Apidologie (1992) 23, 119-132
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Original article

Analysis of alfalfa leafcutter bee *(Megachile rotundata)* provisions pre- and post-sterilization with propylene oxide

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(Received 8 March 1991; accepted 9 December 1991)

Summary — Provisions of *Megachile rotundata* were analyzed for their physical, chemical and microbial properties prior to and following fumigation with propylene oxide. The effects of treated and untreated provisions on larval development and susceptibility to *Ascosphaera aggregata* were also studied. Bacteria, yeasts and filamentous fungi averaged 1.3 x 10^3, 1.5 x 10^2 and 7.5 x 10^3 colony-forming-units g^{-1}, respectively. Fumigation with propylene oxide for 24 h at concentrations of 0.6 and 1.2%, but not 0.1%, effectively sterilized provisions. Untreated provisions consisted of 18.1% water, 56.8% nectar and 25.1% pollen with a pH of 4.1. The sugar content was 65.9% and was comprised of fructose (48.1%), glucose (43.6%), sucrose (1.4%) and turanose/palatinose (1.8%). Pollen contained 6% protein. Pollen was not stratified within provisions and *Medicago sativa* was the most prevalent taxon (66.2%). Propylene oxide treatment had no effect on the protein content or distribution of pollen within provisions but increased the pH by 0.2–0.5. Total sugars and quantities of fructose and glucose were reduced by 3.2, 10.4 and 8.7%, respectively, whereas the caloric content of treated provisions was 1.6% greater than the control treatment. Detoxification under vacuum for 120 h reduced the water content of provisions by 40.3%. Eggs of *M rotundata* did not survive when placed on provisions detoxified under vacuum for 24 h post-fumigation. On provisions detoxified for 120 h, there were no differences in mortality between larvae reared on sterilized, artificial and non-sterilized provisions, but time to prepupation was delayed relative to the non-sterilized treatment. The mean mortalities of larvae inoculated with *A aggregata* were 98.0, 97.8 and 27.4%, respectively on artificial, sterilized and non-sterilized provisions.

Megachile rotundata* / provisions / sterilization / chalkbrood / *Ascosphaera aggregata

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INTRODUCTION

Chalkbrood caused by *Ascosphaera aggregata* Skou is an economically important disease of alfalfa leafcutter bees (*Megachile rotundata* Fabricius) in North America. Epizootics are initiated from ascospores within provisions that are ingested by developing larvae (Vandenberg and Stephen, 1982). Although some work has been done on provisions of other solitary bees (Gilliam *et al,* 1984, 1990), little is known about the physical, chemical and microbial characteristics of alfalfa leafcutter bee provisions. The possibility that the microflora associated with provisions may influence the development of chalkbrood has not yet been explored. Recently it was demonstrated that fewer larvae developed chalkbrood when reared on natural provisions than when maintained on a sterilized artificial diet (Goettel and Vandenberg, unpublished data) raising questions as to the influence of the microflora. We propose to elucidate the role of microorganisms by comparing disease development in inoculated larvae reared on non-sterilized and sterilized natural provisions, and on artificial provisions. The first stage in this process required a sterilant which would kill microorganisms, but be non-destructive to the physical and chemical properties of provisions. Propylene oxide has been found to be an effective sterilant, but all sterilization procedures potentially alter some of the physical and chemical aspects of the substrate. Therefore the objectives of this study were: 1) to evaluate the effectiveness of propylene oxide for sterilization of provisions, 2) to compare the chemical and physical characteristics of untreated and propylene oxide-treated provisions, and 3) to determine the effects of propylene oxide-sterilized, non-sterilized and artificial provisions on larval development and susceptibility to *A aggregata.*

MATERIALS AND METHODS

Collection of alfalfa leafcutter bee cells

Individual nest cells containing eggs were collected at Lethbridge, Alberta from hives in an irrigated field of alfalfa (*Medicago sativa* L). Cells for the fumigation, larval development and *A aggregata* inoculation experiments were collected in July and August, 1990, whereas cells for chemical and physical analyses of provisions were collected in September. Alfalfa was in full bloom during all sampling dates. Following collection, eggs were carefully removed and maintained on 1.5% water agar at 5 °C for not more than 7 days.

Sterilization of provisions with propylene oxide

The efficacy of propylene oxide for the sterilization of provisions was initially tested at concentrations of 0.1, 0.6 and 1.2% (v/v) for 24 h in 1.8-l desiccators. Both the treated and untreated provisions were then aerated for 24 h under vacuum prior to recovery of microorganisms. In a second experiment, provisions were exposed to propylene oxide for 6, 12 and 24 h at a concentration of 0.6%. Subsequently, the treated and untreated provisions were maintained for 120 h under vacuum. A third experiment compared microorganisms from provisions aerated for 120 h under vacuum with non-aerated provisions not fumigated with propylene oxide.

Quantification of microorganisms

Provisions from 5 cells in each of 3–4 replicates per treatment were removed, weighed and homogenized in 0.01 M phosphate buffer (pH 7). Suspensions were then serially diluted 3 times and aliquots of 0.1 ml from each dilution were spread in duplicate onto the surface of nutrient agar modified with 50 mg l^{-1} of nystatin for the recovery of bacteria, and onto sorbose yeast-extract agar (Bandoni, 1981) amended with 100 mg l^{-1} of tetracycline for the recovery of fungi. Cultures were incubated at 25 ± 1 °C for 72-

120 h. Colony-forming-units (cfu) of filamentous fungi, yeasts and bacteria were enumerated at the dilution yielding 20-200 cfu per Petri dish and data were calculated as the mean number of cfu g^{-1} fresh weight of provisions. Experiments were arranged as completely randomized designs (CRD).

