The response of the grape berry moth (Lobesia botrana) to a dietary phytopathogenic fungus (Botrytis cinerea): the significance of fungus sterols

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Abstract

A Tortricidae (Lobesia botrana) has a mutualistic relationship with the fungus (Botrytis cinerea). In this study, we investigated the growth, survival, fecundity and amount of sterols and steroids in larvae of this vineyard pest reared on artificial diets containing mycelium (3%) or purified sterols (0.01%) of the phytopathogenic fungus. Two principal questions related to the physiological and biochemical basis of this mutualistic relationship were addressed: (1) how the fungus influences growth, survival, fecundity, sterol and steroid contents of the insect and (2) are fungal sterols involved in the biochemical basis of mutualism? The presence of fungus in the diet led to a decrease of total duration of larval development (mean gain 5.1–9.4 days compared to the total duration in control of 42.9 days), an increase in survival (mean gain 50–76.3%) and fecundity (gain of 94–102%). These positive effects of the fungus on the biology and physiology of the insect were directly correlated to the presence of fungal sterols in the diet. Fungal sterols are one of the biochemical basis of the mutualistic relationship between L. botrana and B. cinerea. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Insect–fungus interactions; Fecundity; Larval development; Lepidoptera; Sterols; Steroids

1. Introduction

Recent studies on the interaction between the vineyard pest Lobesia botrana (Lepidoptera: Tortricidae) and the phytopathogenic fungus Botrytis cinerea (Deuteromycetes) have demonstrated a mutualistic relationship between these two organisms (Mondy et al., 1998a,b). The larvae act as vectors of B. cinerea inoculum (Fermaud and Le Menn, 1992). In addition, tunneling larvae facilitate rapid penetration and development of the mycelium on the grape berries (Fermaud and Le Menn, 1989). As far as we know, this is one of the few rare mutualistic associations reported between a lepidopteran and a fungus, both being damaging to the same crop, a another one being the European corn borer and stalk rot in corn (Chiang and Wilcoxson, 1961; Carruthers et al., 1986). Generally, the benefits from such associations favour insect development because the fungi are a food source producing vitamins, sterols and other chemical compounds (Beaver, 1989; Kok, 1979). L. botrana reared on a diet including fungal material (grape berries or apple infected with B. cinerea) survived better and had an increased fecundity (Roehrich, 1967; Savopoulou-Soultani and Tzanakakis, 1988), as has been seen for other insects fed fungal material (Kennedy, 1974; Hanski, 1989). Fungi such as yeast have been shown to be a nutrient component which increases egg production in Drosophila (Simmons and Bradley, 1997; Chippindale et al., 1993). One possible explanation for the benefit of mycophagy may be that the ingestion of fungal tissue increases the digestive capacity of the insect because fungal enzymes remain active in the gut following digestion (Abo-Khatwa, 1978; McCulloch Martin, 1992). However, there are uncertainties about the role that fungi play in the insect diet.

Because insects are unable to synthesise sterols de novo, they depend on exogenous sources of sterols for normal growth, development and reproduction (Clark and Bloch, 1959). A phytophagous moth such as L. botrana could use phytosterols of the grape berries to
obtain cholesterol required for moulting hormone biosynthesis (Clayton, 1964; Svoboda et al., 1991). Fungi may play an important, if not an essential role in the nutritional requirements of L. botrana. Fungal sterols may also be involved in this association. Whereas the present understanding of the chemical basis of the insect–fungus interaction and the physiological impact of fungal material in the diet on the development L. botrana are far from complete, a comparison between different fungal supplemented diets was useful in providing insight into the insect–fungus relationship in the vineyard. During the course of this study, the physiological effects, on the insect, of an artificial diet containing mycelium were investigated and we examined whether the fungal sterols were a major biochemical basis of the mutualistic relationship between L. botrana and B. cinerea.

