Research Article

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# Developing effective fumigation protocols to manage strongly phosphine-resistant *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae)

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## Abstract

**BACKGROUND: The emergence of high levels of resistance in *Cryptolestes ferrugineus* (Stephens) in recent years threatens the sustainability of phosphine, a key fumigant used worldwide to disinfest stored grain. We aimed at developing robust fumigation protocols that could be used in a range of practical situations to control this resistant pest.**

**RESULTS: Values of the lethal time to kill 99.9% ($LT_{99.9}$, in days) of mixed-age populations, containing all life stages, of a susceptible and a strongly resistant *C. ferrugineus* population were established at three phosphine concentrations (1.0, 1.5 and 2.0 mg $L^{-1}$) and three temperatures (25, 30 and 35 °C). Multiple linear regression analysis revealed that phosphine concentration and temperature both contributed significantly to the $LT_{99.9}$ of a population ($P < 0.003$, $R^2 = 0.92$), with concentration being the dominant variable, accounting for 75.9% of the variation. Across all concentrations, $LT_{99.9}$ of the strongly resistant *C. ferrugineus* population was longest at the lowest temperature and shortest at the highest temperature. For example, 1.0 mg $L^{-1}$ of phosphine is required for 20, 15 and 15 days, 1.5 mg $L^{-1}$ for 12, 11 and 9 days and 2.0 mg $L^{-1}$ for 10, 7 and 6 days at 25, 30 and 35 °C, respectively, to achieve 99.9% mortality of the strongly resistant *C. ferrugineus* population. We also observed that phosphine concentration is inversely proportional to fumigation period in regard to the population extinction of this pest.**

**CONCLUSION: The fumigation protocols developed in this study will be used in recommending changes to the currently registered rates of phosphine in Australia towards management of strongly resistant *C. ferrugineus* populations, and can be repeated in any country where this type of resistance appears.**


**Keywords:** *Cryptolestes ferrugineus*; strong resistance; phosphine; fumigation protocols

## 1 INTRODUCTION

The fumigant phosphine is used across the globe for disinfestation of stored grains and other durable commodities. Its popularity as a fumigant over several decades can be attributed mainly to its ease of application, use on a wide range of commodities, low cost and, most importantly, its universal acceptance as a nearly residue-free treatment.[1] Although several alternatives have been explored in recent years, they have failed to match the combined advantages offered by phosphine.[2–5] Moreover, the recent phase-out of the ozone-depleter methyl bromide has left phosphine as the only economically and environmentally viable fumigant for the industry for routine disinfestation of stored commodities. However, this overreliance on a single fumigant has resulted in the development of high levels of resistance to phosphine in key storage pest species across the globe.[6–16]

In Australia, a high level of resistance to phosphine has been reported in a strongly resistant strain of *Rhyzopertha dominica* (F.) (600×) over a 48 h fumigation,[6] *Tribolium castaneum* (Herbst) (431×) over a 20 h fumigation[17] and *Cryptolestes ferrugineus* (Stephens) (1450×) over a 72 h fumigation, calculated on the basis of the $LC_{50}$ of each strain relative to their respective susceptible counterparts. The level of resistance in *C. ferrugineus* is the highest ever detected in any stored-product insect species[10] and needs special attention. A proactive approach, however, has resulted in proposed successful management of the strong resistance in *R. dominica*,[18] *Sitophilus oryzae*[7] and *Liposcelis bostrychophila*[11] in Australia through proper characterisation of each of these resistances and development of effective fumigation protocols by manipulation of phosphine concentration, exposure period and temperature. The Australian grain industry is currently facing a problem of highly resistant *C. ferrugineus* surviving currently registered rates of phosphine used in bulk grain storages, particularly in the northern and southern grain belts.[19] Therefore, we aim to investigate the interaction of a range of phosphine concentrations

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and fumigation temperatures against strongly resistant *C. ferrugineus* and to use this information to establish practical fumigation protocols to manage resistance.

## 2 MATERIALS AND METHODS

The response of mixed-age cultures of *C. ferrugineus* to phosphine was evaluated at three temperatures (25, 30 and 35 °C) and three phosphine concentrations (1.0, 1.5 and 2.0 mg $L^{-1}$). In total, nine different combinations of temperature and phosphine concentration were tested for exposure periods of up to 19 days, and each combination was replicated 3 or 4 times.

