

RESEARCH IN CONTEXT: PART OF THE FOCUS ISSUE ON PLANT DEFENCE
AND STRESS RESPONSE

Exploring evolutionary theories of plant defence investment using field
populations of the deadly carrot

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- **Background and Aims** There are a number of disparate models predicting variation in plant chemical defences between species, and within a single species over space and time. These can give conflicting predictions. Here we review a number of these theories, before assessing their power to predict the spatial–temporal variation of thapsigargin between and within populations of the deadly carrot (*Thapsia garganica*). By utilizing multiple models simultaneously (optimum defence theory, growth rate hypothesis, growth–differentiation balance hypothesis, intra-specific framework and resource exchange model of plant defence), we will highlight gaps in their predictions and evaluate the performance of each.
- **Methods** Thapsigargin is a potent anti-herbivore compound that occurs in limited richness across the different plant tissues of *T. garganica*, and therefore represents an ideal system for exploring these models. *Thapsia garganica* plants were collected from six locations on the island of Ibiza, Spain, and the thapsigargin quantified within reproductive, vegetative and below-ground tissues. The effects of sampling time, location, mammalian herbivory, soil nutrition and changing root-associated fungal communities on the concentrations of thapsigargin within these *in situ* observations were analysed, and the results were compared with our model predictions.
- **Key Results** The models performed well in predicting the general defence strategy of *T. garganica* and the above-ground distribution of thapsigargin, but failed to predict the considerable proportion of defences found below ground. Models predicting variation over environmental gradients gave conflicting and less specific predictions, with intraspecific variation remaining less understood.
- **Conclusion** Here we found that multiple models predicting the general defence strategy of plant species could likely be integrated into a single model, while also finding a clear need to better incorporate below-ground defences into models of plant chemical defences. We found that constitutive and induced thapsigargin differed in their regulation, and suggest that models predicting intraspecific defences should consider them separately. Finally, we suggest that *in situ* studies be supplemented with experiments in controlled environments to identify specific environmental parameters that regulate variation in defences within species.

Key words: *Thapsia garganica*, Apiaceae, chemical defence, herbivory, soil, root-associated fungi, fungal inhibition, plant defence model, optimal defence theory, growth rate hypothesis, growth–differentiation balance hypothesis, resource exchange model of plant defence.

INTRODUCTION

How plants optimize and utilize their energy supply under stress has long been of interest to ecologists and evolutionary biologists alike (Thornley, 1972). In this regard, the process of herbivory has been a major focus for the majority of studies, where a plant balances the fitness cost of a loss of photosynthetic capacity against the cost of defence (Tuomi *et al.*, 1988). There is a plethora of specialized metabolites used in chemical defence against herbivores, with compounds making tissue unpalatable due to toxicity, but they all require photosynthates for their production (Gershenson, 2017).

However, when not required, the production of specialized anti-herbivore compounds has repeatedly been shown to negatively impact growth rates, and consequently the level of chemical defences is tightly regulated (Sampedro *et al.*, 2011). Defence compounds can be constitutively produced, but will vary substantially in quantity both within different tissues of an individual plant (Gershenson, 2017) and between individuals of the same species (Maldonado *et al.*, 2017). Alternatively, chemical defences can be induced when the plant is antagonized by specific stimuli (Moreira *et al.*, 2014). Consequently, a number of different models have been developed to predict the resource allocation of plants to specialized

anti-herbivore metabolites. However, different models often give conflicting predictions, with many exceptions observed (Le Bot *et al.*, 2009; Gutbrodt *et al.*, 2012; Kleczewski *et al.*, 2012) and no clear optimum approach established. A better understanding of the resource allocation for plant chemical defence is needed to help guide ecological studies, conservation efforts and sustainable production of medicinal and other useful natural products.

Evolutionary theories of plant defence investment

The optimal defence theory (ODT) was one of the earliest models to consider defence through an evolutionary perspective (McKey, 1974; Rhoades, 1979). It predicts that with increasing herbivory the relative cost of chemical defence is reduced, and this can be over evolutionary time frames or within a single life cycle. While the ODT suggests that there are competing functions for resources, so that defence has a cost in terms of growth and reproductive success, other defensive theories have more explicit expectations in this regard and are preferred (Stamp, 2003). However, it remains the only major model to address *in planta* variation, predicting that tissues of higher value to plant fitness will be better defended. It has performed well in predicting the relative defence levels of flowers (Kessler and Halitschke, 2009) and fruits (Zangerl and Rutledge, 1996), but performs less well for tissues with lower fitness values, like leaves. However, as a general rule, vegetative tissue is suggested to have a lower fitness value than reproductive tissue, and is therefore less protected (Zangerl and Bazzaz, 1992; Hamilton *et al.*, 2001).

The growth-rate hypothesis (GRH), which is sometimes referred to as the resource availability hypothesis (RAH; Coley *et al.*, 1985), expands upon the ODT by suggesting that plant defences and growth rates vary around their ability to regrow after a herbivory event. Plants that have evolved within low-resource or stressful environments have slower growth rates and allocate a higher proportion of photosynthates to defences instead of growth (Karban and Baldwin, 1997; Grime, 2006). Consequently, they are more likely to have evolved a suite of resistance traits, including higher levels of constitutive chemical defences and fewer induced defences than their faster-growing counterparts (Coley, 1988; Grime, 1977, 2006). Similarly, the growth–differentiation balance hypothesis (GDBH) model also predicts a plant's level of chemical defence in response to resource availability. Under the GDBH, two processes regulate the plant's entire photosynthate use. *Growth* refers to any process requiring substantial cell division and elongation, such as the production of roots, stems and leaves, whilst *differentiation* is essentially everything else, including herbivore defence (Loomis, 1932, 1953). Shortages of nutrients and water are thought to slow growth more than photosynthesis. Hence, as carbohydrates accumulate within the plant, the fitness reduction for differentiation processes is lower. Growth and differentiation are hypothesized to be mutually exclusive. Therefore, plants invest in growth-based strategies in resource-rich environments (high competition) and in differentiation-based strategies in low-resource environments (low competition).

Theories of defence investment in response to environmental variation

Both the GRH and GDBH can also be applied to predict the level of defence within a single species, where differences

are more likely derived from environmental rather than evolutionary processes. Unlike the GRH, the GDBH hypothesizes that maximum chemical defence occurs when enough nutrients are present to allow specialized metabolites to be readily synthesized, but before nutrient levels drive growth-based life strategies (as opposed to increasing nutrients leading to decreasing defences under the GRH). This was developed in part due to multiple studies finding that defence compounds were in the highest concentrations at intermediary levels of nutrition (Mihaliak and Lincoln, 1985; Wilkens *et al.*, 1996; Glynn *et al.*, 2007; Scogings, 2018). This suggests that plants form a growth–differentiation continuum over environmental gradients (Herms and Mattson, 1992).

Ecological processes vary over differing geographical scales (Laughlin and Messier, 2015). The macro- and micro-evolutionary processes that shape patterns in chemical defences differently may also vary at different scales in much the same way. For example, well-defended plants were found to reduce chemical defences with increasing soil nutrients (Nerg *et al.*, 1994; Wainhouse and Ashburner, 1996; Woods *et al.*, 2012; Pratt and Mooney, 2013; Couture *et al.*, 2015; Agrawal *et al.*, 2015; Stevens *et al.*, 2016), whilst faster-growing but less defended species showed no variation in chemical defences associated with changing soil nutrients (Hahn and Maron, 2016). Upon noting these likely differences between inter- and intraspecific variation in chemical defences, Hahn and Maron (2016) theorized that broad resource environments select for growth–defence strategies across species (i.e. high- or low-resource environments) as stated in the GRH and GDBH, but extend this to hypothesize that intraspecific growth–resistance relations are selected by smaller-scale environmental factors, such as herbivore community composition (Abdala-Roberts *et al.*, 2015), climatic variation (Woods *et al.*, 2012; Pratt and Mooney, 2013; Anstett *et al.*, 2015) and growing season length (Lehdal and Ågren, 2015), alongside more fine-scale fluctuations in nutrients. They go on to construct a framework for predicting intraspecific patterns of plant growth and defence (intraspecific framework, ISF) within environmental gradients, predicting that increasing resource availability across an environmental gradient will select for faster growth rates within species. They further suggest that constitutive and induced defences are differentially regulated, with constitutive defences indirectly positively correlated with resources within species, while induced defences are favoured in lower-resource environments.

