Rosmarinic acid in *Canna generalis* activates the medial deterrent chemosensory neurone and deters feeding in the tobacco hornworm *Manduca sexta*

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**Abstract.** For all but a very few highly specialized insect feeders, *Canna generalis* L. (Cannaceae) is unacceptable as a food plant and is a highly potent feeding deterrent for the tobacco hornworm *Manduca sexta* Johan. (Lepidoptera). The present study describes the isolation of an active deterrent compound, rosmarinic acid (RA) from the ethanol extract of canna leaves. A two-choice bioassay shows that RA is a feeding deterrent in the concentration range 0.3–3 mm. Bilateral ablation of the oral chemoreceptors eliminates this deterrence. The results reported in the behavioural literature show that only the epipharyngeal sensilla and the medial (but not the lateral) styloconica contribute to this deterrence elicited by canna foliage or extract, and the data obtained in the present study show the same results using RA. Electrophysiological recordings from the medial styloconica show that RA primarily stimulates the ‘deterrent neurone’ in a concentration-dependent manner with a threshold of approximately 0.03 mm and a peak frequency of 69 spikes s⁻¹ at 0.1 mm RA. An extract of canna leaves elicits similar responses. Adding RA to canna extract does not elicit another class of spikes, indicating that both stimuli activate the same neurone. We discuss the significance of the results with respect to furthering our knowledge about the sensory basis of food selection, and specifically about the range of compounds that stimulate deterrent chemosensitive neurones of lepidopteran larvae.

**Key words.** Feeding behaviour, insect neurophysiology, Lepidoptera, phagodeterrents, phytochemistry, sensory coding.

**Introduction**

Diet specialization and the selection of food are important aspects of insect life strategies (Simpson & Raubenheimer, 1993). Such selectivity requires physiological systems to recognize potential food and nonfood. For phytophagous insects, this means that the detection capabilities of the sense organs must match the compounds and their concentrations in the encountered plants. Many phytophagous insects are highly selective in their feeding habits, and so phagodeterrence mechanisms likely play an important role in feeding decisions in these insects (Chapman, 1995). Accordingly, our understanding of food selection depends on our knowledge of the deterrent phytochemicals, the sensory mechanisms by which they are detected and the central nervous system processes that interpret their signals to be a phagodeterrent (Chapman, 1995).

One plant that is rarely damaged by insects is canna (*Canna generalis* L., Cannaceae), an ornamental that is commonly grown in public gardens. It is very strongly phagodeterrent for the tobacco hornworm *Manduca sexta* Johan. (Lepidoptera, Sphingidae). Several behavioural studies using this model insect describe employing canna as the nonhost plant (Jermy *et al.*, 1968; de Boer & Hanson, 1987; Peterson *et al.*, 1993). Canna is such a deterrent that hornworms prefer to starve rather than eat it; however, they will readily feed on it if their taste organs have been removed (de Boer & Hanson, 1987). Canna does not appear to be poisonous: if a diet of canna mixed with host plant is fed to these chemosensory-ablated caterpillars, no toxic effects
are observable; most gain weight, pupate and eclose normally. Thus, it is likely that a phytochemical in canna leaves is deterrent but not toxic to *M. sexta*.

The detection of the phagodeterrent in canna is apparently by contact chemoreception, and not olfaction (de Boer & Hanson, 1987). When hornworms encounter a canna leaf, they take a small test bite and crawl away; removal of all the olfactory organs (antenna and maxillary palps) does not change this. Similarly, the lateral styloconica of the maxilla do not appear to play a role in rejection of canna because their presence or absence makes no difference in these experiments. Removal of the other taste organs, however, has dramatic consequences: ablation of both the medial styloconica of the maxilla and the epipharyngeal sensilla permits larvae to feed on either canna or host plants indiscriminately. Thus, the gustatory receptors in the medial styloconica and epipharyngeal sensilla are clearly the organs responsible for detecting the deterrent(s) in canna.