Component weights, pH determination and sample preparation

Non-treated and propylene oxide-treated provisions (0.6% for 24 h) were removed from 24 cells in each of 4 replicates and weighed. The provisions were then freeze-dried for 24 h, reweighed, homogenized in 5 ml of high performance liquid chromatography (HPLC) quality water and the pH values determined. The homogenates were maintained at 55 °C for 1 h, vortexed at 15-min intervals, and centrifuged at 1 470 *g* for 15 min. The supernatants were removed, the pH values recorded, and the supernatants stored for analysis of the soluble sugars of the nectar fraction. The pollen pellets were freeze-dried for an additional 24 h, weighed, resuspended in 5 ml of deionized water, and the pH values recorded. The effect of the vacuum treatment on the water content of provisions was also investigated. Non-vacuum-treated provisions and provisions maintained under vacuum for 120 h were removed from 24 cells in each of 4 replicates per treatment, weighed, freeze-dried for 24 h and re-weighed.

Analysis of soluble sugars and bomb calorimetry

From each replicate, total soluble sugars in 10- µl aliquots of the water-soluble fractions were determined with a Zeiss Model A refractometer at 25 °C and calculated as percent soluble sugar per g of provision dry weight. In addition, quantities of fructose, glucose, sucrose and turanose/palatinose in each replicate per treatment were determined in 200-µl aliquots of the water-soluble fraction diluted in 800 µl of acetonitrile:water (80:20 v/v) using an HPLC (Low *et al*, 1988). The mixture was filtered initially through a Swinney-25 syringe adapter using a 0.45-µm nylon membrane filter. The HPLC system consisted of a Phenomenex U-Bondapax 300 x 3.9 mm column attached to a Whatman differential refractometer (Waters model R401) maintained at 25.5 °C by a circulating water bath. The sample was introduced by a Rhenodyne (model 7125) injector equipped with a 20-µl injection loop. Elution of the sample was carried out with a mobile phase consisting of acetonitrile:water (80:20 v/v) at a flow rate of 1 ml min^{-1}. Quantities of sugars were calculated as g sugar g^{-1} nectar dry weight.

To determine the caloric content, provisions from 36 cells in each of 4 replicates per treatment were removed, freeze-dried for 24 h and weighed. Approximately 1 g was then oxidized in a Parr (model 1710) adiabatic calorimeter and the caloric content g^{-1} provision dry weight calculated.

Protein content of pollen

Freeze-dried pollen from each of the 4 replicates per treatment were rehydrated in 2.5 ml of 10% sodium dodecylsulfate, then sonicated for 5 min using an Artek (model 300) dismembrator. The suspensions were centrifuged at 15 600 *g* for 6 min and the supernatants collected. Protein in the supernatants was quantified using the Bradford method (Bradford, 1976). Five ml of 10% trichloroacetic acid were added to each supernatant which were re-centrifuged for 10 min. The resulting protein pellets were then dissolved in 5 ml of 0.1 N NaOH at 70 °C; 20-µl aliquots of the protein solutions were added to 5 ml of Bradford's solution and the absorbances measured at 595 nm. Total protein was determined using a bovine albumin standard curve and calculated as g protein g^{-1} pollen dry weight.

Melissopalynology and stratification of pollen

Pollen in each of 4 replicates per treatment was acetolyzed according to the method of Low *et al* (1989). Pellets were suspended in 4 ml of deionized water, centrifuged at 1470 *g* for 5 min, washed with 5 ml glacial acetic acid, recentrifuged and the supernatants discarded. The precipates were maintained in a 1:9 mixture of concentrated sulphuric and acetic anhydride

in a water bath at 100 °C for 3 min, centrifuged and the supernatants decanted. The pellets were then washed in glacial acetic acid, centrifuged, successively washed in 5 ml each of distilled water, ethanol and *t*-butyl alcohol. Following removal of the *t*-butyl alcohol, pollen pellets were resuspended in silicon oil. A drop was mounted for microscopy and a minimum of 200 pollen grains in each of 4 replicates per propylene oxide-fumigated and non-fumigated treatments were identified under phase-contrast microscopy at 640 X.

To determine possible stratification of pollen with nectar in provisions, cells were initially sectioned longitudinally using an Erma freeze microtome, the sections mounted in polyvinyl alcohol and examined with a light microscope at 100 X. Subsequently, cores of provisions were taken using a 3-mm (diameter) cork borer. Cores, 4–5 mm in length, were frozen in liquid nitrogen, removed from the cork borer and cross-sections made at 1-mm intervals beginning at the bottom of the cell and moving upward. Each section was then homogenized in 0.4 ml of the phosphate buffer and the total number of pollen grains per section was determined using a haemocytometer. Quantities of pollen at the bottom of the cell were compared with quantities at the top within and between each of the propylene oxide-fumigated and non-fumigated treatments.

Larval development

Eggs were placed on the surfaces of non-treated and propylene oxide-treated (0.6% for 24 h) provisions in 96-well tissue culture plates. In the first experiment, provisions were maintained under vacuum for 24 h and, in the second experiment, for 120 h prior to egg placement. In addition to the natural provision treatments, an artificial diet, modified from Fichter *et al* (1981) by Vandenberg (personal communication), was included in the second experiment. The artificial diet consisted of: 10 ml honey, 35 g sucrose, 18.6 g macerated honey bee-collected pollen, 18.6 g of a soy milk-yeast mixture, 0.1 g methyl-*p*-hydroxybenzoate, 0.1 g sorbic acid, 1.5 g agar and 80 ml distilled water. The artificial diet was autoclaved at a pressure of 10 psi and dispensed into individual wells. The experiments were arranged as CRD with 8–10 larvae in each of 3 replications. Provisions were maintained at 30 ± 1 °C in the dark. Egg and larval mortality and the time to prepupation (spinning of cocoons) were recorded for 16 days.

