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CHEMICALS INVOLVED IN HONEYBEE–SUNFLOWER RELATIONSHIP

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Abstract—We present a review of work on the plant chemicals involved in the honeybee–sunflower model system. Combined behavioral and chemical analyses were conducted under natural and controlled conditions. First the distribution of forager bees' visits on two pairs of sunflower genotypes producing a different level of hybrid seed yield was recorded under pollen-proof tunnels. Mirasol parental lines producing high seed yields were visited at random, whereas forager bees visited preferentially the female parental line of Marianne, resulting in low seed yield. Nectar samples collected on the genotypes were analyzed by gas chromatography. Fructose, glucose, and sucrose were identified. Parental lines of Mirasol showed similar sugar profiles, whereas the female line of Marianne contained higher amounts of sucrose than the male line. We assume that the bees' preferences between genotypes might rely on differences in the sugar composition of floral nectars, especially in the amount of sucrose. Aromas from headspace collection were compared between pairs and periodically during the flowering period. Of the 144 components indexed for Marianne lines and 136 components for Mirasol lines, 17 of the components for Marianne lines and 18 for Mirasol lines differed significantly according to flowering stage. Significant differences appeared in eight of the 134 components of Marianne lines and in 20 of the 250 components for Mirasol lines. Such differences, even restricted to a few components, might account for honeybees' discrimination between genotypes or flowering stage. Experiments then were conducted in a flight room using an artificial flower device. A total volatile extract was used as a conditioning scent previous to the test where the total extract was successively

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compared to several of its subfractions. Fractions significantly less visited than the total extract were discarded, whereas fractions confused with the total extract were kept. From step to step, a restricted fraction of 28 polar components, among which 15 were identified, was shown to be as active as the initial conditioning extract. These data emphasized honeybees' abilities to generalize from simplified to more complex chemical information. Finally, this work considers the possible use of such plant chemicals, from nectars or aromas, either as targets for genetic modification of crop plants or as direct attractants when sprayed on the crop, for the improvement of entomophilous cross pollination.

Key Words—Sunflower, *Helianthus annuus*, honeybee, *Apis mellifera*, Hymenoptera, Apidae, foraging behavior, aroma, nectar, plant chemicals, conditioning, olfactory discrimination, pollination.

INTRODUCTION

Puzzling questions when considering insects seeking food in their natural environment are: how do they cope with the complexity of the sensory information that is available, and which cues allow them to make their choices? These questions are particularly relevant for insects that have a large food spectrum, such as pollinators. This work is an attempt to answer these questions, using the honeybee–sunflower relationship as a model system.

The sunflower (*Helianthus annuus L.*) currently represents the second most important oilseed crop worldwide (Putt, 1978). The *Helianthus* genus is allogamous, with entomophilous fertilization (Free and Simpson, 1964). Honeybee visits are of great benefit for oilseed production (increased seed yield, oil rate, germination ability) (Parker, 1981). Noticeable improvement of the crop followed the discovery of male cytoplasmic sterility (Leclercq, 1969); commercially controlled hybrids were produced with heterosis benefit at the F_1 generation. Hybrid seed production is strictly dependent on cross-fertilization from male to female lines, which is brought about primarily by honeybees (Radford and Rhodes, 1978), but it is known from field observations that foragers may show a selective preference for certain genotypes, leading to low hybrid seed yields (Freund and Furgala, 1982).

In the honeybee, foraging behavior involves both learning at an individual level (Wenner et al., 1969; Masson, 1982) and communication of information to recruits (Frisch, 1967). When foraging from a flower, visual and olfactory signals (conditioned stimuli) are associated with the food reward, mainly floral nectar (unconditioned stimuli). Conditioned stimuli are memorized and become significant orientation cues. Among these stimuli, olfactory signals have been

shown to be learned particularly rapidly (Menzel, 1967; Koltermann, 1973). Thus, the foraging behavior mainly results from the association of plant allelochemicals acting as chemosensory (olfactory and gustatory) cues for the honeybee.

We have tried to determine: (1) how extensively honeybees are likely to use scents associated with a food reward to discriminate among sunflower genotypes, and (2) if all constituents of the chemical information (food and/or associated signals) are needed for host-plant recognition. Combined behavioral and chemical analyses under natural and controlled conditions were conducted.