Theories of defence investment in response to changing plant-associated microbes

The vast majority of plants form mutualistic associations with microbes (van der Heijden *et al.*, 2015), potentially changing their nutrient profiles (Barrow and Osuna, 2002; Porras-Soriano *et al.*, 2009). Nonetheless, they were not incorporated into models of plant defence until relatively recently (Vannette and Hunter, 2011). The resource exchange model of plant defence (REMPD) looks to integrate nutrient exchange dynamics between plants and soil mutualists in order to predict plant defences within a species, but is the least experimentally validated model (Román *et al.*, 2011; Vannette and Hunter, 2011, 2013; Barber *et al.*, 2013; Xiao *et al.*, 2013; Meier and Hunter, 2018). Finding links between specific plant-associated fungi

and plant chemical defence is complex due to the enormous array of fungi, bacteria and other soil microorganisms that can simultaneously interact with a single plant (van der Heijden *et al.*, 2015). This makes the identification of groups or individuals associated with changing plant chemistry extraordinarily challenging. As a result, support for the REMPD comes predominantly from *in vitro* studies. However, in the last decade high-throughput sequencing techniques have revolutionized the process of characterizing complex microbial communities. This can in turn be used to attempt to understand microbial functioning in processes (Barnes *et al.*, 2018), such as *in situ* plant chemical defence.

Predicting and validating variation of thapsigargin in *Thapsia garganica*

Thapsia (Apiaceae) species are commonly referred to as deadly carrots, since reports of their poisonous qualities have emerged (Gerard *et al.*, 1597). Their toxicity has been known for centuries, with citations dating back to Theophrastus (372–287 BC). It is a small genus of herbaceous perennials (Weitzel *et al.*, 2014), restricted to the Mediterranean from Portugal and Morocco to Turkey and Greece (Smitt *et al.*, 1996), with *Thapsia garganica* being the most widely distributed species (Pujadas-Salvà and Rosselló, 2003). *Thapsia garganica* is reported to contain three major anti-herbivore compounds (thapsigargin): thapsigargin (Rasmussen *et al.*, 1978; Andersen *et al.*, 2015), thapsigarginin (Christensen *et al.*, 1982) and nortrilobolide (Smitt and Christensen, 1991) (Fig. 1). They have been found to be potent inhibitors of the sarco-endoplasmic reticulum Ca^{2+} -ATPase (SERCA) in mammalian cells, leading to cell apoptosis (Thastrup *et al.*, 1990), thereby explaining the remarkable method by which *T. garganica* keeps mammalian herbivores at bay. Accordingly, *T. garganica* is very easy to spot in fields, as its toxicity prevents it from being grazed and it remains tall compared with the surrounding vegetation (Fig. 2A, B, D). The concentration of thapsigargin varies greatly between individuals, with *T. garganica* containing 0.2–1.2 and 0.7–1.5 % of thapsigargin within the roots and the ripe fruits, respectively (Smitt *et al.*, 1995).

Investigations into plant chemical defences are simplified when a small array of compounds are expressed throughout a plant (Zangerl and Rutledge, 1996). Therefore, with its small number of variable thapsigargin present across tissue types, *T. garganica* represents an ideal system for exploring models of plant chemical defence. In this work, we evaluate the predictive

power of the aforementioned five models (ODT, GRH, GDBH, ISF and REMPD) by using them to predict the distribution and variation of the three major thapsigargin within *T. garganica* (Table 1). We subsequently quantified thapsigargin from naturally occurring specimens of *T. garganica*, with populations spanning a mammalian herbivory gradient within its native range on the island of Ibiza. Plants were partitioned into reproductive tissue (flowers, fruits, bracts), vegetative tissue (leaves, senesced leaves, stems) and below-ground tissue (root periderm and inner root). Temporal variation was assessed by repeat sampling once every 3 weeks over a 6-week period (three sampling points). Soil nutrition was recorded for each plant, alongside DNA metabarcoding of the root-associated fungal communities. Using these extensive collections and metadata, predictions from each model were evaluated for their ability to predict the *in situ* variation of thapsigargin between and within individuals of *T. garganica*, and the factors that regulate them.

METHODS

Sample collection

Sampling of *Thapsia garganica* was performed from early May to the end of June 2015 on Ibiza, Spain, during three sampling trips separated by 3-week intervals. Six sampling locations were chosen (Table 2 and Fig. 2C) that were of varying land use and mammalian herbivory pressure. Herbivory pressure was determined from the description of land use given by the landowners as low (fenced fields to keep grazing cattle out), medium (occasionally grazed over the year, but lacking fencing) and high (daily presence of cattle) (see land use descriptions in Table 2).

Sampling was targeted to be approximately before fruiting, when leaves are active, but also during and after fruiting, when leaves have senesced. For every individual we sampled roots and, when possible, stems, leaves (fresh or senesced), bracts, flowers and fruits, but as a result of the above-ground biomass of *T. garganica* senescing over the sampling period, sampling numbers were limited during the second and third sampling times (Table 3). Plant chemical defences can be stored in specialized structures to avoid autotoxicity (Rosenthal and Berenbaum, 1992) and MALDI imaging of the roots has shown thapsigargin to be concentrated near the root periderm of *T. garganica* (Andersen *et al.*, 2017). We therefore partitioned root samples into the outer periderm and inner tissue, sampling 10–20 cm below ground. At each site, five plants were destructively sampled, with a voucher specimen deposited at the herbarium of the Natural History Museum of Denmark (C). Plant tissue samples

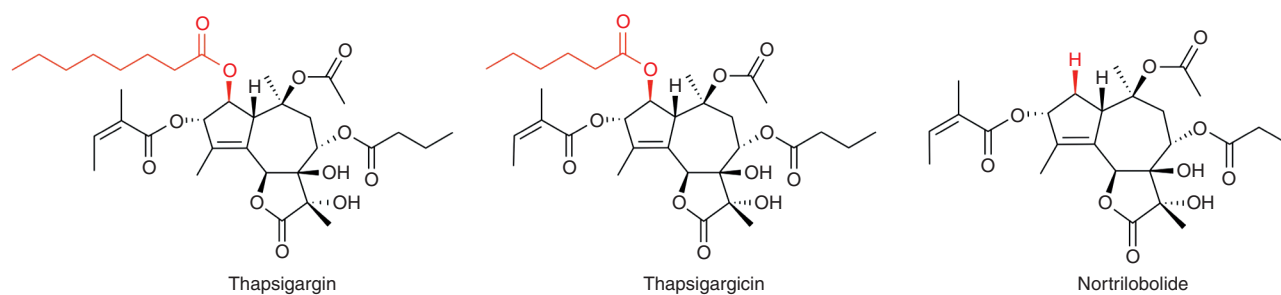


Fig. 1. Chemical structures of thapsigargin, thapsigarginin and nortrilobolide used in this study, with differences in structure highlighted in red.

