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Original article

Microflora associated with the alfalfa leafcutting bee, *Megachile rotundata* (Fab) (Hymenoptera: Megachilidae) in Saskatchewan, Canada

DW Goerzen

Saskatchewan Alfalfa Seed Producers Association 107 Science Crescent, Saskatoon, SK, Canada S7N 0X2

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Summary — The microflora associated with *Megachile rotundata* was isolated from adult bees, larval cadavers, cell provisions, bee cell surfaces, and nest material. Dominant fungi included *Alternaria alternata*, *Aspergillus niger*, *Eurotium chevalieri*, *Mucor* sp, *Penicillium* spp, *Saccharomyces* sp, *Trichoderma citrinoviride*, and *Trichosporonoides* sp. Bacteria commonly isolated included *Bacillus circulans*, *Bacillus mycoides*, *Enterobacter agglomerans*, and *Pseudomonas* spp. These microorganisms may be involved in activities such as spoilage of provisions, production of enzymes, antibiotics, mycotoxins, and growth factors (vitamins, amino acids), metabolic conversion of provisions, and inhibition of competing microorganisms. Microbial succession was observed involving yeast and bacterial fermentation of provisions followed by mould overgrowth of cell contents; this process was often associated with larval mortality. The microflora isolated likely has a range of beneficial and deleterious effects on *M rotundata*. Some fungal species reported here may also be potentially harmful to the health of leafcutting bee managers.

***Megachile rotundata* / associated microflora / fungus / bacteria / Canada**

INTRODUCTION

The alfalfa leafcutting bee, *Megachile rotundata* (Fab), is an important domesticated pollinator of alfalfa and is widely utilized for alfalfa seed production in western Canada (Richards, 1987). Concerns related to the presence of chalkbrood (*Ascosphaera aggregata* Skou) in *M rotundata* populations in the northwestern United States and southern Alberta have led to extensive surveying of Saskatchewan populations. While *A aggregata* has been detected only sporadically and at very low levels in this province over the past 5 years (Goerzen, 1990), numerous other microorganisms have been isolated from various components of these populations. In this paper, the dominant microflora associated with *M rotundata* is reported and the impact of these fungi and bacteria on *M rotundata* populations is discussed.

MATERIALS AND METHODS

Isolates of microorganisms associated with *M rotundata* populations were obtained by sampling adult bees, larval cadavers, spoiled cell provisions (*ie* stored pollen and nectar), bee

cell surfaces, and tunnel surfaces in wood and polystyrene bee nest material.

In order to detect microflora on adult bees, female *M rotundata* adults were captured in the field as they entered or exited nest tunnels and were placed in 1 dram glass vials containing 1 ml sterile distilled water. Individuals were released following 30 s hand-agitation and samples were transferred to the laboratory for culture. As well, male and female *M rotundata* adults newly emerged from cells incubated in the laboratory were individually placed in test tubes containing 1 ml sterile distilled water, agitated in a vortex mixer for 30 s, and the insects removed prior to sample culture. To evaluate microflora on the foliar exterior of leafcutting bee cells, groups of 150 cells from various populations were placed in 30-dram crystallite vials and washed with 25 ml sterile distilled water. Tunnels in leafcutting bee nest material were sampled with sterile cotton-tipped wood applicators wetted with sterile distilled water. Applicators were stored in sterile glass culture tubes for transfer to the laboratory and rehydrated with 2 ml sterile distilled water prior to culture of samples.

All samples collected from adult bees, cell surfaces, and nest tunnels were plated in the laboratory as 0.1 ml aliquots on potato dextrose agar (PDA), nutrient agar (Difco®), or Sabouraud's dextrose agar, incubated at 28 °C, and assessed at 24-h intervals.

Cells containing larval cadavers and spoiled cell provisions were identified from samples of leafcutting bee cells examined in annual surveys of *M rotundata* populations and from material collected in trapnests. Microflora associated with these specimens was plated directly on media *via* sterile loop transfer and incubated under the conditions described above.

