Historic exposure to herbivores, not constitutive traits, explains plant tolerance to herbivory in the case of two *Medicago* species (Fabaceae)

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ABSTRACT

Mechanisms that allow plants to survive and reproduce after herbivory are considered to play a key role in plant evolution. In this study, we evaluated how tolerance varies in species with different historic exposure to herbivores considering ontogeny. We exposed the range-restricted species *Medicago citrina* and its closely related and widespread species *M. arborea* to one and two herbivory simulations (80 % aerial biomass loss). Physiological and growth parameters related to tolerance capacity were assessed to evaluate constitutive values (without herbivory) and induced tolerance after damage. Constitutive traits were not always related to greater tolerance, and each species compensated for herbivory through different traits. Herbivory damage only led to mortality in *M. citrina*; adults exhibited root biomass loss and increased oxidative stress after damage, but also compensated aerial biomass. Despite seedlings showed a lower death percentage than adults after herbivory in *M. citrina*, they showed less capacity to recover control values than adults. Moderate tolerance to *M. arborea* herbivory and low tolerance to *M. citrina* is found. Thus, although the constitutive characteristics are maintained in the lineage, the tolerance of plants decreases in *M. citrina*. That represents how plants respond to the lack of pressure from herbivores in their habitat.

1. Introduction

The interaction between plants and herbivores is considered to be a driver of natural selection [1]. Evolution of the plant defence traits (namely, resistance and tolerance) has enabled plant species to adapt to the presence of herbivores and promoted diversification [1–3]. Plant protection conferred by chemical compounds and physical defence or tolerance strategies are the main mechanisms that support the interaction between plants and herbivores [4]. Plant capacity to defend against herbivores is also classified on the basis of constitutive traits—which are present before predation—and induced resistance or tolerance, which appears after herbivory events [5,6].

Tolerance is considered a good strategy to maintain fitness after herbivory [7,8] and includes a wide variety of mechanisms that act to rapidly compensate for the loss of biomass caused by predation, such as growth compensation, an increase of photosynthetic activity or reallocation of resources from roots to shoots. Some examples of constitutive traits related to tolerance are a high net photosynthetic rate [9], high chlorophyll fluorescence [10] and a high electron transport rate [11]; these features indicate higher herbivory tolerance. In contrast, high constitutive non-photochemical quenching values are related to a low tolerance to herbivory in juvenile plants of endemic species from Hawaii [11]. Moreover, high oxidative basal stress levels are also associated with low tolerance to herbivory [12].

Moreover, plants have developed mechanisms to compensate for the loss of biomass by herbivory. As reviewed by Tiffin [13], the responses executed by plants after predation include an increased photosynthetic rate in the remaining tissues, compensatory regrowth in non-affected meristems, use of storage reserves to generate new biomass, and modification of plant phenology to avoid interactions with herbivores in the future.

In some cases, it has been described that phylogeny determines the impact of plant enemies [14]. But in other cases, is the ecological context (i.e. historic exposure to herbivores) what determines whether plants can tolerate herbivores [5]. A specific analysis could be necessary to elucidate the role of ecological context on how plants tolerate herbivory.
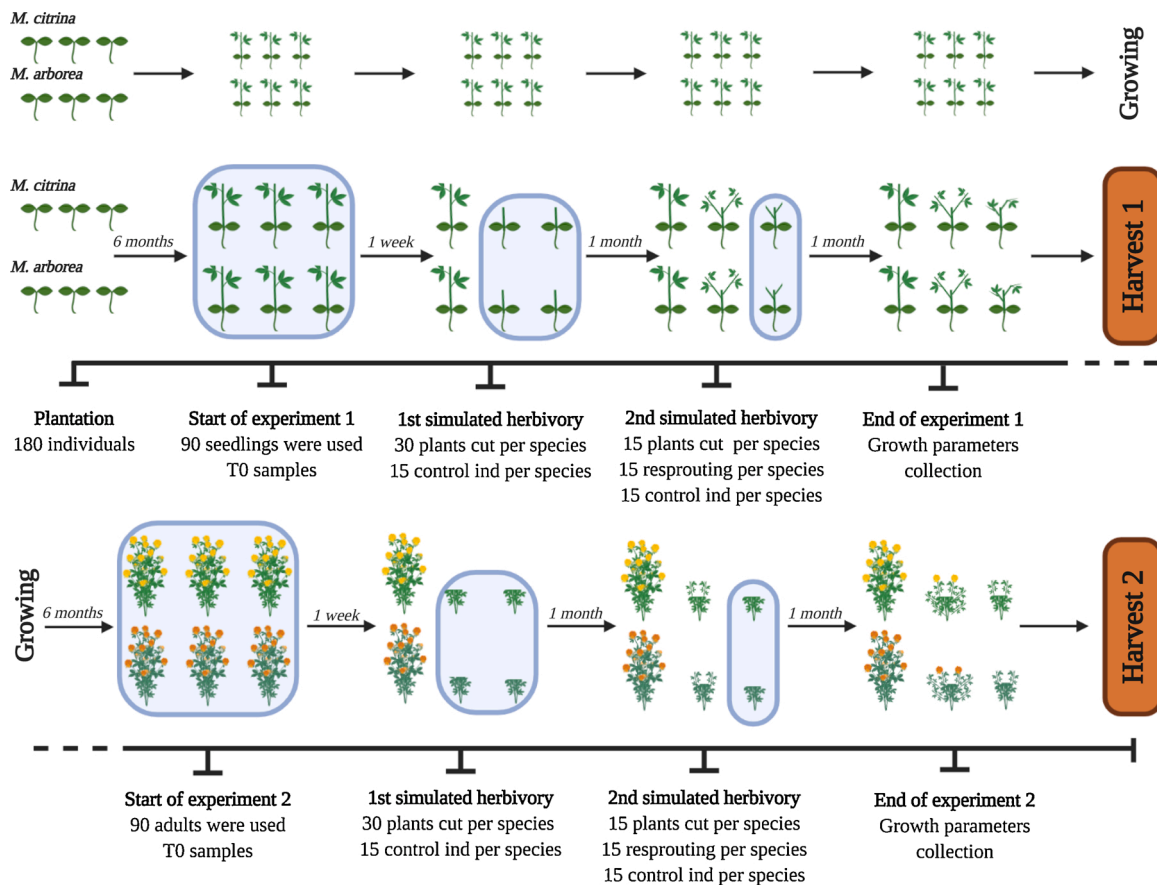

Fig. 1. Experimental design. The black horizontal lines indicate the timeline from planting the seeds (top row) until the two harvest events for the seedling (middle row) and adult plant experiments (bottom row). Blue squares indicate when each treatment started. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The ecological context could be applied to insular endemic plants isolated from predators, which evolved without mammalian herbivores until the arrival of humans [15]. A trade-off exists between growth and plant defence mechanisms [4], which explains the relationship between herbivore exposure and plant features that are not solely related to resistance [3] but also tolerance to herbivory [1]. In fact, the capacity to tolerate herbivores can decrease across evolution in species that have not coexisted with natural herbivores [16]. However, there is little information on how historic exposure to herbivores affects the relationship between constitutive traits and induced tolerance [17]. In the opposite view, some studies have proved that invasive species that have lost their herbivores can relocate their defence resources into other functions in a short-time period [18].

