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Short Communication

Susceptibility of Pupal Stage of Granary Weevil, *Sitophilus granarius* L. (Coleoptera: Dryophthoridae), by Phosphine Fumigation under Oxygen-Enriched Air

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Abstract: There are ongoing global efforts to minimize the use of methyl bromide (MB), which has been in use as an effective fumigant for plant quarantine but was recognized as against environmental protection in the context of ozone layer issues. As phosphine (PH_3) is an alternative fumigant for the granary weevil, *Sitophilus granarius* L., to replace MB, this study examined how the susceptibility of its pupal stage by PH_3 fumigation would be affected under oxygen-enriched conditions. The fumigation dose of 2.0 mg/l of PH_3 at 15°C under various O_2 concentrations and for different fumigation durations revealed that higher O_2 concentration resulted in higher effects of mortality for the same fumigation duration. 100% mortality was demonstrated under 30% and 40% O_2 for 9 days fumigation, and LT_{50}, LT_{95}, and LT_{99} by logit analysis were 0.93, 3.38, and 4.75 days under 40% O_2, respectively. LT_{50} and LT_{95} were half or less than the values observed in the past study under normal atmospheric conditions. It was suggested that PH_3 fumigation under 40% or higher concentration of O_2 was effective treatment to obtain 95% mortality to the pupal stage of the granary weevil. The fumigation at the doses of 0.3, 0.5, and 1.0 mg/l of PH_3 did not result in 100% mortality under 30% and 40% O_2 for 9 days at 15°C . While the same doses were applied, higher mortalities were demonstrated under 40% O_2 than those under 30% O_2, and the 99.7% mortality or higher was demonstrated at the doses of 0.5 and 1.0 mg/l of PH_3 under 40% O_2. From these results, the condition of 40% O_2 was considered a more effective condition than 26% and 30% O_2 to the pupal stage of the granary weevil. Thus, 100% mortality was demonstrated to the pupal stage of the granary weevil by maintaining 2.0 mg/l of PH_3 concentration during fumigation under 30% O_2 concentration or higher for 9 days at 15°C . It was also implied that there would be possibilities to reduce the dose rate of PH_3 as well as shorten the fumigation duration while maintaining 100% mortality for the pupal stage of the granary weevil if 40% O_2 concentration or higher can be applied.

Key Words: phosphine, fumigation, oxygen-enriched, *Sitophilus granarius*, susceptibility

Introduction

The granary weevil, *Sitophilus granarius* L., is one of the quarantine pests recognized by Japanese plant quarantine regulations. Once the granary weevil is detected at import inspection, methyl bromide (MB) fumigation is required for imports, as is currently the only applicable fumigation method. MB, however, was one of the ozone depleting substance designated by Montreal Protocol, which came into force in 1992. Consequently, the Commission on Phytosanitary Measures (CPM), the governing body of International Plant Protection Convention (IPPC), developed and adopted a recommendation in 2008 that encouraged the contracting parties of the IPPC to make efforts to develop and to use alternative techniques to the MB fumigation (IPPC, 2008).

Aluminum phosphide (AlP) is one of the effective fumigants against insect pests in stored grains. It has been revealed that the pupal stage of the granary weevil is less susceptible to phosphine (PH_3), which is an active substance of AlP in regard to mortality effects (Mori and Kawamoto, 1966). Goto *et al.* (1996) examined the susceptibility of the pupal stage of three species of weevils, *Sitophilus granarius*, *Sitophilus oryzae*, and *Sitophilus zeamais*, by PH_3 fumigation. The results showed that the granary weevil was the species most susceptible to PH_3, and that 100% mortality of the granary weevil was demonstrated at a dose of 0.5 mg/l for 10 days at 20°C. And Hayashi *et al.* (2016) examined the various fumigation conditions to ensure 100% mortality of the pupal stage of the granary weevil by PH_3 fumigation, but concluded that 10 days or more were required to ensure 100% mortality at a dose of 2.0 mg/l at 15°C.

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Regarding conditions other than PH_3 doses, fumigation duration, and temperature, it is reported that oxygen (O_2) was an essential element to activate the mortality effect of PH_3 (Bond & Monro, 1967; Bond *et al.*, 1969). According to Liu (2011), super-atmospheric oxygen levels (30-80% of O_2) during the PH_3 fumigation resulted in higher mortality even at low temperature (2-10℃) using larvae and adults of the western flower thrips (*Frankliniella occidentalis*), pupae of the leafminer (*Liriomyza langei*), eggs of the grape mealybug (*Pseudococcus maritimus*), and eggs and pupae of the Indian meal moth (*Plodia interpunctella*).

