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Permethrin Resistance in *Lycoriella mali* (Diptera:Sciaridae) Populations from *Agaricus* Mushroom Farms

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ABSTRACT: *Lycoriella mali* is the major pest of commercial mushrooms. From 1980 until the present, the most widely used adulticide has been permethrin. Resistance rapidly evolved to this compound through oxidative metabolism of the toxin. Significant variation in resistance/susceptibility exists between farms in different geographical regions depending, in part, on pesticide use patterns. Resistance levels ranged from 4 to 47 fold (LC_{50}) compared to a susceptible reference strain. The farm with highest resistance levels had a population with two resistance mechanisms, oxidative metabolism and nerve insensitivity. Resistance through oxidative metabolism appears to relatively unstable. Flies on one farm which stopped applications of permethrin became as susceptible as the reference strain. The use of fly monitors to estimate numbers of flies in production houses and gauging pesticide applications to match pest pressure is essential to reducing the risk of resistance development.

1 INTRODUCTION

Each year the fungus gnat, *Lycoriella mali* (Fitch) (Diptera: Sciaridae), causes approximately $100 M damage to mushroom crops (Cantwell and Cantelo 1984). Permethrin was introduced into mushroom agriculture to control *L. mali* in 1981. By 1984, permethrin was not producing the level of control that it had when first introduced. Growers continued to increase the amount and frequency of insecticide applications to attempt fly control (Brewer and Keil 1989a). Resistance levels measured in the field and laboratory confirmed that populations were resistant to permethrin and that resistance was increasing (Keil 1986, Brewer and Keil 1989a). By the late 1980s, some growers had

abandoned permethrin because of low efficacy. Preliminary experiments by Brewer and Keil (1989b) implicated an oxidative component to permethrin resistance. This oxidative component of permethrin resistance was investigated using the oxidative enzyme inhibitor piperonyl butoxide. Identification and characterization of specific oxidative resistance mechanisms revealed variation in amounts of oxidative enzymes and probable forms of enzymes present (Bartlett and Keil 1996a). Pyrethroids can also be detoxified by a variety of non-oxidative mechanisms. These mechanisms may involve enzymatic detoxication by esterases (Rose *et al.* 1990) and/or glutathione S-transferases (Bull and Pryor 1990) or nerve insensitivity such as knockdown resistance (*kdr*) (Bloomquist and Miller 1985).

2 MATERIALS AND METHODS

2.1 *Test Insects*

Lycoriella mali were collected from mushroom farms in Kennett Square (KN1 & KN2), Avondale (AV), and Reading (RD) Pennsylvania. The KN1 and AV populations had been exposed to permethrin until about 1989. The KN2 population was exposed to permethrin until the middle of 1991. Flies at the RD farm were being treated with permethrin through 1994.

2.2 *Concentration Mortality Testing*

Permethrin stock solutions (5 M) were made from technical grade permethrin [3-phenoxybenzyl [±]-3-[2,2-dichlorovinyl]-2,2-dimethylcyclopropanecarboxylate; *cis/trans* 41/59], dissolved in acetone. Dilutions ranged from 0.05 mM to 100 mM for adults and 0.1 mM to 3000 mM for larvae.

2.3 *Application of permethrin*

Flies (female) were anesthetized with a 10-20 second carbon dioxide exposure. Topical applications of 0.6 µl were made using a microapplicator. Flies were removed to a growth chamber (18°C) for recovery. Mortality was assessed 24 hours after application. Larvae were also treated using the microapplicator. For synergism tests, piperonyl butoxide (0.06 mM in acetone) was applied about two hours prior to exposure to permethrin.

2.4 *Preparation of microsomes*

One gram of flies was homogenized in 5 ml of 4°C buffer. Homogenates were filtered through glass wool and centrifuged at 4°C and 10,000 g for

15 minutes. Supernatants were centrifuged at 4°C and 100,000 g for 1.25 hours. Pellets were frozen at <-40°C and resuspended in Lee and Scott (1989) buffer. Protein content was measured via a bicinchoninic acid (BCA) method (Smith *et al.* 1985).

2.5 *Analysis of microsomal enzymes using model substrates*

N-demethylation activity was measured with the model substrate *p*-chloro-N-methylaniline (Sigma) (Yu 1982). The reaction mixture consisted of the substrate (1.5 µl of 3 mM), NADPH (1.5 µl of 2 mM) and 1 ml microsomes. After incubation at 34° C for 30 minutes, 6% *p*-dimethylaminobenzaldehyde in 3N sulfuric acid (0.25 ml, Sigma) stopped the reaction. The product, *p*-chloroaniline, was recovered with the supernatant after centrifugation. Absorbance was measured at 445 nm and conversion of substrate to product was calculated using a standard curve.

