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Scaled-up ethyl formate fumigation to replace methyl bromide on traded mushroom to disinfest mushroom fly (*Lycoriella mali*)

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Abstract

Mushroom fly, *Lycoriella mali* (Diptera: Sciaridae), is the primary pest in imported mushrooms. The amount of *Tricholoma matsutake* imported from China increases every fall when it is harvested. When importing *T. matsutake,* disinfestation using methyl bromide (MB) or phosphine (PH_3) is performed to prevent the introduction of *L. mali*. However, MB will be phased out due to ozone-depletion, chronic toxicity to workers, and residual issues. PH_3 fumigation in mushroom disinfestation requires a long exposure time (24 h). In this study, we used ethyl formate (EF), which can replace MB and reduce exposure time. The efficacy of EF, PH3 and EF + PH3 on *L. mali* was evaluated. Using 4-h EF fumigation at 5 °C, the 3rd and 4th instar was the most tolerant stage in terms of 99% killed lethal concentration × time products ($LCt_{99\%}$). When 4-h EF fumigation at 5 °C was applied on all stages of *L. mali*, the $LCt_{99\%}$ values of EF were 73.1 g h/m^3 to the 1st and 2nd instar, 112.9 g h/m^3 to the 3rd and 4th instar, 68.9 g h/m^3 to pupae, and 20.1 g h/m^3 to adult. It was confirmed that combination treatment with EF + PH_3 had a synergistic effect on *L. mali*. The $LCt_{99\%}$ of EF + 0.5 g/m^3 of PH_3 to the 3rd and 4th instar was 48.3 g h/m^3. When only 140 g/m^3 of EF was applied for 4 h at > 5 °C and 35 g/m^3 of EF + 0.5 g/m^3 of PH_3 for 4 h at > 5 °C in commercial trials containing *T. matsutake*, proven efficacy (100%) on *L. mali* was confirmed. In the case of EF treatment only, phytotoxic damage occurred due to high Ct products, and there was no phytotoxic damage in combination treatment with EF + PH_3. This study provides a new guideline for EF + PH_3 combination treatment within a shorter exposure time (4 h) than existing PH_3 treatment (24 h) and replacement of MB use.

Keywords: *Lycoriella mali*, Ethyl formate, Phosphine, Alternatives, EF + PH_3, Synergistic effect

Introduction

Flies of the family Sciaridae occur almost worldwide in many different cultivating and perishable commodities [1]. Significant loss of cultivating mushrooms caused by several species such as *Lycoriella mali* and *Lycoriella ingenua* of sciarid in the mushroom industry has been reported from the USA, UK, and South Korea [2–5]. Efficacy of synthetic pesticides such as diflubenzuron, diazinon, methoprene, and phosphate insecticides such as dimethoate and acephate in *L. mali* has been reported [2].

In the mushroom trade among several countries including China and Korea, *Lycoriella* sp. is classified as a quarantine pest in some countries, including South Korea, and must be treated by phytosanitary disinfestation at ports. According to KATI [6], South Korea exported 7584 t of *Pleurotus eryngii*, the main exported mushroom, and imported 145 t of *Tricholoma matsutake* from mainly China in 2019.

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According to the phytosanitary guideline in Korea, imported mushrooms infested with *L. mali* must be chemically treated with methyl bromide (MB). In the case of exported mushrooms, pest free inspection was the last option to avoid rejecting them in countries where they are imported with the option not to treat with MB because it caused loss of quality. As well as phytotoxic damage to mushrooms treated with MB, its use has been phasing out because of ozone depletion properties and chronic toxicity to human in Korea [7]. MB fumigation on food commodities could be more difficult in the future because there is a need to update residual bromide ion and MB itself post all type of food commodities associated with the new Positive List System (PLS) in Korea [8]. In Korea, MB fumigation on treaded mushrooms will be discontinued after 2022 (Personal communication with MG Park), because there is currently no consumer safety data supported in Korea. In the case of PH_3 fumigation, as current alternative options, commercial adaptation might be difficult because a long exposure time is required in grains (> 5 days) and mushrooms (> 1 days), which could shorten the shelf life of mushrooms [9].

Ethyl formate (EF), an alternative to MB, is known to be safer than other fumigants in the workplace and there is residual free regulation in many countries because it is globally classified as a food additive. In practice, EF fumigation has been used in imported commodities such as fruits, vegetables, nursery plants, etc. [10, 11]. A new concept of EF application technology with N_2 (Non-CO_2) was developed and used commercially in Korea [9].

Although EF was effective fumigant, it has high sorption [12] to commodities like perishable fruits and vegetables and more less vaporization at low temperature [13]. PH_3 was good at permeability to commodites like timber [14]. Thus the new concept of fumigation has been studied that combined EF and PH_3, which was more effective to insect pests and less phytotoxic damage to commodities [15, 16].

No study evaluating the efficacy of EF on *L. mali* has been reported. Herein, we suggest new disinfestation guidelines for disinfestation of *L. mali* using EF and EF + PH_3, which are a replacement for MB.

We evaluated (1) Efficacy of EF for 4 h-fumigation on *L. mali* in lab studies, (2) sorption studies on several types of mushroom and gas penetration under imported conditions, (3) Synergestic effect of EF and PH_3 to *L. mali* and Phytotoxic damage to mushroom with EF + PH_3 fumigation. (4) application of liquid EF with N_2 application on a commercial scale for confirmation on imported mushrooms.

Materials and methods

Insects and chemicals

Lycoriella mali was collected from a mushroom farm in Yeongcheon, Gyeongbuk, South Korea during 2020. *L. mali* were transferred and reared in an insect rearing room at Gyeongsang National University. *L. mali* was maintained in the insect rearing room at 24°C and 60–70% relative humidity (RH) with a 16:8 [L:D] h. *Pleurotus eryngii* was provided as a food source. Female adults of *L. mali* lay eggs on water agar (2%) in the insect breeding dish (100 mm × 40 mm). Larvae pupated within 5–6 days, and adults emerged within 25 days; 1st, 2nd, 3rd and 4th instar larvae, pupae, and adults were used in this study. EF (Fumate™, > 99% purity; Hoengseong, Korea) was supplied by Safefume Co. Ltd in Korea. Phosphine was purchased as ECO_2Fume (2% PH_3 + 98% CO_2) from Cytec (Sydney, Australia).

