III. ORIENTATION BEHAVIOR OF LEAF-CUTTING ANT WORKERS *ATTA SEXDENS* TOWARDS A CO_2 SOURCE

Worker of *Atta sexdens* carrying a leaf fragment home to the nest

Introduction

As was shown in chapter one, the ability to perceive and react to CO_2 concentrations is important for microclimatic control in the nests of the leaf-cutting ant *Atta vollenweideri*. In chapter two, one possible behavioral reaction to increased CO_2 concentrations inside the nest was already discussed: the building of turrets on the nest mounds in order to facilitate nest ventilation. Besides in microclimatic control, behavioral reactions of ants to CO_2 have been described in several other contexts. Some ants show aggressive and/or alarming behavior, e.g. *Messor wasmanni* and *Megaponera foetens* as response to CO_2 (Harkness and Harkness 1988; Hölldobler *et al.* 1994), and CO_2 can modulate the response to other stimuli (Just 1998; Raub 1998). The digging behavior of *Solenopsis geminata* which has been described as a response to rescue nestmates, is induced by an increased CO_2 concentration of the soil (Hangartner 1969). A further well known phenomenon is aggregation at places with higher CO_2 concentration which has been reported e.g. for *Solenopsis saevissima* (Wilson 1962). For *Lasius fuliginosus* and *Pheidole pallidula* increased activity has been observed nearby the nest entrance and at elevated CO_2 concentration (Burkhardt 1991; Lechner 1995).

Aggregation and increased activity at the nest entrance have been discussed in terms of olfactory orientation, but none of the studies showed how the ants assess information about the direction towards a CO_2 source.

Olfactorial orientation can be defined as behavior performed in order to obtain information about an odor and the direction of the odor source. Perception of direction is difficult in olfaction, since in general the olfactory organs do not show directional sensitivity. Thus, direction information has to be gathered either indirectly by measuring wind direction or directly by successively or simultaneously measuring concentration differences in space.

The use of wind direction in olfactorial orientation has been described for numerous insects, mostly for flying species (Murlis *et al.* 1992; Kaissling 1997). In an environment where no wind occurs an odor gradient is established and different orientation strategies have to be applied. By moving the antennae differences in odor concentration can be perceived successively which e.g. is used by ants following a trail pheromone (Hangartner 1969). This strategy is also termed klinotaxis (Schöne 1983). The use of differences in odor concentration, simultaneously measured between the two antennae is termed tropotaxis. The ability to discriminate between two odor concentrations by klinotaxis or tropotaxis has been investigated in honey bees (*Apis mellifera*) (Martin 1964). Each orientation strategy puts different demands on the sensory organs and the receptor cells. This aspect will be examined in chapter five.

The present chapter examines whether ants (*Atta sexdens*) are able to orient in a CO_2 gradient and discusses the biological significance of such orientation behavior. Orientation towards a CO_2 source might play an important role in colony organization of leaf-cutting ants: workers returning from a foraging trip might use CO_2 as (additional) cue for orientation inside the (dark) channel system of the nest in order to bring their collected leaf fragments to the fungus chambers. The experiments presented in this chapter test this hypothesis.

Methods

Workers of *Atta sexdens rubropilosa* were obtained from a laboratory colony collected in Botucatú, São Paulo, Brazil. The colony was approx. 3 years old and its fungus garden occupied a volume of about 12 l. The colony was reared at 25°C and 50% relative humidity in a 12 h / 12 h photoperiod and fed predominantly with privet leaves (*Ligustrum vulgaris*).