Loss of defence mechanisms in some insular species makes the plants more vulnerable to predators than phylogenetically related species that have coexisted with herbivores [15], even though the constitutive traits of the species could be similar. Thus, islands and islets provide a suitable setting to study how the historic absence or presence of herbivores can determine plant tolerance to herbivory and whether the historic context has a more evident effect on the responses to predation than constitutive tolerance traits.

However, the tolerance capacity of a species is not uniform across time, since tolerance response varies depending on the life stage of the plant [19]. Moreover, Gruntman and Novoplansky [20] demonstrated that ontogeny had a strong effect on tolerance to herbivory, predation in the vegetative phases induced greater meristem activation whereas predation in the reproductive phases induced investment in late-determined reproductive traits such as fruit and seed biomass. In this sense, the responses to herbivory can vary over time, and data

obtained soon after damage can vary from the values measured at later sampling periods [11,21]. Therefore, in order to adequately describe the herbivory tolerance capacity of a plant species, it is necessary to consider different stages of ontogeny [22,23].

The model species assessed in this work were *Medicago critina* (Font Quer) Greuter and *Medicago arborea* L. Unlike most species from the same genus, *M. citrina* and *M. arborea* are perennial woody shrubs that can grow up to 3 m tall and wide [24]. *M. citrina* is endemic to a few small islets in the Balearic Sea and close to the Iberian Peninsula Coast, and a low number of isolated individuals also occur on the Spanish mainland and Western Ibiza [25]. The plant is included in the Spanish Catalogue of Threatened Species as 'Vulnerable' (RD 139/2011), and considered one of the most threatened species in the Mediterranean [25]. The presence of *M. citrina* on other islets or bigger islands is likely to have been strongly limited by the presence of introduced herbivores, mainly goats, rats and rabbits [25–28]. Latorre et al. [23] observed that this species suffers from the severe combined effects of predation by rats and rabbits, which damage the early recruitment stages (i.e. seeds, seedlings and saplings). At present, due to various rabbit and rat eradication programs in some islets, the presence of *M. citrina* has increased substantially in both the original distribution area and in successfully translocated zones [25]. In contrast, *M. arborea* L., a species closely related to *M. citrina*, is widespread throughout Europe and is commonly used as fodder in many areas, indicating mature plants have a good capacity to tolerate herbivory [29]. Interestingly, both species have a different herbivory context: *M. arborea* is a widely distributed species historically more exposed to herbivory than *M. citrina*, which is a range-restricted species that occurs in free areas of herbivores. Hence, we tested the hypothesis that the species under herbivore pressures

Table 1

Constitutive values of mechanisms related to tolerance to herbivory for studied species.

Parameters related to tolerance	Seedling			Adult		
	M. citrina	*M. arborea*	*t*-test	*M. citrina*	*M. arborea*	*t*-test
Chlorophyll fluorescence (Fv/Fm')	0.726 (± 0.004)	0.698 (± 0.005)	**	0.736 (± 0.010)	0.728 (± 0.014)	ns
Lipidic peroxidation (MDA)	–	–	–	18.64 (± 2.40)	44.50 (± 4.01)	**
Constitutive non photochemical quenching (NPQ)	0.578 (± 0.023)	0.596 (± 0.034)	ns	0.627 (± 0.017)	0.641 (± 0.022)	ns
Maximum electron transport rate (ETR)	98.26 (± 8.74)	89.08 (± 8.77)	ns	144.52 (± 21.42)	116.90 (± 14.41)	ns
Root C:N ratio	14.86 (± 0.57)	12.60 (± 0.63)	*	21.75 (± 1.79)	17.52 (± 0.81)	ns
Shoot C:N ratio	17.72 (± 0.43)	13.69 (± 0.93)	**	17.36 (± 1.44)	14.75 (± 0.77)	ns
R:S ratio	0.524 (± 0.033)	0.386 (± 0.024)	**	0.432 (± 0.056)	0.404 (± 0.064)	ns
Total root biomass (g)	13.91 (± 1.08)	14.05 (± 1.41)	ns	181.74 (± 25.27)	100.44 (± 14.22)	*
Total shoot biomass (g)	19.76 (± 1.73)	23.82 (± 1.39)	ns	431.50 (± 37.24)	280.80 (± 38.80)	*
Nodules biomass (mg)	197.36 (± 47.76)	98.18 (± 16.63)	ns	–	–	–
Specific leaf area (cm2/g)	–	–	–	105.53 (± 5.96)	159.07 (± 6.925)	***

Fv/Fm' and MDA shown corresponds to the last sampling time (T6). Standard error of each parameter is shown between brackets. Significant differences between species for each life stage are shown with asterisks, and indicate significance strength.

* < 0.05.
** < 0.01.
*** <0.001.

Table 2

Temporal variation on chlorophyll fluorescence (Fv/Fm) and lipidic peroxidation (MDA) during the experiment.

	Fv/Fm	MDA
Time	**58.9 (<0.001***)**	**24.84 (<0.001***)**
Treatment	**6.38 (0.001***)**	**5.00 (0.007**)**
Species	0.53 (0.464)	**4.92 (0.027*)**
Life stage	0.91 (0.340)	–
Species × treatment	0.47 (0.610)	**9.14 (0.001***)**
Treatment × life stage	2.75 (0.064)	–
Species × life stage	1.50 (0.219)	–

Numbers indicate Fisher's F-scores (and *p*-values). Asterisks indicate significance.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$); significant differences are marked in bold. Time was considered as smooth term in GAMM models. MDA refers to 3,4-methylene dioxyamphetamine.

present higher herbivory tolerance, although differences in constitutive traits related to herbivory tolerance among species were expected to be low due to their close phylogenetic relationship and their similar plant architecture. Also, changes in tolerance would be observed across ontogeny as observed in many other species.

To validate this hypothesis, we aimed to (1) assess whether constitutive traits are good indicators of plant tolerance to herbivory in two closely related species with different historical contexts of herbivore pressure; (2) evaluate whether induced tolerance after herbivory is herbivore context-dependent, and (3) determine if the differences in constitutive traits and induced tolerance between species are maintained during ontogeny.

2. Materials and methods

2.1. Plant material

Plants from the widespread *M. arborea* and the range-restricted *M. citrina* were cultivated from seeds provided by the Centre Forestal de les Illes Balears (IBANAT, Conselleria de Medi Ambient i Territori) at Finca Pública de Menut (Mallorca Island, western Mediterranean Basin, 39°49′44.78″N 2°54′0.46″E). Seeds were germinated on February 2017, after 30-min scarification with 98 % sulfuric acid to improve the germination rate (though we found the scarification step was not necessary for the seeds to germinate). Seeds were planted in forest germination tablets [30] and when cotyledons emerged, the seedlings were transferred to 3 dm³ pots. Seven months later, the seedlings were transferred to larger pots (6 dm³). Plants were grown outdoors in a cage to protect against all types of herbivores and watered daily to field capacity during the entire experiment.

