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Effects of combinational mycelia obtained via in vitro culture on ageing and reproduction in *Drosophila melanogaster* and their mechanisms

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Abstract

This research serves to investigate the effects of mycelium combinations obtained by in vitro culture of *Amanita virgineoides* Bas, *Phallus rubicundus* (Bosc) Fr., and *Lepista nuda* (Bull.) Cooke fruiting bodies on ageing and reproduction of *Drosophila melanogaster* and their underlying mechanisms. The ageing effect was most pronounced in the *A. virgineoides* + *P. rubicundus* combination group, with a maximum mortality rate of 18.33% at 6 h. The catalase (CAT) and superoxide dismutase (SOD) activities, as well as the transcripts of the longevity genes *Nrf2* and *Hep*, were significantly downregulated to minima at 9 and 18 h, respectively. The malondialdehyde (MDA) content and transcripts of the signalling pathway-related genes *Keap-1*, *TOR*, and *S6K* as well as the longevity gene *MTH* were significantly upregulated to maximum levels. This corrected mortality reached 55.49%, with the lowest performance in terms of mean lifespan, maximal mean lifespan, median lethal time and flyability (15.00%) observed in the offspring at 24 h. In the group receiving a combination of *A. virgineoides* + *P. rubicundus* + *L. nuda*, the adult pupae quantity, adult fly quantity, and adult fly rate were the lowest at 14.33%, 4.00%, and 27.63%, respectively. In addition, the adult fly rate reached only 32.32% of that of the control group, and the highest aberration rate (47.78%) was also observed in the offspring. The mycelia of *A. virgineoides*, *P. rubicundus*, and *L. nuda* all demonstrated toxicity against *D. melanogaster*, and the combinations of different macrofungal mycelia had a synergistic effect regarding their toxicity. The data reported in the present study—particularly when using *A. virgineoides* and *P. rubicundus* with potential insecticidal activity against *D. melanogaster*—are novel.

Keywords Mycelium, Biopesticide, Ageing, Reproduction, Oxidative stress, Longevity gene

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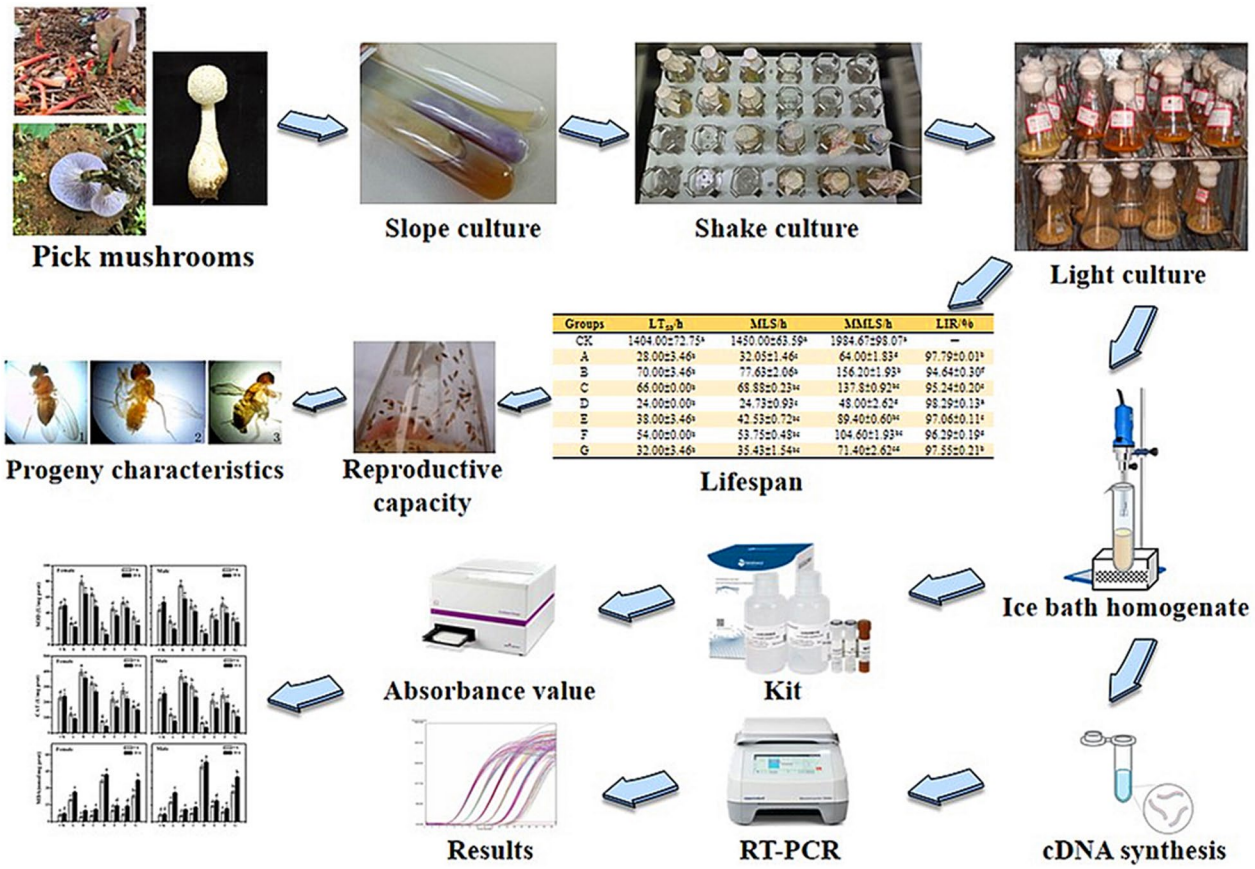
Graphical Abstract



Introduction

The insect pest *Drosophila melanogaster* (Diptera, Drosophilidae) has a wide host range, reproduces rapidly, and has the capability of surviving under various climatic conditions. This species has been conducive to the worldwide spread of economically harmful insects [1]. Chemical fly pesticide utilization is not a sustainable strategy because of administrative limitations and the potential for resistance to insecticides [2, 3]. Many plant and fungal compounds possess marked biological activities related to the presence of secondary metabolites [4, 5]. *Amanita muscaria* (L.: Fr.) Pers. ex Hook. (Agaricales, Amanitaceae) (fly agaric) is capable of catching flies while soaked in water or milk [6]. *A. muscaria* is used against the mosquito *Culex quinquefasciatus* (Diptera, Culicidae) [7]. In addition, in 175 tested species of fungi, 79 were discovered to impede insect development [8]. The observed results prove that some fungi include antifeedant, repellent and even poisonous compounds that act against *Sitophilus zeamais* [9], *Drosophila melanogaster, Tribolium castaneum* [10], *Leptinotarsa decemlineata* [11], and *Plutella xylostella* [12].

One uncommon amino acid of *Amanita virgineoides* Bas impedes the spore germination of *Pyricularia oryzae* Cav., the cause of rice blast disease [13]. The secondary metabolites of *Phallaceae* have anti-inflammatory [14], immune-enhancing [15], and anticancer activities [16], as well as α-glucosidase inhibitory potential [17]. Recent studies have shown that *Lepista nuda* (Bull.) Cooke possesses a variety of pharmacological functions, such as anti-tumour activity [18]. *L. nuda* extracts have already been reported to impede biofilm production [19] and HIV-1 reverse transcriptase [20] to some degree. In *Clitocybe* genus fruiting bodies, extracts have already been reported to show powerful insecticidal activation [21]. Moreover, *L. nuda* has shown insecticidal activation against the larvae of *D. melanogaster* [22].