2. Materials and methods

2.1. Insects

Insects were obtained from INRA strains (Institut National de Recherche Agronomique-Bordeaux) and were reared under controlled conditions (16L:8D; 22±1°C, 60±10% RH) on an artificial diet containing 4.6% casein, 4% cellulose, 2.3% glucose, 1.3% mineral salts, 2.6% of Vanderzant vitamin mixture, 3.3% agar, 0.1% maize oil (containing 0.3% of phytosterols, i.e. 0.0003% in artificial diet) and 0.08% cholesterol as described previously (Mondy et al., 1997).

2.2. Fungus

Botrytis cinerea isolated from a vineyard in 1991 was used. Stock cultures were maintained on 1.5% (w/v) malt–agar medium. Mycelia were obtained in liquid medium containing yeast (0.2%) and glucose (1%) after two weeks of incubation at 22°C. After filtration, mycelia were freeze dried and incorporated into the artificial insect diet, or used for sterol extraction.

2.3. Developmental effects

Newly hatched larvae were reared individually in test tubes containing 1 ml of artificial diet. The artificial diets contained 0.08% cholesterol supplemented with mycelium of B. cinerea (3% of dry weight) or with purified sterols from fungal material (0.01% of dry weight). Two replicates of 60 tubes were used in each experiment and tubes contaminated with micro-organisms were discarded. Larvae were examined daily and their larval stage determined by measuring the cephalic capsule size. The percentage of mortality was calculated at each stage. For all experiments, pupae were weighed and the sex was determined. For the fecundity bioassay, one newly hatched female from an experimental diet treatment was paired with two hatching males reared on the same diet as a single pair to facilitate mating and subsequent oviposition by the female. The total number of eggs laid were monitored after five days of oviposition (the period when the mated females deposited 80% of their eggs). The hatchability of eggs was recorded in all oviposition trials after six days of incubation and females were dissected to verify the presence of spermatheca and to record the ovocyte number.

2.4. Sterol and ecdysteroid analyses

2.4.1. Cholesterol content

Freeze-dried insects were homogenised in dichloromethane-methanol (2:1) by sonication (5 min, three times). After solvent evaporation, the extract was saponified using KOH (6%) in methanol. The following steps were similar to those described by Corio-Costet et al. (1989). The unsaponifiable material was extracted three times with hexane, and the pooled extracts were dried. The residue was chromatographed on Merck HF 254 TLC plates with dichloromethane as the developing solvent. The 4-desmethylsterols were scraped and eluted in dichloromethane. After acetylation, samples were analysed by GC. For each experiment, sterols were determined for six males and six virgin females one day after adult ecdisis.

2.4.2. Ecdysteroid content

Ecdysteroid analyses were performed on whole insects, extracted by sonication in methanol 100% and stored at −20°C until assayed. After centrifugation and evaporation, dried extracts were suspended in 0.1 M phosphate buffer (pH 7.4) and analysed using an enzyme immunoassay (EIA) adapted from Porcheron et al. (1989), which employed a peroxidase conjugate of 20E as a tracer (De Reggi et al., 1992). All assays were performed at least in duplicate. The ecdysteroid titres were expressed in 20-hydroxy-ecdysone equivalents because this ligand was used for standard curves. Ecdysteroid contents were determined daily during the pupal stage for males and females.

2.4.3. Extraction and purification of fungus sterols

Dried mycelium was saponified in methanolic KOH (6%) under reflux for 2 h. The unsaponifiable lipids were extracted with hexane and sterols were purified by silica gel TLC with dichloromethane as developing solvent (Costet et al., 1987). The residue was chromatographed and thesterol band was scraped and eluted in dichloromethane. The compounds were acetylated and samples were analysed by GC (Quartz capillary column (30 m×0.25 mm) coated with OV-1 (J & W Scientific, Folsom, CA). An internal standard of cholesterol was used and identi-
fication was performed by GC–MS (column SE 30 heated at 280°C, Varian 3300, GC with Ross injector linked to a Nermag R10-10C) out at an ionising energy of 70 eV as described previously (Chapuis et al., 1996).