### 2.1 Test insects and preparation of mixed-age cultures

A reference phosphine-susceptible strain (QCF31, population collected from Cecil Plains in Queensland in 1998) and a strongly phosphine-resistant strain (QNCF73, collected from a central storage at Edgeroi in New South Wales in 2007) of *C. ferrugineus* were studied.[10] These reference populations of *C. ferrugineus* were identified by a professional taxonomist within Agri-Science Queensland, Department of Agriculture, Fisheries and Forestry. Cultures of susceptible and strongly resistant *C. ferrugineus* were maintained on a food medium consisting of rolled oats and cracked sorghum (95% w/w), wheat germ (4.5% w/w) and torula yeast (0.5% w/w) at 32 °C and 70% relative humidity (RH) and a photoperiod of 12:12 h light:dark.

To simulate the exposure of the insect population in the field, mixed-age populations were prepared by adding 50 adults of each strain in a jar containing 60 g of the culture medium described above over a 3 week period. All the life stages were examined for their presence in the mixed-age culture jar prior to fumigation. For each temperature and concentration combination, five mixed-age culture jars of susceptible *C. ferrugineus* and eight mixed-age culture jars of strongly resistant *C. ferrugineus* were prepared. Each mixed-age culture jar represented a different phosphine exposure period.

### 2.2 Lethal time to kill 99.9% of population ($LT_{99.9}$) assays

#### 2.2.1 *Fumigation of mixed-age cultures and monitoring*

Response to phosphine was measured by exposing mixed-age cultures of *C. ferrugineus* to fixed concentrations of phosphine in gas-tight desiccators (6 L capacity). Phosphine concentrations of 1.0, 1.5 and 2.0 mg $L^{-1}$ were tested at 25, 30 and 35 °C and 55 ± 5% RH. Data loggers (I-buttons; Maxim Integrated Products, Inc., San Jose, CA) were kept inside each of the test and control desiccators for monitoring of temperature and humidity.

Three of the five mixed-age jars of the susceptible strain were exposed to phosphine for 1, 2 and 5 days at all three temperatures and concentrations. Six of the eight mixed-age jars (see Section 2.1) of the resistant strain were exposed within a range of 7–19 days at 25 °C, 3–18 days at 30 °C and 3–16 days at 35 °C, depending on the phosphine concentration. For example, at 25 °C, a total of six jars were exposed for 14, 15, 16, 17, 18 and 19 days at 1.0 mg $L^{-1}$, whereas at 2.0 mg $L^{-1}$ six jars were fumigated for 7, 8, 9, 10, 11 and 12 days. The remaining two mixed-age jars from each strain were used as controls, and these were exactly the same as the treatment jars except that they were not dosed with phosphine. The range of phosphine concentrations and temperatures tested represents those likely to be used in practice by industry.

Phosphine was generated in a collection tube containing aluminium phosphide introduced in sulphuric acid (5%).[20] The phosphine concentration was measured at the start of the experiment on a Clarus® 580 gas chromatograph (PerkinElmer, Waltham, MA) using a thermal conductivity detector with nitrogen as the standard.[21] $LT_{99.9}$ bioassays were undertaken by placing the mixed-age culture jar containing all the life stages and culture medium inside gas-tight desiccators (6 L) and injecting phosphine through a rubber septum in the lid with a gas-tight syringe to give the required concentration. The phosphine concentration inside the desiccators was monitored daily throughout the experiment using a Clarus® 580 gas chromatograph (PerkinElmer) fitted with a flame photometric detector to confirm that there was no loss of phosphine during the predetermined exposure periods. The approximately 10–15% loss in phosphine concentration was topped up every 3 days.

