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LOW OXYGEN DISINFESTATION OF GRAIN: EXPOSURE PERIODS NEEDED FOR HIGH MORTALITY

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ABSTRACT

Three common grain pests, *Rhyzopertha dominica, Sitophilus oryzae,* and *Tribolium castaneum* were exposed to low-oxygen (O_2) concentrations in nitrogen (N_2) in the range of 0-3% O_2. *T. castaneum* was the most susceptible of these three species and it was possible to obtain very high mortality in these with a treatment of 3% O_2 for 30 days. Very high mortality (>99%) in *R. dominica* and *S. oryzae* cultures, containing all developmental stages, was achieved only at 25°C with less than 1% O_2, although 1 to <2% O_2 for 50 days did cause high mortality and 2 to <3% for 50 days caused some mortality. On the basis of these results, it is concluded that low O_2 atmospheres may, like any other treatment, have specific applications in grain treatment, although their comparative slowness and low efficacy at >1% O_2 will exclude their use in some situations.

INTRODUCTION

Controlled atmospheres (CA) are one of the few non-chemical methods for grain disinfestation and protection. These atmospheres are based on changing the ratios of the respiratory gases, O_2 and/or carbon dioxide, (CO_2) in the storage atmosphere. Both high CO_2, typically > 35%, and low O_2, typically <1%, have been proposed as useful atmospheres for CA storage. Both types of atmosphere have been shown to work well in large-scale field trials and in limited commercial practice (Banks *et al* .,1980; Annis, 1990). To date, however, the cost of supplying the gases needed to create and maintain the atmospheres have been too expensive for the techniques to be used routinely in more than a few special cases (e.g., Shejbal, 1979; Conway *et al.,* 1990).

Recent developments in gas generation technology are reducing the costs of low O_2 atmosphere production and have rekindled interests in the method (Banks *et al.,* 1991). The predicted lower cost in atmosphere generation, along with the considerable laboratory work that has been carried

out on the effects of low O_2 atmospheres on the common insect pests of stored grain, suggest that this technology may be reconsidered shortly for widespread use. However, laboratory work has concentrated mainly, but not exclusively, on the effects of atmospheres in the range of 0 - 1% O_2 (Annis, 1987) on adult insects. Atmospheres at the 1 - 5% O_2 level are more likely to be economic to create and maintain in the field. Furthermore, in any real infestation, there is likely to be a mixture of developmental stages. The experiments reported here are designed to help close the gap in knowledge between existing laboratory studies and what is likely to be feasible in the field.

Although the published work is inadequate to make firm dosage recommendations for low O_2 CA, in the range of 1 - 5% O_2, the following general conclusions can be drawn from it:

* There is a wide difference in the sensitivity of different species to low-O_2 atmospheres.
* Within a single species, there is a wide range of sensitivity to low-O_2 between developmental stages.
* For some species, exposure periods of longer than 20 days are required for concentrations of >1% O_2 at 25°C.
* Existing data for 1.0% or more O_2 is inadequate as a basis for dosage recommendation.
* More laboratory studies are needed before sound recommendations for exposure periods to concentrations of 1.0% or more O_2 can be made.

Conventional dosage response studies are very time consuming and, if carried out on the whole range of possible species and on each stage within the species, would consume many person-years of laboratory time (Annis, 1987). In an attempt to reduce the amount of work and to cover a range of possible responses, three species were used to obtain more information. Background data on the species used are given below.

Sitophilus oryzae is known to be tolerant to low O_2 atmospheres. The effects of low O_2 on adult insects have been studied by many workers, but the response of the other stages to low O_2 atmospheres other than 100% CO_2 are not well-documented.

Less is known on the response of *Rhyzopertha dominica* to low O_2 atmospheres than is the case for *S. oryzae*. It has been stated that 95% mortality can be obtained in adult insects using 100% N_2 for 2 days (Annis, 1987) and that 1% O_2 and higher do not induce 95% mortality at 4 days (Annis, 1987). Again, the effects on immature stages are not well-documented, although it is known that about 76% of eggs survive 4 days at 2% O_2 (Calderon and Navarro, 1980).

