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RESEARCH ARTICLE

Plant-mediated interactions between specialist insect herbivores of *Melaleuca quinquenervia*: Implications for management of invasive populations

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Abstract

1. Classical weed biocontrol involves release of host-specific herbivores from the weed's native range into invaded ecosystems. Often multiple herbivores are released, assuming additive interaction between them. However, herbivory exerted by one species can modify the host quality and defence of the weed species thereby altering the damage by the other herbivore.
2. Using an experimental community module, we studied how plant-mediated interactions between insect herbivores influence their relative effects on plant growth, using *Melaleuca quinquenervia* as a model system. Native to Australia, *M. quinquenervia* is an invasive weed in Florida, including Everglades National Park. Two congeneric herbivores *Lophodiplosis trifida* and *L. indentata* from Australia have been introduced as biocontrol agents against this weed.
3. We investigated if interactions mediated by *M. quinquenervia* influenced the effects of *L. trifida* and *L. indentata* on plant growth and nutrient dynamics, and if the order of plant–herbivore interactions altered these effects.
4. Both *L. trifida* and L. indentata reduced the growth and biomass of *M. quinquenervia*, with *L. trifida* exerting a stronger suppressive effect than *L. indentata*. Interactions between the two herbivores were either additive or independent. The addition of *L. trifida* enhanced the negative impact of *L. indentata* on plant growth, whereas the presence of *L. indentata* did not affect the impacts of *L. trifida*.
5. Both *L. trifida* and *L. indentata* induced similar effects on nutrient allocation. Similar changes in nutrients but differential phenotypic changes suggest that the effect of *L. indentata* was probably compensated for by *M. quinquenervia*. Nutrient allocation responses were generally additive, irrespective of the order of herbivore occurrence, and the consequences of herbivory were more pronounced in seedlings than in saplings.
6. Practical implication: Additive or independent interactions between *L. trifida* and *L. indentata* suggest that their combined effect does not diminish the effect of either herbivore as biocontrol agents. However, if only one agent was to be

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prioritized (e.g. due to limited resources), *L. trifida* can be prioritized as it exerts a stronger suppressive effect on *M. quinquenervia*, relative to *L. indentata*. If both species are used, releasing *L. trifida* before *L. indentata* may be advantageous.

KEYWORDS
biocontrol, community-level interaction, compensation, enemy release, herbivory, plant–insect interaction

1 | INTRODUCTION

Insect herbivory is among the key factors regulating plant community structure (Agrawal et al., 2012; Carson & Root, 2000; Maron 1998; McFadyen, 1998). Invasive plants in novel ecosystems, however, are not associated with co-evolved specialist insects. Such escape from herbivory allows invasive plants to reallocate resources from traits otherwise conferring anti-herbivore defence to those that enhance competitiveness, thereby enabling them to overcome any biotic resistance imposed by other co-occurring plant species in the recipient plant community (Blossey & Notzold, 1995; Crawley, 1989; Keane & Crawley, 2002; Stutz et al., 2016). The enemy release hypothesis posits that this enemy-free status and increased competitiveness are key characteristics of invasive species and are considered main reasons for the elevated abundance of invasive plants in the introduced range relative to their native range (Agrawal et al., 2012; Carson & Root, 2000; DeWalt et al., 2004; Maron, 1998; McFadyen, 1998; Xu et al., 2021).

Classical biocontrol approaches aim to re-establish the link between invasive species and their specialist natural enemies. In weed biocontrol, host-specific insects or pathogens associated with the weed species in the native range are assessed for their direct non-target effects and subsequently released into the introduced range, after regulatory review (Hinz et al., 2019; McFadyen, 1998; Morin, 2020). Upon release, these natural enemies (referred to as biocontrol agents or 'agents') establish self-sustaining populations on their host plants. Ideally, the suppressive effects of this trophic interaction on the focal invasive weed help regulate its abundance in the invaded ecosystem. The top-down regulation by natural enemies is highly dependent on the feeding pressure exerted and consequent negative effects on the growth, fitness, and recruitment of weeds, integration of herbivory with other methods of management, and the environmental context (Hovick & Carson, 2015; Lake & Minteer, 2018; Myers & Sarfraz, 2017).

