

Leaf trichomes and foliar chemistry mediate defence against glasshouse thrips; *Heliothrips haemorrhoidalis* (Bouché) in *Rhododendron simsii*.

Running headline: Chemical and trichome defence in *Rhododendron*

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## Abstract

Herbivore defence mechanisms are a costly diversion of resources away from growth and reproduction. Thus time-limited and tissue specific expression in critical plant parts is more efficient as defined by optimal defence theory. Surprisingly little is known about *Rhododendron* herbivore defence but it may be mediated by combined chemical and physical mechanisms. *Rhododendron simsii* survives cyclic infestations of a leaf-feeding thrips, *Heliothrips haemorrhoidalis*, which severely damage mature leaves but avoid terminal young leaves suggesting specific, localised defence expression. We examined correlations between the distribution of thrips and feeding damage with density of trichomes and the concentration of the diterpenoid, grayanotoxin I, a compound implicated in but not previously reported to mediate invertebrate defence in *Rhododendron*. Our data show that as leaves matured the number of thrips and area of feeding damage increased as trichome density and grayanotoxin I concentration decreased, this inverse correlation suggesting trichomes and grayanotoxin I mediate defence in younger leaf tissue. Grayanotoxin I was tested against *H. haemorrhoidalis* and was toxic to immature life stages and repellent to the adult thrips, reducing numbers of larvae emerging on leaves when applied at ecologically relevant concentrations. This work demonstrates that the pattern of defensive traits in foliage of a species of *Rhododendron* is key to its ability to tolerate cyclic infestations of a generalist herbivore, effectively conserving vital tissues required for growth and reproduction.

**Additional keywords:** Defense, glasshouse thrips, grayanoid diterpene, leaf hairs, plant tolerance, secondary plant compounds.

## Introduction

Plants growing in environments where they are frequently or sporadically threatened by herbivorous insects require mechanisms to protect vital tissues important for continuing growth and reproduction. However, production and expression of plant defence mechanisms

uses resources essential for these life processes and therefore are not always constant and are often concentrated in specific tissues; this being defined by the optimal defence theory (McKey 1974; 1979; Rhoades 1979; Krischik and Denno, 1983; McCall and Fordyce 2010). While variation in chemical defence within individual plants has been documented (McKey 1974; Feeny 1976; Rhoades and Cates 1976; Raupp and Denno 1983; Zangerl and Rutledge 1996; Schoonhoven *et al.* 2005), fewer studies have investigated the distribution of physical defence traits within individuals (Woodman and Fernandes 1991, De Dobbelaere *et al.* 2010). While there is evidence that this too is consistent with the optimal defence theory and defence distribution according to tissue value (Ågren and Schemske 1993; Dalin and Björkman 2003; Björkman *et al.* 2008; Dalin *et al.* 2008), we found gaps in the literature which considers both chemical and morphological characteristics of resilient plant specimens simultaneously when defining i) factors which account for the ability of a species to survive or tolerate invasion by opportunistic polyphagous insects or ii) tissue specific expression of defence mechanisms that might balance the trade-off between the costs of defence and survival.

Botanic glasshouse collections provide a unique opportunity to observe and record host selection, plant tolerance and resistance among a diverse range of plant families and species which are subjected to influxes of the same invasive insect population. The collections in the glasshouses at the Royal Botanic Gardens, Kew are frequently damaged by a polyphagous leaf-feeding thrips; *Heliothrips haemorrhoidalis* (Bouché) (Thysanoptera: Thripidae) but tolerance of different species of plants to infestations of thrips is highly variable. *H. haemorrhoidalis* causes widespread damage to economically important plantation crops such as avocado (Goodall *et al.* 1987; Dennill and Erasmus 1992) and citrus (Holt 1989) by feeding on the contents of epidermal cells of mature leaves (Kirk 1997; Mound 1997) and fruit exocarp (Childers 1997). This results in a silvering of leaf surfaces, followed by leaf desiccation and defoliation on plants intolerant to high infestations. The pest status of this thrips species is exacerbated by parthenogenetic reproduction, resulting in rapid fluctuations in populations over a relatively short period of time when conditions are optimal. The recent

loss of several species within the genera *Rhododendron* in Kew's collections through severe defoliation by *H. haemorrhoidalis* prompted the present study to identify characters within surviving species that might explain their ability to tolerate high levels of thrips infestation. *Rhododendron simsii* Planch (subgenus: Tsutsusi), an evergreen shrub native to China, Taiwan and N.E Burma (Young and Chong 1980; eFlora of China), supported large populations of *H. haemorrhoidalis* on three representative specimens and their clones maintained under glass, suffering significant leaf loss due to the thrips feeding on the mature leaves during the winter months when growth was minimal. However, *H. haemorrhoidalis* appeared unable to infest the youngest leaves on individuals of *R. simsii*, resulting in insect numbers declining on less mature leaves enabling the *R. simsii* specimens to restore foliar mass in spring.

*Heliethrips haemorrhoidalis* selects and feeds on mature leaves of its hosts which has been linked to nutritional quality (Fennah 1963; Lewis 1973; Kirk 1997; Scott-Brown *et al.* 2002) but the influence that the presence of defence metabolites may have on the distribution of these thrips among foliage of hosts has not been previously studied. Species in the genera *Rhododendron* and *Kalmia* contain grayanoid diterpenes (Hegnauer 1966; Qiang *et al.* 2011) and our preliminary examination of the foliage of *R. simsii* indicated that leaves contained a compound from this group; grayanotoxin I, but this compound was not evenly distributed in the plant. Diversity of leaf surface morphology has been reported for *Rhododendron* species (Cowan 1950), with more recent suggestion of a defensive function against insect pests and pathogens (Doss 1980; 1984; De Dobbelaere *et al.* 2010). However, the extent to which chemical and morphological traits of *Rhododendron* contribute to herbivore defence was not known. The objectives of this study were i) evaluate the distribution of thrips and damage across a severely infested plant; ii) examine the distribution of grayanotoxin I in leaves of *R. simsii* and determine any relationship between concentration levels and thrips numbers on foliage; iii) isolate grayanotoxin and determine any behaviour modifying or toxic effects on *H. haemorrhoidalis*; iv) determine any correlations between density of glandular and non-

glandular trichomes on *R. simsii* leaves and between number of thrips and the area of feeding damage, thus examining the hypothesis that chemical or morphological traits are influencing the distribution of thrips among the foliage of *R. simsii* and contributing to the protection of new vegetative growth and flower bud development.

## **Methods and materials**

### ***Plant Material and Thrips.***

Three specimens of *Rhododendron simsii* Planch (Ericaceae) (Kew accession numbers: 1973.12215 and 1978.6211) were located in the Kew Temperate House, in 2014, and the foliage used in the following experiments were taken from these. All life stages of *H. haemorrhoidalis* used in the insect bioassays were collected from infestations on a specimen of *R. simsii* (accession no. 1978-6512) maintained under laboratory conditions  $25^{\circ}\text{C} \pm 1^{\circ}\text{C}$ , with a photoperiod of L16:D8.

***Leaf Surface morphology.***

Leaves from a single specimen of *R. simsii* were detached and ordered by age. Leaf lamina sections (5mm x 5mm) were cut from each leaf and placed in a glass vial and covered with 100% formalin-acetic-alcohol (FAA) fixative and left to stand for 1 week. The fixative was poured off and replaced with 70% ethanol for a further week. The concentration of ethanol was taken up to 90% for 1 hour, followed by 2 changes of 100% ethanol. Each square was dried using a Tousimis Autosamdri-815 Critical Point Dryer (Tousimis, USA). The samples were then mounted on SEM stubs with double sided sticky tape (Agar Scientific Ltd., Stanstead, UK.), abaxial surface exposed and covered with a layer of gold using a Quorum Q150R ES sputter coater (Quorum, East Sussex, UK). The leaf sections were then examined in a Hitachi S3400N variable pressure scanning electron microscope (Hitachi, High Technologies Corporation, Berkshire, UK) and digital images of leaf surface structures were recorded.

***Density of thrips, leaf trichomes and percentage area damage caused by feeding and oviposition.***

Three branches (length 14 cm) were removed from a single specimen of *R. simsii* and the leaves were detached from each branch and labelled (youngest to oldest leaf). The number and life stage of thrips were recorded on lower (abaxial) and upper (adaxial) leaf surfaces before the percentage feeding damage area was estimated and trichome density on leaf surfaces were measured under a high-powered microscope (Leica M165 FC, Leica Microsystems Ltd, Milton Keynes, UK). The observed percentage feeding and oviposition damaged area on the abaxial surface of each leaf was estimated using image analysis software, Fiji (Schindelin, Arganda-Carreras and Frise 2012). This was possible by adjusting the contrast to measure the pale areas of 'silvering' caused by thrips against darker healthy tissue and calculating damaged area as a percentage of total lower leaf surface (to the nearest 5%, 0 to 100%). The number of non-glandular and glandular trichomes present on the lower

surface of excised leaves were recorded within a 25mm<sup>2</sup> area located in the central point of the lamina adjacent to the main vein.