Workers of the colony were allowed to leave the nest for foraging in a connected foraging arena. The nest entrance, a PVC pipe with a diameter of 3 cm, vertically connected to the nest. To separately test each individual at the same point (decision point) when returning to the nest from foraging, a decision box was mounted above the nest entrance as shown in Fig.3.1. The decision box was of Plexiglas and measured $19*9*6$ cm^3. It connected to the nest entrance through a hole in the ground. A partition divided the entrance (E) at the ground of the box and separated two chambers (C1; C2) at the back. The volume of the chambers C1 and C2 up to the open side, facing the bridge (B1), measured 243 ml each ($4.5*9*6$ cm³). A L-shaped wooden stick (diameter 0.3 cm) presented the first part of the bridge to the foraging arena. The vertical axis of B1 measured 8 cm, the horizontal axis 10 cm. From thereon a second bridge (B2) led 80 cm to the foraging arena.
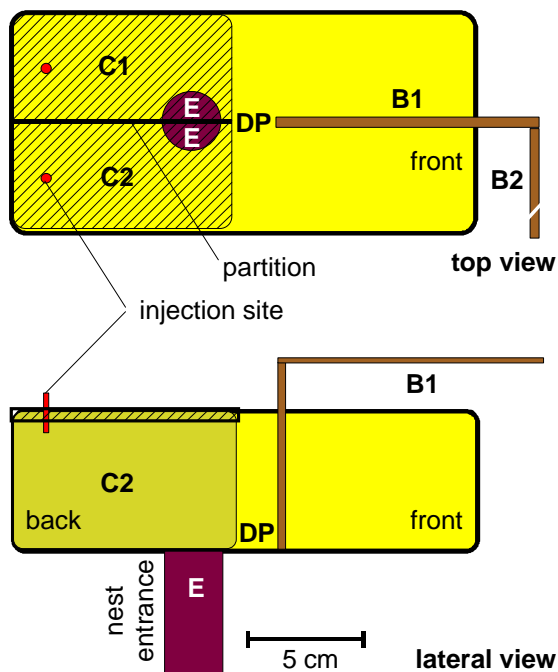

Fig.3.1 Modified nest entrance with decision box. When returning from the foraging arena the workers had to pass the bridge B2 and B1 (left turn), enter the decision box from above to the decision point (DP) from where they choose the chamber C1 or C2 and were removed by the experimentator. E: nest entrance which is closed for the returning workers.

Thus, in the experiment workers of the colony got access to a foraging arena by leaving the nest chamber through the entrance tunnel (E), entering the Plexiglas box (decision box), climbing the wooden stick B1, walking to its end and passing the 80 cm long bridge B2 to enter the arena. The two bridges (B1; B2) were connected either in line or with a turn of 90° to the right or left, respectively, which revealed to be decisive for choice of the workers when coming back to the nest (Fig.3.1; top view with left turn).

In the foraging arena (0.12 m²) fresh leaves were offered to the workers. As soon as the first worker started to carry a leaf fragment back to the nest the connection between entrance and decision box (DP) was cut by sliding a glass plate below the bottom of the decision box. At that time 600 to1000 workers had entered the foraging arena. All workers on the bridge and in the decision box were removed. In the following, each worker which arrived at the decision point was observed whether it entered the right (C1) or the left chamber (C2). After entering one of the two chambers the worker was removed. Thus, in each experiment each worker was tested only once.

Injection of CO_2

The CO_2 concentration inside the chambers C1 and C2 could be increased by injecting synthetic air containing either 10%, 2% or 0.1% CO_2, 21% O_2, and the remaining volume N_2. CO_2 was injected through the cover at the back of the chambers (Fig.3.1 injection site) and controlled with a magnetic valve (Lee Co, LFYX0500250AB). In order to avoid air jets in the chamber the injection was into a small piece of cotton wool. Using different pressure, air flow rates of 1.8 mlmin^{-1} and 7.2 mlmin^{-1} were injected. The resulting flow rates of CO_2 were 180 µlmin^{-1}, 144 µlmin^{-1}, 36 µlmin^{-1} and 10 µlmin^{-1}, respectively (Table 3.1). Injection of 10 µlmin^{-1} CO_2 was used as experiment to control for air flow.

Syn. air [CO_2] air flow rate	10%	2%	0.1%
1.8 mlmin^{-1}	180 µlmin^{-1}	36 µlmin^{-1}	---
7.2 mlmin^{-1}	---	144 µlmin^{-1}	10 µlmin^{-1}

Table 3.1 Resulting CO_2 flow rates in dependency of the used air flow and CO_2 concentration in the pressure tank

Injection into one of the two chambers was continuous, but the site of injection was altered between the two chambers (C1; C2) every 10 minutes. Injection sites were altered in order to exclude other olfactory cues and signals (trail pheromones). Since the chambers were closed except for the front side the establishment of a CO_2 gradient inside the chamber where injection took place can be expected, with higher concentration at the back than at the front side.