In a first step, the distribution of forager visits on pairs of genotypes producing commercial hybrid seeds has been observed under outdoor conditions. Honeybees visit sunflowers mainly seeking nectar; little pollen is gathered (Delaude et al., 1979). Most studies dealing with the role of nectar production in the attractiveness of sunflower crops have been concerned with the relationship between the amount of nectar or total amount of sugar and bee visits (Tepedino and Parker, 1982). The quality of sugars and their relative proportions have not been taken into account. Among the four genotypes considered, no correlation has been established between the total amount of nectar or the total sugar fraction and the attractiveness of the genotypes (Fonta et al., 1985). Here we focused on the sugar composition of the nectars related to the honeybees' foraging behavior.

Little work has been done on sunflower aroma chemistry. Different types of terpenoids have been identified in the sunflower volatile fraction (Eckert et al., 1973; Gershenzon et al., 1981), but otherwise, only the aromatic value of sunflower oil has been investigated (Popescu, 1982). Our aim was first to investigate the qualitative differences between the aromas of the various sunflower genotypes and, second, to define possible cues for intergenotype discrimination by the honeybees.

Among hundreds of components detected by gas chromatography in a crude sunflower extract, 84 were indexed, among which 58 were identified, both in the polar and the apolar fraction (Etievant et al., 1984). In order to define how honeybees use such plant volatiles as orientation cues, the natural foraging situation was reproduced in a flight room, using an artificial flower feeder device (Pham-Delegue and Masson, 1985). The olfactory choices performed by the foragers were recorded subsequent to a conditioning procedure using sunflower aroma extracts. From the bees' responses, it was possible to identify those constituents among the complex volatile blend that influenced bee behavior.

From these combined studies we were able to define molecular criteria for plant attractiveness that are likely to become tools for plant improvement through entomophilous pollination.

METHODS AND MATERIALS

Chemical Cues Involved in Discrimination between Genotypes

Foraging Behavior. The experiments were conducted on two pairs of genotypes that produce commercial hybrids named Marianne and Mirasol. The seed yield reported for these hybrids differs strongly (respectively 5 – 6 quintal/ha and 10 – 13 quintal/ha). Marianne production was stopped in France and Mirasol is considered as the reference variety for sunflower breeders. Male and female lines, as well as phenological stages (stage I, II, III corresponding, respectively, to 1/3, 2/3, 3/3 of disk florets in blossom) were noted.

Observations were carried out under pollen-proof tunnels. In each tunnel a hive containing about 10,000 workers, a 1-year-old queen, brood, and food combs was placed in front of a pair of sunflower genotypes. The number of forager bees per sunflower head, as well as the stage of blossom was recorded on 40 heads per genotype, with three recordings per day, every two days during the flowering period (i.e., six days of observation on each genotype). The attractiveness of a line was reported as the mean number of foragers per 10 sunflower heads. Student t tests were used to compare the attractiveness between the parental lines of a pair.

Collection and Analysis of Nectar. Nectar samples were collected using glass pipets, from 20 florets per head and 10 heads per genotype, throughout the flowering period. The samples of nectar collected were kept in a freezer (−20°C).

The sugar composition was determined using a gas chromatography technique applied to partially dehydrated and derivatized samples (Fonta et al., 1985). To measure the real proportion of the constitutive sugar, an internal standard (triphenylethylene) was added to the samples (Black and Bagley, 1978).

Collection and Analysis of Aromas. Plant volatiles were collected and concentrated using a head space trapping method adapted to sunflower heads (for details, see Pham-Delegue et al., 1989). Aroma collection was carried out (1) on the four genotypes at three phenological stages (I, II, III), to evaluate the stage effect on the production of volatiles and (2) on the four genotypes at stage II, to focus on the sex effect (female versus male lines).

Statistical analysis of the stage effect was performed on the integrated area of each constitutive peak as a ratio of the total area of all indexed peaks of the chromatogram of a given genotype. Data were then compared for each pair of sunflower lines (two repetitions per stage and two injections per sample), using an univariable analysis of variance followed by a multiple comparison of means (Scheffe method).