### ***Chemical analysis.***

Three branches (length 14 cm) were removed from individual specimens of *R. simsii* and ordered by age with oldest leaves furthest from the meristem. Feeding and oviposition damaged area was scored and trichome density recorded (see above) prior to weighing, freeze-drying and grinding individual leaves to a fine powder with a pestle and mortar. The powdered material was extracted in 80% methanol (100 ml per 10 g leaf powder) for 48 hr and filtered. Aliquots (1 ml) of the acetone and aqueous methanol extracts were centrifuged prior to analysis using LC-MS. LC-MS analysis was carried out using a Waters Alliance LC solvent delivery system with a ZQ MS detector on a Phenomenex Luna C18(2) column (150×4.0 mm i.d., 5 µm particle size) operating under gradient conditions, with A=MeOH, B=H<sub>2</sub>O, C=1% HCO<sub>2</sub>H in MeCN; A=0%, B=90% at t=0 min; A=90%, B=0% at t=20 min; A=90%, B=0% at t=30 min; A=0%, B=90% at t=31 min; column temperature 30°C and flow rate of 0.5 ml min<sup>-1</sup>. Grayanotoxin I [(3b,6b,14R)-Grayanotoxane-3,5,6,10,14,16-hexol-14-acetate] was obtained from our own natural products collection as isolated and reported previously (Tiedeken *et al.* 2014; 2015) and used as a chromatographic standard to generate a calibration curve for this compound by quantification of the [M-H]<sup>-</sup> pseudomolecular ion in negative mode which had a *m/z*=411.1 corresponding to the molecular weight of grayanotoxin I and eluting at 8.1 min. The concentration of grayanotoxin I could be calculated from the peak areas and comparison made against standard concentrations as described previously (Tiedeken *et al.* 2014). In order to clarify whether grayanotoxin I was present in trichome exudate or on the surface of the leaf, surface wash extracts were made by dipping whole undamaged young leaves into a beaker containing 200 ml diethyl ether for two minutes and extracts dried and taken up in 80% methanol prior to LC-MS analysis. In addition, trichomes on the abaxial surface of several leaf samples were removed carefully with a razor blade,

freeze dried and examined for the presence of grayanotoxin I using the same extraction process described above for the leaf material.

***Effect of grayanotoxin I on feeding and oviposition site preference (Duel choice test).***

Uninfested mature leaves of *R. simsii* were collected, rinsed in distilled water, dried, and cut into sections (2 × 2 cm) that were dipped in grayanotoxin I at concentrations of 1000, 100 or 10 ppm (treatment), or acetone only (control). Two leaf discs; one treatment and a control, were placed at opposite sides of a Petri-dish (9 cm) on top of filter paper dampened with distilled water. Five *H. haemorrhoidalis* pupae were placed in the centre of the Petri dish, the lid was sealed with parafilm and the locations of the thrips were recorded after 24 h and at regular intervals until 312 h, maintained at 27°C, L16:D8 (n=10 for each concentration). All pupae had eclosed prior to the 24 hr assessment. In order to assess the level of oviposition the number of immature thrips that emerged from leaf discs treated with grayanotoxin I was recorded at weekly intervals for four weeks and compared with numbers emerging on control discs. Living thrips on filter paper or sides of Petri-dish were not included in the selection preference data.

***Mortality of thrips exposed to grayanotoxin I***

Grayanotoxin I was dissolved in acetone to concentrations of 1000, 100, and 10 ppm, and added to small glass vials (2 ml) which were then turned manually with lids in position for 2 min. The lids were removed and excess test solution poured off and the vials were then air dried for 20 min providing a uniform coating of the compound on the internal surfaces of the vial and lid. A small section of avocado fruit skin (5 x 5 mm) was added to the vial as a food source and to maintain humidity. The avocado section was also dipped in the grayanotoxin I treatment at the relevant concentration and air dried on filter paper for 30 min before being placed in the glass vial. A single first stadium thrips was introduced on to the vial internal surface and lids were secured. Vials were maintained at 27°C, L16:D8 throughout the duration of the experiments. Control vials and avocado skin sections were dipped into acetone only.



Mortality of thrips was monitored at 24, 48 hrs and 72 hours after the introduction of the thrips larvae (n=16).

### *Statistical Analysis*

To examine the distribution of the thrips on the leaves of varying age we used a generalized linear model (GLM) that allows for response variables that have error distribution models other than a normal distribution, with quasi-Poisson errors. We grouped the leaves into seven sets relating to leaf maturity before conducting multiple comparisons using Tukey's HSD to determine where the differences between the thrips distribution on grouped age-sets arose. To visualise trends in trichome density and grayanotoxin I concentration in leaves with increasing age the branches data was combined where data demonstrated no significance with Kruskal–Wallis one-way analysis of variance (KW) applying Shapiro–Wilk test to test first that the data did not sit within the normal distribution range. Correlations between the number of thrips on leaves along branches and grayanotoxin I concentration and density of trichomes on ageing leaves were analysed using GLMs with negative binomial errors and log it link. Predicted regression lines between the percentage area damage score and each of the plant traits were established using GLMs with quasibinomial errors with a logit link function and a multiple regression of damage (logit scale) predicted whether significance could be assigned to a particular trichome type. To analyse the effects of isolated grayanotoxin I compound on the choice of leaf-discs by *H. haemorrhoidalis*, we compared the percentage of thrips on treated and untreated discs at set time intervals. We applied the Shapiro-Wilk test for normality as before and the comparison was then undertaken with one-way analysis of variance (ANOVA). The same methods were used to make a comparison between mean numbers of immature larvae distributed on treated and untreated leaf discs. The no-choice experiments were analysed using Mann Whitney U test as normality could not be assumed (Shapiro-Wilk,  $P < 0.05$ ) in order to determine if grayanotoxin I was toxic to first stadium larvae at the three concentrations applied. All generalized linear models were run using the

lme4 package in R (R Core Team 2013, Austria). The remaining statistical modelling was carried out in GenStat (GenStat 14.2, 2011, VSN International Ltd).

## **Results.**

### ***Leaf Surface morphology.***

The morphology of *Rhododendron simsii* leaves that were infested and uninfested with *H. haemorrhoidalis* was examined (Fig. 1 a, b). Lateral branches consisted of approximately 20 to 30 leaves with the youngest leaves (position 1) closest to terminal bud. In the year of study it was observed that between 15 to 20 new leaves were produced on each branch. The leaves of *R. simsii* are lanceolate, coriaceous and coated with a sparse indumentum comprised of simple, multi-cellular glandular and strigose (non-glandular) trichomes on both upper (adaxial) and lower (abaxial) surfaces (Fig 2. a, b). Thrips (Fig. 3) were observed more frequently on the abaxial surfaces of the lower leaves on branches and it appeared that the sparsely distributed adult thrips present on leaves towards the terminal bud were smaller in size than those found on mature leaves. The oldest leaves lacked glandular trichomes which were otherwise numerous on younger leaves. Non-glandular trichomes were recorded on the mature leaves and during the study adult *H. haemorrhoidalis* were observed to secrete single faecal droplets on to the terminal ends of these trichomes hairs (Fig. S1). The mature leaves of thrips-infested plants were severely damaged with up to 95% of the abaxial leaf epidermis desiccated due to cell content removal with high numbers of thrips feeding and ovipositing on the lower leaf surfaces.

### ***Density of thrips, leaf trichomes and percentage area damage caused by feeding and oviposition.***

The numbers of thrips (mean $\pm$ se) on the leaves (abaxial surface) on branches of *R. simsii* were counted and presented in grouped clusters of 4 leaves (Figure 4) to demonstrate the distribution of thrips within branches (no significant difference between numbers on leaves of branch 1, 2 and 3; KW  $P > 0.05$ ). The numbers of thrips depended on leaf age group, with an

inverted U relationship ( $P < 0.0001$ ,  $F = 13.7$ , d.f. = 6.20) showing a decline on numbers on leaves in the oldest group where the percentage area of feeding damage exceeds 95% on all three branches (leaf position 25 to 27). The decrease in trichome density as leaves expanded and matured is shown on Figure 5a and in consideration of the high levels of leaf surface damage on the oldest leaves, the following correlations between trichome density and number of thrips and % area feeding damage are assessed on only the first 20 leaves of branch from terminal bud where damaged surface area was recorded as less than 90%. Figures 6 a and b indicates the significant negative interaction between thrips and trichomes on lower leaf surfaces with a decrease in the numbers of thrips observed on surfaces of younger leaves which have an increased density of non-glandular ( $\chi^2=37.16$ ,  $P<0.001$ ) and glandular trichome ( $\chi^2=36.01$ ,  $P<0.001$ ) types. Trichome density is also negatively correlated with feeding damage on abaxial surface (Figures 7 a and b), with higher density of trichomes resulting in lower areas of damage recorded (non-glandular trichomes,  $F=46.52$ ,  $P<0.001$  and glandular trichomes,  $F=57.61$ ,  $P<0.001$ ). While physically isolating and testing each trichome type against the thrips feeding was not feasible a multiple regression of area of damage predicted that it was the density of glandular trichomes that significantly reduced feeding damage on leaf surface (glandular trichomes,  $F=55.69$ ,  $P<0.0001$ , non-glandular trichomes,  $F=0.15$ ,  $P>0.05$ ).