When conducting dosage response experiments, it is usual to use cohorts of insects with bands of known ages each representing different life stages. This is done so that the response curve is simple and not a composite of many different susceptibilities. It is very difficult to produce test cultures

that contain only a single life stage in insects that develop within the grain e.g. *R. dominica* and *S. oryzae*; however it is much easier in the case of *Tribolium castaneum*. In this study, mixed-age cultures containing approximately equal portions of each life stage of the species were used in an attempt to reduce the time needed to complete the study. The use of mixed cultures, while reducing the experimental time significantly, was expected to lead to many complications in the analysis of the results.

METHODS

Culture Preparation

Cultures for *R. dominica* experiments were made up by placing 0.9 g of adult *R. dominica*, approximately 14 days post-emergence (SGRL strain RD2), on 180 g of 12% m.c. ± 0.3% wheat for 7 days at 25°C, and 60% relative humidity (r.h.). The adults were then removed by sieving to leave a culture containing a 7-day cohort of known age. Eight successive known age cultures were then combined to give a mixed-age culture containing approximately similar numbers of all ages from eggs up to approximately 14-day post-emergent adults. The known age cultures for treatment were mixed gently in a bottle before being combined with other cultures by passing through a Boerner Divider to give four equal portions. Two were designated as controls and two as tests.

Production of cultures of *S. oryzae* (SGRL strain LS2) differed only slightly from the above method. For this species, 0.4 g of adult insects were placed on the wheat. Treatment cultures were made up by pooling seven successive weekly cultures.

Cultures for the *T. castaneum* (SGRL strain TC4) experiments were prepared by placing 0.6g of adults on wheat to which flour had been added (two level tablespoons of flour per 1,500 g of wheat). Treatment cultures were made up by pooling nine successive weekly cultures.

Exposure Procedure

One of the four equal portions was put into a culture bottle with a perforated cap as a static control and incubated at 25°C and 60% r.h.. One portion was used as a flow control where air was continuously flushed through the chamber. The two remaining portions were used as tests that were continuously flushed with low oxygen atmospheres ranging from 0% O_2-3% O_2 ± 0.1 obtained by mixing air and N_2. The apparatus used to expose the flow control and tests consisted of 5-litre glass bottles attached to an inlet manifold (gas entered at the base of the bottle and exited through the lid). A raised metal gauze plenum was placed in the bottle before the culture. This ensured non-blockage of the inlet and an even flow of gas through the grain. Each gas inlet and outlet was covered with metal gauze to keep insects from entering the gas lines.

The exposure bottles were kept at 25±1°C in a temperature-regulated cabinet. The gas flow into each manifold was kept to 200 ml/min. The gas mixtures used were obtained by blending fixed flow rates of air (Commonwealth Industrial Gases, Instrument grade) and N_2 (Commonwealth Industrial Gases, High Purity oxygen-free nitrogen). The flow rates were regulated closely using Brooks Mass Flow Controllers. The concentration was monitored with the use of a gas chromatograph; concentrations were taken once each day; any small deviation in concentration was corrected manually.

The gases used were humidified after blending but before entering the exposure bottles by passing the gas through a series of wash bottles containing a water/glycerol mixture designed to give an outlet of 60% r.h. at 25°C.

Assessment Procedure

All controls and tests were put into glass culture bottles with perforated caps after the exposure period, except in the case of the static controls that were placed in the culture bottles from the beginning of the experiment.

The static controls were sieved, and adults were removed and counted, weekly from the beginning of the experiment. The flow controls were sieved weekly beginning on the day the exposure finished except for those exposure times of >20 days where the flow control was removed at 20 days to avoid a build-up of moisture due to the metabolism of the large number of live insects. These flow controls were then sieved as those with an exposure time of ≤ 20 days.

The test cultures were sieved weekly after the exposure period except for the first sieving where they were sieved two days in succession after a 24-hour recovery period. In all cases, large survival numbers were estimated by weighing and comparing with the weighed subsample of 100 insects. Small numbers were counted directly.

Cultures were sieved until the emergence of the F_1 generation that was shown by an increase in the numbers followed by a decrease. In the controls, the decrease was not so well-defined and counting was stopped when the F_1 would normally be expected in the species (e.g. 4 weeks for *S. oryzae*).

