The Role of the Monoterpene Composition in *Pinus* spp. Needles, in Host Selection by the Pine Processionary Caterpillar, *Thaumetopoea pityocampa*

R. Tiberi,[1] A. Niccoli,[2] M. Curini,[3]
F. Epifano,[3] M.C. Marcotullio[3] and O. Rosati[3]

This paper presents preliminary results on attempts to extract and characterize the volatile secondary metabolites contained in needles of different *Pinus* species and to ascertain the role played by these substances on the behavior of *Thaumetopoea pityocampa* (Denis et Schiffermüller) females, which show a marked preference, during the oviposition period, for some indigenous and exotic species of host plants existing in mixed formation. Limonene is the most abundant monoterpene extracted from *P. pinea* needles, the least favored species of pine processionary caterpillar (PPC) females, although it is present in only very low amounts in other *Pinus* species. An increase was observed in limonene production by *P. pinea* at the start of the flight period of the PPC adult, and subsequently at the beginning of the females' oviposition period. Assays carried out in two pine stands in central Italy showed that limonene, emulsified with water and sprayed on foliage of four different pine species plants, *P. sylvestris, P. nigra, P. pinaster* and *P. radiata,* during the PPC oviposition period, provided a satisfactory degree of protection. In fact, the number of egg clusters collected from treated plants was often lower than the number of egg clusters collected from control plants and was comparable to the number of egg clusters laid on *P. pinea* plants. In particular (R)-(+)-limonene, although not produced in nature by pines, was the most effective deterrent. However, the effect of (S)-(-)-limonene, the enantiomer biosynthesized by pines, was also adequate.
KEY WORDS: *Pinus* spp.; *Thaumetopoea pityocampa*; terpenes; limonene; bioassays.

INTRODUCTION

Among pine defoliators living in the Mediterranean area, *Thaumetopoea pityocampa* (Denis et Schiffermüller), best known as the pine processionary caterpillar (PPC), is undoubtedly the most harmful species due to the damage incurred during its periodic outbreaks. Massive attacks of the lepidopteran occur frequently on both indigenous and exotic species of pine. Of the indigenous species in southern Europe (5,8,12,13), the defoliator shows a marked preference for pines belonging to the *nigra* group, and among the exotic species it shows a preference for *Pinus* radiata Don. (6,13,20). This different susceptibility of several *Pinus* species found in mixed formations towards the attacks of the

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[1]Istituto di Patologia e Zoologia Forestale e Agraria, Università degli Studi, 50144 Firenze, Italy [Fax: +39-55-354786; e-mail: tiberi@ipaf.fi.cnr.it].
[2]Istituto Sperimentale per la Zoologia Agraria, 50125 Firenze, Italy
[3]Istituto di Chimica Organica, Facoltà di Farmacia, Università degli Studi, 06123 Perugia, Italy.

PPC, may refer both to selection of a certain plant by females at the moment of oviposition, already emphasized by Calas (4), and to the influence of the quality of food in different pine species on larval mortality and development rate (3,17; Tiberi, unpublished data). However, it is well known that the female PPC's choice of tree for ovipositing is influenced by tree size and position in the stand, the shape of the crown and thickness of the needles (8,12,20), as it does for *T. wilkinsoni* Tams (7,14). To the best of our knowledge, no study has been conducted of other chemicals present in the pine needles to determine what role they may play in the female's choice of plant.

Pinus pinea L. is the least sought pine by the PPC females during oviposition, when found in mixed formation together with other pine species (2,21). It is also the least suitable for larval growth and survival (17). Clearly, chemicals emitted from or contained in the needles may influence the different preferences exhibited by females towards various pine species during the oviposition period, as well as the different rates of mortality and larval development.

The aim of this preliminary study was to isolate and characterize which of the substances emitted from needles of different pine species were capable of influencing the selection of a particular plant by PPC females during the oviposition period. It was therefore limited to the identification of volatile secondary metabolites (particularly the monoterpenes) present in the pine needles, since they represent the site for oviposition by *T. pityocampa*. The importance of terpenes present in coniferous trees as resistance factors and oviposition stimulants or deterrents has already been discussed in studies of insect feeding and oviposition behavior (1,9,16). In other pine defoliators the influence of monoterpenes in host selection has been determined as, for example, in the spruce budworm, *Choristoneura fumiferana* (Clemens) (19) as well as in the pine beauty moth, *Panolis flammea* (Denis et Schiffermüller), in oviposition (10,11) and *Dioryctria abietivorella* (Grote) (18).

