

DIFFERENTIAL METABOLISM OF 1,8-CINEOLE IN INSECTS

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(Received March 21, 2002; accepted September 11, 2002)

Abstract—In order to compare the metabolism of 1,8-cineole in the pyrgo beetle, *Paropsisterna tigrina*, three other herbivorous insect species, *Faex nigroconspersa*, *Chrysophtharta bimaculata*, and *Oxyops vitiosa*, were fed 1,8-cineole leaf diets. *F. nigroconspersa* adults excreted predominantly 9-hydroxy-1,8-cineole (36.2% of the volatile constituents) with some 2 α -hydroxy-1,8-cineole (11.4%). In contrast, larvae excreted predominantly 2 α -hydroxy-1,8-cineole (27.4%) and smaller proportions of 9-hydroxy-1,8-cineole (5.2%) and 3 α -hydroxy-1,8-cineole (4.3%). *C. bimaculata* adults excreted predominantly 3 α -hydroxy-1,8-cineole (16.5%). *Oxyops vitiosa* adults, on a lower 1,8-cineole diet, excreted predominantly 2 α ,9-dihydroxy-1,8-cineole (4.2%) and 2 α -hydroxy-1,8-cineole (3.5%), with smaller proportions of 3 α -hydroxy-1,8-cineole (1.1%) and 9-hydroxy-1,8-cineole (0.5%). This is the first reported occurrence of a dihydroxycineole as an insect metabolite. Gas chromatographic and mass spectral data for hydroxycineoles are recorded and interspecific metabolite variation discussed.

Key Words—*Faex nigroconspersa*, *Chrysophtharta bimaculata*, *Oxyops vitiosa*, *Pergagrapta*, terpenoid metabolites, 1,8-cineole, hydroxy-1,8-cineole metabolites, gas chromatography–mass spectrometry.

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INTRODUCTION

Trees and shrubs of the family Myrtaceae are abundant food sources for numerous herbivores throughout Australia. Many species of the common *Eucalyptus* and *Melaleuca* genera contain an essential oil that frequently comprises substantial concentrations of the cyclic monoterpene ether 1,8-cineole (Figure 1, structure 1) (Boland et al., 1991; Southwell, 1999a). Although the ingestion of neat eucalyptus oil (presumably 1,8-cineole rich oils) has caused death in humans (Tisserand and Balacs, 1995), herbivorous marsupials, such as the possum (Flynn and Southwell, 1979; Southwell et al., 1980; Carman and Klika, 1992; Bull et al., 1993; Carman et al., 1994; Boyle et al., 2000) and koala (Southwell, 1975; Eberhard et al., 1975), mammals, such as the rabbit (Miyazawa et al., 1989) and rat (Madyastha and Chadha, 1986), and invertebrate insects (Morrow and Fox, 1980; Ohmart and Larsson, 1989; Southwell et al., 1995; Schmidt et al., 2000; Fletcher et al., 2000) and microorganisms (MacRae et al., 1979; Nishimura et al., 1982; Carman et al., 1986; Williams et al., 1989; Liu and Rosazza, 1990;

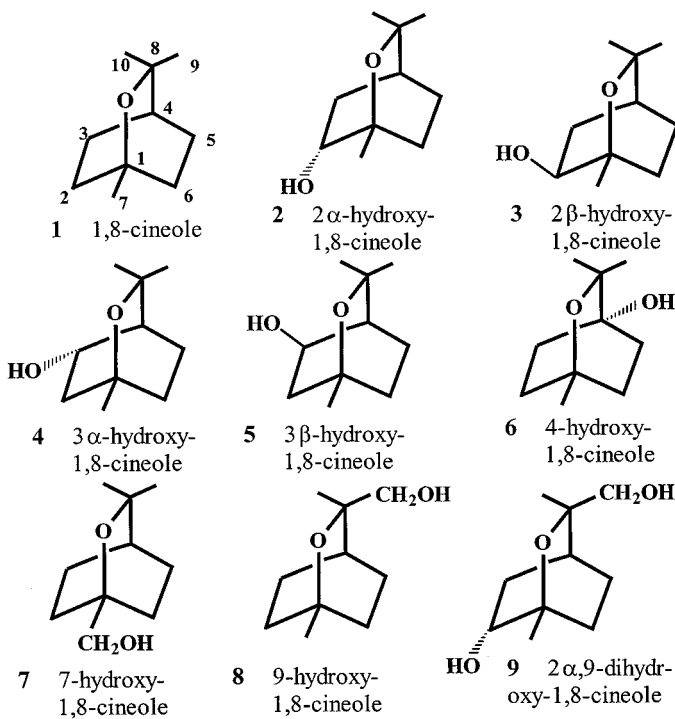


FIG. 1. Structures of 1,8-Cineole and metabolites.