Prior researchers have indicated that *D. melanogaster* exposure to 3 Gy electron beam irradiation, the rare-earth element cerium (Ce) and 15 mM paraquat (Pq) can

trigger oxygen stress, which specifically manifested as an obvious increase in the content of malondialdehyde (MDA). Furthermore, the radiation/Pq/Ce-induced free radicals can in turn impair the antioxidant defence systems, causing a decrease in catalase (CAT) and superoxide dismutase (SOD) activity as well as glutathione (GSH) levels. Moreover, Pq-treated flies showed serious locomotor impairments, with 84% of flies incapable of flying [23]. There was an obvious reduction in maximum lifespan, mean lifespan and reproductive output as doses of cerium increased [24]. Recent studies have shown that metabolic signalling pathways are capable of mediating longevity and age [25]. Target of rapamycin (*TOR*) is an essential regulator of cellular proliferation and impacts senescence, and *S6K* is just one downstream *TOR* kinase effector. *TOR* and *S6K* are involved in the age-related signalling pathway, while *Nrf2*/*Keap-1* is involved in the intrinsic stress protection system [26]. The Jun kinase (*JNK*) signalling pathway indirectly participates in the cell oxygenated stress reaction and increases longevity. *Hep* is a homologue of Jun kinase kinase (*JNKK*), and *D. melanogaster* with the *Hep* mutation was shown to be more sensitive to oxidation stress and to have shorter longevity [27].

To date, the molecular mechanism by which *Amanita* kills flies has not been sufficiently studied, and *Amanita*, being highly toxic, may pose a threat to the environment and humans. It is worth exploring whether there are any macrofungi among edible or medicinal mushrooms that are beneficial to humans and have the same fly killing activity as *Amanita*. Adopting in vitro culture decreases the overexploitation of extremely rare, valuable, or endangered species, presenting a methodology that gives priority to sustainable safeguards and consideration of biodiversity [28]. In this study, the combinational mycelia of *A. virgineoides*, *Phallus rubicundus* (Bosc) Fr., and *L. nuda* were procured by means of in vitro culture and checked for adverse impacts against *D. melanogaster*. In addition, their influences on the lifespan, fecundity, growth and development characteristics of offspring, peroxide content, antioxidant enzyme activity, and transcription of genes related to signalling pathways and lifespan of *D. melanogaster* were contrasted. This research was performed to provide a reference for macrofungus advancement and application.

Materials and methods

Materials

Insects

Captured in one local orchard of cherry (114° E, 31° N), adult wild-type *D. melanogaster* were grown on typical yeast medium with corn and bred inside one light hatcher at 25 ± 1 ℃ under 55% relative humidity with a 12-h light/12-h dark cycle. Virgin flies that had never mated were selected for the study.

Macrofungus strains

A. virgineoides, *P. rubicundus*, and *L. nuda* originated in fallen leaves during the summer of 2021 in the forests of the Chinese Ta-pieh Mountains. These mushrooms were identified in a field survey by comparison with previously prepared herbarium samples. Taxonomic determination was performed by Shanghai Yuanxin Biomedical Technology Co., Ltd.

Methods

Isolation and purification of mycelia

The macrofungal fruiting bodies were germfree, cultured on PDA by isolating the tissue, and grown at 26 ℃. Well-grown slant strains were inoculated into PD in vitro medium with no agar, placed in a rotating shaker, and cultured for 6 days at 28 ℃ with 130 r/min. After in vitro culture, the mycelia were collected by centrifugation at 6000 r/min for 20 min, washed three times with distilled water, and dried to a constant weight at 50 ℃; in addition, the mycelia were weighed.

Combinations and experimental grouping of macrofungal mycelia

The mycelia attained from in vitro culture were blended at a mass proportion of 1:4 with purified water, homogenized by ultrasonic (240 W) crushing for 1 h, sealed, and bottled at 4 ℃. The treatment medium was set based upon the yeast medium with corn, which was regarded as the control (CK). A (*A. virgineoides*), B (*P. rubicundus*), C (*L. nuda*), D (*A. virgineoides* + *P. rubicundus*), E (*A. virgineoides* + *L. nuda*), F (*P. rubicundus* + *L. nuda*), and G (*A. virgineoides* + *P. rubicundus* + *L. nuda*) were established as 7 experimental treatments. Treatments A, B, and C used a single macrofungal mycelium type, while treatments D, E, F, and G used two or more mycelia. The mass ratio of mycelia from different macrofungi was 1:1. In the experiments, 30% of the original mass of distilled water and corn flour in the foundational medium was removed; in addition, the masses were matched to the mass of macrofungal mycelium homogenate. Over the course of configuration, the homogenate was mixed with yeast powder; the medium was separated into 100 mL triangular flasks.

Determination of the ageing effect on D. melanogaster

Two male and two female virgin flies that had been starved for 8 h were placed into the bottles. A total of 80 flies were chosen for every experimental treatment and incubated at 25 ℃. After being placed in bottles, they were surveyed at 6, 12, 18, and 24 h. The number of dead

flies was determined. The corrected mortality rate (CMR, %) and mortality rate (MR, %) were determined on the basis of the formulae below:

$$\mathrm{MR} = \text{number of dead flies/number of test flies} \times 100\% \quad (1)$$

$$\mathrm{CMR} = \left(\text{treatment mortality} - \text{control mortality}\right) / \left(1 - \text{control mortality}\right) \times 100\% \quad (2)$$

Measurement of D. melanogaster lifespan

The virgin flies placed in treatment medium were surveyed and counted every 6 h. After flies had been reared for 24 h with the macrofungal treatment medium, the surviving flies were switched to the yeast medium with corn for additional rearing. The pupae were transferred again to yeast medium with corn, where they remained until their deaths. The median lethal time (LT_{50}, h), mean lifespan (MLS, h), maximum mean lifespan (MMLS, h), and lifespan inhibition rate (LIR, %) were determined using the formulae below:

$$LT_{50} = \text{the time at which half of the fliesdied} \quad (3)$$

$$\mathrm{MLS} = \text{the sum of survival times of allflies in the treatment / quantity of flies in the treatment} \quad (4)$$

$$\mathrm{MMLS} = \text{the mean lifespan of ten longest} - \text{surviving flies in a given treatment} \quad (5)$$

$$\mathrm{LIR} = (\text{mean lifespan of the control flies} - \text{mean lifespan of the experimental flies}) / \text{mean lifespan of the control flies} \times 100\% \quad (6)$$

Measurement of D. melanogaster reproductive capacity

The numbers of newly formed pupae and adult flies per bottle per day were recorded from the day on which the offspring generation formed pupae on the bottle wall until there were no more pupae or flies being formed. The quantity of flies and pupae was determined; in addition, the rate of flies per female was determined by the formulae below:

$$\text{The rate of flies} = \text{number of flies / number of pupae} \times 100\% \quad (7)$$

Measurement of growth and developmental characteristics of D. melanogaster offspring

The offspring of *D. melanogaster* were collected at 24 h after hatching, and their phenotypes were observed under a microscope after anaesthesia with ether. The number of aberrations in female and male flies was recorded, and their body lengths were measured using an eyepiece micrometer. Two hours after awakening, the flies were released outdoors, and those that could take off normally were scored as "flyable". Finally, the sex ratio (♀:♂), body length, aberration rate, and flyability were counted.

Determination of antioxidant enzyme activity and peroxidation product content in D. melanogaster

Next, 100 mg of male and female flies grown with the treatment medium for 9 h and 18 h was weighed. Every group of flies was blended with 0.9 mL of saline solution and homogenized at 2000 r/min for 10 s in an ice bath for an interval of 10 s. The mixture was repeated 3 times to generate a homogenate. The activities of catalase and superoxide dismutase, as well as the content of MDA, were normalized by sample protein values.