MATERIALS AND METHODS

Study sites

For the development of the research program two young planted pine plantations were chosen, one in Monte Senario, near Florence (Tuscany, Italy), at an altitude of *ca* 600 m; the other approximately 15 km northeast of Fondi, near Latina (Latium, Italy), at an altitude of *ca* 550 m. The bioecology of the PPC and its egg parasitoids has been the subject of study in both pine plantations; in these studies evidence of female preferences for oviposition was obtained. The observations showed that reduced infestations by the PPC in the Monte Senario pine stand in 1995 followed 2 years (1993 and 1994) of severe outbreaks, whereas in the Fondi pine stand infestation reached and remained at high levels. In Monte Senario the pine stand, a 16-year-old reafforestation composed mainly of *Pinus nigra* Arn. together with *P. sylvestris* L., lies in a woody area on the hills north of Florence. The Fondi pine stand covers an area of 2 ha and was planted to verify which were the most suitable species for the reafforestation of this area, which had been severely damaged in the past by intensive pasture of sheep and cattle.

From 1997, pine twigs with needle growth of the last 2 years were randomly collected at fixed times from five trees of each species of pine and in each plantation. These needles were extracted with organic solvents and analyzed to determine the qualitative and quantitative composition of secondary metabolites, in particular those able to influence the behavior of PPC females searching for the most suitable plant for oviposition.

Qualitative and quantitative analysis of secondary metabolites

To ascertain a possible influence of monoterpenes produced in needle tissues on the behavior of PPC females during the oviposition period, observations were made of the qualitative and quantitative composition of secondary metabolites produced by five species of pine: *Pinus nigra, P. sylvestris, P. pinaster* Ait., *P. pinea* and *P. radiata.* Branches were collected and stored at 4°C. The following day 10-g samples of needles were selected at random from various parts of each branch and immediately extracted with *n*-hexane (13 ml). Each solution was analyzed by capillary column GC using a Hewlett Packard 5890 gas chromatograph equipped with a 25 m × 0.25 mm MetSil column. The carrier gas was helium and the column temperature was programmed from 50°C to 270°C at 5°C/min. The chromatogram was obtained using a reporting integrator and the composition recorded as percent area, uncorrected for relative response of the standard. Mass spectra were obtained from a GC-MS system, operating in the EI mode at 70 eV, equipped with a 25 m × 0.25 mm MetSil column, using the same chromatographic conditions as reported above. The column was connected to the mass spectrometer ion source *via* an open split interface heated to 250°C. Chiral GC analysis was performed using the same chromatographic apparatus equipped with a 25 m × 0.25 mm 30% 2,3-diethyl-6-*t*-butyldimethylsilyl-β-cyclodextrin type PS086 column. The carrier gas was hydrogen and the column temperature was programmed from 40°C to 190°C at 2°C/min. The quantitative composition was calculated from peak areas using *n*-decane (43.7 μM in *n*-hexane) as the external standard. The identification of the chemical constituents of each extract was based on a comparison of their retention times (R_t) and mass spectra with those obtained from the samples and from the Wiley library spectra.

On the basis of the data obtained from chemical analysis, preliminary biological assays were carried out directly in the pine forest to ascertain the possible ecological role of limonene, one of the monoterpenes found in the needles, as the greatest difference in its concentration depended on the pine species.

Seasonal semiochemical production

The biological assays demonstrated that limonene is capable of affecting the PPC behavior in locating plants for oviposition (see Results). To verify whether this chemical defense of the host plant was of constitutional or induced origin, samples of needles from *P. pinea* were collected between March and October 1998 and subsequently extracted. Extracts were analyzed in the same way as described previously. These studies showed the seasonal variation of limonene content from spring to autumn.

Having defined the variation of limonene concentration in needle tissues of *P. pinea*, the next step was to define the variation of the composition of secondary metabolites in needle waxes of *P. radiata*, the species most preferred by PPC females for oviposition. Therefore, samples of needles from this exotic species of pine were collected in September and October of 1998 and the respective data were compared with those obtained from the samples collected from the same pine species in May and July 1997.