Purified isolates from all sources were subcultured on PDA slants and stored at 4 °C. Isolates were examined using lactophenol cotton blue mounts for preliminary identification; bacterial isolates were Gram-stained. Fungal isolates were submitted to the Biosystematics Research Centre, Ottawa, for identification; selected larval cadaver and cell provision specimens were also submitted. Bacterial isolates were submitted to the Agriculture Canada Research Station, Lethbridge, for identification. Voucher cultures of most isolates reported here have been deposited at DAOM, Ottawa.

RESULTS

The presence and relative abundance of microflora isolated from *M rotundata* adults, larval cadavers, spoiled cell provisions, cell surfaces, and nest material are given in table I. Prevalent microorganisms associated with field-collected *M rotundata* females included *Alternaria alternata*, *Aspergillus niger*, *Enterobacter agglomerans*, *Penicillium* spp, *Pseudomonas solanacearum*, *Rhizopus arrhizus*, and *Trichosporonoides* sp. Likely sources of these microorganisms were alfalfa plant surfaces and the leaves of other plants, including *Chenopodium*, *Fagopyrum*, *Melilotus*, and *Rosa* spp, which are commonly utilized for cell construction (Richards, 1984). The relative abundance of fungal and bacterial species fluctuated with temperature and moisture changes during the field season. Male and female *M rotundata* emerging from laboratory-incubated cells were found to be carrying primarily *A alternata*, *Aspergillus niger*, *Enterobacter agglomerans*, *Eurotium chevalieri*, and *Trichosporonoides* sp. All of these species were isolated frequently from the leaf surfaces of cells and likely picked up by individuals through contact during emergence.

Fungi commonly associated with larval cadavers included *A niger*, *E chevalieri*, *Penicillium purpurogenum*, *P simplicissimum*, other *Penicillium* spp, and *Trichosporonoides* sp, while dominant microorganisms found in conjunction with spoiled cell provisions included *Ascosphaera pollenicola*, *A variegata*, *Aspergillus glaucus*, *A niger*, *Bacillus circulans*, *Enterobacter agglomerans*, *Eurotium chevalieri*, *Penicillium* spp, *Pseudomonas* spp, and *Trichosporonoides* sp. Surfaces of *M rotundata* cells were inhabited by a microfloral complex similar to that found in leafcutting bee nest material, with predominance of *Alternaria alternata*, *Aspergillus niger*, *Enterobacter*

Table I. Microflora isolated from *Megachile rotundata* populations in Saskatchewan [a].

Microorganism	*Adult bee*	*Larval cadaver*	*Cell provision*	*Cell surface*	*Nest material*
Fungi					
Alternaria alternata (Fr: Fr) Keissler	++			++	++
Ascosphaera aggregata Skou		+			
Ascosphaera atra Skou and Hackett			+		
Ascosphaera larvis Bissett		+	+		
Ascosphaera pollenicola Bissett			++		
Ascosphaera variegata Bissett		+	++		
Ascosphaera sp				+	
Aspergillus glaucus Link: Fr		+	++		
Aspergillus niger Van Tiegham	++	++	++	+++	+++
Cylindrocarpon sp				+	+
Eurotium chevalieri L Mangin		++	++	+++	+++
Eurotium sp				+	+
Mucor sp				++	++
Penicillium purpurogenum O Stoll	++				
Penicillium simplicissimum (Oudem) Thom	++				
Penicillium spinulosum Thom					++
Penicillium spp	++	++	++	++	++
Rhizopus arrhizus A Fischer	++		+	+++	+++
Rhizopus sp			+	+	+
Saccharomyces sp			++		++
Trichoderma citrinoviride Bissett					++
Trichosporonoides sp	+++	+++	+++	+++	+++
Trichothecium roseum (Pers: Fr) Link	+			+	
Ulocladium atrum G Preuss			+		
Yeast-like sp NM-K (DAOM 212-057)					+
Yeast-like sp CE-I (DAOM 212-058)				+	+
Unidentified yeast-like sp Y01	+			+	
Unidentified yeast-like sp Y02	+			+	
Bacteria					
Bacillus circulans Jordan			++		++
Bacillus mycoides Flugge					++
Bacillus sp	+				+
Corynebacterium sp	+				+
Enterobacter agglomerans Ewing and Fife	+++		++	+++	+++
Flavobacterium breve (Lustig) Bergey					+
Pseudomonas solanacearum (Smith) Smith	++				
Pseudomonas spp	++		++		++
Unidentified sp B01	+				
Unidentified sp B02				+	