### ***Chemical analysis.***

The LC-MS analysis of leaf extracts of *R. simsii* demonstrated that grayanotoxin I was prevalent in younger leaves but levels of this compound decreased as leaves aged. We found no indication that other closely related grayanoid diterpenes, such as grayanotoxin III were present in the chemical profiles and an example of LC-MS traces collected from an extract of young leaves are provided in the supporting information (S2). The decreasing concentration of grayanotoxin I in increasingly older leaves on branches of *R. simsii* is shown in Figure 5b. As before, to avoid leaves that were showing extensive surface damage, analysis was undertaken on leaves in positions 1 to 20 where damage remained below 90% total abaxial

surface area. Four times as much grayanotoxin I was recorded in the youngest leaves (leaf position 3; 2086.7 ±156.77 ppm) than mature leaves (leaf position 20; 516.7±145.18 ppm). Figure 6c shows that grayanotoxin I influences the number of adult thrips on leaves of this plant species with higher concentrations correlating to lower numbers of thrips on abaxial leaf surfaces ( $\chi^2=42.90$ ,  $P<0.01$ ). Similarly the compound concentration recorded in leaves of *R. simsii* is negatively correlated to the percentage area of thrips damage observed on the abaxial surface of leaves of this species (Figure 7 c,  $F=51.75$ ,  $P<0.001$ ). Grayanotoxin I was not detected in the excised trichomes (glandular and non-glandular) removed from the abaxial leaf surfaces of *R. simsii*, however traces of this compound were detected in the surface-wash extracts (Figure S2, c and d).

#### ***Effect of residues of grayanotoxin I on feeding and oviposition site preference.***

A range of ecologically relevant concentrations of grayanotoxin were selected and applied in the choice tests however residue treatments at the highest concentration tested (1000 pm) resulted in thrips mortality in Petri-dishes with this treatment reaching 92% after 120 hrs from the point of pupae introduction (Fig 8 a) and there was no preference by thrips for treated or untreated discs observed. After a period of 120 hrs thrips exposed to grayanotoxin I treatments of 100 and 10 ppm (ANOVA  $F=7.68$ ,  $P<0.05$  and  $F=27.74$ ,  $P<0.001$  respectively) had shown a preference for untreated leaf discs (Figures 8 b and c) and the selection preference was more pronounced in the lower concentration tested. The number of immature thrips emerging on treated and untreated leaves recorded at 7 day intervals showed that immature stages were significantly higher on control leaves than those treated with 100 ppm and 10 ppm grayanotoxin I (ANOVA;  $F=7.69$ ;  $P<0.05$  and  $F=10.1$ ;  $P<0.01$  respectively,  $n=10$ ; Figures 9 a and b).

#### ***Mortality of thrips exposed to residues of grayanotoxin I***

In no-choice tests grayanotoxin I was highly toxic to first stage larvae of *H. haemorrhoidalis* when the insects came into direct contact with the compound. The mortality of the larvae

exposed to the most concentrated treatment (1000 ppm) exceeded 90% after 24 hours from initial contact (Figure 10). Both 100 and 10 ppm grayanotoxin I residues also resulted in significant levels of toxicity after 24 hours exposure when compared with surviving control (acetone only) larvae ( $MW$ ,  $U=40$ ,  $P<0.001$  and  $U=80$ ,  $P<0.05$  respectively,  $n=16$ ). Grayanotoxin I treatments at concentrations of 100 and 10 ppm resulted in excess of 80% mortality after 72 hours exposed to the compound residues, which again differed from the control larvae mortality monitored after this time ( $MW$ ,  $U=48$ ,  $p<0.001$ , and  $U=56$ ,  $P<0.01$ ). Larvae exposed to grayanotoxin I died on the internal surfaces of the glass vial and not on the food source, whereas in contrast the surviving control thrips were detected on the surface of the avocado sections in all cases suggesting that either contact toxicity or starvation was probable. However, one-off topical applications of grayanotoxin I (1000 ppm) were additionally tested on second stage larvae and the results of this pilot test are shown in the supporting information (Table S3). Mortality levels of thrips briefly in contact with grayanotoxin I in solution increased after 21 days post treatment when compared with mortality levels in controls ( $MW$ ,  $P<0.05$ ) and results indicate that the development time of treated immature thrips was slightly increased.

## **Discussion.**

In this study we identified grayanotoxin I in the leaves of *Rhododendron simsii* and showed that both the concentration of this compound and density of leaf trichomes were not evenly distributed and correlated with the distribution of *H. haemorrhoidalis* infesting this species. Young leaves and buds of *R. simsii* contained the highest concentrations of grayanotoxin I and remained free of thrips in contrast to the most mature leaves which contained little or no grayanotoxin I and suffered the most damage. We found that *R. simsii* had simple glandular and non-glandular trichomes present on both leaf surfaces and trichome density of both forms decreased as the leaves aged. To our knowledge this is the first study that has concurrently

reported the intra-plant distribution of both morphological and chemical defence traits in the genus *Rhododendron*.

Several studies have shown that the concentration of secondary metabolites are highest in buds and then decline as the leaves expand and mature suggesting a higher quantitative investment in chemical defence in the younger tissue (Crankshaw and Langenheim 1981; Palo 1984; Horner 1988; Coley 1983; Mauffette and Oechel 1989; Hatcher 1990; Leiss *et al.* 2009; Wiggins *et al.* 2016). Our findings showed that the concentration of grayanotoxin I demonstrated a similar distribution pattern in the foliage of *R. simsii*. In addition, low molecular-weight phenolics, terpenes and alkaloids are reported to accumulate in early stages of seedling growth and leaf expansion (Dement and Mooney 1974; Cates and Rhoades 1977; Frischknecht *et al.* 1986; Potter and Kimmerer 1986; Puttick 1986; Mauffette and Oechel 1989; Aerts *et al.* 1991; Fujimori *et al.* 1991; Porter *et al.* 1991; Singh *et al.* 1991; also see Herms and Mattson 1992) with accumulation of these compounds occurring in developing epidermal cells (Levin 1976; McClure 1979); the first cells in leaves to mature (Dale 1988) and on which *H. haemorrhoidalis* are known to feed (Kirk 1997; Mound 1997). It is likely that as the thrips progress upwards on leaves on branches of *R. simsii*, that the presence of grayanotoxin I in leaf cells and on the leaf surface is encountered by thrips feeding on younger leaves. While some recent work suggests that the toxicity of grayanotoxin I could underpin its function mediating the behaviour of pollinators (Tiedecken *et al.* 2014; 2016) there is lack of information on the ecological function of this compound in herbivore defence, with only few records documenting activity against model agricultural pest species (Klocke *et al.* 1991; Hu *et al.* 2000; Zong *et al.* 2005; Yi *et al.* 2014). Surprisingly there is little other published work reporting defence effects of grayanoid compounds against *Rhododendron* pests. We found that grayanotoxin I is highly toxic to *H. haemorrhoidalis* through direct contact with the compound residues and in solution. Previously Yi *et al.* (2014) reported that physical contact was essential for adult *Spodoptera litura* (Fabricius) to perceive Rhodojaponin-III and produce the feeding and oviposition deterrent effects against moths and suggested that the

sensilla on tarsus and ovipositor could be chemoreceptor for Rhodojaponin-III. Grayanotoxin I reportedly binds to voltage-gated sodium channels in animal cells, increasing the permeability of sodium ions by inhibiting inactivation of sodium channels (Seyama *et al.* 1985; O'Reilly *et al.* 2014; Kadala *et al.* 2014) with resultant toxic effects on animals (Koca and Koca 2007; Cheng *et al.* 2011; Oliver *et al.* 2015). Klocke *et al.* (1991) demonstrated that grayanotoxin I was deterrent to the Colorado potato beetle; *Leptinotarsa decemlineata* (Say) and the fall army worm; *Spodoptera frugiperda* (J.E. Smith). Similarly our results show that pure grayanotoxin I when applied to leaf discs at concentrations detected in the mature leaves of *R. simsii* (100 and 10ppm) deterred thrips after five days exposure to the compound. Increasing the concentration to 1000 ppm resulted in thrips mortality rapidly exceeding 90% within five days, thus obscuring the deterrent effect seen at the lower test concentrations. While preference for the untreated leaf discs by later life-stages of *H. haemorrhoidalis* was conclusive, the results also suggest that grayanotoxin I may also deter oviposition as fewer immature thrips emerged and developed on leaf discs treated with compound. Interestingly the lowest concentration of grayanotoxin I tested produced the clearest evidence of a deterrent effect against *H. haemorrhoidalis*, thus warranting further investigation into the sub-lethal effects caused by grayanotoxin I in order to fully comprehend the mode of action of this defence mechanism on this generalist herbivore. In our study we show that the profound effects of grayanotoxin I on the mortality and behaviour of the thrips supports our hypothesis that the occurrence and distribution of this compound in the leaves of *R. simsii* is contributing to the preservation of the more valuable young leaf and bud tissue.