In many instances, multiple biocontrol agents are released, assuming additive effects of the herbivores on the focal weed, and limited plant-mediated interactions among them (Denoth et al., 2002). However, herbivory theory predicts that plant-mediated interactions between herbivores are pervasive (Fox, 1981; Gould et al., 1991; Hougen-Eitzman & Rausher, 1994; Strauss, 1991), and that the damage exerted by one insect species can modify the quality and inducible plant defence of host plants that can alter the relative damage exerted by the other species (Agrawal, 1999; Masters, 1995; Moran & Whitham, 1990; Price, 1991; Rodriguez-Saona et al., 2005; Soler et al., 2012). Indirect interactions that are antagonistic can result in lower herbivory relative to the individual effects of each herbivore species on the host plant, thereby disrupting desired outcomes for biocontrol. Therefore, it is important to consider the interactions between biocontrol agents and ascertain whether the combined use of multiple biocontrol agents elicits additive/synergistic suppressive effects on invasive plants prior to their release (Milbrath & Nechols, 2014). Studies on the interactions between biocontrol agents and their impact on weeds reported prior to their release are limited. However, these results indicate that such interactions can be neutral (e.g. Campanella et al., 2009; Gerber et al., 2007; Hunt-Joshi et al., 2004; Hunt-Joshi & Blossey, 2005), additive (Buccellato et al., 2012; Hoffmann & Moran, 1998), antagonistic (Crowe & Bourchier, 2006; Mnqeta & Paterson, 2019), or complex to interpret due to other factors such as the density of the herbivores and plant competition (Milbrath & Nechols, 2004).

Melaleuca quinquenervia (Cav.) S. T. Blake (Myrtaceae) offers an ideal system to examine plant-mediated interactions among biocontrol agents, and their resultant effects on plant growth and survival. This perennial tree species is invasive in natural wetland and upland areas throughout southern Florida, USA, including Everglades National Park, a UNESCO World Heritage Site (Dray et al., 2006; Turner et al., 1997). This tree is attacked by several specialist insect herbivores in its native range in Australia, including a leaf-feeding weevil *Oxyops vitiosa* Pascoe (Curculionidae), a sap-sucking psyllid *Boreoglycaspis melaleucae* Moore (Psyllidae), a bud-gall fly *Fergusonina turneri* Taylor (Fergusoninidae), the stem-galling fly *Lophodiplosis trifida* Gagné (Cecidomyiidae), and the leaf-galling fly *L. indentata* Gagné (Cecidomyiidae). The first four of these species have already been released in Florida as biocontrol agents. *Oxyops vitiosa* (released in 1997) and *B. melaleucae* (released in 2002) significantly reduce the growth, survival, and reproductive potential of *M. quinquenervia*, while *F. turneri* (released in 2005) did not establish in the invaded range (Center et al., 2012). *Lophodiplosis trifida* (released in 2008) and *L. indentata* (release approved in 2022) both oviposit in meristematic buds, but form galls in different tissues (Pratt et al., 2013; Smith et al., 2020). *Lophodiplosis indentata* oviposits on the lamina of expanding leaves and associated axillary buds, and larvae induce blister galls on the leaf lamina. While *L. trifida* uses

similar tissues as *L. indentata* for oviposition, the developing larvae tunnel into green stems near branch apices and induce stem galls (Pratt et al., 2013; Purcell et al., 2007). Since both these species are short-lived as adults (<48 h), almost all interactions between them are likely to be mediated by the host plants that support their larval development.

The goal of this research was to characterize and quantify the plant-mediated interactions between *L. trifida* and *L. indentata*, to predict the consequences for biocontrol of *M. quinquenervia* when both agents are present in the invaded range. Using an experimental community module to test for plant-mediated interactions among these herbivores (Holt & Bonsall, 2017; Holt & Hochberg, 2001; Kaplan & Denno, 2007), we sought to disentangle the relative contributions of each gall insect on their own and in different release orders (Hoffmann, 1990). Specifically, we sought to answer the following questions: (a) Which of the two gall insects has the greatest direct effects on *M. quinquenervia*, and does this change based on plant stage? (b) Are the effects of each of the gall insects on the plant independent of each other? (c) Does the effect of each gall insect depend on the order in which the interaction is established?