[a] Isolation of microorganisms from *M rotundata* populations is rated as occasional (+), frequent (++), or abundant (+++).

agglomerans, *Eurotium chevalieri*, *Mucor* sp, *Penicillium* spp, *Rhizopus* spp, and *Trichosporonoides* sp. Other species commonly isolated from nest material were *Bacillus circulans*, *B mycoides*, *Corynebacterium* sp, *Pseudomonas* spp, and *Trichoderma citrinoviride*. The yeast *Saccharomyces* sp was frequently found in cultures from wood nest material but rarely isolated from polystyrene nest material.

DISCUSSION

The dominant species of filamentous fungi listed (*ie A alternata*, *Aspergillus niger*, *E chevalieri*, *Mucor* sp, *Penicillium* spp, *R arrhizus*, and *T citrinoviride*) are generally saprophytic in nature, growing on available material including cell provisions, larval cadavers, and the foliar surface of cells. However, other fungal species reported here, including *Aspergillus* spp and *Trichothecium roseum*, can be pathogenic on wild bees (Batra *et al*, 1973). While *A aggregata*, the causative agent of chalkbrood in *M rotundata*, is rarely detected in Saskatchewan leafcutting bee populations, several other *Ascosphaera* spp have been isolated and 2 of them, *A pollenicola* and *A variegata*, are frequently found in spoiled cell provisions, usually in conjunction with *E chevalieri* and *Trichosporonoides* sp. The species *A atra* and *A pollenicola* appear to be primarily saprophytic on cell provisions, while *A larvis* and *A variegata* are usually isolated from *M rotundata* larval cadavers and may be pathogenic to the leafcutting bee (Bissett, 1988).

Extensive research in the identification of fungal species associated with the honeybee *(Apis mellifera)* indicated that the most commonly occurring moulds were *Aspergilli*, *Mucorales*, and *Penicillia* (Gilliam and Prest, 1987; Gilliam *et al*, 1989a, 1989b). Richards (1985), in surveys to detect *Ascosphaera aggregata* in *M rotundata* populations, noted the occurrence of *Fusarium*, *Mucor*, *Penicillium*, and *Rhizopus* spp. Except for the absence of *Fusarium* sp, the dominant mould species reported for *M rotundata* in this study are similar, with *Alternaria* and *Eurotium* spp also commonly isolated. As noted by Gilliam *et al* (1989a), moulds merit intensive study due to their activity in the production of enzymes and numerous secondary metabolites including antibiotics, terpenes, and polysaccharides. Research currently underway to investigate possible antagonistic interactions between *A aggregata* and other microflora associated with *M rotundata* (Goettel, Agriculture Canada, Lethbridge, personal communication) is important for the insight it will provide into the complex relationships existing among these microorganisms. Seven of the 22 fungal species reported here are known to produce mycotoxins. These include *Aspergillus niger* (malformin C), *E chevalieri* (gliotoxin), *P purpurogenum* (rubratoxin), *P simplicissimum* (penicillic acid), *P spinulosum* (spinulosin), *T citrinoviride* (T-2 toxin), and *T roseum* (trichothecin) (Wyllie and Morehouse, 1977; Moreau, 1979).

Members of the genus *Trichosporonoides*, which were isolated in abundance from every component of the leafcutting bee system examined here, are yeast-like fungi which are not well known and are in need of taxonomic revision. The strains of *Trichosporonoides* isolated were somewhat variable in cultural characteristics and morphology, and may represent a single species complex which has yet to be described (Bissett, personal communication). The unique morphology and development of this genus was first described by Haskins and Spencer (1967), who isolated *Trichosporonoides oedocephalis* Haskins and Spencer from brood cell material in *Apis mellifera* honeycomb.

Saccharomyces sp was frequently isolated from wood nest material and spoiled

cell provisions, but was never present in abundance. An additional 2 as yet unidentified yeast-like species are currently under evaluation at the Biosystematics Research Centre, Ottawa (DAOM 212-057, DAOM 212-058). These species, along with unidentified yeast-like species designated Y01 and Y02, were isolated occasionally from field-captured adult bees, cell surfaces, or nest material. In work describing yeasts in pollen and bee bread, Gilliam (1979a) isolated 113 yeasts in 7 genera which were associated with almond *(Prunus dulcis)* pollen either from the flower or stored in comb cells of *A mellifera* hives. This demonstrates the diversity of yeast flora which may be associated with bee populations. As noted by Gilliam (1979a), the role of yeasts in processes relating to bee nutrition is as yet poorly defined, but may involve production of necessary growth factors including vitamins and amino acids.