Leaf trichomes have been linked to two functions in rhododendrons; protection from abiotic factors (Cowan 1950; Woodman and Fernandes 1990) and herbivory (Valla 1980; Doss 1984; Balsdon *et al.* 1995). We found that young leaves of *R. simsii* were covered with a matrix of overlapping trichomes on both leaf surfaces, consisting of glandular and non-glandular simple forms, and the density of both forms decreased as leaves aged. Leaf expansion could provide some explanation of the cause of the decrease in trichome density in aging leaves (Valkama *et*

*al.* 2004) from leaf position one to five, after which point leaves are 90-100% expanded and the process of leaf maturation is considered complete (Avery 1933; Isebrands and Larson 1973; Esau 1977; Coleman 1986; Coleman and Leonard 2015). Variation in trichome density in young and mature leaves has been previously reported to occur on individuals of a range plant species and within different populations of the same species (Valverde *et al.*, 2001; Molina-Montenegro *et al.* 2006, Traw and Feeny, 2008, Kobayashi *et al.* 2008, Yamawo *et al.* 2012; 2014, Alba *et al.*, 2014). Evidence suggests that both non-glandular and glandular leaf trichomes provide a defence mechanism against generalist and specialist herbivores (Gibson and Turner 1977; Tingey and Gibson 1978; Becerra and Ezcurra 1986; Gross and Price 1988; Hulley 1988; Woodman and Fernandes 1991; Handley *et al.* 2005; Medeiros and Tingey 2006; Avery *et al.* 2015) and this has been discussed within the framework of the optimal defence theory, although far less frequently than chemical defence strategies (Woodman and Fernandes 1991; Björkman *et al.* 2008; Traw and Feeny 2008; Alba *et al.* 2014). In this study we showed that the numbers of thrips and feeding damage on the leaves of *R. simsii* increased significantly as leaves aged and trichome density decreased. Yet while both glandular and non-glandular trichomes were observed to decrease on aging leaves of this species our evidence suggests that it was the glandular trichomes that were linked to the reduction in thrips feeding damage. In addition to being a physical barrier glandular trichomes can release compounds that can be toxic or repellent to insect herbivores (Kelsey 1983; Goffreda *et al.* 1988; van Dam *et al.* 1998a; 1998b; Ambrósio *et al.* 2008; Kobayashi *et al.* 2008, Tian *et al.* 2012). A study on lepidote rhododendrons by Doss (1984) demonstrated that essential oils extracted from leaf scales of 11 species deterred the black vine weevil, *Otiiorhynchus sulcatus* (F.) suggesting that these volatile compounds may function as a component of plant defence. We observed that the majority of glandular trichomes had ruptured during the process of leaf expansion and maturation. As previous studies have reported that diterpenoids are associated with glandular hairs in some plant species (Kelsey *et al.* 1984) we removed and analysed the glandular trichomes from leaves of *R. simsii* but found no evidence that grayanotoxin I was present in these leaf structures. Although here we focused on the grayanoid diterpene content



of the exudates of the glandular trichomes in our analysis it is possible that compounds from other classes are present and could also be influencing the behaviour of the thrips, thus strengthening the hypothesis that a complexity of biochemical and morphological traits in *R. simsii* contribute to the preservation of young leaves from feeding damage. As damage by herbivores can induce trichome production on leaf surfaces in some species (Agrawal 1999; 2000; Pullin and Gilbert 1989; Traw and Dawson 2002; Rautio *et al.* 2002; Dalin and Björkman 2003; see also Dalin *et al.* 2008) further investigations would be necessary to determine if an increase in trichome density occurred on younger leaves of *R. simsii* as a result of *H. haemorrhoidalis* invading the mature leaves, thus providing a damage –induced mechanism to preserve the more valuable tissue from thrips colonisation.

While plant defence theories (e.g. Stamp, 2003) have helped us understand how pressures of herbivory can influence the diversity of abundance and distribution of defensive traits in plant species, evidence of an evolutionary link between defence traits such as leaf pubescence and secondary metabolites (Agrawal and Fishbein, 2008; Kariñho-Betancourt *et al.* 2015) has highlighted the intrinsic nature of the relationships between these traits in context of resistance and tolerance to herbivores (Rosenthal and Kotanen 1994; Strauss and Agrawal 1999; Carmona and Fornoni 2013). We aimed to establish the variation in distribution of two defence traits in *Rhododendron simsii* and showed that both chemical and physical defence traits are expressed in high value tissue of *R. simsii* and that the concentration of these traits contribute significantly to their preservation from insect feeding damage. The classic indeterminate growth cycle of *R. simsii* follows a pattern of short intensive growth in spring followed by a prolonged period of slow growth, a stage during which plants are known to divert resources to processes other than growth such as defence (Chapin *et al.* 1990; Wardlaw 1990). Slow growth enables a lower trade-off between resource allocation for growth and defence (Tuomi *et al.* 1983; Herms and Mattson 1992). *H. haemorrhoidalis* are first recorded on mature leaves of *R. simsii* in spring with populations increasing into summer until mature leaves defoliate and thrips population rapidly declines, avoiding younger leaves. The

concurrent expression of both morphological and chemical defensive traits in young tissue at the expense of non-essential older leaf tissue illustrates how cost mediates expression of defence traits and is consistent with optimal defence theory enabling a plant to tolerate and survive severe levels of herbivory by preserving vital tissues (McCall and Fordyce 2010). We showed a relationship between each trait and the reduction of thrips and feeding damage and conclude that both trichome density and grayanotoxin I can mediate resistance to or tolerance of *R. simsii* to herbivores. Núñez-Farfán *et al.* 2007 reported that individual plants are often observed to allocate resources to a combination of defence strategies. By further studying the variation of the chemical and morphological traits among natural populations of *R. simsii* and the diversity of consumers (both pollinators and leaf-feeders) they may encounter in these environments which potentially influence the selection of both traits, we can determine if they are complimentary (Fornoni *et al.* 2004) or redundant (Mauricio *et al.* 1997; Tiffin and Rausher 1999; Mauricio 2000; Pilsen 2000), the former resulting in higher benefits from expressing both strategies simultaneously rather than one alone (see also Carmona and Fornoni 2012). Understanding the relevance of physiological traits common to species of plants that are successful in unstable environments can contribute to our knowledge on how some species of plants become invasive and flourish in new habitats and why certain crop species or varieties are more tolerant to pests and disease.

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## Data Accessibility

All data used in this manuscript are present in the manuscript and its supporting information.

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Figure 1. *Rhododendron simsii*; a) before infestation by *H. haemorrhoidalis*, b) defoliation after infestation by glasshouse thrips.

Figure 2. Scanning electron micrograph of a simple non-glandular strigosa trichome (1 mm) and two simple glandular trichomes (100  $\mu$ m) present on the abaxial surface of (a) a mature leaf and (b) a young leaf of *Rhododendron simsii*.

Figure 3. Scanning electron micrograph of an adult glasshouse thrips, *Heliothrips haemorrhoidalis* (Bouché).

Figure 4. Number of thrips (mean $\pm$ se) on both leaf surfaces of *R. simsii*, grouped by age with four leaves in each group; 1-4, youngest leaves to 25-27 oldest leaves (GLM, quasi-Poisson errors). Values with same letter code are not significantly different (Tukey multiple comparison test)

Figure 5: Relative distribution of a) trichomes (density  $\text{mm}^2$ ) on abaxial leaf surfaces and b) concentration of grayanotoxin I (x 1000 ppm) in young and mature leaves on branches of *R. simsii*. Leaf age range recorded by position of leaf from terminal bud (leaf 1) to mature leaves from previous year's growth (leaf 30+).

Figure 6. Number of adult *H. haemorrhoidalis* in relation to leaf traits present in leaves of *R. simsii* a) numbers of adult thrips negatively correlated to the density of non-glandular trichomes on abaxial leaf surface on leaves of *R. simsii*, b) numbers of adult thrips negatively correlated to the density of glandular trichomes on abaxial leaf surface on leaves of *R. simsii* and c) numbers of adult thrips negatively correlated to the concentration of grayanotoxin I present in leaves of *R. simsii*. The regression line is predicted by a generalised linear model with negative binomial errors and a log link.

Figure 7. Percentage score of area of leaf damage (thrips feeding and oviposition) in relation to leaf traits present in leaves of *R. simsii* a) % area of damage on abaxial leaf surface negatively correlated to the density of non-glandular trichomes on abaxial leaf surface on leaves of *R. simsii*; b) % area of damage on abaxial leaf surface negatively correlated to the density of glandular trichomes on abaxial

leaf surface on leaves of *R. simsii* and c) % area of damage on abaxial leaf surface negatively correlated to the concentration of grayanotoxin I present in leaves of *R. simsii*. The regression line is predicted by a generalised linear model with quasibinomial errors and a logit link

Figure 8: Location of five *H. haemorrhoidalis* (inoculated at pupal stage) in dual choice bioassay: % surviving thrips (mean±sem) on treated (grayanotoxin I) and untreated (control) leaf-disc, treatment concentration applied at (a) 1000ppm, (b) 100 ppm and (c) 10 ppm. (ANOVA or *MW*; \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001, *n*=10).

Figure 9. Mean number of immature thrips (mean ± sem) emerging on leaf discs treated with grayanotoxin I (GTX I) (a) 10 ppm or (b) 100 ppm, and untreated (control, acetone only) in dual choice tests recorded over a four weeks after initial introduction of five *H. haemorrhoidalis* pupae. (ANOVA; \**P*<0.05, \*\**P*<0.01, *n*=10).

Figure 10. Percentage mortality of first stadium larvae of *H. haemorrhoidalis* (mean ± sem) exposed to residues of grayanotoxin I (GTX I) at concentrations of 1000, 100, 10 ppm, monitored at 24 hr intervals for three days (Mann Whitney U; \**P*<0.05, \*\**P*<0.01, \*\*\**P*<0.001, *n*=16).