#### 2.2.2 *Post-treatment handling of insects*

After fumigation, the mixed-age culture jars were removed and placed at 30 °C and 55% RH for 7 days to obtain endpoint mortality, when all adults, live and dead, were removed and counted. The mixed-age cultures were then incubated at 30 °C and 55% RH for another 3 weeks and again examined for the presence of live adult insects. Any live adults found were removed and examined, and the mixed-age cultures were further incubated for another 4 weeks, when they were examined for the presence of adult insects. This period of 8 weeks was intended to allow time for surviving immature stages to develop into adults, including those that may have delayed development owing to phosphine fumigation.[22] Experimentally observed values of time to population extinction (TPE), i.e. time to achieve 100% mortality (no survival), to control all life stages of *C. ferrugineus* at each phosphine concentration and temperature regime, were recorded. Time to population extinction is defined as the earliest exposure period from which there is no emergence, provided that this is also true in samples from longer exposure periods.[23]

### 2.3 Statistical analysis

At each phosphine concentration and temperature regime, $LT_{99.9}$ was calculated using the probit method incorporating Wadley's model.[24,25] The 99.9% mortality level was chosen because the aim of phosphine fumigation is to achieve high levels of control, and statistically $LT_{99.9}$ values are more accurate than TPE values. Wadley's model was used to determine the mortality of individuals in the mixed-age cultures comprising an unknown number of immature and hidden life stages. The mortality estimate was obtained by comparison with untreated controls. Wadley's model calculates $LT_{99.9}$ values based on survival data. The analysis was performed using the GenStat 15 statistical package.[26] A lethal time ratio test[27] was undertaken to determine significant differences between $LT_{99.9}$ values for each phosphine concentration across the three temperature regimes, and vice versa. The resulting 95% confidence interval (CI) was used to test the equality of the two $LT_{99.9}$ values (i.e. if the value 1 is contained in the lower and upper levels of CI for the lethal time ratio, then the $LT_{99.9}$ values are not significantly different).

The relationship between $LT_{99.9}$ and the variables phosphine concentration and temperature was examined by multiple linear regression analysis using the GenStat 15 statistical package.[26] A full model was fitted, including two-way interaction. Using backward selection, terms that were not significant at the 5% level were omitted from the final model.

**Table 1.** Effective phosphine protocols to achieve 99.9% mortality in strongly phosphine-resistant *Cryptolestes ferrugineus* in a range of temperatures and concentrations

| Insect | Temperature (°C) | Phosphine concentration mg L$^{-1}$ | Phosphine concentration ppm | Deviation | df | Equation Constant (±SE) | Equation Slope (±SE) | $LT_{99.9}$ (days) (95% fiducial limits)[a] | Time to population extinction (days)[b] |
|---|---|---|---|---|---|---|---|---|---|
| Strongly resistant *Cryptolestes ferrugineus* | 25 | 1.0[c] | 718 | 16.0 | 16 | −6.15(±1.64) | 0.48(±0.11) | 19.26 (17.70–23.06)$^{aA}$ | 18, 15, 16, 16 |
| | | 1.5[c] | 1077 | | | −14.74(±5.54) | 1.53(±0.54) | 11.66 (10.98–15.06) $^{bA}$ | 11, 12, 11, 10 |
| | | 2.0 | 1436 | | | −10.04(±3.54) | 1.32(±0.43) | 9.98 (9.22–13.08) $^{cA}$ | 10, 10, 9 |
| | 30 | 1.0[c] | 718 | 20.0 | 20 | −20.72(±6.69) | 1.69(±0.53) | 14.13 (13.54–16.36) $^{aB}$ | 16, 13, 14, 15 |
| | | 1.5 | 1077 | | | −4.13(±1.07) | 0.68(±0.14) | 10.64 (9.54–13.16) $^{bB}$ | 10, 10, 10 |
| | | 2.0 | 1436 | | | −6.80(±2.32) | 1.47(±0.45) | 6.72 (6.05–9.17) $^{cB}$ | 6, 6, 6 |
| | 35 | 1.0[c] | 718 | 18.1 | 18 | −4.71(±0.92) | 0.55(±0.09) | 14.21 (13.21–16.00) $^{aB}$ | 15, 12, 13, 11 |
| | | 1.5[c] | 1077 | | | −4.05(±0.90) | 0.83(±0.15) | 8.62 (7.87–10.04) $^{bC}$ | 7, 10, 10, 10 |
| | | 2.0[c] | 1436 | | | −5.14(±1.23) | 1.47(±0.29) | 5.59 (5.10–6.64) $^{cC}$ | 5, 7, 7, 4 |

[a] $LT_{99.9}$ values followed by the same lower-case letter across three concentrations for an individual temperature are not significantly different, and $LT_{99.9}$ values followed by the same upper-case letter for an individual concentration across three temperatures are not significantly different, based on the lethal time ratio test [95% confidence intervals (lower and upper levels) for $LT_{99.9}$ are omitted for clarity] (see Section 2.3).
[b] Experimentally observed times to population extinction.
[c] Replicated 4 times (the other treatments were replicated 3 times).