O-demethylation was measured with *p*-nitroanisole (Aldrich) (Yu 1982). The reaction mixture consisted of 1 ml of microsomes, NADPH (2 µl of 2 mM) and the substrate (1.5 µl of 4 mM). The reaction took place at 34°C for 30 minutes. The reaction was stopped with 0.25 ml of 1N HCl. The product, *p*-nitrophenol, was isolated through one chloroform and two sodium acetate extractions. Absorbance of the product (400 nm) was measured and conversion calculated with a standard curve (Yu 1982).

Deethylation of 7-ethoxycoumarin to 7-hydroxycoumarin was measured using the methods of Lee and Scott (1989) and Patil et al. (1990). The reaction mixture included 0.5 mg of microsomal protein in TKEG (50 mM Tris-HCl, pH 8.3; 100 mM KCl; 1 mM EDTA and 10% (v/v) glycerol) buffer (100 µl) and 0.9 ml of the substrate (1.0 mM 7-ethoxycoumarin, Sigma). The reaction was initiated with NADPH (10 µl of 10 mM) and was measured with a spectrofluorometer; excitation 380 nm, emission 460 nm. The results were compared to a product standard curve.

2.6 *Enzyme kinetics of monooxygenases: peroxidase activity against model substrates*

The substrates used were *p*-phenylenediamine (PPD, Sigma) or tetramethylbenzidine (TMBZ, Sigma). The substrate concentrations were between 0.15 mM and 1.5 mM. The reaction mixture consisted of 500 µl of PPD in buffer and microsomal fractions. The reaction was initiated by the addition of 150 µl of 30% hydrogen peroxide. Absorbance of the product was measured at 557 nm. The methods for the TMBZ experiments were identical to those used for PPD, except that the absorbance was measured at 654 nm. The results were used to determine K_m and V_{max} values through Lineweaver-Burk plots (Imberty *et al.* 1984).

2.7 *Carbon monoxide difference spectra to determine microsomal cytochrome P-450*

The amount of cytochrome P-450 enzymes in microsomal preparations was measured through the binding of carbon monoxide by reduced cytochrome P-450. Microsomal suspensions were bubbled with carbon monoxide for 20 seconds. The absorbance was measured between 360 nm and 500 nm. The microsomes were then reduced by addition of 0.5 mg of $Na_2S_2O_4$, and the spectrum was again measured. The difference between the cytochrome P–450 absorbance peak and the absorbance at 490 nm was used to determine the amount of cytochrome P–450 (molar extinction coefficient, 91 cm^{-1} mM^{-1}) (Omura and Sato 1964).

2.8 *Knockdown Resistance*

Twenty-five fourth instar larvae were treated with 0.6 µl of the permethrin LC_{50} for each population. Additional larvae (25) were treated with concentrations above or below the LC_{50} to estimate concentration response lines for paralysis. Ten to thirty minutes after treatment, larvae were touched with a 45°C needle for exhibition of the characteristic curling behavior (Bloomquist and Miller 1985). Those larvae that did not respond were considered paralysed. The values were used for probit analysis and estimation of the effective concentration (EC_{50}, 50% paralysis) for each population. Mortality was measured at 24 hours.

2.9 *Data Analysis*

Permethrin concentration mortality and *kdr* effective concentrations were determined with probit analysis, SAS version 6.0 (SAS Institute 1989). Tests for significant differences between the populations at the LC_{50} and LC_{90} levels were performed with Lethal Dosage Ratio methods (Robertson and Preisler 1992). Slopes were analyzed for statistical significance via a Z test (Keil and Othman 1988). Variations between means for all other experiments were calculated using ANOVA and Ryan's Q multiple range test, $P \leq 0.05$.