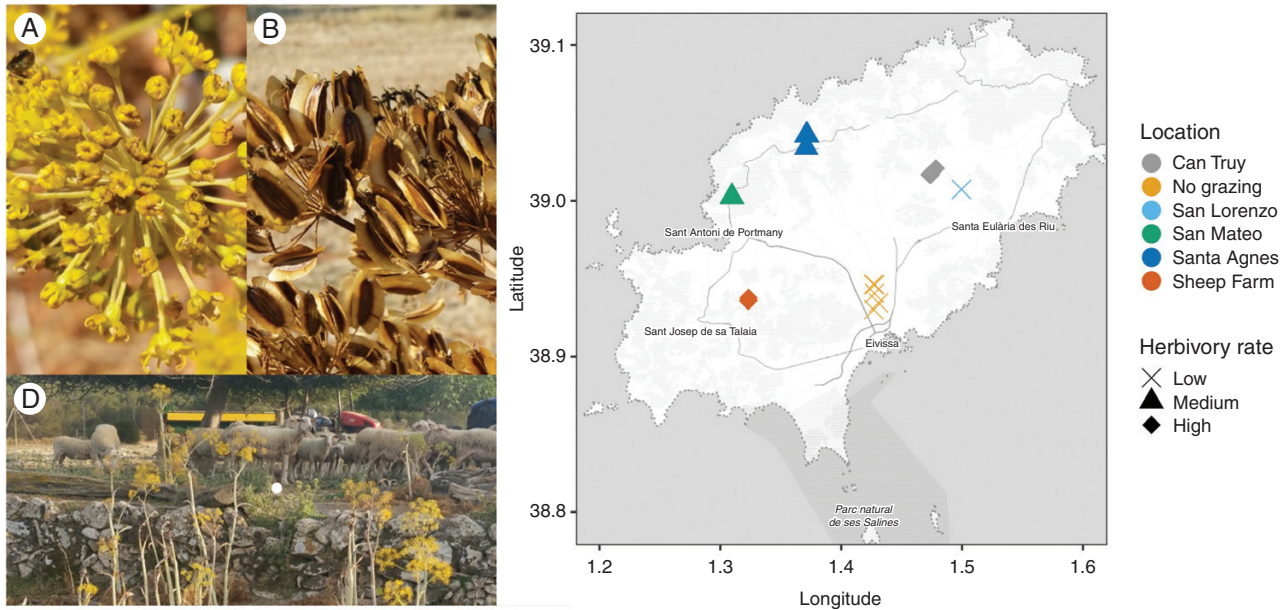


FIG. 2. *Thapsia garganica* (A) flowers and (B) fruits. (C) Map of sampling locations collected across the island of Ibiza. (D) *T. garganica* populations growing under intense herbivory pressure at the sheep farm site.

TABLE 1. Predictions produced from models of plant defences, predicting within- and between-plant variations in thapsigargin within *T. garganica*

(A) Predicting the general defence strategy in <i>T. garganica</i>	Prediction
Theory	A1 <i>T. garganica</i> is highly apparent, and is therefore highly defended
Optimal defence theory (ODT)	A2 <i>T. garganica</i> is adapted to low resources, and therefore has high constitutive defences and low induced defences
Growth rate hypothesis (GRH)	A3 Herbivory is a far greater threat than competition; therefore <i>T. garganica</i> is highly defended
Growth–differentiation balance hypothesis (GDBH)	
(B) Predicting <i>in planta</i> variation of thapsigargin within <i>T. garganica</i>	Prediction
Theory	B1 Thapsigargin vary in concentration between tissue types, and are highest in reproductive tissue
ODT	
(C) Predicting intraspecific variation of thapsigargin within <i>T. garganica</i>	Prediction
Theory	C1a Thapsigargin will increase with increasing herbivory rates
ODT	C1b Thapsigargin will vary in concentration temporally
GRH	C2 Increasing nutrients decrease thapsigargin concentrations
GDBH	C3 Increasing nutrients increase thapsigargin concentrations to a maximum, before declining with further nutrients
Resource exchange model of plant defence (REMPD)	C4 Changing fungal communities are associated with changing thapsigargin concentrations
Intraspecific framework (ISF)	C5a There is a positive correlation between resources and constitutive defences
	C5b Decreasing resources will increase induced defences

for HPLC analysis were collected, dried in silica gel and stored in darkness. Fresh samples for DNA analysis were kept in RNAlater™ (Invitrogen, USA) at 4 °C during the field stay (maximum 3 d) and stored at –20 °C upon return. Additionally, ~200 g of soil was collected from the base of each plant from the upper 20 cm of the soil profile, loosely covered and air-dried.

HPLC analyses

To identify thapsigargin, thapsigargin and nortrilobolide, standards previously extracted from *T. garganica* fruits were used (López *et al.*, 2018). For each compound, standard solutions diluted in 80 % methanol were prepared in triplicate

(dilution concentrations: 5, 25, 50, 200, 400, 500, 600, 800 and 1000 µg mL⁻¹). To quantify the analytes of interest, calibration curves were generated by running the standard dilutions in triplicate on an HPLC. Peak areas were plotted against the concentrations of the standard dilutions; the calibration curve for each standard had a correlation coefficient of 0.999.

Silica-dried plant material was homogenized in liquid nitrogen by mortar and pestle followed by 5 min at 30 Hz in a Tissue Lyser II (Qiagen, Germany) with 2-mm glass beads in 2-mL Eppendorf tubes, before being analysed by HPLC as previously outlined by Mohamed Ibrahim *et al.* (2018) (Supplementary Data Method S1).

TABLE 2. Collection sites chosen in Ibiza, Spain, along with sampling location names

Sampling location	GPS coordinates	Land use	Herbivory level	C:N ratio	Phosphorus (mg kg ⁻¹)	Potassium (mg kg ⁻¹)	pH
No grazing	38°56'47" N, 1°25'36" S	Disused farm land, fenced	Low	38.69 ± 10.27	24.16 ± 7.96	10 233.5 ± 3876.0	8.4 ± 0.2
San Lorenzo	39°0'26" N, 1°29'58" S	Disused almond grove, grass overgrown, not grazed, partially fenced	Low	8.68 ± 1.34	33.68 ± 1.38	5499.1 ± 235.6	8.3 ± 0.1
Santa Agnes	39°0'10" N, 1°18'34" S	Almond grove, ground cleared, infrequently grazed	Medium	35.50 ± 10.09	13.72 ± 6.04	5529.0 ± 417.5	8.3 ± 0.3
San Mateo	39°2'31" N, 1°22'16" S	Olive and almond grove, ground ploughed	Medium	20.04 ± 7.109	23.64 ± 2.34	5096.5 ± 411.2	8.4 ± 0.1
Can Truy	39°1'00" N, 1°28'27" S	Grazing ground for sheep, grazed frequently but not daily	High	27.08 ± 10.01	19.68 ± 8.73	4615.7 ± 534.7	8.4 ± 0.1
Sheep farm	38°56'10" N, 1°19'24" S	Sheep enclosure, cattle present daily	High	33.83 ± 3.78	15.92 ± 5.77	18 285.1 ± 6274.7	8.4 ± 0.2

TABLE 3. Full list of samples that underwent HPLC analysis to quantify thapsigargins, over the 13-week sampling period

Area	Leaves	Flowers	Fruit	Root	Periderm	Bract	Stem	Time point
Sheep farm	4	5	2	5	5			Week 1
CanTruy	5	5		5	5	2		
SanMateo	6	7		5	5			Week 7
SantAgnes	5	10		5	5			
SanLorenzo	3	5	1	5	5	1		
NoGrazing	3	6		5	5			
Sheep farm	2	1	5	4	4			
CanTruy	5	3	5	3	3			Week 13
SanMateo	2	2	2	3	3			
SantAgnes			1	1	1			
SanLorenzo								
NoGrazing		3	3	6	5			
Sheep farm	3	2		1	1	3	3	Week 13
CanTruy	2					3	3	
SanMateo	2			2	2	3	4	
SantAgnes								
SanLorenzo								
NoGrazing	1			1	1	2	2	

Characterizing the abiotic environment

To determine the edaphic properties of the soil, samples were sieved to <2 mm and homogenized by mortar and pestle. The pH of soil samples was determined by mixing 10 mL of soil with 25 mL of Milli-Q water, vigorously mixing for 15 min on a mixing tray at room temperature before immediately measuring the pH with a meter (Mettler Toledo, Denmark). Both the total carbon and nitrogen available to the plant in the soil (CNS analysis) was calculated by mixing 50 mg of soil with 50 mg of tungsten and compressing into a ball in inert foil. Samples underwent a dry combustion procedure on a Vario Macro Cube Elemental analyser (Elementar Analysen Systeme, Germany) using sulphanylamide and acetanilide as standard compounds for the calibration of the results. Phosphorus (P) and potassium (K) were simultaneously measured, with soil digested in a mixture of 2.5 mL of 70 % HNO₃ (nitric acid in distilled water) in a pressurized microwave oven (25 min, 154 bars, 250 °C, 1500 W; Ultrawave, Milestone, USA). Following digestion, samples were diluted in 50 mL of distilled water and underwent a multi-elemental analysis using ICP-OES inductively coupled plasma optical emission spectrometry (Model 5100, Agilent) coupled with a Meinhard nebulizer and cyclonic spray

chamber (flow injection volume 50 µL, flow rate 0.2 mL min⁻¹, mobile phase 3.5 % HNO₃) following Olsen *et al.* (2016). The instrument was run in collision mode with helium gas. Data were acquired and processed using the MassHunter 4.1 chromatographic software package. Elements were calculated as previously described (Olsen *et al.*, 2016) and validated using external calibration standards corresponding to elemental ratios typically found in plants, with data quality ensured using NIST 1515 Apple Leaf certified reference material (National Institute of Standards and Technology, USA). Data that exceeded the limit of quantification were accepted, with accuracy >95 % and the coefficient of variation between five replicates <3 %.

