

# **Odor of achlorophyllous plants' seeds drives seed-dispersing ants**

## **Author information**

Mikihisa YAMADA<sup>1</sup>, Masaru K. HOJO<sup>2</sup>, Akio IMAMURA<sup>1</sup>

1. Hokkaido University of Education, Asahikawa Campus,

Hokumon-cho 9, Asahikawa, Hokkaido 070-8621, Japan

2. Department of Bioscience, School of Science and Technology, Kwansei Gakuin

University, Sanda, Hyogo 669-1337, Japan.

correspondence: Akio Imamura

E-mail: ginryou715@yahoo.co.jp

ORCID ID 0000-0002-5208-1936

## **Declarations**

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### **Author contributions**

A.I. conceived the study, conducted analyses, and wrote the manuscript. K.Y. conducted experiments. M.K. Hojo performed a gas chromatograph analysis and checked the statistical analyses.

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### **Conflicts of interest**

There are no conflicts of interest to declare.

### **Data availability statement**

All relevant data are within the paper and its Supporting Information files.

## Abstract

Seed dispersal by ants is an important means of migration for plants. Although many myrmecochorous plants have seeds containing elaiosome, a nutritional reward for ants, some non-myrmecochorous seeds without elaiosomes are also dispersed by ant species. However, the mechanism by which seeds without elaiosomes enable efficient dispersal by ants is scarcely investigated. The seeds of the achlorophyllous and myco-heterotrophic herbaceous plant *Monotropastrum humile* are very small without elaiosomes and require a fungal host for germination and survival. We performed a bioassay using seeds of *M. humile* and the ant *Nylanderia flavipes* to demonstrate ant-mediated seed dispersal. We also analyzed the volatile odors emitted from *M. humile* seeds and conducted bioassays using dummy seeds coated with seed volatiles. Although elaiosomes were absent from the *M. humile* seeds, the ants carried the seeds to their nests. They also carried the dummy seeds coated with the seed volatile mixture to the nest, and left some dummy seeds inside the nest and discarded the rest of the dummy seeds outside the nest with a bias toward locations with moisture conditions, which might be conducive to germination. We concluded that seeds of *M. humile* were dispersed by the ants, and that seed odors were sufficient to induce directed dispersal even without elaiosomes. It is probable that the fleshy fruit producing genus *Monotropastrum* evolved from the related anemochorous genus *Monotropa*, which produces capsule fruit. This transformation from anemochory to myrmecochory presents a novel evolutionary pathway toward ant-mediated seed dispersal in an achlorophyllous plant.

## KEYWORDS

directed seed dispersal, elaiosome, myco-heterotrophic plant, myrmecochory, seed volatiles

## 1 | INTRODUCTION

Various seed and fruit traits, such as size and color, tend to be correlated, forming what are known as “dispersal syndromes” that may have arisen to attract particular dispersers (Brodie, 2017; Valenta & Nevo, 2020) or to facilitate transport from their parent plants via wind or currents (Nilsson et al., 1991; Bullock & Claeke, 2000; Ohnishi et al., 2008; Nathan et al., 2011). Seed dispersal depending on ants is called myrmecochory. Myrmecochory is widespread among angiosperms and ecologically important; over 11,000 species of myrmecochorous plants in 77 families and 334 genera participate in myrmecochory across various ecosystems (Lengyel et al., 2010).

Seeds of many myrmecochorous plants possess appendages, such as elaiosomes, containing various fatty acids and proteins. These are likely to be nutritional rewards for ants that disperse the seeds (Brew et al., 1989; Lanza et al., 1992). If the seeds are carried to an ant nest, the elaiosomes are generally consumed inside the nest before the seeds are discarded (Culver & Beattie, 1978; Giladi 2006: Fokuhl et al., 2007; Fokuhl et al., 2012). Various studies have shown that nutritional contents, such as lipids, amino acids, and proteins, in the elaiosome are important for inducing seed dispersal (Fischer et al., 2008; Pizo & Oliveira, 2001; Sasidharan & Venkatesan, 2019; Anjos et al. 2020). In contrast, some granivorous ants, such as species of the genera *Messor*, *Pheidole*, and *Tetramorium*, lose or abandon their non-myrmecochorous seeds during transportation and thus disperse seeds without elaiosomes (Retana et al., 2004; Kobayashi, 2009, Ohnishi et al., 2008; Ohnishi et al., 2013; Clemente & Whitehead 2020). Although some chemical signaling might be involved (Youngsteadt et al.,