2.2. Experimental design

The experiment was performed simultaneously for both species following the same procedures (Fig. 1). The plants were randomly allocated to two life stage groups to evaluate how tolerance varies between seedlings (7-months-old) and reproductive adults (16-months-old).

The response of seedlings to herbivory was assessed in an experiment performed during Sep-Dec 2017 (when the first leaves appeared) and the adult response was assessed in an experiment performed in Jul-Sep 2018 (when the first flowers appeared). Three herbivory treatments were applied: (1) no herbivory event as a control ($n = 15$), (2) removing biomass once at the beginning of the experiment (1 st cut, $n = 15$), and (3) removing biomass again 30 days after the first simulation (2nd cut, $n = 15$). Control individuals were used to enable a comparison of constitutive features at the same time-points as the induced tolerance response to simulated herbivory. A severe level of biomass loss was induced at each simulated herbivory event. Considering that moderate herbivory is defined as 50 % defoliation [11], we removed 80 % of the total aerial part of the plants at each herbivory event (including the leaves and stems) to simulate severe herbivory. To ensure standard regrowth after damage, a solution of 0.5 mM jasmonic acid was sprayed onto the incisions after the herbivory simulations [8,11]. All plants were harvested 30 days after the second simulation. However, as some plants died after simulated herbivory, the sample sizes for evaluation of the growth and biochemical parameters varied among treatments (however, all $n \geq 7$).

2.3. Parameters related to physiological and biochemical status

We evaluated the parameters related to tolerance to herbivory described by Strauss and Agrawal [1]. Photosynthetic variables were assessed via pulse-amplitude modulated chlorophyll fluorometry (Junior PAM, Walz) due to its advantages of less time-consuming data collection compared to gas exchange methods, as explained by Barton [11]. Chlorophyll fluorescence (Fv/Fm') was assessed a total of seven times (T0-T6) at 15-day intervals from the beginning of each experiment (before the first herbivory simulation, T0) until harvesting at T6.

The electron transport rate (ETR) in response to various photosynthetic active radiation (PAR) intensities was evaluated at the end of both experiments prior to harvesting. Light curves were performed over 11 steps (PAR 0–1150 µmol photons m^{-2} s^{-1}). Constitutive non-photochemical quenching (NPQ) at the highest PAR intensity was also
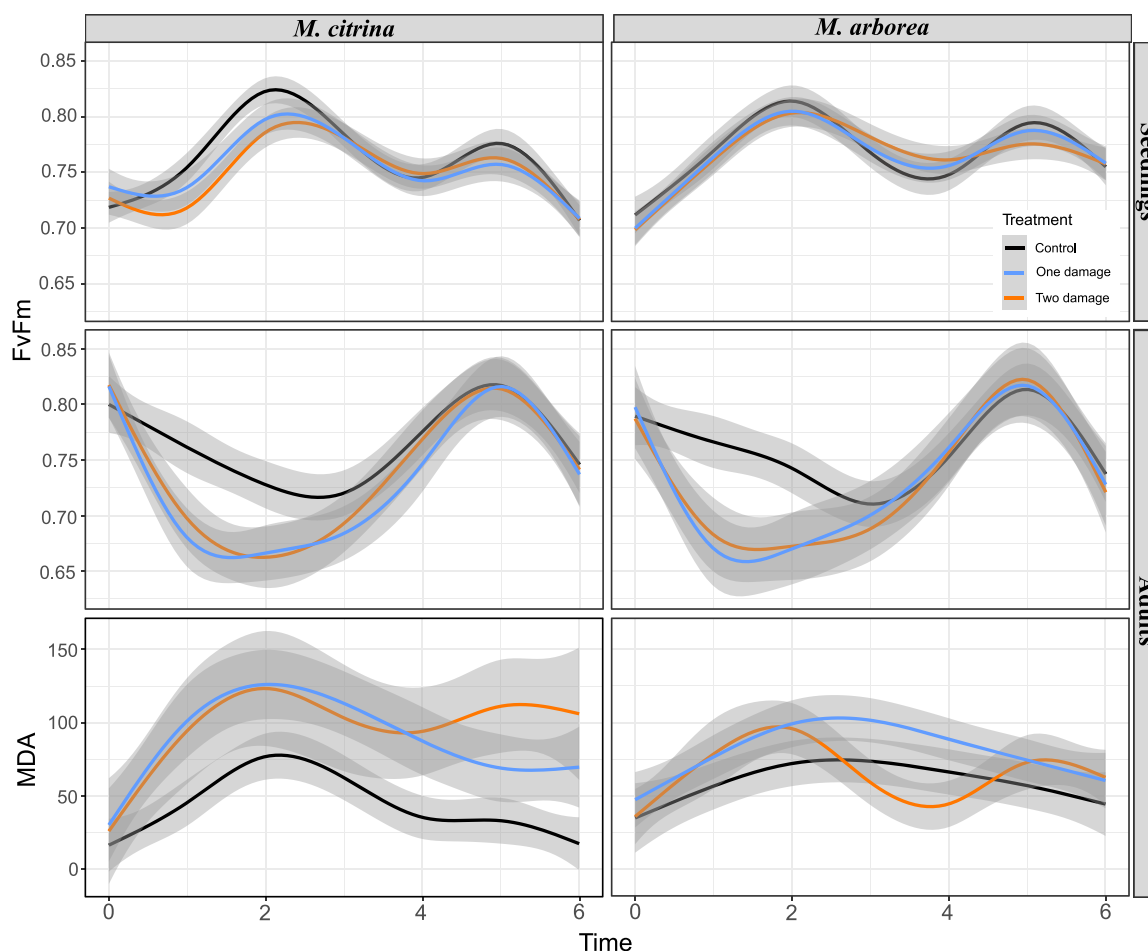
Fig. 2. Temporal series of chlorophyll fluorescence (Fv/Fm') data (top and middle rows) and lipidic peroxidation (MDA) data (bottom row). Data is shown by species (columns) and life stage (rows). Values are mean (± standard error). Control plants are indicated in red circles, one damage treatment event in blue triangles and two damage treatment events in green squares. Blue and green vertical lines indicate the first and second herbivory simulations, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3

Photosynthetic parameters obtained after light curve fitting of the electron transport rate and photosynthetic active radiation (ETR/PAR) curves.

	ETRmax	Alpha	Ek
Treatment	0.51 (0.599)	1.32 (0.274)	0.08 (0.919)
Species	0.32 (0.568)	0.07 (0.789)	0.92 (0.340)
Life stage	**28.62 (<0.001 ***)**	**58.65 (<0.001 ***)**	**11.25 (0.001 **)**
Species × treatment	**3.15 (0.050*)**	**5.45 (0.007**)**	1.44 (0.247)
Treatment × life stage	0.49 (0.615)	0.32 (0.721)	1.24 (0.299)
Species × life stage	3.49 (0.067)	0.46 (0.498)	**4.48 (0.039*)**

Numbers indicate Fisher's F-scores (and *p*-values). Asterisks indicate significance.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$); significant differences are marked in bold.

determined for control plants to evaluate the amount of energy dissipated by heat [11]. The maximum electron transport rate (ETRmax), slope of the light-dependent part of the curve (alpha) and light saturation (Ek) were obtained from the light curves.