Egg hatching test at low temperature

Egg hatching studies of *L. mali* were performed at 5 ± 0.5°C in an incubator. The eggs were collected form rearing cages with 200 mated females on the *Pleurotus eryngii* over 1 day and treated immediately. Before fumigation to eggs, 2% agar medium was laid on the bottom of the breeding dish (50 mm × 15 mm) to maintain moisture and cut pine of *P. eryngii* was placed on the agar medium. Then 20 eggs of *L. mali* were transferred to each cut pine of *P. eryngii*. Because the egg color is transparent, the cut pine of *P. eryngii* was dyed using natural pigments to make observation of the eggs easier. Following placcement in a 5°C incubator, the egg hatching rate was observed for 5 to 14 days. After 72 h of treatment, eggs were checked hatching rate. Treatment was replicated three times, and the control was replicated 10 times at room temperature (24 ± 1°C).

Efficacy to developmetal stages of *L. mali* with EF in a laboratory experiment

Efficacy of EF was evaluated for three different developmental stages (adult, larvae and pupae) of *L mali*. In the case of EF mixed with PH_3 studies, the 3rd and 4th instars of *L mali*, which is the most tolerant stage to EF, was evaluated. Adults of *L. mali* were transferred from a breeding dish (50 mm × 15 mm) using collecting equipment (Fulton, MX-991/U, Georgia) within three days of developing an adult. Adults of *L. mali* were fumigated at 5°C for 4 h with 1.0–9.0 g/m^3 of EF. The 1st and 2nd instar and the 3rd and 4th instar were classified and tested. From 1 to 7 days after hatching, the 1st and 2nd instar and 7 to 14 days were classified into the 3rd and 4th instar. The pupae were used within two days after

pupation. Larvae and pupae stages of *L. mali* were transferred from water agar (2%) to a breeding dish (50 mm × 15 mm). The 1st and 2nd instar of *L. mali* were fumigated at 5°C for 4 h with 1.0–30.0 g/m^3 of EF. The 3rd and 4th instar were fumigated at 5°C for 4 h with 1.0–45.0 g/m^3 of EF. The pupae were fumigated at 5°C for 4 h with 1.0–40.0 g/m^3 of EF.

The concentration of EF was measured using an Agilent portable GC 17A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) after separation on a DB5-MS Column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom CA). The oven temperature was 100°C The injector and detector temperatures were 250 and 280°C, respectively. Helium was used as a carrier gas at the flow rate of 1.5 mL/min. Headspace EF was calculated by the peak area against external EF standards.

After completion of 4 h fumigation, treated *L. mali* was transferred to an insect rearing room at 24°C and 60–70% relative humidity (RH) with a 16:8 [L:D] h. The mortality of the treated *L. mali* larvae and adults were determined by visual inspection of movement using a microscope 1 day after fumigation. The mortality of the treated *L. mali* pupae was determined by checking the number of adults for 5 days after fumigation. Usually, the rate of pupae to adults in the control group was 50% level. All treatments and controls were replicated three times.

The temperature was recorded using data loggers (Thermo Recorder TR-72Ui, T&D Corp., Japan). In this study, a 6.8 L desiccator was used in the experiment as a fumigation chamber. The desiccators were sealed with a glass stopper equipped with a septum (Alltech Associates Australia, Cat. No. 15419). The exact volume of desiccators was measured using weigh of water. The desiccators were tightly sealed with high vacuum grease (Dow Corning, USA). A filter paper (Whatman No. 1) was inserted into the glass stopper to make clear evaporation in the desiccator for the injected EF. A magnetic bar to stir the fumigant was located at the bottom of the desiccator. The dose of fumigant and Ct products was calculated using the method reported by Ren et al. [14].

Efficacy to 3rd and 4th larvae of *L. mali* with EF combined with PH_3 in a laboratory experiment

For EF + PH_3 efficacy studies, the 3rd and 4th instars of *L mali* were trasnffered on agar petri dish in desiccator (6.8 L). The larvae in the desiccators were fumigated at 5°C for 4 h with 4.0–28.0 g/m^3 of EF mixed with 0.5 and 1.0 g/m^3 of PH_3. Analysis of EF concentration was as shown above, the concentration of PH_3 was measured using Agilent 7890A equipped with a flame photometric detector (FPD) and HP-PLOT/Q (30 m × 530 μm × 40 μm, Agilent, Santa Clara, CA) operating in split mode (10:1). The temperature of the injector and the oven was 200℃. The temperature of the detector was 250°C. The injection volumes and flow rate of EF and PH_3 were 60 μl and 20 and 1.5 and 5 ml/min, respectively.

Determination of the synergistic effect of ethyl formate mixed with phosphine against the 3rd and 4th larvae of *L. mali*

After efficacy to EF alone fumigation and EF combined with PH_3 fumigation, we evaluated synergistic effect. A synergistic effect was measured by synergistic ratios (SRs). Synergistic ratios are defined by Hewlett and Plackett [17] and based on equation. 1.

$$SR = \frac{\text{L(Ct) of ethyl formate only}}{\text{L(Ct) of ethyl formate + phosphine}} \quad (1)$$

*SR = 1 describes additive action,
SR < 1 describes antagonism,
SR < 1 describes synergism.

Sorption test of *T. mastutake* for 4 h EF fumigation in a laboratory experiment

Evaluation of sorption of EF on *T. matsutake* was performed in a 6.8 L desiccator under lab condition. Each *T. matsutake* was filled at a 0.5, 1, and 1.5% filling ratio (w/v) for the sorption test; 140 g/m^3 of EF was applied using a syringe for 4 h at 5°C. Gas sampling was performed at time intervals (10 min, 1, 2, and 4 h) and measurements were performed using an Agilent portable GC 17A (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID) after separation on a DB5-MS Column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA).