Miyazawa et al., 1991) are able to cope with substantial quantities of this potential toxin.

In addition, 1,8-cineole has significant bioactivity as a mosquito feeding deterrent and ovipositional repellent (Klocke et al., 1987) and repellent and toxicant against stored-grain beetles (Obeng-Ofori et al., 1997). The use of terpenoids, including trace quantities of 1,8-cineole in the fecal shields of the tortoise beetle, *Eurypedus nigrosignata*, for chemical defense also has been demonstrated (Gomez et al., 1999).

Identification of 1,8-cineole metabolites (+)-2 β -hydroxycineole (**3**) and 2 α -hydroxycineole (**2**) in the frass of pyrgo beetle, *Paropsisterna tigrina* when fed 1,8-cineole-rich *Melaleuca* leaves (Southwell et al., 1995) raised the question: do other insect species metabolize 1,8-cineole in an identical manner? With *P. tigrina*, frass analysis indicated that all terpenoids except 1,8-cineole were being excreted unchanged. In an attempt to acquire evidence more general for insects, the cineole metabolites of other closely and distantly related species were examined, and details of this investigation provided support for a preliminary communication (Southwell, 1999b).

This paper reports the metabolite structures of 1,8-cineole in the frass of chrysomelid beetles, *Faex nigroconsersa* and *Chrysophtharta bimaculata*, and compares the structures with those previously reported for pyrgo beetle, *Paropsisterna tigrina* (Southwell et al., 1995). Recently published 1,8-cineole metabolites reported from both Leichhardt's grasshopper, *Petasida ephippigera* (Fletcher et al., 2000), and from the larvae of *Pergagraptia*, a Pergid sawfly (Schmidt et al., 2000), also are discussed and compared. In addition, the 1,8-cineole frass metabolites of the weevil *Oxyops vitiosa* are described. We recorded chromatographic (GC) and spectral (MS) data useful for identifying 1,8-cineole metabolites.

METHODS AND MATERIALS

Instrumentation. Analytical chromatography was performed on the following: (A) an Hewlett Packard 5890 chromatograph, 3393A Integrator, 7673A auto-sampler and an Alltech AT35 60-m \times 0.25-mm, 0.2- μ m film thickness, midpolarity FSOT column with hydrogen (45 cm/sec) as carrier gas, injection port (split 1:50) at 250°C, flame ionization detector at 300°C and temperature program from 60°C (1 min) to 250°C at 10°C/min; (B) an Hewlett Packard 6890 gas chromatograph, HP Chemstation Rev A.06.03 (509) and autosampler using an Alltech AT35 60-m \times 0.25-mm, 0.25- μ m film thickness, midpolarity FSOT column with hydrogen (55 cm/sec) as carrier gas, injection port (split 1:50) at 200°C, flame ionization detector at 300°C and temperature program from 60°C (3 min) to 240°C at 9°C/min; and (C) a Shimadzu GC14B gas chromatograph with AOC 14 autoinjector, AOC 1400 autosampler using a BP1 50-m \times 0.22-mm, 0.25- μ m film thickness, FSOT

column with hydrogen (35 cm/sec) as carrier gas, injection port (split 1:50) at 250°C, flame ionization detector at 300°C and temperature program from 50°C (1 min) to 240°C at 6°C/min, and a DB WaxETR 60-m × 0.25-mm, 0.25- μ m film thickness, FSOT column with hydrogen (35 cm/sec) as carrier gas, injection port (split 1:50) at 250°C, flame ionization detector at 300°C and temperature program from 50°C (1 min) to 240°C at 6°C/min. Retention indices were measured against *n*-alkanes. For constituent identification, GC-MS investigations were performed similarly using (D) a Hewlett Packard 6890 instrument using an HP5-MS 30.3-m × 0.25-mm, 0.25- μ m film thickness, FSOT column with helium (36 cm/sec) as carrier gas, injection port (split 1:50) at 250°C, mass selective detector (HP 5973) at 250°C (source) and 150°C (quad) with transfer line 280°C, and ion source filament voltage of 69.9 eV. Component identification was made on the basis of mass spectral fragmentation, retention time comparison with authentic constituents, and mass spectral and retention matching with commercial (NIST, Wiley and Adams) libraries. Authentic components were donated by Dr. Ray Carman, Department of Chemistry, University of Queensland, and 4-hydroxy-1,8-cineole was synthesized from terpinolene via the 4,8-epoxide, which, after ring opening with acid and oxymercuration, yielded the desired isomer as previously reported (Carman and Rayner, 1994).