An active principle in canna leaves can be extracted in hot ethanol (Peterson *et al*., 1993). A partially purified fraction tested electrophysiologically elicits large spikes of a single type from the medial styloconicum, the frequency of which correlates well with phagodeterrence in a concentration-dependent manner. The conclusion is that these spikes are produced by a deterrent neurone mediating the behavioural rejection of canna. In view of the importance of understanding phagodeterrence and its role in host selection, the present study isolates and identifies a deterrent phytochemical in canna and confirms its activity using behavioural and electrophysiological bioassays.

**Materials and methods**

**Chemical isolation and identification of rosmarinic acid (RA)**

The procedure described by Peterson *et al*. (1993) was used to obtain an active crude extract of the foliage of *C. generalis*. Leaves were de-veined, weighed, ground in liquid nitrogen and extracted in boiling 95% ethanol; this extract was then filtered, defatted by extracting twice with hexane, reduced in volume by evaporation under reduced pressure, evaporated to dryness under nitrogen and dissolved in 10% ethanol and 0.1 m NaCl. This stock solution was adjusted to 0.84 g fresh weight of canna mL⁻¹, and then stored under nitrogen at −10°C in the dark.

Fractionation was carried out by reverse-phase column chromatography that separates polar compounds on the basis of polarity. Three millilitres of the stock solution was concentrated *in vacuo* at < 30°C and the resulting 0.038 g was dissolved in 0.5 mL of distilled water. This extract was applied to a Sep-Pak cartridge (Waters, Milford, Massachusetts) that had been previously washed with methanol and then water, and elution was performed sequentially with 3 mL of water (fraction 1), 50% methanol (fraction 2) and methanol (fraction 3). Electrophysiological and behavioural assays showed that fraction 2 elicited the greatest response from the deterrent neurone in the medial styloconica (Peterson *et al*., 1993). Therefore, for the present study, this same fraction 2 was used; it was filtered through 0.45 micron filters (Millipore, Burlington, Massachusetts) and analyzed using a high-performance liquid chromatography (HPLC) system consisting of an LC 600 pump, model 717 autosampler and model 996 photodiode array detector (Waters). Samples were injected onto a Lichrospher 5ODS end-capped column (25 cm × 4.0 mm inner diameter) (Millipore) and separated using a binary gradient (solvent A = 2% acetic acid in water; solvent B = 18:1:1 methanol:water:acetic acid) where at T = 0 min, A = 75% and at T = 20 min, A = 0% using a linear gradient with flow rate of 1.0 mL.min⁻¹. The analytical method was adapted for preparative HPLC using a Spherisorb 5ODS column (25 cm × 10 mm inner diameter) (Waters) and a binary gradient (solvent A = 2% acetic acid in water; solvent B = 2% acetic acid in methanol) where at T = 0, A = 80% and at T = 20, A = 0% using a linear gradient with flow rate of 4.73 mL.min⁻¹. Seven subfractions of fraction 2 were collected manually; each subfraction contained either a main peak or a few minor peaks. These fractions were then tested in an electrophysiological bioassay.

**Insects**

For the first cohort of larvae, eggs of *M. sexta* were obtained from the stock USDA colony at Beltsville (Maryland). These larvae were maintained on an artificial diet modified from Yamamoto (1969) and used for the behavioural concentration–response studies and electrophysiology. For the second cohort of larvae used for chemosensory deprivation experiments, eggs were obtained from Carolina Biological (Burlington, North Carolina) and maintained on a commercial tobacco hornworm media (Bio-Serve, Frenchtown, New Jersey) that is essentially the same as the diet used by Yamamoto (1969). For both cohorts, late fourth-instar premoults were removed from the culture daily and isolated at room temperature. After moulting into the fifth instar and just before testing, each animal was given 1 h of *ad libitum* access to six water-saturated filter paper discs to standardize larval hydration. Preliminary experiments showed this procedure to be important: water-deprived animals tolerated some levels of deterrents, presumably to obtain water from the solution.

