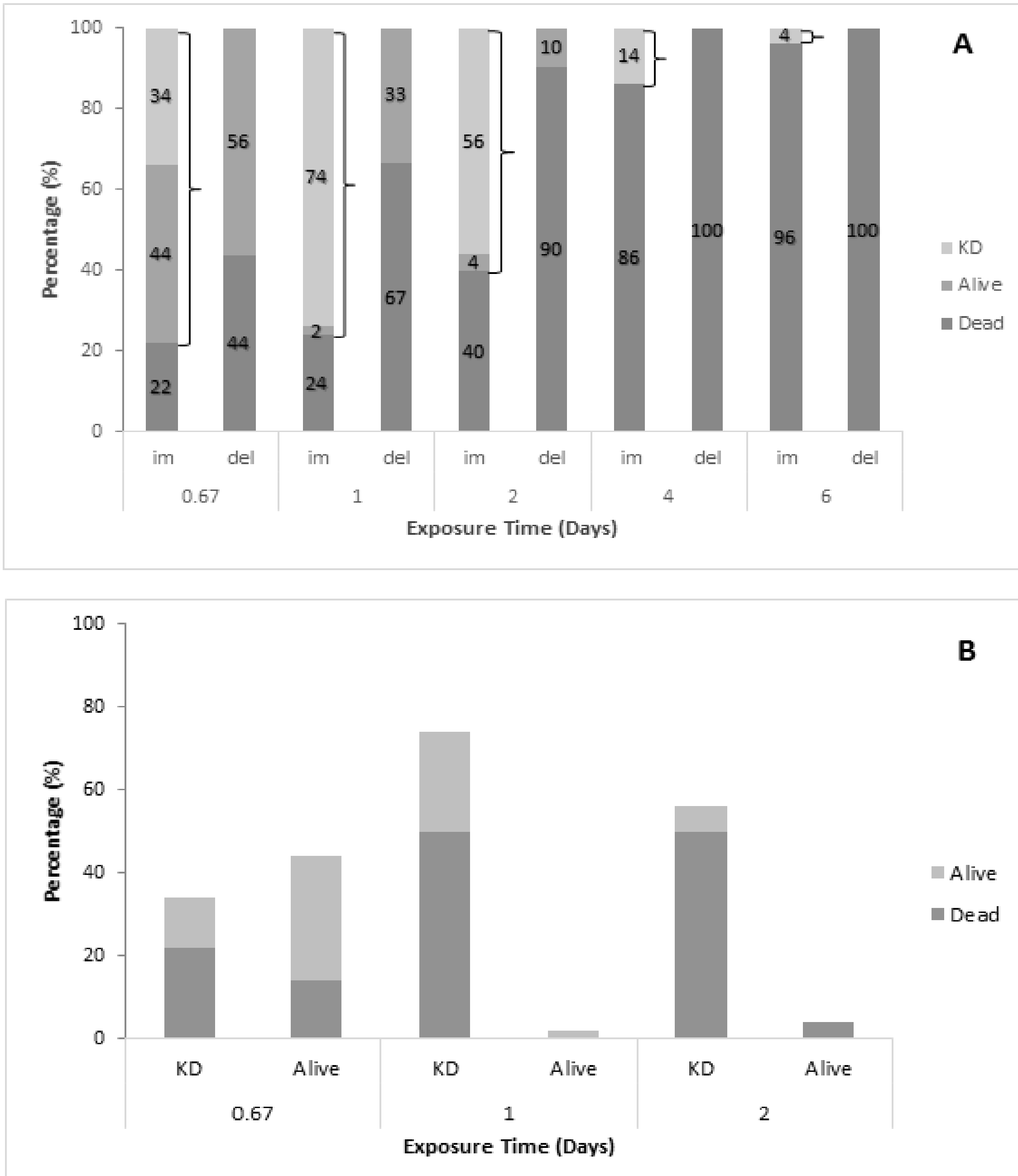
Progeny production after initial exposure										
	T. granarium					*S. zeamais*				
Exposure (d)	0.67	1	2	4	6	0.67	1	2	4	6
Control	3.5	7.0	48.0	34.0	70.0	8.5	6.5	18.5	48.0	65.0
CO_2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Control	0.5	40.5	22.5	24.0	59.0	4.5	15.5	22.5	12.0	57.5
O_2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Progeny production at the 7 d post-exposure period by adults that had been classified as "alive"										
	T. granarium					*S. zeamais*				
Exposure (d)	0.67	1	2	4	6	0.67	1	2	4	6
Control	38.0	34.0	36.0	52.5	24.0	156.5	120.5	87.5	78.5	67.0
CO_2	23.4	0.0	0.0	-	-	-	-	-	-	-
Control	38.0	76.0	35.0	19.0	1.5	72.5	102.5	92.0	38.0	98.5
O_2	10.0	9.6	0.0	-	-	61.2	43.6	-	-	-
Progeny production at the 7 d post-exposure period by adults that had been classified as "knocked down"										
	T. granarium					*S. zeamais*				
Exposure	0.67	1	2	4	6	0.67	1	2	4	6
Control	38.0	34.0	36.0	52.5	24.0	156.5	120.5	87.5	78.5	67.0
CO_2	0.0	8.2	3.4	0.2	0.0	32.4	18.0	0.0	-	-
Control	38.0	76.0	35.0	19.0	1.5	72.5	102.5	92.0	38.0	98.5
O_2	0.8	0.6	0.5	0.0	-	15.0	0.0	41.8	0.0	-