Insect Species and Diets. *Faex nigroconsersa* were collected from a New South Wales (NSW) Agriculture planting of *Melaleuca alternifolia* at Duck Creek, Ballina, NSW, transferred to the Tropical Fruits Research Station, Alstonville, NSW, and maintained on a foliage diet gathered from mature high cineole (59.8%) *M. linariifolia* trees cultivated at Richmond Hill, Lismore.

Chrysophtharta bimaculata adults were obtained as a small population (<50) from a laboratory colony at the Cooperative Research Centre for Temperate Hardwood Forestry at Sandy Bay in Tasmania and prefed on cuttings from preferred field host species *Eucalyptus nitens* and *E. regnans*. Following arrival at the Tropical Fruits Research Station, Alstonville, NSW, the foliage diet was changed to mature high cineole (59.8%) *M. linariifolia* trees cultivated at Richmond Hill, Lismore.

Oxyops vitiosa adults were collected from *M. quinquenervia* trees growing in an abandoned nursery in Fort Lauderdale, Florida, USA, and fed fresh leaves of an intermediate cineole (22.7%) variety of *M. quinquenervia* collected from a field site also in Fort Lauderdale.

Pterygophorus sp. sawfly larvae were investigated in a commercial low cineole (1.6%) *M. alternifolia* natural stand at Greenridge, NSW, where frass was collected during heavy infestation.

Frass Collection, Extraction, and Replication. Samples of frass (10–100 mg) were collected, extracted with absolute ethanol while moist, exposed to microwave irradiation (700 W) (Southwell et al., 1995), and analyzed by GC and GC-MS. In these qualitative investigations, sample size was too small to quantify the amounts

of metabolite excreted with respect to the quantity of cineole ingested, and enantiomeric ratios of the hydroxycineole metabolites were not measured. For all frass samples, the best of several replicates, similar by GC, was used for detailed GC-MS analysis and compared with both the leaf ingesta volatiles and frass volatiles from low to zero cineole diets.

RESULTS

The regiospecific and stereospecific nature of the hydroxylation of 1,8-cineole (**1**) to predominantly (+)-2 β -hydroxy-1,8-cineole (**3**) by the pyrgo beetle (*Paropsisterna tigrina*) feeding on 1,8-cineole-rich *M. linariifolia* leaf (Southwell et al., 1995) raised the question of whether the function of this conversion was detoxification or metabolism for the production of semiochemicals. As a first step in seeking clarification of this issue, the *in vivo* conversion of 1,8-cineole in a number of other insects was investigated and compared with that of pyrgo beetle (Table 1).

F. nigroconsersa (Clark) is a chrysomelid beetle that occupies a similar niche to *P. tigrina* (Southwell et al., 1995) among *M. alternifolia/linariifolia* herbivores. Larvae and adults of both species preferentially attack the terminal foliage and young flush leaf, thus restricting plant growth and regrowth. *F. nigroconsersa* is normally more abundant in drier seasons (Maddox 1996).

When fed 1,8-cineole-rich (60.0%) leaf from *M. linariifolia*, the larvae excreted frass containing volatile constituents that were found, by GC-MS, GC coinjection, and retention index comparison with authentic standards, to consist of 27.4% 2 α -hydroxy-1,8-cineole (**2**), smaller proportions of the 3 α - (**4**) (4.3%) and 9- (**8**) (5.2%) hydroxyl isomers, and a trace of the 2 β -(**3**) (0.5%) hydroxyl isomer. In contrast, the adult insect frass volatile constituents contained the 9-hydroxyl isomer (**8**) as the major component (36.2%), smaller proportions of the 2 α - (**2**) (11.4%) and 3 α - (**4**) (6.7%) hydroxyl isomers, and again a trace of the 2 β - (**3**) (0.5%) hydroxyl isomer. Hence, the *F. nigroconsersa* frass volatile components contrast with *P. tigrina* frass volatile components by varying not only in chemical content, but also in larvae and adults (Table 1).