Lipidic peroxidation was determined as a proxy of biochemical status prior to herbivory [31]. Briefly, the concentration of 3,4-methylene dioxyamphetamine (MDA) was evaluated in fresh leaves from five individuals of each species and treatment in the adult experiments [32].

Leaves were sampled (at the same time that Fv/Fm' was measured) and immediately immersed in liquid nitrogen and stored at −80 °C until analysis [33]. Approximately 0.1 g of frozen leaves were homogenized in 1 ml of 1 % trichloroacetic acid, centrifuged at 10,000 rpm, and 1.5 ml of 0.5 % thiobarbituric acid prepared in 20 % trichloroacetic acid was added to 0.5 ml of the supernatant. The mixtures were heated at 90 °C for 20 min, centrifuged at 10,000 rpm and the absorbance of the samples were measured at 532 nm using a Multiskan Sky Microplate Spectrophotometer (ThermoFisher Scientific) and the non-specific absorption value at 600 nm was subtracted. The amount of MDA-TBA complex was calculated from the extinction coefficient (Cakmak and Horst, 1991).

2.4. Growth parameters related to biomass

Thirty days after the second herbivory simulation and at final harvest, the aerial parts of the plants were dried in an oven at 65 °C for 15 days; the roots were also harvested and dried after removing excess soil and water.

The dry weights of the shoots and roots were obtained to evaluate the root:shoot ratio (defined as the dry weight of root per dry weight of shoot). Total dry biomass was also calculated as the sum of the dried biomass of the shoots, roots and parts removed during the herbivory simulations. Two grams of roots and shoots from each individual were separately ground and passed through a 1-mm mesh sieve. The carbon and nitrogen contents (i.e. C:N ratio) of each sample were analysed following the Dumas method [34] using a LECO TruSpec (Leco®
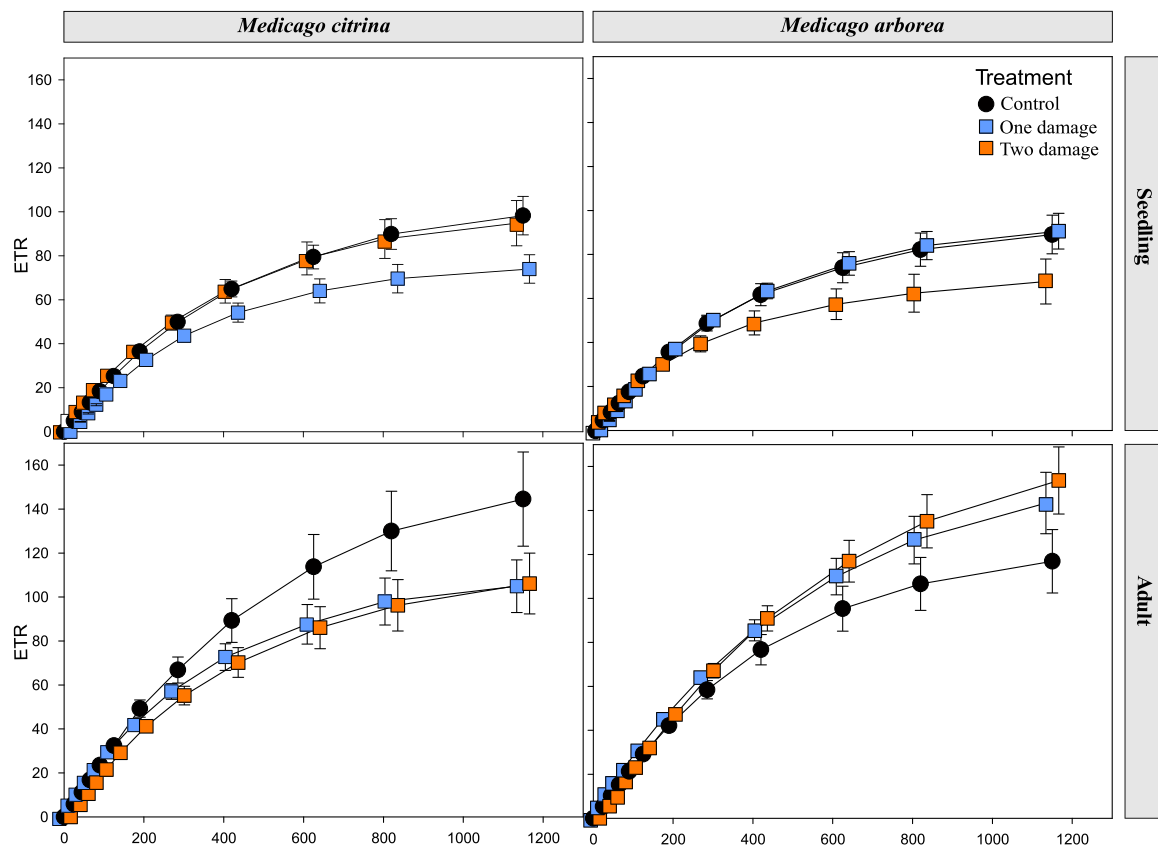
Fig. 3. Light-dependent phase of the light saturated electron transport rate (ETR) curves. Data is shown by species (columns) and life stages (rows). Values are mean (± standard error). Control plants are indicated in red circles, one damage treatment event in blue triangles and two damage treatment events in green squares. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Corporation) to evaluate the ability of plants to store and shunt resources from the roots to shoots after damage. Root nodules were only quantified in seedlings due to the methodological difficulty of separating all nodules from each root in adult plants; the seedling nodules were separated from the roots and weighed.

Finally, five fresh leaves were collected from adult plants at harvest and the foliar area was immediately calculated using ImageJ [35]. Leaves were stored at 65 °C for 15 days and weighed to obtain the specific leaf area (SLA), which was determined as the foliar area divided by the dry leaf biomass.

2.5. Statistical analysis

Statistical analysis was conducted using R software v3.6.1 [36]. To assess constitutive parameters, data from the control individuals was compared between species. The effect of the herbivory simulations was analysed separately for seedlings and adults. Two sample *t*-tests were used to evaluate the significance of the differences between species for each parameter at each life stage.

Generalised Additive Mixed Models were performed using chlorophyll fluorescence and MDA as response variables, treatment, life stage and species as categorical parametric variables, time as linear smooth variable and individuals as random factor. Variance of generated models were analysed using the '*anova*' function of the '*car*' package [37].

Light curves were analysed considering herbivory treatment, life stage and species as explanatory variables and the traits obtained from the fitted curve (ETRmax, alpha and Ek) as response variables. Linear models (LM) were constructed and homoscedasticity and normality assumptions were verified by plotting residuals versus fitted value.

