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Pine monoterpenes and pine bark beetles: a marriage of convenience for defense and chemical communication

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Abstract Pine-feeding bark beetles (Coleoptera: Scolytidae) interact chemically with their host pines (Coniferales: Pinaceae) *via* the behavioral, physiological, and biochemical effects of one class of isoprenoids, the monoterpenes and their derivatives. Pine monoterpenes occur in the oleoresin and function as behaviorally active kairomones for pine bark beetles and their predators, presenting a classic example of tritrophic chemical communication. The monoterpenes are also essential co-attractants for pine bark beetle aggregation pheromones. Ironically, pine monoterpenes are also toxic physiologically to bark beetles at high vapor concentrations and are considered an important component of the defense of pines. Research over the last 30 years has demonstrated that some bark beetle aggregation pheromones arise through oxygenation of monoterpenes, linking pheromone biosynthesis to the host pines. Over the last 10 years, however, several frequently occurring oxygenated monoterpene pheromone components (e.g., ipsenol, ipsdienol and frontalin) have also been shown to arise through highly regulated *de novo* pathways in the beetles (reviewed in Seybold and Tittiger, 2003). The most interesting nexus between these insects and their plant hosts involves the late-stage reactions in the monoterpenoid biosynthetic pathway, during which isomeric dimethylallyl diphosphate and isopentenyl diphosphate are ultimately elaborated to stereospecific monoterpenes in the trees and to hydroxylated monoterpenes or bicyclic acetals in the insects. There is signal stereospecificity in both production of and response to the monoterpenoid aggregation pheromones of bark beetles and in response to the monoterpenes of the pines. In the California fivespined ips, *Ips paraconfusus*, we

Dedicated to Professor David L. Wood on the occasion of his 75th birthday, January 8, 2006

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have discovered a number of cytochome P450 genes that have expression patterns indicating that they may be involved in detoxifying monoterpene secondary metabolites and/or biosynthesizing pheromone components. Both processes result in the production of oxygenated monoterpenes, likely with varying degrees of stereospecificity. A behavioral analysis of the stereospecific response of *I. paraconfusus* to its pheromone is providing new insights into the development of an efficacious bait for the detection of this polyphagous insect in areas outside the western United States. In contrast, a Eurasian species that has arrived in California, the Mediterranean pine engraver, *Orthotomicus* (*Ips*) *erosus*, utilizes both a monoterpenoid (ipsdienol) and a hemiterpenoid (2-methyl-3-buten-2-ol) in its pheromone blend. The stereospecificity of the response of *O. erosus* to the monoterpenoid appears to be the key factor to the improved potency of the attractant bait for this invasive species.

Keywords Aggregation pheromone · Behavior · Biosynthesis · Coleoptera · Host colonization · Ipsdienol · *Ips paraconfusus* · Kairomone · 2-Methyl-3-buten-2-ol · Monoterpene · Myrcene · *Orthotomicus erosus* · *Pinus* · P450 · Scolytidae

Introduction

Bark beetles (Coleoptera: Scolytidae) are a group of subcortical insects that feed as larvae and adults in the phloem of trees and woody shrubs (Wood and Bright 1992). They are closely allied with another group of beetles, ambrosia beetles, which tunnel into the xylem and derive nutrition from associated fungi. Together there are nearly 6,000 species of Scolytidae worldwide, forming one of the most formidable groups of endophytic parasites known to mankind. Although no definitive estimates are available, it is likely that over 500 species of scolytids feed on pine trees in the genus *Pinus*, which is probably the most species-rich group of conifers in the world (Critchfield and Little 1966; Mirov 1967; Price et al. 1998).

Pine bark beetles display a variety of microhabitat associations with pines that include colonization of cones (*Conophthorus* spp.), twigs and small branches (*Pityophthorus* spp.), upper stem and large branches (*Ips* spp., *Orthotomicus* spp., *Pityogenes* spp., *Pityokteines* spp.), main stem (*Dendroctonus* spp., *Ips* spp., *Hylurgops* spp.), and lower stem, root collar, and roots (*Dendroctonus* spp., *Hylurgus* spp., *Hylastes* spp., *Tomicus* spp.) (Fig. 1). Ambrosia beetles (*Gnathotrichus* spp., *Trypodendron* spp., and *Xyleborus* spp.) colonize the sapwood of the lower stem. Many of these species also colonize broken portions of trees that have fallen to the ground or stumps that remain after a tree has been broken or cut. In addition to these spatial patterns related to gross host anatomy, these beetles also partition themselves temporally, with certain genera (e.g., *Dendroctonus*, *Ips*) prefering to colonize recently declining or even healthy trees, whereas other genera prefer to colonize trees in a more advanced state of biodeterioration (e.g., *Hylurgops* or *Hylastes*, the so-called sour cambium beetles).

Host colonization in pine bark beetles involves visual (Strom et al. 1999, 2001), olfactory (DL Wood 1972, 1982), and gustatory signals (McNee et al. 2000, 2003), which in most species culminates in the aggregation of many individuals in the phloem in discrete family units defined spatially by galleries. Aggregation pheromones are used to signal the mass attack of the beetles on pines, allowing the insects to coordinate feeding and mating in time and space (DL Wood 1982; Seybold et al. 2000). The mating systems are varied (Kirkendall 1983; Kirkendall et al. 1997). For example, in *Dendroctonus* spp. the female tunnels through the bark and initiates the construction of a somewhat longitudinally oriented gallery, where she is later joined by a male in a monogynous mating system (Hopkins 1909). The galleries are packed with frass, which is the dust that results from boring activity, and consists of phloem and xylem fragments as well as the feces (Wood et al. 1966). In contrast, in *Ips* spp. the male initiates the construction of a longitudinally oriented gallery, where he is later joined by many females in a polygynous mating system (Struble and Hall 1955). *Ips* spp. push the frass out of the galleries onto the bark surface, resulting in an open gallery system. These galleries assume a

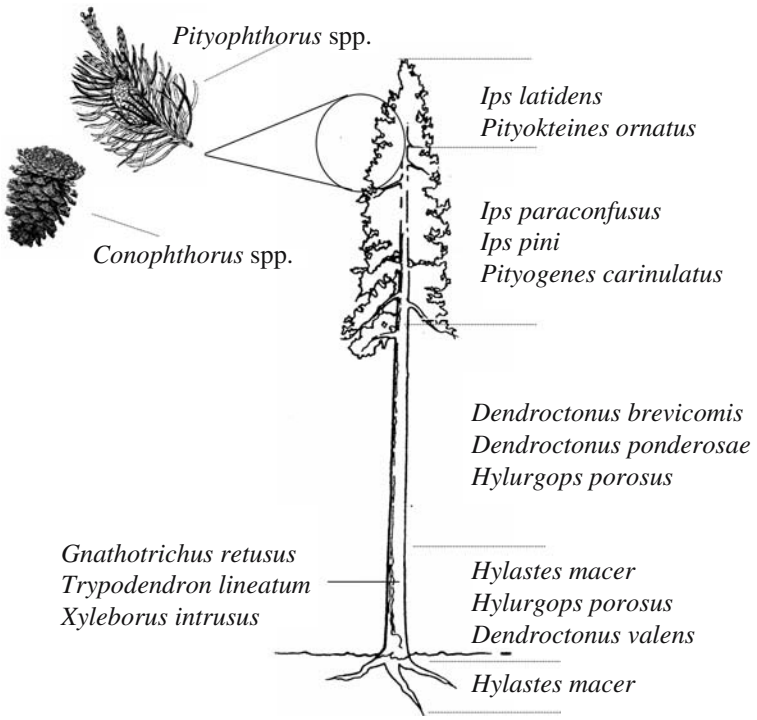


Fig. 1 Spatial colonization patterns of ponderosa pine, *Pinus ponderosa* Dougl. ex Laws., by bark and ambrosia beetles (Coleoptera: Scolytidae) in the central Sierra Nevada of California. Host associations of the species are based on Bright and Stark (1973) and SL Wood (1982). This figure is based on a graphic developed by DL Wood (University of California at Berkeley)

Y- or stellate shape, with a single female in each arm. Hypothetically, the intent of these gallery shapes is to avoid intraspecific competition among the resulting larvae that feed in the phloem away from the egg gallery walls (Poland and Borden 1994; Robins and Reid 1997).

The influence of monoterpenes on pine bark beetles

The behavior and physiology of pine bark beetles during dispersal and at the time of host colonization are largely governed by the interactions of the beetles with monoterpenes (Fig. 2). The relationship between the beetles and these isoprenoids is quixotic, and may have both positive and negative consequences for survival and reproduction (Table 1). Volatile monoterpenes pervade pine forest airspaces throughout the Northern Hemisphere (Tingey and Burns 1980; Guenther et al. 1994; Holzinger et al. 2005a). Kesselmeier and Staudt (1999) estimate that the global carbon input for monoterpenes ranges between 127 and 480 Tg C $year^{-1}$. Monoterpene flux data for pines has been derived from (1) emissions measured around foliage (Litvak and Monson 1998; Litvak et al. 1999; Niinemets et al. 2002) or individual small trees (Tingley et al. 1980; Juuti et al. 1990; Shao et al. 2001) and (2) measurements taken in or above the forest canopy (Schade et al. 1999; Schade and Goldstein 2003; Holzinger et al. 2005a; A Lee et al. 2005). The fluxes are increased by disturbances (Juuti et al. 1990; Strömvall and Petersson 1991; Schade and Goldstein 2003); by temperature (Tingey et al. 1980, 1991; Juuti et al. 1990; Charron et al. 1995; Shao et al. 2001); and by humidity (Schade et al. 1999), leading to dynamic diurnal emission patterns (Schade and Goldstein 2003; Holzinger et al. 2005b). Monoterpene fluxes above a mixed conifer forest containing primarily ponderosa pine, *Pinus ponderosa* Dougl. ex Laws., in California's central Sierra Nevada mountains have ranged seasonally from 0.10 to 0.83 μmol m^{-2} h^{-1} (Holzinger et al. 2005a) with basal emission rates at 30°C in May ranging from 0.05 to 0.38 mg C m^{-2} h^{-1}, depending on the species of monoterpene evaluated (Schade and Goldstein 2003).

Pine bark beetles are thought to generally constrain their dispersal flights within the height

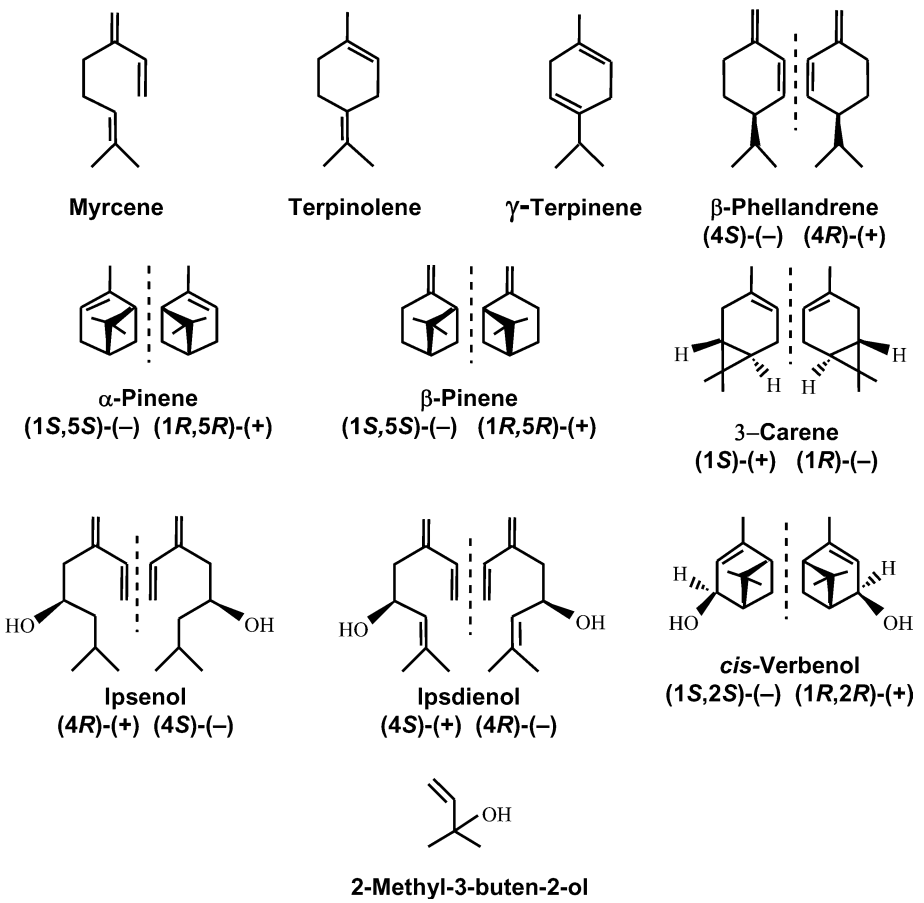


Fig. 2 Behaviorally active isoprenoids for pine bark beetles including myrcene (7-methyl-3-methylene-1,6-octadiene), terpinolene [1-methyl-4-(1-methylethylidene)-cyclohexene], γ-terpinene [1-methyl-4-(1-methylethyl)-1,4-cyclohexadiene], β-phellandrene [methyl-6-(1-methylethyl)-cyclohexene], α-pinene (2,6,6-trimethylbicyclo[3.1.1] hept-2-ene), β-pinene (6,6-dimethyl-2-methylenebicyclo[3.1.1] heptane), 3-carene (trimethylbicyclo[4.1.0]hept-3-ene), ipsenol (2-methyl-6-methylene-7-octen-4-ol), ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol), *cis*-verbenol (*cis*-2,6,6-trimethylbicyclo[3.1.1]hept-2-en-4-ol) [optical rotations of *cis*-verbenol designated as measured in chloroform, enantiomers also referred to as (1*S*,4*S*,5*S*)-(−) and (1*R*,4*R*,5*R*)-(+) by some authors], and 2-methyl-3-buten-2-ol

of the stem of their host trees (Gara and Vité 1962; Schmitz 1980, 1984; Schmitz et al. 1980, 1989; Safranyik et al. 1989, 1992, 2000; Byers 2000; Safranyik and Carroll 2006). A small percentage of the population may disperse above the forest canopy (Furniss and Furniss 1972; Safranyik et al. 1992; Safranyik and Carroll 2006). Thus, monoterpene emissions from the woody portions of stems and branches are more likely to permit focused host-location behavior by pine bark beetles and are likely to be more relevant to their colonization behavior than emissions from foliage. However, very little information appears to be available on these woody emissions or they are presumed to be low under ambient conditions (Schade and Goldstein 2003). When woody tissues are damaged on standing trees or on portions of cut and fallen trees during mechanical disturbances such as forest harvest and thinning operations, total emissions of monoterpenes increase substantially (Strömvall and Petersson 1991; Schade and Goldstein 2003). The three-dimensional aligment of the dispersal space of the beetles with the emerging awareness of the dynamic pool of background monoterpenes in forests has only begun to be explored (Byers et al. 2000).