2 | MATERIALS AND METHODS

Plants used in the experiments were grown in an 80:20 sand: peat mix using *M. quinquenervia* ('MQ' hereafter) seeds field-collected from Peregian, Queensland, Australia. Seedlings were transplanted into 175 mm diameter pots with the same growth mix and 6 g of slow-release fertilizer (Osmocote Controlled Release Fertilizer: Native, Scott's Australia Pty Ltd., Bella Vista, NSW, Australia) prior to the commencement of the trial. *Lophodiplosis indentata* ('LI' hereafter) and *L. trifida* ('LT' hereafter) used in the experiments were sourced from laboratory colonies established from galls derived from field infested *M. quinquenervia* trees in Queensland, Australia. The study was conducted in a glasshouse set at a constant temperature of 22–25°C and 40–70% Relative Humidity. Two separate experiments were conducted: one for seedlings (5–7 cm height) and the other for saplings (50–55 cm height) to understand the role of plant growth stages on the plant-mediated interactions between the herbivores.

2.1 | Herbivory treatments

Ninety-six MQ plants were randomly distributed into six nylon mesh-screened cages with a mesh aperture of ca 1 mm^2 (i.e. 16 plants per cage) and over the course of 2 weeks the following six treatments were imposed: (i) 100 pairs of adult LI on its own (LI), (ii) 100 pairs of LT on its own (LT), (iii) 50 pairs of LI and 50 pairs of LT per week were released simultaneously (LI+LT), (iv) 100 pairs of LI released in the first week and 100 pairs of LT released the second week (LI–LT), (v) 100 pairs of LT released in the first week and 100 pairs of LI released the second week (LT–LI) and (vi) A control treatment with no insects. All cages, except control, received 200 pairs of insects altogether in 2 weeks (i.e. average insect density of 12.5 insect pairs/plant). The plants were held in the treatment cage for one additional week after the two-week inoculation period. This additional period was sufficient to ensure that all the adults had completed oviposition and had died as LI and LT adults are short-lived (<48 h). During the inoculation period, plants were watered regularly during the day to ensure that the soil was saturated and the microclimate in the screened cage maintained a high humidity.

Treatments (i) and (ii) characterized the direct individual effects of LT and LI on MQ. The three combined herbivory treatments (LI+LT, LI–LT & LT–LI) elucidated the interactive effects of LT and LI on MQ, including changes in the individual effects due to plant-mediated interactions between the herbivore species. The treatments LI–LT and LT–LI elucidated the plant-mediated interactions and their effect when one herbivory type is already present while the LI+LT treatment elucidated the interaction when herbivory is imposed simultaneously by both herbivores.

Following this treatment period, each of the MQ plants were randomly allocated to each of four blocks, and were maintained for 20 weeks (the equivalent of three generations of LI/LT). Each block contained a total of 24 plants, for example, four replicate plants from each of the six treatments. Each MQ plant was housed individually in nylon mesh-screened cylindrical cages (2 m in height). The height and basal diameter of each plant was recorded before they were placed inside individual cages. Each of these plants was individually irrigated at the rate of 130 mL/day. At the end of the 20-week period, we collected metrics on plant height, basal diameter, number of tips and root: shoot ratio. Growth data collected prior to herbivory treatments and at the conclusion of the trial were used to calculate growth rates, which were then compared across treatments.

To understand plant response to herbivory, nutrient accumulation or depletion was calculated. For this, the plant material was then broken down into shoots and roots at the end of the trial; roots were carefully separated from the growth medium by gentle washing through a nested series of sieves. Roots and shoots were dried at 60°C over a 2-week period in a fan-forced oven. Because gall insects are known to accumulate nutrients in galls, plant tissues were broken down further into ungalled shoots, LI galls, LT galls and roots to estimated tissue-specific accumulation or depletion of nutrients. The weight of each of these tissue types was recorded for each plant, before the tissues were ground to <1 mm size particles. These ground tissues were then subjected to laboratory analyses to quantify concentrations of macronutrients, micronutrients and toxic substances (e.g. cadmium) in each tissue. All nutrient analyses were performed at the Wagga Wagga Agricultural Institute (accredited by National Association of Testing Authorities, Australia). Percent nitrogen was analysed by AOAC official protein in animal feed method 990.03 (1990) using LECO elemental analysis instruments (AOAC, 2006). Macronutrients and micronutrients were estimated by ICP-20 and Minerals Digest method and analysed based on US EPA Method 6010C (EPA, 2007). All trace elements were detected using the Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES) technique (Lichte et al., 1987).

