Can Batesian mimicry help plants to deter herbivory?

Article in Pest Management Science · June 2007
Impact Factor: 2.69 · DOI: 10.1002/ps.1360 · Source: PubMed

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Can Batesian mimicry help plants to deter herbivores?

Giovanna Massei,* Jane V Cotterill, Julia C Coats, Gareth Bryning and Dave P Cowan
Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK

Abstract: Several authors have suggested that edible plants could avoid herbivory by mimicking olfactory cues of toxic plants. However, very few studies have been carried out to test this hypothesis. The aims of the present study were to identify the volatiles of three clover species and to test whether a species lacking chemical defences, such as red clover, could avoid being grazed by rabbits by mimicking the volatiles of the cyanogenic white clover. Two main volatiles were identified in all three clover species, and a further two volatiles were present in white clover only. Rabbits presented with a choice between white clover, red clover and red clover sprayed with white clover extract ate significantly more red clover than white or white-flavoured red clover. The results suggest that the volatiles of toxic plants could be used and exploited as a source of natural, safe and effective repellents to control the impact of pest herbivores on plants.

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Keywords: Trifolium spp.; Batesian mimicry; mammalian herbivores; natural repellents; plant defences

1 INTRODUCTION
One classic ecological paradigm that has the potential to accelerate the discovery and effective application of novel, safe chemicals to protect vulnerable crops or commodities is the concept of Batesian mimicry. Batesian mimicry occurs when predators learn to avoid attacking a noxious prey characterised by specific visual cues (the model) and also to avoid edible, non-toxic prey producing similar cues (the mimic).1,2 By mimicking key features of toxic organisms, nontoxic prey benefit from reduced predation. Several authors have suggested that some plant species might rely on Batesian mimicry systems to escape herbivore attack.3–5 However, few empirical studies have tested this hypothesis.6,7

When searching for suitable food, most mammalian herbivores acquire aversions to harmful plant species on the basis of odour and taste rather than appearance. Plants containing toxins also frequently contain other secondary metabolites, often volatiles, that herbivores learn to associate with the negative consequences of ingesting such plants.7 These volatiles act as defence signals that advertise the toxicity of the plant,3 thus protecting it from feeding damage and the herbivores from ingesting toxins.8–10 Herbivores that acquire a generalised aversion to the volatiles of toxic plants should also avoid consuming species imitating these warning signals but lacking the associated toxins of the model species. Consequently, these systems are likely to be a readily exploitable, rich source of effective and safe repellents.

The present study aimed to provide proof of the concept that the Batesian mimicry paradigm can be exploited as a basis for developing novel repellents that could be used to protect vulnerable crops or commodities.

A natural Batesian complex was reproduced, comprising one herbivore, the wild rabbit (Oryctolagus cuniculus L.) and three species of clover: red clover (Trifolium pratense L.) and strawberry clover (Trifolium fragiferum L.), which appear regularly in the diet of rodents and lagomorphs,11,12 and white clover (Trifolium repens L.), which contains cyanogenic glucosides known to deter herbivores.13 All three clover species may occur naturally in the same area where they are selectively consumed (red and strawberry clover) or avoided (white clover) by lagomorphs.14 The aims of this study were:

• to use a natural system to identify plant volatiles that could be used by harmful species such as white clover to advertise their toxicity to herbivores;
• to test whether mammalian herbivores avoid consuming plants that lack chemical defences (experimental mimic) but mimic the odour of closely related, visually similar toxic plants (experimental model);
• to assess the persistence of herbivore response to the mimic plant species.

2 METHODS
2.1 Experimental plants and animals
All clover species were grown by sowing seeds (2 g) onto the surface of moist compost (JIC No. 3) in plastic trays (40 × 22 cm). Plants were used approximately
8–10 weeks later. Three species of clover were used in experiment 1: red clover, strawberry clover and two varieties of white clover, *T. repens* var. Herbiseed and *T. repens* var. Aran, the latter well known to contain a high concentration of cyanogenic glucosides. In experiment 2, only one white clover variety, *T. repens* var. Aran, was used, along with red and strawberry clover. Rabbits (n = six males and eight females) were trapped from a wild population and housed singly in outdoor pens (1.8 × 5 m). Rabbits were offered water and rabbit pelleted food (RABMA, Special Diet Services, Witham, UK) *ad libitum* and were given 2 weeks to acclimatise before the beginning of the experiments.