Fungal metabarcoding and toxicity screening

DNA from the periderm and inner root was extracted using a PowerSoil DNA isolation kit (MP Biomedicals, USA) according to the manufacturer's guidelines, with lysis performed by two rounds of mechanical lysis at 30 Hz for 30 s in a TissueLyser II (Qiagen, Germany). DNA extracts underwent DNA metabarcoding as described by Barnes *et al.* (2016), using

the universal fungal internal transcribed spacer (ITS) primers gITS7 (Ihrmark *et al.*, 2012) and ITS4 (Gardes and Bruns, 1993) (Supplementary Data Method S2).

From a total of 1 946 964 reads produced via metabarcoding, a total of just 6121 were assigned to fungal species (0.3 %), with reads derived from both the root periderm and inner root samples primarily consisted of reads from the plant host (0.5 and 0.3 % respectively). Given the lack of fungal reads in the inner roots and periderms of *T. garganica*, the toxicities of thapsigargin, thapsigargin and nortrilobolide were tested *in vitro*. Petri dishes were established by adding 100 μL (1000 mg L^{-1} stock) of one of the three thapsigargin to 20 mL of potato dextrose agar (PDA) giving a final concentration of 5 mg L^{-1} . Additionally, plates containing 100 μL of pooled root extracts (that had been quantified by HPLC) in 20 mL of PDA were also established, giving final concentrations of 3.7 $\mu\text{g L}^{-1}$ thapsigargin, 2.1 $\mu\text{g L}^{-1}$ thapsigargin and 0.8 $\mu\text{g L}^{-1}$ nortrilobolide. Untreated PDA plates served as controls. Common plant-associated fungi from the two most abundant phyla [a *Fusarium* sp. (Ascomycota) and a *Clitopilus* sp. (Basidiomycota)] were initially grown in untreated PDA and incubated until a homogeneous fungal mycelium had formed. Single 4-mm diameter plugs of each fungus were transplanted into each of the four treatment plates and the untreated PDA controls ($n = 15$ for each fungus). For each plate, the diameter of fungal colonies was measured in two places in a cross formation over time, and repeated until the control colonies were spread over three-quarters of the Petri dish.

Statistical analyses

All statistical analyses were performed in statistical computing environment R. Due to sampling time 1 being the most complete, and to avoid the confounding effects of temporal variation, correlations of expression of thapsigargin with locality and mammalian herbivory and between plant parts were investigated from samples collected during this time point only. Correlations between compounds were assessed using Pearson's correlation coefficients. Mixed linear modelling was performed to assess differences in individual thapsigargin between tissue types, sites and mammalian herbivory levels, with the plant of origin serving as the random effect. Significance was calculated for each by likelihood ratio testing (χ^2) performed using the drop1 function in the lme4 package (Bates *et al.*, 2014). Meanwhile, as it was only possible to sample above-ground tissue in some of the sampling times, statistical analysis of temporal variation was limited to the below-ground tissue of time points 1 and 2 only. Significant temporal variation of the three thapsigargin was assessed in the root periderm and inner root independently, using Mann–Whitney tests within R's native statistics package.

As the below-ground tissue from sampling time 1 was ubiquitously available, the effects of environmental variation on the concentration of thapsigargin was investigated using partitioned root periderm and inner root tissue independently. Initially, the co-expression of (\log_{10} normalized) thapsigargin in the root periderm and inner roots was investigated with paired *t*-tests. Individual thapsigargin (\log_{10} normalized) from either the root periderm or inner roots underwent mixed linear modelling to test for environmental variation in each of the three major defence compounds (with soil pH, C:N ratio, P, K and

mammalian herbivory serving as fixed effects and plant part serving as the random effect). As before, likelihood ratio tests (χ^2) were performed using the drop1 function to identify significantly correlated variables.

In assessing fungal toxicity, two-way ANOVAs were performed for each of the *Fusarium* sp. and *Clitopilus* sp. fungi, where treatment (controls, thapsigargin, thapsigargin, nortrilobolide and plant extracts) and time (in hours after inoculation) were analysed against the rank of diameter (due to lack of normality).

RESULTS

Predicting the general defence strategy of *T. garganica*

All three thapsigargin were detectable in some of the sample types, but varied substantially in concentration both between and within tissue types. Thapsigargin itself ranged from being undetectable to 5.76 mg kg^{-1} , and was the most abundant defence compound with a mean concentration of $0.70 \pm 0.07 \text{ mg kg}^{-1}$. Thapsigargin was also extremely variable and the second most abundant of the thapsigargin, which ranged from being undetectable to 8.05 mg kg^{-1} , with a mean of $0.39 \pm 0.07 \text{ mg kg}^{-1}$. Thapsigargin and thapsigargin strongly correlated with each other ($r_s = 0.780$, $P > 0.001$) and were both detectable in the majority of samples analysed (94.6 %). Nortrilobolide had a mean concentration of just $0.12 \pm 0.02 \text{ mg kg}^{-1}$ and was undetectable in 64.3 % of samples. While nortrilobolide was weakly correlated with thapsigargin ($r_s = 0.200$, $P = 0.041$), it was not correlated with thapsigargin ($r_s = 0.186$, $P = 0.060$).

Predicting *in planta* variation of thapsigargin in *T. garganica*

To avoid the confounding effects of temporal variation between sites, the tissue parts were investigated using the samples from the first time point only. The likelihood ratio test (LRT) of mixed linear models revealed significant variation between sites for thapsigargin (LRT = 22.3, $P < 0.001$) and nortrilobolide (LRT = 19, $P = 0.002$) but not for thapsigargin (LRT = 8.908, $P = 0.113$). Thapsigargin and thapsigargin were both at highest concentrations in the San Lorenzo site (low herbivory), averaging 1.00 and 0.75 mg kg^{-1} , respectively, whilst thapsigargin was lowest in the Can Truy site (high herbivory; 0.50 mg kg^{-1}) and thapsigargin in the no-grazing site (low herbivory; 0.14 mg kg^{-1}). Despite significant variation between sites in thapsigargin and nortrilobolide, when sites were analysed as low, medium or high herbivory, herbivory did not significantly influence any of the thapsigargin (LRT = 0.76, $P = 0.684$), thapsigargin (LRT = 2.22, $P = 0.330$) or nortrilobolide (LRT = 1.00, $P = 0.605$) levels (Fig. 3A).

Due to the inconsistent availability of above-ground biomass parts, the analysis of temporal variation in thapsigargin was limited to the below-ground parts (root periderm and inner root tissue; Fig. 3B) from time points 1 and 2. Within the inner roots, thapsigargin significantly decreased in abundance between sampling times 1 and 2, from a mean of $0.88 \pm 0.12 \text{ mg kg}^{-1}$ to $0.46 \pm 0.10 \text{ mg kg}^{-1}$. However, neither thapsigargin ($W = 163.0$, $P = 0.104$) nor nortrilobolide ($W = 146.5$, $P = 0.476$) varied significantly during this period. There were also no significant differences in all three thapsigargin over time in the root

periderm [thapsigargin ($W = 218.5$, $P = 0.903$), thapsigarginic ($W = 230.5$, $P = 0.725$) and nortrilobolide ($W = 123$, $P = 0.280$)].

