TOLERANCE TO CARBON DIOXIDE IN *SITOPHILUS ORYZAE*

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Abstract

Carbon dioxide is a valuable gas for insect control in stored commodities. Dosage regimes for this application have been based on the times needed to obtain high mortality in pupae of *Sitophilus oryzae*; the most tolerant stage of one of the most tolerant species. On the basis of time to obtain a set mortality, pupae of *S. oryzae* are about 10 times more tolerant to 40% carbon dioxide than adults. Previously established dosage recommendations based on this have been shown to be adequate for complete disinfestation in the field. There is, however, a risk in commercial use that the full dosage regime may not always be achieved. In which case the greatest risk of survival, and hence selection for resistance, is likely to be in the most tolerant stages of the most tolerant species. Previous studies on resistance to carbon dioxide in stored product insects have concentrated only on the response within adult populations. Experimental work reported here shows that natural tolerance of pupae to carbon dioxide varies widely over a range of strains of *S. oryzae*. A qualitative increase in tolerance is shown in a single stain selected for survival to carbon dioxide, within pupal populations, over 7 successive selections. However, the increase in tolerance was too small when compared to the variation in the data to allow any estimate in its magnitude to be made.

INTRODUCTION

Controlled atmospheres based on high carbon dioxide concentrations have undergone considerable investigation, in the laboratory, as a means of insect control in cereal grains. Resistance to carbon dioxide has been induced under laboratory conditions (Bond and Buckland 1979, Navarro *et al* 1985, Donahaye 1990) and concern has recently been expressed that this could occur rapidly in commercial practice. Therefore it is important for potential users to realise that the treatment is not going to lose its efficacy in the short term due to the rapid development of resistance in the field. This is especially true as the perceived cost of preparing the storage, and creating and maintaining carbon dioxide rich atmospheres is high when compared with the equivalent cost of treating grain with the conventional fumigant phosphine.

Some understanding of the natural tolerance of species to the treatment is essential to any discussion of resistance. *Sitophilus oryzae* and the *Trogoderma* spp. have been identified as amongst the most tolerant species to carbon dioxide treatments (Annis 1987). *S. oryzae* can be successfully controlled with commercially feasible carbon dioxide dosage regimes (Annis 1990).

Dosage mortality studies for this species should therefore give rise to dosage recommendations adequate for all other species except the diapausing larvae of *Trogoderma* spp. Many studies that give useful background information about the effects of carbon dioxide on *S. oryzae* have been carried out, but these were not designed to define the dosages necessary for the complete mortality of all life stages. A major limitation of many of the studies (Busvine 1942, Marzke et al, 1970; Stoyanova and Shikrenov 1976, Kashi 1981, Navarro et al 1985) is that they are limited to adult insects and for *S. oryzae* these are known to be one of the less tolerant stages to carbon dioxide (Bailey 1956, Lindgren, and Vincent 1970, Stoyanova and Shikrenov 1974, Desmarchelier 1984). A delay of adult emergence has been reported in a variety of species after treating immature stages with carbon dioxide rich and oxygen depleted controlled atmospheres (see Bailey and Banks 1980 for review). Where treatment causes a significant delay in development of a tolerant stage it is not possible to use a treatment strategy such as that currently used in determining phosphine dosages (e.g. Winks 1990). In this strategy normal development allows progress from a tolerant stage to a more susceptible one during the course of treatment.

This paper provides an overview of the dosage mortality response of *S. oryzae* in order to show that the pupae are very much more tolerant to most concentrations of carbon dioxide than other stages. Furthermore a concentration/time combination that will reliably give high mortality for pupae should also give a high mortality for the other developmental stages and for these stages give substantial safety factor. This puts into some doubt the relevance of previous resistance studies to treatments in the field. These studies have concentrated on selection and development of resistance in adults and unless a treatment is far short of specification adults will always be killed. A first stage in obtaining more relevant information was to investigate the natural tolerance spectrum of *S. oryzae* pupae. Secondly an attempt was made to select carbon dioxide tolerance by sub-optimal exposures on successive generations of surviving pupae.