Determination of the corresponding quantity of transcripts in D. melanogaster

Total mRNA was obtained from *D. melanogaster* by the TRIzol approach as described in the TRIzol kit's instructions (Invitrogen). cDNA synthesis was performed according to the instructions for the PrimeScript™ RT–PCR Kit. The primer sequences adopted for quantitative analysis were generated by Shanghai Meiji Biomedical Technology Co. and are listed in Table 1. Fluorescence real-time quantitative PCR was carried out by means of QuantStudio 3 real-time quantitative PCR. The reagents employed were those included in the DyNAmo™ SYBRR Green qPCR Kit; in addition, experimental operations were implemented on the basis of the reagent instructions. Data were collected and managed with CFX-Manager; in addition, corresponding expression levels of internal reference and target genes were determined with the Ct ($2^{-\triangle\triangle Ct}$) approach [29]. Using ribosomal protein (*RP49*) as an internal reference gene, high-throughput fluorescent quantitative PCR was adopted to determine the corresponding quantities of transcripts of lifespan-related genes, such as hemipterus (*Hep*), nuclear factor erythroid-2-related factor 2 (*Nrf2*), and methuselah (*MTH*) and signalling-pathway-related genes.

Table 1 qRT–PCR primer sequences for lifespan-related and signalling pathway-related genes

Gene	Primer sequence (5′–3′)	
RP49	Forward	CCCTCTTCCAGCCATCGTTC
	Reverse	CCACCGATCCAGACGGAGTA
S6K	Forward	CGCAGGACGAGATGATGGA
	Reverse	TGGGATGGGTTGGTTGGT
TOR	Forward	CCATCCAGCGGTATGCGGTTATC
	Reverse	TTCAGTGGCACCTTCTTCTTGTCG
Keap-1	Forward	CCAACTTCCTCAAGGAGCAG
	Reverse	CGGCGACAAATATCATCCTT
Hep	Forward	GTGGATCTGCATGGAGCTGATGTC
	Reverse	CGTTGACCGTCGCCACTGTG
Nrf2	Forward	GGAGATGACGAGGAGGAGAG
	Reverse	GTGGCATAGGAGGCATTG
MTH	Forward	AGCGTATATTAGGAGTGAAGAAGG
	Reverse	CCGTAGGAAGAAGGTGTAAGTC

Statistical analysis

All experiments were repeated 3 times; the data are presented as the mean ± standard deviation (SD) of every sample. For comparisons of the means of treatment groups, one-way analysis of variance (ANOVA) was performed, followed by Tukey's post hoc test, using SPSS v26 software (SPSS Inc., Chicago, USA). In the figures, different lowercase letters between similar columns indicate least significant differences (LSDs) between treatments at the 5% level ($P < 0.05$). The antioxidant levels and gene expression levels were statistically analysed with two-way ANOVA to determine the effect of each factor and the effects of their interactions.

Results

Ageing influences of combinational macrofungal mycelium-supplemented diets on *D. melanogaster*

A longevity assay was performed to determine the influence of mycelia on the ageing of flies. Male and female flies were reared on a diet supplemented with the mycelia of seven combinations of macrofungi. Table 2 shows the numbers of fly deaths that occurred in the seven treatment groups at 6 h. Only treatment B showed no significant difference; the mortality in the other six treatment significantly increased compared with that of the control. The mortality of flies in each experimental group showed an upwards trend with extension of the time spent feeding on macrofungal mycelia. The corrected mortality in treatment D was the most elevated at 24 h (55.49%), and that in treatment B was the least elevated (18.65%) ($P < 0.05$).

Effects of combinational macrofungal mycelium-supplemented diets on the longevity of *D. melanogaster*

The inhibition rates and lifespans of the flies after receiving combinational macrofungal mycelium-supplemented diets for 24 h are shown in Table 3. Table 3 shows that, in contrast to the control, the seven combinations of macrofungal mycelia reduced the LT_{50}, MLS, and MMLS of the flies. Moreover, the differences were significant compared with the control ($P < 0.05$). Nevertheless, there were no obvious differences in LT_{50} and MLS among treatments

Table 2 Mean (± SD) mortality rate (MR) and corrected mortality rate (CMR) of *Drosophila melanogaster* receiving combinational macrofungal mycelium-supplemented diets lasting for 6, 12, 18, and 24 h

Groups	6 h		12 h		18 h		24 h	
	MR/%	CMR/%	MR/%	CMR/%	MR/%	CMR/%	MR/%	CMR/%
CK	0.00 ± 0.00^{e}	–	0.00 ± 0.00^{f}	–	0.00 ± 0.00^{e}	–	1.67 ± 0.72^{f}	–
A	10.00 ± 2.50^{cd}	10.00 ± 2.50^{cd}	27.08 ± 3.15^{b}	27.08 ± 3.15^{b}	36.25 ± 2.17^{b}	36.25 ± 2.17^{b}	41.67 ± 3.15^{c}	40.67 ± 3.27^{c}
B	3.33 ± 1.44^{e}	3.33 ± 1.44^{e}	6.67 ± 2.89^{e}	6.67 ± 2.89^{e}	15.83 ± 1.44^{d}	15.83 ± 1.44^{d}	20.00 ± 2.50^{e}	18.65 ± 2.05^{e}
C	7.50 ± 1.25^{d}	7.50 ± 1.25^{d}	13.33 ± 2.60^{d}	13.33 ± 2.60^{d}	19.17 ± 1.91^{d}	19.17 ± 1.91^{d}	27.08 ± 1.44^{d}	25.84 ± 1.80^{d}
D	18.33 ± 2.60^{a}	18.33 ± 2.60^{a}	35.83 ± 3.15^{a}	35.83 ± 3.15^{a}	43.75 ± 3.75^{a}	43.75 ± 3.75^{a}	56.25 ± 3.75^{a}	55.49 ± 4.11^{a}
E	8.75 ± 2.17^{cd}	8.75 ± 2.17^{cd}	21.67 ± 2.89^{c}	21.67 ± 2.89^{c}	30.83 ± 3.15^{c}	30.83 ± 3.15^{c}	39.58 ± 2.89^{c}	38.54 ± 3.40^{c}
F	11.67 ± 1.91^{bc}	11.67 ± 1.91^{bc}	19.58 ± 2.60^{c}	19.58 ± 2.60^{c}	26.25 ± 1.25^{c}	26.25 ± 1.25^{c}	32.08 ± 2.60^{d}	30.93 ± 2.82^{d}
G	14.17 ± 0.72^{b}	14.17 ± 0.72^{b}	31.25 ± 2.50^{ab}	31.25 ± 2.50^{ab}	38.75 ± 3.31^{b}	38.75 ± 3.31^{b}	48.33 ± 3.82^{b}	47.45 ± 3.96^{b}
F value	31.834		63.999		104.679		114.095	48.091
df	7		7		7		7	6
P value	0.000							

The means labelled by different letters are significantly different between treatment groups at $P < 0.05$ (Tukey's test). CK—the control group—received an ordinary diet with no combinational macrofungal mycelia. Flies in treatments A, B, C, D, E, F, and G received a mycelium-supplemented diet with *Amanita virgineoides* Bas, *Phallus rubicundus* (Bosc) Fr., *Lepista nuda* (Bull.) Cooke, *A. virgineoides* + *P. rubicundus*, *A. virgineoides* + *L. nuda*, *P. rubicundus* + *L. nuda*, and *A. virgineoides* + *P. rubicundus* + *L. nuda*, respectively