Variation in thapsigargin was investigated from the samples collected in time point 1. Mixed linear modelling revealed that thapsigargin (LRT = 34.5, $P < 0.001$), thapsigarginic (LRT = 52.5, $P < 0.001$) and nortrilobolide (LRT = 41.5, $P < 0.001$) all varied significantly between plant parts (Fig. 3C). Thapsigargin and thapsigarginic were highest in the reproductive tissue (mean: fruits, 1.56 ± 0.92 and 0.60 ± 0.29 mg kg⁻¹, respectively; flowers, 0.88 ± 0.10 and 0.43 ± 0.29 mg kg⁻¹; bracts, 1.37 ± 0.63 and 0.59 ± 0.18 mg kg⁻¹) followed by the below-ground tissue (inner roots, 0.84 ± 0.10 and 0.45 ± 0.08 mg kg⁻¹; root periderm, 0.59 ± 0.20 and 0.51 ± 0.29 mg kg⁻¹), and then vegetative tissue (fresh, 0.28 ± 0.06 and 0.12 ± 0.02 mg kg⁻¹; senesced, 0.20 ± 0.03 and 0.07 ± 0.02 mg kg⁻¹). Nortrilobolide was also highest in reproductive tissue (fruits, 0.54 ± 0.35 ; flowers, 0.29 ± 0.08 ; bracts, 0.22 ± 0.16 mg kg⁻¹), but was in significantly higher abundance in vegetative tissues (senesced leaves, 0.03 ± 0.01 ; fresh leaves, 0.07 ± 0.03 mg kg⁻¹) than in below-ground tissue (inner root, $<0.01 \pm 0.02$; root periderm, 0.01 ± 0.01 mg kg⁻¹).

Predicting intraspecific variation of thapsigargin within *T. garganica*: abiotic regulation

As below-ground tissue was present for all samples of time point 1, the effects of environmental variation on thapsigargin concentrations on the inner roots and root periderm were analysed. As before, thapsigargin and thapsigarginic correlated positively in the inner roots ($r_s = 0.904$, $P > 0.001$) and root periderm ($r_s = 0.470$, $P = 0.013$), but nortrilobolide did not significantly correlate with either thapsigargin ($r_s < 0.01$, $P = 0.967$ and $r_s < 0.01$, $P = 0.980$, respectively) or thapsigarginic ($r_s < -0.01$, $P = 0.967$ and $r_s = -0.13$; $P = 0.602$) (Fig. 4A). Surprisingly, whilst thapsigargin concentration correlated significantly between the inner root and the root periderm (d.f. = 25, $t = 2.412$, $P = 0.024$) of the same plants, neither thapsigarginic nor nortrilobolide concentrations correlated.

Given the lack of co-correlation between the different tissue types, the effects of environmental regulation (C:N ratio, P, K, soil pH and mammalian herbivory level) were analysed in the inner root and root periderm independently for each individual thapsigargin. Both thapsigargin (LRT = 5.488, $P = 0.019$) and thapsigarginic (LRT = 5.560, $P = 0.018$) correlated with C:N ratio in the root periderm (Fig. 4B). Soil pH (LRT = 5.869, $P = 0.015$) correlated with thapsigargin in the root periderm (Fig. 4C). Nortrilobolide did not correlate with any explanatory factors in the root periderm, but correlated with K (LRT = 14.663, $P < 0.001$), soil pH (LRT = 6.659, $P = 0.012$) and herbivory (LRT = 11.815, $P = 0.003$) in the inner roots.

Predicting intraspecific variation of thapsigargin within *T. garganica*: root-associated fungi

The root periderm and inner root tissues from sampling time point 1 also underwent DNA metabarcoding to characterize the associated below-ground fungal communities. Despite there being a deliberate poor match between host DNA and

the fungal targeting primers, >99.7 % of reads produced by metabarcoding were assigned to the ITS region for roots of all 30 sampled plants of *T. garganica*, leaving nominal reads assigned to the fungal community. The plant PCR products were ~100 bp shorter than predicted amplicon length for fungi, whilst the PCR products of the surrounding soil were of the expected length. We therefore hypothesized that *T. garganica* could inhibit fungal associations, potentially via thapsigargin, thapsigarginic and nortrilobolide.

The toxicity assay tested the inhibition of growth by *T. garganica*-derived compounds on common plant-associated fungi, a *Fusarium* sp. and a *Clitopilus* sp. Whilst the *Fusarium* sp. grew quicker than the *Clitopilus* sp., the growth of both fungi was significantly reduced by all three compounds and by the raw plant extracts that were previously quantified by HPLC (*Fusarium* sp.: d.f. = 4, $F = 352.7$, $P < 0.001$; *Clitopilus* sp.: d.f. = 4, $F = 415.4$, $P < 0.001$). Fungal colonies at least halved in size during the incubation period (Fig. 5). Tukey tests revealed that the ascomycete fungi were affected most by the plant extract, then by nortrilobolide and thapsigarginic equally, whilst thapsigargin also significantly slowed fungal growth, but least (Fig. 5A). Conversely, *Clitopilus* sp. fungal growth was also significantly slowed, but least by the plant extract, followed by thapsigarginic and nortrilobolide equally (Fig. 5B). Thapsigargin was the most effective in inhibiting *Clitopilus* sp. growth.

DISCUSSION

In our analyses, we show that the three major thapsigargin within wild populations of *T. garganica* vary considerably between different tissue types and spatially, and modestly over time. Here we evaluate how these observations compare with predictions made with theories of plant investment (Table 1).

Predicting the general defence strategy of *T. garganica*

All models predicted that *T. garganica* would be highly defended (Table 1, predictions A). Given that substantial quantities of the potent thapsigargin were observed in nearly all tissue types in most plants, results here suggest the models performed well.

The ODT predicted that *T. garganica* would be well defended due to being highly apparent within the ecosystem (prediction A1; Fig. 2D). However, as a herbaceous plant, *T. garganica* is predicted to invest fewer resources in defence than woody species (prediction A1). These defences are predicted to be in low concentrations and effective against generalist herbivores rather than specialists (qualitative defences rather than quantitative; Smilanich et al., 2006). Thapsigargin are terpenes that are qualitative defence compounds toxic to a wide variety of organisms (Fox, 1981). However, they were also present in relatively high abundance across the different tissues of *T. garganica*, and therefore the ODT performed well in predicting the species' general defensive strategy. The GDBH also predicted high defences (prediction A3), but as a result of herbivory being a far greater threat than competition within the relatively sparsely populated, nutrient-poor Ibiza