### 2.2 Volatile extraction and analysis

Corn oil extracts of white clover were produced using the maceration technique to infuse the oil. Corn oil was used as a carrier because pilot tests had established that corn oil sprayed on clover did not affect its palatability for rabbits (unpublished observations). Furthermore, being non-polar, corn oil is regarded as a good solvent to extract volatiles but not the cyanogenic glucosides, which are soluble in aqueous or polar organic solvents. Freshly cut white clover (55–60 g) was submerged in corn oil (150 mL) in a glass Kilner jar and incubated in the dark at 30 °C for 2 days. The oil was then decanted into a clean jar, and the residual clover was washed twice with fresh oil (2 × 10 mL), adding the washings to the new jar. Another batch of freshly cut clover was added to the oil, incubating at 30 °C for a further 2 days. After this time the oil extract, hereafter called the ‘white clover extract’, was separated from the clover by decantation, filtered through muslin and stored in a sealed jar in the dark at 4 °C for up to 2 weeks before being used.

The analysis of the volatiles released from the white clover (var. Aran) extract and from white, red and strawberry clover plants was carried out by solid-phase microextraction (SPME). Freshly cut clover (2.5 g) was placed in a 250 mL glass beaker sealed over the top with aluminium foil. After 20 min the volatiles were sampled for 20 min using Supelco 75 μm polydimethylsiloxane/carboxen portable SPME field samplers. To assess the impact of damage to clover, the volatiles released from a growing white clover plant (*T. repens* Aran) were also analysed before (15 min) and after (15 min) damaging the plant by cutting off approximately 30% of the leaves with scissors.

The volatiles released from the white clover extract were analysed by pipetting 5 μL of this extract into a 400 mL glass beaker which was then covered with foil and left to equilibrate for 30 min. The volatiles released from the extract were sampled by SPME fibres for 30 min. A control sample (i.e. plain corn oil) was also collected as above, so that the peaks due to the corn oil in the white clover oil extract could be accounted for. Although no specific references were found that cyanogenic glucosides are completely odourless, the volatile components of clover plants (which have the potential to act as olfactory cues) are from a number of chemical classes including alcohols, ketones, aldehydes, esters and acids, furans and terpenes, but not glucosides.

The volatiles were analysed by gas chromatography–mass spectrometry (GC-MS) on a Hewlett Packard HP5890 series II gas chromatograph coupled to a VG Trio-1 mass spectrometer. Compounds were identified from their mass spectra using NIST and NBS mass spectral libraries.

### 2.3 Experiment 1

This experiment assessed rabbit preferences for clover species and varieties in a four-choice trial (strawberry clover, red clover and white clover var. Aran and var. Herbiseed). Rabbits (n = 6) were offered one tray per clover species or variety per test day, and the position of the four trays in each pen was randomised each day. Clover consumption was recorded after 24 h and expressed as estimated proportion eaten. Three independent observers were trained to estimate the proportion eaten, and the difference in the estimates between observers did not exceed 10%. Eight tests (four on week 1 and four on the following week) were carried out. Data on clover consumption were analysed by a repeated measurement analysis using restricted maximum likelihood (REML) with rabbit as random effect and uniform correlation structure over days. Values of *P* < 0.05 were regarded as significant, although the *P* values derived from chi-squares in REML are approximate.

### 2.4 Experiment 2

This experiment determined whether rabbits generalised their aversion for white clover to red clover treated with the white clover extract. A new group of rabbits (n = 8) were offered a choice between three clover ‘types’: white clover sprayed with corn oil, red clover sprayed with corn oil and red clover sprayed with white clover extract. Plants were sprayed with either 6 mL of corn oil or 6 mL of white clover extract per tray using an Ecospray laboratory sprayer (LCF, Aix-en-Provence, France). Rabbits were presented with one tray per clover type per test day, and the position of the three trays in each pen was randomised each day. Each test lasted 24 h and the consumption of clover was expressed as estimated proportion eaten. One test per day was carried out every other day for 2 weeks, for a total of 6 days.

Data on clover consumption were analysed by a repeated measurement analysis using REML with rabbit as random effect and autoregressive correlation structure over days. The study was carried out under a UK Home Office Licence, in accordance with the Animals (Scientific Procedures) Act, 1986, and was approved by an internal ethics panel.

### 3 RESULTS

Four main volatiles were identified in the three clover species (labelled I to IV in Table 1). (E)-3-Hexenyl
acetate and \((E)-3\)-hexenol were present in all species, but in substantially larger amounts in the white clover \(T. repens\) Aran. Two major volatiles of \(T. repens\) Aran, 2-butanone and 2-propanone, were released only by this species. These two volatiles were identified at levels that were more than 100 times the limit of detection of the method.

The analysis of a growing white clover plant showed that one volatile, \((E)-3\)-hexenol, was produced once the clover had been cut, and was not detected in the undamaged growing plant (Table 2). However, the two key compounds unique to \(T. repens\) Aran (2-butanone and 2-propanone) were released even when the plant was undamaged.