2008; Pizo & Oliveira, 2001), the mechanism by which seeds without elaiosomes enable efficient dispersal by ants is less investigated. Ohnishi et al. (2008) and Ohnishi et al. (2013) showed that elaiosome-less seeds of *Chamaesyce* plants were carried by some ant species and that their seed dispersal was effective, although they have not determined the ant-attracting chemical components of the *Chamaesyce* seeds.

One of the evolutionary significances of myrmecochory is directed seed dispersal. The advantages of the directed ant-dispersal hypothesis are that ant nests maintain moist conditions and the seeds carried to the nest can escape desiccation and have a higher seedling survival (Levey & Byrne, 1993, Anjos et al. 2020), and the ants bury the seeds at depths where humidity, temperature, and other conditions are suitable for germination (Gibson, 1993, Anjos et al. 2020). However, it is unclear whether seeds are discarded on suitable sites in a directed manner in species without elaiosomes.

*Monotropastrum humile* (D. Don) H. Hara (Ericaceae) is achlorophyllous—that is, it does not have chlorophyll and does not perform photosynthesis—and inhabits forest floors in the temperate regions of Asia (Ohashi et al. 2016). The plant is approximately 10 cm tall and parasitizes *Russula* and *Lactarius* species of Russulaceae (Bidartondo and Bruns, 2001). *M. humile* is a full myco-heterotrophic species, meaning that it parasitizes fungi throughout its life (Merckx, 2013). Seeds are smaller than 0.3 mm in length and require their host fungi for germination because the seeds have only 12 cells, including the two embryos (Tanaka & Morita 1999). Thus, seed destination is a determinant of survival because such small seeds do not have appendages, such as elaiosomes. Its host, the mycorrhizal fungi of *Russula* species, inhabit the

shallow areas of the forest floor (Rachel, 2004; Courty et al., 2008), and Imamura and Kurogi (2003) reported that *M. humile* roots inhabit the forest floor to a depth of 5–10 cm. For seed dispersal, *M. humile* uses insects wandering on the ground when their berries fall to the ground during the fruiting season. Cockroaches (Uehara & Sugiura, 2017) and camel crickets (Suetsugu, 2017) have been reported as seed dispersers via endozoochory. According to these studies, *M. humile* berries fall to the ground during the fruiting season, and the wandering insects are presumed to consume the pulpy flesh around the seeds and consequently contribute to the dispersal of the seeds involved in their foraging behavior.

Although *M. humile* seeds have no elaiosomes and seem to be non-myrmecocohorous plants (Suetsugu, 2017; Uehara & Sugiura, 2017), Suetsugu (2017) and Uehara and Sugiura (2017) have reported that several ant species, including *Nylanderia flavipes* Smith (Formicidae: Formicinae), frequently visit *M. humile* fruits. In addition, we have observed, using a fixed-point camera, that *N. flavipes* ants are attracted to the fruits and seeds of *M. humile* and that some of the seeds, although not many, were carried by them. Since the seeds are coated with a phlegmatic organic layer, it is possible that *M. humile* attracts ants with fruit- and seed odors and that their seeds are carried by the ants. The seeds might then be abandoned after the outer components are consumed (Sasidharan & Venkatesan 2019). Consequently, the seed may be dispersed to suitable, host-inhabited sites on the forest floor.

In this study, we investigated the seed dispersal of an achlorophyllous plant *M. humile* without specialized nutritional rewards to ants. Since the elaiosome-less seeds of this achlorophyllous plant are very small and difficult to track in the field, we first performed a

bioassay using intact and odorless seeds to verify whether the seed odors of *M. humile* are key signals for dispersal by ants. Afterwards, bioassays were performed using small pieces of paper coated with the odor component of *M. humile* seeds as dummy seeds. We tested whether the dummy seeds were carried into the nests and were discarded to specific locations by the ants.