Fig. 1.



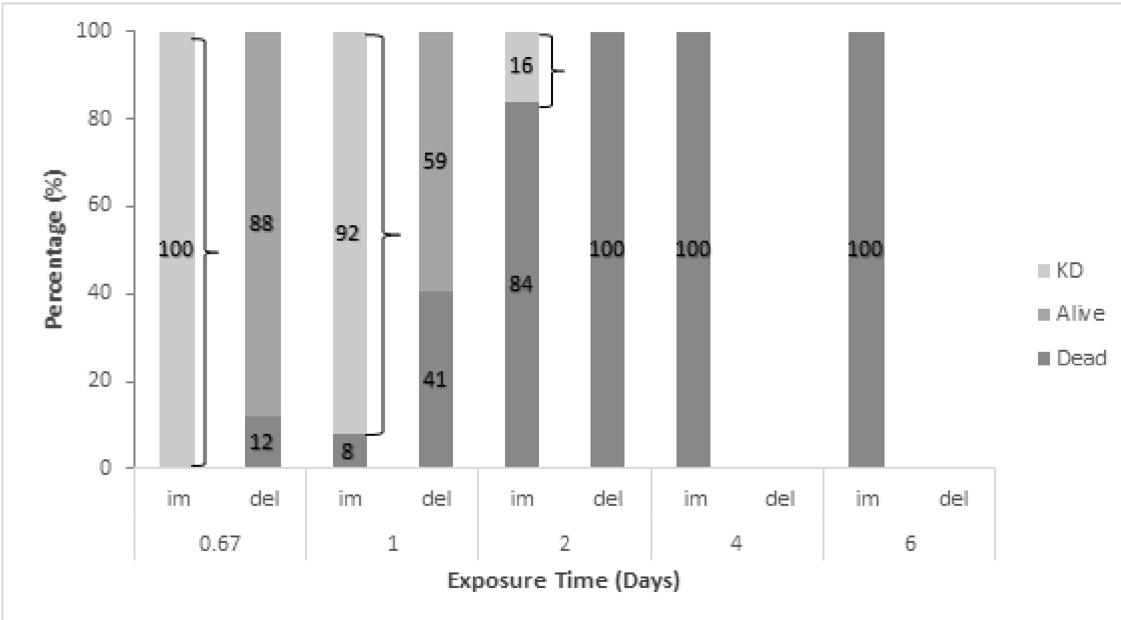
Mean (% ± SE) immediate mortality of *T. granarium* and *S. zeamais* adults exposed to either 70% of CO_2 or 0.1% of O_2, at five exposure intervals (means among treatments within each species and exposure interval followed by the same lowercase letter are not significantly different; where no letters exist, no significant differences were noted; Tukey-Kramer HSD test at 0.05)

Fig. 2.