The data treatment of the sex effect was applied to the values of the integrated area of each indexed peak as a ratio of standard peak area (1 mg

2-hexanone added before concentration of extracts). To compare male to female aromagrams (three repetitions per genotype and two injections per sample), a univariable analysis of variance was used.

For identification of the discriminative components, gas chromatography and mass spectrometry were coupled, following conditions previously described (Etievant and Bayonove, 1983; Pham-Delegue et al., 1989).

Processing of a Complex Chemical Signal

Bioassay. A honeybee colony (about 3000 workers, and 1-year-old queen and brood) was kept in a flight room under controlled conditions (Temperature 25°C; relative humidity 55%; 12-hr light–12-hr dark). Foragers were allowed to visit a device (during a 2-hr period) on which a sugar solution was placed (sucrose 50%, the unconditioned stimulus), and a scent (the conditioned stimulus, successively consisting of the total sunflower extract and its polar fraction, see below).

Bees then were tested, without food reward, in a choice situation between the conditioned scent and an unconditioned scent stimulus. The test period was divided into four trials of 5 min, interspersed with periods in which the conditioned scent was again presented with a food reward. The distribution of visits during the test periods expressed as a percentage of landings for the different stimuli examined, was compared using a χ^2 test, 1 *df.*

Chemical Stimuli. To provide general information on sunflower aroma constituents, a bulk sample was made of 10 flower heads from various cultivars. The sunflower heads were collected and extracted using a dichloromethane solvent extraction method, which has been proven to be as efficient as the headspace method (Etievant et al., 1984). The solvent extract was first separated into polar and apolar fractions, and then the polar fraction was arbitrarily split into three fractions (A, B, C) as described in Pham-Delegue et al,. (1986). Separation and identification methods have been described previously (Etievant et al., 1984).

RESULTS

Chemical Cues Involved in Discrimination between Genotypes (Figure 1)

Foraging Behavior. A foraging preference appeared for the flowering stages I and II, stage III being visited much less often in all genotypes. Moreover, the foragers' visits were randomly distributed between the parental line of Mirasol, whereas the female line of Marianne was visited significantly more often than the male line.