Figure 1. *Rhododendron simsii*; a) before infestation by *H. haemorrhoidalis*, b) defoliation after infestation by thrips.



a)

b)

Figure 2. Scanning electron micrograph of a simple non-glandular strigosa trichome (1 mm) and two simple glandular trichomes (100  $\mu\text{m}$ ) present on the abaxial surface of (a) a mature leaf and (b) a young leaf of *Rhododendron simsii*. Scale bars 100  $\mu\text{m}$ .

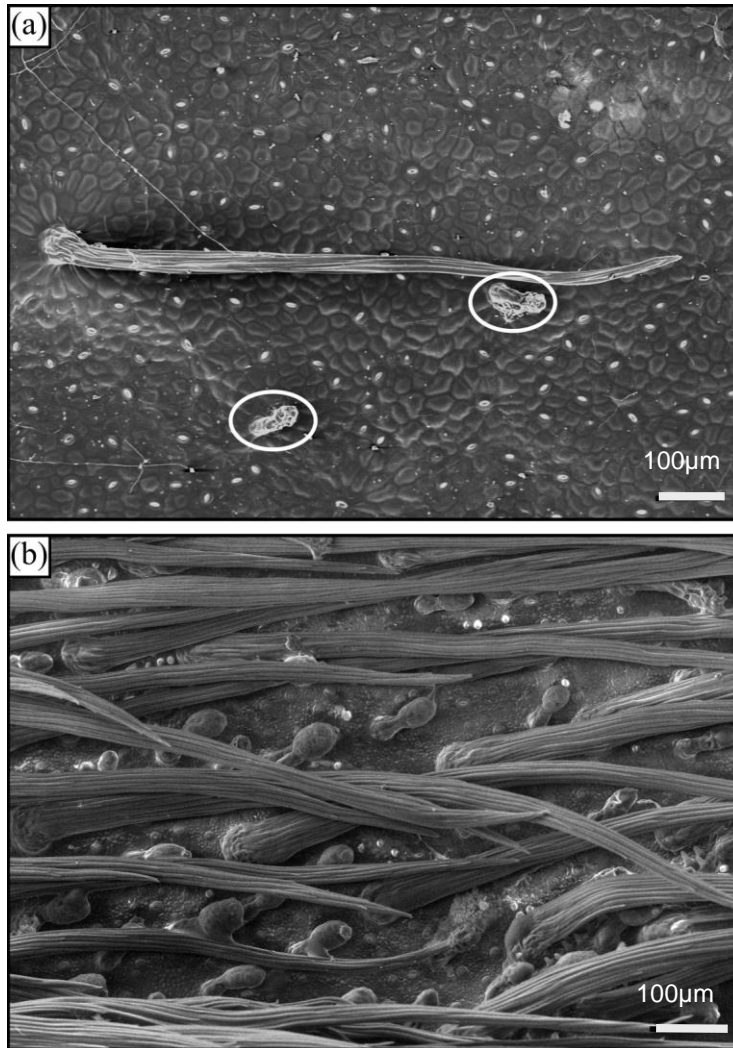


Figure 3. Scanning electron micrograph of an adult glasshouse thrips; *Heliothrips haemorrhoidalis* (Bouché). Body length 1.374 mm.



Figure 4. Number of thrips (mean±se) on both leaf surfaces of *R. simsii*, grouped by age with four leaves in each group; 1-4, youngest leaves to 25-27 oldest leaves (GLM, quasi-Poisson errors). Values with same letter code are not significantly different (Tukey multiple comparison test)

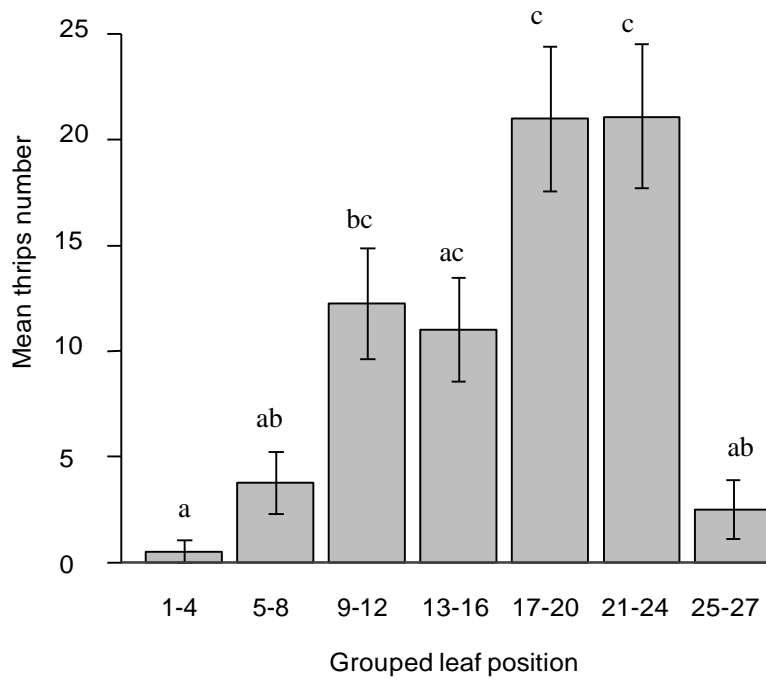
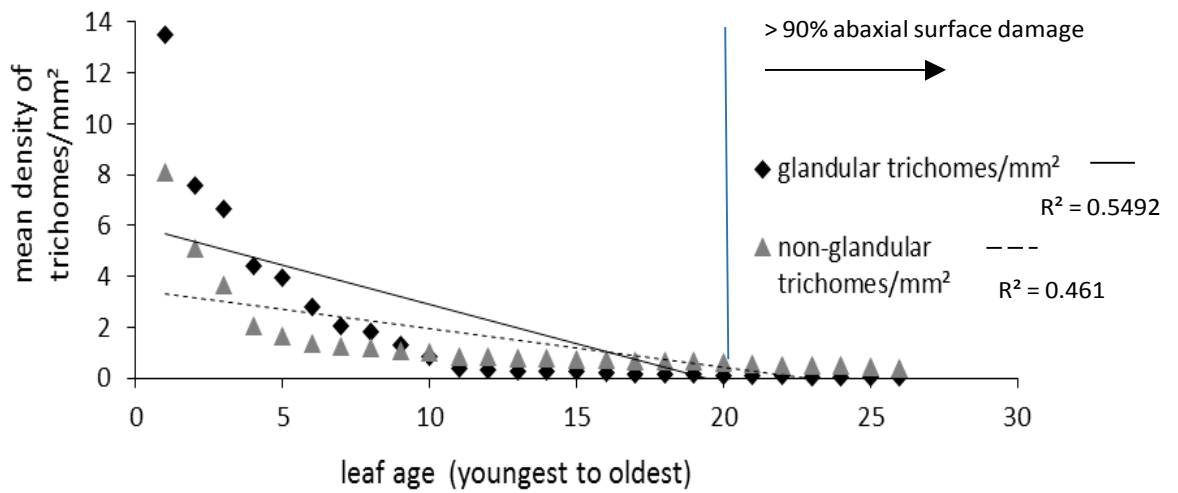




Figure 5: Relative distribution of a) trichomes (density  $\text{mm}^2$ ) on abaxial leaf surfaces and b) concentration of grayanotoxin I (x 1000 ppm) in young and mature leaves on branches of *R. simsii*. Leaf age range recorded by position of leaf from terminal bud (leaf 1) to mature leaves from previous year's growth (leaf 30+).

a)



b)