Chalkbrood bioassay

Sporulating cadavers were supplied by the Canadian Leafcutter Bee Cocoon Testing Center, Brooks, Alberta. Cadavers placed on a fine plastic mesh in a Petri dish were stirred with a magnetic stir bar to release ascospores from subcutaneous ascomata. Ascospores that had passed through the mesh were collected and maintained at 5 °C until required. The viability of ascospores was tested by placing approximately 1×10^6 ascospores into 0.1 ml of Sabouraud's dextrose broth with 2% yeast extract, streptomycin (50 µl ml^{-1}) and penicillin (25 IU.ml^{-1}) in a 16- ml test tube. The tube was then filled with CO_2, sealed and maintained at 30 °C. A germination percentage of 76% was observed after 24 h. Immediately prior to use, ascospores were crushed between 2 sterile glass slides to release ascospores from remaining spore balls. Ascospores were then suspended in 0.017 mM DOSS (dioctyl sulfosuccinate)-saline buffer (pH 7.1) (Stephen *et al,* 1982), enumerated with a haemocytometer and the concentration of spores adjusted to 5×10^8 ascospores ml^{-1}.

The mortality of larvae maintained on propylene oxide-treated, untreated and artificial provisions post-inoculation with *A aggregata* was compared. Cells were fumigated with propylene oxide for 24 h at 0.6% and subsequently detoxified under vacuum for 120 h. Untreated provisions were also maintained under vacuum for 120 h. Following vacuum treatment, an egg was placed onto each of 80 provisions per treatment. Provisions were placed in 96-well plates as a CRD with 5 replicates per treatment. Aliquots of 2 µl containing 1×10^6 ascospores of *A aggregata* were placed on the surface of provisions next to each egg and maintained at 30 ± 1 °C in the dark. After 16 d, the incidence of mortality was recorded.

Data analysis

Computations were performed with Statistical Analysis System Software (SAS Institute Inc,

Cary, NC) using the ANOVA, general linear model and TTEST procedures. Homogeneity of variance was checked using the folded form *F* statistic in conjunction with the TTEST procedure. Normality of data was tested using the univariate procedure and when data did not conform to normality, an appropriate transformation was used. Means are presented as X ± standard error.

RESULTS

Efficacy of sterilization

From provisions fumigated with propylene oxide at a concentration of 0.1% for 24 h, recovered bacterial cfu g^{-1} ranged from 3.8 x 10^2 to 1.1 x 10^3 and fungal cfu g^{-1} ranged from 4.9 x 10^1 to 8.2 x 10^1. After 24 h fumigation at 0.1%, fungal ($P = 0.018$) but not bacterial ($P = 0.15$) cfu were significantly less numerous than those recovered from the control provisions. Bacterial and fungal cfu isolated from the control provisions ranged from 2.2 x 10^3 to 5.5 x 10^3 cfu g^{-1} and 3.9 x 10^2 to 5.9 x 10^3 cfu g^{-1}, respectively.

No mircroorganisms were recovered from provisions treated with propylene oxide for 24 h at concentrations of 0.6 and 1.2%. At the concentration of 0.6%, no viable propagules of filamentous fungi or yeasts were recovered after 6 h exposure. Although some bacterial propagules could be recovered following 12 h exposure, their numbers were greatly reduced (98.6% fewer cfu ($P = 0.05$)) (table I), and provisions were rendered sterile by 24 h. Vacuum treatment alone had no significant effect on bacteria or fungi.

Chemical properties

Propylene oxide treatment increased ($P = 0.0001$) the pH of the water-soluble and pollen fractions and of the whole provi-

Table I. Number of colony-forming-units (cfu) of filamentous fungi, yeasts and bacteria recovered from provisions of alfalfa leafcutter bees *(Megachile rotundata)* treated with propylene oxide (0.6% v/v)[w].

Epoxide Exposure (h)	*Vacuum time (h)*	*cfu.g^{-1} fresh weight*[x]		
		Bacteria	*Yeasts*	*Filamentous*
0	0	1.3 x 10^3a[y]	1.5 x 10^2a[z]	7.5 x 10^3a[z]
0	120	1.7 x 10^3a	3.9 x 10^1a	4.4 x 10^2a
6	120	6.3 x 10^2a	0.0	0.0
12	120	2.4 x 10^1b	0.0	0.0
24	120	0.0	0.0	0.0

[w] Microorganisms were recovered using a dilution plating method. Colony-forming-units of bacteria were recovered on nutrient agar modified with nystatin, whereas cfu of yeasts and filamentous fungi were recovered on sorbose yeast extract agar modified with tetracycline. [x] If possible, cfu g^{-1} provision (fresh weight) were calculated from the dilution yielding 20–200 colonies $dish^{-1}$. [y] Means not followed by the same letter are significantly different ($P \leq 0.05$) according to Duncan's multiple range test of logarithmic-transformed data. Untransformed means are reported. [z] Means followed by the same letter are not significantly different ($P \leq 0.05$) as determined by a *t*-test for logarithmic-transformed data. Untransformed means are presented.