Thus, the honeybees' ability to discriminate between genotypes and flow-

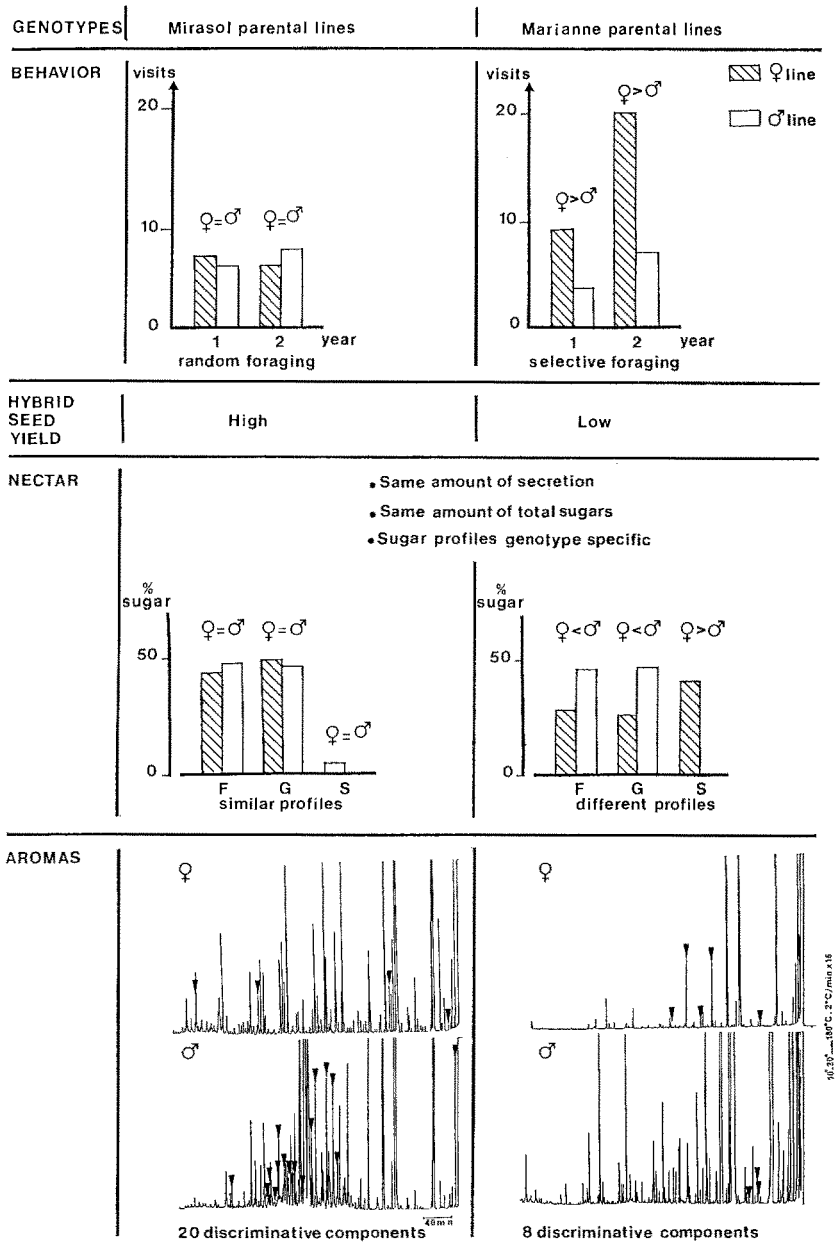

FIG. 1. Synopsis of chemical cues (nectar, aromas) involved in discrimination between genotypes (F: fructose; G: glucose; S: sucrose).

ering stages (Drane et al., 1982) was confirmed. It has to be emphasized that the selective behavior performed on Marianne parental lines resulted in a low seed yield, whereas the random distribution on Mirasol parental lines contributed to a high level of seed production.

Analysis of Nectar. Fructose, glucose, and sucrose were identified. All genotypes present high levels of glucose and fructose that are equivalent (30–50% of the total sugars), but the proportion of sucrose fluctuates strongly between genotypes: 40% sucrose was measured in the female line of Marianne, which was visited far more than the male line, where no sucrose was detected in the nectar. In Mirasol parental lines, only traces of sucrose could be found in both lines, corresponding to an equal distribution of visits. We may thus assume that the sugar composition of nectar, particularly the proportion of sucrose, is a key parameter in the honeybees' selective foraging behavior. However, although bees' preferences correlate well with the quality of the food reward available, bees are able to recognize the preferred genotype using distant chemical cues, i.e., aromas.

Analysis of Aromas. Of the 144 components indexed for Marianne lines and 136 components for Mirasol lines, 17 of the components for Marianne lines and 18 for Mirasol lines differed significantly according to flowering stage. Nine of the 17 variable components in the Marianne lines showed higher relative proportions at stage I, and 13 of the 18 variable components of Mirasol lines showed higher relative proportions at stage II. These data can be related to the behavioral observations in which bees showed preferences for flower at stages I and II for all genotypes.

To compare aromas from male and female lines, 134 and 250 peaks were indexed for Marianne and Mirasol lines, respectively. Significant differences appeared in eight of the 134 components for Marianne lines and in 20 of the 250 components for Mirasol lines. It has to be stressed that differences between genotypes do not necessarily occur in components that are most abundant. Three of the eight and 12 of the 20 variable components for Marianne and Mirasol lines, respectively, appear only in one or the other genotype of a pair, generally in the male line. Moreover, most of the variable components between Mirasol lines have higher amounts in the male line (16 of 20 components). The identification of some of the discriminative components has been reported elsewhere (Pham-Delegue et al., 1989).