Bacteria dominant in this study included *Bacillus circulans*, *B mycoides*, *Enterobacter agglomerans*, and *Pseudomonas* spp. These were isolated most often from adult bees, cell provisions, and nest material. *Enterobacter agglomerans* was also abundant on cell surfaces. Two unidentified bacterial species, designated B01 and B02, were isolated infrequently from adult bees and cell surfaces. In reports of bacteria associated with *A mellifera* (Gilliam, 1979b), the stingless bee, *Melipona fasciata* (Gilliam *et al,* 1990a), and 5 species of solitary bees (Gilliam *et al,* 1984, 1990b), *Bacillus* spp were dominant, and in all cases, *B circulans* was isolated. Gilliam *et al* (1990b) concluded that *Bacillus* spp are common associates of Apoidea and could participate in metabolic conversion of provisions and in inhibition of competing and spoilage microorganisms.

The presence of this diverse group of fungi and bacteria associated with *M rotundata* populations in Saskatchewan probably has a broad range of effects on these populations. As noted, the microorganisms may contribute to the production of many beneficial compounds such as antibiotics, vitamins, amino acids, and antispoilage agents. However, many of the species listed may also harm leafcutting bee populations by interfering with larval development and spoiling cell provisions. In laboratory studies, *B circulans* and *Pseudomonas* spp, in conjunction with *Saccharomyces* and *Trichosporonoides* spp, were observed to cause larval mortality through fermentation of cell provisions. The spoilage of cell provisions often led to subsequent overgrowth of cell contents by *Aspergillus* niger, *Penicillium* spp, and *R arrhizus* (Goerzen, unpublished data). This process of microbial succession in cells was reported previously in the alkali bee, *Nomia melanderi* (Batra *et al,* 1973). In addition to exhibiting antibacterial and fungistatic activity, toxins associated with some fungal species isolated here have been shown to elicit responses including feeding aversion and inhibition of protein synthesis (Moreau, 1979). Any of these processes could contribute to leafcutting bee larval mortality found in populations evaluated in this study. Surveys of Saskatchewan leafcutting bee populations undertaken over the 5-year period 1985 to 1989 have shown the percentage of cells containing dead larvae, mouldy larvae, and spoiled provisions to range from 6.9–11.9% of total cells evaluated (Goerzen, 1990).

Many of the mould species found in association with the alfalfa leafcutting bee may be potentially harmful to the health of leafcutting bee managers as well. *Alternaria*, *Aspergillus*, *Penicillium* and *Rhizopus* spp are among those fungi which are considered most important medically (Aukrust *et al,* 1985). These species have been implicated in allergic reactions and broncho-

pulmonary disease and may cause conditions ranging from pulmonary hypersensitivity disease (*eg* allergy, asthma) to life-threatening infection (Pennington, 1986). During leafcutting bee incubation and cell-harvesting operations, large numbers of emerging bees or loose cells concentrated in confined areas may lead to high levels of airborne spores of these species. Several less frequently isolated mould species reported here, including *Cylindrocarpon*, *Trichoderma*, and *Ulocladium* spp, are not ordinarily associated with human diseases but under certain conditions may act as opportunistic pathogens (Rippon, 1987).

Identification of the microflora associated with *M rotundata* is important in elucidation of the complex interactions between this economically important solitary bee species and the microorganisms present in its environment. A reduction in high levels of mould species which may interfere with larval development and spoil cell provisions will assist in increasing the quality of alfalfa leafcutting bee populations. An awareness of the presence of mould species which have been implicated in human allergic reactions will allow leafcutting bee managers to take measures to reduce contact with potentially harmful spores and to incorporate control techniques which will reduce levels of non-beneficial microorganisms in *M rotundata* populations. However, the beneficial effects associated with the presence of a balanced complement of microorganisms in the leafcutting bee ecosystem are doubtless of great importance and require further study.