## 2 | MATERIALS AND METHODS

### 2.1 | Plant and ant materials

We used the polyphagous ant species *N. flavipes*, which has been shown to collect plant seeds in the field (Tanaka & Tokuda, 2016; Hosoishi et al., 2019). Ten *N. flavipes* colonies were collected from June to July 2019 from sites at Yuminariyama Mountain and the Hokkaido University of Education Asahikawa Campus in Hokkaido, Japan (approximately 43.79N, 142.33E). All of the colonies collected were queenless, consisting of approximately 60 workers and numerous eggs, pupae, and larvae. *M. humile* populations and *N. flavipes* colonies coexist sympatrically at Yuminariyama Mountain. We also collected *M. humile* fruits at Yuminariyama Mountain in August 2019 and stored them in a screw tube at 4 °C.

The ant colonies were kept in a container that consisted of an artificial nest and a foraging arena (Figure 1). A polypropylene box (200 mm length × 136 mm width × 68 mm height) was used as the foraging arena. Fluon was applied to the upper 3 cm inside the arena to prevent ant escape. A vinyl tube (10 mm diameter × 120 mm length) was used as an artificial nest and connected to the side wall of the container at a height of 15 mm. To prevent the

artificial nest from drying out, an absorbent piece of cotton was inserted at the wall-side of the nest and moistened with distilled water. The absorbent cotton was replaced every two weeks.

The foraging arena was divided into four equivalent sections to investigate whether the ants discarded seeds in areas with different conditions. The four sections were filled with dry glass beads (1.5–2.5 mm in diameter), moistened glass beads, dry cotton wool, and moistened cotton wool.

Glass beads simulated an environment **chemically as inorganic** substances, such as sand or small pebbles on the forest floor, and each section was filled with approximately 10 g of beads. Cotton wool simulated an environment **chemically as organic** substances, such as fallen and decomposing litter, and each section was filled with approximately 0.25 g of cotton. The entrance to the nest was placed at the center of the arena so that the four sections were equally aligned for the ants. The entrance of the nest floats was bridged with filter paper (Figure 1a). In the bioassay, the seeds or the dummy seeds were placed at the center of the arena near where the bridge connected the different **sections (Figure 1b)**.

To moisten two of the sections, 5 mL of distilled water was added every three days. All the containers were kept indoors at 25 °C. The ants were fed tuna and honey.

## **2.2 | Volatile collections and chemical analyses**

The volatiles emitted from *M. humile* seeds were collected using solid phase micro extraction (SPME) with a 66-μm PDMS/DVB fiber (Supelco, Bellefonte, PA, USA). The seeds with pulp were placed in 4-mL glass vials and sealed with aluminum foil. The SPME fiber was inserted into the covered vial and volatiles were sampled for 1 h at room temperature. Blank

assays with empty vials were performed before each volatile collection session. Three independent samples were collected and analyzed.

Immediately after sampling, the SPME fiber was injected into the split/splitless injector of a gas chromatograph (GC17A; Shimadzu, Japan) equipped with a DB-WAX column (30 m length  $\times$  0.25 mm inner diameter  $\times$  0.25  $\mu$ m film thickness; J & W Scientific Inc., Folsom, CA, USA) and mass spectrometry detectors (QP5000; Shimadzu, Japan) with electron impact ionization (70 eV). The oven temperature was maintained at 40 °C for 5 min, programmed to increase to 220 °C at a rate of 10 °C min<sup>-1</sup>, and held isothermally for 10 min. The SPME fiber was desorbed in splitless mode for 1 min, while the injector and interface temperatures were at 220 °C. Helium was used as the carrier gas, and the column head pressure was 100 kPa. Volatile compounds were tentatively identified by matches with the NIST mass spectral database, and identifications were confirmed by matching mass spectra and Kovat's retention index with those of commercially available authentic standards.

### 2.3 | Bioassay using *M. humile* seeds

We performed bioassays using seeds of *M. humile*, 20 fresh untreated or solvent-treated seeds (odorless seeds) per turn of the bioassay. For the solvent treated seeds, 20 fresh seeds of *M. humile* were soaked in ethanol (99.5%, Wako Pure Chemical Industries, Ltd.) for 30 min. Four replicate colonies resulted in a total of 80 seeds for each treatment. Untreated and solvent treated seeds were assayed separately to avoid odor transfer. The colonies were starved (only given water) for 72 h before performing the assays. After the seeds were set at the center of the arena, the number of seeds carried to the nest by the ants was recorded every 15 min for

90 min.