The C:N ratio and dry weight of roots and shoots were analysed separately for each species and life stage to evaluate variation between the treatments. LM models were made, checking that the residuals met the criteria of normality and homoscedasticity through validation graphs. The post-hoc Tukey and pairwise tests were used to identify significant differences, by applying the '*lsmeans*' function of the '*lsmeans*' package [38]. The same procedures were followed for the nodule dry weight data for the seedling dataset and SLA data for the adult plants.

3. Results

3.1. Constitutive traits related to tolerance

Comparison of the constitutive traits in the control treatments revealed some significant differences in the analysed variables between *M. citrina* and *M. arborea* (Table 1). In seedlings, *M. citrina* exhibited a significant higher chlorophyll fluorescence value, C:N ratio and root: shoot ratio than *M. arborea* while NPQ, ETR, root, shoot and nodules biomass remained no-significant (Table 1). In adult plants, *M. arborea* had significantly higher MDA and SLA values, *M. citrina* had higher root and shoot biomass and chlorophyll fluorescence, NPQ, ETR, C:N and R:S ratios remained similar between species (Table 1).

3.2. Induced tolerance after simulation of herbivory

The death rate was used as the first proxy of tolerance to herbivory. All *M. arborea* plants in the seedling and adult experiments survived until harvested. However, in the seedling experiment (Fig. 1), 20 % of *M. citrina* plants died after the second simulation. In the adult experiment, 28.5 % of *M. citrina* plants died after the first simulation and 27.2 % died after the second simulation.

Chlorophyll fluorescence differed between treatments and over time during the experiments as shown in Table 2 and Fig. 2. Chlorophyll

Table 4
Growth parameters obtained at the harvest time-point after the herbivory simulations.

| Response variable		Fixed variables	d.f., residual	F	P-value	
C:N ratio	Root	Treatment	2, 48	1.00	0.374	ns
		Species	1, 48	0.91	0.343	ns
		Life stage	1, 48	162.59	< 0.001	***
		Treatment × species	2, 48	8.17	< 0.001	***
		Treatment × life stage	2, 48	0.90	0.411	ns
		Species × life stage	2, 48	1.41	0.239	ns
	Shoot	Treatment	2, 48	38.23	< 0.001	***
		Species	1, 48	18.92	< 0.001	***
		Life stage	1, 48	0.34	0.562	ns
		Treatment × species	2, 48	4.51	0.016	**
		Treatment × life stage	2, 48	2.41	0.099	ns
		Species × life stage	2, 48	0.006	0.968	ns
Dry weight	Root	Treatment	2, 131	104.97	< 0.001	***
		Species	1, 131	0.002	0.987	ns
		Life stage	1, 131	1023.80	< 0.001	***
		Treatment × species	2, 131	6.21	0.002	**
		Treatment × life stage	2, 131	6.57	0.001	***
		Species × life stage	1, 131	39.01	< 0.001	***
	Shoot	Treatment	2, 131	8.05	< 0.001	***
		Species	1, 131	15.93	< 0.001	***
		Life stage	1, 131	846.84	< 0.001	***
		Treatment × species	2, 131	1.13	0.325	ns
		Treatment × life stage	2, 131	2.19	0.115	
		Species × life stage	1, 131	42.22	< 0.001	***
Nodules		Treatment	2, 84	11.44	< 0.001	***
		Species	1, 84	0.34	0.561	ns
		Treatment × species	2, 84	4.00	0.021	*
SLA		Treatment	2, 84	11.44	< 0.001	***
		Species	1, 84	0.34	0.561	ns
		Treatment × species	2, 84	4.00	0.021	*

Asterisks indicate significance strength.
* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$). SLA refers to specific leaf area.
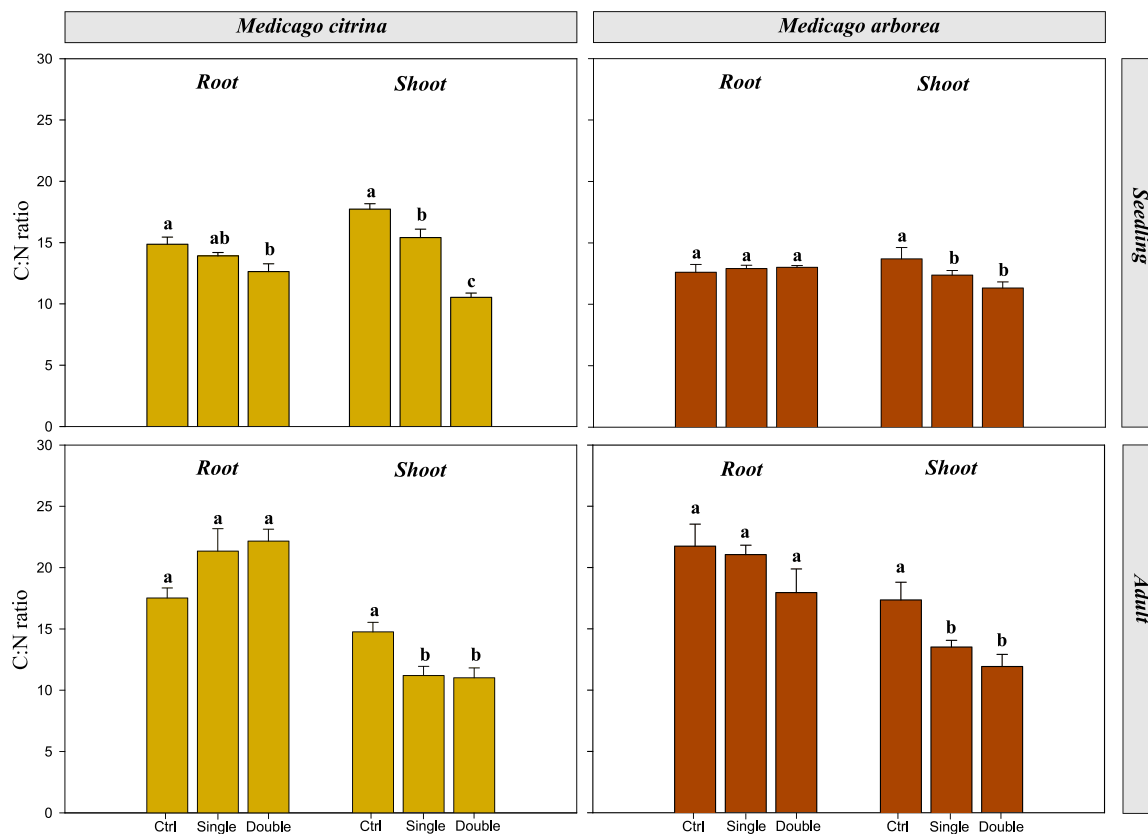
Fig. 4. C:N ratio for each species (columns) and life stage (rows) in control plants (Ctrl), and after the single cut simulation (Single) and double cut simulation (Double). Each graph shows the root and shoot data separately. Values are mean (± standard error). Different letters indicate significantly different groups (posterior Tukey-test).
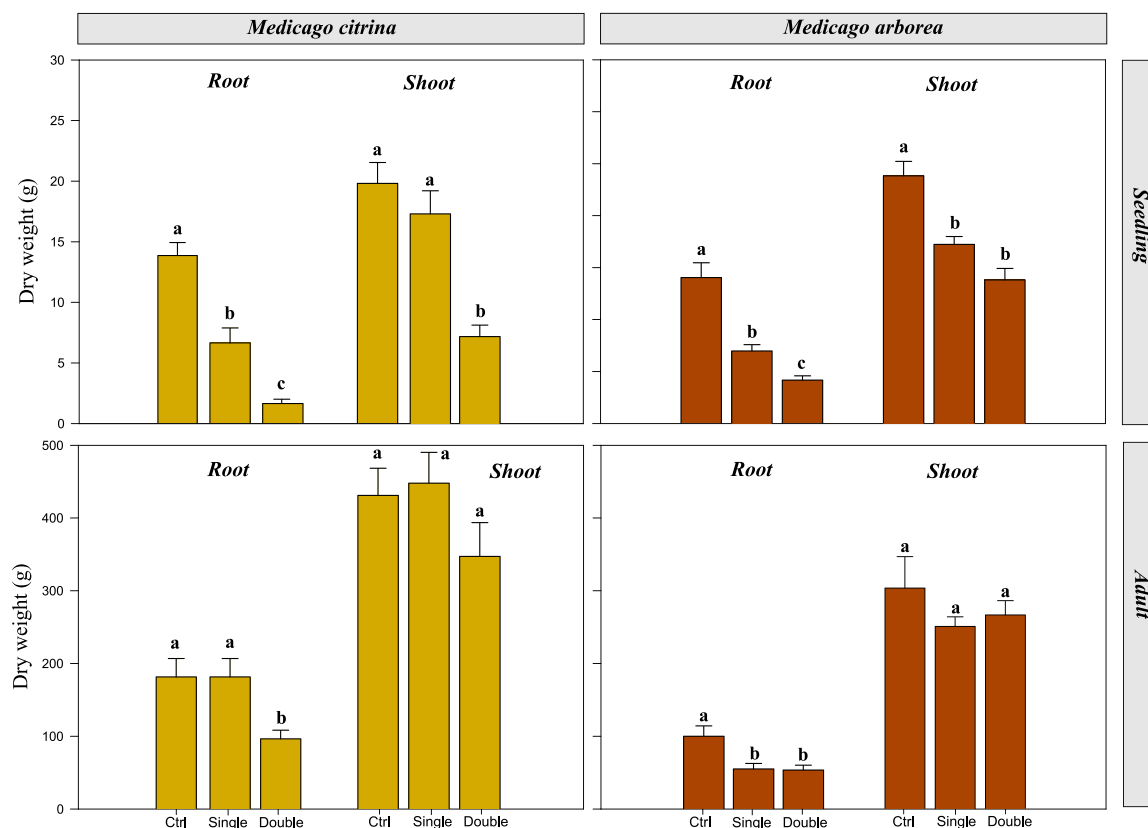
Fig. 5. Dry biomass weight data for each species (columns) and life stage (rows) in control plants (Ctrl), and after the single cut simulation (Single) and double cut simulation (Double). Each graph shows the root and shoot data separately. Values are mean (± standard error). Different letters indicate significantly different groups (posterior Tukey-test).