2.5. Statistical analysis

Depending on strength and normality, Student’s t-test (for development duration and weight analyses), Mann–Whitney U-test (for fecundity and sterol content analyses) and a Chi² test at $P=0.05$ (for mortality analyses) were used to analyse the means of nutritional bioassays (Wonnacott and Wonnacott, 1990). When the same distribution was used through several comparisons, the Bonferroni corrected $P$ values was used to assess the final significance of the test. Computations were carried out using SPSS software (SPSS, 1995). Means were calculated for two experiments of 60 newly hatched larvae.

3. Results

3.1. Sterol composition of B. cinerea

The sterol pattern of the extract of B. cinerea used to supplement diet B was complex. The percentages of sterol composition of the fungus were listed in Table 1. The major sterol identified was ergosterol (85.2%) as seen for most filamentous fungi (Parks and Weete, 1991). In addition to ergosterol, five minor 4-demethylsterols, ergostetraenol [methylcholest-5,7,9(11),22-tetraen-3-βol] (1), methylcholest-5,22-dien-3-βol (2), lichesterol [methylcholest-5,8,22-trien-3-βol] (3), ergosterol [methylcholest-5,7,22-trien-3-βol] (4), fecosterol [methylcholest-8,24(24’)-dien-3-βol] (5), episterol [methylcholest-7,24-dien-3-βol] (6) were present as in the analyses of Loeffler and Hayes (1992). Each accounted for 0.4 to 4.4% of total sterols. Botrytis mycelium contained 2.68±0.02 mg of sterols per gram of dry weight.

3.2. Post-embryonic development of L. botrana in experimental insects

The development of L. botrana is characterised by a succession of five larval and one pupal instars. Rearing larvae on the B. cinerea supplemented diet after hatching immediately affected the chrysalis weight, the duration of development, and the rate of survival. The total duration of development of the insects reared with mycelium (A) or purified sterol fraction (B) was significantly shorter than in the control (Table 2). The duration of male development decreased 4.5 days on B diet ($t=5.01, P<0.001$) to 7.9 days on A diet ($t=6.84, P<0.001$). Similarly, female development duration was decreased 5.7 days on B diet ($t=4.91, P<0.001$) to 10.9 days on A diet ($t=8.14 P<0.001$).

Final chrysalis weight was affected only by A diet. The increase in weight for males was 15.9% ($t=1.13, P<0.04$), and 16% for females ($t=2.86, P<0.013$). Survival was significantly influenced by the fungal material presence in the diets. As shown in Table 2, the experimental insects had a sharply decreased mortality as compared to control insects. At the end of development, the relative percentage of insect mortality fed on diets B or A was 56.8% and 78.4% lower than for control insects, respectively (diet B: $\chi^2=16.12, P=0.01, DF=1$; diet A: $\chi^2=32.7, P<0.001, DF=1$). Mortality remained low during larval instars for the insects reared on diets A and B. The mortality of insects reared on control diet during the second larval instar (8% mortality) was significantly higher than the mortality of insects reared on supplemented diets ($\chi^2=16.2, P<0.001, DF=1$) (Fig. 1). Of the control insects, 17.4% did not complete adult ecdysis (diet A: $\chi^2=8.9, P<0.003, DF=1$ and diet B: $\chi^2=14.8, P<0.002, DF=1$). Conversely the presence of mycelium or purified sterol fraction led to drastically reduced pupal mortality.