## 2.4 | Bioassay using the dummy seeds made of filter paper

We performed a bioassay using dummy seeds made of a square bit of filter paper (2 mm × 2 mm) to track the dummy seeds with marks by carbon pencil. The dummy seeds were soaked with the volatile compounds of *M. humile* seeds (Table 1). These compounds were purchased from Wako Pure Chemical Industries (Osaka, Japan) or Tokyo Kasei (Tokyo, Japan) with >98.0% purity.

In this dummy-seed assay, 20 pieces of filter paper were numerically numbered with a carbon pencil to track the outcome of the dummy seeds that were used. The odor treatment in this assay consisted of eight conditions: each of the six odors identified alone (six conditions), a mixture of equivalent amounts of the six odors (one condition), and the control condition (solvent alone). Each odor component was diluted to a final concentration of 100 ng/μL using 99.5% ethanol as a solvent. Twenty pieces of filter paper were soaked in 20 μL odor solution (1 μL for each filter paper). Dummy seeds soaked with solvent were used as the control. Each of the odor treatments was replicated six times using six different colonies, and each colony experienced each treatment only once. The colonies were starved (only given water) for 72 h before performing the assays.

The order of the odor treatments that were served to each colony was randomized using the 'sample' function of the base package of R 3.5.2 for Mac OSX (R core team, 2018). Dummy seeds were placed at the center of the arena, and the number of dummy seeds carried to the nest by the ants was recorded every 15 min for 90 min. After 72 h from setting the dummy

seeds, the number of dummy seeds inside the nest and discarded outside the nest was recorded. For discarded dummy seeds, we also recorded their destination and numerical number on the dummy seeds.

## 2.5 | Statistical analysis

All analyses were executed using R 3.5.2 for Mac OSX. The package 'tidyverse' was used for data shaping and arrangement. The factors affecting the number of seeds (or dummy seeds) carried by ants were analyzed using a generalized linear mixed model (GLMM) and likelihood ratio test. We used the zero-inflated Poisson distribution with the function glmmTMB from the package 'glmmTMB'. In this analysis, colony identity was set as the random effect and odor conditions were the explanatory variables. Chi-square tests were performed to examine whether the destination of the dummy seeds was biased.

## RESULTS

### 3.1 | Volatile chemical composition of *M. humile* seeds

Chemical analyses of seed volatiles revealed the presence of several substances. By comparing the results with blank analyses, we found eight compounds that were derived from *M. humile* seeds (Figure 2). Among the eight peaks, six were identified as isobutyl alcohol, isoamyl alcohol, isoamyl hexanoate, linalool, isobutyric acid, and  $\alpha$ -terpineol (Table 1). Peaks 2 and 8 could not be identified from the obtained mass spectra.

### 3.2 | Bioassay using *M. humile* seeds

The ants carried away **fresh** *M. humile* seeds in 90 min. The number of *M. humile* seeds carried to the nest within 90 min is shown in Table 2. Nine of the 80 untreated seeds were carried to the nest, although none of the **solvent-treated** seeds were carried to the nest (Figure 3, Table S1). GLMM analysis revealed that untreated seeds were carried away significantly more than treated seeds (Table S2).

### 3.3 | Bioassay using filter paper as dummy seed

Ants did not carry the dummy seeds **treated with odor components** to the nest in the observed 90 min but did in 72 h (Table S3). A total of 30 out of 840 odor-treated dummy seeds were carried to the nest. After comparing the number of removed dummy seeds for each odor treatment, the six-odor-mixture treatment was carried most to the nest according to the GLMM analysis (Table 2, Figure 4).

**Of the 30 dummy seeds collected by the ants, 17 were discarded (Figure 5). The 13 seeds not discarded were left inside the nests.** A chi-square test was performed on the following six categories: the four sections of the foraging field, the empty area at the center of the field, and inside the nest. Based on this analysis, there was a significant bias toward discarding to a specific location ( $\chi^2 = 20$ ,  $df = 5$ ,  $P < 0.001$ ). When we performed a chi-square test on the four categories regarding the composition of the discard location, that is, glass beads, water-moistened glass beads, cotton wool, and water-moistened cotton wool, the distribution was also significantly biased ( $\chi^2 = 10$ ,  $df = 3$ ,  $P < 0.01$ ), and most of the seeds were discarded on moistened glass beads (Figure 5).