Biological assays

Biological assays were carried out to determine a possible ecological role of limonene in the behavior of PPC females during the phase of host plant location for egg deposition in 1997 in the Monte Senario and Fondi pine plantations. To evaluate a probable deterrent effect of limonene towards PPC ovipositing females on the basis of the number of egg clusters laid on each pine, the flight period of *T. pityocampa* was monitored using pheromone traps. The traps were placed in the pine forests from early July to mid-September. In Monte Senario, PPC flight began in mid-July and continued until the end of August; in Fondi it began about one week later and continued until the first week of September. During these periods pine foliage was sprayed with (R)-(+) or (S)-(-)-limonene emulsified with water (500 mg/*l*) (Table 1). In the Fondi pine stand, *P. pinea* trees obviously did not undergo this kind of treatment and were considered as controls. In both pine stands, treated and control plants occupied marginal positions or were isolated specimens; thus they were situated in the best position to be located and used as hosts for oviposition by PPC females.

The choice to use also (R)-(+)-limonene in our biological assays, although not naturally produced by *P. pinea*, as previously mentioned, was justified by the fact that cases in which the enantiomer of a semiochemical not produced in nature exerts a biological effect are well documented in the literature (15). The dates of treatments during summer 1997 are given in Table 1.

TABLE 1. Details of treatments of pine trees in the Monte Senario and Fondi plantations (in parentheses, dates of treatments)

Location	Species	No. of trees treated with		No. of control trees
		(R)-(+)-Limonene	(S)-(-)-Limonene	
Monte Senario, Tuscany	*P. nigra*	6	6	21
(July 18, 28; Aug. 6, 14)	*P. sylvestris*	6	6	9
Fondi, Latium	*P. radiata*	2	2	2
(July 23, 31; Aug. 10, 19)	*P. pinaster*	2	2	2
	P. pinea	–	–	24

Egg clusters were collected and counted on September 17 in Monte Senario and on October 8 in Fondi. The quantitative analysis of the samples collected in the two pine plantations in 1997 showed clear distinctions in the composition of the monoterpenes in the different pine species.

RESULTS

Monoterpene composition in needles

Table 2 and Figure 1 give the concentration values (in ppm) of the most abundant components of pine needle extracts. From the data reported, it is evident that the most significant differences in the composition of secondary metabolites contained in needle tissues concern limonene. In fact, the concentration of this monoterpene in needle extract of *P. pinea* is 24 to 70 times higher than that determined for other pine species. This finding led to the hypothesis that limonene might be one of the possible defense mechanisms by which *P. pinea* protects itself from PPC attacks.

Limonene is produced naturally in both the enantiomeric forms, (R)-(+) and (S)-(-); prior to carrying out biological assays, chiral GC analysis showed that *P. pinea* produces only (S)-(-)-limonene (Table 3). Data on the seasonal variations encountered in limonene production in *P. pinea* are given in Table 4 for 1998.

TABLE 2. Retention time (R_t) and quantitative composition (expressed in ppm) of the most abundant monoterpenes extracted from pine needles

		Concentration (ppm)				
	R_t	*P. sylvestris*	*P. pinaster*	*P. radiata*	*P. nigra*	*P. pinea*
α-Pinene	3.84	22.10±0.08	44.55±0.10	12.43±0.09	35.19±0.07	3.59±0.05
β-Pinene	5.01	3.59±0.03	22.50±0.28	43.86±0.06	1.32±0.04	1.57±0.01
Mircene	5.87	3.19±0.03	7.13±0.04	traces	0.88±0.02	1.58±0.04
σ^3-Carene	6.01	5.83±0.04	2.84±0.05	traces	traces	–
Limonene	6.09	0.89±0.03	1.48±0.02	2.57±0.07	1.91±0.03	61.92±0.07
β - Fellandrene	6.52	0.62±0.03	2.58±0.05	–	traces	4.24±0.03

TABLE 3. Comparison between chiral GC retention time (R_t) of limonene extracted from *Pinus pinea* and standards of pure enantiomers

	R_t (in minutes)
P. pinea extract	15.45
Standard (S)-(-)	15.47
Standard (R)-(+)	16.39

TABLE 4. Limonene concentration in *Pinus pinea* needle extracts during the period March–October 1998

Date	Limonene (ppm)
March 19	82.39±0.06
April 28	70.24±0.05
May 19	59.95±0.06
July 6	62.39±0.03
July 16	70.92±0.03
July 31	72.44±0.07
September 17	69.42±0.02
October 1	67.12±0.06