The reports above imply that the effectiveness of PH_3 against various insect pests is enhanced under oxygen-enriched air. Susceptibility of the granary weevil by PH_3 fumigation was, therefore, examined under the oxygen-enriched conditions in order to identify effective and appropriate fumigation conditions.

Materials and Methods

1. Test insect

The granary weevil, *Sitophilus granarius* L., was originally derived from Great Britain with permission (Permit No. 12Y-336) from MAFF, and then reared on brown rice at the National Food Research Institute in Tsukuba, Ibaraki Prefecture. Some part of the rearing colony was introduced into the Research Division of Yokohama Plant Protection Station with the ministerial permission of MAFF (Permit No. 13Y-894). They were then reared on domestic wheat in a climate chamber with conditions of 25℃, 60% RH, 16L8D. The emerged adults (approximately 700-800, 10-14 days after emergence) were placed on wheat grains (150 g) to lay eggs in the chamber for 3 days, and then the adults were removed. 30 days after the removal of adults, 15 g portions of wheat grains infested with the pupal stage of the granary weevil were weighed and put into small plastic containers (8.3 cm diameter × 4.6 cm high) covered with mesh lids. These containers with test insects were acclimated at 15℃ (fumigation temperature) in a fumigation room overnight.

2. Fumigation

Fumigation was conducted using acrylic resin boxes of 6.0-liter volume (equipped with gas application and sampling ports, gas exhaust ports, and a temperature probe) in a temperature-controlled room at 15℃. O_2 gas cylinders (standard gas; Taiyo Nippon Sanso Corp.), which were adjusted at 30.1%, 40.1%, and 59.8% of O_2 concentrations (v/v, nitrogen balanced) were prepared. Mixture gas of the cylinders (30.1% and 40.1% of O_2) was flowed into the fumigation box at 2.0-2.5 l/min. for 15 minutes before dosing of PH_3 at the conditions of 30% and 40% O_2 concentration. An appropriate amount of mixture gas of the cylinder (59.8% O_2) was introduced into the decompressed box using a gas-tight syringe at the condition of 26% O_2 concentration. Then an appropriate volume of PH_3 gas from cylinder (10.2% v/v, nitrogen balanced; Takachiho Chemical Industrial Co. Ltd.) was applied into the box using the gas-tight syringe. Mixture gas in the box was circulated using a magnetic stirrer for 3-4 hours after dosing of PH_3. Temperatures in the boxes were monitored using Ondo-tori WL (RTR-71; T&D Corp.) during fumigation. After the fumigation was completed, the mixture gas was exhausted using an aeration system for 1 hour, and the plastic containers were moved to the rearing conditions. Two series of experiments were carried out. The first group was to evaluate LT values and at the dose of 2.0 mg/l of PH_3 for 1, 2, 3, 6, and 9 days under the conditions of 26%, 30%, and 40% O_2 concentration at 15℃. The second group was to confirm the feasibility of this method and at the doses of 0.3, 0.5, and 1.0 mg/l of PH_3 for 9 days under the conditions of 30% and 40% O_2 concentration at 15℃.

3. Measurements of gas concentration of O_2 and PH_3

O_2 gas concentrations at the commencement of the fumigation were measured using a gas chromatograph (GC-2014 with TCD; Shimadzu Corp.). Analytical conditions were as follows: Carrier gas: He 15ml/min., Column: Molecular sieve 5A 60-80 mesh φ 3 mm × 3 m, Oven temp.: 40℃, Det. temp.: 120℃, Current: 120 mA. PH_3 gas concentrations at the commencement and the end of the fumigation were measured by a gas chromatograph (SRI 8610C with TCD; SRI Instruments). Residual gas rate of PH_3 at the end of fumigation was

Table 1. Number of replications, pupation rate, total number of pupae tested, and oxygen concentration of fumigation.