## 4 | DISCUSSION

### 4.1 | Dispersal of elaiosome-less seeds by ants

In this study, we first verified that *M. humile* seeds without elaiosomes indeed attracted ants. We confirmed that 9 out of 80 untreated seeds were carried to the ant nest, whereas no solvent-treated seeds were collected (Table S1). We also found that by using dummy seeds coated with the odor components of *M. humile* seeds, dummy seeds treated with a mixture of six major components were carried to the nest by the ants, and the dummy seeds treated with the solvent were not carried away (Table S3). These results indicate that volatile seed odors are involved in attracting ants and inducing seed-carrying behavior.

Among the six main odor components, linalool and  $\alpha$ -terpineol were identified as floral scents of *M. humile*, but the other four components were not identified (Kubo & Ono, 2014). These results indicate that *M. humile* seeds attract ants via their seed or fruit-specific odor mixture. In the pepper family plant, *Peperomia macrostachya*, a mixture of seed odors efficiently attracts the ant, *Camponotus femoratus* (Youngsteadt et al., 2008). Since the volatile seed odors would have disappeared in 72 h, the dummy seeds were abandoned outside the nest. Similarly, the seeds are likely to be abandoned after the outer fruity coating components are consumed. Therefore, even in the absence of elaiosomes, it is possible that *M. humile* could disperse their seeds through ants using a specific blend of odor components.

This plant does not depend solely on myrmecochory, as Suetsugu (2017) has reported that many seeds have been recovered from the excretion of herbivorous insects. Hence, ants are

thought to participate in the seed dispersal of *M. humile*, in addition to herbivorous insects, such as cockroaches and camel crickets (Suetsugu, 2017; Uehara & Sugiura, 2017). Since the number of seeds carried away in our assay was relatively small, myrmecochory of the species could be just one aspect of their zoochory.

#### 4.2 | Implication by tracking of dummy seeds

Seed dispersal by ants could be beneficial for achlorophyllous species. Although we could not track where the real seeds were discarded because of their small size, the author M. Y. observed that some of the real seeds carried into the nests were left in a corner of the nest (data not shown). Thus, the ant nest could be one of the seed destinations, although granivory inside the nest by ants cannot be rejected. Dummy seeds coated with mixed odors were carried into the nest and subsequently discarded outside of the nest by the ants. Despite the fact that 13 dummy seeds were left inside the nest (17 were discarded outside), it appears that seeds left in the nest can also be considered dispersed because *N. flavipes* nests are located under litter or on rotting trees (Kallal & LaPolla, 2012). Although we did not copy the true conditions of forest floors, the target of discards was biased, and the dummy seeds were discarded mostly onto the water-moistened glass beads. In the ant species *Myrmica rubra*, it is known that workers discard inert items and corpses in different destinations (Diez et al., 2012). In our study, it was also observed that ant corpses were disposed of in cotton-wool compartments, where the dummy seeds were not discarded. These results suggest that the ants might differentiate between discard destinations depending on the material, and that the target of ants for discarded seeds may be areas like the water moistened sections in our experiments.

Mycorrhizal fungi of *Russula* species as hosts of *M. humile* inhabit the top shallow layer of the forest floor (Rachel, 2004; Courty et al., 2008). Thus, where the ant nests are located and where the seeds are discarded (moist soil near the surface) is considered to be close to their hosts and to be suitable for germination, growth, and survival of *M. humile* seeds, although mycorrhizal roots have not been quantified before. If biased discarding, as suggested by this study, also occurs under natural conditions, it would support two seed-dispersal hypotheses: 1) seeds relocated to moist environments, including ant nests, can escape desiccation and have enhanced germination and seedling survival; 2) seeds relocated to ant nests can also escape predators (modified from Hanzawa et al., 1988; Boyd, 2001). Further experiments with intact seeds under simulated natural conditions are needed to confirm the directed dispersal of *M. humile* seeds by ants.