Small numbers of undetected survivors are very significant in doses giving high mortality; therefore, *S. oryzae* exposures that had initially 10 or less insects alive were then sieved for five weeks. If no further emergence occurred, they were held for another five weeks, then sieved and resieved again a week later to ensure there was no delay in emergence. *R. dominica* and *T. castaneum* test cultures were treated in a similar way except for the length of sieving time and holding time that were varied accordingly. Test cultures showing no survival were sieved for eight weeks and held for an additional eight weeks to ensure there was no survival.

RESULTS

Control emergence was always high but variable (Table 1) and there was no evidence of significant control mortality (i.e., no excessively low emergence or numerous dead adults). Static and flow controls pairs tended to give the same number of surviving and emergent adults.

Table 1: The average number of adults harvested from the untreated controls (static and flow included). This number is taken as an estimate for the number of individuals treated in each test chamber (usually 2 chambers used).

Species	Average number per chamber	n	SD
R. dominica	7118	34	1697
S. oryzae	6195	32	2947
T. castaneum	341	18	108

The data in Fig. 1 has been plotted to reflect one possible and useful model of dosage/mortality - the decimal reduction model used in microbiology to describe the effects of sterilisation doses where, after an initial period, the logarithm of the number of individual organisms surviving decreases approximately linearly with dose (Pflug, 1987). The reported data are of inadequate quality to confirm this model in the current case but the semi-logarithmic plot is still a useful basis for further presentation of the data as there is equal spacing on the survival axis for each tenfold reduction in survival.

Treatment times were determined in an *ad hoc* fashion on the basis of results of previous treatments. The exposure period for any concentration was extended to a maximum of 53 days. Very high mortality was found only at all concentrations in the range 0-3% O_2 in *T. castaneum*. High mortalities were also found with <1% O_2 in *R. dominica* , and in *S. oryzae* with 1% O_2 or less (Fig. 1).

The treatments nearly always caused a significant and sometimes a prolonged delay in adult emergence of surviving insects. This varied in duration from close to zero to approximately 50 days depending on the treatment and species concerned (Figure 2). No such delay was observed in either of the static or flow control insects.