Monoterpenes as attractive kairomones for pine bark beetles

Within the dynamic aerial sea of monoterpenes and other volatile organic compounds that characterize pine ecosystems, some species of dispersing adult pine bark beetles manage to focus their olfactory system on specific monoterpenes that emanate from specific pines. In these cases, monoterpenes function as essential host attractants (kairomones) that enhance the reproduction and survival of the beetles (reviewed in Seybold et al. 2000). Researchers have tested the behavioral impact of monoterpenes by placing them in discrete release devices (i.e., near-point sources) from which the monoterpenes elute on the order of 10 to 1,000 mg/day. For example, when tested individually, (*S*)-(−)-*β*-pinene, (*R*)-(+)-α-pinene, and (*S*)-(+)-3-carene (Fig. 2) all attracted the red turpentine beetle, *Dendroctonus valens* LeConte, to multiple funnel traps in the mixed conifer forest of California's central Sierra Nevada mountains (Hobson et al. 1993). These authors also demonstrated that the three monoterpenes were present in the oleoresin of two of the pines colonized in this area by *D. valens*, *P. ponderosa*, and sugar pine, *Pinus lambertiana* Dougl. Other pine-infesting bark beetles that respond in flight significantly to monoterpenes alone include the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (to *γ*-terpinene) (Miller and Borden 2003); the western pine engraver, *Ips latidens* (LeConte), and the pine engraver, *Ips pini* (Say) (both to *β*-phellandrene) (Miller et al. 1986; Miller and Borden 1990a, b, 2000); and the pine shoot beetle, *Tomicus piniperda* L. [to (*R*)-(+)-α-pinene, (*S*)-(−)-α-pinene, (*S*)-(+)-3-carene, and terpinolene] (Byers et al. 1985; Schroeder and Eidmann 1987; Schroeder 1988; Schroeder and Lindelöw 1989; Byers 1992; Czokajlo and Teale 1999; Poland et al. 2003, 2004). Both sexes of the southern pine beetle, *Dendroctonus frontalis* Zimm., responded to increasing doses of α-pinene relative to a solvent control in a laboratory walking bioassay (McCarty et al. 1980), but the response to α-pinene alone was not confirmed with flight behavior in a controlled field experiment (Payne et al. 1978).

Monoterpenes as pine bark beetle pheromone co-attractants

Monoterpenes may also work in concert with beetle-produced compounds to enhance the responses to aggregation pheromones (Table 1, Vité 1970). A research team led by DL Wood and RM Silverstein first discovered this phenomenon with the western pine beetle, *Dendroctonus brevicomis* LeConte (Silverstein et al. 1968, Bedard et al. 1969, 1970, 1980; Wood et al. 1969; Silverstein 1970a, b; Wood 1970, 1972). Using a benzene extract of the frass from unmated females feeding in *P. ponderosa*, laboratory assays of the walking behavior of both sexes of *D. brevicomis* revealed that the response to female-produced *exo*-brevicomin was synergized by a hydrocarbon fraction that was inactive alone (Silverstein et al. 1968; Silverstein 1970a, b); one of the synergistic components of the hydrocarbon fraction was isolated and identified as myrcene (Fig. 2) (Silverstein 1970a, b). Myrcene, which is present in the host volatiles from oleoresin of *P. ponderosa* (Hobson et al. 1993) and Coulter pine, *P. coulteri* D. Don (Smith 2000), also acted synergistically with *exo*-brevicomin to attract both sexes of the beetle in flight (Bedard et al. 1969, 1970). The synergistic effect of myrcene was less evident when the monoterpene was tested with the binary mixture of *exo*-brevicomin and male-produced frontalin (Bedard et al. 1980). Nonetheless, further field tests with *exo*-brevicomin, frontalin, and six monoterpenes (each presented individually in the experiments) confirmed that the combination with myrcene elicited the highest trap catches (Wood 1972; Bedard et al. 1980). Distilled oleoresin (turpentine, whose chemical composition was unreported) enhanced the flight response to *exo*-brevicomin and frontalin to a greater extent than myrcene (Wood 1972; Bedard et al. 1980). In these tests the release rate of synthetic myrcene alone was equivalent to its release rate from the turpentine [24 mg/day, Bedard et al. (1980)] or likely exceeded its release rate from the turpentine [96 mg/day vs. 48 mg/day, assuming a 10% myrcene content of the turpentine, Wood (1972)]. In another study, freshly tapped oleoresin from *P. ponderosa* was unattractive alone to

Table 1 A critical survey of research on pine-feeding bark beetles and their physiological and behavioral responses to monoterpenes

Species	Responses to selected monoterpenes
Conophthorus coniperda	(±)-α-Pinene increases male flight response to racemic *trans*-pityol in one of four experiments; individual monoterpenes or mixtures are not attractive to males or females in the absence of pityol (De Groot et al. 1998); flight response to α-pinene is dose- and enantiospecific when combined with racemic *trans*-pityol (Miller et al. 2003).
Conophthorus ponderosae	(−)-α-Pinene increases male flight response to racemic *trans*-pityol-baited traps (Miller et al. 2000).
Conophthorus resinosae	(±)-α-Pinene, (−)-*β*-pinene, (+)-α-limonene, and myrcene in various combinations did not enhance the flight response of males to racemic *trans*-pityol in a series of three experiments; the pinenes were not attractive alone (De Groot and Zylstra 1995)
Dendroctonus brevicomis	Limonene is toxic (Smith 1965a, b) and *Pinus ponderosa* trees from areas with historically high populations of *D. brevicomis* have relatively high concentrations of limonene, myrcene, and *β*-pinene, but low concentrations of α-pinene (Sturgeon 1979); myrcene metabolized by adults of both sexes to myrcenol (Renwick et al. 1976b) and by males to ipsdienol (Renwick et al. 1976b; Byers 1982; Seybold et al. 1992); camphene metabolized by adults of both sexes to 6-hydroxy-camphene (Renwick et al. 1976b); α-pinene metabolized by immature (teneral) adults to *trans*-verbenol at lower rates than by mature adults; α-pinene also metabolized by mature, but not teneral, adult males to verbenone (Byers 1983b); myrcene enhances the walking and flight responses to the pheromone component *exo*-brevicomin (Bedard et al. 1969, 1970; Silverstein 1970a, b) and the flight response to *exo*-brevicomin and frontalin (Wood 1972; Wood et al. 1976; Bedard et al. 1980). Other monoterpenes (camphene, 3-carene, limonene, α-pinene, and *β*-pinene), *P. ponderosa* oleoresin, or distilled *P. ponderosa* oleoresin (turpentine) either do not increase or weakly increase the flight response to *exo*-brevicomin and frontalin (Vité and Pitman 1969; Wood 1972; Bedard et al. 1980).
Dendroctonus frontalis	Limonene, *β*-pinene, α-pinene, and camphene are toxic, in descending order of toxicity (Cook and Hain 1988); camphene metabolized by adults of both sexes to 6-hydroxy-camphene (Renwick et al. 1976b); α-pinene metabolized by larvae and adults, but not by pupae, to *trans*-verbenol and by adult males to verbenone (Hughes 1975); α-pinene metabolized to *trans*-verbenol, verbenone, and myrtenol by teneral adult females (*trans*-verbenol conversion enhanced by methoprene treatment) (Bridges 1982); antennal responses of both sexes to α-pinene and 3-carene (Dickens and Payne 1977); *Pinus taeda* turpentine enhances response to aggregation pheromone (Billings 1985); α-pinene elicited a significant and dose-dependent response from both sexes in a laboratory walking bioassay (McCarty et al. 1980), but the additive effect of α-pinene to the pheromone component frontalin was not tested directly in the experiment; α-pinene may or may not enhance the flight response to frontalin as the experimental evidence is either not analyzed statistically (Renwick and Vité 1969), not present (Payne et al. 1978), or confounded by the presence of turpentine in the experiment (Billings 1985).
Dendroctonus ponderosae	α-Pinene metabolized by mature females and males to *trans*-verbenol (Hughes 1973b); (−)-α-Pinene metabolized by mature females to verbenene, *p*-mentha-1,5,8-triene, and *o*- and *p*-cymene (deuterated substrate and products), (−)-, (+)-, and (±)-α-pinene metabolized by females to *trans*-verbenol in a dose-dependent manner (Gries et al. 1990); antennal responses to α-pinene, *β*-pinene, myrcene, (*E*)-ocimene, *β*-phellandrene, limonene, camphene, sabinene, 3-carene, α-terpinene, *p*-cymene, *γ*-terpinene, and terpinolene (Huber et al. 2000; Pureswaran et al. 2004a); *γ*-terpinene attractive relative to an unbaited trap in field flight assay (Miller and Borden 2003) and attractive at high release rates (~52 and 1,110 mg/day) when combined with *exo*-brevicomin and *trans*- and *cis*-verbenol (=aggregation pheromone) (Miller and Borden 2000); α-pinene combined with *trans*-verbenol found to be more attractive than camphene or myrcene, which were more attractive than either limonene, 3-carene, or *β*-pinene in an uncontrolled flight assay in which the results were not analyzed statistically (Pitman 1971); myrcene and terpinolene found to be more attractive when combined with *trans*-verbenol than were 3-carene, limonene, α-pinene, or *β*-pinene (Billings et al. 1976); myrcene found to be more attractive than α-pinene and other monoterpenes in presence of pheromone components (Borden et al. 1983; Conn et al. 1983; Miller and Lindgren 2000); high release rates of 3-carene (~600 mg/day) (Miller and Borden 2000) and myrcene (900–6,500 mg/day) (Borden et al. 1987; Miller and Borden 2000) each increased flight response to the aggregation pheromone; *β*-phellandrene elicited a dose-dependent and increasing flight response to the aggregation pheromone, but no release rates significantly increased trap catch relative to the pheromone alone (Miller and Borden 2000), however, it was attractive over a range of doses in combination with ipsdienol (Miller and Borden 1990a); myrcene synergized attraction to aggregation pheromone, but there was no primary attraction detected to a variety of mixtures of monoterpenoids (Pureswaran and Borden 2005); myrcene may be superfluous in inciting attack on standing *Pinus contorta latifolia* with *trans*-verbenol and *exo*-brevicomin (Borden et al. 1990).

Table 1 continued

Species	Responses to selected monoterpenes
Dendroctonus terebrans	α-Pinene metabolized by larvae and adults, but not by pupae, to *trans*-verbenol (Hughes 1975); α-pinene metabolized by microsomal fraction of larvae and adults to α-pinene oxide (White et al. 1979); dose-dependent antennal responses to α- and β-pinene and *P. taeda* turpentine (Delorme and Payne 1990); turpentine is attractive in the field (reviewed in Nation et al. 1996); ethanol synergizes the flight response to turpentine from *Pinus elliottii elliottii* Engelm. and *P. palustris* Mill. (contained α- and β-pinene, camphene, limonene, β-phellandrene, and myrcene) when the two materials were combined in one solution, but not when they were released from individual devices (Phillips et al. 1988).
Dendroctonus valens	α-Pinene metabolized by male adults to *cis*- and *trans*-verbenol and *cis*-3-pinen-2-ol, β-pinene metabolized by males to pinocarvone and *trans*-pinocarveol (Hughes 1973a); antennal responses by both sexes to (*R*)-(+)- and (*S*)-(−)-α-pinene, (*S*)-(−)-β-pinene, (*R*)-(+)- and (*S*)-(−)-limonene, (*S*)-(+)-3-carene, myrcene, β-phellandrene, and terpinolene (White and Hobson 1993). Responses by antennae to (*R*)-(+)- and (*S*)-(−)-α-pinene suggest different receptors for each enantiomer (White and Hobson 1993). (+)-α-Pinene, (−)-β-pinene, and 3-carene are attractive in flight assays of North American populations (Hobson et al. 1993; Fettig et al. 2004), but 3-carene is the best attractant in introduced populations in China (Sun et al. 2004). High release rate of (+)-α-pinene (estimated at 12,375–16,500 mg/day) is attractive, but flight response to the attractant (12,375 mg/day) is interrupted by (−)-α-pinene (4,125 mg/day) (Hobson et al. 1993); (−)-β-Pinene with ethanol was more attractive than (−)-α-pinene alone or with ethanol (Petrice et al. 2004); high release rate of ethanol increased trap catch to (±)-α-pinene and (−)-β-pinene (Joseph et al. 2001).
Gnathotrichus retusus	α-Pinene is neither a host attractant alone nor a synergist of the aggregation pheromone component sulcatol (Borden et al. 1980, 1981; Liu and McLean 1989); high release rate of ethanol increased trap catch to (±)-α-pinene and (−)-β-pinene (Joseph et al. 2001).
Hylastes angustatus	α-Pinene and myrcene were tested in combination with ethanol in choice assays with *Pinus patula* bark, but the experiments were not designed to test for the effect of the monoterpenes relative to either ethanol or bark alone (Erasmus and Chown 1994).
Hylastes ater	β-Pinene with ethanol, and raw turpentine with ethanol increased trap captures relative to an unbaited trap; α-pinene (with or without ethanol) did not increase trap captures relative to an unbaited trap (Reay and Walsh 2002).
Hylastes brunneus	(−)-α-Pinene increases attraction to ethanol (Schroeder and Lindelöw 1989).
Hylastes cunicularis	(−)-α-Pinene increases attraction to ethanol (Schroeder and Lindelöw 1989).
Hylastes longicollis	Attracted to myrcene, β-pinene, terpinolene, β-phellandrene, and 3-carene, each in combination with ipsenol (Miller and Borden 1990b); high release rate of ethanol increased trap catch to (±)-α-pinene and (−)-β-pinene (Joseph et al. 2001).
Hylastes macer	High release rate of ethanol increased trap catch to (±)-α-pinene and (−)-β-pinene (Joseph et al. 2001).
Hylastes nigrinus	Attracted to α-pinene (Witcosky et al. 1987); high release rate of ethanol increased trap catch to (±)-α-pinene and (−)-β-pinene (Joseph et al. 2001).
Hylastes opacus	Attracted to (−)-α-pinene and (−)-α-pinene with ethanol (Schroeder and Lindelöw 1989) or to α-pinene alone or to β-pinene with ethanol relative to a trap baited with *Ips typographus* pheromone (Petrice et al. 2004). Attracted more to the combination of nonanal and (−)-α-pinene than to (−)-α-pinene alone (De Groot and Poland 2003).
Hylastes salebrosus	Ethanol increases flight response to *P. elliottii*/*P. palustris* turpentine (contained α- and β-pinene, camphene, limonene, β-phellandrene, and myrcene). Experiment lacked an unbaited control to prove response to turpentine alone (Phillips 1990).
Hylurgops palliatus	Attracted to a mixture of 3-carene, (+)- and (−)-α-pinene and terpinolene with ethanol, but not to the monoterpene mixture alone and only weakly to ethanol alone (Byers 1992); not attracted to (−)-α-pinene alone (Schroeder 1988) or attracted to one release rate of (−)-α-pinene (Schroeder and Lindelöw 1989), but attracted to (−)-α-pinene and ethanol (Schroeder 1988, 2003; Schroeder and Lindelöw 1989); attracted to β-pinene or β-pinene and terpinolene, but not terpinolene alone when these components were combined with ethanol (Volz 1988); trapped in response to terpinolene and (±)-α-pinene (individually and combined) when these components were combined with ethanol (no negative control) (Vité et al. 1986).