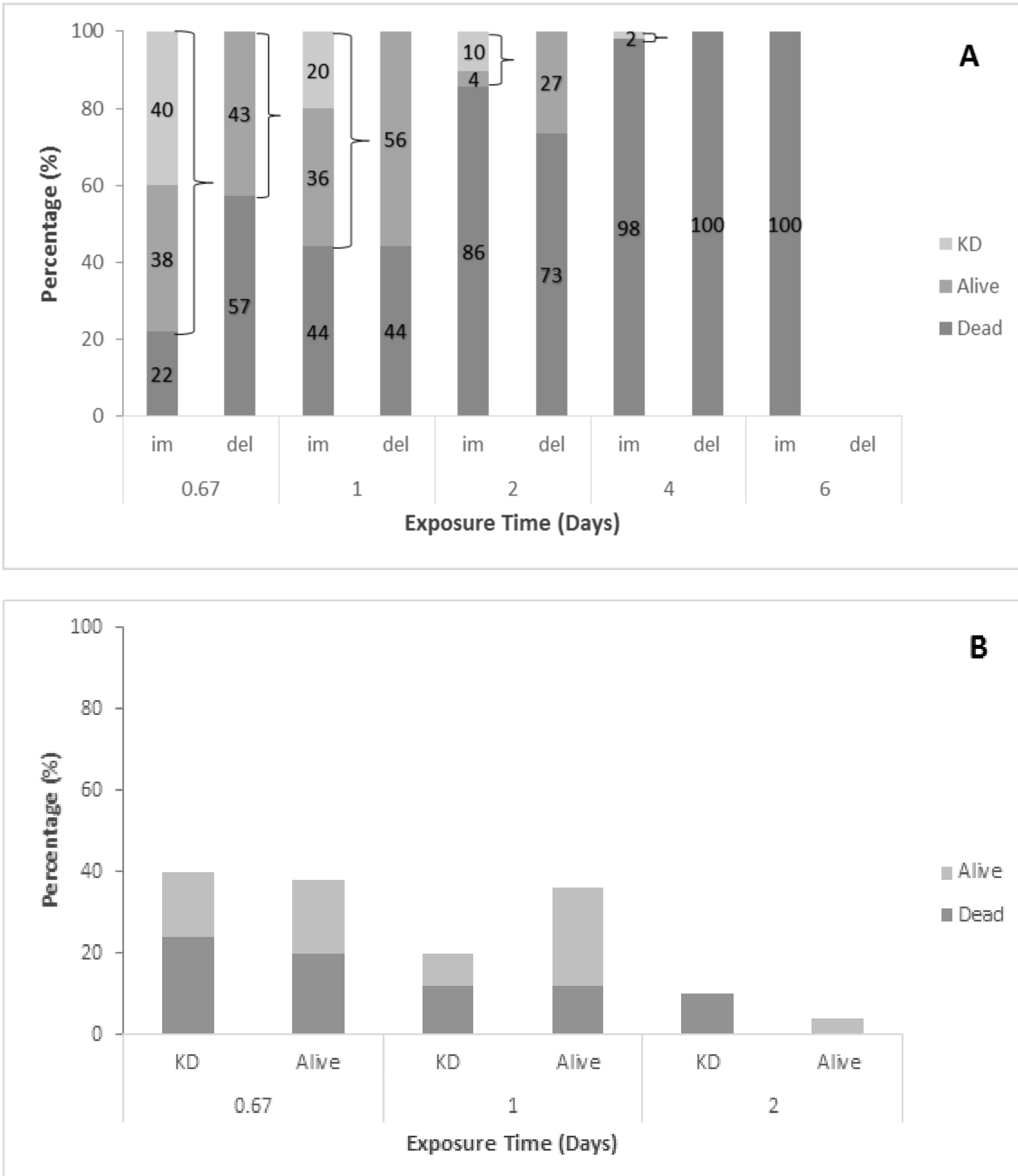
Mean (%) immediate (im) percentages of dead, alive and knocked down adults of *T. granarium* exposed to 70% CO_2 for five exposure intervals, and the delayed (del) percentages (%) of alive or dead adults at the post exposure period (7d) of the alive and knockdown (KD) adults (inside brackets) that had been transferred after each exposure interval to normal conditions (A). Mean (%) percentages of dead and alive adults at the post exposure period of Alive or KD adult after their immediate exposure (B).

Fig. 3.