A further gradient can be expected at the decision point where air of the two chambers joins. Since the site of injection was altered, the gradient is inverted at the DP with an undefined delay. Shortly after changing the site of injection the CO_2 concentration is higher in the chamber with previous injection than in the chamber with actual injection.

No attempt was made to measure the actual concentration or the slope of the gradient at the decision point.

Data capture

Side preference

Side preference of returning workers was tested without injection of CO_2 into a chamber. In the experiments the connection between the two bridges was either in line or with a left or a right turn, respectively. The number of workers entering chamber C1 or C2 was counted over a period of 40 min. Workers were removed without counting when they failed to enter C1 or C2 during 5 s after arriving at the decision point. This occurred only in very few cases. The differences in side preference between the three experiments were tested with the Chi^2-test.

CO_2 preference

In the experiments with CO_2 injection into the two chambers the two bridges were connected in line. Data were taken throughout alternation of the injection site. In each experiment the site of injection was changed four times and the number of workers entering each chamber was counted. As soon as the site of injection was changed the decision of a worker to the site without injection was counted as decision for lower CO_2 concentration and vice versa. Number of workers choosing the chamber with CO_2 injection and without CO_2 injection, respectively, were pooled for the four alternations.

Workers were categorized concerning their load. Two categories were used, one for workers carrying a leaf fragment (loaded workers) and the other for workers carrying no leaf fragments (unloaded workers).

The differences in number of workers entering the chamber without injection of CO_2 and the chamber with CO_2 injection were tested with the Chi^2-test and with Fisher's exact test for small sample size (n<80) as was the case for the sample of loaded workers (Sokal and Rohlf 1995).

Side preference vs. CO_2 preference

In order to test whether the side preference of workers is influenced by CO_2 concentration the two bridges were connected as shown in Fig.3.1 (left turn). Site of injection of CO_2 was alternated as described above and the number of workers entering the right box were tested (Chi^2-test) with respect to the site of CO_2 injection.

All graphs show relative numbers of workers (%) since the number of workers arriving at the decision point per unit time (10 min) decreased throughout the experiments.

Results

Side preference

Workers returning from the foraging arena showed no side preference when returning in line to the decision box (114 workers to the left and 115 to the right) as shown in Fig.3.2. A strong side preference was found depending on the connection between the two bridges. Workers which had to turn left before arrival at the decision point ($n_1 = 142$) preferred the right box compared to workers which arrived in line ($n_2 = 229$). This preference is highly significant ($Chi^2 = 23,04$; $p < 0.01$). A preference to the opposite side was found for workers which had to turn right ($n_3 = 173$) before arrival at the decision point compared to workers which arrived

in line. This preference was highly significant as well ($Chi^2 = 15.82$; $p < 0.01$). The side preference of workers was inverse to the direction they had turned in shortly before, irrespective of whether it was a left or a right turn. The strength of the side preference to the inverse side was not different between 'right turn' and 'left turn' workers ($Chi^2 = 1,14$; $p = 0,28$).

It has to be mentioned that the nest and the foraging arena were at the same place in all three experiments. Only the decision box was turned around the nest entrance and the bridge B2 was moved parallel.
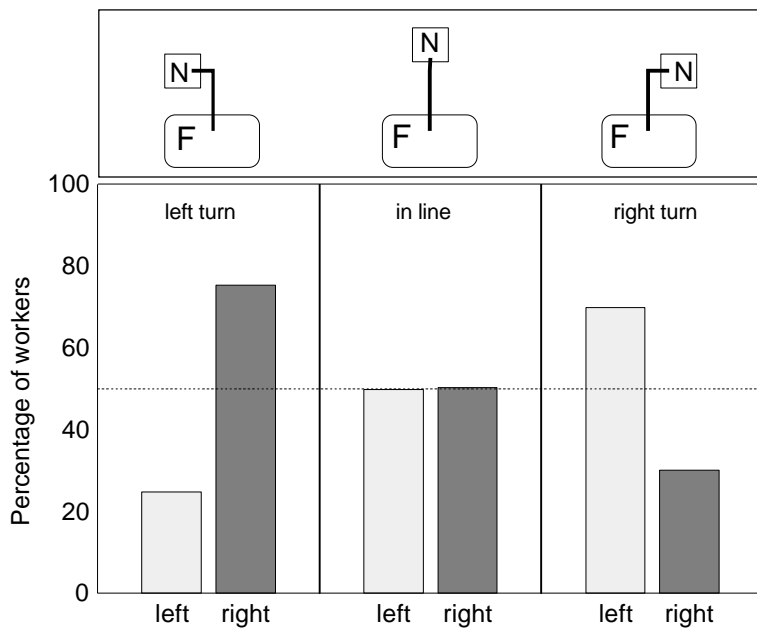

Fig.3.2 Side preference of workers returning to the nest in dependency of the experimental design (upper graph). Workers returning from the foraging arena (F) had to either turn left before entering the decision box (left turn), enter the decision box straight (in line) or turn right (right turn). Number of workers tested was in the range of 142 to 229 individuals.