METHODS

CULTURE PREPARATION

Approx 1.1g of adult *S. oryzae* (SGRL strain LS2) were left on 180g of 12%±0.3% moisture content wheat for 24 hours at 25°C. The adults were then removed by sieving. The sieved wheat was then incubated at 60% RH and 25°C until it contained a culture of insects containing the age class required. The age classes were 1, 8, 15, 29, 36 and 50 days old at the start of exposure and nominally represented the life stages; eggs, early larvae, late larvae, pupae, young adults and adults. When required the culture was passed through a Borner divider twice and split into four 45g sub-samples made up of two pairs. One of each pair was designated as test and the other as a corresponding control. This procedure typically produced 200 - 300 insects per sub-sample.

EXPOSURE PROCEDURE

Test and control cultures were put into cages in a chamber continuously flushed with a low flow of either carbon dioxide at the concentration under test, or air in the case of controls. The chambers were immersed in a water bath regulated to 25±0.1°C and the in-coming gas (air or carbon dioxide/air mixture) humidified to 60±1% relative humidity at 25°C. The carbon dioxide concentrations were produced by closely controlling the flow ratio of food grade carbon dioxide and dry, instrument grade air. The actual concentrations of the gases used were analysed at the beginning and end of each day at which time any necessary adjustments were made to ensure the concentration was maintained within ±0.5% of its nominal value during the exposure period.

Exposure periods for the base-line time/mortality curves were established by a trial and error method. The aim of this method was to obtain at least three sets of results with mortalities greater than 0% and less than 100%, and at least one set with 100% mortality. In order to ensure the 100% mortality results were not simply obtained with excessive exposure periods, an arbitrary goal was set to have at least one 100% mortality observed at no more than 10% longer than the longest time showing some survival. In order to complete the experiments in a reasonable time and to maximise the use of equipment, it was necessary to abandon attempts to obtain data with 100% mortality for any concentration for which substantial mortality for any stage was unlikely in less than 40 days.

EMERGENCE AND MORTALITY ASSESSMENT PROCEDURE

Immediately after the exposure period the culture was transferred from the exposure cage to a 125ml bottle with a well perforated cap. The culture was then gently sieved each day until no further F_1 emergence was evident for three successive sievings. In the case of zero or very low (<10 insects) emergence sieving was continued for 30 days. In the case of zero emergence the culture was examined again after a further 30 days incubation to ensure there had not been any extreme delay in emergence. At each sieving the numbers of insects were recorded. All live insects were collected into a second culture that was assessed for post treatment mortality at 7, 14, and 21 days after the start of emergence. In cases where emergence had started before the end of exposure assessment was made at 7, 14, and 21 days after the end of treatment. Dead insects were recognized by their lack of movement despite a range of stimuli.

STATISTICAL ANALYSIS

Over the range of carbon dioxide concentrations used mortality could be due to oxygen depletion, carbon dioxide toxicity or an interaction of both. Furthermore, there is evidence that low oxygen is antagonistic to the effects of carbon dioxide (Annis 1987). The above influences taken together may well lead to a curve relating mortality to exposure period having at least two components; an early phase where carbon dioxide toxicity dominates and a later phase where oxygen depletion may be a contributor. If this is the case its is unlikely that probit analysis would be an appropriate method of describing the relationship. Furthermore, the actual number of individuals exposed is not known and has to be estimated from the numbers in the controls (Wadley's problem, Finney 1971). It was therefore essential to devise an appropriate statistical method for this non-standard problem.

All analyses were carried out using a generalized linear model package, **GLIM,** with user supplied subroutines. Using this package the data was fitted by equations giving the logit of survival as a cubic polynomial of time for each stage and concentration. The **GLIM** formulation allowed for the non-standard nature of the analysis. Logits were used rather than probits because they were more easily compatible with this use of **GLIM**. The use of a cubic polynomial does not preclude the possibility that a linear relationship may exist, as the equation for the cubic polynomial curve includes that of the linear curve.

The standard methods of calculating confidence intervals around any LT_x (cf Finney 1971) would not be applicable even if probits were used instead of logits because there is no reason to believe the line is straight, the number of treated individuals is estimated, not known, and extra-binomial variation may be present. The method devised used an approach analogous to the confidence interval based on deviance. In this approach the best cubic fit was obtained for a series of tentative LT values around the LT_x value. If the fit, as judged, by residual deviance, was significantly worse according to $P < 0.05$ the value of the tentative LT is rejected as plausible. The range of LT which was acceptable then forms the 95% confidence limits. Fuller details of the statistical technique used are planned for publication at a later date

RESULTS

The calculated LT_{99} for 40, 65 and 95% carbon dioxide are shown in figure 1. The most important feature of these results is that pupae were generally the most tolerant stage displaying significantly more tolerance at 95% and 65% carbon dioxide than the next most tolerant stage, early larvae.