**Behavioural assays**

Behavioural responses of the larvae were determined using the two-choice disk test modified from de Boer & Hanson (1987). Briefly, discs of 1.5 cm in diameter were punched from edible glass-fibre filter paper (GF/A; Whatman, U.K.) and pinned into a styrofoam floor at a height of 1 cm. Six discs were arranged in a circle of 10 cm in outer diameter and covered by the lid of a Petri dish (diameter 12 cm) to form a chamber. Three discs treated with 0.1 mL of test solution were alternated with three control discs wetted with 0.1 mL of distilled water. The test began when one larva was placed with random orientation in the center of the chamber and allowed to feed until approximately half of the disc area of the most-eaten choice, either treated or control, was consumed. The area that was eaten for all six
discs was estimated visually to ±5% or by software analysis of
digital photos spot-checked visually and summed within each
category (treated or control). An index was computed such that
phagodeterrence is positive (0 to 100) and phagostimulation
negative (0 to −100):

\[
\text{Deterrence index} = \frac{\text{control} - \text{treated}}{\text{control} + \text{treated}} \times 100
\]

Tests were conducted at room temperature (approximately
22 °C) using RA (Sigma-Aldrich, St Louis, Missouri) as the
treatment. Larvae were tested only once and then discarded.

Chemosensory deprivation

Early fifth-instar larvae from the second cohort were anaes-
thetized by immersion in water for 10–20 min (Gothilf &
Hanson, 1994). Control larvae were anesthetized but not
subjected to surgery. Control and experimental larvae were
tested at the same time under the same conditions. Bilat-
eral extirpations were performed on the experimental larvae
using the techniques described by de Boer & Hanson (1987)
employing small iridectomy scissors for amputations com-
prising the lateral styloconica of the maxilla; both medial and
lateral styloconica by removal of the galea of the maxilla;
and epipharyngeal sensilla by removal of the epipharynx.
Verification of successful bilateral organ removal without dam-
age to nontarget organs was conducted microscopically after
recovery. After an overnight recovery period and the water
pre-test described above, one larva was placed into a two-choice
disk test with 1 mM RA (ICN, Costa Mesa, California) on the
treatment discs and distilled water on the control discs.
The significance of differences in responses between groups
was determined by a nonparametric Kruskal–Wallace test
(alpha = 0.05).

Electrophysiology

The intact animal preparation of Gothilf & Hanson (1994)
was used for the electrophysiological recordings. An anaes-
thetized larva was inserted backwards into a glass vial with
only its head protruding through a hole in a thin rubber gasket
and the vial cap. The vial contained a Ag-AgCl wire refer-
ence electrode and 0.1 m KCl that provided electrical connection
between this reference electrode and the larval body surface.
In addition, the maintained immersion suppressed the animal’s
movements throughout the approximately 4-h experiment with-
out affecting the chemosensory responses. At the end of the
experiment, the animal was released from the vial and observed
to be mobile and feeding the next day. Each animal was used
only once.

Neural responses of the styloconica were obtained electrophysiologically
using the standard tip recording technique (Frazier & Hanson, 1986; Gothilf &
Hanson, 1994). A micropipette with a tip diameter of approximately 50 μm
containing the stimulus was placed over the tip of a maxillary
styloconicum, allowing the stimulus solution to contact the
sensory dendrite through the apical pore. The micropipette
also contained an electrolyte (0.1 m KCl) in contact with a
Ag-AgCl surface that served as the active electrode. This was
connected to a high impedance preamplifier (George Johnson,
Baltimore, Maryland) (Frazier & Hanson, 1986) with a rapid
baseline-restoring circuit that minimized contact artefacts and
permitted undistorted action potentials to be recorded within
10 ms after contact. The signal was amplified with a bandpass
of 100 Hz to 3 kHz and saved in digital format using data acqui-
sition software (‘STA’; E. Städler, Federal Research Station,
Switzerland) sampling at 10 kHz. A single trial consisted of
sequentially contacting the tip of each lateral and medial stylo-
conica bilaterally with the stimulating/recording micropipette
for 1.5 s. Three of these trials were conducted with each stim-
ulus; inter-trial intervals of 3 min permitted full recovery of
the sensory neurones between trials. A 0.1 m KCl control trial
was performed at the beginning and end of the experiment
and after each change of stimulus for a total of nine or more
control trials. Action potentials were classified and counted for
1 s with custom software using digital filtering to isolate spikes
and align their peaks, and then a template matching procedure
was used to sort them (Stitt et al., 1998; as modified by
M. Rowley, Louisville, Kentucky). Classification was
spot-checked manually. We recorded from eight larvae, all
of which provided similar responses. From these, we analyzed
data from six medial and lateral styloconica pairs that provided
good recordings from both styloconica and signal-to-noise
ratios suitable for spike classification.