sions by 0.2–0.5. The mean pH of the sterilized water-soluble fraction, pollen fraction and whole provisions was 4.3 ± 0.0, 4.5 ± 0.0 and 4.3 ± 0.03, respectively. In comparison, the mean pH of the non-sterilized water-soluble fraction, pollen fraction and whole provisions was 4.1 ± 0.03, 4.2 ± 0.02 and 4.1 ± 0.03, respectively. The mean pH of the artificial provision was 5.0 ± 0.02. Total soluble sugars were reduced (P = 0.0160) by 3.2% (table II). HPLC analysis of the water-soluble fraction indicated that sugars accounted for 94.9% (w/w) of the water-soluble fraction (table II): fructose and glucose comprised 91.7%, the oligosaccharides, sucrose and turanose/palatinose comprised 3.2%. Quantities of fructose and glucose in non-sterilized provisions were 10.4% (P = 0.0132) and 8.7% (P = 0.0259) greater, respectively, than in propylene oxide-fumigated provisions but the ratio of fructose to glucose (1.1:1) was the same for both treatments. There were no significant differences in the quantities of sucrose (P = 0.7432) and turanose/palatinose (P = 0.0799) between the two treatments. The mean caloric content of sterilized provisions was 4501.1 ± 12.1 cal g^{-1}, 1.6% greater (P = 0.0099) than the caloric content of non-sterilized provisions (4427.2 ± 15.8 cal g^{-1}). The mean protein content of pollen from non-sterilized provisions was 6.0 ± 0.4%. There was no significant difference between the protein content of pollen from non-sterilized and propylene oxide-fumigated (6.5 ± 0.3%) provisions.

Physical properties

Detoxification for 120 h under vacuum reduced (P = 0.0001) the water content of provisions from 18.1 ± 0.01 to 10.8 ± 0.3%. However, there were no differences (P = 0.05) in the moisture, water-soluble fraction or pollen content of non-sterilized and sterilized provisions following detoxification for 120 h. The solid components of provisions were comprised of 58.6–70.4% nectar (water-soluble fraction) and 23.6–34.7% pollen (non-water-soluble fraction). Characterization of pollen from both treatments indicated no significant differences in the incidence of taxa. Pollen consisted of 58.6–70.4% *M sativa*, 23.6–34.7% non *Medicago* legume, 0.4–6.3% Brassica-

Table II. Some chemical characteristics of non-sterilized (control) and propylene oxide-sterilized provisions of alfalfa leafcutter bee *(Megachile rotundata)*[v].

Treatment	*pH*	*Calories*[w] *(Cal g^{-1})*	*Pollen*[x] *protein (%)*	*Total sugars*[y] *(%)*	*Fructose*[z] *(%)*	*Glucose*[z] *(%)*	*Sucrose*[z] *(%)*	*Turanose*[z] *(%)*
Sterilized	4.3 ± 0.03	4501.1 ± 12.1**	6.0 ± 0.3	63.8 ± 0.1*	43.1 ± 1.1*	39.8 ± 1.2*	1.7 ± 0.4	1.5 ± 0.1
Control	4.1 ± 0.03	4427.2 ± 15.8	6.5 ± 0.4	65.9 ± 0.6	48.1 ± 0.8	43.6 ± 0.5	1.4 ± 0.5	1.8 ± 0.1

[v] Provisions were sterilized with propylene oxide at a concentration of 0.6% (v/v). [w] Calories g^{-1} provision dry weight. [x] Percentage protein content of pollen dry weight. [y] Percentage sugars g^{-1} provision dry weight. [z] Percentage sugars (w/w) calculated as weight sugar per dry weight of water-soluble fraction. The turanose fraction may include quantities of palatinose. * Propylene oxide-sterilized treatment significantly different from the control treatment at $P < 0.05$. ** Propylène oxide-sterilized treatment significantly different from the control treatment at $P < 0.01$.

ceae, 0–0.4% Asteraceae, 0–0.4% Chenopodiaceae and 0–9% unidentified. Although the high densities of pollen in each longitudinal section made enumeration impossible, there was no evidence of stratification of pollen. This observation was supported by examination of provision cores ranging from 4–5 mm in depth. Pollen in the top 1 mm of non-sterilized provisions ranged from 1.6 x 10^4 to 3.1 x 10^4 pollen grains mm^{-3}, whereas pollen from the bottom 1 mm ranged from 2.7 x 10^4 to 3.8 x 10^5 pollen grains mm^{-3}. There was no difference (P = 0.0794) in the density of pollen between the top and bottom of the provisions and fumigation of provisions with propylene oxide had no significant effect on the distribution of pollen within the provision profiles.

Development and mortality of larvae

On provisions sterilized with propylene oxide for 24 h at a concentration of 0.6%, followed by a 24-h detoxification period, no eggs survived, whereas mean egg mortality on non-sterilized provisions detoxified for 24 h was 45.2 ± 7.6%. When provisions were detoxified for 120 h post-treatment with propylene oxide, there were no significant differences in the incidence of egg mortality (P = 0.1242) between non-sterilized (41.7 ± 8.3%), artificial (33.3 ± 4.2%) and propylene oxide-sterilized (58.3 ± 8.3%) provisions. However, the time to prepupation relative to non-sterilized provisions was delayed by 3.8–5.8 days on artificial and by 6.8-7.7 days on propylene oxide-sterilized provisions (fig 1). Following inoculation of provisions with ascospores of *A aggregata*, the mortality of larvae averaged 97.8 ± 2.2% on propylene oxide-sterilized, 98.0 ± 2.0% on artificial and 27.4 ± 3.1% on non-sterilized provisions.