3 RESULTS

3.1 *Resistance levels*

The RD population had the highest level of permethrin resistance. The resistance level for this strain was elevated approximately 47 fold (LC_{50} = 4.21 mM) as compared to a susceptible strain (LC_{50} = 0.09 mM) (Brewer and Keil 1989a). The next most resistant population, KN2 (LC_{50} = 0.93 mM), had an approximately 10 fold greater permethrin resistance than the reference strain. The LC_{50} values for the remaining two populations, AV and KN1 (0.35 mM and 0.30 mM respectively), were approximately 4 and 3 fold higher than the LC_{50} for the reference strain (not significantly different from each other) (Table 1). Slopes of the probit mortality lines ranged from 0.80 to 1.47. The slope of the RD population line was significantly different ($Z > 2.80$) as compared to the other populations. The populations were divided into three resistance levels; RD-most resistant, KN2-intermediate, and AV/KN1-least resistant (Table 1).

Table 1. Permethrin mortality in *Lycoriella mali* adults.

Population	*n*	LC_{50}[a,b]	LC_{90}[a,b]	Slope[b] ± SE	RR_{50}[c]	RR_{90}[c]
ONTARIO	404	0.09a (0.07-0.11)	0.71a (0.62-3.48)	1.47x ± 0.23	—	—
AVONDALE	2155	0.35b (0.30-0.40)	5.67b (4.28-7.96)	1.06x ± 0.06	3.9	8.0
KENNETT 1	2193	0.30b (0.22-0.39)	4.46b (2.88-8.23)	1.09x ± 0.08	3.3	6.3
KENNETT 2	2396	0.93c (0.82-1.06)	10.49c (8.10-14.3)	1.21x ± 0.06	10.3	14.8
READING	640	4.21d (3.05-5.82)	171.60d (93.8-385)	0.80y ± 0.07	46.8	241

[a]Concentration in mM.
[b]95% Fiducial Intervals and slopes calculated with SAS Probit Analysis.
[c]Resistance Ratio: LC_{50} or LC_{90} divided by appropriate Ontario value.
a-d LC values followed by same letter represent no significant difference at the P=0.05 level. Lethal Dose Ratio with adjusted 95% Confidence Intervals (Robertson and Preisler 1992).
x,y Slope values followed by same letter represent no significant difference at the P=0.025 level (Keil and Othman 1988).

3.2 *Piperonyl butoxide synergism of permethrin in adults*

Reduction of permethrin resistance by a fixed dose of piperonyl butoxide (PB) was significant for each population, except RD. Application of PB decreased the RD population LC_{50} to 2.29 mM (1.9 fold) and the LC_{90} to 73.5 mM (2.1 fold); neither reduction was significant. Treatment with PB significantly decreased the LC_{50} 3.9 fold (0.21 mM) and the LC_{90} 2.7 fold (4.18 mM) in the KN2 population. In the AV population, PB treatment caused a significant reduction in LC_{50} to 0.1 mM (2.9 fold) and LC_{90} to 1.52 mM (3.2 fold). Treatment caused a significant decline in LC_{50} to 0.09 mM (2.7 fold), but not in LC_{90} (1.64 mM, 3.1 fold) for the KN1 population. No significant differences between probit line slopes for the PB treated and untreated groups was seen (Table 2).

Table 2. Permethrin toxicity: effects of the synergist piperonyl butoxide.

Population*	*n*	LC_{50} (95% FL)[a,b]	LC_{90} (95% FL)[a,b]	Slope ± SE[b]	SR[c,d]	Z^2
AVONDALE/PN	419	0.29 (0.20-0.41)	4.85 (2.73-11.3)	1.04 ± 0.12	2.99	40.96
AVONDALE/MX	313	0.10 (0.61-0.15)+	1.52 (0.85-3.71)+	1.08 ± 0.14	3.19	
AVONDALE/PB	148	3.28 (1.30-9.61)	248 (50.2-1.09 x 10^4)	0.68 ± 0.15		
KENNETT 1/PN	332	0.24 (0.15-0.35)	5.04 (2.56-14.2)	0.96 ± 0.12	2.67	24.68
KENNETT 1/MX	293	0.09 (0.05-0.14)+	1.64 (0.82-4.98)	1.00 _ 0.15	3.07	
KENNETT 1/PB	138	3.31 (1.31-9.93)	286 (54.6-1.53 x 10^3)	0.66 ± 0.15		
KENNETT 2/PN	437	0.83 (0.58-1.16)	11.2 (6.69-23.3)	1.13 ± 0.12	3.95	18.66
KENNETT 2/MX	298	0.21 (0.13-0.33)+	4.18 (2.28-10.2)+	0.99 ± 0.12	2.67	
KENNETT 2/PB	208	5.64 (2.67-12.8)	346 (100-3.13 x 10^3)	0.72 ± 0.11		
READING/PN	369	4.36 (2.71-7.04)	154 (71.5-467)	0.83 ± 0.09	1.90	13.20
READING/MX	310	2.29 (1.34-3.76)	73.5 (35.4-214)	0.85 ± 0.10	2.09	
READING/PB	178	8.58 (4.33-18.1)	281 (94.9-1.95 x 10^3)	0.85 ± 0.14		

*Treatments: PN-permethrin PB-piperonyl butoxide MX-permethrin and piperonyl butoxide

[a]Concentration in mM.