Treatment plot			Replication	Pupation rate[1)] (mean% ± SD)	Total number of pupae tested[2)] (n)	Actual O_2[3)] (mean% ± SD)
Target O_2 (%)	Dose rate (mg/l)	Duration (day)				
26	2.0	1, 2, 3, 6, 9	3	84.0 ± 1.3	974	26.5 ± 0.2
30			2	83.9 ± 1.0	598	29.5 ± 0.1
40			3	86.9 ± 3.8	1,200	39.3 ± 0.2
30	0.3, 0.5, 1.0	9	3	82.4 ± 3.1	1,022	29.7 ± 0.1
40			3	83.2 ± 0.5	861	39.4 ± 0.2

[1)] Wheat infested with granary weevil were frozen at the day of dosing and then pupation rates were examined.
[2)] Total number of pupae tested is the number of one treatment plot at the start of fumigation.
[3)] O_2 concentration was measured at the start of the fumigation.

calculated by a formula as follows:

$$\text{Residual gas rate (\%)} = \frac{\text{Final gas concentration (mg/l)}}{\text{Dose rate (mg/l)}} \times 100$$

4. Evaluation of mortality

Control plot of the dose of 2.0 mg/l of PH_3 was moved to the rearing conditions a day after dosing. Control plot of the doses of 0.3, 0.5, and 1.0 mg/l of PH_3 was moved to the rearing conditions at the end of fumigation. Emerged adults of control and treatment plots were observed periodically, and when adults were detected, they were counted and removed from the containers. Each treatment plot was continuously observed for adult emergence until 30 days after the last emergence for the each control plot. A 7.5 g sample of infested wheat grains was prepared for each replication, and frozen at -30℃ at the day of dosing. Pupation rate was estimated by dissecting this wheat. The number of pupae at the commencement of the fumigation was estimated by the number of emerged adults of the control plot multiplied by the pupation rate. Mortalities of treatment plots were evaluated by a formula as follows:

$$\text{Mortality (\%)} = \left(1 - \frac{\text{Number of adults of treatment plot}}{\text{Number of adults of control plot}}\right) \times 100$$

Mortality (%) of these tests were transformed by arcsine $\sqrt{x}$, and then analyzed by two-way analysis of variance (ANOVA) and Tukey-Kramer HSD test using computer program, JMP (SAS Institute). And the results of the dose of 2.0 mg/l of PH_3 were analyzed by logit analysis using a computer program, Polo Plus ver.1.0 (LeOra Software, Petaluma, CA, USA).

Table 2. Residual gas rate of PH_3 at the end of fumigation.

Treatment plot			Residual gas rate[1)] (mean% ± SD)
Target O_2 (%)	Dose rate (mg/l)	Duration (day)	
26, 30, 40	2.0	1	96.4 ± 1.7
		2	98.6 ± 1.1
		3	95.2 ± 2.6
		6	86.6 ± 2.1
		9	85.9 ± 9.2
30, 40	0.3	9	84.8 ± 2.8
	0.5		91.7 ± 10.0
	1.0		83.3 ± 4.1

[1)] Residual gas rate (%) = (Final gas concentration / Dose Rate) × 100

Results and Discussion

1. Conditions of fumigation experiments

The number of replications, pupation rate, total number of pupae tested, and actual O_2 concentration at the start of the fumigation are shown in Table 1. Averages of pupation rates were 82.4-86.9%. Actual O_2 concentrations at the commencement of the fumigations were 26.5%, 29.5%, 39.3%, 29.7%, and 39.4% in each treatment plot, respectively. Residual gas rates of PH_3 at the dose of 2.0 mg/l for 1-9 days in Table 2 were 98.6-85.9%. The same figures at the doses of 0.3, 0.5 and 1.0 mg/l of PH_3 for 9 days were 91.7-83.3%. Average temperatures during the fumigation were 14.6-14.9℃ in all fumigation experiments.