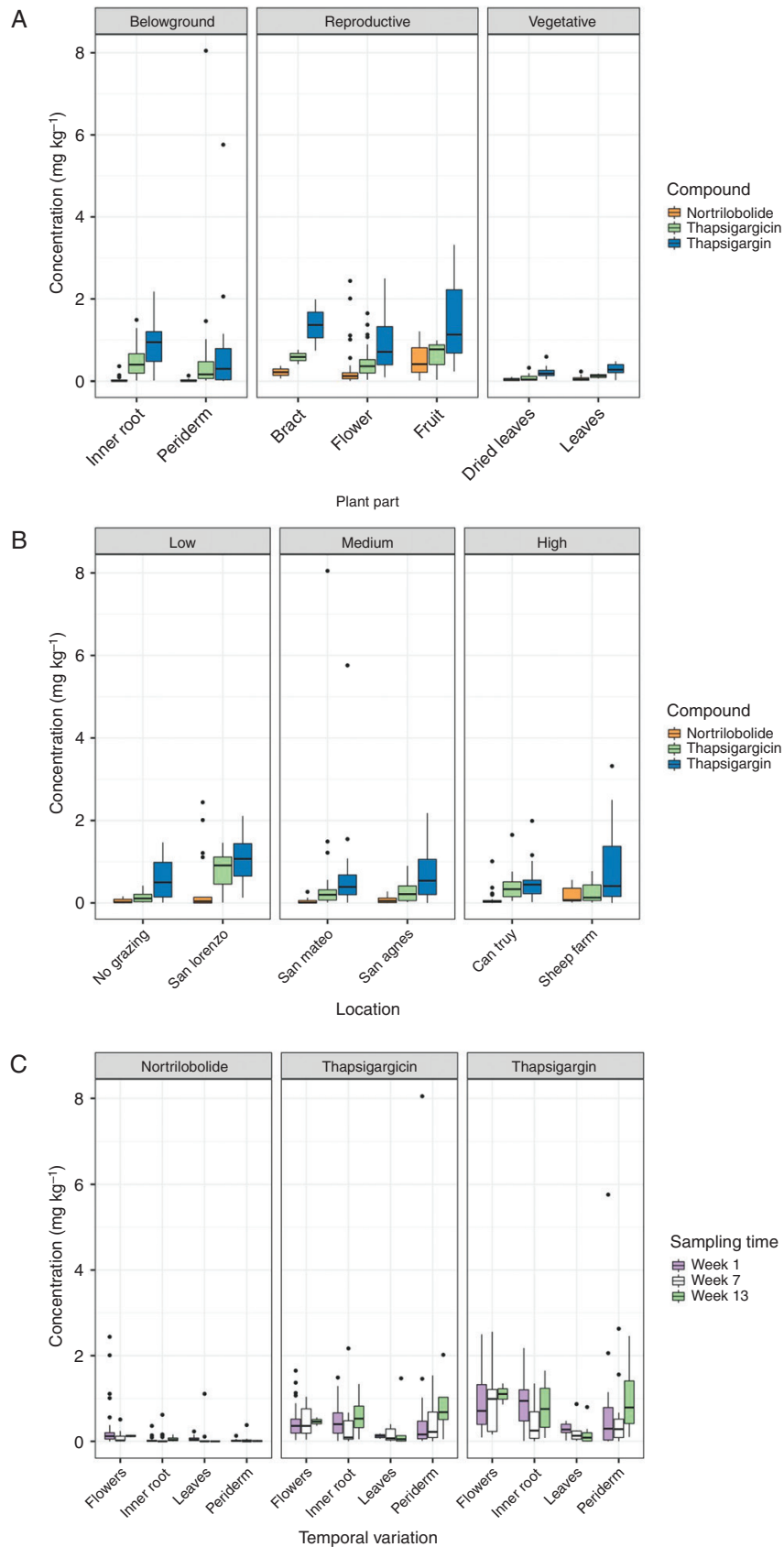


FIG. 3. Relative distribution of thapsigargin, thapsigargin and nortrilobolide from *Thapsia garganica* (A) within the different plant tissues, (B) between different sampling locations that had differing intensities of mammalian herbivory, and (C) over time. There was significant variation in all three thapsigargins between plant parts, while significant variation between sites was limited to thapsigargin and nortrilobolide. Due to sampling limitation, only variation in the inner roots and root periderms from time points 1 and 2 underwent statistical analyses for temporal variation, with only thapsigargin in the inner roots varying significantly temporally.

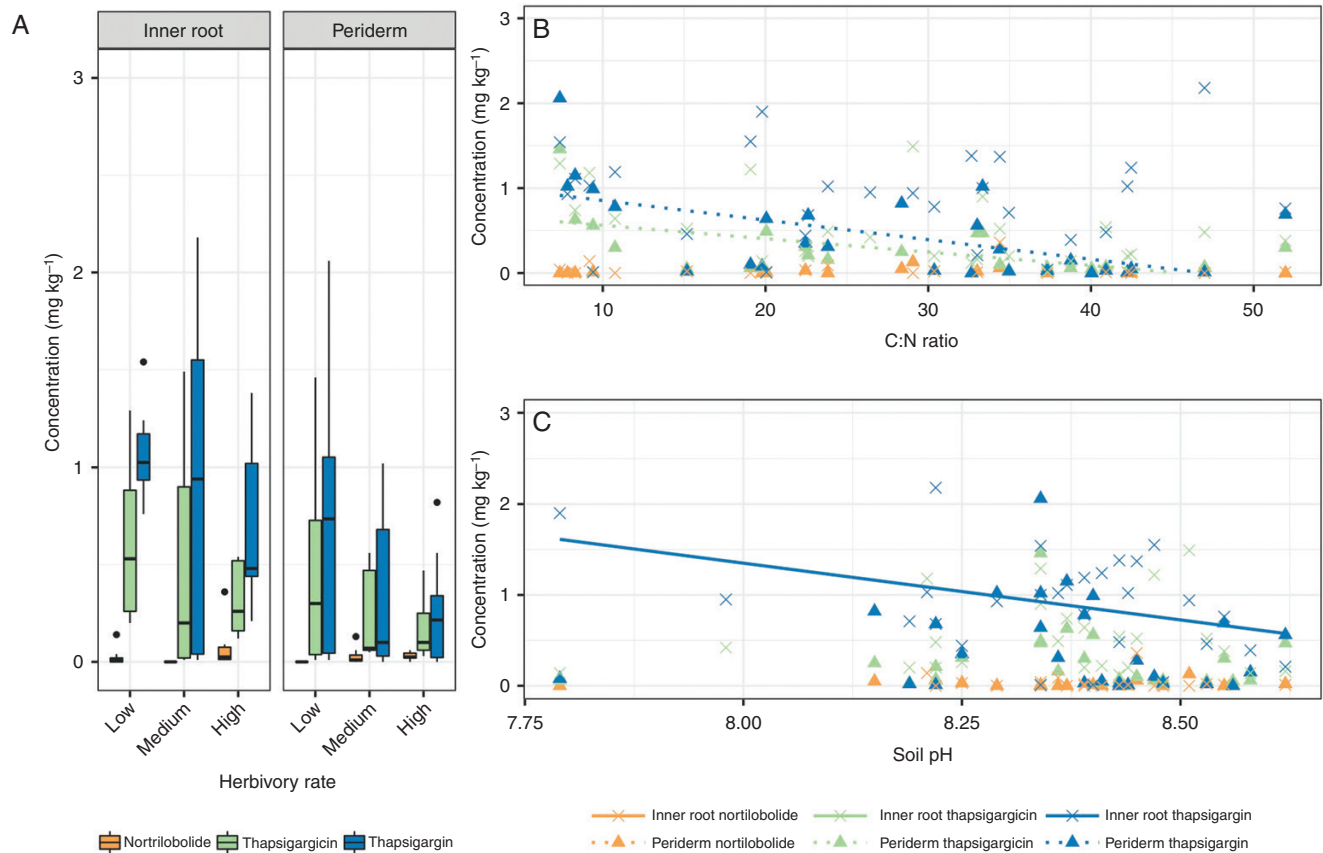


FIG. 4. Relative distribution of thapsigargin, thapsigarginin and nortrilobolide in inner roots and root periderms of *Thapsia garganica* (A) across the herbivory gradient, (B) with changing C:N ratio and (C) with changing soil pH. Lines of best fit are drawn with significant interactions. The C:N ratio was significantly correlated with thapsigargin and thapsigarginin in the root periderm, and soil pH was significantly correlated with thapsigargin in the inner root. Note that for Fig. 4 only, a single sample has been excluded as it contained exceptionally high thapsigargin concentrations (5.8 mg kg^{-1} of thapsigargin and 8.0 mg kg^{-1} of thapsigarginin).

soils. The GRH similarly predicted considerable chemical defences within *T. garganica* that are produced constitutively, as it is adapted to a low-resource environment (prediction A2), and therefore adopts a defence-based life strategy rather than a growth-based strategy to overcome its decreased ability to compensate for tissues lost to herbivory. Logically, these concepts are likely interlinked in many ecosystems, with increasing soil nutrients increasing the biomass an ecosystem can support (de Graaff *et al.*, 2006), which in turn increases competition and reduces how apparent individual plants are.