## 3 RESULTS

The mortality of mixed-age populations of susceptible *C. ferrugineus* that were fumigated for 1, 2 and 5 days at all temperature and concentration combinations was 100% for all exposure periods. No emergence of susceptible adults was observed after 8 weeks for any temperature and concentration combinations in any exposure periods. Therefore, data obtained from the susceptible strain were not suitable for probit analysis using Wadley's model to calculate $LT_{99.9}$ values.

Probit analysis using Wadley's method of the mixed-age populations of strongly resistant *C. ferrugineus* at each phosphine concentration and temperature provided $LT_{99.9}$ values that decreased concomitantly as phosphine concentration and temperature increased from 1.0 to 2.0 mg L$^{-1}$ and from 25 to 35 °C (Table 1, Fig. 1). In general, experimentally observed TPEs for the strongly resistant *C. ferrugineus* were shorter than $LT_{99.9}$. Probit analysis also revealed that efficacies of phosphine concentrations (1.0, 1.5 and 2.0 mg L$^{-1}$) were significantly different, i.e. efficacy was highest at 35 °C, as confirmed by 95% CI ratio test. However, mortalities for 2.0 mg L$^{-1}$ were significantly different across all temperatures. The shortest $LT_{99.9}$ (5.59 days) was recorded at the highest phosphine concentration (2.0 mg L$^{-1}$) and highest temperature (35 °C). The longest time to kill 99.9% of the strongly resistant *C. ferrugineus* population was 20 days at 25 °C for fumigation with 1.0 mg L$^{-1}$ of phosphine. No significant difference was observed in 99.9% mortalities of populations at 30 to 35 °C with 1.0 mg L$^{-1}$ of phosphine, as confirmed by 95% CI ratio test (Table 1). The differences between the longest and shortest times to kill 99.9% of a population were 6, 3 and 5 days at 1.0, 1.5 and 2.0 mg L$^{-1}$, respectively, across all temperatures. However, within each temperature the difference between the longest and shortest times to population extinction were 10, 8 and 9 days at 25, 30 and 35 °C, respectively, across all three phosphine concentrations.

Multiple linear regression analysis of the strongly resistant *C. ferrugineus* data revealed that phosphine concentration and temperature both contributed significantly to $LT_{99.9}$ ($P < 0.003$, $R^2 = 0.92$), with concentration being the dominant variable and accounting for 75.9% of the variation. The two-way interaction ($P = 0.523$) did not contribute significantly to the model and so was omitted.

The resulting regression equation is as follows:

$$LT_{99.9} = 27.51\ (\pm 3.35) - 0.38\ (\pm 0.11)\ (\text{temperature}) - 27.41\ (\pm 3.61)\ (\log\ \text{concentration})$$

Based on this equation, an increase in concentration and temperature lowers the $LT_{99.9}$ of strongly resistant *C. ferrugineus* (Table 1).

## 4 DISCUSSION

The aim of the present research was to develop robust fumigation protocols that could be used in a range of practical grain storage situations to control strongly resistant *C. ferrugineus* populations. We have established fumigation protocols in the laboratory for three phosphine concentrations (1.0, 1.5 and 2.0 mg L$^{-1}$) and three temperatures (25, 30 and 35 °C) for the control of all life stages of strongly resistant *C. ferrugineus*. Our results follow an existing trend of either increasing phosphine concentration or increasing fumigation period for the control of strongly resistant populations. Moreover, it was also established that successful control of resistant *C. ferrugineus* can be achieved using shorter fumigation periods at elevated grain temperatures, irrespective of the concentration used. Based on our data, mixed-age populations of strongly resistant *C. ferrugineus* can be successfully controlled using a 20 day fumigation with 1.0 mg L$^{-1}$ at 25 °C, which is similar to the results reported by Wang *et al.*[28] According to Wang *et al.*,[28] an initial concentration of 1.0 mg L$^{-1}$ of phosphine with a further requirement to maintain a concentration above 0.4–0.7 mg L$^{-1}$ for 16–25 days was required to control strongly resistant *C. ferrugineus* in warehouses. In another study, Li and Yan[29] recommended a phosphine concentration of 0.3 mg L$^{-1}$ for more than 28 days to control strongly resistant *C. ferrugineus* in warehouses. Nayak *et al.*[10,30] reported that 1.0 mg L$^{-1}$ of phosphine maintained for 24 days at 20 °C was required to attain population extinction of strongly resistant *C. ferrugineus*. The $LT_{99.9}$ times established in the present study for the various temperature