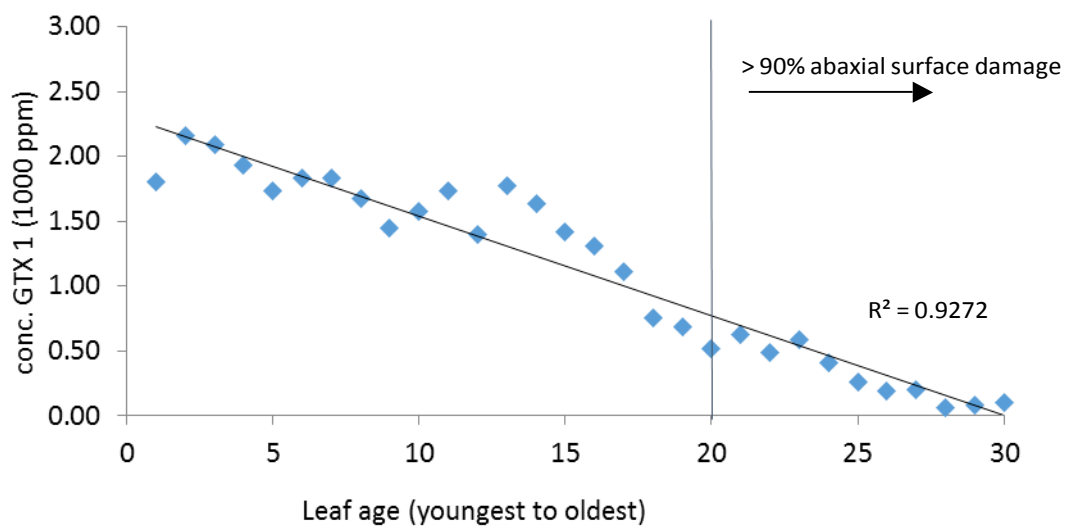
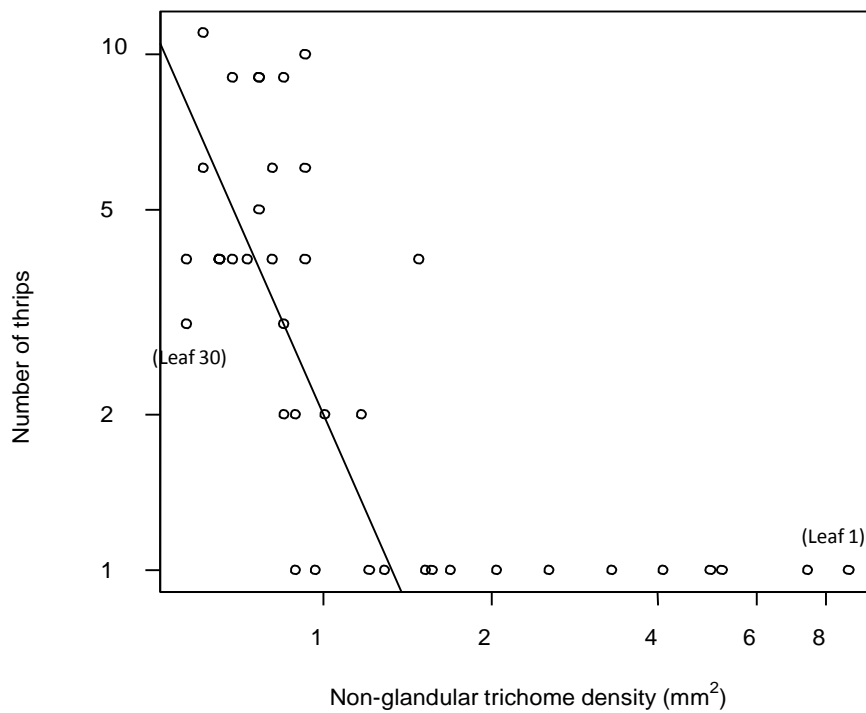
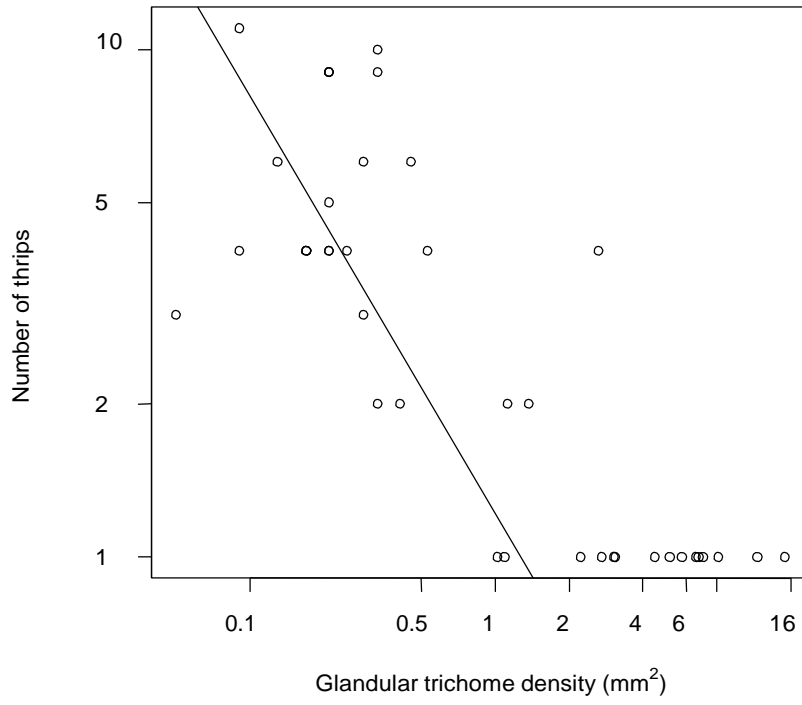


Figure 6. Number of adult *H. haemorrhoidalis* in relation to leaf traits present in leaves of *R. simsii* (leaf position 30, oldest leaf to leaf position 1, youngest leaf) a) numbers of adult thrips negatively correlated to the density of non-glandular trichomes on abaxial leaf surface on leaves of *R. simsii*, b) numbers of adult thrips negatively correlated to the density of glandular trichomes on abaxial leaf surface on leaves of *R. simsii* and c) numbers of adult thrips negatively correlated to the concentration of grayanotoxin I present in leaves of *R. simsii*. The regression line is predicted by a generalised linear model with negative binomial errors and a log link, noting there is some evidence of an asymptotic trend at high trichome densities.

a)



b)



c)

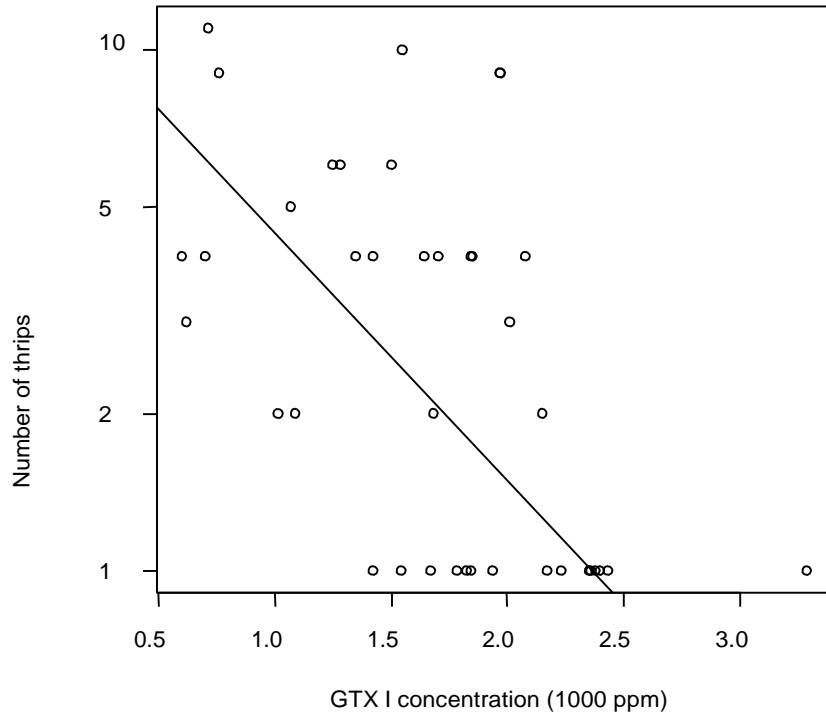
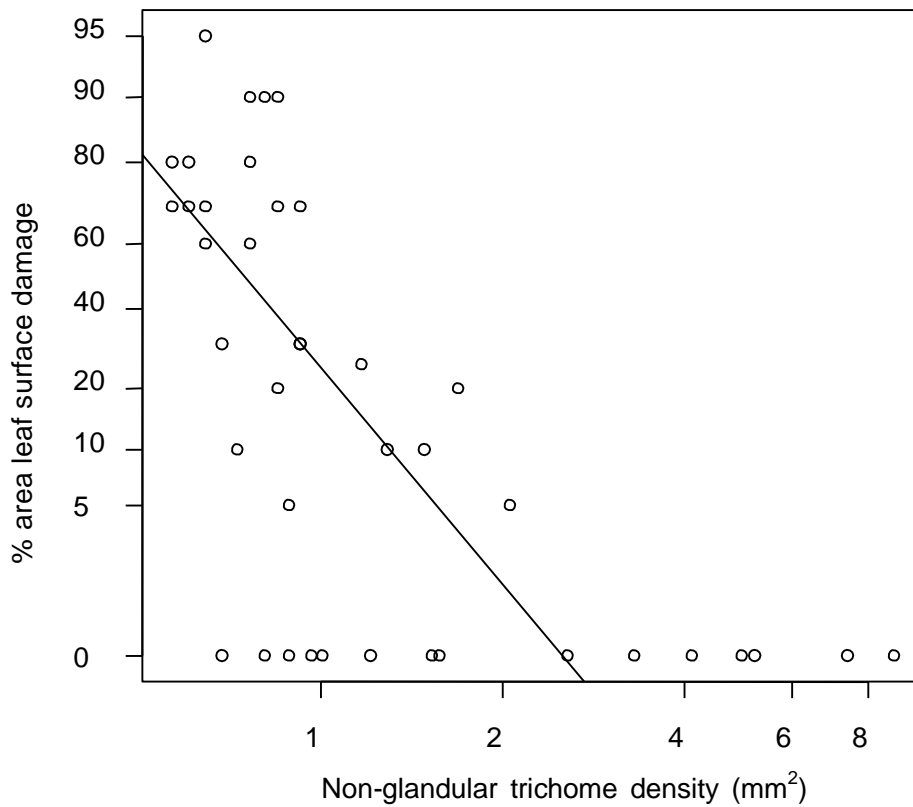
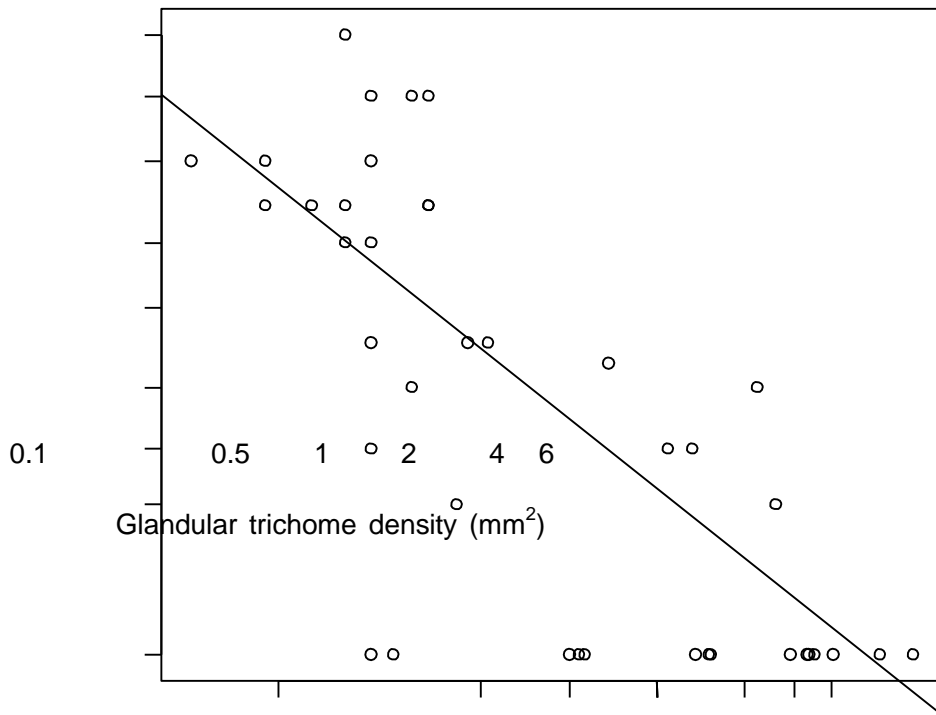