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Résumé — Étude de la microflore associée à la mégachile *Megachile rotundata* (Fab) (Hymenoptera: Megachilidae) au Saskatchevan, Canada. Des isolats de champignons et de bactéries, associés à des populations de *Megachile rotundata*, ont été obtenus par échantillonnage d'abeilles adultes, de cadavres de larves, de provisions endommagées contenues dans des cellules, de la surface de cellules de couvain, et de la surface des tunnels présents dans le nid. Les abeilles adultes et les surfaces des cellules ont été traitées par lavage dans de l'eau distillée stérile, alors que les prélèvements des tunnels ont été réalisés avec des tiges de bois munies de coton à leur extrémité. Au laboratoire, les échantillons ont été recouverts avec de l'agar dextrose de pomme de terre, de l'agar nutritif, et de l'agar dextrose de Sabouraud, et incubés à 28 °C. La microflore associée aux cadavres de larves et aux provisions endommagées a été transférée dans les milieux au moyen d'un instrument stérile. Les isolats purifiés de champignons ont été examinés en utilisant du lactophénol, et les isolats de bactéries ont été colorés par la coloration de Gram.

La présence et l'abondance de la microflore isolée de populations de *M rotundata* sont données dans le tableau I. Les microorganismes dominants sont : les champignons *Alternaria alternata*, *Aspergillus niger*, *Eurotium chevalieri*, *Mucor* sp, *Penicillium* spp, *Rhizopus arrhizus*, *Saccharomyces* sp, *Trichoderma citrinoviride*, and *Trichosporonoides* sp; les bactéries *Bacillus circulans*, *B mycoides*, *Enterobacter agglomerans*, and *Pseudomonas* spp. Un grand nombre des espèces de champignons présents sont saprophytes, et crois-

sent sur les provisions et les cadavres de larves. Cependant, *Aspergillus* spp et *Trichothecium roseum* sont connus pour être pathogènes pour les abeilles sauvages.

Les champignons peuvent également jouer un rôle important pour leur activité dans la production d'enzymes, d'antibiotiques et de mycotoxines. Le champignon, voisin des levures, *Trichosporonoides* a été isolé dans tous les types d'échantillons analysés. Le rôle des levures dans la nutrition de l'abeille peut consister en la production de vitamines et d'acides aminés. Les bactéries dominantes dans cette étude peuvent participer à la transformation métabolique des provisions et à l'inhibition des microorganismes en compétition avec elles.

La microflore isolée des populations de mégachiles a vraisemblablement à la fois des effets bénéfiques et nuisibles. Tandis que beaucoup d'espèces de champignons et de bactéries peuvent contribuer à la production de composés utiles, d'autres peuvent interférer avec le développement larvaire et abîmer les provisions. Cette microflore est souvent associée à des mortalités larvaires. Les moisissures, incluant *Alternaria*, *Aspergillus*, *Penicillium* et *Rhizopus* spp pourraient également être potentiellement nocives pour les éleveurs de ces abeilles. Pendant les opérations de récolte en particulier, un très grand nombre d'abeilles et de cellules, qui sont maintenues dans des zones confinées, peuvent émettre dans l'air ambiant, de grandes quantités de spores provenant de ces moisissures, qui ont été impliqués dans des réactions allergiques et des maladies bronchopulmonaires. L'identification de la microflore associée à *M rotundata* est importante pour la compréhension des intéractions complexes existant entre cette importante espèce d'abeilles solitaires et les microorganismes présents dans l'environnement. Tandis qu'une réduction du niveau élevé d'espèces de moisissures nuisibles pour *M rotundata* permettrait d'accroître la qualité des populations de cette abeille, les effets bénéfiques de quelques microorganismes pourraient être d'une grande importance, et nécessitent des études ultérieures.