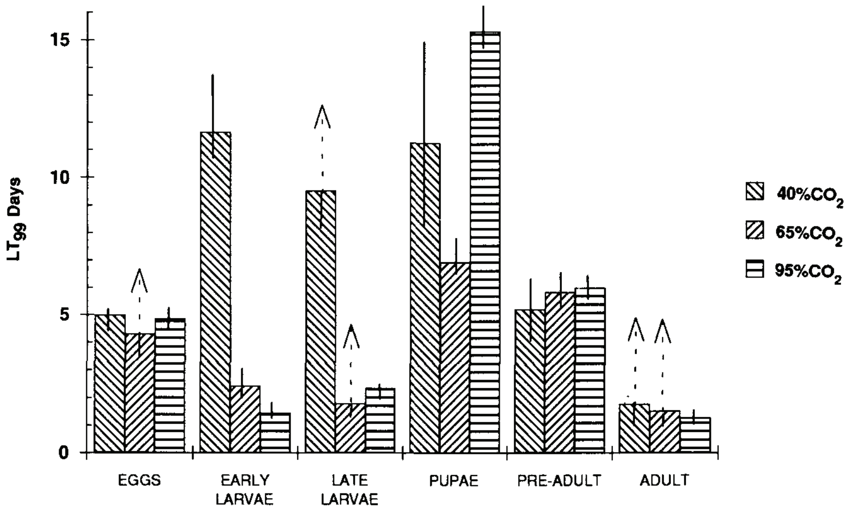

Figure 1. Calculated LT_{99}, for nominal developmental stages of *S oryzae* in 40, 65 and 95% carbon dioxide, in air, at 25°C. The vertical bar indicates the 95% confidence interval, a dashed line indicates that the upper confidence limit was not assessable.

Two additional observation from the results were:

1. there was no additional post treatment mortality during 21 days of post-emergence observations, in the case of pre-emergent stages, or during post-treatment observations, in the case of adults
2. in almost all treatments emergence was delayed when compared to the controls, this delay varied up to about 20 days depending on the length of treatment and stage treated.

There was a significant increase in survival during selection for survival of LS2 pupae to 40% and 65% carbon dioxide, after 7 successive selections. Seven out of 8 points showed lower mortality ($p<0.05$, one tailed sign test) in the selected pupae than in the F_1 data (Figures 2 and 3). However there was no indication of the magnitude of this shift as some of these points do not fall outside the upper confidence interval of the F_1 curve.

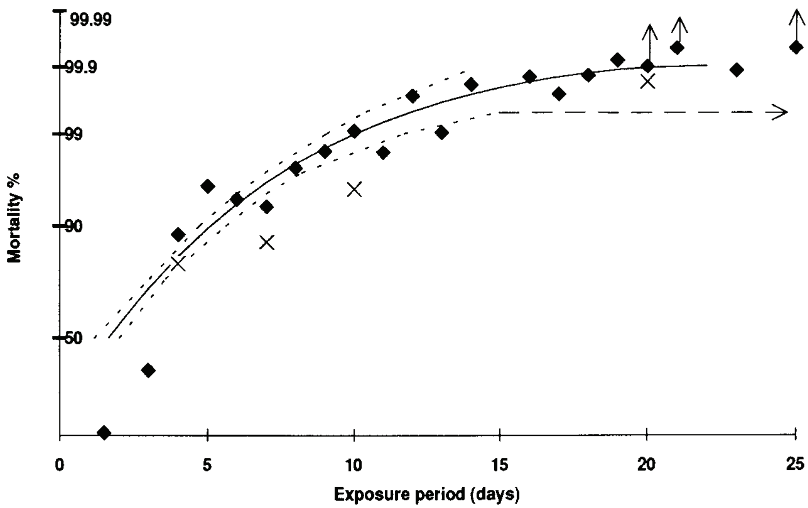

Figure 2. Observed mortality in *S. oryzae* pupae when treated for various times with 40% carbon dioxide, ◆ F_1, X after 7 generations of selection for pupal survival to 40% carbon dioxide, symbols with solid line arrows show the minimum mortality possible at that period and the direction of the actual value. Dashed lines show the 95% confidence limits for the response curve calculated from the F_1 data (solid line), the dashed arrow shows that the upper confidence limit is unassessable above 99.5% mortality.