The white clover extract was dark-green in colour and had a strong and distinctive odour. Once sprayed onto the trays of clover, the odour was still detectable to the human nose after 12 h, indicating that the corn oil acted as a slow-release matrix.

### Table 1. Peak area \((\times 10^6)\) of the main volatiles released from 2.5 g of white, strawberry and red clover

<table>
<thead>
<tr>
<th>Chemical</th>
<th>White clover T. repens</th>
<th>Strawberry T. fragiferum</th>
<th>Red clover T. pratense</th>
</tr>
</thead>
<tbody>
<tr>
<td>I 2-Propanone</td>
<td>28.7</td>
<td>0.5 (^a)</td>
<td>0.3 (^a)</td>
</tr>
<tr>
<td>Pentane</td>
<td>12.4</td>
<td>3.7</td>
<td>2.0</td>
</tr>
<tr>
<td>Acetic acid</td>
<td>0.7</td>
<td>0.2</td>
<td>0.3</td>
</tr>
<tr>
<td>II 2-Butanone</td>
<td>78.0</td>
<td>N/D (^b)</td>
<td>N/D</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>0.9</td>
<td>0.4</td>
<td>0.1</td>
</tr>
<tr>
<td>3-Pentanone</td>
<td>1.8</td>
<td>0.04</td>
<td>0.03</td>
</tr>
<tr>
<td>Heptane</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>((E)-3)-Hexenal</td>
<td>0.3</td>
<td>0.04</td>
<td>0.01</td>
</tr>
<tr>
<td>III ((E)-3)-Hexenol</td>
<td>79.8</td>
<td>3.0</td>
<td>1.4</td>
</tr>
<tr>
<td>((E)-2)-Hexenol</td>
<td>2.9</td>
<td>0.01</td>
<td>N/D</td>
</tr>
<tr>
<td>1-Octen-3-ol</td>
<td>0.6</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>IV ((E)-3)-Hexenyl acetate</td>
<td>127.7</td>
<td>0.2</td>
<td>9.2</td>
</tr>
<tr>
<td>((E)-2)-Hexenyl acetate</td>
<td>10.7</td>
<td>N/D</td>
<td>N/D</td>
</tr>
</tbody>
</table>

\(^a\) Background levels from laboratory air.
\(^b\) N/D = not detected.

### Table 2. Peak area \((\times 10^6)\) of the main volatiles released by a white plant before (15 min) and after (15 min) damage

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Before damage</th>
<th>After damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Propanone</td>
<td>7.8</td>
<td>18.1</td>
</tr>
<tr>
<td>2-Butanone</td>
<td></td>
<td>33.9</td>
</tr>
<tr>
<td>((E)-3)-Hexanol</td>
<td></td>
<td>1.7</td>
</tr>
</tbody>
</table>

\(^a\) N/D = not detected.

Key \(T. repens\) Aran volatiles (2-butanone and 2-propanone) were released in significant quantities from the oil extract, as well as smaller amounts of the damage-induced volatile, \((E)-3\)-hexenol, and three other volatiles identified in cut \(T. repens\) Aran, \((E)-3\)-hexenal, 1-octen-3-ol and \((E)-3\)-hexenyl acetate (Table 3).

In experiment 1, one rabbit refused to eat any clover for three consecutive tests and was thus removed from the trial. Rabbits preferred strawberry and red clover to both varieties of white clover (Wald statistic = 94.46, df = 3, \(P < 0.001\)) (Fig. 1). There were no differences in total consumption between days (Wald statistic = 4.14, df = 7, \(P = 0.763\)) and in the interaction between day and clover type (Wald statistic = 24.95, df = 21, \(P > 0.05\)).

In experiment 2, two rabbits refused to eat clover for three consecutive tests and were thus removed from the trial. Rabbits ate significantly different proportions of the three clover types (white clover, red clover and red clover sprayed with white clover extract) (Fig. 2). Rabbits ate more untreated red clover than white or white-flavoured red clover (Wald statistic = 24.91, df = 1, \(P < 0.001\)), and the consumption of white clover did not differ from that of white-flavoured red clover (Wald statistic = 0.21, df = 1, \(P = 0.643\)).

### Table 3. Peak area \((\times 10^6)\) of the main volatiles released by 5 \(\mu\)L of the white clover extract

<table>
<thead>
<tr>
<th>Chemical</th>
<th>2-Propanone</th>
<th>2-Butanone</th>
<th>((E)-3)-Hexenal</th>
<th>((E)-3)-Hexenol</th>
<th>1-Octen-3-ol</th>
<th>((E)-3)-Hexenyl acetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>681.6</td>
<td>100.2</td>
<td>1.1</td>
<td>5.2</td>
<td>3.0</td>
<td>0.3</td>
<td></td>
</tr>
</tbody>
</table>

\(\mu\)L = microlitres.