Table 1 continued

Species	Responses to selected monoterpenes
Hylurgops porosus	Attracted to terpinolene, β-phellandrene, and 3-carene, each in combination with ipsenol (Miller and Borden 1990b); high release rate of ethanol increased trap catch to (±)-α-pinene and (−)-β-pinene (Joseph et al. 2001).
Hylurgops reticulatus	High release rate of ethanol increased trap catch to (±)-α-pinene and (−)-β-pinene (Joseph et al. 2001).
Hylurgops subcostulatus	High release rate of ethanol increased trap catch to (±)-α-pinene and (−)-β-pinene (Joseph et al. 2001).
Hylurgus ligniperda	α-Pinene, β-pinene, and raw turpentine when combined with ethanol increase trap captures relative to an unbaited trap; α-pinene alone and β-pinene alone also increase trap captures (Reay and Walsh 2002); α- or β-pinene when combined with ethanol or α-pinene alone increase trap captures relative to a trap baited with *Ips typographus* pheromone (Petrice et al. 2004).
Ips avulsus	Myrcene metabolized by males to ipsdienol (Hughes 1974); dose-dependent antennal responses of both sexes to α-pinene (Smith et al. 1988); turpentine presented at a high release rate reduced trap catch to a generic pheromone bait for *Ips* spp. (Billings 1985).
Ips calligraphus	Camphene, limonene, and β-pinene are toxic at 40 and 100 ppm; α-pinene is toxic at 100 ppm; limonene is the least toxic and camphene is the most toxic of the monoterpenes at 100 ppm (Cook and Hain 1988); dose-dependent antennal responses of both sexes to α-pinene (Smith et al. 1988).
Ips grandicollis	Dose-dependent and similar antennal responses by both sexes to α-pinene (Smith et al. 1988; Ascoli-Christensen et al. 1993); α-pinene, β-pinene, myrcene, limonene, camphene, and carene are attractants (Werner 1972; Chénier and Philogéne 1989; Erbilgin and Raffa 2000); camphene, limonene, and myrcene appeared to enhance flight responses to an extract of *I. grandicollis* frass, but the results were not analyzed statistically (Werner 1972); turpentine presented at a high release rate enhanced trap catch to a generic pheromone bait for *Ips* spp. (Billings 1985). α-Pinene when combined with ethanol or α-pinene alone increased trap captures relative to a trap baited with *Ips typographus* pheromone (Petrice et al. 2004).
Ips latidens	At some release rates β-phellandrene (~200 mg/day) and β-pinene (~240–1,200 mg/day) increase attraction to ipsenol, but high release rates (100–2,000 mg/day) of α-pinene, 3-carene, terpinolene, and myrcene, and one release rate of β-phellandrene (~2,100 mg/day) interrupt the flight response to ipsenol (Miller and Borden 1990b, 2000).
Ips mexicanus	Attracted to β-phellandrene and 3-carene, each in combination with ipsenol (Miller and Borden 1990b).
Ips paraconfusus	3-Carene metabolized by males to 1-methyl-5-(α-hydroxy-isopropyl)-cyclohexa-1,3-diene (Renwick et al. 1976b); myrcene metabolized by mature adult males, but not females or immature (teneral) males, to ipsdienol and ipsenol (Hughes 1974; Byers et al. 1979; Hendry et al. 1980; Byers 1983b); α-pinene metabolized to *cis*- and *trans*-verbenol by mature and teneral, adult males and females (Renwick et al. 1976a; Byers 1981, 1983b); (−)-α-pinene metabolized dose-dependently to *cis*- and *trans*-verbenol and myrtenol by males and females, males produce more of these compounds (Byers 1981); myrcene induces a "coma" in beetles at high headspace concentrations (Byers et al. 1979); α-pinene causes mortality at high headspace concentrations (Byers 1981); dose-dependent antennal responses to myrcene and (+)-α-pinene (Light 1983).
Ips pini	3-Carene metabolized by males to 1-methyl-5-(α-hydroxy-isopropyl)-cyclohexa-1,3-diene (Renwick et al. 1976b); myrcene metabolized to ipsdienol (Vanderwel 1991); antennal responses to β-phellandrene and limonene (Huber et al. 2000); (−)-, (+)-, and (±)-α-pinene, (±)-β-pinene, and (±)-limonene at increasing concentrations generally inhibit postlanding behaviors (initial gallery entry, within tissue orientation, and gallery extension) by males (Wallin and Raffa 2000); β-phellandrene is weakly attractive alone in flight behavioral assays (Miller and Borden 1990a); various high release rates of 3-carene (~200–1,200 mg/day), β-phellandrene (~5, 40–1,000 mg/day), and β-pinene (~240–1,200 mg/day) increase flight response to ipsdienol (Miller and Borden 1990a, 2000, 2003); high release rates (~200–340 mg/day) of myrcene, (−)- and (+)-α-pinene, and (−)-β-pinene reduce flight response to ipsdienol and lanierone (Erbilgin and Raffa 2000); (±)-α-pinene elicits a dose-dependent flight response from both sexes when combined with ipsdienol and lanierone, attractive at ~60 mg/day, interruptive at ~350 mg/day (Erbilgin et al. 2003); high release rates of myrcene (~50–650 mg/day) and terpinolene (~340–2,100 mg/day) interrupt the flight response to ipsdienol (Miller and Borden 2000, 2003).

Table 1 continued

Species	Responses to selected monoterpenes
Ips stebbingi (=schmutzenhoferi)	(−)-α-Pinene [> 68.5%-(−)] was tested in combination with different concentrations of (±)-ipsenol in a flight bioassay. Because of the absence of a negative control, there was no definitive proof of a response to α-pinene alone. In one experiment, the response to α-pinene and ipsenol did not differ from the response to α-pinene alone (Kohnle et al. 1988).
Pityogenes bidentatus	(−)-α-Pinene, (+)-α-pinene, and terpinolene at high release rates (144 mg/day) interrupt attraction to *cis*-verbenol and grandisol (El-Sayed and Byers 2000). (−)-α-Pinene (33.6 mg/day), (+)-α-pinene (33.6 mg/day), (−)-*β*-Pinene (23.5 mg/day), (+)-3-carene (51.1 mg/day) and terpinolene (18.7 mg/day) or the combination of (±)-α-pinene, (+)-3-carene, and terpinolene (60 mg/day total release) interrupt attraction to *cis*-verbenol and grandisol (Byers et al. 2000).
Pityogenes knechteli	3-Carene and (−)-α-pinene interrupt attraction to ipsdienol (Miller and Borden 2003).
Tomicus minor	Antennal responses to (+)-α-pinene, (−)-α-pinene, (+)-3-carene, myrcene, and terpinolene (Lanne et al. 1987).
Tomicus piniperda	Antennal responses to (+)-α-pinene, (−)-α-pinene, (+)-3-carene, myrcene, and terpinolene (Lanne et al. 1987). Attracted to various combinations of 3-carene, (±)-α-pinene, (+)-α-pinene, (−)-α-pinene, and terpinolene with and without ethanol (Byers et al. 1985; Klimetzek et al. 1986; Vité et al. 1986; Schroeder and Eidmann 1987; Byers 1992); attracted to (−)-α-pinene (Schroeder 1988; Schroeder and Lindelöw 1989; Czokajlo and Teale 1999; Poland and Haack 2000; Poland et al. 2003); attracted to *β*-pinene and terpinolene when combined with ethanol (Volz 1988); attracted to a 1:7 mixture of (+)-α-pinene and ethanol (Zumr 1989); to 1:0.1, 1:0.9, and 1:9 mixtures of racemic α-pinene and ethanol (Czokajlo and Teale 1999); to a 1:10 mixture of (−)-α-pinene and ethanol (Schroeder 2003); and to various combinations of α-pinene, *β*-pinene, and ethanol (Petrice et al. 2004). Addition of *trans*-verbenol to (−)-α-pinene alone or to the blend of (−)-α-pinene and other semiochemicals was attractive in Michigan, US and Ontario, Canada (Poland et al. 2003, 2004), but not significantly more attractive than a host compound blend (ethanol, *β*-pinene, terpinolene) in Germany (Volz 1988).
Trypodendron lineatum	(−)-α-Pinene is not attractive alone, but it increased attraction to ethanol (Schroeder 1988; Schroeder and Lindelöw 1989), whereas in another study the combination of (±)-α-pinene (30 mg/day) and ethanol (120 mg/day) was not attractive (Borden et al. 1981); α-pinene and ethanol combined enhance the response to the pheromone component lineatin (Vité and Bakke 1979; Shore and McLean 1983; Paiva and Kiesel 1985), at low release rates (28.8–33.6 mg/day) α-pinene (unreported enantiomeric composition) increased the response to lineatin and ethanol (12.0–14.4 mg/day), but at higher α-pinene release rates (86.4–100.8 mg/day) the increase was less pronounced (Bakke 1983). (−)-α-Pinene alone (413 mg/day) elicited significantly higher trap catches than myrcene alone (281 mg/day) (Miller and Lindgren 2000).

Associations of these scolytid species with *Pinus* have been documented in Bright and Stark (1973); SL Wood (1982); Wood and Bright (1992); and Bright and Skidmore (2002)

D. brevicomis, but enhanced the flight response to *exo*-brevicomin and frontalin three-fold (Vité and Pitman 1969).

Other examples of the positive influence of monoterpenes as co-attractants on the response to aggregation pheromone include (1) the eastern fivespined ips, *Ips grandicollis* (Eichhoff), and camphene, limonene, or myrcene (Werner 1972), α-pinene (Erbilgin and Raffa 2000), or turpentine from loblolly pine, *Pinus taeda* L. (Billings 1985); (2) *I. pini* and 3-carene, β-phellandrene, or β-pinene (Miller and Borden 2000, 2003) or certain release rates of α-pinene (Erbilgin et al. 2003); and (3) *D. ponderosae* and α-pinene (Pitman 1971, but see Table 1 about the quality of this experiment), myrcene (Borden et al. 1983, 1987; Conn et al. 1983; Miller and Lindgren 2000; Pureswaran and Borden 2005), myrcene or terpinolene (Billings et al. 1976), or 3-carene, myrcene, or β-phellandrene (Miller and Borden 2000). The role of α-pinene (Renwick and Vité 1969) as a co-attractant in the pheromone of *D. frontalis* is confounded by laboratory experiments that have not tested directly the comparative responses to frontalin with and without the monoterpene (McCarty et al. 1980); by field experiments with a minor treatment effect but no statistical analysis (Renwick and Vité 1969); by field experiments with no treatment effect related to α-pinene (Payne et al. 1978); or by field experiments where the individual monoterpene was also tested in conjunction with high release rates of α-pinene-containing turpentine from the host *P. taeda* (Billings 1985). Further work in this system is necessary. Recently, Poland et al. (2003, 2004) concluded that *trans*-verbenol is an aggregation pheromone component for immigrant North American populations of *T. piniperda* and that (−)-α-pinene, attractive by itself, is also a host-produced co-attractant with *trans*-verbenol. Byers (2004) has hypothesized that monoterpenes may also regulate proximal behavior of bark beetles; specifically, to enhance entry rates into already initiated galleries. Similar to the instances of long-range attraction noted above, the proximal activity of monoterpenes in this case would be in the context of the aggregation pheromone emanating from the bark surface or from the gallery itself. Thus, in contrast to the views of early workers in the field, who considered monoterpenes as ''replaceable'' in the phenomenon of bark beetle aggregation (Renwick 1970), a review of the modern literature shows that for some species they appear to be essential as co-attractants.