3.3. The effect of B. cinerea on fecundity

Egg production significantly increased in response to a diet containing mycelium or purified sterols of B. cinerea (Table 3). Females issuing from the diets A and B produced more eggs (64–71%) than control females (control females versus A and B females: $U=5, P=0.0009$, and $U=20, P=0.0002$, respectively). No significant difference was observed between the number of eggs laid by A or B female ($U=69, P=0.37$). No significant difference was observed between the number of ovocytes and the egg hatchability for the different diets. As the chrysalis reared on diet A (containing 3% of mycelium) had a higher weight than the control (Table 2), the relation between the weight and the egg pro-

<table>
<thead>
<tr>
<th>Sterol</th>
<th>Sterol composition RRt</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) ergostetraenol</td>
<td>1.136</td>
<td>2.1</td>
</tr>
<tr>
<td>(2) methylcholest-5,22-dien</td>
<td>1.143</td>
<td>3.9</td>
</tr>
<tr>
<td>(3) lichesterol</td>
<td>1.149</td>
<td>4.0</td>
</tr>
<tr>
<td>(4) ergosterol</td>
<td>1.176</td>
<td>85.2</td>
</tr>
<tr>
<td>(5) fecosterol</td>
<td>1.205</td>
<td>0.4</td>
</tr>
<tr>
<td>(6) episterol</td>
<td>1.228</td>
<td>4.4</td>
</tr>
</tbody>
</table>

a (1) ergostetraenol [methylcholest-5,7,9(11),22-tetraen-3-βol], (2) methylcholest-5,22-dien-3-βol, (3) lichesterol [methylcholest-5,8,22-trien-3-βol], (4) ergosterol [methylcholest-5,7,22-trien-3-βol], (5) fecosterol [methylcholest-8,24(24’)-dien-3-βol], (6) episterol [methylcholest-7,24-dien-3-βol].

b RRt: relative retention time to internal standard cholesterol.
Table 2
Nutritional impact of mycelium (A) or fungal sterols (B) on insect development and mortality. Mean (± SE) of total duration of development (days), chrysalis weight (mg) and total mortality (%)a

<table>
<thead>
<tr>
<th>Diet</th>
<th>Duration of insect development (days)</th>
<th>Chrysalis weight (mg)</th>
<th>Mortality (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Control</td>
<td>40.9±0.8</td>
<td>44.9±1.1</td>
<td>8.8±0.3</td>
</tr>
<tr>
<td>(A) mycelium</td>
<td>33.0±0.8***</td>
<td>34.0±0.8***</td>
<td>10.2±0.4*</td>
</tr>
<tr>
<td>(B) sterols</td>
<td>36.4±0.4***</td>
<td>39.2±0.4***</td>
<td>8.8±0.4</td>
</tr>
</tbody>
</table>

a Significant levels: *P<0.05, **P<0.01, ***P<0.001 (A and B insects compared to control insects).
b Total mortality from hatching to emergence for males and females.

Fig. 1. Effect of various diets on the percentage of mortality during larval development of Lobesia botrana (means ± SE). Control insects reared on artificial diet (□); experimental insects reared on mycelium of B. cinerea supplemented diet A (■); or on B. cinerea purified sterols supplemented diet B (□).

Table 3
Mean (±SE) of fecundity, hatchability and ovocytes number per female of Lobesia botrana reared on diet supplemented with fungus (A and B) and controla

<table>
<thead>
<tr>
<th>Diet</th>
<th>Fecundity (eggs laid per female)</th>
<th>Ovocyte number (ovocytes per female)</th>
<th>Hatchability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>71±10.6</td>
<td>6±2.5</td>
<td>86.6±2.3</td>
</tr>
<tr>
<td>Mycelium</td>
<td>138±10***</td>
<td>6±2.8</td>
<td>90.1±2.5</td>
</tr>
<tr>
<td>Purified sterols</td>
<td>144±16 ***</td>
<td>8±2.6</td>
<td>95.0±6.5</td>
</tr>
</tbody>
</table>

a Significant level: *P<0.05, **P<0.01, ***P<0.001 (treated insects compared to control insects).

3.4. Effect of B. cinerea on sterol and ecdysteroid content

Fig. 2 shows that egg production of females of similar weight differs depending on the presence of fungus in the diet. B. cinerea in the diet (mycelium or sterols) improved egg production compared with control insects (diet A: U=43, P=0.004**; diet B: U=15, P=0.0009***). There were no difference between the insects issuing of supplemented diets (A and B, U=58.5, P=0.037 ns).