Gas penetration test of EF on packed mushrooms with logistic consideration (Styrofoam box)

In the case of import and export *Tricholoma matsutake,* a styrofoam box with an ice pack was used as packing materials. Evaluation of gas penetration of EF on packed mushrooms (*Tricholoma matsutake*) was performed in a 0.275 m^3 fumigation chamber at 5°C. Each mushroom was filled at a 1.0% filling ratio (w/v) for the gas penetration test; 35, 70 and 140 g/m^3 of EF was applied using a special vaporizer (supplied by Safefume Co.) for 4 h at 5°C. The two conditions (inside, outside of the Styrofoam box) of EF concentration were analyzed. Gas sampling was performed at time intervals and measurements were performed using an Agilent portable GC 17A.

Commercial scaled-up fumigation

EF alone fumigation on Tricholoma matsutake

Scale-up fumigation was performed using a 25 m^3 Tarp-fumigation chamber in the Incheon airport warehouse in South Korea. *Tricholoma matsutake* were placed in a styrofoam box (1.7% f.r.) and the cover of the box was opened, which is based on commercial use. Then, 140 g/m^3 of EF was applied for 4 h at 5°C. Liquid EF was vaporized with SFM-I (supplied by Safefume Co.) with N_2 as the carrier gas and a fan was placed at the bottom of the Tarp-chamber for efficient gas circulation. The concentrations of the inside and outside of the styrofoam box were analyzed. Three of the insect breeding dishes containing > 300 larvae of *L. mali* (total 1079) were then located in the styrofoam box. Gas sampling was collected at time intervals (0, 1, 2, and 4 h) and EF concentration inside and outside of the styrofoam box was measured using GC-FID. Because measurement with GC-FID directly at the field is impossible, the concentration was checked in advance at the field using a gas analyzer (IBRID MX6; Industrial Scientific, Pittsburgh, PA, USA). A previous study found no sigfinicant difference in the measured concentration of IBRID and GC-FID. Fumigated larvae of *L. mali* were transferred to an insect rearing room. The mortality of larvae was determined 1-d after fumigation. All treatments and controls were replicated three times.

EF fumigation mixed with PH_3 on Tricholoma matsutake

Scale-up fumigation was performed using a 5 m^3 fumigation chamber in the same APQA site in South Korea. *Tricholoma matsutake* were placed in a styrofoam box (0.5% f.r.) and the cover of the box was opened, which is based on commercial use. Then, 35 g/m^3 of EF mixed with 0.5 g/m^3 of PH_3 was applied for 4 h at 5°C. Liquid EF was vaporized using a vaporizer (supplied by Safefume Co.) with N_2 as the carrier gas and a mini fan was placed at the bottom of the chamber for efficient gas circulation. The concentration of PH_3 was achieved by injecting 125 ml of 2% PH_3 into a 5 m^3 fumigation chamber. The concentrations of the inside and outside of the styrofoam box were analyzed. Three insect breeding dishes containing > 300 larvae of *L. mali* (total 1,097) were then located in the styrofoam box. Gas sampling was collected at time intervals (0, 1, 2, and 4 h) and EF and PH_3 concentrations inside and outside of the styrofoam box were easured using GC-FID and GC-FPD. Fumigated larvae of *L. mali* were transferred to an insect rearing room. The mortality of larvae was determined 1-d after fumigation. All treatments and controls were replicated three times.

Phytotoxic assessment

The phytotoxic damage of EF on mushrooms (*Pleurotus eryngii, Tricholoma matsutake*,) was evaluated in scaled-up studies (5 m^3 fumigation chamber). Two types of mushrooms were filled at a 1.5% filling ratio; 140 g/m^3 of EF for *Pleurotus eryngii* and 140 g/m^3 of EF and 35 g/m^3 of EF + 0.5 g/m^3 PH_3 for *Tricholoma matsutake* were applied using a special vaporizer (supplied by Safefume Co.) for 4 h at 5°C. After completion of fumigation, mushrooms were transferred to storage at 5 ± 0.8°C. The deterioration degrees were classified according to symptoms of the water condensation surface of the cap and changing a soft cap, grill, stem parts (0: Non, 1: water condensation, 2: water condensation following changing a soft cap and grill parts 3: water condensation following changing a soft cap, grill and then stem part). A color change (hue value) using a color meter (TES 135A, Taiwan), weght loss (%, weight differences before and after treatment) and market value based on the hardness of head parts (the harder the best, the softer the worst) were evaluated 3-d after fumigation.

Statistical analysis

Analysis of the toxicological dose response to EF by *L. mali* was based on a Probit analysis (Finney, 1971). As part of the analysis, the slopes of the Probit transformations were determined as well as Chi-square tests of data homogeneity for different treatments. The indices of toxicity measurement derived from this analysis were $L(Ct)_{50\%}$ = median lethal concentration that causes 50% response (mortality) and $L(Ct)_{99\%}$ = lethal concentration that causes 99% response (mortality) of exposed *L. mali* determined from a range of at least 10 different Ct products to ensure that the observed data covered mortality from 0 to 100% and adequately covered the intermediate range. For analysis of the hatchability of eggs on two temperatures and phytotoxic damage assessment of EF fumigation, on mushrooms, a T-test procedure was used to compare the two sample means. The EF alone fumigation and EF combined with PH_3 fumigation against phytotoxicity of mushrooms were compared using Tukey's test in scaled-up trials. All statistical analyses were performed using SAS (ver. 9.4; SAS Institute Inc.) [18].