Nearly all research on the effect of monoterpenes as attractants or as bark beetle pheromone co-attractants has bypassed the procedure of sequential fractionation and assay of oleoresin volatiles that might reveal potential synergisms and the behavioral activity of minor components (see Silverstein et al. 1967 for the methodology; Silverstein 1970a, b; Byers et al. 1985; Hobson et al. 1993 for attempts at the application). Instead, the majority of studies have presented beetles in the field with individual synthetic monoterpenes or simple blends based on the most abundant monoterpenes in host pine oleoresin. Most recently, the selection of which compounds to test has been guided by antennal responses in combined gas chromatography-electroantennographic detection (GC-EAD) (e.g., Pureswaran et al. 2004a). However, this approach has perhaps prematurely removed the key monoterpenes from the context of the quantitatively and qualitatively complete odor of wound oleoresin from the pine hosts. In a debate over the experimental approach used to isolate and identify monoterpenes that enhanced the response of *D. brevicomis* to its pheromone, Bedard et al. (1970) wrote, ''There is no logic whatever in the a priori assumption favoring a 'predominant' [quotation marks of Bedard et al. (1970)] component over a minor one.'' Indeed 35 years later, it is very intriguing that certain monoterpenes that are relatively minor components of the volatile fraction of the oleoresin of pine hosts play a major role in the attraction of certain bark beetle species that colonize those hosts. For example, myrcene occurs as 7% (*P. ponderosa*, Hobson et al. 1993), 1.4–15.4% (*P. ponderosa*, Smith 1977), 20.3–20.7% (*P. coulteri*, Smith 1967, 2000), 3.9% [Sierra Nevada lodgepole pine, *P. contorta murrayana* (Balfour) Critchfield, Smith 1964], 2.6% [Rocky Mountain lodgepole pine, *P. contorta latifolia* (Engelmann) Critchfield, Pureswaran et al. 2004b], 1.9–3.9% (both subspecies of *P. contorta*, Smith 1983, 2000), and 4.4% (limber pine, *P. flexilis* James, Zavarin et al. 1993) of the monoterpenes in extracted oleoresin, xylem, or

combined outer bark, phloem, and xylem. Yet myrcene appears to be the most efficacious co-attractant for the pheromone of *D. brevicomis* (Wood 1972; Bedard et al. 1980), which colonizes *P. ponderosa* and *P. coulteri*, and for the pheromone of *D. ponderosae* (Billings et al. 1976; Miller and Borden 2000; Miller and Lindgren 2000; Pureswaran and Borden 2005), which can colonize all of the above hosts. Terpinolene, which is generally present in even lower quantities than myrcene in the pines noted above, is also a highly effective co-attractant for *D. ponderosae* in the Cascade Mountain (Billings et al. 1976) and central and southern Rocky Mountain regions (Seybold et al. unpublished data) of the western United States (US). Pureswaran (2003) has speculated that with *D. ponderosae* the response to myrcene as a pheromone co-attractant may be a vestigial behavioral trait that reflects an earlier, more prominent association with hosts that produced more myrcene (e.g., whitebark pine, *Pinus albicaulus* Engelmann or its progenitor). Presumably, similar evolutionary hypotheses could be posited for *D. brevicomis* and myrcene, and *D. ponderosae* and terpinolene as well.

Some studies have evaluated the role of monoterpenes as behavioral chemicals for bark beetles in a more natural context. In a tree-baiting study in Dalarna, Sweden, Schroeder and Eidmann (1987) found that 14-cm diameter Scots pine, *Pinus sylvestris* L., trees were colonized at significantly higher rates by *T. piniperda* when the trees were baited for one day with (−)-α-pinene, (+)-3-carene, terpinolene, or the combination of all three monoterpenes (each released at an estimated 5 μl/h). In a similar study in British Columbia, Canada with *D. ponderosae*, Borden et al. (1990) reported that *P. contorta latifolia* were colonized whether or not myrcene was included in the inciting bait of female-produced *trans*-verbenol and male-produced *exo*-brevicomin. Presumably, myrcene or other monoterpenes volatilizing naturally from oleoresin released from the newly infested trees replaced the need for myrcene in the synthetic attractant. Pureswaran and Borden (2005) also attempted to evaluate the co-attractant role of myrcene for *D. ponderosae* in a more natural context. They reported that the addition of myrcene (95 mg/day) enhanced the flight response of *D. ponderosae* to its aggregation pheromone more than a blend of the five most abundant monoterpenes in *P. contorta latifolia* stem volatiles (which did not contain myrcene). Myrcene as a co-attractant with *trans*-verbenol for *D. ponderosae* was also numerically (but not statistically) more efficacious than a blend of six *P. contorta latifolia* monoterpenes in funnel trapping (Conn et al. 1983) and baited tree (Borden et al. 1983) studies.

Finally, there is a semantic issue related to the role that host-derived monoterpenes play relative to bark beetle aggregation pheromones in the ensemble of attractive semiochemicals. A pheromone is defined as ''a substance secreted by an animal to the outside that causes a specific reaction in another member or members of the same species'' (Nordlund and Lewis 1976). When a bark beetle colonizes a pine, monoterpenes can be emitted from wounded tree tissue or oleoresin flowing from the wound, from boring dust that passes around the beetle during excavation, from undigested tree tissue in fecal material that passes through the alimentary canal of the beetle, and from potentially sequestered host monoterpenes that are re-released by the beetle. Not all of these cases are congruent with the phrase 'secreted by an animal', so whether a monoterpene emanating from a colonization site is a kairomone or an aggregation pheromone component is a matter of debate (Silverstein 1977; Browne et al. 1979; Borden 1985). The recent discovery of a monoterpene synthase enzyme activity in male *I. pini* (Martin et al. 2003) with the implication that bark beetles may indeed biosynthesize monoterpenes may ultimately resolve this nomenclatural dilemma in certain species. Whatever functional designator we assign to the attractive monoterpenes that are newly released during bark beetle colonization, in the forest airspace they join the background flux of monoterpenes that has originated from foliage and to a lesser extent from unwounded outer bark before and during colonization.

Monoterpenes as behavioral interruptants

Monoterpenes may also have negative consequences for the survival and reproduction of pine bark beetles. In some instances, and often at high

release rates (approx. 100–2,000 mg/day), monoterpenes act as repellents (interruptants) to reduce the flight responses to other behavioral chemicals (Miller and Borden 1990a, b, 2000, 2003; Hobson et al. 1993; Byers et al. 2000; El-Sayed and Byers 2000; Erbilgin and Raffa 2000; Erbilgin et al. 2003). Although the release rates were not explicitly stated, Hobson et al. (1993) demonstrated that the addition of 0.33 equivalent of (*S*)-(−)-α-pinene (an estimated 4,125 mg/day) to one equivalent of attractive (*R*)-(+)-α-pinene (an estimated 12,375 mg/day), significantly reduced the flight response of *D. valens*, providing an example of stereospecific interruption of one monoterpene by another (see below). In British Columbia, terpinolene (approx. 340–2,100 mg/day) and myrcene (approx. 60–1,300 mg/day) interrupted the flight responses of *I. latidens* and *I. pini* to their respective pheromones; terpinolene (approx. 2,100 mg/day) did the same for *D. ponderosae* (Miller and Borden 2000).

As is the case with the attractive effects of monoterpenes, little is known of the interruptive effects in the quantitative and qualitative context of the complete odor of wound oleoresin from an infested pine. It is first perhaps of interest to ask whether monoterpene release rates on the level of thousands of mg are biologically relevant for trees in pine ecosystems. Most attempts to quantify monoterpene release rates from woody branches or stems of pines have, for simplicity, involved small cut logs [e.g., Browne et al. 1979, 24.2 mg/day for myrcene from cut logs of *P. ponderosa* (75 cm × 25 cm); Byers et al. 1985, 30 mg/day for individual monoterpenes from cut logs of *P. sylvestris* (28 × 13 cm); Pureswaran et al. 2004b, 10–1,200 μg/g dry tissue for individual monoterpenes in *P. contorta latifolia*] or bark chips [e.g., Byers et al. 2000, 48μg/day to 3.84 mg/day from *P. sylvestris* or Fettig et al. 2006, 10 mg/day to 55 mg/day from whole chipped trees from *P. ponderosa* (in both cases the quantities eluted depended on the type of monoterpene)]. These lower end estimates and the likely higher release rates of monoterpenes from larger sections of fallen trees, large stump cross sections, and standing large trees characteristic of western North American forests suggest that monoterpenes are released from pine tissue in nature at rates that match or exceed those that have interrupted the flight of beetles experimentally. Indeed, in a study of volatiles released from three to five m of the main stem of *P. ponderosa* during colonization by several hundred *D. brevicomis* in the Sierra Nevada of California (Madera County), Browne et al. (1979) found that two trees released myrcene at 50.4–112.8 mg/day/m stem length, respectively.

It is also interesting to consider whether or not the attractive olfactory stimuli provided by monoterpenes that have functioned in behavioral trapping assays as important attractants or pheromone co-attractants (but are released as minor components of wound oleoresin) could be drowned out in the natural context by the cacophony of more abundant, interruptive monoterpenes. *Dendroctonus valens* was highly attracted in flight to a distillation fraction presumably containing most of the monoterpenes in the oleoresin of *P. ponderosa*, even though the relative abundance of an interruptant [(*S*)-(−)-α-pinene, 14.3%] exceeded that of one of the principal attractants [(*R*)-(+)-α-pinene, 0.9%] (Hobson et al. 1993). Apparently the presence of two other attractants [(*S*)-(−)-*β*-pinene, 35.8% and (*S*)-(+)-3-carene, 34.4%] overcomes the interruptive stimulus in the oleoresin. It is tempting to hypothesize that the high release rate interruptive effects of monoterpenes may simply reflect an experimental artifact, i.e., generic biological or behavioral saturation at artificially high levels (e.g., see parabolic response curve for *I. pini* to racemic α-pinene in Erbilgin et al. 2003). However, the interruptive effects depend on the type (species) of monoterpene, and Miller and Borden (2000) show that in *I. pini* and *D. ponderosae* some monoterpene co-attractants continue to elicit increasingly attractive responses, even at extremely high release rates.

The synchrony and relevance of interruption of flight behavior by higher release rates of certain monoterpenes with the various phases of host colonization (DL Wood 1982) is also poorly understood. If long-range interruption occurs soon after the bark is ruptured by invading beetles and early in the concentration phase of host colonization, when high density intraspecific competition is not a factor and mates are left unjoined, then the interruptive signals may have a

net negative impact on beetle survival and reproduction. If interruption occurs later during the establishment phase of colonization and dispersing beetles are re-directed to alternative hosts where the phloem is less fully occupied, then the opposite impact may pertain. Interruption of proximal host selection behavior of bark beetles during the selection and concentration phases may also be regulated by host monoterpenes. From a laboratory assay, Wallin and Raffa (2000) concluded that as concentrations of (−)-, (+)-, and (±)-α-pinene, (±)-β-pinene, and (±)-limonene increased in the assay medium, initial gallery entry of male *I. pini* decreased, the beetles were more likely to move from amended to non-amended portions of the medium, and gallery length decreased. The male responses of host entry and gallery length extension to α-pinene were heritable traits (Wallin et al. 2002).

Monoterpenes as behavioral chemicals for predators of pine bark beetles

Monoterpenes also influence the behavior of insects that prey on pine bark beetles, providing an indirect impact on the survival and reproduction of the scolytids. In this instance the pine bark beetle herbivores occur in the middle of a tritrophic ''sandwich'' between the plants and the carnivores, and the semiochemical signals move freely across the trophic levels. The documented effects on predators involve monoterpenes alone and as co-attractants with bark beetle pheromones (i.e., multicomponent kairomones with components derived from both of the lower trophic levels). In one of the first reported cases where monoterpenes alone elicited a flight response from the carnivores, Rice (1969) noted that two voracious predators of California pine bark beetles, *Temnochila chlorodia* (Mann.) (Coleoptera: Trogositidae) and *Enoclerus lecontei* (Wolc.) (Coleoptera: Cleridae), responded to α- or β-pinene in uncontrolled experiments in which the data were not analyzed statistically. These effects need to be re-examined using modern methodology. With the checkered beetle, *Thanasimus dubius* (F.) (Coleoptera: Cleridae), a key predator of *D. frontalis* in *P. taeda* in the southeastern US, Mizell et al. (1984) reported that the predator responded in a dose-dependent manner in a laboratory flight assay to α- and β-pinene, both of which occur in *P. taeda* turpentine. In a field assay, Billings (1985) found that *Temnochila virescens* (F.) responded significantly in flight to *P. taeda* turpentine. In several tests of a blend of monoterpenes representative of the Pinaceae occurring in eastern Canada, Chénier and Philogène (1989) found that the checkered beetles, *T. dubius*, *Enoclerus nigripes rufiventris* (Spinola), and *E. nigrifrons gerhardi* Wolcott responded significantly, although in low numbers, to the full blend of monoterpenes (with and without ethanol) and generally to treatments containing (±)-α-pinene. The Eurasian predator, *Thanasimus formicarius* (L.), responded at significantly higher levels in flight to (−)-α-pinene relative to an unbaited trap (Schroeder 1988; Schroeder and Lindelöw 1989), and at significantly higher levels to the combination of (−)-α-pinene and ethanol relative to both an unbaited trap and to the aggregation pheromone of *Ips typographus* (L.) (Schroeder 2003). Another group of predaceous beetles, the dead log beetles (Coleoptera: Rhizophagidae = Monotomidae), appear to be variously attracted to (−)-α-pinene [*Rhizophagus depressus* (F.)] or (−)-α-pinene and ethanol [*R. ferrugineus* (Payk.)] (Schroeder 1988; Schroeder and Lindelöw 1989).