Solutions of pure chemicals used for electrophysiological
testing were RA (Sigma-Aldrich Chemicals) and caffeine
(Sigma-Aldrich) dissolved in 0.1 m KCl. Canna stimuli were
obtained from the ethanol extract of canna leaves adjusted to
the original leaf concentration (reported as ‘1×’, as in Peterson
etal., 1993). Dilutions of the 1× were carried out with 0.1 m
KCl to maintain electrolyte concentration. Mixtures of RA and
canna extract were made by mixing equal volumes of selected
RA concentrations and canna extract dilutions. Thus, if each
solution contained the same chemical, its concentration in the
mixture would be maintained, whereas the concentration of
dissimilar chemicals would be halved.

Results

Identification of a phagodeterrent chemical in canna

HPLC analysis of the biologically active 50% methanol frac-
tion (fraction 2) of the crude ethanol extract of canna revealed
a major peak at 14.52 min. This peak and other minor peaks
were collected by HPLC into seven subfractions, each of which
was assayed for activity by electrophysiology. The most active
subfraction contained a peak that was identified as RA (Fig. 1),
an ester of caffeic acid and 3,4-dihydroxyphenylactic acid. The
identification of RA was confirmed by co-chromatography
and ultraviolet-spectral comparison with an authentic standard (Apin
Chemicals, U.K.). RA was present at 1.6 mg g⁻¹ fresh weight of
foliage (4.4 mm, assuming all fresh weight is active solution)
and constituted 85% of the ultraviolet-absorbing components in the 50% methanol fraction.

**Behavioural responses to RA**

RA is clearly a strong phagodeterrent for hornworms; the concentration–response curve of two-choice bioassays of RA versus water showed that the deterrence threshold is approximately 0.3 mM RA (the concentration at which deterrence by RA is significantly greater than that of water, \( P = 0.025, t\)-test). Deterrence is almost complete at 1 mM RA (Fig. 2).

Differential ablation experiments provide an indication of the sensory pathway of deterrence by RA. First, removing only the lateral styloconica did not significantly reduce aversion to RA (lateral (Lat) versus not ablated (None), \( P = 0.746, \text{Kruskal–Wallace test} \) (Fig. 3). This indicates that the laterals are not a major contributor to the RA deterrence pathway, and that ‘Lat’ may also be regarded as a sham control for these experiments. Second, when the medial styloconica were also removed, deterrence was significantly reduced compared with larvae that still possessed them (lateral and medial (ML) and epipharynx, medial and lateral (EML) versus None, Lat and epipharyngeal sensilla (Epi), \( P < 0.0001, \text{Kruskal–Wallace} \) (Fig. 3). Third, larvae with lateral and medial ablations were significantly less deterred than those with only laterals ablated (ML versus Lat, \( P = 0.044, \text{Kruskal–Wallace} \) (Fig. 3); because the only difference here is the absence of the medial, the evidence indicates that it is the primary organ mediating aversion to RA.

A caveat here is that the concentration–response experiments were performed with a different cohort of larvae than those used in the ablation experiments. However, both cohorts show strong deterrence at the same concentration of RA (1 mM) (Fig. 2 versus Fig. 3, None), suggesting that there was little if any difference between these cohorts.

**Chemosensory neurone responses**

**Responses to 0.1 m KCl.** Because all electrophysiological stimuli contained 0.1 m KCl, this was used as the control stimulus for the electrophysiological recordings. It elicited low frequency responses (approximately 20 s\(^{-1}\)) of two neurones in both the medial and lateral styloconica (Fig. 4, trace 1; Fig. 5 at 0 concentration of RA and canna). These responses remained constant in the nine or more control trials throughout the 4 h experiment.