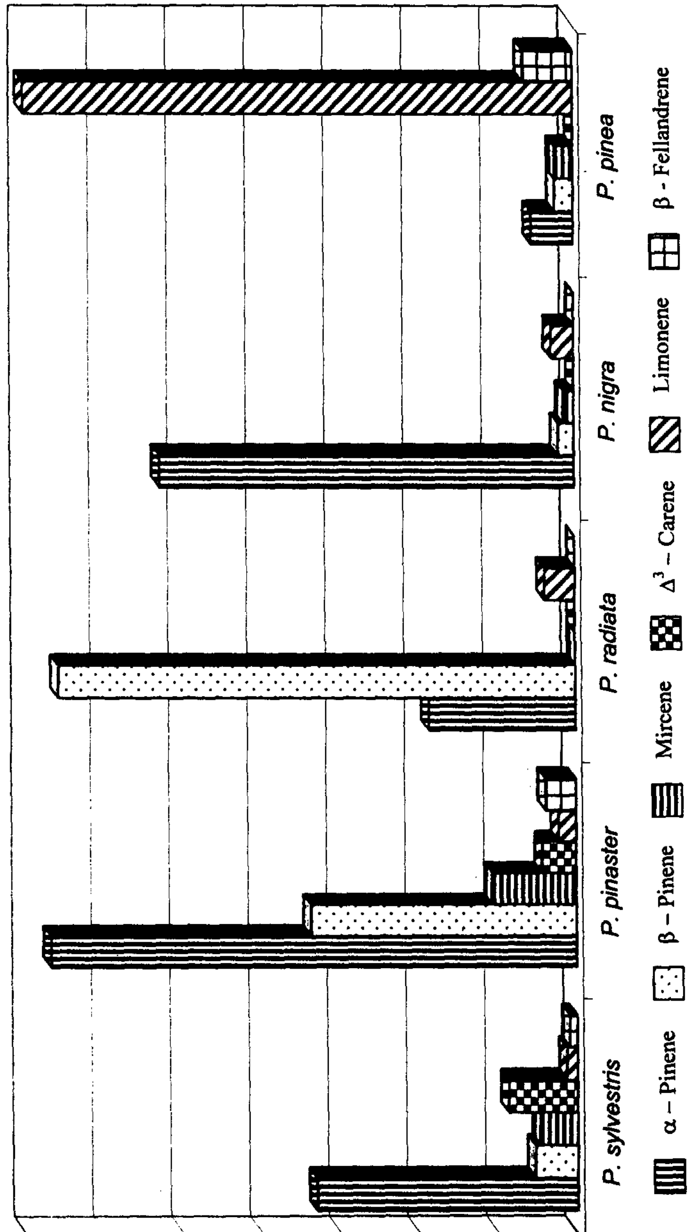

Fig. 1. Histograms of the quantitative composition (expressed in ppm) of the most abundant monoterpenes extracted from pine needles.

The data reported in Table 4 show that limonene production undergoes a progressive decrease from March until May, when it reaches the lowest levels. This phenomenon may be due to diminished biosynthesis by the plant and to higher evaporation of limonene from leaf surfaces, due to an increase in seasonal temperatures. In summer, the limonene concentration in needle tissues tends to increase, only to decrease in September and October. The greatest increase is recorded in the second half of July, just when the flight period of *T. pityocampa* adults starts. Therefore, it seems that in the spring limonene biosynthesis in *P. pinea* increases when PPC females begin their search for the most suitable plants for oviposition. It is necessary to assess whether such a phenomenon is in any way related to a defense mechanism of *P. pinea* to protect itself from ovipositing females of the PPC, or to other factors such as temperature. Although limonene biosynthesis increased, there were no evident changes in the biosynthesis of any other monoterpenes, such as α- and β-pinene, which are found in the needle tissue of *P. pinea*. Over the same period the concentrations of these substances showed no relevant differences in comparison with the levels reported in Table 2 and Figure 1, varying from a minimum of 3.4 ppm to a maximum of 3.6 for α-pinene and from 1.3 to 1.6 for β-pinene.

TABLE 5. Seasonal variations in limonene, α- and β-pinene production in *Pinus radiata*

	Concentration (ppm)			
	May 1997	July 1997	Sept. 1998	Oct. 1998
α-Pinene	2.80±0.03	12.43±0.03	17.11±0.07	16.91±0.01
β-Pinene	13.10±0.04	43.86±0.04	85.33±0.02	85.72±0.04
Limonene	2.53±0.03	2.57±0.04	4.41±0.04	4.22±0.03

Limonene concentration in *P. radiata* remained constant at very low levels in comparison with the limonene concentration in *P. pinea* (Table 4). On the other hand, α- and β-pinene concentrations underwent a substantial increase from July to October, when the PPC began its flight period and oviposition.