fluorescence was reduced by the treatments and over time in both *M. citrina* and *M. arborea*. Despite that, the reduction was only detected after the first herbivory simulation. After the second herbivory event, the Fv/Fm' values recovered to control values. Specifically, lower values than control were observed for chlorophyll fluorescence in *M. citrina* seedlings after the first cut, but not after the second. In contrast, no differences were observed among treatments for *M. arborea* seedlings, even though chlorophyll fluorescence fluctuated over time in both species.

Lipidic peroxidation increased in the treatment groups after the herbivory simulations, while control plants maintained baseline levels in both species (Table 2). Specifically, the herbivory treatments significantly increased the MDA values in *M. citrina* compared to control plants during the whole experiment, whereas treated and control *M. arborea* plants had similar MDA values (Fig. 2).

The light curves indicated strong variation in all variables between the seedlings and adults. Furthermore, life stages exhibited different photosynthetic performance in terms of ETRmax and alpha after herbivory damage (Table 3).

In seedlings, *M. citrina* exhibited reduced photosynthetic activity after the second herbivory damage, whereas *M. arborea* showed reduced photosynthetic activity after the first herbivory simulation, but recovered after the second damage (Fig. 3). Different patterns of variation in the light curves of adult plants were observed among treatments for both species. In *M. citrina*, the treatments led to lower photosynthetic capacity than control plants, whereas *M. arborea* exhibited increased photosynthetic activity after damage (Fig. 3).

Complete analysis of the growth parameters is summarized in Table 4. The C:N ratio after herbivory varied between species and life stages, as shown in Fig. 4. The root C:N ratios were not affected by the herbivory simulations and both species showed similar root C:N ratios,

with the exception of *M. citrina* in the seedling experiment. In contrast, significant reductions in the shoot C:N ratio were found among treatments, with greater reductions in *M. citrina* than *M. arborea* and in seedlings than adults. Generally, the treatment affected the C:N ratio in shoots, but not in roots, with stronger effects observed in *M. citrina* than *M. arborea*.

Biomass produced by plants (also considering the parts removed during the herbivory simulations in the total outcome) indicated tolerance response mechanism varied between both the species and life stages (Fig. 5; Table 4). The roots were severely affected by damage, even though only the aerial parts of the plant were removed during the herbivory simulations. Seedlings exhibited a greater reduction in root biomass than adults in both species. *Medicago citrina* demonstrated a varied response to damage across time. The seedling roots were initially affected as root biomass decreased after the first cut, and a further reduction was observed after the second cut. In contrast, adult plants maintained root biomass after the first cut, with a reduction in biomass only observed after the second cut. In *M. arborea*, seedling root biomass decreased in a similar pattern to *M. citrina*; however, adult *M. arborea* root biomass also reduced after the first cut, but not between the first and second cuts. On the other hand, the seedling shoot biomass of both species strongly decreased after the herbivory simulations. The adults of both species resprouted and recovered the initial amount of shoot biomass, with both the single and double damage treatments achieving the same levels as controls.