[b]95% Fiducial limits and slopes calculated with SAS probit procedure. + Significant difference in LC value (calculated by the lethal dose ratio method of Robertson and Preisler 1992).

[c]Synergism ratio: LC of untreated adults divided by LC of PB treated adults. First value in population group is LC_{50}, second value is LC_{90}.

[d]Predicted versus observed Z^2 values, df = 5, $P < 0.05$.

3.3 *Enzyme activity against model substrates*

N-demethylase activity (0.67 nmol/mg protein/min) from the RD population was significantly higher than in the other three populations. The KN2 population was significantly different from the AV and KN1 populations, which had similar resistance levels. Flies from the RD population also had the highest level of O-demethylase activity (0.16 nmol/mg protein/min) which was significantly higher than the other populations. The remaining populations showed no significant difference between their mean O-demethylation values. O-deethylation activities of the AV and KN1 populations were the lowest and not significantly different. The activity for the KN2 population was significantly higher than AV or KN1. The highest O-deethylase activity was from the RD population (0.14 nmol/mg protein/min), significantly higher than the other populations.

3.4 *Microsomal enzyme einetic activity*

Kinetic activity reaction velocity (V_{max}) for the substrate PPD was highest in the RD population (0.69 nmol/mg/min). This activity level was 1.8 fold greater than the next highest population, KN2. The AV and KN1 populations had V_{max} values that were about 3.5 and 4.2 fold lower respectively. The mean V_{max} of KN1, AV and KN2 were all significantly smaller than

RD, but the KN2 V_{max} mean was significantly larger than AV and KN1. V_{max} for KN1 and AV were also significantly different. Measurements of substrate affinity (K_m) varied from 1.77 µM (RD) to 2.08 µM (KN1). The RD population had a significantly smaller mean K_m (smaller K_m = higher affinity) than the other populations. The remaining populations had no significant differences in K_m means.

Enzyme activity was also calculated using the substrate TMBZ. The RD population had the highest V_{max} activity (0.61 nmol/mg/min) and was significantly different compared to the other populations. The KN2 population was significantly different than KN1 and AV, which were not significantly different. The K_m values ranged from a high of 1.49 µM (KN1) to a low of 1.02 µM (RD). Significant differences between K_m means were grouped with KN1 and AV in one group and KN2 and RD in the other group.

3.5 *Carbon monoxide difference spectra*

The RD population possessed a significantly higher level of cytochrome P-450 than the next highest population, KN2 (0.19 and 0.11 nmol/mg protein respectively). The KN1 (0.07) and AV (0.08) populations had significantly lower mean cytochrome P-450 levels, but were not significantly different from each other.

3.6 *Knockdown resistance*

Non-significant EC_{50} values were found for the AV, KN1 and KN2 populations. The EC_{50} value of treated RD larvae (158 mM) was significantly larger (2.2 fold) than the LC_{50} value of untreated larvae. Comparisons of probit line slopes revealed no significant differences between the LC and EC lines among populations ($Z < 0.50$).

4 DISCUSSION

4.1 *Permethrin resistance*

At the LC_{50} and LC_{90} levels, the RD population was significantly more resistant to permethrin than the other populations. The KN2 population had significantly higher permethrin resistance than KN1 and AV (not significantly different from each other). Insect populations can be genetically tiered with levels of resistance differing by number of homozygous susceptibles, heterozygous individuals, and homozygous resistants. Resistance

to propoxur in the mite, *Metaseilus occidentalis* (Nesbitt) is an example of a single gene controlling resistance. Distinct separation of homozygous susceptibles and homozygous resistants from the heterozygotes was demonstrated by dose-mortality experiments (Roush and Plapp 1982). In *L. mali*, a possible explanation for the three levels of permethrin resistance may be due to the populations possessing different genetic complements. The KN2 population was under selection pressure longer than the less resistant populations. The proportion of susceptible individuals in this population was probably lower than either KN1 or AV. This population may also be affected by different environmental influences since oxidative resistance was not completely overcome by piperonyl butoxide and this population showed no evidence of possessing an alternative permethrin resistance mechanism. The RD population had the highest level of permethrin resistance and may have a larger number of individuals carrying alleles for oxidative resistance. An additional mechanism (knockdown resistance) may also enhance permethrin resistance for the RD population. These patterns may be due to differing frequencies of resistant alleles in each population or by the presence of unique sets of alleles.