Figure 7. Percentage score of area of leaf damage (thrips feeding and oviposition) in relation to leaf traits present in young and mature leaves on branches of *R. simsii* a) % area of damage on abaxial leaf surface negatively correlated to the density of non-glandular trichomes on abaxial leaf surface on leaves of *R. simsii*; b) % area of damage on abaxial leaf surface negatively correlated to the density of glandular trichomes on abaxial leaf surface on leaves of *R. simsii* and c) % area of damage on abaxial leaf surface negatively correlated to the concentration of grayanotoxin I present in leaves of *R. simsii*. The regression line is predicted by a generalised linear model with quasibinomial errors and a logit link

a)



b)



c)

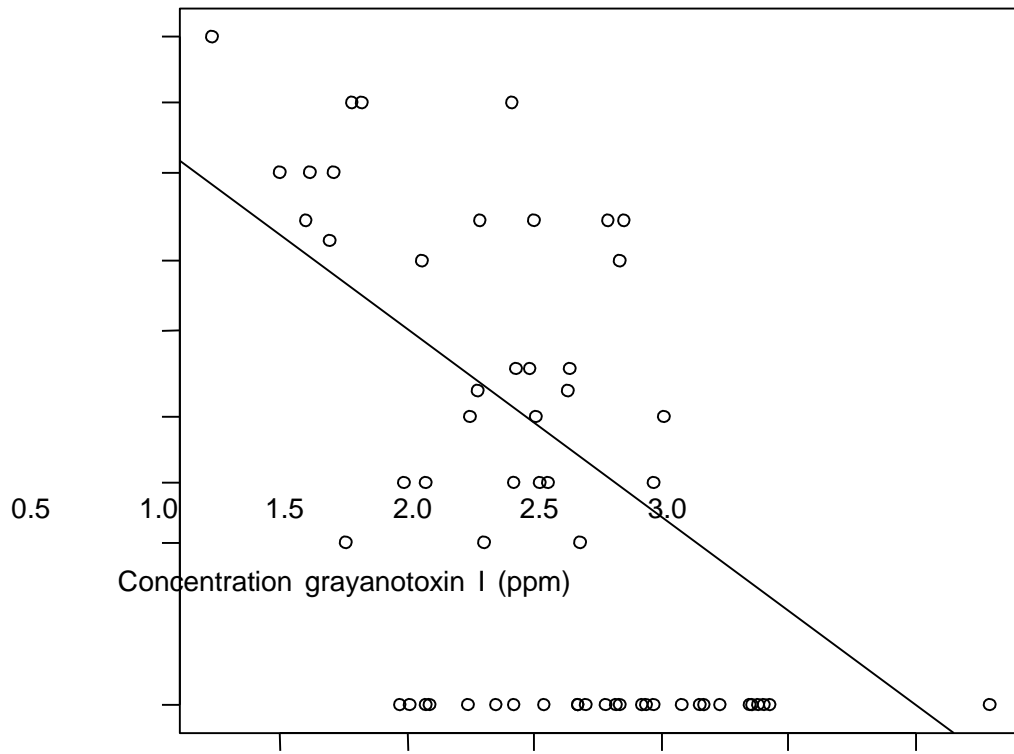
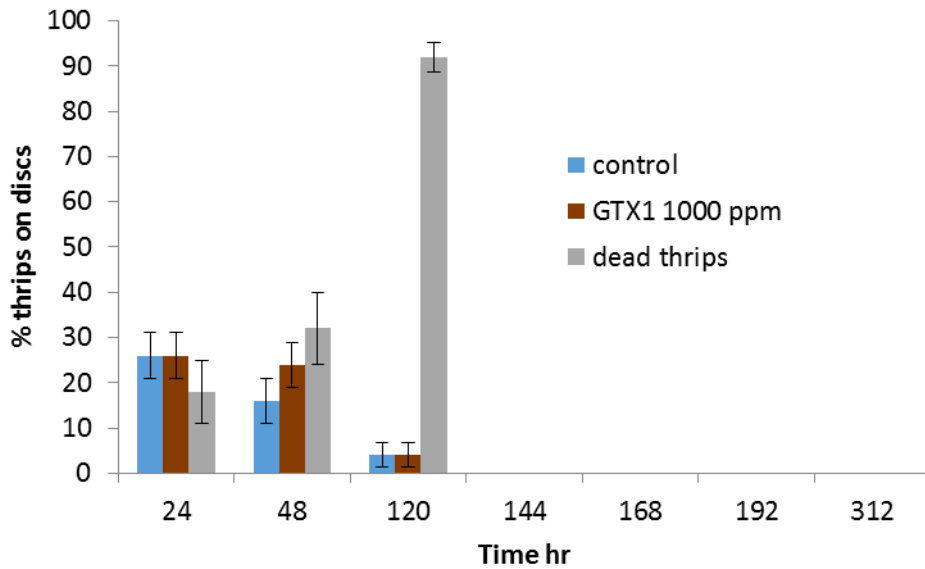
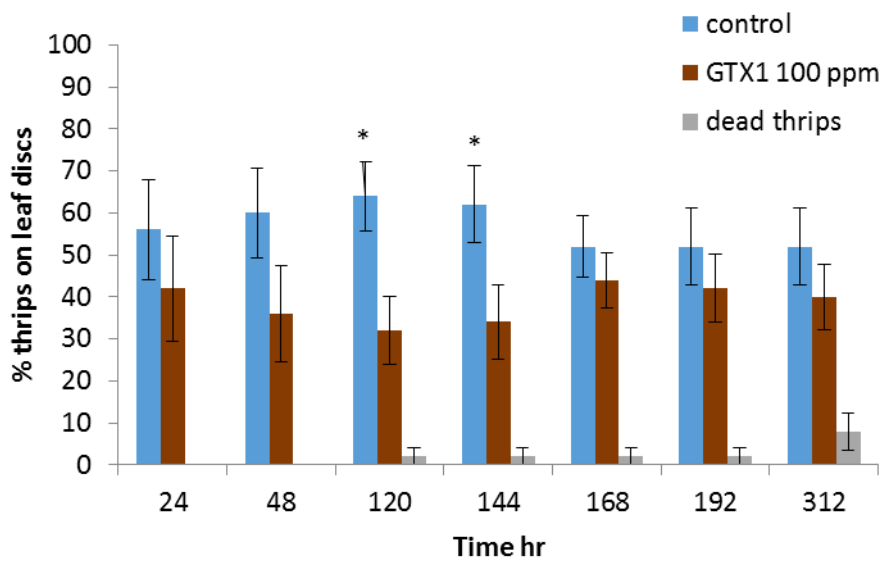


Figure 8: Location of five *H. haemorrhoidalis* (inoculated at pupal stage) in dual choice bioassay: % surviving thrips (mean±sem) on treated (grayanotoxin I) and untreated (control) leaf-disc, treatment concentration applied at (a) 1000ppm, (b) 100 ppm and (c) 10 ppm. (ANOVA; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, n=10).