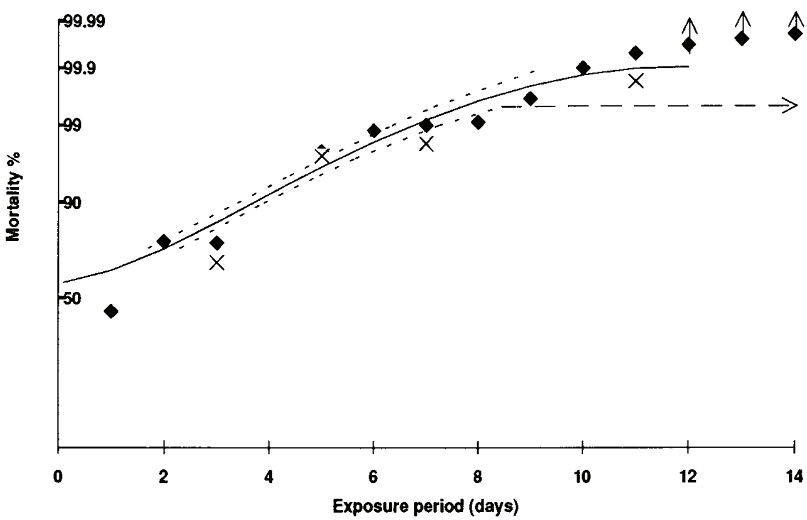

Figure 3. Observed mortality in *S. oryzae* pupae when treated for various times with 65% carbon dioxide, ◆ F_1, X after 7 generations of selection for pupal survival to 65% carbon dioxide, symbols with solid line arrows show the minimum mortality possible at that period and the direction of the actual value. Dashed lines show the 95% confidence limits for the response curve calculated for the F_1 data (solid line), the dashed arrow shows that the upper confidence limit is unassessable above 99.5% mortality.

Screening of the tolerance of the pupae of 9 strains of *S. oryzae* produced an interesting set of results. The target mortality in the screening was based on the times to give 75% mortality in the reference strain LS2 with 40% (3.30 days) and 65% (2.03 days) carbon dioxide. There was a general scatter of results around 75% mortality. The major exceptions to this were almost zero mortality in CSO416 at 40% carbon dioxide and almost no survival in the strain GSO at both 40 and 60% carbon dioxide (figure 5).

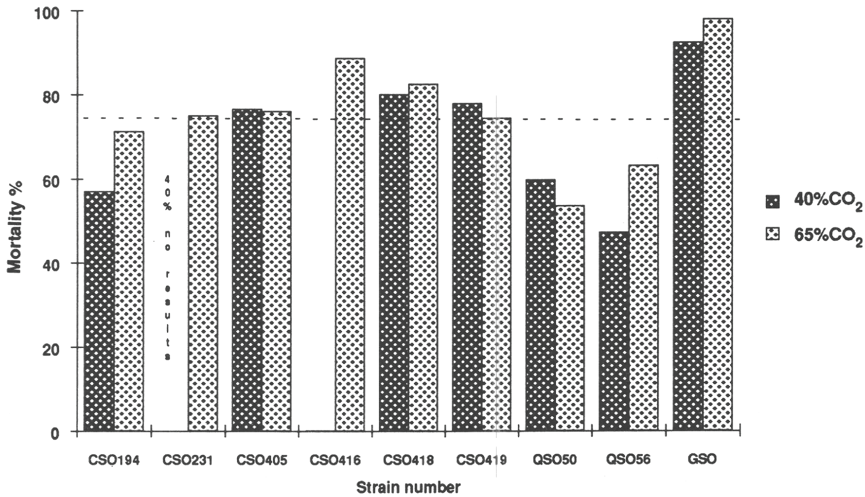

Figure 4. Response of several strains of *S. oryzae* pupae to exposures of 40 and 65% carbon dioxide that would give 75% mortality (dotted line) to F_1 pupae of strain LS2.