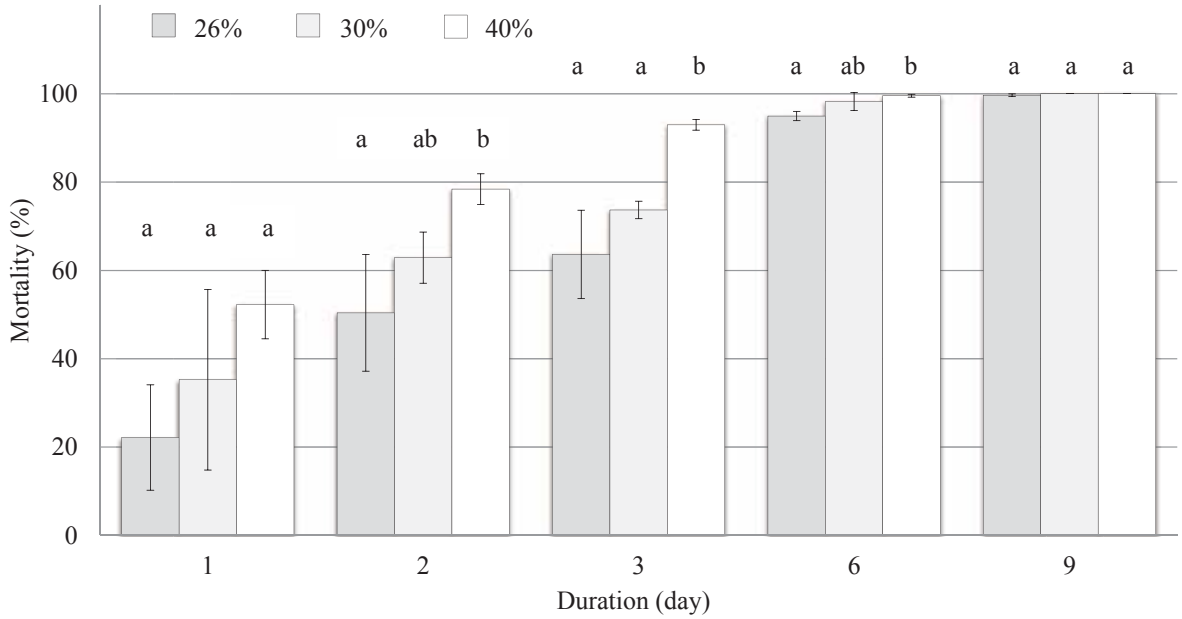

Fig. 1 Mortality of the pupal stage of *Sitophilus granarius* at the dose of 2.0 mg/l of PH_3 for 1, 2, 3, 6 and 9 days under 26, 30 and 40% of O_2 at 15℃ (mean% ± SD).

The mortality data were transformed by arcsin $\sqrt{x}$ before ANOVA. Different letters among different O_2 concentrations within the same duration were significantly different ($P < 0.05$; Tukey-Kramer HSD test).

Table 3. Estimated LT values of the pupal stage of *Sitophilus granarius* at the dose of 2.0 mg/l of PH_3 under 26, 30 and 40% of O_2 at 15ºC.

Target O_2 (%)	LT value[1)] (day)			Slope ± SE
	LT_{50}(95%CL)	LT_{95}(95%CL)	LT_{99}(95%CL)	
26	2.22 (1.86-2.56)	5.82 (5.04-7.12)	7.84 (6.66-9.83)	0.82 ± 0.03
30	1.58 (1.03-1.99)	4.99 (4.13-6.75)	6.90 (5.56-9.73)	0.86 ± 0.04
40	0.93 (0.74-1.08)	3.38 (3.12-3.72)	4.75 (4.32-5.33)	1.20 ± 0.05

[1)] LT values were culculated using logit model.

2. Fumigation at the dose of 2.0 mg/l of PH_3 under 26%, 30%, and 40% O_2

Effects on mortalities of the pupal stage of *Sitophilus granarius* at the dose of 2.0 mg/l of PH_3 at 15℃ are shown in Fig. 1. Higher concentration of O_2 showed higher mortality at the same duration. The two-way ANOVA did not find significant interactions between the O_2 concentration and the fumigation duration ($P = 0.0732$). Mortalities among different O_2 concentrations within the same duration were then analyzed by Turkey-Kramer HSD test. Since mortalities varied among the different concentrations of O_2, there was no significant difference for 1 day fumigation ($P > 0.05$). Mortalities of 2, 3, and 6 days fumigations under 40% O_2 were significantly higher than those under 26% O_2 ($P < 0.05$). 100% mortalities were demonstrated under 30% and 40% O_2 for 9 days, but not under 26% O_2. Since there was no significant difference in mortalities for 9 days fumigation among various concentrations, it was implied that fumigation duration was a more affecting factor on mortality than O_2 concentration. According to Hayashi *et al.* (2016), mortality of the pupal stage of the granary weevil was 99.6% at a dose of 2.0 mg/l of PH_3 for 9 days at 15℃ under normal atmospheric conditions. It was confirmed that the effectiveness on mortality of PH_3 was enhanced by the fumigation under 30% and 40% O_2.