CO$_2$ preference

Workers returning from the foraging arena showed a preference toward higher CO_2 concentration as shown in Fig.3.3. When CO_2 was injected with a flow rate of 180 µlmin^{-1} or 144 µlmin^{-1} unloaded workers significantly preferred the site of injection ($Chi^2 = 31.98$; $p < 0.01$ for 180 µlmin^{-1} and $Chi^2 = 31.98$; $p < 0.01$ for 144 µlmin^{-1} CO_2) compared to the preference shown by workers in the control experiment (with an injection of 10 µlmin^{-1} CO_2). Workers loaded with leaves also showed a significant preference at the same CO_2 flow rates towards the site of injection ($p < 0.01$; Fisher's exact test for 180 µlmin^{-1} and $p < 0.01$; Fisher's exact test for 144 µlmin^{-1}).

The preference of loaded workers was even stronger than the preference of unloaded workers ($p < 0.01$; Fisher's exact test for 180 µlmin^{-1} and 144 µlmin^{-1} CO_2). Injection of 36 µlmin^{-1} CO_2 did not influence the side preference, neither for loaded nor for unloaded workers compared to the control experiment ($Chi^2 = 73.97$; $p < 0.01$ for unloaded and $Chi^2 = 12.67$; $p < 0.01$ for loaded worker). The experiments with injection of 180 µlmin^{-1} and 144 µlmin^{-1} CO_2 were repeated twice and revealed similar (not significantly different) results. In the control

experiment unloaded and loaded workers showed a significant preference toward the site of injection compared to an expected symmetrical distribution for each of the four injection intervals ($Chi^2 = 73.97$; $p < 0.01$ for unloaded and $Chi^2 = 12.67$; $p < 0.01$ for loaded worker).
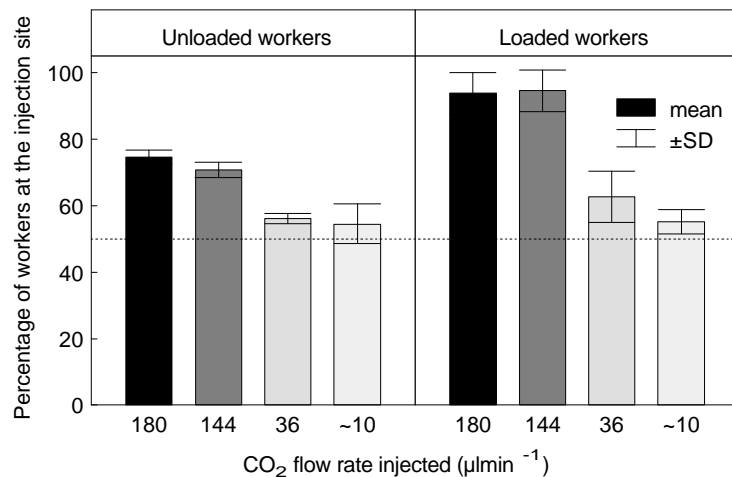

Fig.3.3 Preference of workers toward CO_2 injection site (higher CO_2 concentration) when returnng from the foraging arena. Workers carrying leaf fragments were counted as loaded workers, workers without fragments as unloaded workers. Total number of unloaded workers was in the range of 256 to 399 and of loaded workers in the range of 40 to 68 individuals for each experiment. The site of injection was changed four times during each experiment and the standard deviation presents the variability in these four intervals.