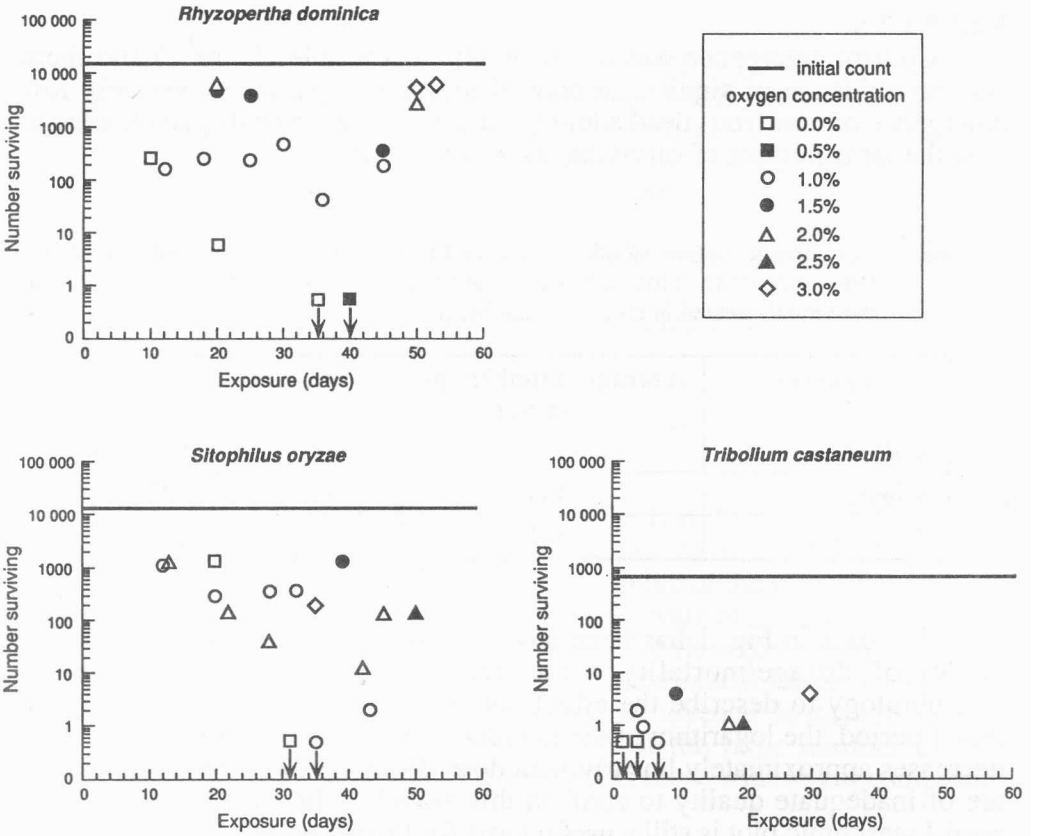

Fig. 1: Number of adults emerging from cultures when treated with low-oxygen atmospheres at 60% r.h. and 25°C. Cultures were *Tribolium castaneum*, (approximately 700 mixed-age insects, a mixture of 9 equally-sized sequential weekly cohorts), *Rhyzopertha dominica* (approximately 2 by 7,000 mixed-age insects, a mixture of 8 equal sized sequential weekly cohorts) and, *Sitophilus oryzae* (approximately 2 by 6,000 mixed-age insects, a mixture of 7 equally-sized sequential weekly cohorts).

DISCUSSION

As expected during the planning phase, statistical analysis of the data was difficult. The reasons for this can be seen in the wide variation in the observed results (Fig. 1). Although there is an obvious trend of increasing mortality, with increasing time and decreasing O_2, there is considerable scatter around any notional line representing a continuous mathematical function. This is not surprising not only because of the mixture of susceptibilities, due to different stages, but also due to the cumulation of errors associated with the original production of cohorts, sampling errors in their splitting and recombination to produce composite test cultures.