Estimated LT values and slopes of regression lines are shown in Table 3. 95% confidence limits of LT_{50}, LT_{95}, and LT_{99} under 26% and 30% O_2 overlapped, and the slopes of those were almost the same. 95% confidence limits between the condition of 40% O_2 and others did not overlap, except for LT_{50}, and the slope under 40% O_2 was larger than the others. Goto *et al.* (1996) reported that LT_{50} and LT_{95} of the pupal stage of the granary weevil were 1.9 and 8.5 days at a dose of 2.0 mg/l at 15℃ under normal atmospheric conditions, respectively. Substantially lower LT_{50} and LT_{95} values were observed under 40% O_2, about half or less than those under normal atmospheric conditions. It was implied that the condition of 40% or higher concentration of O_2 was more effective to obtain 95% mortality or more of the pupal stage of the granary weevil by PH_3 fumigation.

3. Fumigation at the doses of 0.3, 0.5, and 1.0 mg/l of PH_3 under 30% and 40% O_2

In the fumigation tests with PH_3 at a dose of 2.0 mg/l, the sorption of PH_3 by commodities during fumigation was not considered. Fumigation tests at the doses lower than 2.0 mg/l of PH_3 were, therefore, conducted considering the resultant decrease of PH_3 gas concentration

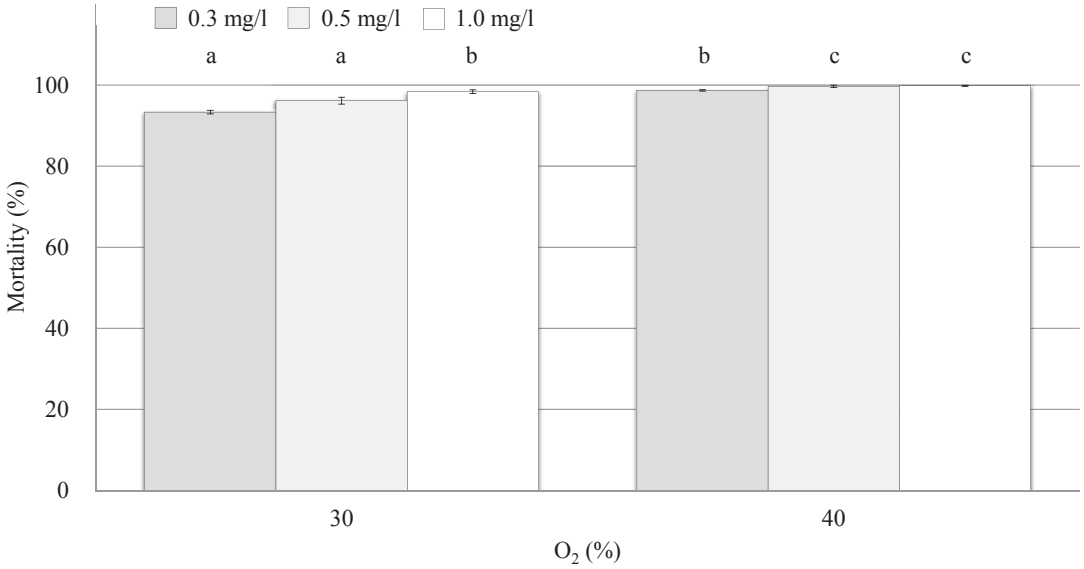

Fig. 2 Mortality of the pupal stage of *Sitophilus granarius* at the doses of 0.3, 0.5 and 1.0 mg/l of PH_3 for 9 days under 30 and 40% of O_2 at 15℃(mean% ± SD). The mortality data were transformed by arcsin $\sqrt{x}$ before ANOVA. Different letters were significantly different ($P < 0.05$; Turkey-Kramer HSD test).