In many instances, monoterpenes enhance the responses of pine bark beetle predators to the pheromones of the herbivores. Billings (1985) reported that *P. taeda* turpentine significantly increased both the flight response of *T. virescens* to a generic bait for *Ips* spp. (in two experiments) and of *Thanasimus dubius* to the *D. frontalis* attractant, frontalure. The flight responses of *T. dubius* to pheromone components of various pine-infesting *Ips* spp. were also increased significantly by (−)-α-pinene, (+)-α-pinene, and 3-carene in a series of studies in Wisconsin in the Great Lakes Region of the US (Erbilgin and Raffa 2001). The response of *T. dubius* to the *I. pini* aggregation pheromone (ipsdienol and lanierone) in Wisconsin was significantly and dose-dependently enhanced by the addition of racemic α-pinene (Erbilgin et al. 2003). In British Columbia, the responses of less aggressive predaceous beetles such as the wrinkled bark beetle,

Lasconotus complex LeConte (Coleoptera: Colydiidae), and darkling beetles, *Corticeus* sp. Piller and Mitterpacher (Coleoptera: Tenebrionidae), to the kairomone ipsdienol have also been enhanced significantly by the addition of 3-carene or β-phellandrene or γ-terpinene, and 3-carene or β-phellandrene or α- or β-pinene, respectively (Miller and Borden 1990a, 2000, 2003). Another checkered beetle, *T. undatulus* (Say), responded at an increased level to ipsdienol in these studies when 3-carene supplemented the bait (Miller and Borden 2003). In Wisconsin, (−)-α-, (+)-α-, and (−)-β-pinene increased responses of the predaceous hister beetle, *Platysoma cylindrica* (Paykull) (Coleoptera: Histeridae), to various pheromone components of *Ips* spp., whereas (−)- and (+)-α-pinene increased responses of *Corticeus parallelus* (Melsh) (Erbilgin and Raffa 2001). Interestingly, in several instances in these experiments the monoterpene myrcene interrupted the response of *T. dubius* to *Ips* spp. pheromone components (Erbilgin and Raffa 2001), potentially representing a net beneficial impact on the herbivore from the presence of this monoterpene in the semiochemical message.

Monoterpenes, pine defenses, and effects on bark beetle physiology

Monoterpenes are also detrimental physiologically to pine bark beetles as a consequence of their role in defense of pines. Defense of these long-lived trees consists of anatomical and chemical components that are both constitutive and inducible (Nebeker et al. 1993; Langenheim 2003; Franceschi et al. 2005). Pines have vertical and horizontal interconnected resin canal systems that span both the xylem and the phloem (Langenheim 2003). As a consequence, pines defend themselves against breaches in their outer bark by bark beetles and other invaders to a greater degree from their constitutive or preformed defenses than they do from their induced defenses (Nebeker et al. 1993). Further, sapling pines have a high level of monoterpene cyclase (monoterpene synthase) activity in the constitutive resin canal system that does not increase significantly upon wounding of the stem (Lewinsohn et al. 1991). Whether this biochemical effect holds for larger trees typically colonized by bark beetles remains to be established. Treatment of *P. contorta latifolia* and *P. taeda* with bark beetle-associated fungi results in hypersensitive response lesions whose oleoresin appears to contain quantitatively and qualitatively different monoterpene compositions than constitutive oleoresin (Shrimpton 1973; Raffa and Berryman 1982, 1983; Paine et al. 1987; reviewed in Nebeker et al. 1993).

When the outer bark is opened, the defense system of pines manifests itself in both physical and chemical terms through the release of oleoresin from severed resin canals. Stark (1965) defined oleoresin as "...the non-aqueous secretion of resin acids dissolved in a terpene hydrocarbon oil which is (a) produced in or exuded from the intercellular resin ducts of a living tree; ....." For example, when *D. ponderosae* colonizes *P. ponderosa*, *P. contorta latifolia*, or other species, the first few pioneers are often killed or driven out by the mass flow of oleoresin that emanates from the resin canal system and pours out the nascent entrance tunnel (Beal 1939). This is especially evident if the host tree has adequate moisture and oleoresin exudation pressure (Stark 1965). Blackman (1931) described the elaborate behavior of female *D. ponderosae* during the early stages of colonization. The female alternatively bites tree tissue from the phloem–xylem interface and retreats frequently to the outside surface of the bark where she spreads and disposes of masses of oleoresin adhering to her body. This historical description underscores the lengthy contact period during which female *Dendroctonus* spp. are exposed to the physical obstacle presented by oleoresin as well as its potentially toxic hydrocarbons (Nebeker et al. 1993). Since insects take oxygen into their bodies through pleural spiracles (lateral aperatures) along the thorax and abdomen, immersion of bark beetles in oleoresin may have a suffocating as well as a toxic effect. Hodges et al. (1977, 1979) reported that the resistance of four native pine species in the southeastern US to colonization by *D. frontalis* was strongly related through a discriminant analysis to physical properties of the oleoresin such as total flow, flow rate, viscosity, and time to crystallization.

The role of monoterpenes in the chemical defense of pines rests on the experimental

evidence that upon prolonged exposure at close range, monoterpenes can be insecticidal to pine bark beetles (Smith 1961, 1965a, b; Cook and Hain 1988, Table 1). Specifically, at high doses in closed containers, they exhibit a fumigant toxicity effect (Smith 1961, 1965a, b; Byers et al. 1979; Byers 1981; Cook and Hain 1988). Byers et al. (1979) reported that after an 18 h exposure, the percentage of ''comatose'' male California fivespined ips, *Ips paraconfusus* Lanier, increased sharply when the headspace concentration of myrcene in a sealed glass bottle reached approx. 4 μg/ml. Similar studies with α-pinene resulted in mortality in the 40–50% range when the headspace concentration reached approx. 18 μg/ml (Byers 1981). Byers and Birgersson (1990) estimated that the vapor concentration of myrcene in an *I. paraconfusus* nuptial chamber in *P. ponderosa* was 0.028 μg/ml. Thus, whether the volatile insecticidal effects measured in closed containers in the laboratory pertain in the more open system of a gallery whose volatiles are exhausted by ventilation through an entrance hole, or perhaps through the somewhat porous bark surface, has yet to be examined experimentally. However, given the descriptions of Blackman (1931) and Beal (1939) noted above, *Dendroctonus* spp. adults may come in prolonged contact with high concentrations of monoterpenes dissolved in liquid oleoresin during attempts at host colonization. Smith (1966) reported that even brief immersion of *D. brevicomis* adults in fresh resin had a deleterious effect on ability to feed subsequently in pine phloem, and resins of nonhost pines increased the rate of mortality of the adults relative to resin of *P. ponderosa*.

The influence of oxygenated monoterpenes on pine bark beetles

In addition to large-scale emissions of monoterpenes *sensu stricto* from vegetation in pine forests, there is a growing realization that most monoterpenes emitted in these forests may undergo rapid oxidation through exposure to frequently encountered atmospheric oxidants such as hydroxyl (OH^-) and nitrate (NO_3) radicals, and ozone (O_3) (Atkinson and Arey 2003; Holzinger et al. 2005b; Lee et al. 2006). These landscape-level oxidation products may influence the host colonization behavior of pine bark beetles, but appear upon first analysis (AH Goldstein, personal communication) to be of much smaller molecular weight than most oxygenated monoterpenes that elicit behavioral responses from bark beetles (Seybold et al. 2000).

Oxygenated monoterpenes and pheromone biosynthesis

Monoterpene oxidation also occurs on a more localized scale, driven by biological rather than physical chemical processes. Perhaps the most intimate relationship between pines, their monoterpenes, and pine bark beetles is the involvement of the isoprenoids in pheromone biosynthesis by the beetles. In male *I. paraconfusus*, the (−)-enantiomer of α-pinene is converted to *cis*-verbenol (Fig. 2) (Renwick et al. 1976a), a key component of the three-part aggregation pheromone (Silverstein et al. 1966). Another monoterpene, myrcene, is converted into the other two pheromone components, ipsdienol (2-methyl-6-methylene-2,7-octadien-4-ol) and ipsenol (2-methyl-6-methylene-7-octen-4-ol), by this species (Byers et al. 1979; Hendry et al. 1980). Similar conversions of monoterpenes to behaviorally active oxygenated compounds also occur in other coniferophagous species (Hughes 1973a, b, 1974, 1975; Renwick et al. 1973, 1976b; Klimetzek and Francke 1980; Byers 1982, 1983a, b; Hunt et al. 1986; Pierce et al. 1987; Hunt and Smirle 1988; Lindström et al. 1989; Gries et al. 1990; Vanderwel 1991; Seybold et al. 1992; Barkawi 2002). In addition to enzymatic transformations endogenous to bark beetles, other potential sources of behaviorally active oxygenated monoterpenes include autoxidation (Hunt et al. 1989; Grosman 1996) and conversions that are mediated by bacteria or fungi that are symbiotic with the beetles (Brand et al. 1975, 1976; Byers and Wood 1981; Conn et al. 1984; Hunt and Borden 1989a, b). In all of these cases, the origin of these monoterpenes has been thought to be the oleoresin associated with the phloem or outer xylem in pines or other conifers.

Over the last 10 years the research on biosynthesis of pine bark beetle aggregation pheromones has shifted the focus to *de novo* pathways present endogenously in the beetles (Seybold et al. 1995; Ivarsson et al. 1997; Tillman et al. 1998, 2004; Barkawi et al. 2003). It has become clear that evolution has provided bark beetles with an elaborate mechanism for self-contained synthesis of these critically important colonization and reproductive signals to guide their assemblages (Seybold and Tittiger 2003). For example, male *I. pini* synthesize ipsdienol *de novo* through the regulatory control of juvenile hormone (JH III), which appears to act primarily on HMG-CoA reductase in the mevalonate (MVA) pathway (Tillman et al. 2004). Multiple enzymes in this pathway are upregulated during pheromone biosynthesis in several bark beetle species (Tillman et al. 1998; Tittiger et al. 2000; Martin et al. 2003; Gilg et al. 2005), and gene expression for these enzymes is coordinated (Keeling et al. 2004). With *I. pini*, cell-free extracts of male tissue will also convert geranyl diphosphate (GDP) to the monoterpene myrcene in a regulated fashion, providing the first biochemical evidence for a monoterpene synthase in the Metazoa (Martin et al. 2003), and explaining successful pheromone biosynthesis in *Pinus* spp. that appear to contain insufficient quantities of available host myrcene (Byers and Birgersson 1990). Tissue from female *I. pini* does not carry out this conversion. The synthesis of myrcene is stimulated by both feeding on host pine phloem and treatment with JH III, which are both correlates of pheromone biosynthesis.

In biochemical terms, there is a rather remarkable nexus of the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in pines with the MVA pathway in pine bark beetles (Fig. 3). The pathways overlap when the pines and beetles convert isomeric dimethylallyl diphosphate and isopentyl diphosphate to GDP; they are joined when the beetles utilize myrcene from the host and/or *de novo* synthesized myrcene to form the pheromone alcohol endproducts. Thus, pheromone synthesized from pine-based myrcene originates from the MEP pathway, whereas pheromone synthesized from beetle-based myrcene originates from the MVA pathway.

Oxygenated monoterpenes and cytochrome P450s

In the last stages of pheromone biosynthesis, the monoterpene alcohol and ketone pheromone end products in pine bark beetles are likely formed through the catalytic activity of cytochrome P450 enzymes (P450s) (White et al. 1979, 1980; Hunt and Smirle 1988). These enzymes may form enantiospecific oxygenated products from prochiral monoterpenes (e.g., myrcene) or from chiral monoterpenes (e.g., α- or β-pinene). P450s occur ubiquitously in organisms ranging from bacteria to fungi to plants to animals (Omura 1999). In eukaryotes, they catalyze NADPH-dependant oxidations on an extremely diverse array of substrates. In animals, they are involved in detoxification of plant secondary metabolites, hormone biosynthesis and degradation, pheromone biosynthesis and degradation, and metabolism of fatty acids (Feyereisen 1999; Omura 1999).

There have been only a few studies directly targeting P450-related physiology or biochemistry of pine bark beetles. White et al. (1979) found that microsomes isolated from larval and adult black turpentine beetles, *Dendroctonus terebrans* (Olivier), converted α-pinene to α-pinene oxide and other oxidation products. Further, they reported that although α-pinene induced cytochrome P450 activity in rat liver microsomes, it did not do so in *D. terebrans* microsomes. In experiments with *D. ponderosae*, females and males treated with the P450 inhibitor, piperonyl butoxide, yielded abdominal extracts that displayed a reduced conversion of α-pinene and myrcene to *trans*-verbenol and ipsdienol, respectively, as well as an accumulation of the monoterpene precursors (Hunt and Smirle 1988). The biosynthesis of *exo*-brevicomin by male *D. ponderosae* involves the incorporation of molecular oxygen during the epoxidation of (*Z*)-6-nonen-2-one (Vanderwel and Oehlschlager 1992), and this reaction is likely catalyzed by a P450. Also using *D. ponderosae* as a model, Pierce et al. (1987) outlined the pathways for *P. ponderosa* and *P. contorta latifolia* monoterpene metabolism through oxygenation (Fig. 4). The conversions involve mainly allylic hydroxylation and hydration reactions focused on double bonds