2.2 | Statistical analysis

Statistical analyses were performed in R 4.4.0 (R Core Team, 2025). The data on growth parameters were subjected to two-way ANOVA with plant stage and treatments as fixed factors; post hoc pairwise comparisons were made using Tukey's HSD at $\alpha = 0.05$. For significant two-way ANOVA results, means within factors were compared using univariate ANOVA. The data on nutrients were subjected to multivariate ANOVA with plant stage, herbivory treatments and plant tissues as fixed factors, and for significant MANOVA results, means within factors were compared using univariate ANOVA and Tukey's significance test at $\alpha = 0.05$.

3 | RESULTS

We use the following terminology (and notation) to indicate the direct herbivore effects: 'negative' (↓) if there was a reduction in plant growth or depletion of nutrients, 'positive' (↑) if there was an increase in growth or accumulation of nutrients or 'neutral' (Θ) if there was no change in growth or nutrient dynamics when compared with the control treatment (Figure 1; Table 1). For the combined treatments LI–LT and LT–LI, interaction between LI and LT (mediated by MQ) is referred to as 'independent' (⇊) if the addition of the second herbivore did not change the effects induced by the first herbivore, 'additive' (++) if the release of the second herbivore induced greater impacts than the individual herbivore treatments or 'antagonistic' (−−) if the release of the second herbivore resulted in a lower impact than the individual herbivore treatments. For the LI+LT treatment, the resulting impacts are referred to as 'additive' (++) if the effect is greater than the individual herbivore treatments, 'antagonistic' (−−) if the growth reduction was lower than the individual herbivore treatments or 'independent' (⇊) if the combined effect is similar to the effect exerted by individual herbivores (Figure 1; Table 1). The terminologies employed were partially adapted from Hatcher (1995).

3.1 | Individual and combined effects of *L. trifida* and *L. indentata* on *M. quinquenervia*

Herbivory effects of LI and LT on growth of MQ seedlings differed significantly among individual and combined treatments (Height: $F_{5,90} = 61.81$, $p < 0.01$; Basal stem diameter: $F_{5,90} = 17.24$, $p < 0.01$;