**Responses to RA.** Responses from the medial styloconica to RA consisted mainly of large spikes of uniform shape and inter-spike interval, indicating a single neurone source (Fig. 4A, traces 2 and 3). The activity of this ‘RA neurone’ was concentration-dependent, with a threshold of <0.03 mM and mean peak frequency of 69 ± 7 spikes s\(^{-1}\) at 0.1–1 mM (Fig. 5A, solid lines and symbols). A second, smaller spike was also seen (Fig. 4A, trace 2) that decreased in frequency at higher RA concentrations (Fig. 5A, open symbols). These might be responses of the ‘salt neurone’ to the 0.1 m KCl in the stimulus solution.

Responses to RA from the lateral styloconica (Fig. 4B, traces 2 and 3) are similar to those of the medial styloconica, although with somewhat higher spike frequencies of the small spike (Fig. 5A). Interestingly, the average frequencies of the large spikes of the deterrent neurones were very similar in the two styloconica (Fig. 5A).

**Responses to canna extract.** Responses from the medial styloconica elicited by canna extract (Fig. 4A, traces 4 and 5) were very similar to those elicited by RA (Fig. 4A, traces 2 and 3). Responses from the lateral styloconica to canna (Fig. 4B, traces 4 and 5) appeared to be more heterogeneous than those of the medial styloconica (Fig. 4A, traces 4 and 5), presumably as a result of more types of sensory neurones firing. As with the RA stimuli, the average frequencies of the large spikes were comparable in the two styloconica (Fig. 5B).

**Responses to mixtures of RA and canna extract.** Adding RA to the canna stimuli did not elicit a new class of spikes from the medial styloconica, comprising evidence that the neurone responding to RA is also the primary neurone responding to canna (Fig. 4A, traces 2 and 3 versus 6 and 7). As with the canna extract, responses from the lateral styloconica to the mixtures (Fig. 4B, traces 6 and 7) were more heterogeneous than those of the medial styloconica (Fig. 4A, traces 6 and 7). The average responses to the mixtures (Fig. 5C) were generally comparable with those elicited by their constituents at similar concentrations (Figs 5A, B).

**Responses to caffeine.** This well-known phagodeterrent for *M. sexta* was included here as a comparison with RA. Both chemicals elicited the same large spike, presumably from the same deterrent neurones (Fig. 4, traces 3 and 8). One difference between the effects of the two stimuli is that caffeine elicited a higher frequency of large spikes from the lateral than the medial styloconica, whereas responses to RA were similar in both styloconica (Fig. 5).

**Discussion**

The results of the present study strongly indicate that RA is an active principle in extracts of canna leaves. The calculated RA concentration found in the leaf is approximately 5 mM, which is five times the concentration that substantially inhibited feeding on it in the two-choice test. RA is a derivative of caffeic acid; some other derivatives of caffeic acid modulate the feeding of many species of lepidopteran larvae. For example, chlorogenic acid acts as a mild feeding deterrent to *Spodoptera litura* and is considered to play a role in the resistance of peanuts to this insect (Stevenson et al., 1993). It also enhances the antifeedant activity of *Cicer* isoflavonoids (Simmonds & Stevenson, 2001).
Fig. 1. High-performance liquid chromatography (HPLC) evidence that rosmarinic acid (RA) is in canna. HPLC trace at 310 nm of the 50% methanol eluted Sep-Pak fraction of the hot ethanol extract of foliage of canna. The large peak is identified as RA.

Fig. 2. Behavioural concentration–response curve to rosmarinic acid (RA). Symbols are the mean ± SE of the Deterrence index for n larvae in two-choice tests comprised of solutions of RA versus distilled water.