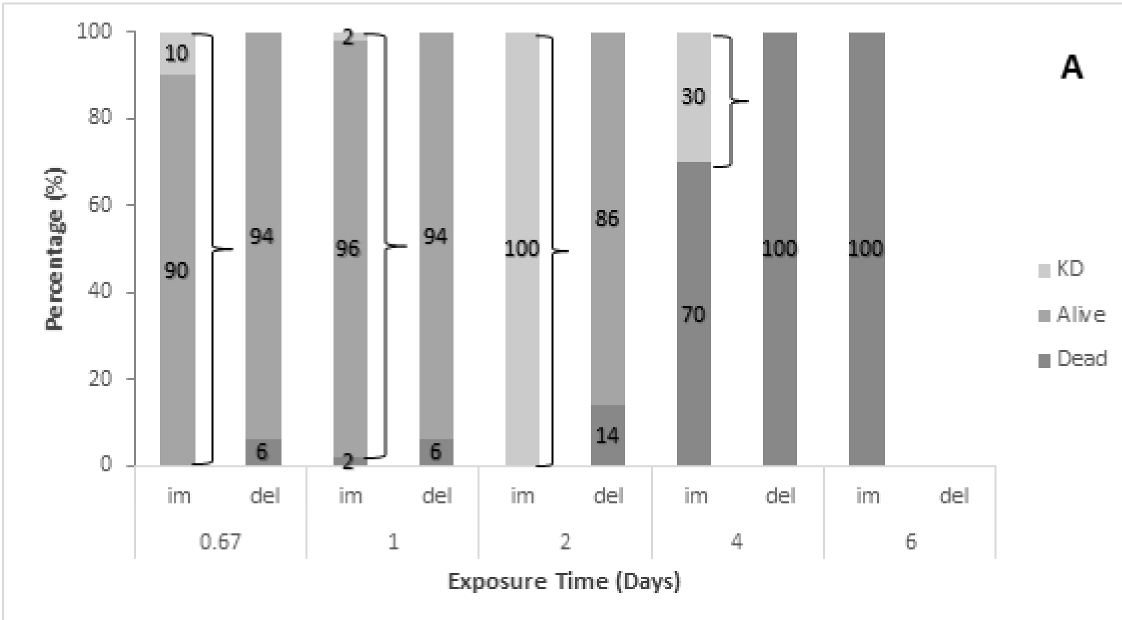
Mean (%) immediate (im) percentages of dead, alive and knocked down adults of *S. zeamais* exposed to 70% CO_2 for five exposure intervals, and the delayed (del) percentages (%) of alive or dead adults at the post exposure period (7d) of the alive and knockdown (KD) adults (inside brackets) that had been transferred after each exposure interval to normal conditions.

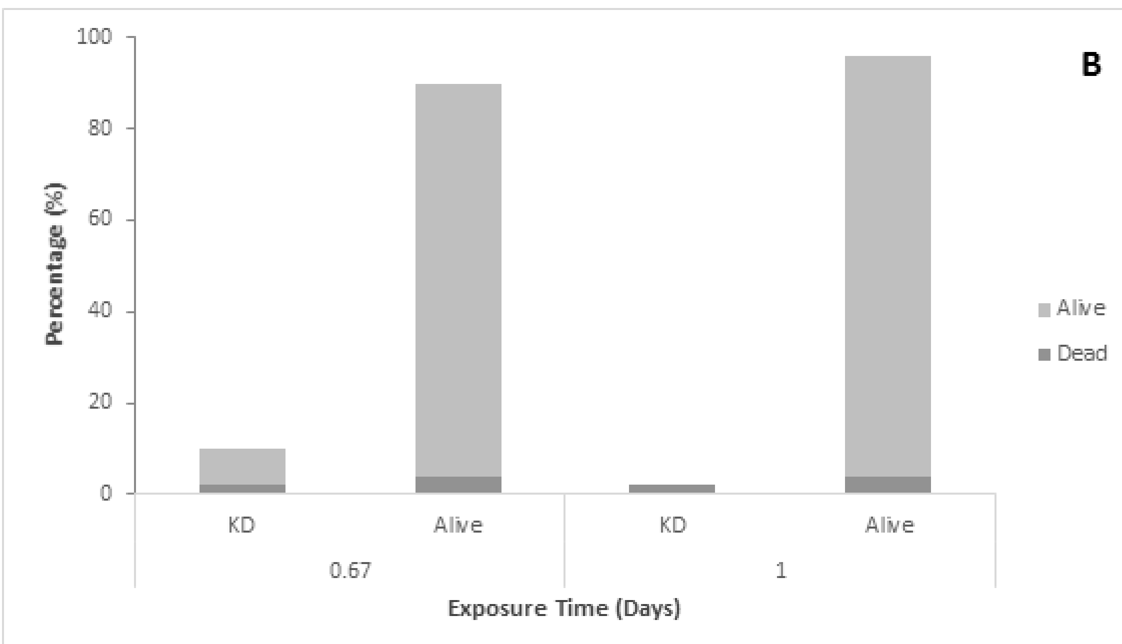
Fig. 4.