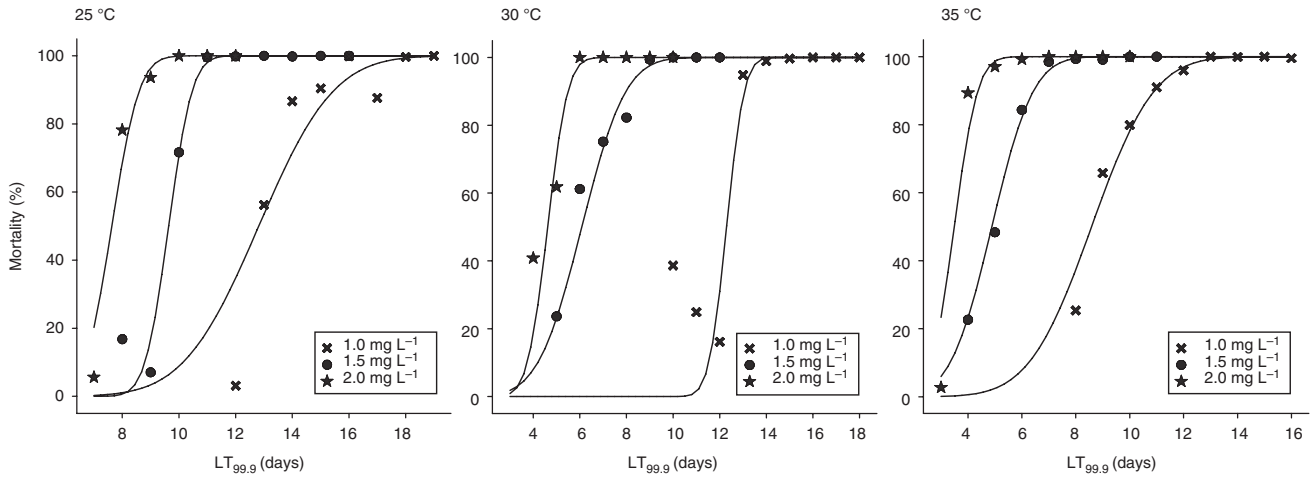


**Figure 1.** Response of mixed-age populations of strongly resistant *Cryptolestes ferrugineus* to phosphine at three concentrations and temperatures. Curves are presented as mortality calculated by probit analysis using Wadley's model.

and concentration combinations for strongly resistant *C. ferrugineus* are much longer than previously established times for other strongly phosphine-resistant stored-product pests. For example, fumigation periods of 5 and 7 days were required for 1.0 mg $L^{-1}$ of phosphine at 25 °C to attain population extinction of mixed-age populations of strongly resistant *R. dominica* from Australia[18] and India.[31] In the Philippines, Sayaboc *et al.*[32] reported 98.3 and 99.1% mortality of a strongly resistant population of *R. dominica* in 3 and 7 days at 1.0 and 0.71 mg $L^{-1}$ respectively, whereas Liang *et al.*[33] reported that 99.9% mortality of a strongly resistant Chinese strain was achieved at 0.4 mg $L^{-1}$ in 9.8 days. Similarly, it was reported that, to achieve population extinction of strongly resistant *S. oryzae* at 25 °C, protocols of 4 days at 1.0 mg $L^{-1}$ and 7 days at 0.25 mg $L^{-1}$ were required.[7,31] According to Nayak and Collins,[11] at 15 °C and 70% RH, 19 and 11 days are required to control the phosphine-resistant psocids *L. bostrychophila* using 0.1 and 1.0 mg $L^{-1}$ respectively. At a higher temperature of 35 °C and 55% RH, however, only 4 and 2 days of fumigation were required at the respective phosphine concentrations to achieve population extinction of this strongly resistant pest.