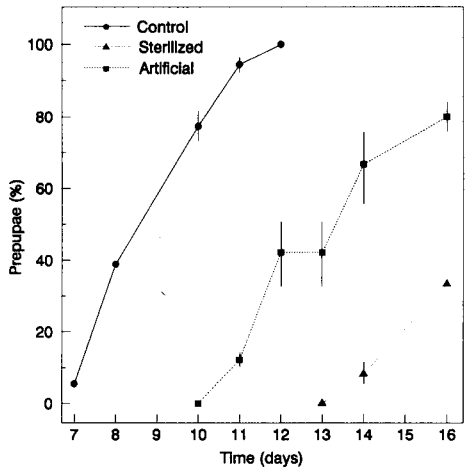

Fig 1. Time (days) to prepupation when eggs of *Megachile rotundata* were placed on non-sterilized provisions (control), propylene oxide-sterilized provisions (sterilized) and artificial provisions (n = 24–30 eggs/treatment). All provisions were maintained under vacuum for 120 h prior to egg placement. Vertical bars represent standard error of the means for arcsine transformed data. Untransformed means are presented.

DISCUSSION

Klostermeyer *et al* (1973) determined the composition of leafcutter bee provisions to be 64% nectar and 36% pollen. We found that provisions removed from bee cells were comprised of 18.1% water, 56.8% nectar and 25.1% pollen. Provisions of another solitary bee, *Centris pallida*, contained 60–70% dissolved solids, 15.3–16.3% water and 13.7–24.7% pollen (Gilliam *et al*, 1984). Leafcutter bees are primary pollinators of alfalfa in North America, and pollen of *M sativa* was the most prevalent taxon recovered from provisions. However, a large number of non-*Medicago* pollen grains (23.6–34.7%), likely *Melilotus* (sweet clover) was also identified. The

high quantities of clover pollen in provisions may have been influenced by the senescing state of the alfalfa at the September sampling date and the relative abundance of sweet clover in the pathways surrounding the field rather than indicating a preference for sweet clover. The different taxa of pollen in provisions likely indicates a similarly mixed origin of floral nectar with alfalfa being the primary source.

The majority of nectars from vascular plants contain, almost exclusively, the sugars fructose, glucose and sucrose (Baker and Baker, 1983). The sugars in nectar of alfalfa have been quantified as 5-68% sucrose, 14–38% fructose, 10–19% glucose and 0–11% maltose (Furgala *et al,* 1958; Low *et al,* 1988), although the relative amounts of these sugars are influenced by environmental conditions and the genomic composition of alfalfa (Walker *et al,* 1974). Our study showed fructose and glucose to be the primary sugars of leafcutter bee provisions, comprising 91.7% of the water-soluble fraction. Similarly, Low *et al* (1988) determined that the main sugars found in honey with an alfalfa or sweet clover nectar source were fructose and glucose, but approximately 3% consisted of oligosaccharides, the majority of which did not originate with the nectar. We found that the oligosaccharides sucrose and turanose/palatinose comprised 3.2% of the water-soluble fraction of provisions. Most oligosaccharides, including turanose and palatinose in honey, are formed by the activity of α- and β-glucosidase on nectar sugars by *Apis* spp (Low *et al,* 1988). With the HPLC method used, the retention times of turanose and palatinose were the same and therefore these sugars could not be distinguished from each other; further the HPLC lacked the sensitivity to detect additional, minute quantities of oligosaccharides. However, the finding of either turanose or palatinose in alfalfa leafcutter bee provisions suggests that *M rotundata*, like *A mellifera*, transglycosylates saccharides in alfalfa nectar with the α-glucosidase enzyme.

Although floral nectar has been shown to contain small quantities of amino acids (Gilliam *et al,* 1980, 1981), pollen appears to be the main source of nitrogen for developing larvae. Pollen from various sources contains protein in the range of 5.9–28.3% (Stanley and Linskens, 1974; McCaughey *et al,* 1980; Standifer *et al,* 1980), whereas we found that total protein recovered from sonicated pollen from provisions contained only 4.9–6.8% protein. The ability of honey bee adults to digest cytoplasmic contents of alfalfa pollen has been documented (Peng *et al,* 1986). The digestive capability of alfalfa leafcutter bee larvae is unknown but fragmented pollen grains were observed in samples of frass (data not presented). Klostermeyer *et al* (1973) reported that leafcutter bees carried mostly pollen during initial provisioning trips; in subsequent trips, the bees gradually increased the amounts of nectar relative to pollen and the final loads consisted entirely of nectar. Similarly, Gilliam *et al* (1984) observed a layer of nectar on the surfaces of larval provisions of the solitary bees, *C pallida* and *Anthophora* sp. If the provision of leafcutter bees were stratified with a higher concentration of nectar at the surface, it would suggest that the initial nutrition of larvae consists almost entirely of saccharides. However, direct microscopy and enumeration of pollen from the top and bottom layers of the provision cores indicated that pollen and nectar were not stratified vertically. Whether the bees mix the pollen and nectar within the cells or whether the resolving powers of the techniques we employed were inadequate to detect increased quantities of nectar at the surface of provisions is unknown.

The influence of microorganisms associated with provisions of leafcutter bees on

chalkbrood is unknown. Fungi associated with bee bread and honey of *A mellifera* produced substances inhibitory to *A apis in vitro* (Gilliam *et al,* 1988), but their activity *in vivo* was not tested. Some studies have shown that antimicrobial activity *in vitro* does not necessarily correlate with efficacy *in vivo* (Andrews, 1985; Hentges and Freter, 1962), but many workers opt for *in vitro* methods since living biological systems are difficult to manipulate. The presence of microorganisms in provisions does not necessarily indicate that the microflora has any influence on chalkbrood. The pH of leafcutter bee provisions was 4.1; this pH would inhibit the growth of many bacteria but generally not fungi. However, the osmotic pressure exerted by the high concentration of sugars (65.9%) in provisions would inhibit most microbial development. Therefore, many of the microorganisms recovered from provisions likely represent dormant propagules.