TABLE 6. Number of egg clusters collected from control trees and from pines treated with limonene

Location	Species	Egg clusters collected on					
		Trees treated with:				Control trees	
		(R)-(+)-Limonene		(S)-(-)-Limonene			
		No.	Mean/tree	No.	Mean/tree	No.	Mean/tree
Monte Senario	*P. nigra*	0	0	0	0	5	0.23
(Tuscany)	*P. sylvestris*	0	0	1	0.16	2	0.22
Fondi	*P. radiata*	5	2.5	14	7.0	10	5.0
(Latium)	*P. pinaster*	1	0.5	6	3.0	10	5.0
	*P. pinea**	–	–	–	–	12	0.5

* *P. pinea* trees were not treated with limonene.

Results of biological assays

The results of the biological assays with limonene are illustrated in Table 6, which shows the number of egg clusters collected in the two pine forests for each pine species. The small number of egg clusters collected from *P. nigra* and *P. sylvestris* as well as from the control plants in Monte Senario can be explained by the fact that, as previously mentioned, in this pine plantation the population of PPC had been in a latent state since 1995. The data reported in Table 6 suggest that limonene is able to provide a good level of protection from PPC ovipositing females. The best effect is provided by (R)-(+)-limonene, the enantiomer not produced by *P. pinea*. In fact, in both pine stands the number of egg clusters collected from pines treated with this compound was always lower than that collected from pines treated with (S)-(-)-limonene and from control plants. Nevertheless, the treatment with (S)-(-)-limonene also had a positive effect in deterring oviposition by PPC females in all cases except one: in the case of *P. radiata* in the Fondi pine plantation, (S)-(-)-limonene was not successful in preventing PPC oviposition, as shown by the comparison between the number of egg clusters collected from pines treated with this enantiomer and that collected from control pines.

CONCLUSIONS

The results of our research suggest that the preference shown by PPC females for oviposition on certain species of pine may be related to the influence exerted by some volatile secondary metabolites dissolved in pine needle tissue. Among these, limonene seems to play a decisive role, as shown by the smaller number of egg clusters laid on *P. pinea*, the species that produces a higher quantity of this monoterpene, and by biological assays carried out in the two pine plantations. On plants of *P. nigra*, *P. sylvestris*, *P. radiata* and *P. pinaster* treated with (R)-(+) and (S)-(-)-limonene emulsified with water during the flight period of the PPC, a smaller number of egg clusters was collected than from control plants in all cases except one. The best 'protective' effect is exerted by (R)-(+)-limonene, the enantiomer not naturally produced by *P. pinea*. Nevertheless, (S)-(-)-limonene also seems to be effective, except in one case on *P. radiata* in the Fondi pine forest. In fact, 14 egg clusters were collected from two plants treated with (S)-(-)-limonene, as opposed to ten egg clusters from control plants and five egg clusters from plants treated with (R)-(+)-limonene. On the whole, (R)-(+)-limonene provided a degree of protection similar to or even higher than that determined for *P. pinea*. By comparing the number of egg clusters collected from plants treated with (R)-(+)-limonene with the number of egg clusters collected from control plants and *P. pinea* (Table 6), it is evident that (R)-(+)-limonene provided a protective cover to pines against ovipositing PPC females.

Another interesting result was obtained from the analysis of the seasonal production by *P. pinea* and *P. radiata* (Tables 4 and 5) of the most abundant volatile secondary metabolites, limonene and α- and β-pinene, respectively. The production of limonene in *P. pinea*, following a decrease during spring, increases remarkably at the start of the flight period of the PPC and remains at high levels until the beginning of autumn. However, in *P. radiata*, the quantity of this monoterpene, which is very low, does not undergo relevant changes over the same period, whereas the production of α- and β-pinene shows a considerable increase from July to October. This phenomenon does not occur in *P. pinea*: although the production of limonene increases, changes in α- and β-pinene production are not evident.

The strong increase in α- and β-pinene production in *P. radiata*, if compared with the course of limonene production in *P. pinea*, is a very interesting finding. In fact, the most significant aspect is represented by the qualitative and quantitative differences between secondary metabolite production during the flight period and the oviposition of PPC in the preferred species of pine in comparison with the lepidopteran's least favored species.

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