Instances of variable resistance development due to variable insecticide application practices and differences in resistance due to population isolation have been seen in numerous insect species. Variable levels of azinphosmethyl resistance were seen in codling moth, *Cydia pomonella* (L.), populations that were geographically isolated but had similar control histories (Knight *et al.* 1994). Differential exposure to pesticides have resulted in different levels of resistance at the farms. Although permethrin was utilized at each of the farms, the duration of permethrin use and the application intensity varied for each farm. Variation in the slopes of the concentration mortality probit lines was not significant among the farms except for the RD population (significantly steeper). The heterogeneity suggested by the low probit mortality slopes indicates the potential for permethrin resistance in each population to increase and be reestablished.

4.2 *Piperonyl butoxide: synergism of permethrin*

Treatments of adults with PB reduced permethrin resistance less than four fold. These reductions were much less than the 12.4 fold reductions seen by Brewer and Keil (1989b) using the same concentration. Data from Brewer and Keil (1989b) indicated that the application of PB to permethrin resistant adults completely reversed resistance in comparison to the susceptible reference strain. A fixed application rate of piperonyl butoxide (sublethal) was used to avert complete inhibition of the oxidative enzymes. Permethrin/PB ratios have been shown to favor synergism at

lower concentrations of toxin (Brewer and Keil 1989b), as at higher concentrations increased toxicity of the insecticide may not be seen due to interference by PB. In insects, cytochrome P-450 enzymes convert dietary sterols (i. e., ergosterol in mushrooms) into essential metabolic products that cannot be synthesized *de novo* (Feldlaufer and Svoboda 1991). The high levels of PB necessary for ratio testing may cause mortality in the insects due to blockage of these essential cytochrome P-450 enzymes not directly involved in xenobiotic metabolism.

Application of a fixed amount of PB significantly reduced permethrin resistance in KN1 and AV to near that of the reference strain. The largest reduction of permethrin resistance at the LC_{50} was found in the KN2 population. Application to KN2 diminished permethrin resistance 3.9 fold, but this level of resistance was still over 2 fold higher than the reference strain. The KN2 population may possess an underlying mechanism which does not employ oxidative metabolism processes. Another possibility may be that the KN2 population had a cytochrome P-450 which was not as easily inhibited by PB. Resistance in the RD population was reduced only 1.9 fold, a change lower than would be expected for a fixed PB treatment (Brewer and Keil 1989b). This suggests that the RD population had a resistance mechanism that is not oxidative in nature. Significant synergism was observed when results were compared with expected mortality. In the AV population, this suggests that the level of the oxidative enzymes being inhibited by PB has remained essentially the same over the last four to five years.

4.3 *Oxidative metabolism variation*

The conversion of *p*-chloro-N-methylaniline has been used to study metabolic resistance in many insects. Permethrin resistance in the diamondback moth, *Plutella xylostella* (L.), was correlated with a 3 fold higher N-demethylase activity in a resistant strain with a 1.3 fold increase in cytochrome P-450 content (Yu and Nguyen 1992). In this study, the RD population had the highest level of *p*-chloro-N-methylaniline conversion followed by KN2. Differences in permethrin resistance between these populations were similar in magnitude to the variation in N-demethylation. The KN1 and AV populations also had similar abilities to convert the substrate. N-demethylation activity was comparable to the level of permethrin resistance in these two populations.

Measurement of *p*-nitroanisole O-demethylation has been used to show variation in xenobiotic metabolism. Biotransformation of the insecticide parathion is known to yield *p*-nitrophenol as an end product via cytochrome P-450 oxidative metabolism (Soranno and Sultatos 1992).

O-demethylation by cytochrome P-450 enzymes may play a role in permethrin resistance in *L. mali*, but this mechanism may not be as important as N-demethylation, especially in the KN2 population. Variation in enzyme content or form in the KN2 population may have reduced the efficiency of conversion by O-demethylation. The KN2 population may have had lower amounts of a cytochrome P-450 enzyme specific for O-demethylation than for N-demethylation.