Processing of a Complex Chemical Signal (Figure 2)

After being conditioned to the total extract, the bees were placed in a choice situation between the conditioned stimulus and either the polar or the apolar fraction of the total extract. Behavioral responses showed that the apolar frac-

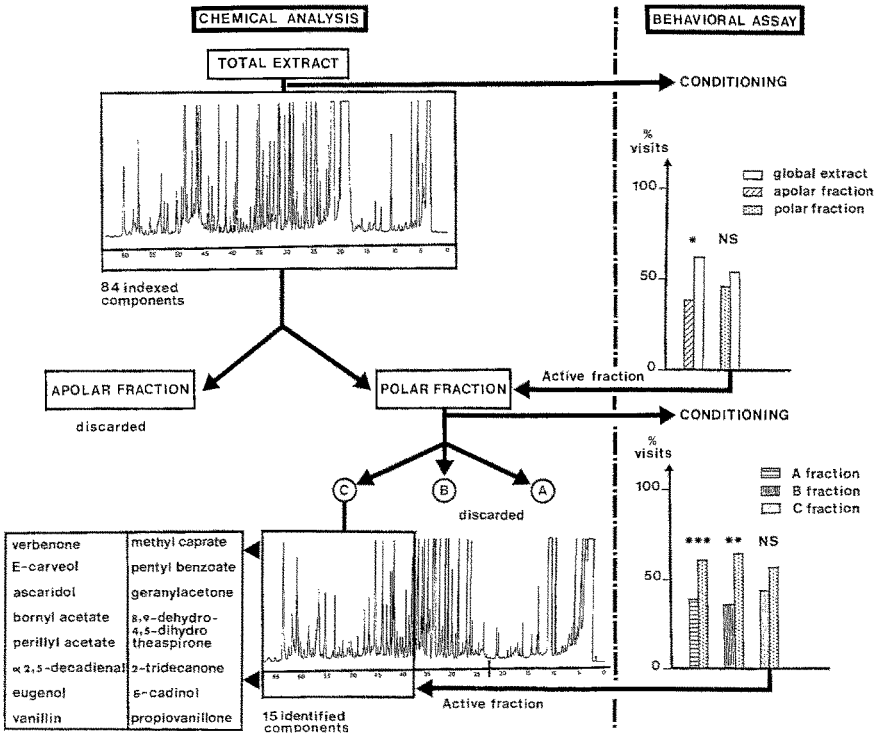

FIG. 2. Scheme of the combined chemical and behavioral procedures leading to the identification of active components that allowed the recognition of the sunflower volatile blend by the forager bees (χ^2 test, 1 *df*. NS: not significant; *P < 0.05; **P < 0.01; ***P < 0.001).

tion was significantly discriminated from the conditioning scent, while the polar fraction was confused with the global aroma. Thus the polar fraction was considered by the bees as very similar to the global extract.

Following the same procedure, the three fractions of the polar extract were presented to the foragers previously conditioned to the polar extract. It appeared that only the C fraction was statistically confused with the conditioned aroma, whereas the A and B fractions were significantly less attractive ($P < 0.001$ and $P < 0.01$, respectively) than the whole polar fraction. The C fraction includes 28 constituents, among which 15 were chemically identified, the others being sesquiterpenes (Pham-Delegue et al., 1986). This C fraction, the so called active fraction, represents the most restricted fraction of the sunflower volatile blend that elicits the same level of attraction as the total extract in our experimental conditions.

DISCUSSION

From the study of the honeybee–sunflower model, some information can be gained on the role and the nature of plant chemicals involved in host plant selection and on subsequent applications.

Nectars appear to be the key factor influencing honeybee choices. As is the case in all floral nectars, sunflower nectars consist mainly of sugars with simple qualitative profiles. Glucose, fructose, and sucrose were identified, in proportions characteristic of the genotype (Pham-Delegue et al., 1987); this was later confirmed within a larger range of sunflower genotypes (Vear et al., 1990). Moreover, the level of visits appeared to be positively correlated with the amount of one particular sugar sucrose (Pham-Delegue et al., in preparation). Thus we may assume that a plant breeding program to increase the amount of sucrose would lead to an increased attractiveness of the genotypes towards forager bees. An increased level of pollinator visits would increase the speed of seed maturation and the oil rate (Parker, 1981). Furthermore, the sugar composition of nectars in the genotypes to be paired for hybrid seed production could be screened out easily using gas chromatography (Fonta et al., 1985) or liquid chromatography methods (Erickson et al., 1979; Severson and Erickson, 1983; Vear et al., 1990) in order to avoid the pairing of genotypes with different amounts of sucrose.