at the end of fumigation. As shown in Fig. 2, average mortalities were 93.3%, 96.2%, and 98.4% at the doses of 0.3, 0.5, and 1.0 mg/l of PH_3 under 30% O_2, respectively. And these were 98.7%, 99.7%, and 99.8% under 40% O_2, respectively. The two-way ANOVA did not find significant interactions between the O_2 concentration and the dose rate ($P = 0.0912$). As mentioned above, no significant difference was found between 30% and 40% O_2 at the dose of 2.0 mg/l of PH_3 for 9 days. Then, mortalities of all the combinations of PH_3 dose rates and O_2 concentrations were analyzed by Turkey-Kramer HSD test to evaluate the effectiveness of O_2 concentration. No significant difference was observed between 1.0 mg/l of PH_3 under 30% O_2 and 0.3 mg/l of PH_3 at 40% O_2 ($P > 0.05$). This implies that the condition of 40% O_2 is more effective than 30% O_2. Mortality effects of 0.5 and 1.0 mg/l of PH_3 at 40% O_2 were significantly higher than the conditions above ($P < 0.05$). It was demonstrated that PH_3 fumigation at doses of 0.5 mg/l or higher of PH_3 under 40% O_2 for 9 days could result in 99.7% mortality or more against the pupal stage of the granary weevil.

In this study, it was revealed that PH_3 fumigation under the condition of oxygen-enriched air had a higher mortality effect on the pupal stage of the granary weevil than that under the normal atmospheric conditions. If 2.0 mg/l of PH_3 gas concentration is maintained for more than 9 days under the condition of 30% or more O_2, granary weevils will be killed completely. It was also implied that there would be possibilities to reduce the dose rate of PH_3 as well as the fumigation duration while maintaining 100% mortality for the pupal stage of the granary weevil if 40% O_2 concentration or higher can be applied. It is necessary to further clarify the relationship between grains and their sorption of PH_3 in order to establish the fumigation schedule for PH_3 fumigation under oxygen-enriched air in the future.

4. Flammable nature of PH_3

The flammable nature of PH_3 is a practical concern in the actual application of PH_3 fumigation, as it easily catches fire at normal temperatures. Kondo *et al.* (1995) reported that when O_2 concentration (%) becomes higher, the lowest PH_3 limit (%) to catch fire also becomes higher, which implied that higher O_2 concentration had a suppressing effect on the flammable nature of PH_3. At the same time, it is essential to avoid dust explosions with maximum care in PH_3 fumigation under oxygen-enriched air because oxygen has auxiliary combustive properties.

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和 文 摘 要

高酸素濃度下でのリン化水素くん蒸に対するグラナリアコクゾウムシ蛹の感受性（英文）

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臭化メチルは、長年植物検疫の効果的なくん蒸剤として使われてきたが、オゾン層保護の観点からその使用を最小限にとどめる努力が国際的に進められている。日本の検疫病害虫の一つであるグラナリアコクゾウムシに対しては、臭化メチルの代替剤としてリン化水素が有効であるが、本調査においては、酸素濃度を高くした条件下でリン化水素くん蒸によるグラナリアコクゾウムシ蛹の感受性がどう影響を受けるかについて試験を実施した。さまざまな酸素濃度とくん蒸日数において、リン化水素 2.0mg/l、15℃でくん蒸した結果、同じくん蒸期間でみると、より高い酸素濃度で殺虫効果がより高くなった。100% の殺虫効果が酸素濃度 30 及び 40%、9 日間で達成された。ロジット解析による酸素濃度 40% での LT_{50}、LT_{95} 及び LT_{99} は、それぞれ 0.93、3.38 及び 4.75 日であり、LT_{50} 及び LT_{95} は通常大気条件下で行われた過去の調査結果の値の半分以下であった。酸素濃度 40% 以上でのリン化水素くん蒸が、グラナリアコクゾウムシ蛹に対して 95% 以上の殺虫効果を得るために効果的であると考えられた。リン化水素 0.3、0.5 及び 1.0mg/l、酸素濃度 30 及び 40%、9 日間、15℃でのくん蒸の結果、100% の殺虫効果は得られなかった。同じ薬量で比較すると酸素濃度 40% で殺虫効果は高く、酸素濃度 40%、薬量 0.5 及び 1.0mg/l では、99.7% 以上の殺虫効果が得られた。

以上の結果から、グラナリアコクゾウムシ蛹は、酸素濃度 30% 以上、9 日間、15℃で、くん蒸中リン化水素濃度 2.0mg/l を維持することにより 100% 殺虫されることが明らかとなった。また、酸素濃度を 40% よりも高くすることにより、グラナリアコクゾウムシ蛹を 100% 殺虫するリン化水素の投薬量及び処理期間を、低下、短縮することが可能であることが示唆された。

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