Results and discussion

Egg hatchability test at low temperature

This study was conducted to investigate eggs hatching in low temperature conditioned mushrooms. According to previous ecological studies on *L. mali*, the average length of a generation from egg to adult was 28 d at 21°C [19]; it

Table 1 Egg hatchability of *Lycoriella mali* under two conditions (5 ± 0.5℃, 24 ± 1.0℃)

Day after oviposition	Temperature (mean ± SE, ℃)	No. of tested	No. of hatched	*p*-value	Hatchability (mean ± SE, %)
5	5 ± 0.5	60	0	< 0.0001	0.0 ± 0.0
	24 ± 1.0	60	52		88.3 ± 1.7*
6	5 ± 0.5	60	0	< 0.0001	0.0 ± 0.0
	24 ± 1.0	60	60		100.0 ± 0.0
7	5 ± 0.5	60	0	< 0.0001	0.0 ± 0.0
	24 ± 1.0	60	60		100.0 ± 0.0
8	5 ± 0.5	60	0	–	0.0 ± 0.0
9	5 ± 0.5	60	0	–	0.0 ± 0.0
10	5 ± 0.5	60	0	–	0.0 ± 0.0
11	5 ± 0.5	60	0	–	0.0 ± 0.0
12	5 ± 0.5	60	0	–	0.0 ± 0.0
13	5 ± 0.5	60	0	–	0.0 ± 0.0
14	5 ± 0.5	60	0	–	0.0 ± 0.0

* All the unhatched eggs were emergedll at 6-d after oviposition

- impossible to check

Table 2 LCt (Lethal Concentration × time) value of EF fumigation for 4 h expoure on *Lycoriella mali* at 5 ± 0.5 ℃

Stage	$L(Ct)_{50\%}$ (95% CL)	$L(Ct)_{99\%}$ (95% CL)	Slope ± SE	*df*	χ^2
1st, 2nd instar	43.6 (37.1–53.3)	73.1 (68.4–100.2)	3.1 ± 0.3	21	28.9
3rd, 4th instar	27.84 (22.53–33.01)	112.9 (90.70–152.25)	3.2 ± 0.3	28	48.47
Pupae	36.8 (28.9–46.1)	68.9 (57.6–97.1)	4.3 ± 0.3	15	40.2
Adults	7.8 (5.8–13.9)	20.1 (16.6–26.2)	2.0 ± 0.4	13	32.1

took five days from eggs to hatching. The egg hatchability under normal temperature condition (24 ± 1°C) were 88.3 ± 1.7% and 100.0 ± 0.0% at the day of the 5th and 6th day after oviposition (Table 1). However, we confirmed that the eggs under low temperature (5.0 ± 0.5°C) did not hatch at all. Based on logistic distribution of imported *T. matsutake* from China, it takes at least five days from harvest to consumers, all logistics is under < 5°C, meaning that survival of the egg might be diffidult under cold temperature conditions if found in imported mushrooms.

Efficacy to developmetal stages of *L. mali* with EF in a laboratory experiment

The efficacy of 4 h EF fumigation (practical exposure condtion using EF fumigations in Korea) on larvae, pupae, and adults stages of *L. mali* at 5℃ is shown in Table 2. For the 1st and 2nd of *L. mali* larvae, the $L(Ct)_{50\%}$ and $L(Ct)_{99\%}$ values of EF were 43.6, 73.1 g h/m^3. For the 3rd and 4th of *L. mali* larvae, the $L(Ct)_{50\%}$ and $L(Ct)_{99\%}$ values of EF were 25.0 and 112.9 g h/m^3. The $L(Ct)_{50\%}$ and $L(Ct)_{99\%}$ values of EF on *L. mali* pupae were 36.8 and 68.9 g h/m^3 andadults were 7.8 and 20.1 g h/m^3 at 5°C (Table 2), respectively. Thus, the order of susceptively

Table 3 Efficacy of PH3, EF and EF + PH_3 fumigation for 4 h expoure on *Lycoriella mali* at 5 ± 0.5℃

Fumigant dose	Ct products (g h/m^3)	Mortality ± SE (%)
Control	0.0	0.0 ± 0.0
PH_3 0.5 g/m^3	1.8	0.0 ± 0.0
PH_3 1.0 g/m^3	3.5	0.0 ± 0.0
EF 6.0 g/m^3	14.8	20.0 ± 2.9
EF 10.0 g/m^3	26.4	46.7 ± 1.7
EF 18.0 g/m^3	44.9	86.7 ± 1.7
EF 26.0 g/m^3	65.5	95.0 ± 0.0
PH_3 0.5 g/m^3 + EF 6.0 g/m^3	1.8 + 15.2	41.7 ± 1.7
PH_3 0.5 g/m^3 + EF 10.0 g/m^3	1.8 + 26.6	40.0 ± 2.9
PH_3 0.5 g/m^3 + EF 18.0 g/m^3	1.8 + 45.7	100.0 ± 0.0
PH_3 0.5 g/m^3 + EF 26.0 g/m^3	1.8 + 65.7	100.0 ± 0.0
PH_3 1.0 g/m^3 + EF 18.0 g/m^3	3.6 + 47.0	100.0 ± 0.0
PH_3 1.0 g/m^3 + EF 26.0 g/m^3	3.5 + 65.6	100.0 ± 0.0

Table 4 LCt (Lethal concentration x time) value of EF mixed with 0.5 g/m^3 PH_3 for 4 h exposure on *Lycoriella mali*

Temp (℃)	Stage	$L(Ct)_{50\%}$ (95% CL)	$L(Ct)_{99\%}$ (95% CL)	Slope ± SE	*df*	χ^2
5	3rd, 4th instar	34.4 (33.3–35.7)	48.3 (44.9–53.9)	15.8 ± 1.8	16	77.59

Table 5 Syngeristic efficacy of EF mixed with 0.5 g/m^3 PH_3 on 3rd–4th larvae stage of *Lycoriella mali*

Temp (℃)	Stage	[a]Synergistic ratios $L(Ct)_{50\%}$	[b]Synergistic ratios $L(Ct)_{99\%}$
5	3rd–4th larvae	0.81	2.34

[a] Synergistic ratios (SRs): $L(Ct)_{50\%}$ of ethyl formate only/ $L(Ct)_{50\%}$ of ethyl formate + 0.5 g/m^3 phosphine

[b] Synergistic ratios (SRs): $L(Ct)_{99\%}$ of ethyl formate only/ $L(Ct)_{99\%}$ of ethyl formate + 0.5 g/m^3 phosphine

of *L. mali* life stages to EF based on $L(Ct)_{99\%}$ values was adults > pupae > 1st and 2nd instar > 3rd and 4th instar larvae. *Drosophila suzukii* (Diptera: Drosophilidae) of the other flies was also most tolerant larval stage except for eggs [20].