Importance of CO_2 for orientation

The two prior experiments showed that CO_2 induces a side preference toward the site of CO_2 injection and that workers prefer a side depending on the direction they turned into shortly before reaching the decision point. In this experiment CO_2 preference vs. the found side preference was tested. The side preference toward the right chamber (C1) after a left turn at the B2-B1 connection of the bridge was used in order to investigate whether the preference to the site of CO_2 injection could override this side preference. Irrespective of whether CO_2 was injected into the left chamber (C2) or into the right chamber (C1) the side preference to the right chamber remained high (76% vs. 73%). No significant difference in the number of workers choosing the right side was found ($Chi^2 = 1.48$; $p = 0.22$). Thus, in this experiment side preference dominates over orientation toward CO_2.
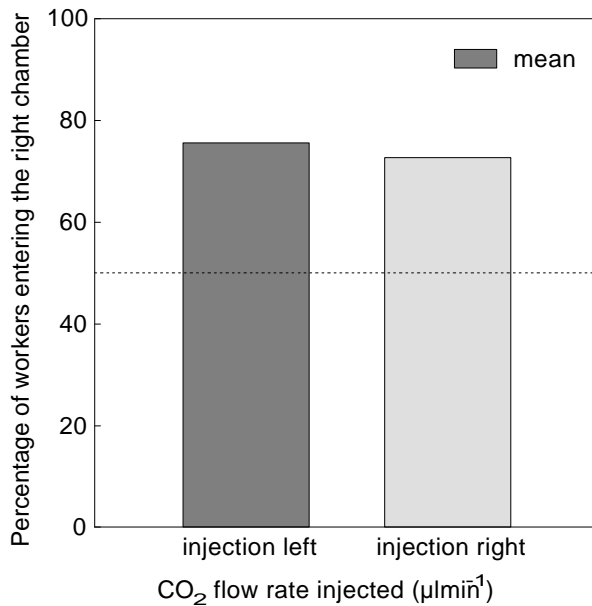
Fig.3.4 Side preference shown in Fig.3.2 (left turn) in dependency of increased CO_2 concentration in the left chamber (injection left) or in the right chamber (injection right). A total number of 259 workers was tested and injection site was changed four times.

Discussion

The described experiments revealed that leaf-cutting ants *Atta sexdens* can orient in a CO_2 gradient. When returning from a foraging trip a high percentage of more than 90% of workers carrying a leaf fragment chose the higher CO_2 concentration (injection of 144 $\mu lmin^{-1}$). Workers without leaf fragments showed the same kind of preference but to a lower extent (75%). This is the first evidence for orientation in a CO_2 gradient for ants.

For other insects orientation toward a CO_2 source has already been described in various contexts. Haematophagous flies use CO_2 to find their hosts (Anderson and Olkowski 1968; Warnes and Finlayson 1985). In wind tunnel experiments upwind flight behavior (anemotaxis) is elicited with changes in CO_2 concentration, but not at a constant concentration of 0.5% (Gilles 1980; Warnes and Finlayson 1985). The combination of host odor and CO_2 is often even more effective. For instance, fire bugs need host odor and elevated CO_2 concentration to be attracted (Núñez 1982). Adding CO_2 to baits for capturing biting insects can increase the catch by a factor of 70 (Kline *et al*. 1991).

CO_2 can also be of significance for host finding in phytophagous insects. This has been demonstrated with larvae of the wood-boring beetle *Orthosoma* (Coleoptera) moving in a gradient (Paim and Beckel 1964; White *et al*. 1974) to higher concentrations in expectation of finding fungi for feeding. Why the larvae of the corn earworm *Helicoverpa* (Lepidoptera) prefer higher CO_2 concentrations is less obvious (Rasch and Rembold 1994).

Elevated CO_2 concentrations also affect the selection of an oviposition site by adult females in the wood-boring beetle mentioned above (Paim and Beckel 1964). A higher concentration might indicate a suited substrate for the development of the larvae. Also for adult lepidopteran species CO_2 has been discussed as cue for finding host plants (Bogner 1990).

The orientation mechanisms used in these diverse contexts are understood only in a few cases. Adult Lepidoptera can measure small differences in CO_2 concentration with their specialized labial pit organ. Results obtained from electrophysiological recordings suggest that the lepidopteran CO_2 sense can readily detect the presumably small CO_2 gradients associated with plant metabolic activity (Stange 1992). A remarkable oviposition site selection has been described for *Cactoblastis* (Lepidoptera) where the females can detect the quality of the leaf by successive probing the air above the leaf surface with their labial palps (Myers *et al*. 1981; Stange 1996). Since the host plant *Opuntia stricta* is a CAM plant CO_2 fixation takes place at night. Thus, above the leaf surface the CO_2 concentration is slightly reduced at night. Although the concentration differences are minute, *Cactoblastis* can detected these differences due to the incredibly high resolution of their CO_2 receptor cells (about 0.5 ppm CO_2)(Stange *et al*. 1995). The CO_2 gradient is perceived by successive probing (klinotaxis) the air in different heights above the leaf surface.