Table 3 Mean (±SD) median lethal time (LT_{50}, h), mean lifespan (MLS, h), maximum mean lifespan (MMLS, h), lifespan inhibition rate (LIR, %) of *D. melanogaster* receiving combinational macrofungal mycelium-supplemented diets lasting for 24 h

Groups	LT_{50}/h	MLS/h	MMLS/h	LIR/%
CK	1404.00 ± 72.75^{a}	1450.00 ± 63.59^{a}	1984.67 ± 98.07^{a}	–
A	28.00 ± 3.46^{b}	32.05 ± 1.46^{c}	64.00 ± 1.83^{d}	97.79 ± 0.01^{b}
B	70.00 ± 3.46^{b}	77.63 ± 2.06^{b}	156.20 ± 1.93^{b}	94.64 ± 0.30^{f}
C	66.00 ± 0.00^{b}	68.88 ± 0.23^{bc}	137.8 ± 0.92^{bc}	95.24 ± 0.20^{e}
D	24.00 ± 0.00^{b}	24.73 ± 0.93^{c}	48.00 ± 2.62^{d}	98.29 ± 0.13^{a}
E	38.00 ± 3.46^{b}	42.53 ± 0.72^{bc}	89.40 ± 0.60^{bc}	97.06 ± 0.11^{c}
F	54.00 ± 0.00^{b}	53.75 ± 0.48^{bc}	104.60 ± 1.93^{bc}	96.29 ± 0.19^{d}
G	32.00 ± 3.46^{b}	35.43 ± 1.54^{bc}	71.40 ± 2.62^{cd}	97.55 ± 0.21^{b}
F value	1039.572	1456.686	1113.185	162.606
df	7	7	7	6
P value	0.000			

The means labelled by different letters are significantly different between treatment groups at $P < 0.05$ (Tukey's test). CK—the control group—received an ordinary diet with no combinational macrofungal mycelia. Flies in treatments A, B, C, D, E, F, and G received a mycelium-supplemented diet with *A. virgineoides*, *P. rubicundus*, *L. nuda*, *A. virgineoides* + *P. rubicundus*, *A. virgineoides* + *L. nuda*, *P. rubicundus* + *L. nuda*, and *A. virgineoides* + *P. rubicundus* + *L. nuda*, respectively

A, C, D, E, F, and G. The LT_{50}, MLS, and MMLS in treatment D were the lowest, while those in treatment B were the highest, with differences of 46, 52.9, and 108.2 h, respectively. The lifespan inhibition rate in the seven treatments was 94.64 ~ 98.29%. There was no obvious difference in the lifespan inhibition rate between treatments A and G, but there were significant differences between the others.

Influences of combinational macrofungal mycelium-supplemented diets on the fecundity of *D. melanogaster*

A reproduction assay was performed to determine the influence of mycelia on the fertility of flies. The pupa number, adult fly number, and adult fly rate decreased significantly after the flies received combinational macrofungal mycelium-supplemented diets compared with the control ($P < 0.05$) (Table 4). As shown in Table 4, the toxicity of treatment G was the strongest, and the numbers of pupae and adult flies and the adult fly rate were the lowest at 14.33%, 4.00%, and 27.63%, respectively—with the last value being only 32.32% of that in the control. Treatment B had the weakest inhibitory effect on the fecundity of flies, with an adult fly rate of 62.17–72.72% of that in the control.

Table 4 Means (±SD) the pupa number, adult fly number, and adult fly rate of *D. melanogaster* receiving combinational macrofungal mycelium-supplemented diets

Groups	Pupae number	Adult flies number	Adult flies rate/%
CK	67.33 ± 6.03^{a}	57.67 ± 7.02^{a}	85.49 ± 3.08^{a}
A	23.00 ± 2.65^{ef}	10.67 ± 1.53^{ef}	46.28 ± 1.55^{d}
B	42.33 ± 2.52^{b}	26.33 ± 2.08^{b}	62.17 ± 2.14^{b}
C	34.67 ± 2.52^{c}	20.67 ± 2.31^{bc}	59.52 ± 3.30^{b}
D	17.67 ± 1.53^{gf}	6.00 ± 1.00^{fg}	33.81 ± 2.83^{e}
E	25.67 ± 1.53^{de}	13.33 ± 1.53^{de}	51.85 ± 3.21^{cd}
F	30.67 ± 2.08^{cd}	17.33 ± 2.31^{cd}	56.37 ± 3.78^{bc}
G	14.33 ± 1.53^{g}	4.00 ± 1.00^{g}	27.63 ± 4.17^{e}
F value	101.979	98.897	99.862
df	7		
P value	0.000		

The means labelled by different letters are significantly different between treatment groups at $P < 0.05$ (Tukey's test). CK—the control group—received an ordinary diet with no combinational macrofungal mycelia. Flies in treatments A, B, C, D, E, F, and G received a mycelium-supplemented diet with *A. virgineoides*, *P. rubicundus*, *L. nuda*, *A. virgineoides* + *P. rubicundus*, *A. virgineoides* + *L. nuda*, *P. rubicundus* + *L. nuda*, and *A. virgineoides* + *P. rubicundus* + *L. nuda*, respectively

Influences of combinational macrofungal mycelium-supplemented diets on the growth and development characteristics of *D. melanogaster* offspring

Microscopy and behavioural assays were performed to determine the influence of mycelia on the growth and development characteristics of offspring. Table 5 shows that in contrast to that in the control, the sex ratio in treatment A was as high as 2.17, while those in the other six treatments showed no significant differences compared to that in the control. The body length of flies in treatment E was the smallest, at only 2.14 mm, which was 0.32 mm less than that of flies in the control. The flyability of flies in the seven treatment groups decreased significantly compared with that in the control group; furthermore, treatment D had the lowest value, at only 15.00%. The aberration rate of the seven treatment groups increased compared with that in the control group, with treatment G presenting the highest rate (47.78%). In addition, in contrast to that in the control, the aberration rates in treatments A, D, E, and G increased significantly.

Influences of combinational macrofungal mycelium-supplemented diets on the activity of antioxidant enzymes and the content of peroxides in *D. melanogaster*

An antioxidant assay was performed to determine the influence of mycelia on the oxidation level of flies. A

Table 5 Mean (±SD) sex ratio, body length, flyability, aberration rate of *D. melanogaster* receiving combinational macrofungal mycelium-supplemented diets

Groups	Sex ratio	Body length/mm	Flyability/%	Aberration rate/%
CK	1.01 ± 0.04^{b}	2.46 ± 0.05^{ab}	84.43 ± 7.62^{a}	2.34 ± 1.04^{c}
A	2.17 ± 0.76^{a}	2.29 ± 0.20^{ab}	31.31 ± 3.50^{cd}	37.12 ± 4.22^{a}
B	1.14 ± 0.05^{b}	2.50 ± 0.15^{ab}	62.30 ± 7.56^{b}	6.24 ± 1.80^{bc}
C	1.28 ± 0.10^{b}	2.51 ± 0.03^{a}	54.88 ± 4.58^{b}	11.28 ± 2.28^{bc}
D	1.61 ± 0.35^{ab}	2.31 ± 0.07^{ab}	15.00 ± 13.23^{d}	38.73 ± 4.89^{a}
E	1.24 ± 0.14^{b}	2.14 ± 0.08^{d}	48.16 ± 9.33^{bc}	14.91 ± 7.50^{b}
F	1.48 ± 0.19^{b}	2.26 ± 0.06^{cd}	48.75 ± 8.20^{bc}	11.67 ± 1.44^{bc}
G	1.59 ± 0.15^{ab}	2.28 ± 0.15^{cd}	45.40 ± 13.51^{bc}	47.78 ± 13.47^{a}
F value	3.954	4.155	15.490	23.043
df	7			
P value	0.011	0.009	0.000	0.000