With *F. nigroconsersa* and the other species investigated, the enantiomeric excess of the metabolites derived from achiral 1,8-cineole was not investigated. Even though chiral standards of the synthetic hydroxycineole metabolites were available, they could not be separated on two different chiral GC columns. The chirality of the major *P. tigrina* metabolite was determined by optical rotation measurement of a substantial quantity (57.4 mg) of (+)-2 β -hydroxy-1,8-cineole (Southwell et al., 1995).

C. bimaculata (Olivier) is another paropsine chrysomelid beetle and a major flush leaf defoliator in *Eucalyptus* plantations and native regrowth forests of Tasmania (Greaves 1966; De Little, 1983). When fed 1,8-cineole-rich (59.8%)

TABLE 1. PROPORTIONS (%) OF 2 α -, 2 β -, 3 α -, 3 β -, AND 9-HYDROXY AND 2 α , 9-DIHYDROXY METABOLITES OF 1,8-CINEOLE DETECTED IN FRASS VOLATILES OF INSECTS FEEDING ON 1,8-CINEOLE-RICH *Eucalyptus* OR *Melaleuca* LEAF

Hydroxy-1,8-cineole isomer	Coleoptera						Orthoptera		Hymenoptera	
	<i>Paropsisterna tigrina</i> (pyrgo beetle) ^e		<i>Faex nigroconspersa</i> ^{b,c}		<i>Chrysophtharta bimaculata</i> adults ^{b,c}		<i>Oxyops vitosae</i> ^c (melaleuca snout beetle adults)	<i>Petasida ephippigera</i> ^d (Leichhardt's grasshopper adults) ^d	<i>Perga grapta</i> sp. ^{e,f} (Sawfly larvae)	<i>Pterygophorus</i> sp. ^c (sawfly larvae)
	Adults	Larvae	Adults	Larvae	Larvae	Larvae				
2 α -OH (2)	1.7	2.0	11.4	27.4	0.6	27.4	3.5	5		
2 β -OH (3)	38.7	39.8	0.5	0.5	0.8	0.5		0.5		
3 α -OH (4)	1.6	1.9	6.7	4.3	16.5		1.1	34		tr
3 β -OH (5)								3.5		
9-OH (8)	1.7	0.7	36.2	5.2	3.4		0.5	7		
2 α ,9-di OH (9)							4.2			
1,8-cineole (unreacted)	10.1	7.4	6.9	19.2	28.0		5.8	0		0.2

^a Southwell et al. (1995).

^b Preliminary communication, Southwell (1999b).

^c This communication.

^d Extrapolated from Fletcher et al. (2000).

^e Reexamination of Schmidt et al. (2000) data.

^f Moore (2002, personal communication).

leaf from *M. linariifolia*, the adults excreted frass containing volatile constituents found, by GC-MS, GC coinjection, and retention index comparison with authentic standards, to consist of 16.5% 3 α -hydroxy-1,8-cineole (**4**), a smaller proportion of the 9- (**8**) (3.4%) hydroxyl isomer, and traces of the 2 β - (**3**) (0.8%) and 2 α - (**2**) (0.6%) hydroxyl isomers (Table 1). The larvae of this species were unavailable for investigation.