In relation to $LT_{99.9}$ of the strongly phosphine-resistant *C. ferrugineus* and the interactions of the two variables investigated in this study (temperature and concentration), phosphine concentration exerted the maximum effect, accounting for 75.9% of the variation in response. Irrespective of the phosphine concentrations used, $LT_{99.9}$ and TPE in strongly resistant *C. ferrugineus* were longer at lower temperature and shorter at higher temperature. This observation of increased phosphine toxicity with increasing temperature is in accordance with previous studies undertaken on a range of stored-grain beetles, moths and psocids.[11,16,34–36] Based on our data, for example, 1.0 mg $L^{-1}$ of phosphine is required for 20, 15 and 15 days, 1.5 mg $L^{-1}$ for 12, 11 and 9 days and 2.0 mg $L^{-1}$ for 10, 7 and 6 days at 25, 30 and 35 °C, respectively, to attain 99.9% mortality of the strongly resistant *C. ferrugineus* population. This phenomenon of increased phosphine toxicity with increasing temperature has been correlated with increase in insect respiratory rate, metabolic rate and oxygen consumption in response to increasing temperature, which increases the uptake of phosphine and leads to higher mortalities.[34,36] Bond *et al.*[37] also demonstrated that environmental factors that lower the rate of metabolism, such as reduced oxygen atmosphere or decreased temperature during fumigation, would lead to increased tolerance to phosphine in insects. Further evidence of this phenomenon was observed in studies of resistant *Caenorhabditis elegans* (Maupas), where it was found that an increase in metabolic rate conferred increased susceptibility to phosphine,[38] while a constitutively lowered metabolic rate conferred resistance.[39]

The protocols developed in this study aimed at recommending practical minimum fumigation periods and phosphine concentrations that would control all life stages (eggs, larvae, pupae and adults) of strongly resistant *C. ferrugineus* in Australia. The $LT_{99.9}$ data give us guidelines for achieving a successful fumigation. They can be used to determine the phosphine concentrations required to attain complete control of resistant populations within a certain time period needed for a specified temperature. Failure of fumigation normally occurs when phosphine concentrations are not maintained at the required levels, and generally this is the case for large bunker (pad) storages or old leaky silos, where it is difficult to achieve airtightness. There have been changes to the cylinder phosphine label in Australia over the last decade to address the development of strong phosphine resistance in *R. dominica*. For example, the current label of phosphine ($ECO_2FUME^{®}$) in Australia recommends that, to control a strongly resistant population of *R. dominica*, fumigation with a concentration of 1 mg $L^{-1}$ of phosphine should be undertaken within a gas-tight storage structure for 10, 9, 5 and 3 days at temperatures of 20, 25, 30 and 35 °C respectively. Given that they are much higher than the current registered protocols, we suggest that the protocols developed in the present study to manage strongly resistant populations of *C. ferrugineus* need to be incorporated in the label through changes to the current registration. To achieve this, it is imperative that industry-scale trials be undertaken.

In conclusion, protocols developed in the present study provide industry with some flexibility in application of phosphine at a range of temperatures of the stored commodity for management of infestations of strongly resistant *C. ferrugineus*. This type of flexibility allows grain storage managers to operate more economically, provided that the storage structures are properly sealed and gas is monitored during the course of fumigation. In large commercial bulk storage structures, high concentrations of phosphine such as the ones we are suggesting from this study (e.g. 2 mg $L^{-1}$ for 10 days) can be practically maintained by using a cylinderised formulation (e.g. $ECO_2FUME^{®}$ and $VAPORPH_3OS^{®}$). Moreover, connecting a recirculation system enables rapid and

even distribution of the gas throughout the storage structure. This approach also has the advantage of topping up the gas during the fumigation period if the monitoring system indicates the loss of gas. Phosphine is generally considered to be a cheap fumigant, and managing strongly resistant *C. ferrugineus* far outweighs the additional costs involved in the higher dosages such as 2 mg $L^{-1}$ for 10 days. Moreover, additional costs in the form of higher phosphine dosages are sufficiently justified in the case of fumigating oilseeds and pulses, where use of an alternative fumigant such as sulfuryl fluoride is not applicable. We conclude that, given these advantages, the research output from the present study will help to sustain the usefulness of phosphine into the foreseeable future.

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