***Megachile rotundata* / microflore associée / champignons / bactéries / Canada**

Zusammenfassung — Die mit der Luzerne-Blattschneiderbiene *(Megachile rotundata)* in Saskatchewan, Kanada, assoziierte Mikroflora. Durch Probenentnahme von erwachsenen Bienen, toten Larven, verdorbenen Zellvorräten, der Oberfläche der Bienenzellen sowie des Materials der Neströhre wurden Isolate von Pilzen und Bakterien gewonnen, die zusammen mit den Populationen von *Megachile rotundata* vorkommen. Die Proben von den Bienen und der Zelloberfläche wurden durch Waschen in destilliertem Wasser gewonnen, während die Proben von dem Material der Neströhren mittels eines sterilen Wattebausches entnommen wurden. Die Proben wurden im Laboratorium auf Kartoffel-Dextrose-Agar, Nähragar oder Sabouraud's Dextrose-Agar ausgestrichen und bei 28 °C inkubiert. Die Mikroflora in toten Larven oder verdorbenen Zellvorräten wurden mittels einer sterilen Öse auf das Nährmedium übertragen. Gereinigte Pilzisolate wurden in mit Lactophenolblau gefärbten Präparaten untersucht, Bakterien-Isolate wurden gramgefärbt. Die Art und relative Häufigkeit der von *M rotundata*-Populationen isolierten Mikroflora ist aus Tabelle I ersichtlich. Unter den vorherrschenden Mikroorganismen befanden sich die Pilze *Alternaria alternata, Aspergillus niger, Eurotium chevalieri, Mucor* sp, *Penicillium* spp, *Rhizopus arrhizus, Saccharomyces* sp, *Trichoderma citrinoviride* und *Trichosporonoides* sp; an Bakterien

wurden *Bacillus circulans*, *B mycoides*, *Enterobacter agglomerans* und *Pseudomonas* spp gefunden. Viele von den Pilzarten sind Saprophyten, die auf derartigen Materialen wie Zellvorräten und toten Larven wachsen. Von *Aspergillus* spp und *Trichothecium roseum* ist jedoch bekannt, daß sie für Wildbienen pathogen sind. Pilze können auch für die Produktion von Enzymen, Antibiotika und Mykotoxinen wichtig sein. Der Hefe-ähnliche Pilz *Trichosporonoides* wurde von allen komponenten des Blattschneiderbienen-Systems isoliert. Die Rolle von Hefen in Bezug auf die Ernährung der Bienen kann die Erzeugung von Vitaminen und Aminosäuren einschließen. Die in dieser Untersuchung vorherrschenden Bakterien können bei der Umwandlung der Zellvorräte im Stoffwechsel und bei der Hemmung von anderen Mikroorganismen von Bedeutung sein.

Die von den Populationen der Luzerne-Blattschneiderbiene isolierte Mikroflora besitzt wahrscheinlich Effekte, die von nützlich bis schädlich reichen. Während viele Pilz- und Bakterienarten zu der Erzeugung nützlicher Verbindungen beitragen mögen, können andere die Larvenentwicklung stören und die Zellvorräte verderben. Die mikrobielle Entwicklung, wie sie im Laboratorium beobachtet wurde und welche die Hefe- und bakterielle Gärung der Vorräte und eine Schimmelüberwucherung des Zellinhalts betraf, war oft mit dem Absterben der Larven verbunden. Schimmelpilze, einschließlich *Alternaria*-, *Aspergillus*-, *Penicillium*- und *Rhizopus*-Arten können potentiell auch für die Betreuer der Blattschneiderbienen schädlich sein. Während des Ausbrütens der Bienen und der Tätigkeiten der Ernte reifer Zellen, können große Mengen von Bienen oder Bienenzellen in geschlossenen Räumen zu einer hohen Sporenkonzentration dieser medizinisch wichtigen Schimmelpilze in der Luft führen und in der Folge zu allergischen Reaktionen und bronchopulmonalen Erkrankungen.

Die Bestimmung der mit *M rotundata* assoziierten Mikroflora ist für die Aufklärung der komplexen Wechselwirkungen zwischen dieser wirtschaftlich wichtigen solitären Bienenart und den in ihrer Umwelt vorhandenen Mikroorganismen wichtig. Während die Reduktion einer Überzahl für *M rotundata* schädlicher Schimmelpilze die Qualität der Populationen von Blattschneiderbienen verbessern wird, kann der günstige Einfluß einiger Mikroorganismen von so großer Bedeutung sein, daß weitere Untersuchungen gerechtfertigt werden.

***Megachile rotundata* / assoziierte Mikroflora / Pilz / Bakterien / Kanada**

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