O. vitiosa is an Australian weevil species that has become widely established in Florida, USA, since its release in 1997 for the biological control of *Melaleuca quinquenervia* (Center et al., 2000). Invasive populations of *M. quinquenervia* threaten the biodiversity of the Florida Everglades (Turner et al., 1998). Both the larvae and adults of *O. vitiosa* feed on the emerging flush foliage of *M. quinquenervia* and a few related taxa (Wheeler, 2001). When fed *M. quinquenervia* leaves with intermediate levels of 1,8-cineole, the adults excreted frass containing volatile constituents found, by GC-MS, GC coinjection, and retention index comparison with authentic standards, to consist of 4.2% of 2 α ,9-dihydroxy-1,8-cineole (**9**). This is, to the best of our knowledge, the first report of a dihydroxycineole from insect frass. This diol has been identified previously as a 1,8-cineole metabolite of the brushtail possum, *Trichosurus vulpecula*, and subsequently synthesized (Carman et al., 1994). Other hydroxylated cineoles detected were 2 α -hydroxy-1,8-cineole (**2**) (3.5%), a smaller proportion of 3 α -hydroxy-1,8-cineole (**4**) (1.1%), and a trace (0.5%) of the 9-hydroxyl isomer (**8**) (Table 1). Although the larval frass of this weevil was unavailable, a shiny oil secretion from the cuticle has been the subject of a separate investigation (Wheeler et al., 2002) and was also available for hydroxycineole determination. This analysis showed the presence of trace (0.1%) quantities of 2 α -hydroxy-1,8-cineole (**2**) and none of the other hydroxyl cineoles.

No further investigations of the *Petasida ephippigera*, Leichhardt's grasshopper, were undertaken (Fletcher et al., 2000) other than an extrapolation of the contribution of the hydroxycineoles to the total frass volatiles, given that the hydroxycineoles contributed 50% (Fletcher, personal communication, 2001). Hence, the contribution to the total frass volatile fraction from each of the hydroxycineoles identified is shown in Table 1 for comparison. Fletcher et al. (2000) were incorrect in claiming "the first occurrence in which the 3 α -isomer (9) [our (**4**)] is the predominant isomer formed and only the second reported occurrence of cineole oxidation in insects." We had described cineole metabolites from two other species in a preliminary report (Southwell, 1999b) (including the 3 α -isomer as the predominant isomer from *C. bimaculata* adults) published prior to the submission of their report.

Two other earlier investigations reported, but did not identify, cineole metabolites from insect frass. The published chromatograms (Ohmart and Larsson, 1989) for the larval frass oils of *Paropsis atomaria* indicate substantial modification of the 1,8-cineole in their *Eucalyptus blakelyi* diet to compounds with similar

retention times to the above hydroxycineoles. 1,8-Cineole from *M. quinquenervia* was modified by *Pergagrapt*a sawfly larvae (Hymenoptera, Symphyta, Pergidae) to an unassigned hydroxycineole (Schmidt et al., 2000). The published mass spectrum indicates one of the 2-hydroxy-1,8-cineoles but does not distinguish between the α -**(2)** and β -**(3)** isomers. Coinjection of 2 β -hydroxy-1,8-cineole **(3)** did not give peak enhancement, but showed that the sawfly metabolite eluted 10 sec. later when injected on a HP5 stationary phase column (Moore, personal communication, 2001), indicating that the metabolite was almost certainly 2 α -hydroxy-1,8-cineole **(2)**.

The usefulness of this GC-MS method for the determination of cineole metabolism in insects was established by detecting as little as 0.09% of 3 α -hydroxy-1,8-cineole **(4)** as the only 1,8-cineole metabolite from a different sawfly species, *Pterygophorus* sp. (also Hymenoptera, Symphyta, Pergidae), feeding on the terpinen-4-ol chemotype of *M. alternifolia* containing only 1.6% 1,8-cineole. This is a relative of *Pergagrapt*a sp. (Schmidt et al., 2000) and is also known to defoliate *Eucalyptus*. This is consistent with the detection of trace quantities of 2 β -hydroxycineole in the frass of the pyrgo beetle, *P. tigrina*, adults and larvae fed *M. alternifolia* leaf containing 2% 1,8-cineole in the volatile extract (Southwell et al., 1995). The major component of the *Pterygophorus* frass volatiles was viridiflorol (24.6%). This sesquiterpene alcohol was present in only trace quantities in the *M. alternifolia* on which the larvae were feeding and, hence, accumulated from either this food source or another local food source, such as *M. quinquenervia* that can contain higher concentrations of viridiflorol (Brophy, 1999). The *Pergagrapt*a sawfly was not seen as accumulating viridiflorol in frass (Schmidt et al., 2000) when feeding on *M. quinquenervia*, whereas the weevil, *O. vitiosa*, does seem to sequester this sesquiterpenoid in cuticular secretions (Wheeler et al., 2002).