Efficacy to 3rd and 4th larvae of *L. mali* with EF combined with PH_3 in a laboratory experiment

The efficacy of EF + 0.5 g/m^3 PH_3 fumigation for 4 h on the 3rd and 4th of *L. mali* at 5°C is shown in Table 3. The mortality of *L. mali* was no effect with PH_3 of 0.5 and 1.0 g/m^3 for 4 h fumigation, and was 86.7% with EF of 18.0 g/m^3 for 4 h fumigation, Otherwise, EF combined with PH_3 of 0.5 g/m^3 fumigation for 4 h was controlled 100% against 3rd and 4th instar of *L. mali* (Table 3). The $L(Ct)_{50\%}$ and $L(Ct)_{99\%}$ values of EF + 0.5 g/m^3 PH_3 against the 3rd and 4th of *L. mali* were 34.4 and 48.3 g h/m^3 at 5 °C, respectively (Table 4). According to the results of a preliminary experiment at 5°C, the 3rd and 4th instar were the most tolerant stages of *L. mali*. It was confirmed that application of EF + 0.5 g/m^3 PH_3 to *L. mali* had a synergistic effect when treated $L(Ct)_{99\%}$ at 5°C (Table 5). The fumigant, PH_3 was required more longer exposure times to kill insect pests than EF [21] and EF was needed to high concentration to control insect pests at low temperature [22]. But mixed usage of these fumigants would be better to control insect pests than alone [15, 16].

Sorption test of *T. mastutake* for 4 h EF fumigation in a laboratory experiment

Based on the efficacy studies resulting in $L(Ct)_{99\%}$ values of EF on *L. mali* larvae, which is the most tolerant to EF fumigation in this study, estimated applicable schedules (140 g/m^3 EF for 4 h exposure) were performed on *T. matsutake*. The losses of EF inside the fumigated desiccators and their adsorption are shown in Fig. 1.

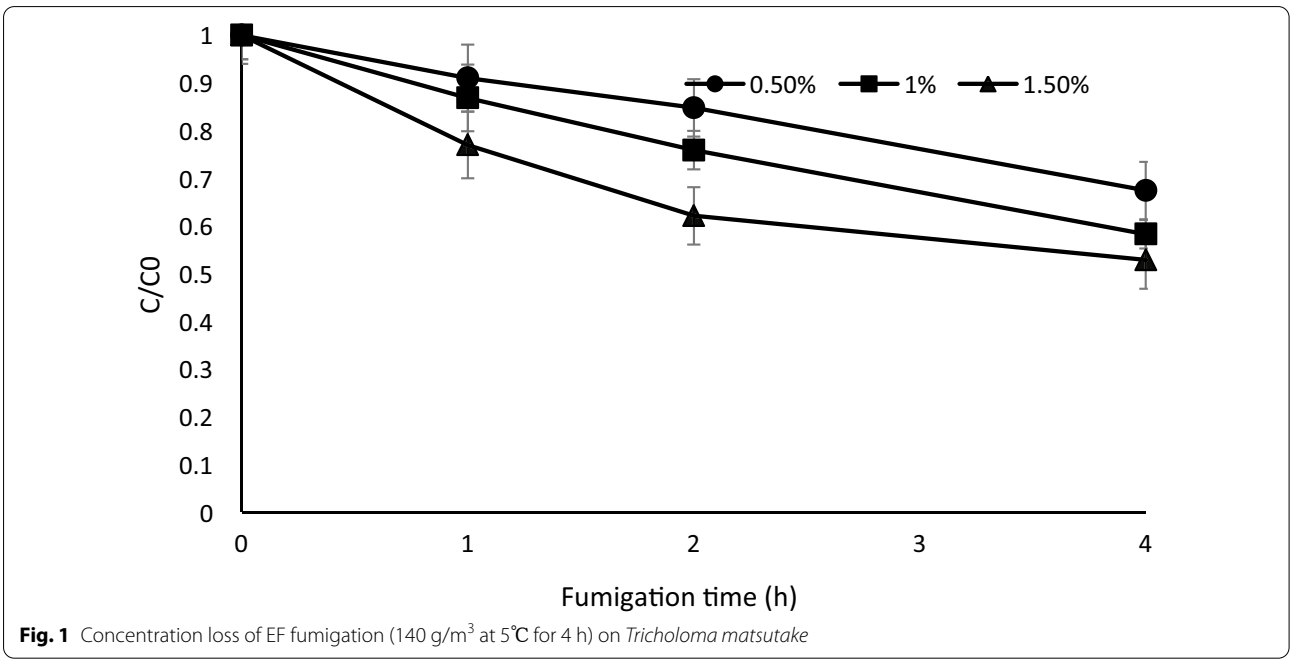

Fig. 1 Concentration loss of EF fumigation (140 g/m^3 at 5℃ for 4 h) on *Tricholoma matsutake*

The concentration of EF decreased during 4 h of exposure on mushrooms. Loss rate of EF on *T.matsutake* was relatively low, approximately 30% at f.r. 1.5%. But EF was required high concentration due to their high sorption to commodities like fruits and vegetable, which can cause to phytoxic damage to commodities [23–25].