The means labelled by different letters are significantly different between treatment groups at $P < 0.05$ (Tukey's test). CK—the control group—received an ordinary diet with no combinational macrofungal mycelia. Flies in treatments A, B, C, D, E, F, and G received a mycelium-supplemented diet with *A. virgineoides*, *P. rubicundus*, *L. nuda*, *A. virgineoides* + *P. rubicundus*, *A. virgineoides* + *L. nuda*, *P. rubicundus* + *L. nuda*, and *A. virgineoides* + *P. rubicundus* + *L. nuda*, respectively

two-way ANOVA with three factors could fully explain the main effects of sex, time and treatment as well as the interaction effect among the three variables (R square > 0.98). Moreover, the effects of sex, time, treatment, sex*treatment and time*treatment were significant for SOD, CAT and MDA ($P < 0.05$), while the interaction effects of sex*time and sex*time*treatment were not significant for CAT and MDA ($P > 0.05$) but were significant on SOD ($P < 0.05$).

Figure 1 shows that, compared to those in the control, the CAT and SOD activities in treatments A, D, and G decreased significantly, while those in treatment B increased significantly after flies received combinational macrofungal mycelium-supplemented diets for 9 and 18 h. The antioxidant enzyme activities in treatments C, E, and F were different due to different culture times, sexes, and enzyme types. The SOD activity in treatment D was the lowest at 18 h, with values of 12.83 U mg^{-1} for female flies and 13.89 U mg^{-1} for male flies. Moreover, the CAT activity in treatment D was also the lowest, with values of 43.06 U mg^{-1} for female flies and 36.76 U mg^{-1} for male flies.

The MDA content of flies in the seven treatments increased significantly at 18 h, and the MDA content in treatment D was the highest at 42.06 nmol/mg for female flies and 53.66 nmol/mg for male flies. Treatment G increased the most at 18 h compared with the values found at 9 h, with a 60.95% increase in female flies and a 48.2% increase in male flies.

Influences of the combinational macrofungal mycelium-supplemented diets on the transcription levels of genes related to signalling pathways in *D. melanogaster*

A real-time PCR assay was performed to determine the influence of mycelia on the transcript levels of genes related to signalling pathways of flies. A two-way ANOVA with three factors could fully explain the main effects of sex, time and treatment as well as the interaction effect among the three variables (R square > 0.95). Moreover, the one-way main effect of sex was nonsignificant for *TOR*; the two-way interaction effect of sex*treatment was not nonsignificant for *S6K* and *TOR*; sex*treatment had a nonsignificant effect on *Keap-1*; and the three-way interaction effect of sex*time*treatment was nonsignificant for *S6K* and *Keap-1* ($P > 0.05$).

Figure 2 shows that the levels of *Keap-1*, *TOR*, and *S6K* gene transcripts in female and male flies increased at 18 h compared with 9 h. In addition, the levels of *S6K*, *TOR* and *Keap-1* transcription among treatments A, C, D, E, F, and G were significantly increased at 18 h compared to those in the control. The levels of *S6K*, *TOR*, and *Keap-1* transcription in treatment D were the highest, and the levels in male flies were 0.24, 0.25, and 0.56 times greater than those in female flies, respectively. The level of *TOR* transcription increased most significantly among the three signalling pathway genes. The fold changes in *TOR* transcripts in female and male flies in treatment D compared with the control were 3.42 and 3.67 at 18 h, respectively, having increased by 0.8 and 1.45 times compared with those found at 9 h.

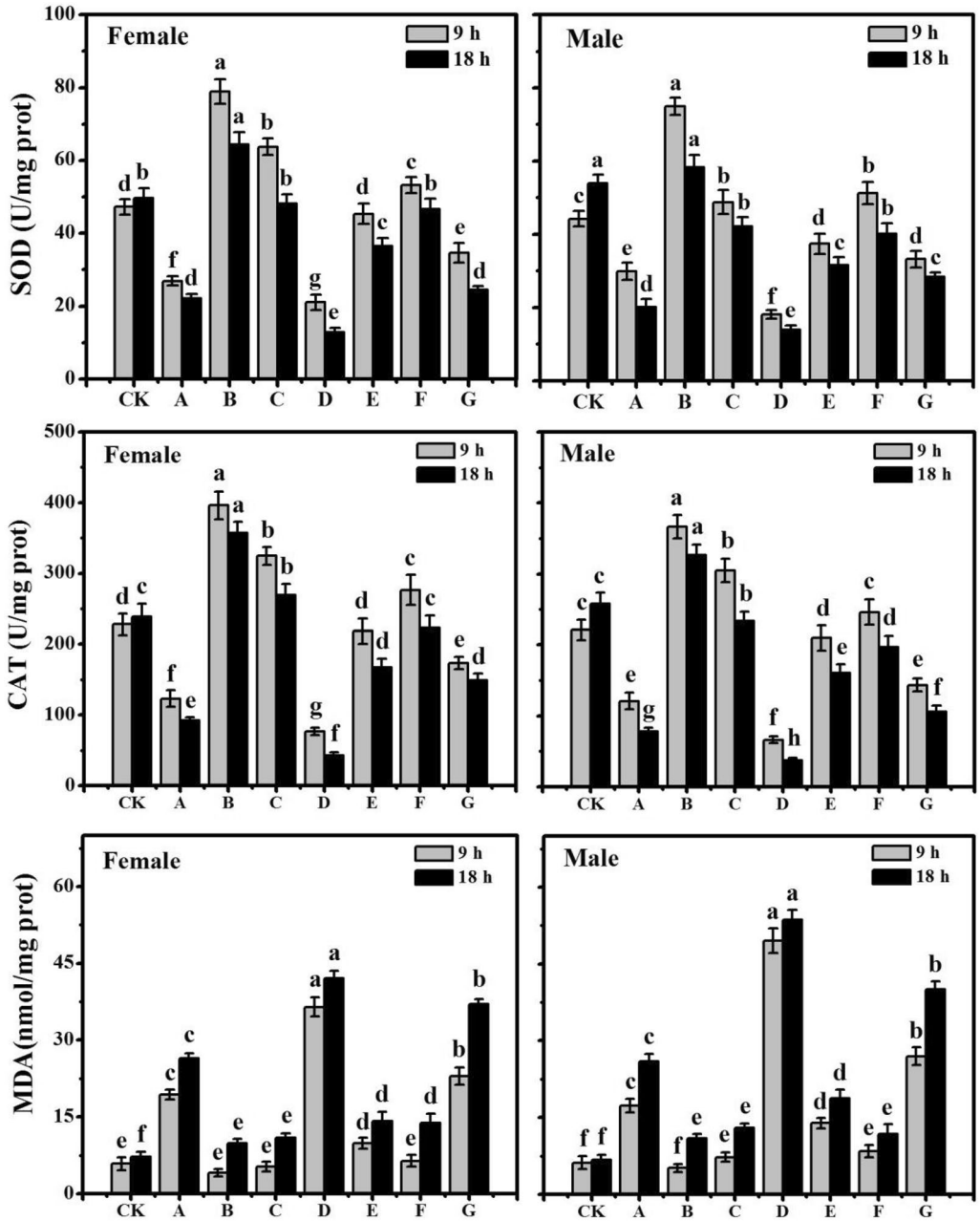


Fig. 1 Mean (± SD) the activity of CAT, SOD, the content of MDA of *D. melanogaster* receiving combinational macrofungal mycelium-supplemented diets for 18, 9 h. The means labelled by different letters are significantly different between treatment groups at $P < 0.05$ (Tukey's test). CK—the control group—received an ordinary diet with no combinational macrofungal mycelia. Flies in treatments A, B, C, D, E, F, and G received a mycelium-supplemented diet with *A. virgineoides*, *P. rubicundus*, *L. nuda*, *A. virgineoides* + *P. rubicundus*, *A. virgineoides* + *L. nuda*, *P. rubicundus* + *L. nuda*, and *A. virgineoides* + *P. rubicundus* + *L. nuda*, respectively