RA can be isolated from many species within the Boraginaceae and the subfamily Nepetoideae of the Lamiaceae; it can also be isolated from ferns belonging to the family Blechnaceae, as well as hornworts and species belonging to the sea grass families Zosteraceae and Potamogetonaceae (Peterson & Simmonds, 2003). To our knowledge, none of the species known to contain RA are hosts of M. sexta. Further work should be undertaken to determine whether the medial styloconic sensillum is responsive to other caffeic acid derivatives. This could provide more information about the specificity of the neurone, as well as the role that this widely distributed group of compounds plays in the feeding behaviour of the hornworm.

Previous research on the effect of differential ablation of chemoreceptors on feeding behaviour shows that a single medial (but not lateral) styloconic sensillum alone is capable of blocking feeding on canna (de Boer & Hanson, 1987). Similar ablation experiments in the present study using RA instead of canna show equivalent results: the medial (but not lateral) styloconica are important in rejection of RA. Therefore, the discovery that RA elicits a classic, single-deterrent neurone response in the medial sensillum is significant. In the M. sexta literature, this neurone is characterized by its response to partially purified fractions of canna leaves, and associated behavioural assays show that the activity of this neurone correlates well with phagodeterrence (Peterson et al., 1993). RA elicits almost identical responses in the present study, thus supporting the hypothesis that RA is the primary active principle in canna for deterring hornworm feeding. This exemplifies one generally accepted mechanism of deterrence, namely that rapid activity of a deterrent neurone blocks feeding. This and other mechanisms of deterrence are discussed by Bernays & Chapman (2000) and Glendinning et al.
Fig. 4. Electrophysiological responses of the maxillary gustatory neurones to deterrents. (A) Medial styloconicum. (B) Lateral styloconicum. The duration of the traces shown is 600 ms, starting from the initial contact artefact at the left of each trace. Canna concentrations listed are relative to full strength extract ( = 1x). All solutions contained 0.1 M KCl as an electrolyte. All responses depicted are from the left maxilla of one animal. Note that the responses to rosmarinic acid (RA) are very similar to those of canna, especially in the medial styloconica.

Although, in the present study, the lateral styloconica are shown to be quite responsive to both RA and canna, this sensillum does not appear to mediate deterrence to canna (de Boer & Hanson, 1987), nor to RA (Fig. 3). Presumably, the multi-neuronal response to RA from the lateral sensillum is not a signal that is interpreted by the central nervous system as a phagodeterrent.

The potency of RA as a deterrent for the tobacco hornworm (behavioural threshold approximately 0.3 mm and electrophysiological threshold < 0.03 mm) is comparable with that of many phytochemical deterrents tested in other insects (Schoonhoven & van Loon, 2002). The phagodeterrence of RA varies from threshold to maximum in less than a decade of concentration range. This steep concentration–response curve is also characteristic of the responses of other insects to strong deterrents, such as the response of the locust Schistocerca gregaria to azadirachtin (Mordue et al., 1998).

When a chemosensitive neurone is characterized as either a deterrent or phagostimulant neurone, the question arises as to whether it is a generalist or a specialist. The fact that only a small percentage of the total number of plant species is consumed by most phytophagous insects suggests that caterpillar deterrent neurones must be generalists because they are the gatekeepers of feeding that must exclude the vast majority of plants (Schoonhoven, 1982). To date, however, only two compounds, aristolochic acid (Glendinning et al., 2002) and now RA, are known to stimulate this ‘RA neurone’ in the medial styloconica of M. sexta, and only a few more deterrent stimuli (caffeine and salicin and related compounds, plus aristolochic acid) are known for the ‘caffeine neurone’ in the lateral styloconica (Schoonhoven, 1972; Glendinning et al., 1999; Cocco & Glendinning, 2012). The responses to RA and caffeine in the present study (Fig. 4) exemplify the two known deterrent pathways, although presumably there are others as well. Their limitations, interactions and mechanisms of behavioural control are not well understood. For example, there are many phytochemicals that are deterrent for other species but not for M. sexta (Wrubel & Bernays, 1990); it is not known whether these phytochemicals simply go undetected by the sensory neurones of M. sexta, or whether the hornworm’s deterrent pathways are more permissive. Clearly, more information about deterrent compounds and their effects on insect sense organs and behaviour is needed to provide a better understanding of food selection mechanisms.

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