Gas penetration test of EF on packed mushroom with styrofoam box

A gas penetration test was performed using a 0.275 m^3 fumigation chamber with different filling ratios of *T. matsutake.* As a result of EF gas penetration, there was a difference between the inside and outside of the styrofoam box (Fig. 2). When EF 35, 70, 140 g/m^3 was applied, Ct products inside the styrofoam box were 60.2 ± 2.4, 95.0 ± 2.9 and 167.1 ± 6.1 g h/m^3 at f.r. 1.0%, respectively. However, EF Ct products outside the styrofoam box were 89.4 ± 2.2, 136.3 ± 2.3 and 219.7 ± 4.1 g h/m^3 at f.r. 1.0%, respectively, shown in Fig. 2. Regarding the initial concentration, the concentration of inside was 15–40% of the concentration of outside. After 2 h of treatment, the inside and outside concentrations were similar. The calculation of Ct products in the lab might be different when applied in actual scaled-up trials because it is denpendent on sealing conditions, temperature, and condition of commodities (water content, logistic packing, etc.). According to our data when determing EF concentration to achieve target Ct products (112.9 g h/m^3, Probit estimation for 99% mortality on *L. mali*) with 4 h exposure, 100 g/m^3 of EF might be applicable. However, > 100 g/m^3 of EF could be obtained with proven efficacy (> 99%) under the worst circumstance of commercials. EF concentration inside the styrofoam box containing *T. matsutake* was lower than outside. The difference of EF concentration inside and outside the box might be different depending on fanning conditions (running time, numbers of fans, locations of fan, electrical capacity, etc.) during the EF fumigition. Based on unpubuished data, we suggested using a fan for more than 2 h at low-middle capactity for even distribution of gas.

Commercial sized EF fumigation

EF alone fumigation on Tricholoma matsutake

The scaled-up EF fumigation to confirm disinfestation on *L. mali* was performed using a 25 m^3 Tarp-fumigation chamber filled with *T. matsutake* (1.7% f.r. w/v). When 140 g/m^3 was applied for 4 h at 5°C, *L. mali* achieved 100% mortality (total 1079 of fumigated *L. mali* larvae used). The acculatumed Ct products of EF were 159.0 ± 3.1, 147.2 ± 2.8 g h/m^3 inside, 195.3 ± 4.3 g h/m^3 outside the styrofoam box (Fig. 3, Table 6). We confirmed more than achievable $L(Ct)_{99\%}$ values and the loss rates of EF were approximately 70–80% for 4 h exposure, which was similar to previous lab condition studies. When conducting this test, we tried to confirm the mortality for *L. mali* naturally infested in *T. matsutake*, however collection of a large amount of *T. matsutake* was impossible and the number of infested *L. mali* per mushroom was too small. For a small number of *L. mali*, 100% mortality

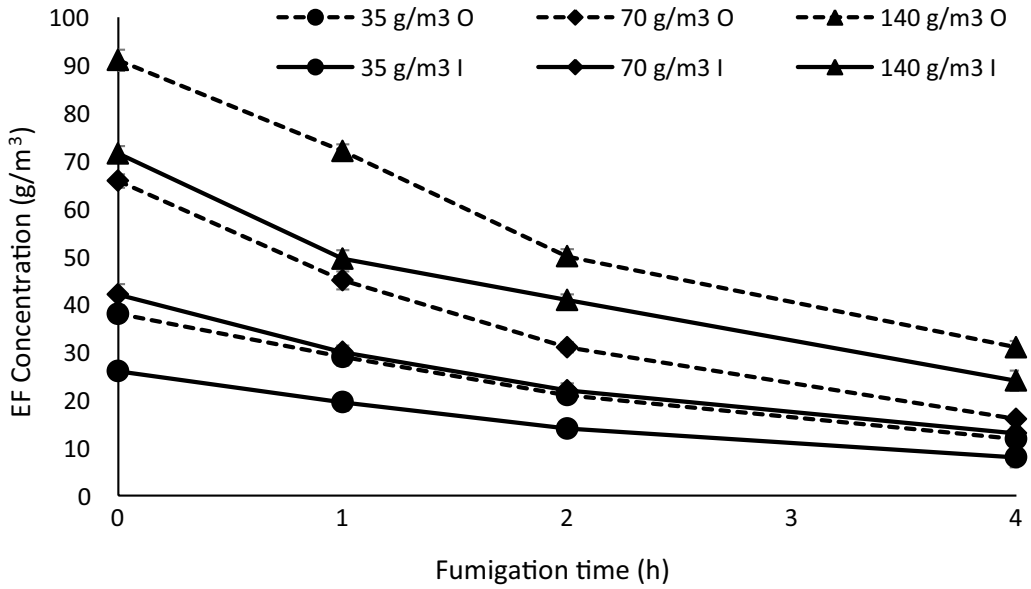

Fig. 2 Gas concentration, when fumigated with 35, 70, and 140 g/m^3 EF for 4 h inside (I) and outside (O) of styrofoam box containing 1.0% f.r. of *Tricholoma matsutake* (temp. 5.1 ± 0.3℃)