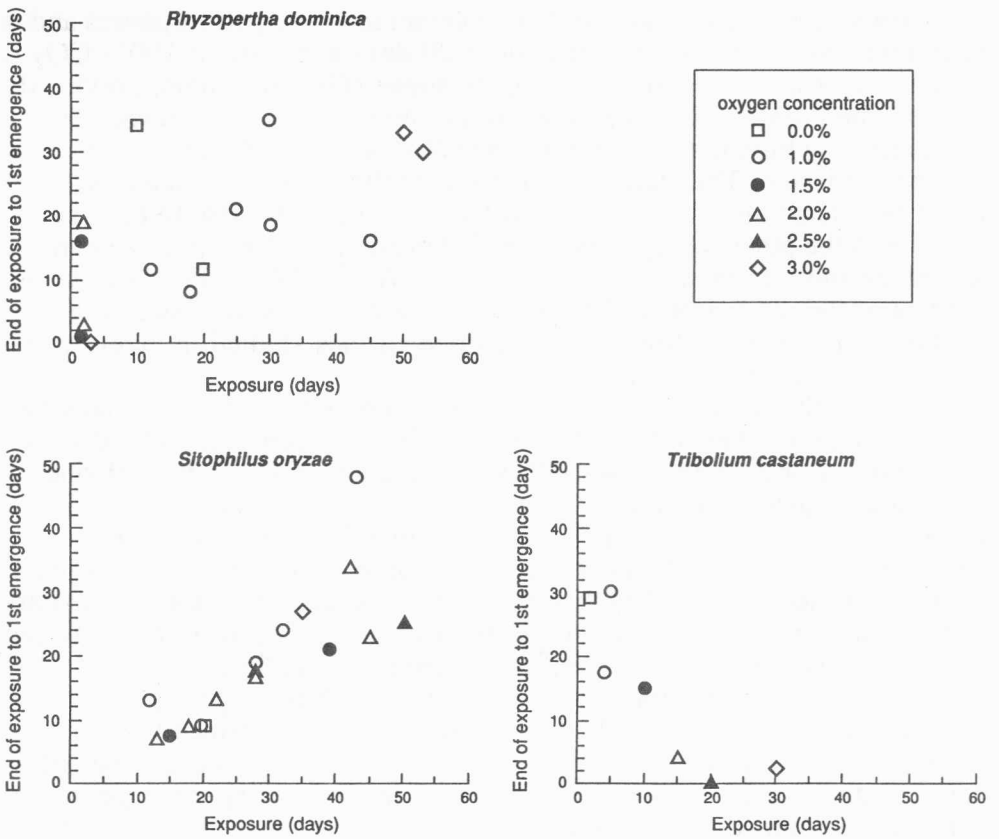

Fig. 2: Time between end of treatment and first emergence of adults in cultures of mixed stages of *Rhyzopertha dominica, Sitophilus oryzae*, and *Tribolium castaneum,* exposed to various low-oxygen (high-nitrogen) atmospheres. Treatment and incubation at 60% r.h. and 25°C.

Furthermore, the *ad hoc* method used to determine dosages means that any form of comparative statistics is of little direct value in analysis of results.

Despite the statistical difficulties, the results contain enough information about mortality and delay in emergence to be useful in discussing low O_2 treatment regimes. *Tribolium castaneum* is known to be a relatively susceptible species to low O_2 atmospheres. Simple inspection of the findings confirm the conclusion that of the three species tested, *T. castaneum* is by far the most susceptible. Of the three species studied in the present experiments it showed the best documented response to low O_2, with some information available for all life stages and limited information on concentrations from 2-3% O_2 (Table 2). The main reason for inclusion of this species was to provide a link with previously published results.

Sitophilus oryzae is known to be tolerant to low O_2 atmospheres and it has previously been shown that at least 20 days exposure to 100% CO_2 is needed to obtain >95% mortality in its pupae (Fig. 3) (Annis, previously unpublished data). Furthermore, it is obvious that *S. oryzae* has a comparatively high level of tolerance and *R. dominica* is the most tolerant of the species tested. The high level of susceptibility of *T. castaneum* is not of much practical value for insect control as it would be unwise to assume the absence of the other two species or for that matter any other species, many of which are more tolerant than *T. castaneum* (Annis, 1987). For this reason, the rest of the discussion will be based on the conservative assumption that *R. dominica,* the most tolerant of the three species studied, is likely to be present in most real infestations.

Although formal analysis of the data is impossible, it is clear that when treating large numbers (approximately 10,000) of mixed-age individuals of *R. dominica* or *S. oryzae* (at 1.0% O_2 or higher), the survival of one to many individuals is possible at very long exposure periods (up to 53 days). Furthermore, it is clear from the data that merely extending the exposure period by a further few days is not going to cause total mortality. How much longer is needed for complete disinfestation is not clear from the current data as this will depend on the nature of the mathematical relationship between mortality and dose (or its components, O_2 depletion and time).

It appears that the only way a statistically based dosage regime can be defined is by carefully controlled exposure of a closely defined target group (most tolerant stage of the species likely to be present) to a graded set of doses. Data from the current study does not clearly identify the target group but it can be implied from the delay in emergence data that adults of all three species are often the most susceptible of the stages. However, at lower doses (smaller products of O_2 deficit [21-O_2%] and time) the delay was sometimes close to zero, thus implying that at least some adults or pre-adults survived these treatments. The variable delay until emergence makes any better determination of the most tolerant developmental stage especially difficult. For example, delays of 45 days may imply (but not confirm) that all other stages than eggs are killed but the very much shorter delays observed under other conditions counter this. Any better resolution of the most tolerant stage and subsequent statistically-based dosage regime must await experimentation designed specifically for this purpose.

The current findings, pose two interesting and related problems concerning the role of CA and quite possibly other insect control measures. These problems stated simply are how many insects are to be treated and how many survivors can be tolerated.

Insect control in grain seems to have three possible primary goals and one secondary one. The primary goals are:

- To minimise damage and loss as a result of insect activity and/or presence. This implies that direct damage/loss due to insects eating grain shall

Table 2: Time in days required to induce at least 95% mortality in *Tribolium castaneum* (at 20–29°C) at various concentrations of oxygen balance nitrogen, (Annis, 1987, synthesis of works of many authors).