(a)



(b)





(c)

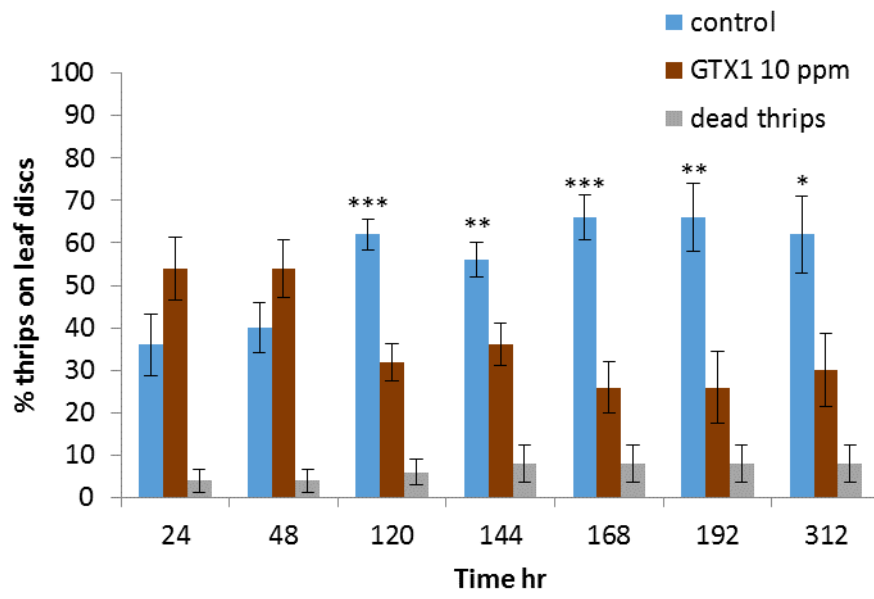
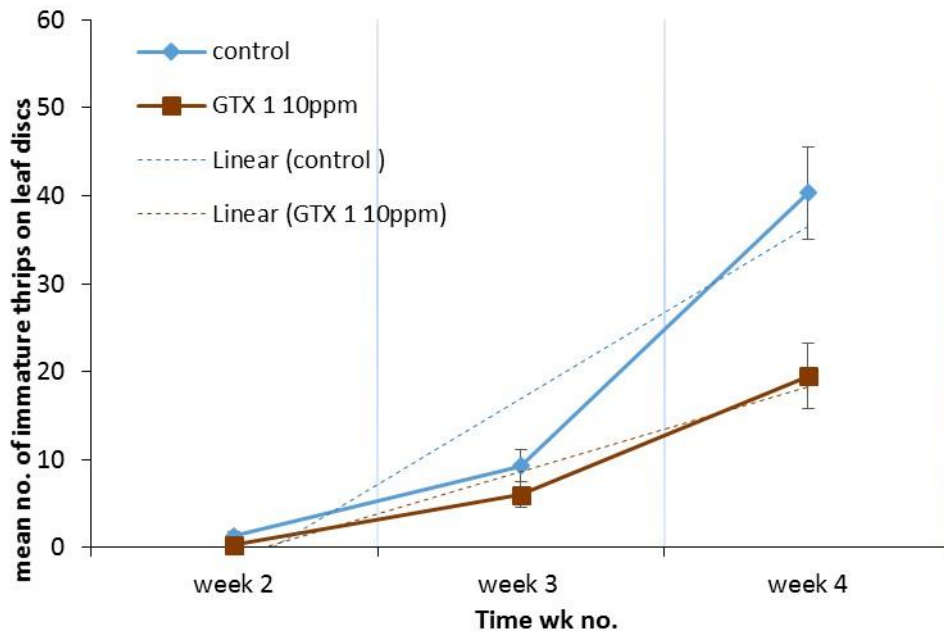


Figure 9. Mean number of immature thrips (mean  $\pm$  sem) emerging on leaf discs treated with grayanotoxin I (GTX I) (a) 10 ppm or (b) 100 ppm, and untreated (control, acetone only) in dual choice tests recorded over a four weeks after initial introduction of five *H. haemorrhoidalis* pupae. (ANOVA; \*P<0.05, \*\*P<0.01, n=10).

(a)



(b)

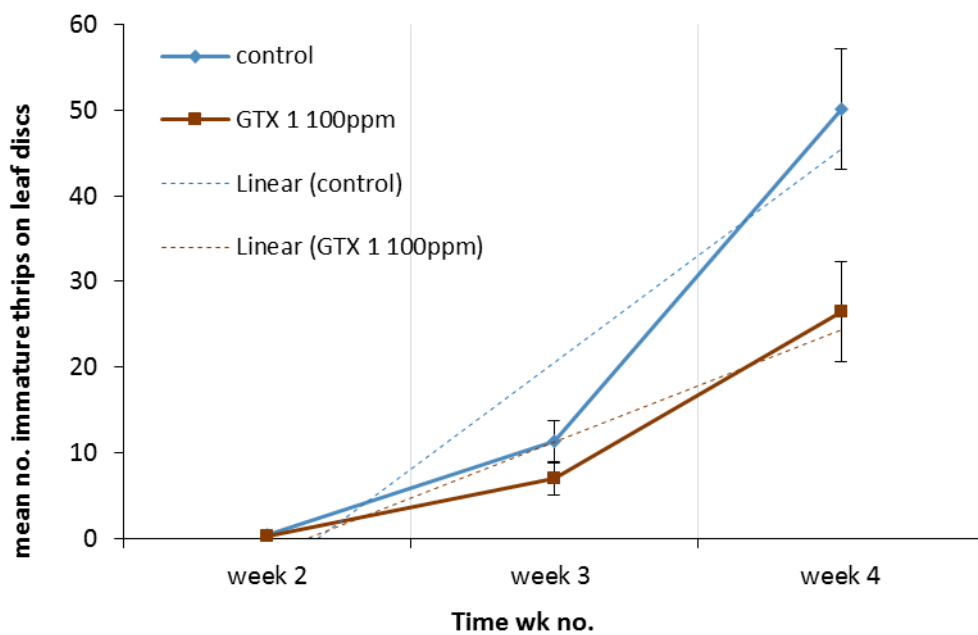
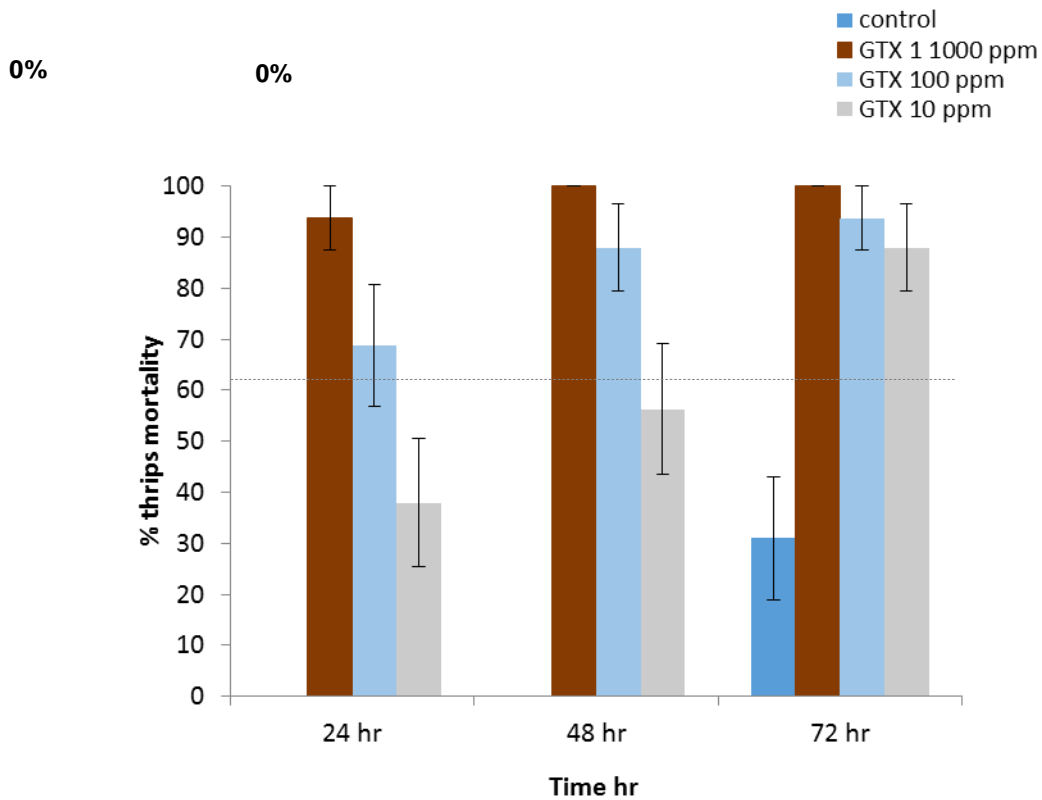


Figure 10. Percentage mortality of first stadium larvae of *H. haemorrhoidalis* (mean  $\pm$  sem) exposed to residues of grayanotoxin I (GTX I) at concentrations of 1000, 100, 10 ppm, monitored at 24 hr intervals for three days (Mann Whitney U; \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, n=16).



### **Supporting information**

S1: Figure S1a: Abaxial surface of leaf of *R. simsii* showing 90% area damage caused by *H. haemorrhoidalis* through feeding and oviposition. Figure S1b. Immature stages of *H. haemorrhoidalis* on abaxial leaf surface of *R. simsii*. Non-glandular trichomes with fecal droplet deposited on terminal tip.

S2: LCMS traces of extract of whole young leaf, excised trichomes and leaf surface wash of *Rhododendron simsii*.

S3: Effects of a single topical application of grayanotoxin I isolated from the leaves of *R. simsii* on the mortality level of second instars of *H. haemorrhoidalis*.

S4: Measurement and bioassay data to examine the relationship between concentration of grayanotoxin I, trichome type density and damage caused by thrips feeding on the abaxial leaf surface of leaves of *R. simsii* (abaxial data only included).