Predicting in planta variation of thapsigargin in *T. garganica*

Unlike the other theories, the ODT can also be used to predict how defences are distributed within plants, stating that the most valuable tissues, or those at greatest risk of herbivory, are better defended (prediction B1; Rhoades, 1979). This led to the general statement that vegetative parts are easier to replace than reproductive parts (Zangerl and Bazzaz, 1992; Hamilton *et al.*, 2001). Here we find that fruits and flowers are particularly well protected, fitting well with this prediction. Furthermore, *T. garganica* has a very low germination rate, only 15 % in some instances (Makunga *et al.*, 2003); low reproductive success might therefore explain the high investment in protecting

reproductive tissue. Meanwhile, the low concentration of thapsigarginin found in the leaves suggests they are of lower fitness value to the plant. This too, seems logical, given that *T. garganica* is distributed only in areas of high solar radiation without competition for light. Meanwhile, the below-ground biomass had greater concentrations of thapsigarginin than leaves, but less than the reproductive tissue, and represents a considerable sink of photosynthates. Below-ground plant chemical defences can play integral roles in ecosystems, shaping soil microbial communities (van der Putten, 2003), yet their variation between and within species remains poorly characterized and they are generally not well integrated within current chemical defence theories (Tsunoda *et al.*, 2017). Further studies comparing the plant's above- and below-ground tissue value and vulnerability, coupled with chemical defences, are required to evaluate whether the ODT is applicable to below-ground plant biomass (Tsunoda and van Dam, 2017).

Predicting intraspecific variation of thapsigargin within *T. garganica*: abiotic regulation

Predicting intraspecific variation of thapsigargin within *T. garganica* was more challenging than predicting the general defensive strategy of *T. garganica*. Models sometimes

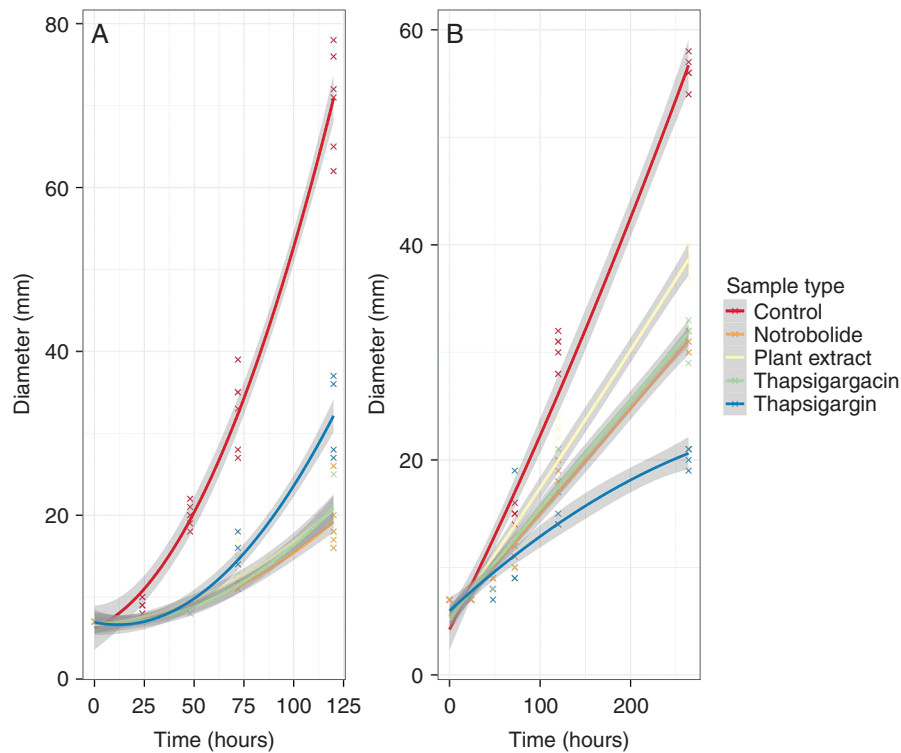


FIG. 5. (A) *Fusarium sp.* and (B) *Clitopilus sp.* fungi were grown on PDA. The medium was untreated in the control or had 100 μL of 1000 p.p.m. standards added to 25 mL of liquid medium before setting. Additionally, 100 μL of methanol plant extract (from roots) was added to 25 mL of liquid medium before setting to screen for alternative fungal inhibitors. Both fungi had their growth significantly reduced by all treatments.

gave conflicting and generally fewer specific predictions about the within-species regulation of thapsigargin when compared with the inter-specific models, and the observed variation of thapsigargin was compatible with multiple conflicting models.

The ODT predicts that increasing herbivory will increase chemical defences (Rhoades, 1979; prediction C1a). In this study, we observed variation between sites, but limited support for this was derived from mammalian herbivory, with effects limited to nortrilobolide in the inner root. According to the land use strategies given (Table 2), the herbivory pressure of the different sites has been maintained for many years (D. Marban, Thapsibiza S.L., Spain, pers. comm.), which could explain the lack of correlation between mammalian herbivory and the concentrations of thapsigargin. Furthermore, whilst herbivory pressure is the basis for its chemical defence mechanisms, in fact local cattle do not eat *T. garganica* and it is usually found in large numbers and intact, demonstrating an acquired knowledge of the post-ingestive effects of the species. Despite the toxicity of thapsigargin to a wide range of eukaryotes (Thastrup *et al.*, 1990; Vaca *et al.*, 1994; Fiandra *et al.*, 2006; Johnson *et al.*, 2009), other types of herbivory cannot be ruled out. Literature on insect herbivory within *T. garganica* is scarce, but evidence of insect damage, observed as damaged leaves and bored fruits and stems, was observed in the field. In addition, the larvae of the Mediterranean moth *Agonopterix thapsiella* have been reported to be specialists in feeding on the young leaves and developing flower buds of *T. garganica*, and those of *Depressaria veneficella* on the stems (Stainton, 1869). The ODT additionally states that plants will be most protected mid-season (Zangerl and Rutledge, 1996; prediction C1b), as

the cost of herbivory is greatest when compared with the early and late seasons; however, we found limited temporal variation in thapsigargin. It should be noted that there was temporal variation in the presence of above-ground biomass (i.e. stems, leaves, bracts, flowers and fruits), which were all only intermittently present during our sampling, and therefore total thapsigargin within a plant may still vary considerably over time. Given the toxicity of these compounds, the plant may also simply be inedible throughout the sampling period, leading to limited differences in chemical defences associated with neither the herbivory gradient nor time.

Unlike the ODT, the GRH and GDBH make explicit expectations about nutrient gradients affecting plant defences. The GRH predicts that increasing nutrients, increases growth and reduces defences (prediction C2; Grime, 2006), whilst the GDBH predicts there is an optimal level of nutrients where defences peak, before a growth strategy is preferred and defences decrease (prediction C3 not compatible with the GRH). Optimum chemical defences have been observed with intermediate nutrient levels within single species (Mihaliak and Lincoln, 1985; Wilkens *et al.*, 1996; Glynn *et al.*, 2007; Scogings, 2018) and the GDBH is viewed as the most advanced model of plant investment in defence. Results here further support the idea that the GDBH should subsume the GRH in explaining intraspecific variation in chemical defences (Stamp, 2003), despite the notable exceptions to the GDBH that have been observed (Le Bot *et al.*, 2009; Kleczewski *et al.*, 2012; Gutbrodt *et al.*, 2012).