Root nodules were only sampled during the seedling stage, and their biomass reduced after the simulations of herbivory in both species (Fig. 6A; Table 4), although the effect was significantly higher in *M. citrina* than *M. arborea*. Specifically, after the first simulation, root nodule biomass decreased in *M. citrina* and was maintained in *M. arborea*. After the second simulation, root nodules were almost absent
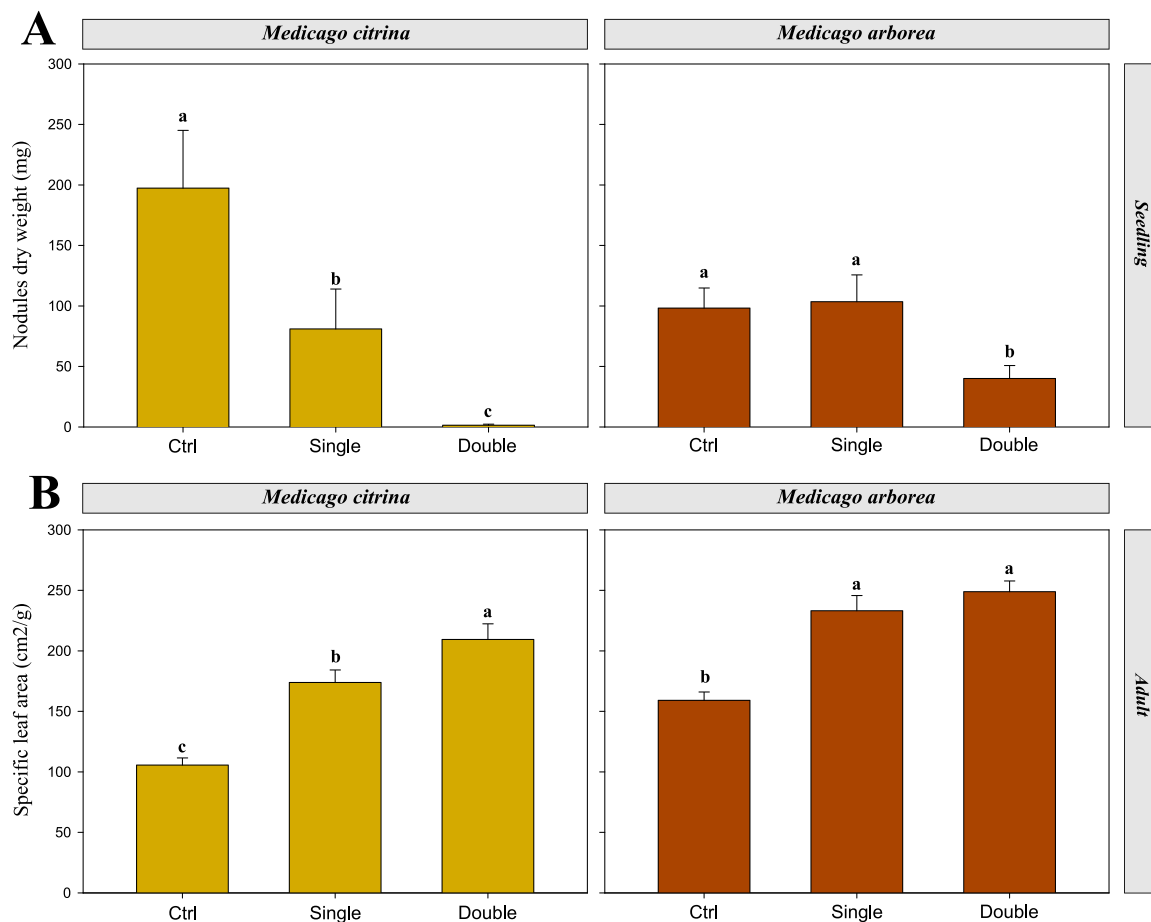
Fig. 6. Nodule dry biomass for seedlings (A) and specific leaf area for adults (B) in controls and the (Ctrl), single cut (Single) and double cut (Double) treatments. Each graph shows the root and shoot data separately. Values are mean (± standard error). Different letters indicate significantly different groups (posterior Tukey-test).

in *M. citrina* and reduced by around 50 % in *M. arborea* compared to control plants. On the other hand, SLA, which was only sampled in the adult stage (Fig. 6B; Table 4), increased after damage in both species, with a greater extent of damage in *M. arborea*.

4. Discussion

This study partially supports the hypothesis that the range-restricted species *M. citrina* has a lower tolerance to herbivory than the widespread, closely related species *M. arborea,* independent of ontogeny. However, *M. citrina* exhibited better values for constitutive traits related to tolerance than *M. arborea*. Thus, in this case, the relationship between constitutive tolerance traits and the responses to herbivory are only observed when herbivores are present in as selective pressure, as observed in other cases [1,39] at both the seedling and adult stages.

4.1. Are constitutive tolerance traits related to herbivore pressure?

The range-restricted species *M. citrina* exhibited better values for constitutive traits than the widespread, closely related, *M. arborea*, including better physiological activity lower oxidative stress and a higher R:S ratio and root C:N ratio. This would suggest that range-restricted species are more tolerant to herbivores [11–13]. Thus, *M. citrina* may have retained good constitutive traits related to tolerance to herbivores, even though the species does not coexist with herbivores in its natural distribution and it only occurs in isolated areas.

The lack of herbivores as a selective pressure has been described as a cause of loss of defence in various species [15], based on the idea that

there is a trade-off between growth and defence [40]. Following this argument, the maintenance of constitutive characteristics that improve the response to herbivores, despite the absence of herbivory pressure, can be explained by either an absence of a trade-off between growth and the development of such constitutive characteristics, or that these characteristics are related to tolerance to other types of stress. Indeed, the same constitutive trait could be related to both biotic and abiotic stresses [41–43]. For example, *M. citrina* is well adapted to severe saline-exposure water-stress, which is related with high C:N or root: shoot ratios [44,45]. Moreover, coastal species usually present lower SLA than their inland relatives, as happened with our study species [46]. So, some constitutive traits related with herbivory tolerance in other species [1,13], would be related to tolerance to salinity in the case of *M. citrina* in comparison with *M. arborea*, which is known to be poorly salt-tolerant [47]. As a result, constitutive traits related with resistance to several stress are not always good predictors of herbivory tolerance. Adult plants of *M. citrina* are a clear example since plants died after damage despite resprouting through nutrient reallocation was expected due to the high nitrogen content of roots.