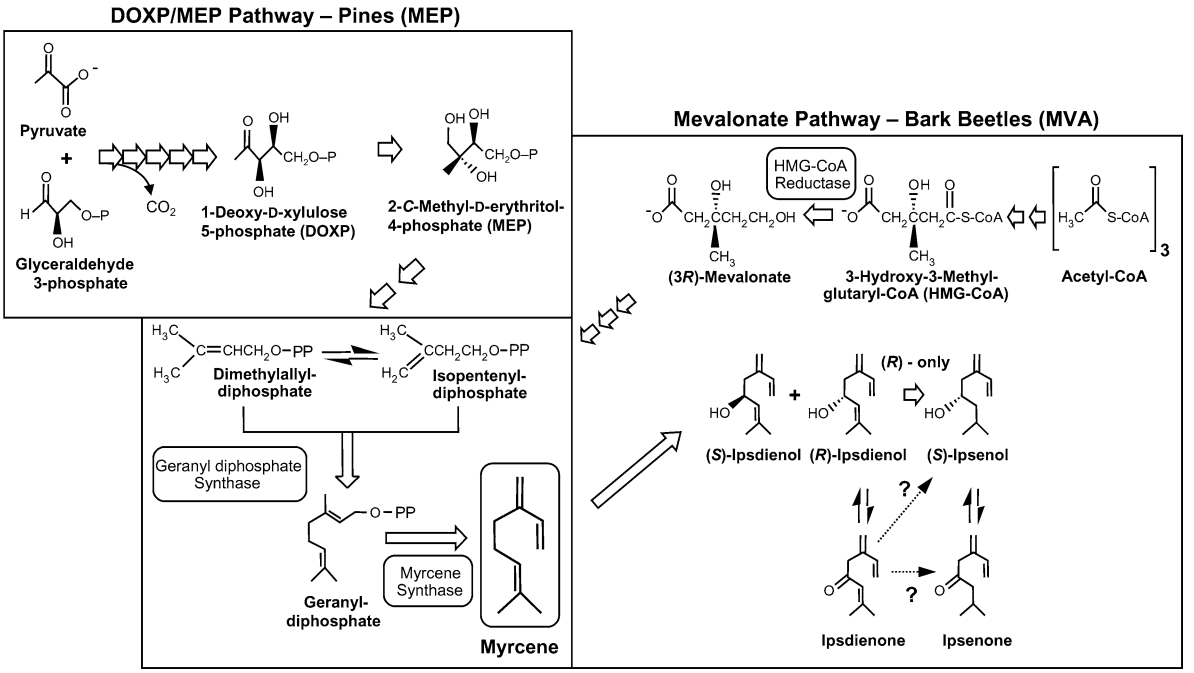
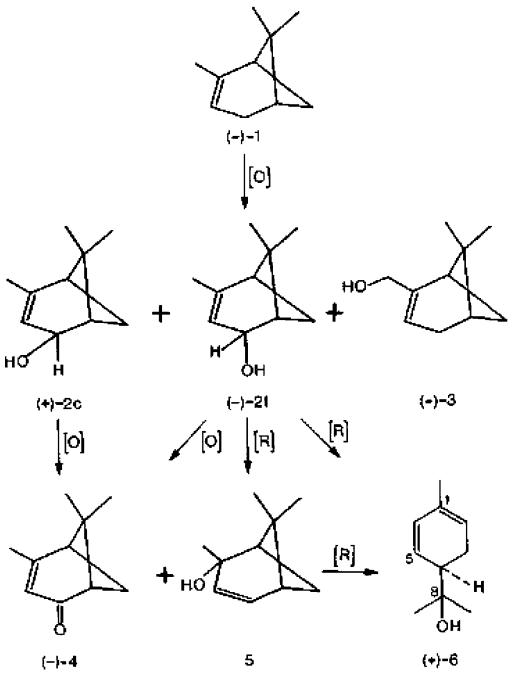


Fig. 3 Proposed interaction of monoterpenoid biosynthetic pathways in pines and pine bark beetles showing different origins of C5 units from the 2-C-methyl-D-erythritol-4- phosphate (MEP) and mevalonate (MVA) pathways for the convergent synthesis of myrcene. Myrcene is oxidized to ipsdienol (likely by P450s) in pine bark beetles. Figure reproduced in modified form from Fig. 1 on page 174 in Martin et al. (2003) with kind permission of Springer Science and Business Media

in the carbon skeleton; epoxidation reactions may also occur. These allylic hydroxylations and epoxidations, which likely involve molecular oxygen, serve as a prelude to the isolation and characterization of P450s from pine bark beetles by illustrating the scope of functionalities necessary for beetles during host colonization. For example, as noted above, in *I. paraconfusus* and other *Ips* spp. it is likely that the final or penultimate biosynthetic reaction in pheromone production, the conversion of myrcene to ipsdienol, is catalyzed by a P450 (Fig. 4C). Because in many cases the final pheromone product consists of blends of both (+)- and (−)-ipsdienol, it is possible that two separate P450s catalyze the enantiospecific reactions.

In an attempt to find the P450s potentially involved in pheromone biosynthesis in *I. paraconfusus*, and to set a foundation for a deeper understanding of the plethora of events during bark beetle colonization of host tissue, we have used degenerate PCR techniques to identify and clone 14 P450s from cDNA derived from RNA isolated from male and female *I. paraconfusus* fed in *P. ponderosa* phloem for 20 h. Further rapid amplification of cDNA ends has allowed the isolation of full length cDNAs of eight of the 14 P450s. We are continuing work on obtaining full length cDNAs for the other six P450s for use in functional characterization of the enzymatic activity of their protein products. Other insects whose genomes have been more extensively characterized (e.g., the fruit fly, *Drosophila melanogaster* Meigen and the mosquito, *Anopheles gambiae* Giles) have approx. 100 P450 genes (Adams et al. 2000; Gomez et al. 2005). Since endophytic pine bark beetles have an intimate interaction with a plant host defense system, we might anticipate that they have at least as many, if not more, P450 genes than these Diptera. Thus, we have isolated perhaps 10 to 15% of the ensemble of P450s present in *I. paraconfusus*.

All but two of the 14 P450s seem to belong to the Cyp4 family of P450s, the most common subfamily of insect P450s. One is a member of the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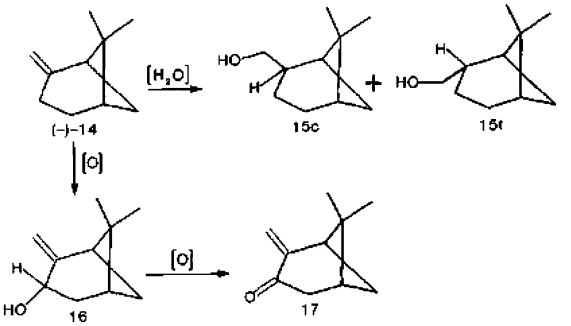
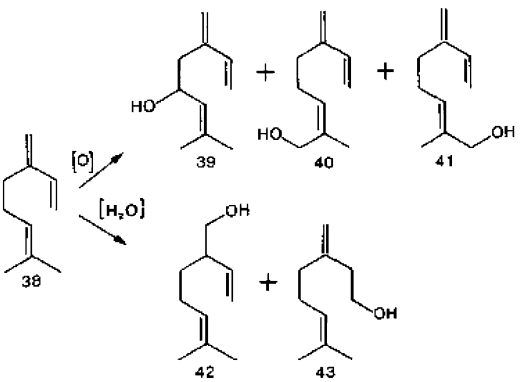
(+)-2c
(-)-2t
(-)-3
[O]
[O]
[R]
[R]
(-)-4
5
(+)-6

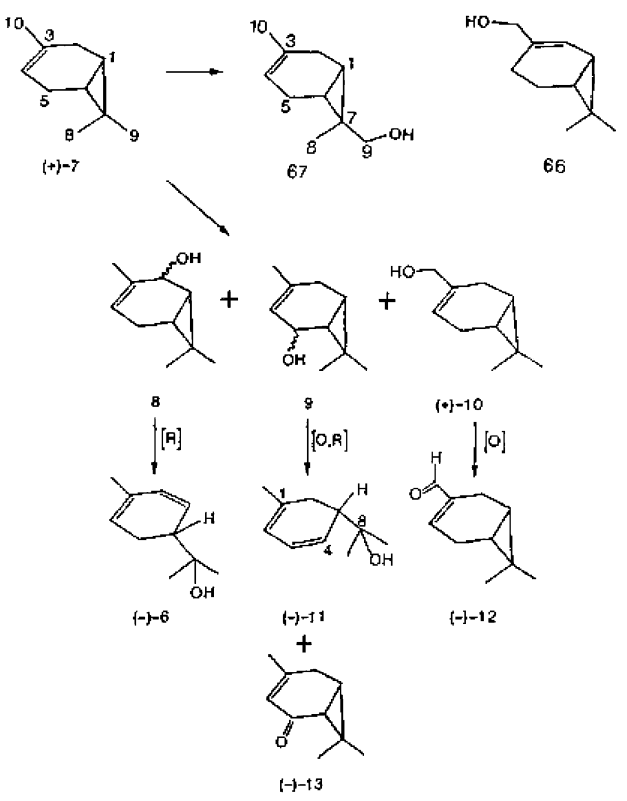
A


(-)-14
[H2O]
15c
15t
[O]
16
[O]
17

B


[O]
[H2O]
38
39
40
41
42
43

C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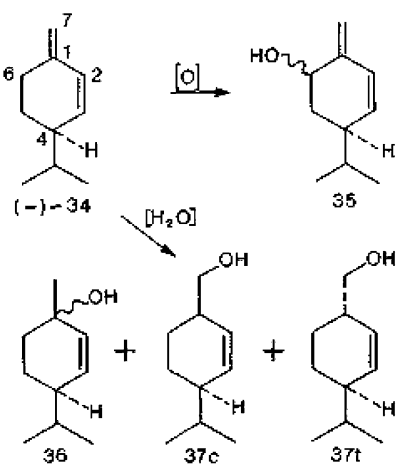
67
66
8
9
(+)-10
[R]
[O,R]
[O]
(-)-6
(-)-11
(-)-12
(-)-13

D


(-)-34
[O]
35
[H2O]
36
37c
37t

E

◀**Fig. 4** Theoretical oxidative transformations of monoterpenes from ponderosa pine, *Pinus ponderosa*, and Rocky Mountain lodgepole pine, *Pinus contorta latifolia* (Engelmann) Critchfield by the mountain pine beetle, *Dendroctonus ponderosae* Hopkins (modified from Pierce et al. 1987). (**A**) Allylic oxidation and rearrangement products of (−)-α-pinene; (**B**) allylic oxidation and hydration products of (−)-β-pinene; (**C**) allylic oxidation and hydration products of myrcene; (**D**) allylic oxidation and rearrangement products of (+)-3-carene; and (**E**) allylic oxidation and hydration products of (−)-β-phellandrene. Numerical identification of structures as in Pierce et al. (1987)

Cyp9 family; another is a member of the Cyp31 family and is likely not of insect origin (i.e., contamination from nematodes or mites, which are internally and externally phoretic, respectively, with pine bark beetles, Kinn 1971; Massey 1974; Stephen et al. 1993). All 14 P450s were subjected to quantitative, real-time PCR-based expression analyses. Individual male and female *I. paraconfusus* were fed for 0, 8, or 24 h in fresh *P. ponderosa* phloem. Each sex and time point was represented by 12 individual insects (i.e., 12 replicates, each consisting of one insect). Following feeding, HMG-CoA reductase transcript levels [used as a control because the expression pattern of this gene in *I. paraconfusus* is well characterized (Tillman et al. 2004)] increased dramatically in males fed for 8 and 24 h, but as expected did not change in females that do not produce pheromone, providing evidence that the insects had responded appropriately to their exposure to host tissue. In addition, 10 of the 14 P450s showed statistically significant differential transcript accumulation in males and/or in females, usually, but not always, within 8 h of initial contact with host phloem. The Cyp31 gene was among the four that did not show differential transcript accumulation in either sex following feeding. The differential expression responses that we have observed may be classified into six groups.

First, we observed three genes that were upregulated in males, but whose expression levels did not change in females. We hypothesize that these may be involved in pheromone biosynthesis (a potential substrate is myrcene), male-specific juvenile hormone (JH) biosynthesis (Feyereisen et al. 1981) (potential substrates are methyl farnesoate or farnesoic acid), or detoxification of constitutive defenses encountered by pioneering males making the first encounter with a host tree (potential substrates include various terpenoids or plant ecdysteroids).

Second, we observed some genes that were downregulated in males, but did not change in females. Genes of this class would include genes that were no longer required, or whose expression would be detrimental for males that had successfully located and colonized a pine host. We hypothesize that these genes may be involved in degradation of pheromones (Wojtasek and Leal 1999; Maïbèche-Coisne et al. 2004) or host kairomones that directed the insects to the tree in the first place (potential substrates would include pheromone components or host compounds that are behaviorally-active to foraging bark beetles). Upon arriving and colonizing a suitable pine host, male behavior may be altered if persistent foraging-related signals were received and processed. In addition, after males arrive at a host and begin to feed, their juvenile hormone titers should increase (Tillman et al. 1998). Thus genes that degrade JH may also be downregulated at this point. Such enzymes would likely have JH, methyl farnesoate, farnesoic acid, farnesol, or farnesal as substrates (Sutherland et al. 1998).

Third, we observed P450s that were upregulated in both sexes after feeding. These could be involved in *cis*-verbenol biosynthesis, as it is produced by both sexes (Byers 1981, 1983b). The most likely substrate in this case would be α-pinene. The generality of this transformation of α-pinene is illustrated by its widespread occurrence in nature, ranging from bacteria and fungi (Brand et al. 1975; Prema and Bhattacharyya 1962) to human tissues (Eriksson and Levin 1990); the latter followed by the excretion of the conjugated alcohol in the urine. Because both sexes of *I. paraconfusus* are confronted with toxins from pines, and because both sexes require high titers of JH during host colonization, this class of P450s might be involved in xenobiotic detoxification or JH biosynthesis. Flight muscle degradation, which begins in both sexes of *I. paraconfusus* immediately after host colonization (Borden and Slater 1969; Bhakthan et al. 1970), and is stimulated by JH (Borden and Slater 1968; Unnithan and Nair 1977), is another process

that might involve P450s upregulated in both sexes following feeding. In addition, reproductive activity likely increases metabolic requirements dramatically, and thus fatty acids may be a substrate for these enzymes (Aoyama et al. 1990; Feyereisen 1999; Omura 1999). One P450 in our study, the Cyp9 family gene, showed upregulation after feeding on the order of almost $10^5\times$ in males but only $10^2\times$ in females, both compared to non-fed insects. A P450 involved in myrcene detoxification and in conversion of myrcene to ipsdienol might show such a pattern in that males would need to rapidly clear myrcene that had been synthesized in the midgut, as ipsdienol, both to allow survival (clearance of a toxin) and to attract mates and conspecific males, whereas phloem-mining females would have to detoxify the tree-produced myrcene. Thus, in such a situation, both males and females might produce transcripts of the same gene, but at different levels reflecting the different roles played by the protein product of the gene in host colonization and reproductive-related activity.