Currently plant breeders, who face many other agronomic constraints, do not consider the sugar composition of nectars as an attractant to pollinators, either for screening procedures of genotypes or for more basic genetic studies. However, the effects of plant breeding on nectar secretion to improve entomophilous cross-pollination are taken into account in some species such as oilseed rape *Brassica napus* L. In this species, genotypes with initial depressed nectar production had their secretion restored through selection to increase pollinator attraction (Mesquida et al., 1988). We consider work directed towards increased nectar amounts or sugar amounts in nectar secretions as the most promising way to improve cross-pollination using insects. Other parameters, such as composition of amino acids, proteins (Baker and Baker, 1976), and ions (Waller et al., 1972), also may have an important influence on bees' choices.

Long-distance orientation cues such as aromas are associated with the food reward. In the sunflower, as in many other species [e.g., cotton (Hedin, 1976) or maize (Buttery et al., 1978)], the aromas are very complex mixtures whose composition is plant-specific; most constituents are common to all sunflower genotypes. From a restricted, active fraction of the common constituents, bees are able to generalize the total plant aroma. However, slight differences between the aromas of the different genotypes, as shown by comparing their chromatographic profiles, may be used as discriminative cues that enable foragers to select the best food source. The identification of active fractions and of com-

ponents specific to given genotypes would be of interest for plant breeders. It may be possible to combine plant genetic and chemical analyses to elucidate the genetic basis of volatile biosynthesis. This may aid the development of plants that produce an attractive aromatic blend, or, alternatively, to develop plants that are chemically resistant towards pest insects. Many studies have been conducted to identify plant chemicals acting as attractants or repellents towards pest insects (e.g., Finch, 1977; Visser et al., 1979; Gershenzon et al., 1981; Guérin et al., 1983; Dawson et al., 1989), but very limited work has been devoted to the chemicals involved in pollinator attraction. By combining behavioral and chemical analyses, we have shown that honeybees could use a reduced fraction of the total aroma to recognize the sunflower blend. Further studies using a direct coupling between gas chromatography and electroantennogram recordings have indicated that a limited range of components elicit the highest antennal responses (Thiery et al., 1990). As a matter of fact, investigations to identify volatile components involved in bee attraction may result in the identification of a range of active components but probably not as limited as plant chemicals involved in host recognition in pest insects. This may depend on the different behavioral repertoires of both kinds of insects. Taking into account the learning abilities of the honeybees, we may assume that they will not limit their response to very specific plant volatiles since they can learn to respond to other less specific components when they are associated with a food reward.

Elsewhere, attempts were made to use these learning abilities to improve the visits of forager bees to a target plant using either conditioning of bees to floral scents (Gühler, 1930; Koch, 1931; Burgett, 1980) or attraction mediated by pheromonal blends (Williams et al., 1981a,b). Such methods are of interest, particularly when a great amount of honeybee visits is needed on a crop during a short period of time. However, if long and regular foraging behavior is needed to obtain cross-pollination, then the use of olfactory stimuli will not be efficient if the food provided by the crop does not reward the bees. Another way of using the behavioral plasticity of honeybees would be to modify their olfactory preferences at early stages of their development. It has been shown that the honeybee antennal system becomes functional three days before emergence and that olfactory rearing conditions during preimaginal stages to early adult day-life could affect antennal sensitivity (Masson and Arnold, 1984) and synaptic density in the antennal lobe (Gascuel and Masson, 1987). Olfactory exposure at early adult stages was shown to induce behavioral changes towards the exposed odorant (Pham-Delegue et al., 1990). These data suggest that changes in the olfactory environment applied during a sensitive period may lead to predictable and durable behavioral changes. The possible application of this for controlled pollination using floral components as exposure stimuli must be considered.

Finally, a better knowledge about the allelochemicals involved in plant–

pollinator relationships will result in the determination of molecular criteria influencing honeybee selective foraging behavior. Such chemicals could be used either sprayed on the crop to attract pollinators with or without previous conditioning or produced by the target plants after integration into plant breeding programs. This latter field of experiments, which requires cooperation between geneticists, plant breeders, insect physiologists, and biochemists, seems to be the most promising prospect for new applications in the control of entomophilous pollination.

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