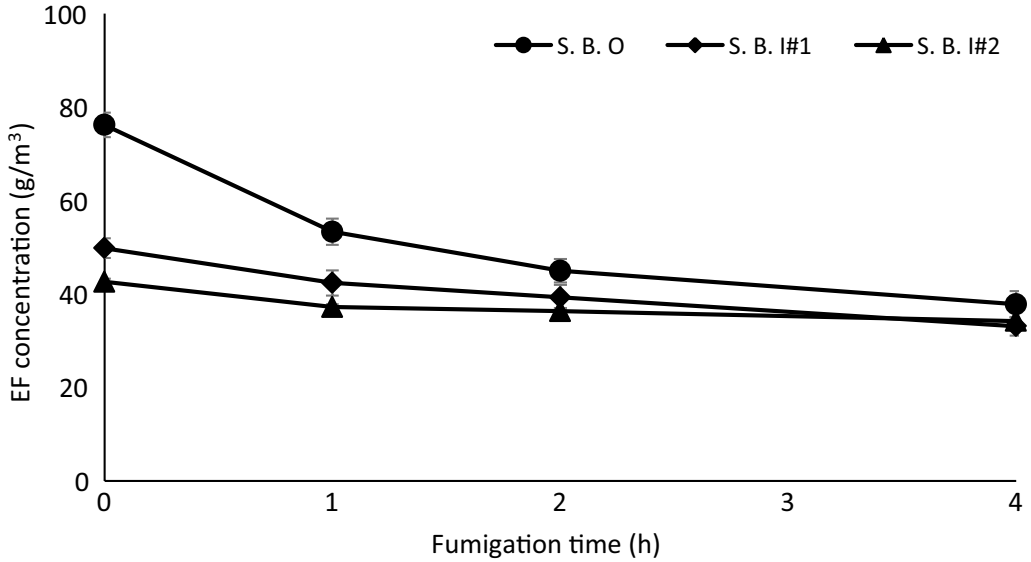

Fig. 3 Gas concentration when fumigated with 140 g/m^3 EF for 4 h on *Tricholoma matsutake* (f.r. 1.7%) in scaled-up trials (25 m^3 Tarp-fumigation chamber, temp.: 7.3 ± 0.4 ℃). *Gas concentration inside (S. B. I) and outside (S. B. O) the styrofoam box

Table 6 Scaled-up trials of liquid EF-fumigaion and EF + PH3 fumigation on *Tricholoma matsutake*

Type and size of fumigation	Appl. Dose (g/m^3)	Packing and gas sampling	Ct products (g h/m^3)	Mort. (%) (dead/total)	Deterioration degree[1]	Market value[2]
Fumigation chamber[1] (25 m^3)	–	Untreated	–	0 (0/159)	0 a	B1 B2 C
	EF 140.0	S.B.(I#1)	159.0 ± 3.1	100 (331/331)	3 b	D D D
		S.B.(I#2)	147.2 ± 2.8	100 (387/387)	3 b	D D D
		S.B.(O)	195.3 ± 4.3	100 (361/361)	3 b	D D D
Fumigation chamber[2] (5 m^3)	–	Untreated	–	0 (0/159)	0 a	B1 B2 C
	EF 35.0 + PH_3 0.5	S.B.(I#1)	50.6 ± 0.6	100 (397/397)	0 a	B1 B2 C
		S.B.(I#2)	50.2 ± 1.1	100 (356/356)	0 a	B1 B2 C
		S.B.(O)	66.4 ± 0.1	100 (344/344)	0 a	B1 B2 C
				p-value	< 0.0001	< 0.0001

S.B: Styrofoam box, Inside (I), Outside (O)

1. Condition: f.r. 1.7%, 7.33 ± 0.39 ℃

2. Condition: f.r. 0.5%, 4.94 ± 0.21 ℃

[1] The deterioration degrees. 0: Non, 1: water condensation, 2: water condensation following changing soft a cap and grill parts 3: water condensation following changing soft a cap, grill and then stem part

[2] Market value based on commercial grades. A: button, B1: young mushroom, B2: mature mushroom, C: overmature mushroom, D: not for sale

was achieved. Regarding weight loss, in assessment of 3 d after fumigation, *T. matsutake* was not significantly different (*df*: 16, *p*-value: 0.8062) (Fig. 4). Likewise, regarding color change, *T. matsutake* was not significantly different (*df*: 16, *p*-value: 0.8198) (Fig. 5.).

EF fumigation mixed with PH3 on Tricholoma matsutake

We proposed EF + PH_3 combination fumigation. It was performed using a 5 m^3 container filled with *T. matsutake* (0.5% f.r. w/v). When 35 g/m^3 of EF + 0.5 g/m^3 of PH_3 was applied for 4 h at 5 °C, *L. mali* achieved 100% mortality (total 1,097 of fumigated *L. mali* larvae used). The acculatumed Ct products of EF were 50.6 ± 0.6, 50.2 ± 1.1 g h/m^3 inside, 66.4 ± 0.1 g h/m^3 outside the styrofoam box (Fig. 6, Table 3). We confirmed more than achievable $L(Ct)_{99\%}$ values and the loss rates of EF were approximately 60–70% for 4 h exposure, which was similar to previous lab condition

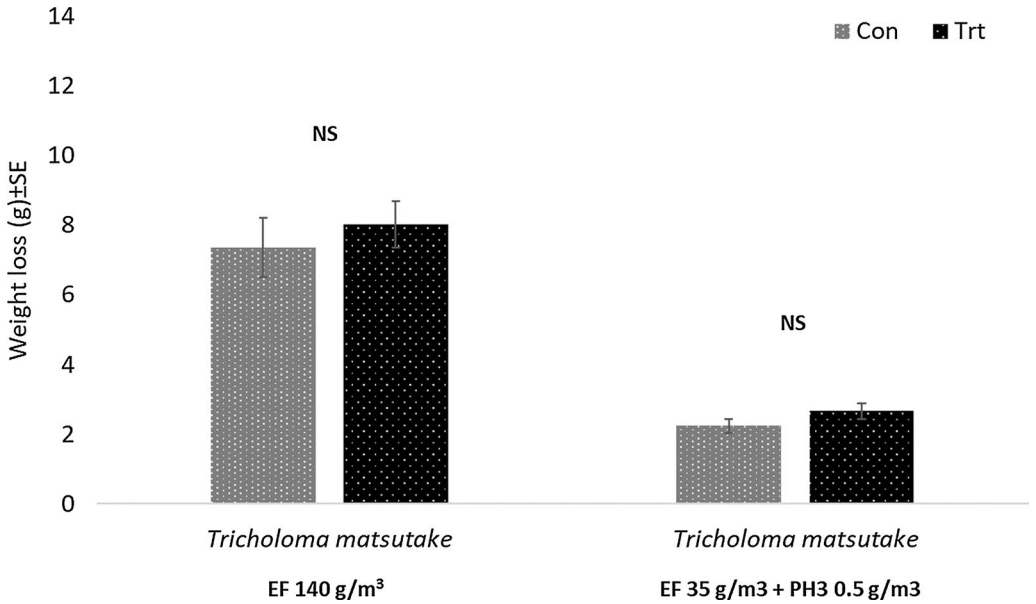

Fig. 4 Weight loss after 3-d fumigation on mushrooms fumigated with 140 g/m^3 EF only and 35 g/m^3 EF mixed with 0.5 g/m^3 PH_3 (4 h exposure). NS: Not significant