Propylene oxide has been used effectively for the non-destructive sterilization of a variety of substrates including soils (Bartlett and Zelazny, 1967; Skipper and Westermann, 1973), flower petals (Inglis and Boland, 1990), agar media (Klarman and Craig, 1960) and dietary components for mites (Douglas and Hart, 1989). However, some physical and chemical alterations have been reported in soils following fumigation: the pH increased from 0.4 to 1.1 (Bartlett and Zelazny, 1967; Skipper and Westermann, 1973; Wolf *et al,* 1989), surface area decreased, and levels of extractable Mn increased (Wolf *et al,* 1989). Similarly, we found the pH of provisions increased by 0.2–0.5, total soluble sugars and quantities of fructose and glucose were reduced, but the caloric content increased following propylene oxide treatment. We found no alterations in the stratification or protein content of pollen. The epoxides, including propylene oxide, act by alkylating carboxyl, phenolic, sulfhydryl, amino and hydroxyl groups of protein (Phillips, 1949). The hydrogen atoms from hydroxyl groups in saccharides of provisions could become neutralized by binding to the oxygens at the unstable C-O bonds in the epoxide, resulting in an increased pH. Alkylation of hydroxyl groups would also reduce the polarity of saccharides, which is compatible with our findings of reduced quantities of sugars in treated provisions. Conversely, the higher caloric content of fumigated provisions is also indicative of alkylation of hydroxyl functional groups. Although the alkylation of sugars appeared to be small (3.2–10.4%), the ratio of fructose to glucose was the same in provisions from both treatments suggesting that the monosaccharides were alkylated at equal rates. Amino groups are alkylated by propylene oxide (Phillips, 1949) but we did not detect any difference in the quantities of total protein from pollen between the 2 treatments using the Bradford technique. Overall, physical and chemical alterations to alfalfa leafcutter bee provisions sterilized with propylene oxide were minimal.

Similarly to studies which showed that residual propylene oxide in soils inhibited plant growth (Bowen and Rovira, 1961; Bartlett and Zelazny, 1967), we found that inadequate detoxification increased bee egg mortality. Following detoxification of provisions for only 24 h, 100% mortality of bee eggs was observed on provisions sterilized with propylene oxide whereas 45.2% mortality was observed on non-sterilized provisions. The relatively high mortality of eggs on the non-sterilized provisions is attributed to their maintenance at 5 °C for up to 7 d and not to the reduced moisture content of provisions since no differences were observed in the mortality of eggs maintained on non-sterilized provisions aerated for 120 h (41.7%) with those aerated for 24 h (45.2%). Furthermore, there

were no differences in mortality of eggs reared on sterilized and non-sterilized provisions aerated for 120 h. However, the development time of larvae on sterilized provisions was markedly delayed relative to non-sterilized provisions. We also found increased mortality of larvae inoculated with *A aggregata* reared on sterilized provisions. We conclude that the delayed development of larvae and increased mortality are due to inadequate detoxification and/or to the absence of viable microorganisms in provisions.

The artificial diet utilized in studies of pathogenesis by *A aggregata* (Vandenberg and Stephen, 1982, 1983) was included here as a comparative treatment. The artificial diet possessed no living microorganisms and the composition of the diet differed substantially from that of natural provisions. Similarly to previous findings (Goettel and Vandenberg, unpublished data), we found increased mortality in larvae inoculated with *A aggregata* reared on artificial provisions than on non-sterilized natural provisions and larval development was slowed. However, larvae reared on the artificial provisions developed faster than those raised on propylene oxide-treated provisions, further suggesting that residual propylene oxide was responsible, at least in part, for the delayed development.

Although propylene oxide effectively eliminated microbial populations within provisions of alfalfa leafcutter bees, the results indicate that improvements to the detoxification step are necessary before propylene oxide can be considered as the sterilant of choice for our system. Increasing the time for vacuum treatment may render the provisions free of residual propylene oxide but increasing the detoxification to 120 h already reduced the water content of provisions by 40.3%, suggesting that rehydration of provisions would be necessary if the detoxification time were increased.

ACKNOWLEDGMENTS

This research was funded by a Farming for the Future grant from Alberta Agriculture. Several individuals provided expert assistance in various aspects of the project: 1) JF Gusse, Department of Plant Science, University of Alberta with the quantification of pollen protein and of total sugars in the nectar fraction of provisions, 2), M Fierheller and J Gelfand, Alberta Agriculture Food Processing Center with the caloric determinations and 3) H Friehe, Department of Anthropology, University of Alberta with the acetolysis and identification of pollen. Special thanks are also due to A Warren Elgersma, Department of Chemistry, University of Alberta and P Sporns, Department of Food Science, University of Alberta for their assistance with the HPLC analyses.