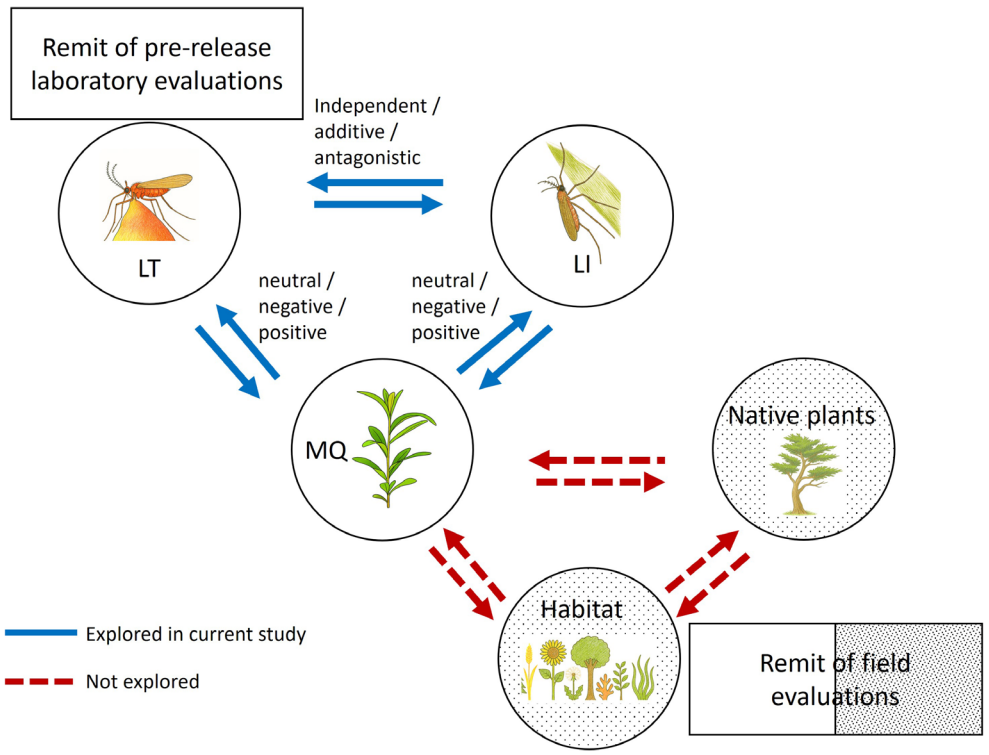


FIGURE 1 Schematic representation of interaction between *Lophodiplosis trifida* (LT), *L. indentata* (LI) and *Melaleuca quinquenervia* (MQ) in a habitat. Interaction was denoted as 'negative' when there was a clear reduction in plant growth, 'neutral' when there was no reduction or 'positive' if there was an increase in growth. Interactions between *L. trifida* and *L. indentata* were referred to as 'independent' if there was no difference in reduction in plant growth between combined treatments (LI+LT, LI–LT and LT–LI) and individual treatments (LT or LI), 'additive' if the growth reduction in combination treatments was greater than the individual treatments or 'antagonistic' if the growth reduction in combination treatments was lower than the individual treatment.

TABLE 1 Growth dynamics and nutrients dynamics in *M. quinquenervia* seedlings and saplings in response to individual and combined effects of *L. trifida* and *L. indentata* 'Θ' no reduction in growth or no change in nutrients allocation relative to control (neutral); '↓' decrease in growth or depletion of nutrients relative to control (negative); '↑' increase in growth or accumulation of nutrients relative to control (positive); '⫫' the combined effect is not greater or lesser than the individual effects (independent); '++' the combined effect is greater than the individual effects (additive); '−−' the combined effect is lower than the individual effects (antagonistic).

	Seedlings					Saplings				
	Individual effects		Combined effects			Individual effects		Combined effects		
Parameters	LI	LT	LI+LT	LI−LT	LT−LI	LI	LT	LI+LT	LI−LT	LT−LI
Growth parameters										
Plant height	Θ	↓	⫫/++[a]	++	⫫	−	−	⫫	⫫	⫫
BSD	↓	↓	⫫/++[a]	⫫	⫫	↓	−	⫫	−−	⫫
Root:Shoot	Θ	↓	⫫	⫫	⫫	−	−	⫫	⫫	⫫
Tips	Θ	↓	⫫/++[a]	++	⫫	−	↑	⫫/++[a]	++	⫫
Macronutrients										
Nitrogen	↑	↑	++	++	++	↑	↑	++	⫫	++
Phosphorous	↑	↑	++	++	⫫	↑	↑	++	++	++
Potassium	↑	↑	++	++	++	↑	↑	++	++	++
Micronutrients										
Boron	Θ	↑	++	++	⫫	−	−	⫫	⫫	⫫
Calcium	↑	↑	++	⫫	⫫	↑	↑	++	++	++
Cobalt	Θ	↑	++	⫫	⫫	−	−	⫫	⫫	⫫
Copper	Θ	Θ	⫫	⫫	⫫	−	−	⫫	⫫	⫫
Iron	Θ	↓	⫫	⫫	−−	−	−	⫫	⫫	⫫
Magnesium	Θ	↑	++	++	⫫	−	−	⫫	⫫	⫫
Manganese	Θ	↑	⫫/++[a]	++	⫫	−	−	⫫	⫫	⫫
Molybdenum	↑	↑	++	++	⫫	−	−	⫫	⫫	⫫
Sodium	↑	↑	++	++	⫫	−	↑	++	++	++
Nickel	↑	↑	⫫	⫫	⫫	−	−	⫫	⫫	⫫
Sulphur	↑	↑	++	++	⫫	↑	↑	++	⫫	⫫
Zinc	↑	↑	⫫/++[a]	++	⫫	−	−	++	⫫	++
Cadmium	Θ	↑	⫫/++[a]	⫫	⫫	−	−	⫫	⫫	⫫
Chromium	Θ	↓	⫫	⫫	⫫	↓	↓	⫫	⫫	⫫
Aluminium	Θ	Θ	⫫	⫫	⫫	−	−	⫫	⫫	⫫

[a]'Independent' when the combined effect was compared with the effect exerted by LT and 'additive' if the combined effect was compared with LI.