Influences of the combinational macrofungal mycelium-supplemented diets on the transcript levels of lifespan-related genes in *D. melanogaster*

A real-time PCR assay was performed to determine the influence of mycelia on the transcription of genes related to the lifespan of flies. A two-way ANOVA with three factors could fully explain the main effects of sex, time and treatment as well as the interaction effect among the three variables (R square > 0.95). Moreover, the two-way interaction of sex*time had a non-significant effect on *Hep*; the three-way interaction of

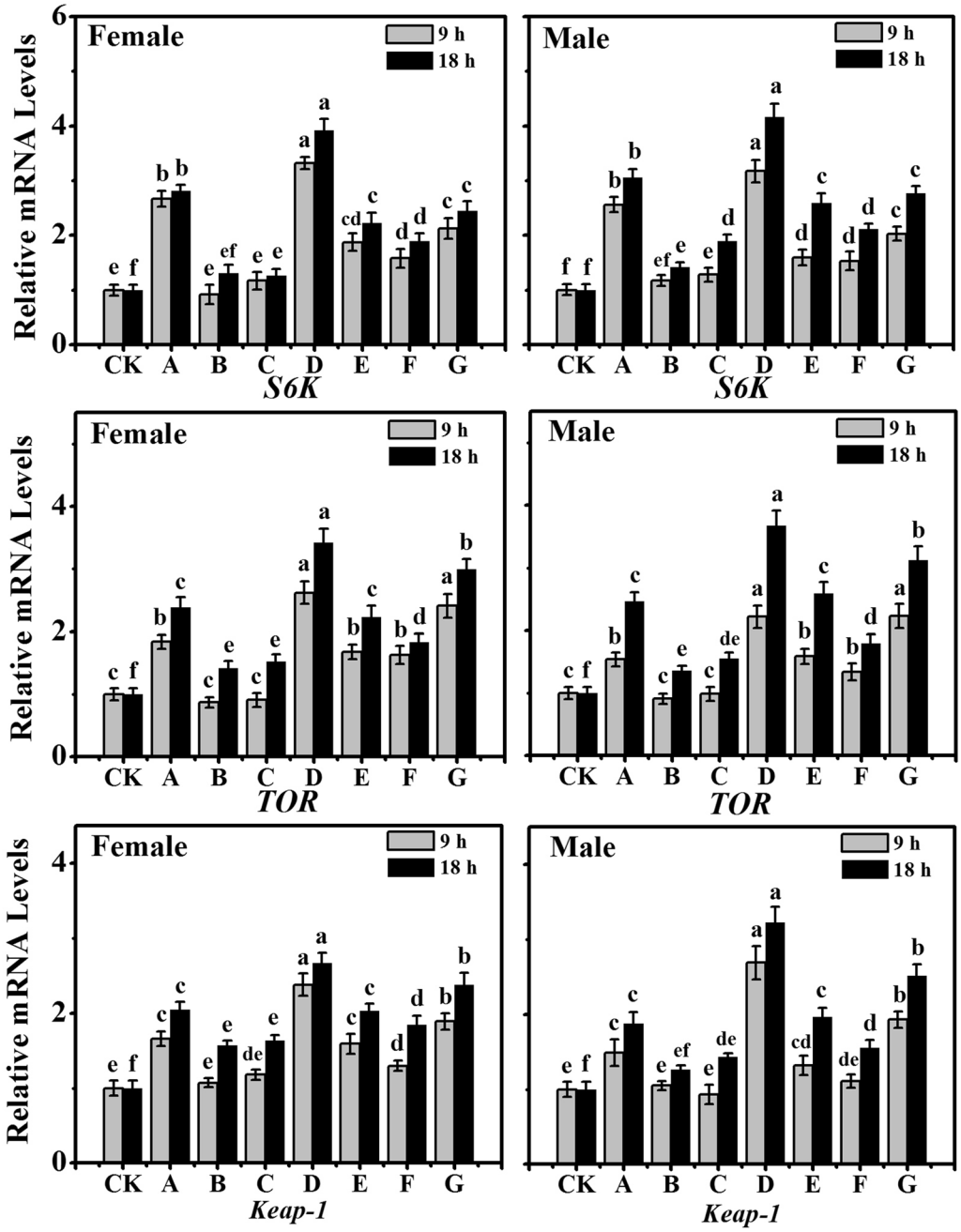


Fig. 2 Mean (±SD) the corresponding quantities of the transcriptions of the signalling pathway-related genes receiving combinational macrofungal mycelium-supplemented diets for 9, 18 h. The means labelled by different letters are significantly different between treatment groups at $P < 0.05$ (Tukey's test). CK—the control group—received an ordinary diet with no combinational macrofungal mycelia. Flies in treatments A, B, C, D, E, F, and G received a mycelium-supplemented diet with *A. virgineoides*, *P. rubicundus*, *L. nuda*, *A. virgineoides* + *P. rubicundus*, *A. virgineoides* + *L. nuda*, *P. rubicundus* + *L. nuda*, and *A. virgineoides* + *P. rubicundus* + *L. nuda*, respectively