Fourth, we observed P450s that were downregulated in both sexes after feeding. As with the P450s that were downregulated in males only after feeding, these may be involved in degradation of behaviorally active chemicals in the antennae or in degradation of JH.

Fifth, we observed a gene that was downregulated in males but upregulated in females. This gene may be involved in female-specific JH or ecdysone production (Tillman-Wall et al. 1992; Blomquist et al. 1994) in preparation for reproductive activity, with possible substrates including methyl farnesoate, farnesoic acid, or a number of candidates from the ecdysone biosynthesis pathway (Warren et al. 2002). Alternately, this gene may be involved in ''heavy duty'' detoxification of host secondary metabolites. Because the female is the later-arriving sex, and she carries out more extensive boring activity in the phloem than the male, she may be confronted with constitutive or fungally stimulated induced defense responses that differ in quantity and quality from those presented to the male. Thus, females are likely assaulted at the site of infestation with particularly toxic secondary metabolites in large quantities, and they may express a special ensemble of P450s that is able to deal with such major threats to their reproductive success.

Finally, we observed some genes that were constitutively expressed in both sexes at what seem to be high levels at all time points before and after feeding. For example, because we detected a consistent signal for one of the P450 genes in over 98.6% of all samples regardless of sex or feeding status, it was chosen as the housekeeping gene for the quantitative analysis of expression. This and similar genes could be involved in constitutive detoxification of host secondary metabolites or basic and relatively continuous metabolic processes, e.g., fatty acid metabolism. The functions of such constitutively expressed genes could be highly varied and will possibly be quite difficult to predict.

The primary amino acid sequences of P450s do not provide information that allows precise prediction of their function. Thus, while our work to date has set a firm foundation for the study of P450s in bark beetles, further research will require functional characterization of each of the P450s that we have thus far cloned from *I. paraconfusus*. Functional characterization, combined with further expression analyses of these and other P450s following treatment of the insects with hormones, plant secondary metabolites, or at different insect life stages, will provide a much better understanding of the important events just prior to and following host colonization by these ecologically- and economically important insects.

Oxygenated monoterpenes and stereospecific responses by pine bark beetles

The enantiomeric composition of kairomone and pheromone components of pine bark beetles is a critical determinant of behavioral activity (Wood et al. 1976; Birch et al. 1980; Francke and Vité 1983; Francke et al. 1986; Seybold 1993). With *D. valens*, Hobson et al. (1993) clearly showed a strong preference in flight response to the kairomone (*R*)-(+)-α-pinene; the antipode interrupted the response to the (+)-enantiomer. Strangely, the enantiomeric composition of α-pinene in the oleoresin of *P. ponderosa*, one of the primary hosts in this region, was 95%-(−). With oxygenated monoterpene pheromones, perhaps the best

example involves western populations of *I. pini*. Birch et al. (1980) found that ethanol solutions of (*R*)-(−)-ipsdienol were attractive in the lab and field, whereas solutions of (*S*)-(+)-ipsdienol were interruptive. As little as 5–10% of the (+)-enantiomer caused a significant reduction in trap catch in response to the (−)-enantiomer. Below we discuss two current projects in our laboratory in which the flight responses of *I. paraconfusus* and the Mediterranean pine engraver, *Orthotomicus* (*Ips*) *erosus* (Wollaston), are governed by the enantiomeric composition of the oxygenated monoterpene pheromone components.

Ips paraconfusus

The California fivespined ips is an important and polyphagous pest of pines in Oregon and California (Struble and Hall 1955; Schultz and Bedard 1987). Its broad host range and capacity to thrive in coastal as well as montane climates make it a potential for concern as an invasive species in other parts of North America and other continents. It is very abundant on adventive plantings of Monterey pine, *Pinus radiata* D. Don, in urban landscapes in coastal California, and *P. radiata* is the most widely planted pine in the world with plantations covering nearly 4 million ha in southern hemisphere locations such as Australia, Chile, New Zealand, and South Africa (Lavery and Mead 1998). Thus, an efficacious aggregation pheromone bait for *I. paraconfusus* would be an important detection tool for international pest management programs.

As noted above, the male-produced pheromone of *I. paraconfusus* is a synergistic blend of three monoterpene alcohols, ipsenol, ipsdienol, and *cis*-verbenol (Silverstein et al. 1966; Wood et al. 1967, 1968). The predominant naturally occurring enantiomers isolated from males were (4*S*)-(−)-ipsenol, (4*S*)-(+)-ipsdienol, and (1*S*,2*S*)-(+)-*cis*-verbenol, which occurred in a ratio of 100:10:2 (Wood et al. 1967). The optical rotation of *cis*-verbenol varies depending on the solvent in which it is measured [acetone or methanol, (1*S*,2*S*)-(+); chloroform, (1*S*,2*S*)-(−)]. Although Silverstein et al. (1966) reported the original natural product as $[\alpha]_D^{21} = +4°$, measured in acetone, most literature subsequent to Mori et al. (1976) and commercial vendors refer to (1*S*,2*S*)-*cis*-verbenol as the (−)-enantiomer, i.e., as measured in chloroform. The commercially available pheromone for *I. paraconfusus* is an equal (racemic) mixture of the optical isomers of ipsenol (220 μg/day), a highly-enriched blend (approx. 97%) of (+)-ipsdienol (110 μg/day), and 83%-(1*S*,2*S*)-(−)-*cis*-verbenol (300–600 μg/day) (Phero Tech Inc., Delta, British Columbia, Canada, all release rates measured at 25°C) (Fig. 2). Thus, the stereochemistry of the components of the commercially available pheromone matches, in part, the naturally occurring compounds; the relative release rates do not match the naturally occurring component ratios.

In 2004 and 2005 we used multiple funnel traps and pheromone components from Phero Tech Inc. and ChemTica Internacionale S.A. (Heredia, Costa Rica) in modern release devices to test the preference of *I. paraconfusus* for the various enantiomers in three sequential experiments at the University of California, Blodgett Research Forest in El Dorado Co., California (Table 2). This was the site of the historic first field study of this pheromone system in June of 1966 (Wood et al. 1967). Treatments were organized in a randomized complete block design of four blocks, and checked and re-randomized every few days (nine, seven, and thirteen times in experiments 1–3, respectively). In experiment 1, the enantiomers of ipsdienol [97%-(+) and 97%-(−)] were tested in combination with racemic ipsenol and 83%-(−)-*cis*-verbenol. The experiment also included conophthorin, a spiroacetal that is known to interrupt the flight response of other species of *Ips* (Huber et al. 2001; Zhang 2003). Conophthorin has been isolated from a wide range of natural sources, including cone beetles, twig beetles, wasps, and angiosperm tree bark (Huber et al. 1999, 2000; Francke and Kitching 2001; Zhang and Schlyter, 2004).

We found that *I. paraconfusus* had a strong preference for the bait containing (+)-ipsdienol (Table 2). A 2× release rate of racemic ipsdienol attracted fewer *I. paraconfusus* than the 1× release rate of (+)-ipsdienol; this indicates that the (−)-enantiomer of ipsdienol interrupts the attractive response, confirming previous California

Table 2 Progression of experiments to demonstrate the enantiospecific response of the California fivespined ips, *Ips paraconfusus*, to pheromone components, Blodgett Forest Research Station, El Dorado Co., California, 2004–2005 (Seybold et al. unpublished data)

Experiment	Dates	Goals	Treatments[a]	Outcomes
1	27 August–22 September, 2004	Optimize the enantiomeric composition of ipsdienol[b, c, d] Test interruption by conophthorin	Ipsdienol in various blends [racemic 1× and 2×, (+)-1×, (−)-1×] while keeping 83%-(−)-*cis*-verbenol and racemic ipsenol constant in each treatment, conophthorin alone, conophthorin added to (+)-ipsdienol & *cis*-verbenol & ipsenol, unbaited trap (7 treatments)	Treatment with (+)-ipsdienol attractive; with (−)-ipsdienol not attractive; racemic 1× and 2× partially attractive due to interruption by (−)-ipsdienol; conophthorin interruptive
2	28 July–12 August, 2005	Optimize the enantiomeric composition of *cis*-verbenol	*cis*-Verbenol in blends [(+), (−), and 83%-(−)] or absent while keeping (+)-ipsdienol and racemic ipsenol constant in each treatment, unbaited trap (5)	Treatments with (−)-*cis*-verbenol attractive; with (+)-*cis*-verbenol weakly attractive; higher response to 83%-(−)-*cis*-verbenol vs. (−)-*cis*-verbenol due to a higher release rate
3	12 August–19 September, 2005	Optimize the enantiomeric composition of ipsenol[b, d]	Ipsenol in blends [(+)-1×, (−)-1×, racemic 1× and 2×] or absent while keeping (+)-ipsdienol and 83%-(−)-*cis*-verbenol constant in each treatment, unbaited trap (6)	Treatments with (−)-ipsenol attractive; with (+)-ipsenol not attractive, racemic 2× is most economical and effective form of ipsenol in the experiment
Future directions	2006	Test varying ratios of ipsdienol, *cis*-verbenol and ipsenol Test the effect of α-pinene	Ipsdienol, *cis*-verbenol, ipsenol in 10:2:100 ratio to mimic natural pheromone, and in varying component ratios, unbaited trap Three-part blend alone and combined with commercially available α-pinene, and racemic 2×, (+)-1×, (−)-1× blends, unbaited trap (6)	

[a] All materials from Phero Tech, Inc. unless otherwise indicated. For all enantiomeric mixtures of ipsdienol, 1× release rates are 0.11 mg/day. 83%-(−)-*cis*-Verbenol release rate is 0.3–0.6 mg/day at 25°C, and (+)- and (−)-*cis*-verbenol rates are 0.08 mg/day at 20°C (ChemTica, Internacionale S.A.). For all enantiomeric mixtures of ipsenol, 1× release rates are 0.22–0.24 mg/day. Racemic conophthorin release rate is 3.0 mg/day.

[b] Light and Birch (1979).

[c] Paine and Hanlon (1991).

[d] Kohnle et al. (1994).

studies from Siskyou Co. (Light and Birch 1979), San Diego Co. (Paine and Hanlon 1991), and Nevada Co. (Kohnle et al. 1994). Conophthorin also interrupted the response of *I. paraconfusus* to the attractant blend containing (+)-ipsdienol, suggesting that in addition to aiding in avoiding non-host angiosperms (Huber et al. 1999, 2000; Zhang and Schlyter, 2004), it may also aid in maintaining species specificity in pheromone communication with pine-infesting cone and twig beetles that use it as a pheromone component (Birgersson et al. 1995; Pierce et al. 1995; Dallara et al. 2000).

In experiment 2, various enantiomeric blends of *cis*-verbenol were tested in combination with (+)-ipsdienol and racemic ipsenol. The blend with (−)-*cis*-verbenol was highly attractive to *I. paraconfusus*, whereas the blend with (+)-*cis*-verbenol was only weakly attractive and not different from the two-component blend without any *cis*-verbenol (Table 2). We observed differences in flight responses to (−)-*cis*-verbenol from the two commercial vendors that are likely due to the substantial differences in the release rates of the formulations (Phero Tech: 300–600 μg/day at 25°C versus ChemTica: 80 μg/day at 20°C). Since an enantiomeric blend of *cis*-verbenol that contained 17% of the (+)-enantiomer was quite attractive, (+)-*cis*-verbenol is likely not interruptive. However, this needs to be confirmed with a trial comparing responses to racemic *cis*-verbenol released at 1× and 2× with responses to (+)- and (−)-*cis*-verbenol released at 1×. A review of the literature reveals that no previous studies have attempted to determine the impact of the enantiomeric composition of *cis*-verbenol on the flight response of *I. paraconfusus*.

In experiment 3, the enantiomers of ipsenol [97%-(+) and 97%-(−)] were tested in combination with (+)-ipsdienol and 83%-(−)-*cis*-verbenol. The blend with (−)-ipsenol attracted *I. paraconfusus*, whereas the blend with (+)-ipsenol did not (Table 2). Since the response to the 2× release rate of racemic ipsenol was similar to the response to the 1× release rate of (−)-ipsenol, (+)-ipsenol is likely not interruptive. Our results suggest that a higher release rate of racemic ipsenol relative to (+)-ipsdienol would be a more efficacious attractant for *I. paraconfusus*. Ten release devices of the currently available formulation of racemic ipsenol to one of (+)-ipsdienol [1100:110 μg/day, (−)-ipsenol:(+)-ipsdienol] would most accurately align the synthetic bait with the naturally occurring component ratios. A review of the literature revealed only one study that investigated the impact of the enantiomeric composition of ipsenol on the flight response of *I. paraconfusus*. Light and Birch (1979) reported that in Siskiyou Co. (+)-ipsenol did not reduce the flight response to a *P. ponderosa* log infested with male *I. paraconfusus* (i.e., the naturally produced aggregation pheromone), and this is consistent with the results of our experiment 3. The sexes responded in the same patterns for all treatments and experiments outlined above.

Future research on the enantiospecific response of *I. paraconfusus* to its three-component pheromone blend will involve a more controlled study of the impact of the enantiomeric composition of *cis*-verbenol (see above); a study that varies the individual components in tandem and separately, and a study that investigates the role of the enantiomers of α-pinene and perhaps other monoterpenes as co-attractants.

Orthotomicus erosus

The invasion of exotic species of plants and animals has led to major ecological and economic problems (Pimentel et al. 2000). From an insect pest management perspective, worldwide commerce and transport of wood packing and plant materials are resulting in the homogenization of the bark beetle fauna across international borders (Wood and Bright 1992). In a 15-year survey, many scolytids have been intercepted in barked rough wood associated with packing materials that carry tiles, marble, machinery and other construction goods to US ports (Haack 2001, 2006). This growing problem is especially notable in California where the number of established exotic bark beetle species has doubled to nearly 20 species in the last few years (Penrose et al. in preparation).