Oxidative metabolism measured by O-deethylation of 7-ethoxycoumarin (ECOD) has been used to determine variation in insecticide resistance. Cytochrome P-450 enzymes that possess ECOD are also known to metabolize a variety of insecticides. Due to overlap of substrate specificities seen in cytochrome P-450 enzymes that metabolize insecticides, correlations can be seen between ECOD and permethrin resistance levels (Patil *et al.* 1990) and may also apply to *L. mali*. The RD population had a significantly higher level of ECOD and this was correlated with an increase in cytochrome P-450 enzyme content. Scott et al. (1990) observed a 1.7 fold increase of cytochrome P-450 enzyme in an insecticide resistant strain of house fly which possessed a 5 fold larger ECOD level than a susceptible strain.

4.4 *Enzyme kinetic Studies*

Each population was significantly different from the others in regard to the apparent V_{max} for conversion of *p*-phenylenediamine (PPD). Since V_{max} is related to the amount of enzyme participating in the reactions (Reidy *et al.* 1990), each of populations possessed different levels of enzyme involved in the conversion of PPD. The RD population possessed approximately twice the enzyme level as the next highest population, KN2. This result correlates with the level of total cytochrome P-450 enzyme found in these two populations. In many insects, differences in the amount of enzyme involved in xenobiotic metabolism have been correlated with resistance differences between susceptible and resistant strains. In *L. mali*, the differences in V_{max} for PPD reflect variations in specific cytochrome P-450 content for each of the populations. This variation is partially responsible for the differences in permethrin resistance. Unlike the results for V_{max}, only the RD population had a K_m significantly different from the other populations. Reidy *et al.* (1990) found that flour beetle strains (susceptible and resistant) that were different in V_{max} were not different for K_m. As K_m is related to substrate binding properties of the enzyme, the substrate was bound with similar affinities in three of the populations. In this case, the enzyme(s) metabolizing PPD have a slightly greater affinity for the substrate in the RD population and may contribute to higher permethrin resistance levels.

Similar to the findings for PPD, the differences in permethrin resistance for the populations paralleled the differences in V_{max} for reactions with tetramethylbenzidine (TMBZ). The populations were in the same groupings as seen for N- and O-demethylase activity and cytochrome P-450 content. Differences in permethrin resistance between the populations were again larger than differences in V_{max}. Measurements of TMBZ K_m were different than that seen for PPD. Kennett 2 and RD were now in the same significance category with KN1 and AV in the other significance category. Kennett 2 and RD have a cytochrome P-450 enzyme with a higher affinity for binding TMBZ.

4.5 *Microsomal fraction levels of cytochrome P-450*

Although the variation for CO difference spectra was not as great as that seen with the enzyme activities, the populations could again be divided into three groups for amount of total cytochrome P-450 enzymes. The RD population had a lower ratio of cytochrome P-450 to enzyme activity. The cytochrome P-450 enzyme(s) possessed by this population are more efficient at conversion of substrate. Alternatively, cytochrome P-450s specific for certain reactions exist in greater amounts in some strains than others while variation in total amounts of cytochrome P-450 are not as great (Hodgson 1983).

4.6 *Knockdown (kdr) resistance*

Three of the populations were not significantly different for effective concentrations (EC_{50}). The RD population had a considerably larger EC_{50} value. Cross-resistance to DDT is also afforded via *kdr* and flies that had prior exposure usually possess some level of *kdr* resistance (Sawicki 1978). *Lycoriella mali* may have been pre-selected for permethrin resistance because DDT was used in the mushroom industry until 1972. The *kdr* gene is recessive and may have persisted at low frequency in the populations. Introduction of permethrin in the early 1980s would have renewed selection pressure. This may have led to the reappearance of the *kdr* gene and increased DDT (Brewer and Keil 1989b) and pyrethroid resistance. Oxidative inhibitors of monooxygenase activity did not completely nullify permethrin resistance in some of the populations. This suggests that the *kdr* gene may still be present. The RD population was more resistant to knockdown by permethrin than other populations. This information, coupled with the finding that the level of permethrin resistance in this population was reduced the least by pretreatment with PB, suggests that *kdr* may play a proportionally larger role in permethrin resistance.

Two alternative resistance mechanisms, esterases and glutathione S-transferases, were also investigated and found not to be involved with permethrin resistance in *L. mali* (Bartlett and Keil 1996b).

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