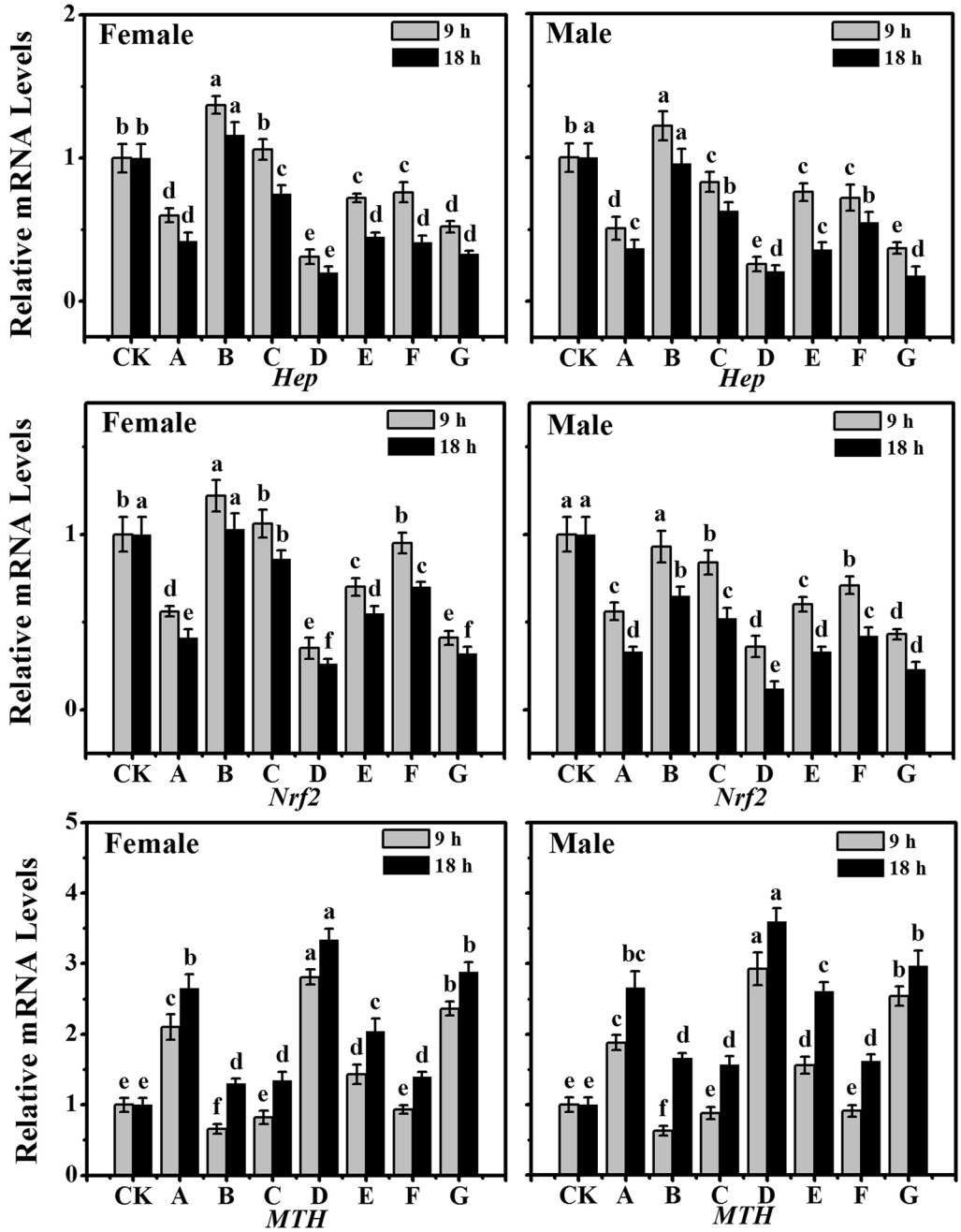


Fig. 3 Mean (± SD) the corresponding quantities of lifespan-related genes transcriptions, like hemipterus (*Hep*) of *D. melanogaster*, nuclear factor erythroid-2 related factor 2 (*Nrf2*), methuselah (*MTH*) receiving combinational macrofungal mycelium-supplemented diets for 18, 9 h. The means labelled by different letters are significantly different between treatment groups at $P < 0.05$ (Tukey's test). CK—the control group—received an ordinary diet with no combinational macrofungal mycelia. Flies in treatments A, B, C, D, E, F, and G received a mycelium-supplemented diet with *A. virgineoides*, *P. rubicundus*, *L. nuda*, *A. virgineoides* + *P. rubicundus*, *A. virgineoides* + *L. nuda*, *P. rubicundus* + *L. nuda*, and *A. virgineoides* + *P. rubicundus* + *L. nuda*, respectively

sex*time*treatment had a nonsignificant effect on *Hep, Nrf2* and *MTH* ($P > 0.05$).

Figure 3 shows that the transcription levels of the lifespan-related *Nrf2* and *Hep* genes were reduced significantly in treatments A, D, E, and G at 9 and 18 h compared with the control. Furthermore, they showed differences in treatments B, C, and F due to different feeding times, sexes, and gene types. The levels of *Hep*

and *Nrf2* transcripts decreased and the level of *MTH* transcripts increased at 18 h compared with those observed at 9 h. The level of *MTH* transcripts in all seven treatment groups increased significantly at 18 h compared with that in the control, with that in treatment D—3.34 and 3.6 times higher for the female and male flies, respectively—being the highest.

Discussion

In this study, the mycelium of *A. virgineoides*, which is a common local species, was added to treatment medium and then fed to *D. melanogaster*. Both the experimental group fed only *A. virgineoides* mycelium (treatment A) and its combinational treatment groups (treatments D, E, and G) showed an ageing effect on *D. melanogaster* (Table 2). Therefore, *A. virgineoides*, which is similar to the other macrofungi of *Amanita*, can disrupt the antioxidant system, upregulate the transcription of genes related to signalling pathways, inhibit the expression of longevity-related genes, and reduce the lifespan and fertility of *D. melanogaster*. The toxic peptides amanitin and phalloidins identified in a case of phalloides syndrome due to poisoning caused by mushrooms [30] were found not only in *A. phalloides* but also in 3 other *Amanita* species, 8 species of *Galerina*, and 11 *Lepiota* species [31]. Our results are similar to those of previous studies showing that low α-amanitin concentrations negatively impact larva-to-adult progression time, pre-adult viability, adult body size, and adult longevity [32]. These results may be because pantheric acids A–C in poisonous mushrooms promote lipid accumulation in adipocytes [33]. It has already been supported that activation of *TOR* [34] and *S6K* [35] expression is capable of decreasing the lifespan by means of rapamycin in *D. melanogaster*, which corresponds to the results of the current research. The *Methuselah* (*MTH*) gene has long been thought to be a key longevity element. Moreover, diverse studies have shown that overexpression of the *MTH* gene in *D. melanogaster* can shorten lifespan [36].

To date, researchers have isolated and identified the structures of compounds, such as metalloprotease [37], bisabolane sesquiterpene [38], chloro-substituted pentenamide [39], lignoren and harziandion [38] from *A. phalloides*. In Korea, *A. phalloides* is commercially available and cooked in water for consumption due to its high content of glutamic acid, monosodium glutamate-like compounds, and total 5′-nucleotides [40]. This indicates that *A. phalloides* is safe for humans and the environment, provided it is appropriately handled.

It is evident that *A. phalloides* possesses promising applications for the development of biogenic insecticides. However, *D. melanogaster* has developed ibotenic acid [41] and α-amanitin resistance over a long period of evolution. These characteristics are associated with cytochrome P450 activity [42] and not with the gut microbiota [43]. A former study revealed that toxin entry blockage by means of the cuticle, stage I and II detoxification, and sequestration inside lipid particles, along with the proteolytic cleavage of α-amanitin, contribute to the quantitative trait [44].

To identify more medicinal fungal resources that can be used to kill flies and thus solve the problem of α-amanitin resistance in *D. melanogaster*, this study also selected the mycelium of *P. rubicundus* and *L. nuda*, which are common locally. Combinational treatment media with the mycelium of *A. virgineoides* were configured and then fed to *D. melanogaster*. The results showed that the ageing effect of the D (*A. virgineoides* + *P. rubicundus*) combinational treatment was the strongest, with the highest mortality rate of 18.33% in *D. melanogaster* at 6 h. After receiving a mycelium-supplemented diet for 24 h, flies exhibited a mortality rate as high as 55.49% (Table 2), with the lowest LT_{50}, MLS, and MMLS (Table 3) and the lowest flyability (15.00%) found in the offspring generation (Table 5). Recent studies have shown that lactones [45], lucidenic acid O, lectins [46], fungal cyclic peptides [47], and haemolysins in mushrooms are potential natural insecticides. Cnispin, a protease inhibitor that impedes the serine protease trypsin, is poisonous to *D. melanogaster*. Clitocine, a new nucleoside, has shown powerful insecticidal activation against the pink bollworm *Pectinophora gossypiella* [48]. If the compounds in macrofungi with insecticidal activity can be isolated and identified in the future, this information will help reveal the molecular mechanism underlying the synergistic action of several key molecules in killing flies and provide a reference for the development of fly killing pesticides.