With metabolites of 1,8-cineole becoming increasingly significant in chemical ecology, ease of detection is imperative. NMR assignments of both ^1H and ^{13}C signals are available (Carman et al., 1994). A comprehensive investigation into the biotransformation of 1,8-cineole in the brushtail possum, *Trichosurus vulpecula*, (Boyle et al., 2000), has recorded MS and GC retention data for the trimethylsilyl ethers of metabolites of undefined stereochemistry. Consequently, for the ease of identification of small quantities of metabolites in mixtures, we now summarize the GC retention indices on four stationary phases, along with the MS data of the eight most significant ions from eight known hydroxycineole isomer metabolites from insects (Table 2). These data are based on the authentic, synthetic hydroxycineoles as measured in our laboratory and are identical to the insect metabolites where identified.

DISCUSSION

This investigation has shown that the metabolism of 1,8-cineole in insects is extremely variable, with variation occurring both between species and, in at

TABLE 2. GAS CHROMATOGRAPHIC RETENTION INDEX AND MASS SPECTRAL DATA FOR 1,8-CINEOLE METABOLITES

1,8-Cineole metabolite	GC retention index				Mass Spectral m/z (abundance %)							
	RI ^a ₁	RI ^b ₂	RI ^c ₃	RI ^d ₄	M ₁	M ₂	M ₃	M ₄	M ₅	M ₆	M ₇	M ₈
2 α -OH (2)	1300	1228	1359	2081	170(15)	155(2)	137(3)	126(70)	111(53)	108(100)	71(70)	43(75)
2 β -OH (3)	1288	1217	1345	1951	170(12)	155(2)	137(3)	126(78)	111(52)	108(100)	71(68)	43(78)
3 α -OH (4)	1317	1246	1381	2133	170(26)	155(27)	137(25)	127(28)	108(48)	93(62)	87(53)	43(100)
3 β -OH (5)	1327	1259	1400	2160	170(5)	155(100)	137(28)	127(30)	108(36)	93(80)	85(48)	43(91)
4-OH (6)	1258	1187	1316	1993	170(5)	155(8)	142(10)	124(5)	112(100)	97(67)	69(58)	43(100)
7-OH (7)		1263			170(10)	155(59)	137(18)	111(100)	93(66)	79(47)	69(70)	43(74)
9-OH (8)	1340	1267	1400	2091	170(0)	155(0)	139(100)	121(6)	95(35)	81(13)	71(21)	43(67)
2 α ,9-diOH (9)	1692	1470	1688	2801	186(0)	170(0)	155(83)	124(18)	109(22)	93(28)	84(25)	43(100)

^aBP1 50-m column.

^bHP5 30-m column.

^cAT35 60-m column.

^dDB WaxETR 60-m column.

least one case (*F. nigroconspersa*), between the larval and adult forms. In contrast, however, the adult frass from another species (*O. vittosa*) was found to contain the same major cineole metabolite, 2α -hydroxy-1,8-cineole, as the cuticular secretion of the larvae of the same species. Hydroxylation, presumably catalyzed by microsomal, cytochrome P-450-dependent enzymes (Gershenson and Croteau, 1991; Miyazawa et al., 2001) favored, with the possible exception of *F. nigroconspersa* and *O. vittosa* adults, ring carbon oxidation rather than the exposed methyl oxidation more common with vertebrates (Carman and Klika, 1992; Bull et al., 1993; Carman et al., 1994; Southwell et al., 1995).

Although these hydroxycineole metabolites have been positively identified as metabolites of the stated species, and dual oxygenase roles of both detoxification and semiochemical production are possible (Gershenson and Croteau, 1991), bioassay investigations are needed to test the activity of these compounds before it is known whether they actually induce a response in the insect.

Acknowledgments—The authors are indebted to Dr. Ray Carman and Dr. Mary Fletcher (Chemistry Department, University of Queensland) for providing hydroxycineole samples and unpublished data, respectively, and Dr. Chris Moore (Queensland Department of Primary Industries) for unpublished data on the cineole ingestion in *Pergagrapta* sawfly larvae investigation (Schmidt et al., 2000). We also thank Dr. Anthony Clarke (Cooperative Research Centre for Temperate Hardwood Forestry, Sandy Bay, Tasmania) for providing a small population (<50) of *C. bimaculata* adults for a laboratory colony, and George Wagner, Greenridge, New South Wales, for collecting frass from a field population of the *Pterygophorus* sp. sawfly larvae.

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