![Figure 1](image-url)Consumption, expressed as proportion eaten, of white clover (Aran and Herbiseed), strawberry clover and red clover by rabbits \(n = 5\) in eight tests. Each test lasted 24 h.

DOI: 10.1002/ps
although consumption of clover type varied with time (day) (Wald statistic = 11.43, df = 5, P = 0.043).

4 DISCUSSION

The results of this study confirmed that edible plants that mimic the flavour of toxic species might significantly reduce herbivore attack. In particular, the study identified a natural system in which a model species, the white clover *T. repens* var. Aran, could advertise its toxicity to herbivores by using specific volatiles that are not found in other non-toxic, sympatric clover species. The study also demonstrated that, by producing these volatiles, a palatable, visually similar and closely related species such as red clover would significantly decrease being consumed by rabbits. The aversion persisted for 2 weeks, although some variation in the strength of the aversion was observed between days.

One of the volatiles found in white clover, (E)-3-hexenol, was only produced once the clover had been damaged. ‘Wound volatiles’ are released from clover plants when they are damaged, either artificially or by herbivores. Conversely, the two compounds unique to *T. repens* Aran, 2-butanone and 2-propanone, appear to be released even when the plant is undamaged. These two compounds might therefore be perceived by herbivores as warning signals indicating the plant’s toxicity. Other mammalian herbivores have been shown to use volatiles as olfactory cues advertising the toxicity of a plant. For instance, brushtail possums (*Trichosurus vulpecula* Ker) learn that the concentration of strongly aromatic terpenes in the leaves of *Eucalyptus* species is related to the levels of odourless, illness-inducing secondary compounds. Thus, by developing a conditioned aversion to foliar terpenes, possums avoid ingesting the plant’s toxins. The results of the present study suggest that the characteristic odour of white clover might have evolved as the olfactory concomitant of odourless toxins to ‘warn’ herbivores to search for food elsewhere.

Little evidence has been gathered so far on the natural occurrence of olfactory Batesian mimicry in plant–herbivore interactions. Previous experiments conducted in simulated Batesian mimicry systems showed that sheep could transfer their aversion from cinnamon-flavoured rice (the model) to cinnamon-flavoured wheat (the mimic) and that the aversion persisted for at least two days. Several authors have suggested that it is unlikely that edible plants could avoid herbivory by mimicking the odour of toxic plants, because herbivores constantly sample food and are likely to overcome their initial aversion if the warning cues are not reinforced by the presence of the toxin. However, the willingness of a herbivore to resample a plant that induced illness might be directly related to the risks associated with reingesting the toxins again (i.e. the higher the risk, the lower is the probability of resampling). For instance, conditioned aversion to larkspur persists for at least 3 years without further reinforcement when averted cattle are grazed separately from non-averted cattle. It is also possible that some plants might exploit Batesian mimicry only for relatively short periods during which they are most susceptible to herbivory. Future research should identify the factors affecting the persistence of aversion towards ‘mimic’ plants.

Ideally, both plants and herbivores would benefit from preingestive olfactory cues, such as volatiles, that prevent animals from feeding on toxic plants and plants from being damaged. However, the present study indicated that, when plants cannot rely on preingestive cues, they might still use post-ingestive warning signals to reduce intake. In this context, taste might potentiate the role of odour by acting as a ‘reminder’ of the plant’s toxicity and ultimately lead to decreased herbivory.

The present study indicated that, although the effect of olfactory mimicry persisted for at least 2 weeks, rabbits consumed the cyanogenic white clover in small but increasing amounts over time. Other authors found that herbivores can detoxify cyanogenic glycosides and develop a degree of habituation to these toxins. However, the animals in the present study, as well as those in the above studies, consistently consumed more acyanogenic than cyanogenic clover, suggesting that herbivores may be unable to detoxify large amounts of toxins.
These results suggest that warning signals of toxic plants could potentially be exploited by other plant species that lack chemical defences but use the same volatile signals as used by toxic plant species. As the acquisition and transfer of food aversion is strongly associated with the degree of toxicosis,29,30 further studies could explore the volatiles of highly toxic plants. These chemicals are likely to be a readily exploitable, rich source of effective, safe and natural repellents that could be used to protect species of economic interest, such as crops and ornamentals, against pest species.

ACKNOWLEDGEMENTS
The authors thank the Department for Environment, Food and Rural Affairs (Defra) for funding this work. They are grateful to Carola Deppe for statistical advice and to John Chambers and Roger Quy for useful discussion.

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