	% oxygen			
Stage	**0.0**	**1.0**	**2.0**	**3.0**
Eggs	2.5	1.5	3.0	4.0
Larvae	1.5	6.5	>14	>14
Pupae	4.0	>3.0	—	—
Adults	4.0	>3.0	—	—

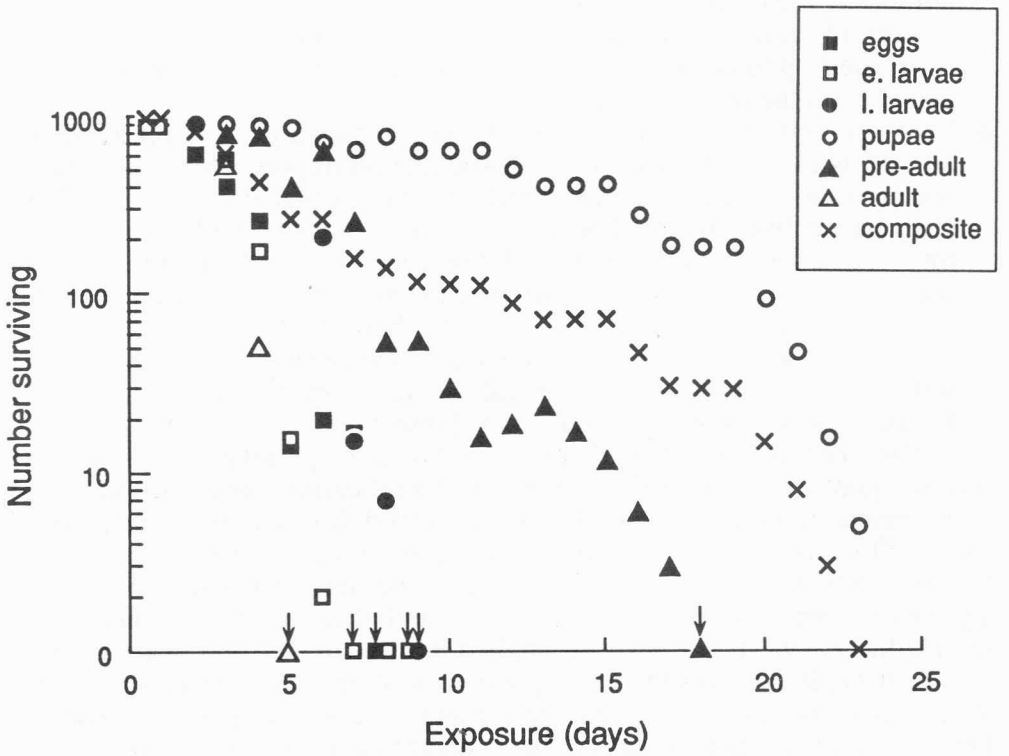

Fig. 3: The effects of exposure to 0% oxygen (100% carbon dioxide) on approximately 1,000 *Sitophilus oryzae* at different developmental stages at 25°C and 60% r.h. Symbols with arrows indicate that no survivors were seen at this exposure period, e. larvae = early instar larvae, l. larvae = late instar larvae.

be kept below an economic threshold and that insect populations shall be kept below the level at which significant grain heating occurs. This is the standard likely to be applied, rightly or wrongly, by a farmer or a supplier of animal feed. The presence of more than a few visible surviving adult insects indicates that the goal has not been met or may shortly no longer be met. The survival of the less-obvious immature stages is considered rarely.

- To ensure that the commodity meets "client" requirements of freedom from insects. This is a very much more stringent goal than the previous one. It usually implies that, at the time of transfer to the "clients'" care, there will be no detectable live (most probably adult) insects. This is a variable goal depending on the nature of the supplier/client relationship. Two examples illustrating this relationship are the requirement that a farmer, in Australia, deliver "insect-free" grain to a bulk handling authority, meaning in practice 0 - 8 insects/t (White, 1985) and that a packer of domestic food have no visible insects when the food is consumed up to 6 months after packing (suggestive of true zero insects per tonne at the time of packing).
- To meet legislative requirements e.g., in Australian grain, a nil tolerance of insects in grain for export, or quarantine on import. This is a difficult question and it is clear that the overall objective is that after treatment there shall be **no** live insects. The verification of this standard is practically impossible for obvious reasons. The best that can be done practically in the case of a nil tolerance on export is to ensure that no adult insects are found during a reasonable inspection before dispatch. Quarantine fumigations aim to ensure that fumigations are carried out to standard that will, if followed, ensure theoretically a very low risk of survival (i.e., killing all insects out of 300,000 treated insects).