Tips growth: $F_{5, 90} = 13.28$, $p < 0.01$; root: shoot ratio: $F_{5, 90} = 6.20$, $p < 0.01$).

LT exerted negative effects on all growth parameters recorded. In contrast LI had a neutral effect on the growth in plant height and the number of growing tips, and a negative effect on basal stem diameter and root: shoot ratio (Figure 2). All the combined treatments (i.e. LI+LT, LI−LT and LT−LI) significantly reduced MQ growth compared with the control treatment. The reduction in height, basal stem diameter, root: shoot ratio and tips growth noticed in seedlings due to LT herbivory was similar when LI herbivore was present at the same time (LI+LT) or when introduced a week later (LT−LI). This suggests an independent effect of LT and LI (Figure 2; Table 1; Table S1). However, the effect of LI on MQ growth was greater when combined with LT signalling additive interaction (Figure 2; Table S1).

3.2 | Response of *M. quinquenervia* to independent and combined effects of *L. trifida* and *L. indentata*

Seedlings accumulated more nitrogen, phosphorus, and potassium in response to LI and LT damage compared with the control treatment (nitrogen: $F_{5, 18} = 44.14$, $p < 0.01$; phosphorus: $F_{5, 18} = 24.81$, $p < 0.01$; potassium: $F_{5, 18} = 53.28$, $p < 0.01$). This response was greater for LT than LI damage (Figure 3). It is important to note that despite the neutral interaction, seedlings accumulated greater amounts of nitrogen and potassium due to LI damage compared with the control treatment (Figure 3; Table S2).

The greater amount of nutrient accumulation for LI+LT, LI−LT and LT−LI treatments compared with the LI alone treatment indicating significant interaction between LT and LI. Greater amounts of