DISCUSSION

A common approach to investigating controlled atmosphere dosages has been to identify a scale of susceptibilities covering developmental stages and species in an attempt to define the targets for setting dosages. It is clear from the data in this paper that the difference in susceptibility to carbon dioxide between stages in a single species can be very large and it is therefore important to be sure that the correct stage is targeted. This is especially important in the case of the action of carbon dioxide on *S. oryzae,* and quite possibly with many other species, where it appears that carbon dioxide arrests development, thus leading to much reduced progress from a less susceptible stage to a more susceptible stage during treatment.

If resistance studies have any value in determining the medium to long term prospects of a treatment method it is important that they are carried out on the species and stage most likely to be selected. In the case of carbon dioxide treatment of stored grains this is the pupae of *S. oryzae.* This paper has shown that, in at least one laboratory strain, 7 selections of pupae for survival does produces an increase in their tolerance. It is not possible to determine magnitude of this increase as it is small compared to the observed variation in the dosage/mortality response. It does, however, appear there is a wide variation of pupal tolerance to carbon dioxide over a range of strains. Screening only 9 strains with a dose designed to give 75% mortality at 40% carbon dioxide gave a response varying between 2% and 95% mortality, while 65% carbon dioxide gave between 55 and 98% mortality. These results, especially the two extremes, require further investigation before any statements can be made about the incidence or absence of resistance. It is interesting to note, however, that CSO416, the most tolerant, was a survivor from a long-term underground storage experiment while GSO, the most susceptible, was recently collected from wheat in a domestic kitchen. All strains, including the reference strain LS2 but excluding GSO, had been kept in laboratory culture for many years. This makes it likely that they were subject to higher than atmospheric carbon dioxide concentrations (Williams and Wilbur 1968) for long periods, thus possibly resulting in some selection for carbon dioxide tolerance.

CONCLUSIONS

Pupae are the only stage of *S. oryzae* in which selection for survival is likely to occur during treatment with carbon dioxide carried out with the recommended dosage regime, where there is an initial concentration starting about 80% and decaying to >35% carbon dioxide in 15 days. Attempted selection of pupae of a single strain has shown that, at least in this strain, selection for pupal survival is not rapid. There is however a wide range of inherent tolerance amongst a small collection of strains screened. The reason for this range is not clear and is unlikely to be further clarified unless a wide range of strains with a limited history of laboratory culturing are screened.

One of the laboratory strains so far screened has a significantly higher level of tolerance to 40% carbon dioxide than the others examined. A more detailed investigation of this strain is needed to decide if its level of tolerance is significant as far as commercial treatments are concerned.

Finally it appears that if carbon dioxide treatments are carried out in well sealed enclosures, to the recommended concentration/time regime, there will be little chance of survival in any stage and therefore there should be minimal risk of selection for resistance.

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LA TOLERANCE DE *SITOPHILUS ORYZAE* AU DIOXYDE DE CARBONE

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RESUME

L'utilisation du dioxyde de carbone constitue une méthode valable de désinsectisation des denrées entreposées. Le calcul des doses a été, à cet effet, largement basé sur la période nécessaire à l'obtention d'une forte mortalité de la nymphe de *S. oryzae*, stade le plus tolérant d'une des espèces les plus difficiles à détruire par ce procédé. Les nymphes de *S. oryzae* sont à peu près dix fois plus tolérantes à une teneur de 40 % en dioxyde de carbone que les adultes, en se basant sur le temps. Les doses recommandées se sont montrées efficaces pour une désinsectisation complète en pratique. Cependant, pour tout traitement commercial, il existe un risque, celui de ne pas pouvoir atteindre la dose complète. Dans cette situation, les chances de survie augmentent et, avec elles, le risque de voir apparaître des individus résistants, chez les espèces ou les stades de développement de l'insecte présentant déjà une forte tolérance au produit. Des études antérieures entreprises sur le développement d'une tolérance de *Sitophilus spp* au dioxyde de carbone se sont portées sur la réponse des populations adultes à la sélection. Cet article rapporte les essais entrepris pour augmenter le niveau de tolérance de la nymphe de *S. oryzae* par une sélection appliquée sur plusieurs générations de nymphes.