4.2. Is induced tolerance to herbivory lower in species with less exposure to herbivores?

Medicago citrina and *M. arborea* responded differently to the herbivory simulation events. First, a considerable proportion of *M. citrina* individuals died after simulation (20 % of seedlings and 55 % of adults), whereas all *M. arborea* individuals survived. Considering the main traits related to compensation [1], *M. citrina* almost lacks tolerance to
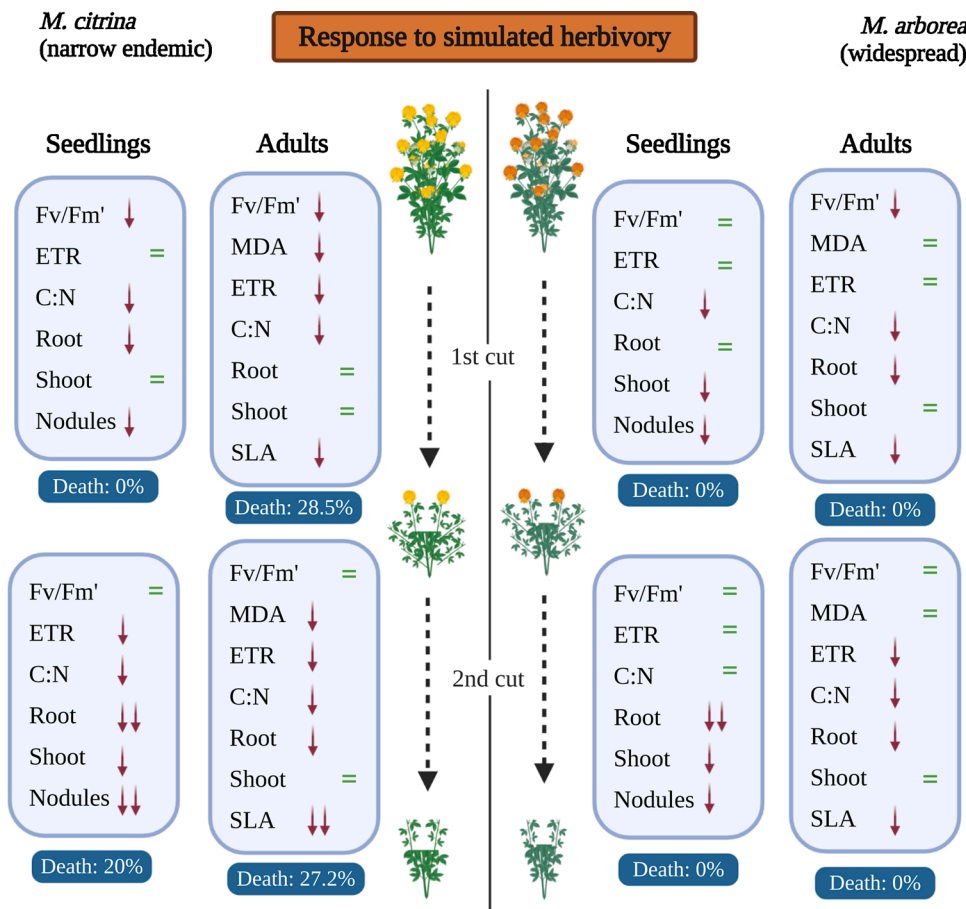
Fig. 7. Induced tolerance of *Medicago citrina* and *Medicago arborea* in the seedling and adult life stages to simulated herbivory after the first cut and second cut. Negative effects compared to control plants are marked in red arrows; double arrows indicate where the double cut treatment lead to a greater effect than the single cut; green equal symbols indicates where the values were similar in the treatment and control. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

herbivory, whereas *M. arborea* tolerates slight herbivory. For both species, none of the parameters measured were higher after damage compared to control plants (Fig. 7), as previously observed for some herbivory-tolerant species [21,48–50]. Tolerant species exhibit improvements in many parameters after damage, such increased biomass overall [51] or an increase in the C:N ratio of shoots that emerge after herbivory events [13]. However, these effects were not observed in either of the studied species, which indicates *M. citrina* and *M. arborea* do not overcompensate after herbivory [52].

Additionally, *M. arborea* recovered its chlorophyll fluorescence value after damage without increasing lipidic peroxidation and maintained the number of live root nodules, while the opposite trends were observed for all of these variables in *M. citrina*. Indeed, even though herbivory was only performed on the aerial parts of the plants, indirect biomass loss was observed for the underground parts of both species (i.e. roots and nodules), which confirms that neither species can use root storage to compensate for herbivory.

However, the second cut did not synergistically increase the effects of herbivory, except for the increase in SLA in *M. citrina*, which can be related to optimization of the foliar area and investment of resources for further development [53]. Interestingly, both species were able to recover shoot biomass after the first and second damage, even though root biomass reduced. This observation would predict tolerance capacity for both species, in which resource allocation from the roots to shoots indicates a mechanism of compensation for herbivory biomass loss [54, 55]. Nevertheless, this pattern was not observed for the C:N ratios of either roots or shoots, as the C:N ratios of both plant structures decreased after herbivory. Despite this, we predict the plants could gradually recover over a longer period of time, and probably reach control values. However, increased lipidic peroxidation indicates long-term damage to biochemical status, and may imply indirect effects

in the future [31,56]. Further studies of biochemical disturbances after damage are necessary to evaluate how herbivory induces oxidative stress in tolerant plant species, especially in the context of tolerance to introduced herbivores. In contrast, *M. arborea* was able to recover damage-induced changes in lipidic peroxidation and chlorophyll fluorescence, and damages in light caption were detected later in *M. arborea* than *M. citrina,* as also observed in other species such as endemics from the Hawaii Islands [11].

Evolution leads to reduce defence allocation in those species that lose their natural enemies or that have been introduced in other ecological contexts where predators are absent [57]. The present study is an example of that process since the range-restricted species is less tolerant than the widespread.

4.3. Does ontogeny affect tolerance capacity patterns?

Medicago citrina was more affected by herbivory than *M. arborea* in both life stages. This lack of tolerance to defoliation in both life stages may explain why the entire *M. citrina* populations disappeared from islands and islets when herbivores were introduced [27]. Indeed, the dead risk after herbivory is higher in *M. citrina* than in *M. arborea* as proved in this study. Adults of *M citrina* showed a higher death percentage than seedlings after herbivory simulations, indicating higher damage by herbivores. However, evaluating individuals that survived, seedlings showed less capacity to recover control values than adults, in terms of root C:N ratio, biomass or even the parameters derived from the light curves (ETRmax, alpha and Ek), as observed in similar experiments using seedlings of endemic species from Hawaii [11]. *Medicago citrina* seedlings exhibited a poorer response in terms of root biomass and regrowth capacity compared to adults from the same species. This observation indicates it would be harder for seedlings to resprout and

grow after herbivory [58] but see [21]. Moreover, damage during the seedling stage can affect the development of further life stages [59]. An incremental increase in tolerance to herbivory has been reported during ontogeny [60]. In this study, the seedlings of both species were vulnerable to herbivory, and the adult life stage of both species exhibited improved responses despite many *M. citrina* adult individuals still died. Indeed, death events of *M.* citrina may plausibly explain the constrained distribution mediated by predation of the seeds and seedlings by introduced mammalian herbivores [27].

4.4. Implications for the conservation of threatened species

The evolutionary context of *M. citrina* and *M. arborea* may be the main factor that explains the varied tolerance to herbivory among the seedling and adult plants of each species. Assessing if endemic plants from islands can tolerate herbivory will be essential to evaluate the impact of introduced mammal herbivores in insular communities. As observed in this study, seedlings are the most threatened stage of the population structure and therefore the recruitment of the population might be severely affected. Also, *M. citrina* adults die after predation, despite individuals that survived were able to recover successfully. In this sense, future conservation strategies must focus on protecting natural populations from introduced herbivores, and considering this threat when implementing reintroduction or reinforcing measures.

5. Conclusions

Even though its constitutive defence traits predicted *M. citrina* could tolerate herbivory, the opposite was observed as the species exhibited poor induced tolerance after damage. Our study is an example of how the herbivore pressure may play a more essential role in determining plant tolerance to herbivory than constitutive defences. Therefore, if induced tolerance fails to compensate for herbivory, the species must rely on the escape strategy in areas where herbivores are absent, as is the case of the islets where *M. citrina* occurs. Assessment of ontogeny is crucial when evaluating tolerance to herbivory; seedlings recover slower than adults, and *M. citrina* showed high death percentages in both stages. Therefore, lack of herbivory pressure in the historic context leads to a loss of induced tolerance, but not constitutive tolerance traits.

Declaration of Competing Interest

The authors declare they have no potential conflict of interest

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