The analysis of cholesterol content (Table 4) did not show a significant difference between control insects and
insects reared on diets A or B. In all experimental diets, adult males of L. botrana contained higher amounts of cholesterol than females (for wet weight $U=54.5$, $P=0.01^{**}$; and for dry weight $U=69$, $P=0.011^{*}$).

The male ecdysteroid titre showed a similar pattern, peaking at 3150 to 4000 pg 20E eq/mg. The peak occurred 4 days (on the control diet) or five days (on diet A) after pupal ecdysis (Fig. 3). The pattern obtained was different for females. Two peaks were detected, one 4 days after pupal ecdysis, peaking at 2900 pg 20E eq/mg, and a second 9–10 days after pupal ec dysis, peaking at 5200 (in control insects) to 6950 in diet A insects. The ecdysteroid titre in females reared on diet A showed a low SE value for all measurements. This fact emphasised the best synchronisation of insect development as described above. The synchronisation effect suggested a direct or indirect action of fungus sterols on the regulation of development mechanisms of the insect.

4. Discussion

It has been recently shown that the presence of the fungus on the grape berry cluster modifies the behaviour of L. botrana (Mondy et al., 1998a) and favours its development and survival on grapes infected with B. cinerea (Mondy et al., 1998b). Larval diet plays an important role in determining the size and fecundity of most insects, the Lepidoptera in particular, because the adults are unable to assimilate food and their eggs must be derived from reserves laid down by the larvae. In the present experiments, diet supplemented with fungus induced physiological effects on the insects such as an increase in weight and fecundity and a decrease in the total duration of development and mortality. Fungi on host plants or in an artificial diet are a possible nutritional source for insects (Beaver, 1989; Berryman, 1989). Many insects have a symbiotic relationship with fungi such as the ambrosia beetle, leaf-cutting ants and macrotermitididae. They require fungi as essential nutrient sources for compounds such as sterols (Chu et al., 1970; French and Roeper, 1973; Quilan and Cherrett, 1978). Many studies have shown the importance of fungus such as yeast in artificial diets. Yeast is required in the diet for egg production in Chrysoperla carnea (McEwen and Kidd, 1995) and yeast favours oogenesis and eggs laid after starvation in Drosophila melanogaster (Bownes et al., 1988; Chippindale et al., 1993; Simmons and Bradley, 1997). However, the active compounds implicated were not specified. In that insects are not capable of synthesising the steroid nucleus de novo and therefore require a dietary source of sterol for their survival, sterols derived from B. cinerea may have positive effects on development. Purified fungal sterols added to the diet led to similar effects as noted with mycelium-supplemented diet. This fact confirmed the involvement of fungal sterols in the mutualistic relationship between B. cinerea and L. botrana. Indeed, both diets induced a decrease in the larval development duration and larval mortality. An increase in chrysalis weight was recorded only when the larvae were fed mycelium, but the increase of female fecundity was...
observed with both mycelium and purified sterol diets. It is generally accepted that insect fecundity is proportional to adult size (Tanaka, 1981; Karlsson, 1987). In the *B. cinerea–L. botrana* relationship, the results indicate that the increase in fecundity was not correlated with the increase in size of insects reared with mycelium or purified sterols. Purified sterols of *B. cinerea* are involved in the determination of fecundity of *L. botrana* and an increase in fitness.