Integration between the imput of both antennae (tropotaxis) was so far tested only with stable stimulus intensities at the antennae. Martin found that in honey bees a 2:1 ratio of odor concentration between the two antennae was needed to elicit tropotaxis (Martin 1964). Under natural conditions such gradients rarely occur more than a few centimeters from the source (Neuhaus 1965). However, by moving both antennae in a gradient, klinotaxis is possible based on the information acquired by each antenna separately. In addition, by integration of such information from both antennae (tropotaxis) the orientation ability might be increased. This would be simultaneous tropotaxis and klinotaxis.

The mechanisms underlying the described orientation of *Atta sexdens* in a CO_2 gradient is not clear. Anemotaxis can be excluded, since the flow rate of air at the decision point can be expected to be far below the smallest flow rates detectable for insects (about 1.5 $cmsec^{-1}$)(Bell and Kramer 1979; Böhm 1995). 7.2 $mlmin^{-1}$ were injected at most which results in a flow rate below 0.1 $mmsec^{-1}$ through the open front side of the chambers. Injection was vertical and into a piece of cotton wool, thus air jets at the decision point cannot have occurred. Whether the ants use tropotaxis or klinotaxis or a combination of both remains to be tested.

The preference of the ants for the site of injection in the control experiment has to be considered as preference for an unknown odor in the air used from the pressure tank or for a higher CO_2 concentration as well (unfortunately no data were available for the room CO_2 concentration).

The CO_2 flow rates (up to 0.18 $mlmin^{-1}$) used in this experiments were lower than the CO_2 production rate in one chamber with fungus and ants (about 1 $mlmin^{-1}$; see chapter one). Therefore the stimulus intensity in this experiment did not exceed naturally encountered intensities in the channel system of the giant nests. For foraging workers of leaf-cutting ants moving through the dark nest channels a CO_2 gradient might indicate the proximity of a large chamber with fungus or a chamber where high metabolic activity takes place. Finding the right place for depositing leaf fragments in a short time increases foraging efficiency. In order to evaluate places for deposition of leaf fragments CO_2 concentrations and CO_2 gradients might help the ants.

This hypothesis is supported by the finding that workers orientate in a CO_2 gradient towards higher concentrations and by the fact that workers carrying leaf fragments choose the site of higher CO_2 concentration with a higher probability than unloaded workers.

The preference to higher CO_2 concentrations shows that the leaf-cutting ants can use a CO_2 gradient as cue for orientation. Under certain circumstances, however, this preference is superimposed by other orientation strategies. This was illustrated in the experiment were an existing side preference of the workers could not be influenced by CO_2 injection. The existing side preference can be explained by orientation to visual cues in the surrounding of the experimental set-up. In social hymenopteran visual orientation has been intensively studied in honey bees, but also in ants, e.g. for the tropical ant *Odontomachus bauri* the use of the light pattern of the forest canopy for orientation has been shown (Oliveira and Hölldobler 1989).

Therefore, a possible explanation for the side preference irrespectively of the CO_2 gradient could be use of landmarks. A second possibility explaining the side preference is path integration with a systematical error (Wehner 1984).

Thus, when other (visual) orientation cues are available, orientation towards a CO_2 source seems to be superimposed. This is an interesting finding in light of the question whether there is a hierarchy in orientation cues. However, in the natural context of CO_2 orientation towards fungus chambers inside the dark nest visual cues are lacking.

In conclusion, it could be demonstrated that leaf-cutting ants can orientate in a CO_2 gradient. When returning from foraging the ants turn toward the higher CO_2 concentration. This effect was found in all workers, but it was pronounced for workers carrying leaf fragments compared to workers without leaf fragments. The findings reported in this study support the hypothesis that CO_2 gradients are used as a cue inside the nest to find suited fungus chambers for unloading the leaf fragments.