Résumé — Analyse des provisions de *Megachile rotundata* avant et après stérilisation à l'oxyde de propylène. Cette étude évalue l'efficacité de l'oxyde de propylène pour stériliser les provisions de la mégachile de la luzerne *(Megachile rotundata)*, compare les caractères physicochimiques des provisions traitées et non traitées et détermine l'action des provisions stérilisées ou non et des provisions artificielles sur le développement larvaire et la sensibilité à *Ascosphaera aggregata*, agent du couvain plâtré. L'oxyde de propylène a d'abord été testé à différentes concentrations (0,1; 0,6 et 1,2% (v/v) pendant 24 h) et à différentes durées d'exposition (0; 6 et 12 h à 0,6%). Les microorganismes des provisions non stérilisées et des provisions traitées ont été récupérés sur un mélange sorbose-extrait de levure-agar additionné de tétracycline (pour les champignons filamenteux et les levures) et sur un milieu nutritif à l'agar additionné de

nystatine (pour les bactéries). Les bactéries, les levures et les champignons filamenteux des provisions non stérilisées ont atteint respectivement en moyenne 1,3 x 10^3, 1,5 x 10^2 et 7,5 x 10^3 unités formant des colonies (g^{-1} de poids frais) (tableau I). La fumigation à l'oxyde de propylène pendant 24 h aux concentrations de 0,6 et 1,2% a stérilisé les provisions, mais la concentration de 0,1% n'a pas eu d'effet. À la concentration de 0,6%, une exposition de moins de 6 h a tué les propagules de champignons, mais il a fallu entre 12 et 24 h d'exposition pour tuer les bactéries (tableau I). La fraction hydrosoluble des provisions (nectar) a été séparée de la fraction non hydrosoluble (pollen) et l'on a mesuré le poids des composants et leur pH. Les provisions étaient constituées en moyenne de 18,1% d'eau, 56,8% de nectar et 25,1% de pollen; leur pH était de 4,1. Les sucres totaux et la composition en sucres de la fraction nectar des provisions ont été déterminés respectivement par réfractométrie et HPLC (80:20 acétronitrile/solvant aqueux). Les sucres totaux représentaient 65,9% et comprenaient du fructose (48,1%), du glucose (43,6%), du saccharose (1,4%) et du turanose/palatinose (1,8%) (tableau II). La présence de turanose et/ou de palatinose dans les provisions suggère que *M rotundata* effectue la conversion enzymatique des saccharides du nectar. Le pollen des provisions a été identifié, après acétolyse, en microscopie optique et la teneur en protéines du pollen traité aux ultrasons mesurée par la technique de Bradford. Le pollen renfermait 6% de protéines; *Medicago sativa* était le taxon dominant (66,2%), mais une autre légumineuse était également fréquente (23,6-34,7%). Afin de déterminer une éventuelle stratification du pollen et du nectar dans les provisions, des grains de pollen, prélevés dans le haut et dans le bas de profils verticaux, ont été comptés à l'hémacytomètre. Aucune différence dans la densité du pollen n'a été observée. L'oxyde de propylène n'a pas eu d'effet sur la teneur en protéines ni sur la distribution du pollen. Le pH des provisions s'est élevé de 0,2 à 0,5 à la suite de la stérilisation (tableau II). L'oxyde de propylène a provoqué l'alkylation des groupes hydroxyles, diminuant ainsi la polarité des saccharides, et les sucres totaux, ainsi que la teneur en fructose et en glucose des provisions stérilisées, ont respectivement diminué de 3,2%, 10,4 et 8,7% (tableau II). À l'opposé, la valeur calorique des provisions traitées a été supérieure d'1,6% à celle des témoins, autre preuve de l'alkylation.

Le développement larvaire a été comparé sur des provisions non stérilisées, des provisions traitées à l'oxyde de propylène (0,6% pendant 24 h) et des provisions artificielles passées à l'autoclave. Les provisions ont été maintenues sous vide durant 24 ou 120 h, puis des œufs récoltés au champ ont été placés à la surface des boulettes de provisions. Le tout a été mis en étuve à 30 °C et examiné quotidiennement pendant 16 j. Les œufs placés sur des provisions stérilisées puis détoxifiées sous vide pendant 24 h n'ont pas survécu. Sur les provisions détoxifiées durant 120 h, on n'a pas observé de différences dans la mortalité des larves élevées sur provisions stérilisées (58,3%), sur provisions artificielles (33,3%) et sur provisions non stérilisées (41,7%). Pourtant la prénymphose a été retardée de 6,8–7,7 j sur provisions stérilisées et de 3,8–5,8 j sur provisions artificielles par rapport au témoin (provisions non stérilisées) (fig 1).

Une suspension de 1 x 10^6 ascospores d'*A aggregata* a été placée à côté de chaque œuf et le taux de mortalité a été relevé au bout de 16 j. La mortalité moyenne des larves inoculées a été de 98,0% sur provisions artificielles, de 97,8% sur provisions stérilisées et 27,4% sur provisions non stérilisées. L'inhibition du développement et

l'augmentation de la mortalité des larves inoculées sur les provisions stérilisées à l'oxyde de propylène sont dues à une détoxication insuffisante des provisions et/ou à l'absence de microorganismes. Bien que l'oxyde de propylène soit efficace pour éliminer les microorganismes des provisions avec des altérations physico-chimiques apparemment minimes, la détoxication constitue le principal facteur limitant.

Megachile rotundata* / stérilisation / provisions / couvain plâtré / *Ascosphaera aggregata