With regards to myco-heterotrophic plants, large parts of them produce dust-like and anemochorous seeds, which require host fungus in their germination, and which can disperse over long distances (Merckx, 2013). Thus, there is a trade-off in seed dispersal between distance and direction. The genus *Monotropastrum*, which produces fleshy fruits, is thought to have evolved from the related anemochorous genus *Monotropa*, which produces capsule fruit (Bidartondo & Bruns, 2001). This speciation is considered to be from anemochory (distance dispersal) to zoochory, including myrmecochory. Therefore, the verification of the role of myco-heterotrophic herbaceous species in the temperate forests of Asia and of the role of seeds without specialized rewards for ants, presents a new aspect of directed seed dispersal of achlorophyllous and myco-heterotrophic plants involving ants.

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315 **5 | CONCLUSION AND CAVEATS**

316 We verified that reward-less seeds of achlorophyllous and myco-heterotrophic *M. humile* were  
317 dispersed by ants. We found that seed-specific volatile mixtures are involved in the attraction of  
318 ants and subsequent seed dispersal. A part of the dummy seeds was left in the ant nests and the  
319 other part may be discarded outside the nest directionally, although we could not evaluate the  
320 fate of real seeds. In future studies, we must track the fate of genuine seeds and investigate the  
321 spatial distribution of the plants and ant nests to validate the directed seed dispersal of the  
322 achlorophyllous plant *M. humile*. An expected method to perform this bioassay is to copy  
323 various conditions of the forest floors and examine the directed dispersal of seeds by ants in  
324 detail.

325

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330

331 **Author contributions**

332 A.I. conceived the study, conducted the analyses, and wrote the manuscript. K.Y.  
333 conducted the experiments. M.K. Hojo performed a gas chromatograph analysis and  
334 checked the statistical analyses.

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### Conflict of interests

There are no conflicts of interest to declare.

### Data availability

All relevant data are within the paper and its Supporting Information files.

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 449

Table 1 Odor components of *M. humile* seeds identified by GC/MS analysis. Six identified components and their mixture were used in our bioassays.

Peak No.	Components	Molecular formula	Molecular weight	Retention Index	Diagnostic Ions (m/z)
1	Isobutyl alcohol	C <sub>4</sub> H <sub>10</sub> O	74.1	1073	33, 43, 55, 74
2	unidentified	-	-	1186	43, 55, 70
3	Isoamyl alcohol	C <sub>5</sub> H <sub>12</sub> O	88.2	1208	42, 55, 70
4	Isoamyl hexanoate	C <sub>11</sub> H <sub>22</sub> O <sub>2</sub>	186.3	1480	43, 70, 99, 117
5	Linalool	C <sub>10</sub> H <sub>18</sub> O	154.2	1573	41, 43, 55, 71, 93, 121, 136, 154
6	Isobutyric acid	C <sub>4</sub> H <sub>8</sub> O <sub>2</sub>	88.1	1588	43, 73, 88
7	α- terpineol 98.0%	C <sub>10</sub> H <sub>18</sub> O	154.2	1725	43, 59, 81, 93, 121, 136
8	unidentified	-	-	1852	41, 60, 69, 93, 123

Peak numbers correspond to those in Figure 2

Table 2 Results of the generalized linear mixed model (GLMM) and likelihood ratio test on the number of dummy seeds carried by ants with a zero-inflated Poisson distribution. The colony identity was set as the random effect. The odor component was the explanatory variable.

Conditional model	Variance	Std. Div.		
(Intercept)	8.52e-12	2.92e-06		
Components	Estimate	Std. Error	z value	Pr(> z )
Isobutyl alcohol	-22.325	3.17e+04	-0.001	0.999
Isoamyl alcohol	-0.866	7.78e-01	-1.113	0.266
Isoamyl hexanoate	-0.866	7.78e-01	-1.113	0.266
Isobutyric acid	-22.325	3.17e+04	-0.001	0.999
Linalool	-0.228	5.54e-01	-0.411	0.681
$\alpha$ - terpineol	-0.920	7.50e-01	-1.226	0.220
Mixture of six components	1.370	2.31e-01	5.927	< 0.001
Ethanol as control	-22.325	3.17e+04	-0.001	0.999
	Estimate	Std. Error	value	Pr(> z )
Estimate of Zero-inflation model				
(Intercept)	-1.52	1.14	-1.33	0.18