Cholesterol will generally satisfy this dietary requirement for sterol, and many phytophagous insects are capable of converting the phytosterols commonly found in their dietary plant material to cholesterol. It is then used as the precursor for moulting hormones (Svoboda and...
A concentration of 0.08% cholesterol was used in the control diet because previous data had indicated that a minimum of 0.05% was required for complete development (unpublished data). The quantity of added sterol whether from mycelium or purified sterol was very low (8.1–10 mg per 100g of diet) and was not a real nutritional source. Indeed, the entire diet contained 80 mg of cholesterol and 0.3 mg of phytosterols per 100 g of food. The level of Δ5-sterols (essentially cholesterol 99.6%) in the artificial diet can play a major role in two physiological processes. The bulk of dietary sterol is allocated for structural components in cell membranes and it serves as precursors for steroid hormones. The EIA measurements obtained on L. botrana showed the presence of 20E and emphasised the role of cholesterol as a potential precursor of ecdysteroids in this insect. L. botrana is able to convert dietary phytosterols from grape berries into cholesterol, but not the fungal sterols of B. cinerea (data not show). The role of B. cinerea sterols in the decrease of pupal mortality or the increase of egg production is in agreement with the results of Chu et al. (1970) obtained with Xyleborus ferrugineus. Our experiments showed that B. cinerea sterols can act on the duration of the larval stage (especially at the second instar and pupal instar), on survival of the larvae and fecundity of females. It will be interesting to identify which Botrytis sterols are able to induce these effects. The fungus contains six 4-demethylsterols which are methylated at C24 position. We found different unsaturated positions on ring B and on the side chain: Δ5, Δ7, Δ9(11) and a Δ22(23) in ergostatetraenol (1), Δ6 and Δ22(23) in sterol (2), Δ5,Δ8 and Δ22(23) in lichesterol (3), Δ5,7 and Δ22(23) in ergosterol (4), Δ5,8 and Δ24(241) in fecosterol (5) and Δ5,7 and Δ24(241) in episterol (6). The next step in this study will be to determine the role of each fungal sterol in the development of the insect, particularly ergosterol. This sterol is methylated at C24 with a double bond at C22–C23 and two double bonds at C5–C6 and C7–C8. Even though, in general, insects are capable of removing the methyl at C24, a number of insects cannot reduce the double bond between C22–C23. The goal of this transformation, for the insect, is to synthesise cholesterol. It will be of interest to determine how the insect goes about this transformation. On the other hand, other sterol(s) saturated at the 22 position and present at low concentrations may also play a role.

We have quantified the sterol and the ecdysteroid contents in males and females. No important qualitative or quantitative differences were found in the sterol contents. Insect vitellogenesis and egg production are under endocrine control. In species where vitellogenesis begins in the last larval instar (e.g. Lymantria dispar, Bombyx mori), low JH titre and/or high ecdysteroid titre are required for vitellogenesis (Tsuchida et al., 1987; Davis et al., 1990). There may be a nutritional effect of fungal sterols on ovogenesis mediated by changing levels of ecdysteroid secreted by the ovary in response to the diet and a difference may eventually be detected in the eggs. The ecdysteroid analysis showed no difference except for the second peak which was observed in females 9–10 days after pupal ecdisys. This peak corresponded to ovogenesis and the insects reared on fungus showed ecdysteroid titre peaking at 6700± pg 20Eeq/mg versus 5800± pg 20E Eq/mg in the control females. As shown, the experimental females laid more eggs than control females and the increase in ovogenesis peak seemed in relationship with the increase of eggs laid. This was verified by an analysis of egg weight and ecdysteroid content in control and experimental eggs. The control and experimental eggs had the same weight (7.06±0.75 and 6.17±0.65 μg/egg, respectively). The level of ecdysteroid in the eggs showed no significant difference (9.17±2.84 μg/egg in the control and 10.30±3.06, in experimental eggs, respectively).

During insect development on the experimental diet, ecdisys synchronisation was better than in insects fed the control diet. This observation was reinforced by the ecdysteroid analysis in the females, the standard error of which was very low compared to the control females. A direct or indirect action of the fungal sterols on endocrine regulation can be expected. B. cinerea contained in the diet of L. botrana increased survival and fecundity of insects and decreased the total duration of development. The mutualistic relationship between this phytophagous insect and this filamentous fungus is hinged, on fungal sterols. The sterols are the biochemical basis of this relationship having profound physiological effects on the insect.

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