The **secondary goal** of insect control is to prolong the life of the control agent by ensuring that the mode of application does not lead to the development of resistance to the treatment method. It is not clear what level of insect kill is needed to fulfill this goal and current logic assumes that if there are no survivors, then there can be no development of resistance. Dosage regimes known to leave survivors are a potential cause of resistance (Dyte and Halliday, 1985). The survival implied in some of the roles of fumigation shown in Table 3 means there is a possibility of development of some form of increased tolerance or resistance to the treatment with repeated application. The risk of this happening has not been quantified and there are currently no plans to do so.

Results of this study show that 1 - 3% O_2 atmospheres can not fulfill all of these roles on every occasion. Their main value appears to be in meeting the first two or three of the objectives in Table 3.

All concentrations up to about 2% O_2 induce a substantial reduction in insect numbers, and concentrations between 2-3% appear to stop population

increase. This should mean that if incoming grain has a low level of infestation, atmospheres with an O_2 concentration of < 3% should ensure that the level of infestation does not increase (depending on the actual concentration and exposure time, the level of infestation may decrease).

Concentrations of less than 2% will give a significant degree of disinfestation and are likely to allow considerable post-treatment storage times without need for further treatment, as not only do they reduce numbers significantly but they also cause considerable delay in adult emergence e.g., 1% O_2 for 45 days leaves 100 survivors out of 12,000 *S. oryzae* treated with the first of these appearing 50 days after the end of treatment or 95 day after the start of treatment.

Table 3: Spectrum of disinfestation requirements graded from least to most stringent objectives.

Objective of treatment	Acceptable level of survival
Commodity protection	Many insects surviving but below the threshold of economic damage
Long-term uninspected storage with constant gas addition	Dependent on initial population but must reduce population to, and/or not allow population to exceed, the threshold of economic damage during the treatment
Client requirements	Less adult insects than the limit of inspection or the stated standard
Client requirements (retail package)	No visible insects at the time of resale in the vast majority of packages
Long term (sealed) uninspected storage with single gas addition	Reduction of population to below a self-sustainable level
Quarantine	Demonstrated survival of less than 1 out of 300,000 in mixed-age target insect(s)
Prevention of the development of resistance	Conventionally zero, no matter what stage, species or starting number. The level of kill actually needed is undefined at present
Absolute mortality	Zero, no matter what stage, species or initial number

Concentrations of less than 1% can give complete disinfestation (out of 14,000 insects) in 35 days at 25°C and 60% r.h.. The main limitation is that 35 days may be considered slow and the period is presumably longer below 25°C (approximately twice the exposure period per 10°C decrease, Banks and Annis, 1990). This limitation makes such atmospheres totally unsuitable for situations where rapid complete disinfestation is required.

CONCLUSION

Low O_2 controlled atmospheres are useful for specific roles in insect control in stored-grain. These roles depend on the users' requirement in terms of the extent of original infestation, tolerable level of survival, O_2 concentration attainable, storage time, and minimum time between treatment and end use. At 25°C, these roles are dictated by the following limits based on the results of this study:

* No survival acceptable out of >10,000 individuals treated
  Exposure period is undefined but known to be somewhat longer than 35 days at 0.0% O_2 and longer than 40 days at 0.5% O_2.
* No survivors acceptable out of 10,000 individuals treated.
  35 days at 0.0% O_2
  40 days at 0.5% O_2
* Tens of survivors acceptable out of 10,000 individuals treated
  45 days at 1.0% O_2
  > 5 days at 1.5% O_2
* Reduction to 10% of original numbers is acceptable
  Exposure period: > 50 days at 2-3% O_2

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