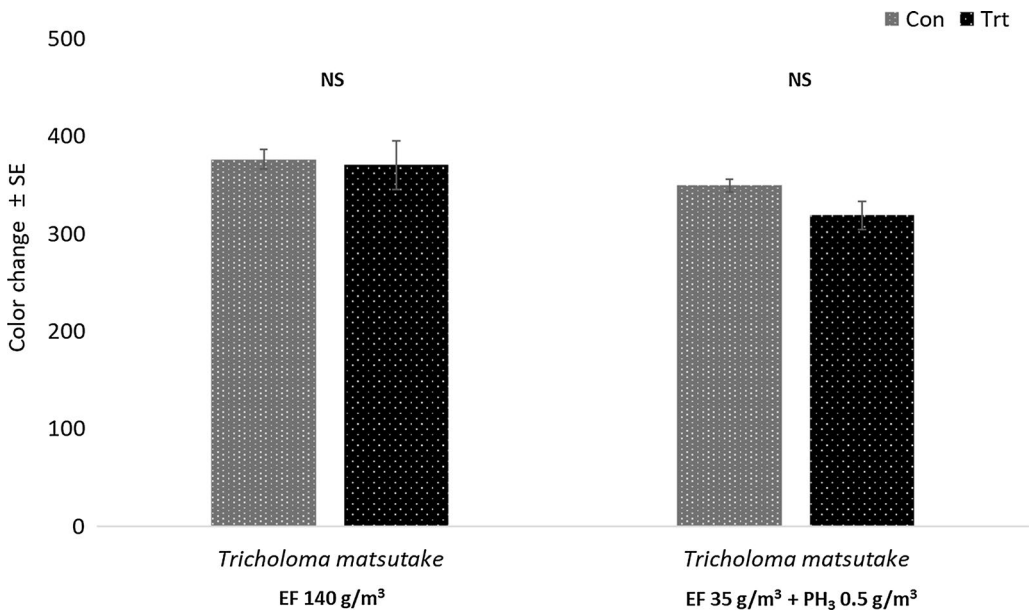

Fig. 5 Color change after 3-d fumigation on mushrooms fumigated with 140 g/m^3 EF only and 35 g/m^3 EF mixed with 0.5 g/m^3 PH_3 (4 h exposure). NS: Not significant

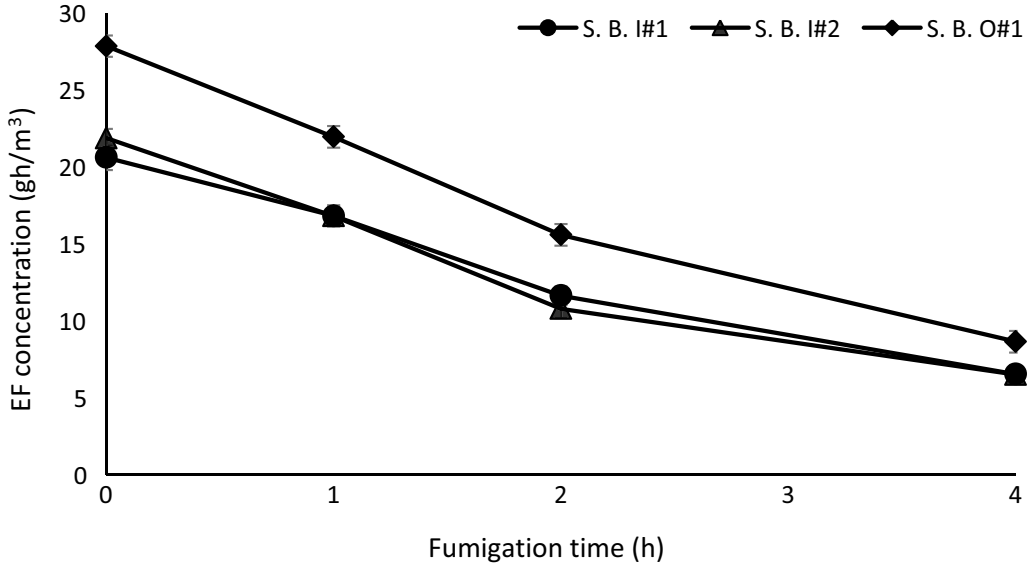

Fig. 6 Gas concentration when fumigated with 35 g/m^3 EF + 0.5 g/m^3 PH_3 for 4 h on *Tricholoma matsutake* (f.r. 0.5%) in scale-up trials. (5 m^3 fumigation chamber, Temp.: 5.0 ± 0.2 ℃) *Gas concentration inside (S. B. I) and outside (S. B. O) the styrofoam box

studies. Regarding weight loss, in assessment of 3-d after fumigation, *T. matsutake* was not significantly different (*df*: 18, *p*-value: 0.4423) (Fig. 4). Regarding color change, *T. matsutake* was not significantly different (*df*: 16, *p*-value: 0.3473) (Fig. 5). Thus, EF combined with PH_3 fumigation was no damage to mushrooms unlike EF alone fumigation. There were previous studies reported that EF + CO_2 [26], PH_3 + CO_2 for reducing LT (Lethal time) values [27] and PH_3 + O_2 [28] for increasing efficacy and decreasing phytotoxic damage to commodities.

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Authors' contributions
B.H.L, K.W.K and M.G.P designed the experiments. T.H.K and D.B.K conducted experiments, results analysis and interpretation. G.H.R and T.H.K wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are avaliable from the corresponding author on reasonable request.

Declarations

Competing interests
The authors declare that they have no competing interests.

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