The ISF model predicted a positive correlation between constitutive defences and resources (prediction C5a) and a negative correlation for induced defences (prediction C5b). Results

here further support previous findings that thapsigargin and thapsigargin are constitutively biosynthesized in *Thapsia* plants (Christensen *et al.*, 1997; Drew *et al.*, 2009; Pickel *et al.*, 2012; Andersen *et al.*, 2017) while suggesting that nortrilobolide is induced by environmental factors. Given the observed differences in factors regulating these ‘constitutive’ and ‘induced’ defences, partitioning defences, as only the ISF model does, may improve future predictions. Furthermore, the ‘constitutive’ defences (thapsigargin and thapsigargin) fit well with this model, increasing with a decreasing C:N ratio (increasing soil N). The key difference between predictions from the GDBH and the ISF theory is that the GDBH predicts an eventual decline in chemical defences with increasing nutrients whereas the ISF model does not, and sampling over a wider nutrient gradient is required to disentangle these two predictions. In contrast, the ‘induced’ defences (nortrilobolide) did not correlate with soil properties in either the root periderm or inner root and is therefore either less sensitive to abiotic environmental variation, or even not effected by them, and this is in contrast to what the ISF predicts. Alternatively, nortrilobolide may have been induced by one of the many environmental stimuli not tested here and, given the higher quantities observed in the above-ground biomass, insect herbivory would be of particular interest in further experiments.

Whilst soil pH is not a nutrient, it was found to significantly influence thapsigargin and thapsigargin in the root periderm in a non-linear fashion, as predicted by the GDBH. Yet its impact on plant chemical defences is much less explored than soil nutrients. Experimental manipulations need to be performed to validate whether pH does regulate thapsigargin, but results here highlight the potential of *in situ* studies to find novel mechanisms of regulation that can be explored within more controlled environments.

Predicting intraspecific variation of thapsigargin in *T. garganica*: root-associated fungi

Here, we found that the non-specific binding of primers to *T. garganica* DNA led to nominal numbers of reads being fungal-derived through metabarcoding. Our subsequent fungal toxicity assay found that all three defence compounds, as well as the crude plant extracts, significantly inhibited plant-associated fungal growth. Other plant defence compounds have been shown to inhibit fungal colonization, most notably in the glucosinolates found within the Brassicaceae (Pedras and Hossain, 2011; Hu *et al.*, 2015). In doing so, these plants inhibit potential beneficial symbioses with mycorrhizal fungi and dark septate endophytes that can enhance nutrient uptake (Barrow and Osuna, 2002; Porras-Soriano *et al.*, 2009). However, mycorrhizal fungi function parasitically on their hosts under certain circumstances (Johnson *et al.*, 2009). Pathogenic fungi are one of the greatest threats to a plant’s vitality (Dean *et al.*, 2012), and these associations were also inhibited. Soil microorganisms interact with the outermost surfaces of roots, and many fungi can penetrate into or between root cells themselves (Vierheilig *et al.*, 2001). MALDI-TOF imaging revealed large quantities of thapsigargin near the outermost surface of the root of *T. garganica*, and results here suggest this would likely

strongly inhibit fungal interactions (Andersen *et al.*, 2017). Furthermore, glucosinolates have also been shown to inhibit parasitic nematodes in Brassicaceae (Ntalli and Caboni, 2017) and thapsigargin might be employed as part of the below-ground defences against a wide range of below-ground biotic interactions. Although it cannot be ruled out that thapsigargin are moved to the root periderm as part of a storage strategy to avoid autotoxicity, in both scenarios there seems to be no fitness cost incurred by not having below-ground fungal associations, and this could even increase fitness. Whilst the REMPDP was developed to characterize chemical defences in response to changing numbers of mutualists within a species, understanding the evolutionary and ecological determinants of plants that strongly inhibit root fungal associations could provide further insight into cost-benefits of root-associated fungal associations, and in turn give a better understanding of their influence on plant chemical defences.

Future perspectives

Here we assemble a framework to highlight available models and their practical application in predicting chemical defences at the *in planta*, intraspecific and interspecific levels (Table 4). Additionally, for future studies we have a few main suggestions. Initially, we propose that multiple models of chemical defence be assessed simultaneously, as each has a different utility and this will ultimately assist in the development of an integrated model able to predict chemical defences across levels. We also believe that significant improvements could be achieved by supplementing targeted analyses with untargeted metabolomic screenings, as more complex responses, including responses of non-target compounds, may be overlooked in traditional approaches (Zangerl and Berenbaum, 1997; Stamp, 2003, 2004; Gutbrodt *et al.*, 2012). We further suggest that *in situ* studies could supplement experiments in controlled environments (such as reciprocal transplant studies and common garden studies), so that relevant specific environmental regulators of chemical defence can be identified before hypothesis testing, echoing Hahn and Maron (2016). Most importantly, we recommend the establishment of a gold standard of best practice for testing intraspecific variation of chemical defences in plants, as differing experimental designs and methodologies might in part be responsible for conflicting results (Scogings, 2018), and therefore hinder the development of an all-encompassing theory of plant chemical defences.

Conclusions

In this work we show that *T. garganica* was constitutively defended by potent anti-herbivore and anti-fungal compounds, thapsigargin, which were highly variable both within and between plants. We found 10-fold differences in thapsigargin concentrations associated with tissue type. We also found 2-fold differences associated with spatial variation, which were linked to soil properties, whilst there was limited temporal variation in thapsigargin. We found that the theories predicting interspecific variation in plant defences effectively predicted the

TABLE 4. A framework briefly outlining the available models, the experimental setup required and the future improvements needed to predict and validate in planta, intraspecific and interspecific variation in plant chemical defences

	<i>In planta</i> variation	Intraspecific variation
Types of experimental setup	<ul style="list-style-type: none"> ○ Sampling of different tissue types from the same individuals. ○ Repeated sampling of a population over time. 	<ul style="list-style-type: none"> ○ Sampling of the same species between different populations. ○ Sampling single species over environmental gradients.
Relevant theories	<ul style="list-style-type: none"> ○ Optimal defence theory (ODT). 	<ul style="list-style-type: none"> ○ Growth rate hypothesis (GRH). ○ Growth–differentiation balance hypothesis (GDBH). ○ Resource exchange model of plant defence (REMPD). ○ Intraspecific framework for variation in plant defences (ISF).
Current status	<ul style="list-style-type: none"> ○ The ODT is the only major theory to predict in planta variation. ○ The level of chemical defences is hypothesized to vary with the value of the tissue to the plant, as well as with the level of herbivory. ○ This ‘value’ is not well defined, but generally reproductive tissue is thought to be of higher value than leaves and is therefore better defended. ○ Its applicability to below-ground defences has not been tested thoroughly. 	<ul style="list-style-type: none"> ○ The ISF is the most advanced theory in predicting chemical defences at the intraspecific level, expanding upon the GRH by partitioning induced and constitutively active defences. ○ However, the ISF suggests a linear relationship between defences and resources, whilst the GDBH suggests there is an optimum. ○ The specific environmental factors that regulate chemical defences remain poorly defined.
Future improvement	<ul style="list-style-type: none"> ○ More studies are needed to determine the relative value of root tissue to plants. ○ Further quantification of below-ground herbivory rates are required. ○ At the intraspecific level, other theories are considered superior to the ODT, therefore the <i>in planta</i> aspects of the ODT should be amalgamated with these more sophisticated models of intraspecific variation. 	<ul style="list-style-type: none"> ○ Further experimental observations are required to determine whether partitioning-induced and constitutive defences are valid. ○ Experimental <i>in situ</i> observations should be performed to identify putative environmental determinants of chemical defences, before validation within glasshouse studies.

growth strategy and level of defences within *T. garganica* (Koricheva, 2002). We also found that one theory, the ODT, effectively predicted the distribution of thapsigargin in the above-ground but not below-ground biomass. Models performed least well in predicting intraspecific variation in chemical defences, but results here further suggest that the GRH should be subsumed by the GDBH. We could not differentiate between the ISF model and the GDBH in performance; however, sampling over a wider nutrient gradient would allow this to be resolved. Ultimately, we are some way from a ‘grand unifying theory’ in predicting intraspecific variation in plant chemical defences, but with the increasing availability of high-throughput analyses of DNA (both of plants and their microbial symbionts) and chemistry, and databases such as WorldClim and the Global Biodiversity Information Facility (GBIF), we can inch ever closer.

SUPPLEMENTARY DATA

Supplementary data are available online at <https://academic.oup.com/aob> and consist of the following. Method S1: HPLC analysis. Method S2: fungal sequencing and analysis.

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