The discovery of the Mediterranean pine engraver, *Orthotomicus erosus*, in California in May of 2004 (JC Lee et al. 2005) is an example of one new invasive species that raises serious

concerns. It is a generalist pest of pines in its native range in the Mediterranean, Middle East, and Central Asia, and in introduced areas of Chile, Fiji, and South Africa (Eglitis 2000). Generally, *O. erosus* infests standing pine trees under stress, recently fallen trees, broken branches or logging debris. Besides causing feeding damage, *O. erosus* has vectored some ophiostomoid fungi that are pathogenic to pines (Wingfield and Marasas 1980). In the southern Central Valley of California, this beetle has been found infesting pine trees and cut logs in parks, golf courses, and other urban landscapes.

The chemical ecology of *O. erosus* has been studied in Europe, Israel, and South Africa. Giesen et al. (1984) used combined gas chromatography–mass spectrometry to analyze the headspace gas from hindguts dissected from male *O. erosus* that had infested logs of maritime pine, *Pinus maritima* Lamarck (=*P. pinaster* Ait.). The chemical analysis and subsequent field test in South Africa confirmed that ipsdienol and 2-methyl-3-buten-2-ol (MB) (Fig. 2) were major components of the aggregation pheromone. The combination of ipsdienol and MB was necessary to attract *O. erosus*; traps baited with ipsdienol alone (Giesen et al. 1984; Serez 1987) or MB alone (Klimetzek and Vité 1986; Mendel 1988) attracted few beetles. These authors suggested that ipsdienol was a long-distance signal, whereas MB influenced landing behavior of *O. erosus*. Other common bark beetle pheromone components, such as ipsenol, *cis*-verbenol, *trans*-verbenol, and frontalin did not appear to influence the flight behavior of *O. erosus* near Bordeaux, France (Klimetzek and Vité 1986). At a field site near Lisbon, Portugal, verbenone and possibly *cis*-verbenol inhibited *O. erosus* attraction to ipsdienol and MB (Paiva et al. 1988). The release rates (Klimetzek and Vité 1986) and enantiomeric composition (Kohnle 1991) of ipsdienol were studied for their effect on *O. erosus* flight response, but the results were inconclusive. The impact of the release rate of MB on the response of *O. erosus* has not been evaluated previously. Therefore, release rates, enantiomeric composition, and the effect of host monoterpene co-attractants (e.g., α-pinene) are all research questions that need to be addressed to optimize the attractant bait to improve detection of this beetle in North America.

In a series of experiments in 2005 in Fresno and Tulare Cos., California, we tested the flight response of *O. erosus* to pheromone and host compounds using baited multiple funnel traps (Table 3). Treatments were organized in a randomized complete block design of four blocks, and checked and re-randomized once or twice every week. In experiment 1, responses were evaluated to racemic ipsdienol, MB, and (−)-α-pinene, alone and in combination. *Orthotomicus erosus* responded at very low levels to each of the components alone, but responded synergistically to racemic ipsdienol and MB (Table 3). Experiments 2 and 3 optimized the release rates of MB and racemic ipsdienol, respectively. The results of experiment 4 indicated that beetles were strongly attracted to (−)-ipsdienol, whereas (+)-ipsdienol was interruptive, making the racemic blend of ipsdienol inappropriate for an optimal attractant. Results of experiment 5 confirmed that *O. erosus* responded synergistically to (−)-ipsdienol and MB. The efficacy of the bait was proven in experiments 4 and 5 where the responses to (−)-ipsdienol and MB exceeded the responses to male pheromone produced naturally in small cut logs of Aleppo pine, *Pinus halepensis* Miller, each containing 25 feeding males. The sexes responded in the same patterns for all treatments and experiments.

Future research on the enantiospecific response of *O. erosus* to its two-component pheromone blend will involve a study that varies the release rate of (−)-ipsdienol; a study that varies the release rates of (−)-ipsdienol and MB in tandem; and a study that investigates the role of the enantiomers of α-pinene as co-attractants. In experiment 1, the role of (−)-α-pinene was not clear because only one trap on one date had excessively high captures in the bait containing racemic ipsdienol, MB, and (−)-α-pinene (Table 3).

2-Methyl-3-buten-2-ol has been shown to be a major volatile released by pine needles (foliage) from ten species of pines from western North America, including seven that occur in California (Harley et al. 1998). Fluxes of oxygenated volatile organic compounds above a *P. ponderosa* plantation in California were dominated by MB and

Table 3 Progression of experiments to demonstrate the enantiospecific response of the Mediterranean pine engraver, *Orthotomicus erosus*, to various pheromone and host compounds in Fresno and Tulare Cos., California, 2005 (Lee et al. unpublished data)

Experiment	Dates	Goals	Treatments[a]	Outcomes
1	7 February–18 March, 2005	Test synergism of 2-methyl-3-buten-2-ol (MB), racemic ipsdienol, and α-pinene [b,c]	MB, racemic ipsdienol, and α-pinene alone and in combination, unbaited trap (6 treatments)	MB & racemic ipsdienol synergistic; in one case MB & rac. ipsdienol & α-pinene attractive
2	28 March–15 July, 2005	Optimize release rate of MB	MB at 0.5–1.8, 17–60, 81–271, 810–2710 mg/day while keeping racemic ipsdienol release constant in each treatment, unbaited trap (5)	MB release rate of 0.5–60 mg/day & racemic ipsdienol most attractive
3	15 July–2 September, 2005	Optimize release rate of racemic ipsdienol[c]	Racemic ipsdienol at 0.11, 0.55, and 5.55 mg/day while keeping MB constant in each treatment, unbaited trap (4)	No difference in response to low and high ipsdienol release rates & MB; low release device more economical
4	2–23 September, 2005	Optimize enantiomeric composition of ipsdienol[d] Compare to natural pheromone of 25 males	Ipsdienol in blends [racemic 1× and 2×, (+)-1×, (−)-1×] while keeping MB constant in each treatment, male-infested log, unbaited trap (6)	(−)-Ipsdienol & MB attractive and the combination outperforms natural pheromone; racemic 1× and 2× partially attractive due to interruption by (+)-ipsdienol
5	23 September–18 October, 2005	Test synergism of (−)-ipsdienol and MB Compare to natural pheromone of 25 males	MB, (−)-ipsdienol alone and in combination, male-infested log, unbaited trap (4)	(−)-Ipsdienol & MB synergistic and the combination outperforms natural pheromone
Future directions	2006	Optimize release rate of (−)-ipsdienol	(−)-Ipsdienol at four release rates while keeping MB constant, unbaited trap (5)	
		Test effect of α-pinene	(−)-Ipsdienol & MB alone and combined with commercially available, racemic 2×, (+)-1×, (−)-1×- α-pinene, unbaited trap (6)	

[a]All materials from Phero Tech, Inc. unless otherwise indicated. For all enantiomeric mixtures of ipsdienol, release rates are 0.11 mg/day unless otherwise specified. MB release rates are 17–60 mg/day unless otherwise specified. MB from Sigma Aldrich was delivered from 400 μl plastic Eppendorf tubes to produce a release rate of 0.5–1.8, and from 15 ml plastic bottles to produce release rates of 81–271, and 810–2710 (all mg/day). Commercially available 95%-(−)-α-pinene was released from 15 ml plastic bottles at 150 mg/day at 23°C.

[b]Giesen et al. (1984); Mendel (1988).

[c]Klimetzek and Vité (1986).

[d]Kohnle (1991).

methanol (ca. 1.3 mg C m^{-2} h^{-1}) (Schade and Goldstein 2001). Thus, an intriguing possibility with *O. erosus* in California is that the high emission rates (>5 μg C g^{-1} h^{-1}) of MB from the foliage of *P. contorta murrayana*, *P. coulteri*, Jeffrey pine, *P. jeffreyi* Balfour, *P. ponderosa*, Bishop pine, *P. muricata* D. Don, gray pine, *P. sabiniana* Dougl. ex D. Don, and Torrey pine, *P. torreyana* Parry ex Carr., may play a role in the chemical ecology of this immigrant bark beetle species (Harley et al. 1998). *Pinus coulteri* (70.6 μg C g^{-1} h^{-1}), *P. sabiniana* (67 μg C g^{-1} h^{-1}), and *P. torreyana* (37.3 μg C $g^{-1}h^{-1}$), whose native populations are all distributed in relative proximity to the introduced population of *O. erosus*, had particularly high emission rates of MB (Harley et al. 1998). Widely planted *P. radiata* had an intermediate emission rate of this hemiterpenoid in the survey. Although MB released from foliage may be an ecologically inappropriate context for host colonization by *O. erosus*, the high vapor phase concentrations of MB in forests containing these hosts may serve as a general attractant for *O. erosus*, specifically in instances when ipsdienol-producing native *Ips* spp. (e.g., *I. latidens, I. spinifer* (Eichhoff), *I. mexicanus* (Hopkins), *I. plastographus maritimus* Lanier, *I. pini*, or *I. paraconfusus*) are colonizing the branches or main stems of these trees. The behavioral activity of MB has been tested recently in the mixed conifer forest of California's Sierra Nevada (Gray 2002), prior to the potential invasion of this forest by *O. erosus*. In that study, two native, pine-infesting species, *I. paraconfusus* and *D. brevicomis*, and their common predators (beetles in the families Trogositidae and Cleridae that were not determined to species) did not appear to respond significantly to MB as an attractive or interruptive signal.

The introduction of *O. erosus* into California also provides an opportunity to study the biosynthesis of MB in a pine-infesting bark beetle species and provides new motivation to study the formation of MB in pine host trees (Fig. 5). This hemiterpenoid is a relatively unusual and infrequently occurring pheromone structure among the bark beetles (Seybold and Vanderwel 2003). Its biosynthesis in bark beetles has been studied briefly in the Eurasian spruce engraver, *Ips*

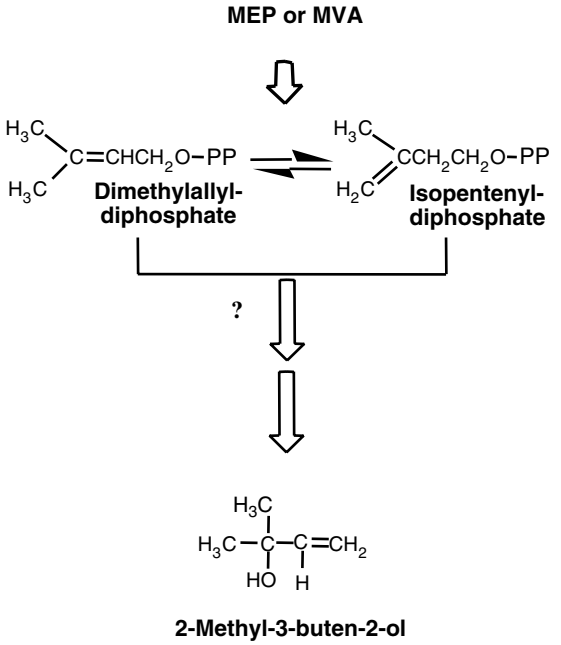


Fig. 5 Biosynthesis of the hemiterpenoid 2-methyl-3-buten-2-ol (MB) has not been completely elucidated in bark beetles, but likely involves modifications of dimethylallyl diphosphate (DMAPP) or isopentenyl diphosphate (IDP), either of which could be derived from either the 2-C-methyl-D-erythritol-4-phosphate (MEP) or mevalonate (MVA) pathway. Based on initial labeling studies (Lanne et al. 1989), the latter pathway is the more likely route to 2-methyl-3-buten-2-ol in bark beetles. In *Pinus ponderosa*, MB is derived from the MEP pathway (Zeidler and Lichtenthaler 2001)

typographus L. (Lanne et al. 1989), but nothing is known of how the biosynthesis of MB is regulated in bark beetles or what role, if any, terpene synthases or P450s may play in the conversion of dimethylallyl diphosphate (DMAPP) to the alcohol endproduct. In contrast, regulation of the formation of MB has been studied to some extent in pines. For example, in *P. ponderosa* needle tissue the formation of MB occurs via the MEP pathway (Zeidler and Lichtenthaler 2001). While it is possible that P450s could be involved in the oxidation of DMAPP in pine bark beetles, there is evidence from research with needle tissue of *P. sabiniana* that MB is formed instead by a terpene synthase enzyme activity (Fisher et al. 2000). The reaction mechanism of pine MB synthase in the formation of MB from DMAPP could be similar to the formation of the monoterpene alcohol linalool from GDP in Norway spruce,

Picea abies L. Karst, and other plant species (Martin et al. 2004). The role of a terpene synthase in the formation of the related hemiterpene isoprene from DMAPP is also well established in plants (Miller et al. 2001). In the case of the formation of MB from DMAPP the reactive carbocation intermediate in the terpene synthase reaction would be quenched by the addition of water, instead of proton elimination as occurs in the formation of isoprene.

Conclusions

Pine bark beetles are significant forest pests with an interesting reproductive biology that is guided in many cases by host monoterpenes and isoprenoid aggregation pheromones. In a few species, host monoterpenes are attractive alone as long-range signals, but they have been recognized repeatedly in many species as essential co-attractants with aggregation pheromones. The monoterpenes arise in the pines *via* the MEP pathway. Some pheromones can arise both from host monoterpenes and through *de novo* synthesis in the beetles *via* the MVA pathway. Both production of pheromones and flight response to pheromones are stereospecific processes. Research currently underway on bark beetle pheromone biosynthesis will broaden our understanding of the role of P450's in stereospecific oxygenation reactions of monoterpenes and in hemiterpenoid biosynthesis. Ongoing research on stereospecific responses of *I. paraconfusus* and *O. erosus* will optimize the efficacy of commercial baits to detect these species as potential invaders in ports and forested regions in other continents (*I. paraconfusus*) and within North America (*O. erosus*).

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