The lowest numbers of adult pupae and adult flies and the lowest adult fly rate were recorded in the G (*A. virgineoides* + *P. rubicundus* + *L. nuda*) combinational treatment, at 14.33%, 4.00%, and 27.63%, respectively (Table 4). This was in addition to the highest aberration rate of 47.78% observed in the offspring generation (Table 5), which showed that the combination of different macrofungal mycelia could have a synergistic toxic effect. Although the fungi of *Phallaceae* have a special strong flavour that attracts flies and insects to feed on their spores (which then spread them through faeces, thus forming a mutually beneficial relationship between the fungus and the insect), this study concluded that a *P. rubicundus* mycelium-supplemented diet was adverse to *D. melanogaster*, especially when combined with *A. virgineoides* mycelium. This may be because the feeding stimulants were used together with insecticides to

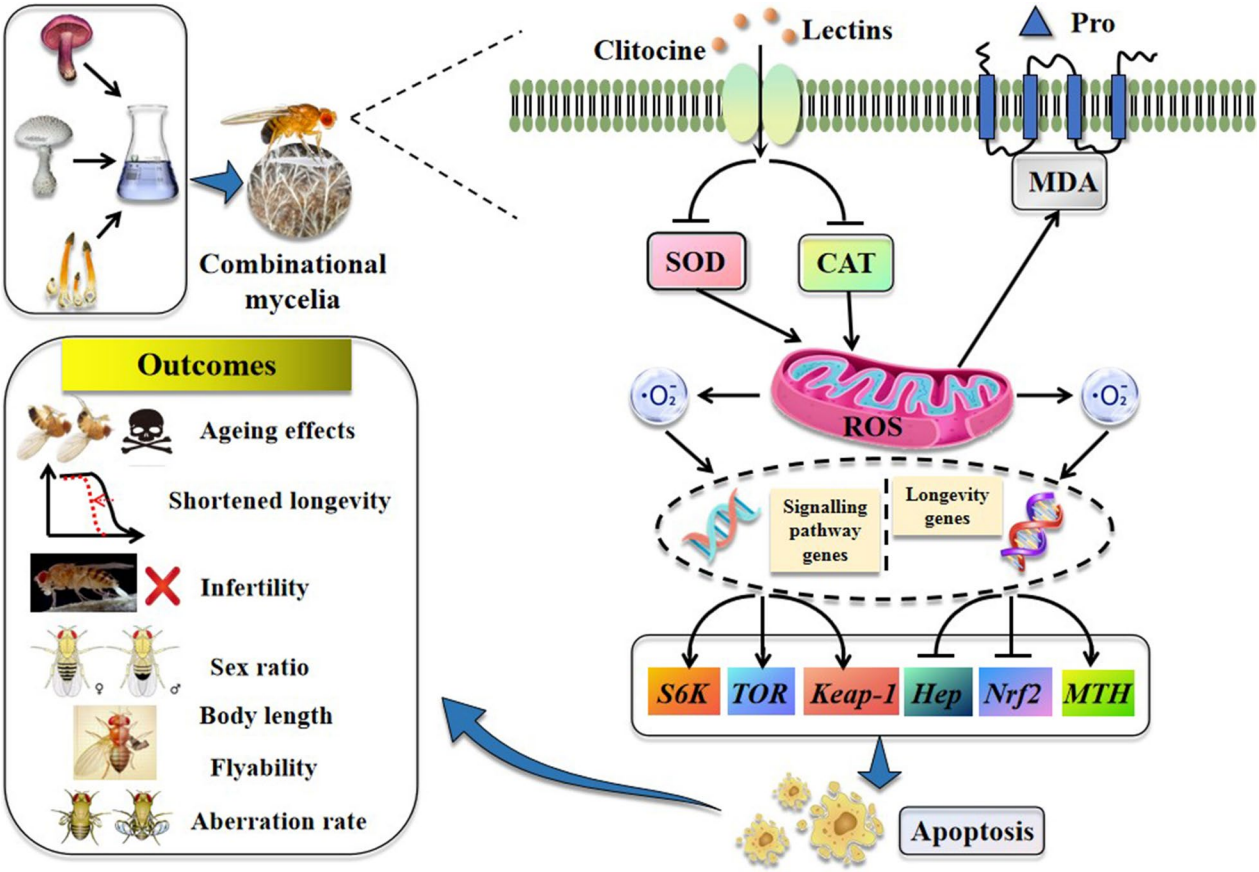


Fig. 4 Schematic diagram of effects of combinational mycelia obtained via in vitro culture on ageing and reproduction in *D. melanogaster* and their mechanisms

increase the dosage and treatment effects. The addition of 0.1% sucrose to acetamiprid, spinetoram, spinosad, and cyantraniliprole was demonstrated to decrease the LC_{50}, leading to higher and earlier adult SWD mortality and a reduction in larval infestation [49].

In this study, the mycelia of *A. virgineoides*, *P. rubicundus*, and *L. nuda* were selected for the ageing test and yielded certain effects. These research results filled a knowledge gap in this field (Fig. 4). Previous research on fly mortality was limited to large fungal fruiting bodies. This study provides an application idea for the exploitation of macrofungi that cannot yet be cultivated on a large scale in plantations.

Conclusions

In summary, the findings of this research, which examined the effects OF diets supplemented with mycelia of *A. virgineoides*, *P. rubicundus,* and *L. nuda* and their combinations on *D. melanogaster*, showed that they all exerted ageing effects on *D. melanogaster*, with the most pronounced ageing effect found for the *A. virgineoides*+*P. rubicundus* combination. This combination was able to significantly reduce the activity of antioxidant enzymes, increase the accumulation of peroxidation products, upregulate the transcription of genes associated with age-related signalling pathways, inhibit the expression of longevity genes, reduce the lifespan and fertility of *D. melanogaster*, and adversely affect the offspring. These achievements provide a reference for the application of macrofungi for the discovery of biogenic fly killing pesticides.

Abbreviations

HIV-1	Human immunodeficiency virus-1
Pq	Paraquat
Ce	Cerium
MDA	Malondialdehyde
SOD	Superoxide dismutase
CAT	Catalase
GSH	Glutathione
MR	Mortality rate
CMR	Corrected mortality rate
LT_{50}	Median lethal time
MLS	Mean lifespan
MMLS	Maximum mean lifespan
LIR	Lifespan inhibition rate
cDNA	Complementary DNA

RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
RNase	Ribonuclease
PCR	Polymerase chain reaction
RT–PCR	Reverse transcription–polymerase chain reaction
MTH	Methuselah
Nrf2	Nuclear factor erythroid-2-related factor 2
Hep	Hemipterus
S6K	RPS6-p70-protein kinase
TOR	Target of rapamycin
Keap-1	Kelch-like ECH-associated protein 1
ANOVA	Analysis of variance
LSD	Least significant differences
SWD	Spotted wing *Drosophila*
D. melanogaster	*Drosophila melanogaster*
A. virgineoides	*Amanita virgineoides* Bas
P. rubicundus	*Phallus rubicundus* (Bosc) Fr.
L. nuda	*Lepista nuda* (Bull.) Cooke
A. muscaria	*Amanita muscaria* (L.: Fr.) Pers. ex Hook

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Author contributions
YH performed the lab experiments, project administration and writing—original draft. JL performed the field experiments, analyzed the all data (statistical analyses) in study. DW supervised the study and writing of the manuscript. NZ contributed in review and editing of the manuscript. XQ performed project administration, arranged funding sources. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All the authors gave their consent for publication of this article.

Competing interests
The authors declare that there is no competing interest among the authors.

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