Mean (%) immediate (im) percentages of dead, alive and knocked down adults of *T. granarium* exposed to 0.1% O_2 for five exposure intervals, and the delayed (del) percentages (%) of alive or dead adults at the post exposure period (7d) of the alive and knockdown (KD) adults (inside brackets) that had been initially transferred after each exposure interval to normal conditions (A). Mean (%) percentages of dead and alive adults at the post exposure period of Alive or KD adult after their immediate exposure (B).

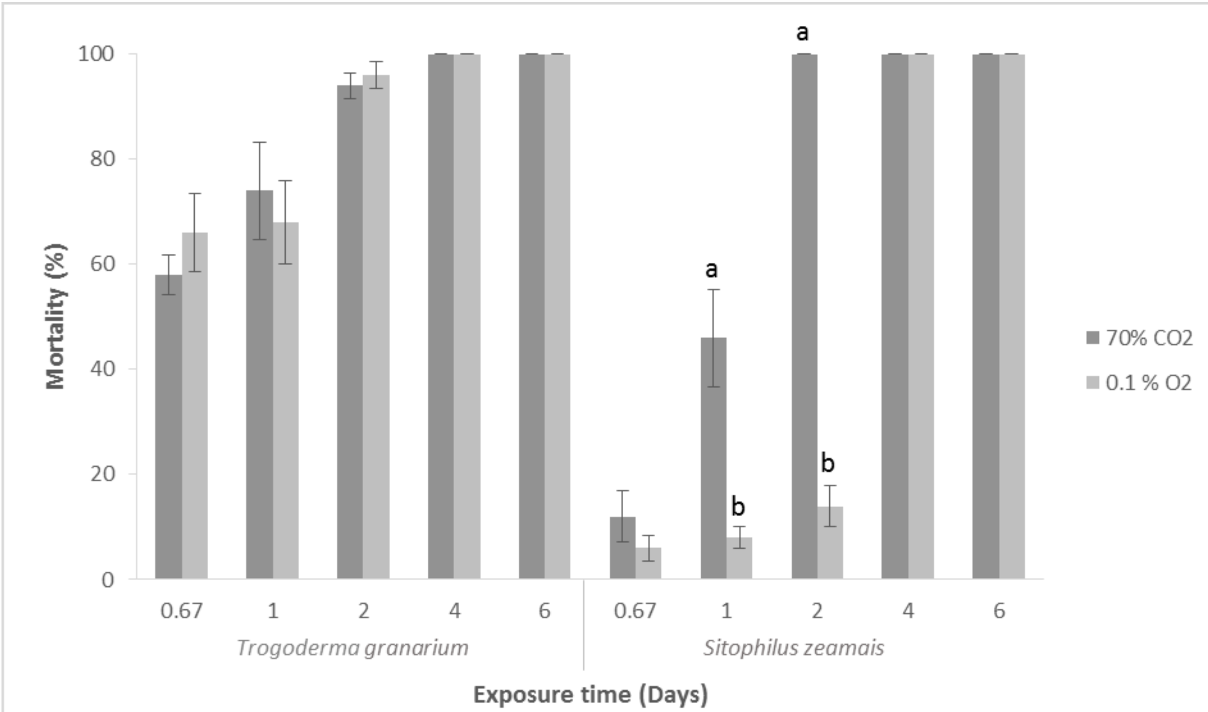
Fig. 5.





Mean (%) immediate (im) percentages of dead, alive and knocked down adults of *S. zeamais* exposed to 0.1% O_2 for five exposure intervals, and the delayed (del) percentages (%) of alive or dead adults at the post exposure period (7d) of the alive and knockdown (KD) adults (inside brackets) that had been initially transferred after each exposure interval to normal conditions (A). Mean (%) percentages of dead and alive adults at the post exposure period of Alive or KD adult after their immediate exposure (B).

Fig. 6.



Mean (% ± SE) delayed mortality of *T. granarium* and *S. zeamais* adults exposed to either 70% of CO_2 or 0.1% of O_2, at five exposure intervals (means among treatments within each species and exposure interval followed by the same lowercase letter are not significantly different; where no letters exist, no significant differences were noted; Tukey-Kramer HSD test at 0.05)