Zusammenfassung — Analyse der Vorräte der Luzerne-Blattschneidebiene *(Megachile rotundata)* vor und nach Sterilisierung mit Propylenoxyd. Diese Untersuchung bewertet die Wirksamkeit von Propylenoxyd zur Sterilisierung der Vorräte der Luzerne-Blattschneidebiene *(Megachile rotundata)*, vergleicht die chemischen und physikalischen Eigenschaften von unbehandelten und mit Propylenoxyd behandelten Vorräten und bestimmt die Wirksamkeit von propylenoxydsterilisiertem, nicht sterilisiertem und künstlichem Futter auf die Entwicklung der Larven und die Empfänglichkeit für *Ascosphaera aggregata*. Propylenoxyd wurde zunächst in verschiedenen Konzentrationen (0,1, 0,6 und 1,2% (v/v) für 24 Stunden) und Expositionszeiten (0, 6 und 24 Stunden bei 0,6%) getestet. Mikroorganismen von nicht sterilisierten und behandelten Vorräten wurden auf Sorbose-Hefeextrakt-Agar mit Tetrazyklinzusatz (für Fadenpilze und Hefen) und auf Nähragar mit Nystatinzusatz (für Bakterien) ausgebracht. Bakterien, Hefen und Fadenpilze von unsterilisierten Vorräten erreichten im Durchschnitt je 1,3 x 10^3, 1,5 x 10^2 und 7,5 x 10^3 koloniebildende Einheiten (g^{-1} Frischgewicht) (Tabelle I). Begasung mit Propylenoxyd für 24 Stunden bei Konzentrationen von 0,6 und 1,2 – aber nicht von 0,1% – sterilisierte die Vorräte. Konzentrationen von 0,6% töteten bei einer Einwirkung von weniger als 6 Stunden die Sporenbildner der Pilze, aber es war eine Einwirkung von 12–24 Stunden nötig, um Bakterien zu töten (Tabelle I). Es wurden der wasserlösliche Anteil des Futters (Nektar) von der nicht-wasserlöslichen Fraktion (Pollen) getrennt und das Gewicht und die pH-Werte der Komponenten gemessen. Das Futter bestand im Durchschnitt zu 18,1% aus Wasser, 56,8% Nektar und 25,1% Pollen mit einem pH von 4,1. Der Gesamtzucker und die Zusammensetzung der Zucker in der Nektarfraktion des Futters wurde mit einem Refraktometer, bzw mit HPLC (System: 80:20 Azetonitril:wässriges Lösungsmittel) bestimmt. Der Gesamtzuckergehalt betrug 65,9% und bestand aus Fruktose (48,1%), Glukose (43,6%), Sukrose (1,4%) und Turanose/Palatinose (1,8%) (Tabelle II). Die Anwesenheit von Turanose und/oder Palatinose im Futter läßt vermuten, daß in *M rotundata* eine enzymatische Umwandlung von Nektar-Sacchariden stattfindet. Der Pollen in den Vorräten wurde in acetolysiertem Zustand unter einem Lichtmikroskop bestimmt und sein Proteingehalt nach der Bradfordtechnik gemessen. Der Pollen enthielt 6% Protein; Luzerne *(Medicago sativa)* war mit 62,2% die vorherrschende Pflanzenart, aber es kam auch Pollen einer anderen Leguminosenart häufig (23,6–34,7%) vor. Um eine eventuelle Ablagerung des Pollens in Schichten festzustellen, wurden Pollen von oberen und unteren Lagen in einem Hämatozytometer getrennt ausgezählt. Zwischen oberen und unteren Lagen wurden keine Unterschiede in der Dichte festgestellt. Propylenoxyd hatte keinen Einfluß auf Proteingehalt oder Dichte des Pollens. Die Sterilisierung erhöhte jedoch den pH der Vorräte um 0,2–0,6 (Tabelle II). Propylenoxyd alkylierte Hydroxylgruppen, senkte dadurch die Polari-

tät der Saccharide und verminderte den Gesamtzucker wie die Quantität von Fruktose and Glukose der begasten Vorräte um jeweils 3,2, 10,4 und 8,7% (Tabelle II). Im Gegensatz dazu war der Kaloriengehalt der behandelten Vorräte um 1,6% größer (Tabelle II) als bei den Kontrollen, was ebenfalls für eine Alkylierung spricht. Die Larvenentwicklung wurde bei nichtsterilisiertem, mit Propylenoxyd sterilisiertem (0,6% für 24 Stunden) und im Autoklaven behandeltem künstlichen Futter verglichen. Nach Lagerung aller Vorräte im Vakuum für 24 oder 120 Stunden wurden im Feld gesammelte Eier auf die Oberfläche eines jeden Vorratsballens gelegt, bei 30 °C inkubiert und 16 Tage lang täglich untersucht. Die Eier überlebten nicht auf Propylenoxyd-sterilisiertem, im Vakuum für 24 Stunden entgiftetem Futter. Wurden diese Vorräte aber 120 Stunden lang entgiftet, dann ließ sich kein Unterschied in der Mortalität zwischen Larven auf sterilisiertem (58,3%), künstlichem (33,3%) und nichtsterilisiertem Futter (41,7%) erkennen. Es war jedoch die Zeitspanne bis zur Vorpuppe auf sterilisiertem und künstlichem Futter gegenüber dem Versuch ohne Sterilisierung um jeweils 6,8–7,7, bzw 3,8–5,8 Tage verzögert (Abb 1). Die Empfänglichkeit der Larven für *A aggregata* (Kalkbruterreger) wurde bei den drei Behandlungsarten des Futters ebenfalls geprüft. Es wurden eine Suspension von 1 x 10^6 Askosporen neben jedes Ei ausgebracht und nach 16 Tagen die Mortalitätsrate bestimmt. Für das künstliche, sterilisierte und das nichtsterilisierte Futter betrug die mittlere Mortalität jeweils 98,0, 97,8 und 27,4%. Sowohl gehemmte Entwicklung wie erhöhte Mortalität der inokulierten Larven auf mit Propylenoxyd sterilisiertem Futter waren Folge ungenügender Entgiftung der Vorräte und/ oder des Fehlens von Mikroorganismen. Obwohl Propylenoxyd Mikroorganismen im Futter bei minimalen physikalischen und chemischen Veränderungen sehr wirkungsvoll beseitigte, scheint die wesentliche Einschränkung der Methode in der anschließenden Entgiftung zu liegen.

Megachile rotundata* / Sterilisierung / Vorräte / Kalkbrut / *Ascosphera aggregata

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