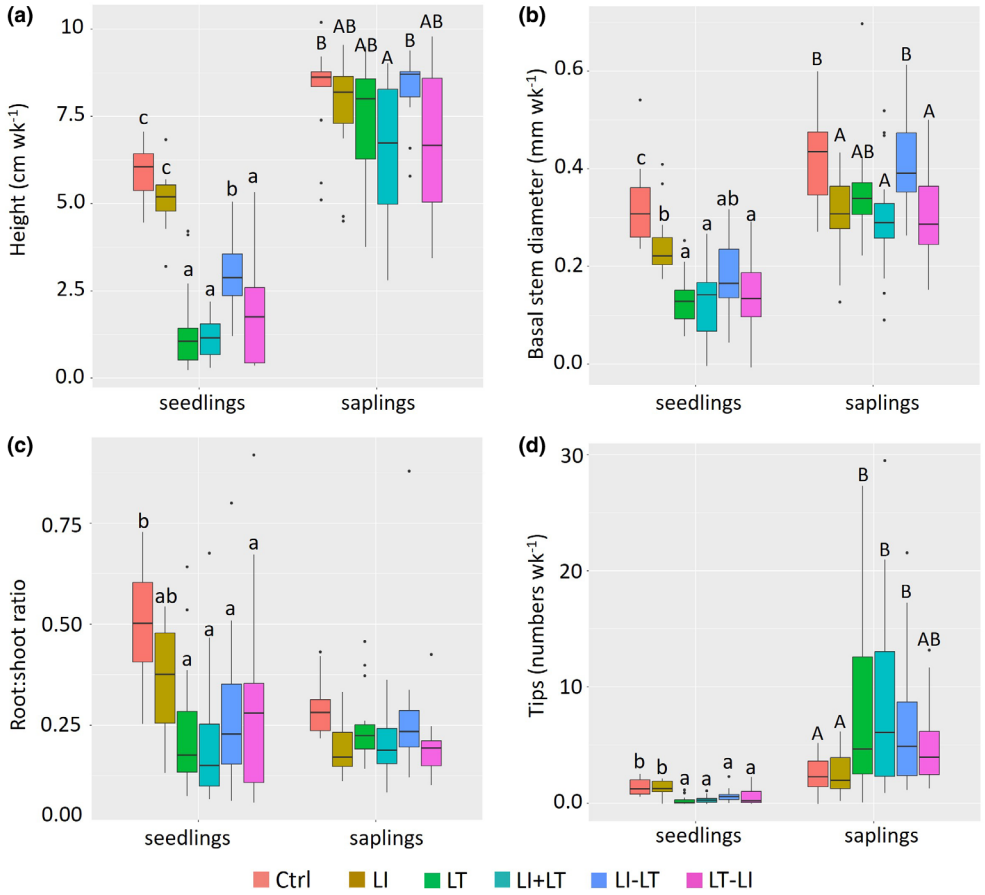


FIGURE 2 Interaction between *Lophodiplosis trifida* and *L. indentata* and its effect on the growth of *Melaleuca quinquenervia* seedlings and saplings. (a) Height; (b) Basal diameter; (c) Root:Shoot ratio, and (d) Tips. Different letters on adjacent boxes (lowercase for seedling and uppercase for saplings) indicate significant differences ($p < 0.05$) among treatments. CTRL—Control, LI—*Lophodiplosis indentata* alone, LT—*L. trifida* alone, LI+LT—*L. indentata* and *L. trifida* released together, LI–LT—*L. indentata* released prior to *L. trifida*, LT–LI—*L. trifida* released prior to *L. indentata*. Boxplots are presented which indicate the interquartile range (box), the median (line within the box) and the range of the data.

nitrogen and potassium were accumulated when both LT and LI were present at the same time (LI+LT) than for the individual treatments (LT and LI alone), possibly as a result of increased response from MQ to the additive herbivory effect from LI and LT (Figure 3; Tables S2–S4). There was a greater accumulation of nitrogen and potassium when LI was released after LT (LT–LI) when compared with the LT alone treatment (Figure 3a,c). While LI alone accumulated fewer macronutrients than the LT treatment, accumulation was greater when LT was released after LI (i.e. in the LI–LT treatment; Figure 3; Tables S2–S4).

A response similar to macronutrients was detected for micronutrients as well. Seedlings accumulated or depleted several micronutrients in herbivory treatments compared with control treatment, and this response was noticed in a greater number of nutrients for the damage exerted by the LT herbivore than the LI herbivore (Tables S5–S7). Enhanced response from MQ was noticed in nutrients dynamics of boron, calcium, magnesium, molybdenum, sodium and sulphur when LI and LT released together (Figure 4). The greater accumulation or depletion of nutrients when LT was released after LI (LI–LT) compared with LI alone has also suggested increased response from MQ to the combined effects. The accumulation or depletion of several micronutrients was similar when LI was released after LT (LT–LI) compared with LT alone treatment (Figure 4).

Tissue-wise analyses revealed that seedlings accumulated or depleted nutrients in root, shoot, and gall tissues in response to LT herbivory, and the accumulation was greater in gall and shoot tissues than the root tissues (Figures S1–S7). Accumulation or depletion was detected only in gall tissues for LI herbivory but not in roots or shoot tissues (Table 1). Tissues in which nutrients accumulated or depleted (roots, shoots, LI galls, or LT galls) did not change due to the interaction between LT and LI.

3.3 | Life stage specific response of *M. quinquenervia* to the interaction between *L. trifida* and *L. indentata*

The herbivory effects differed between plant life stages with greater effect on seedlings than on saplings (Height: $F_{1,36} = 526.92$, $p < 0.01$; Basal stem diameter: $F_{1,36} = 153.73$, $p < 0.01$; root: shoot ratio: $F_{1,36} = 17.11$, $p < 0.01$; tips growth: $F_{1,36} = 73.24$, $p < 0.01$). Neither LT nor LI affected the height and root: shoot ratio when released alone

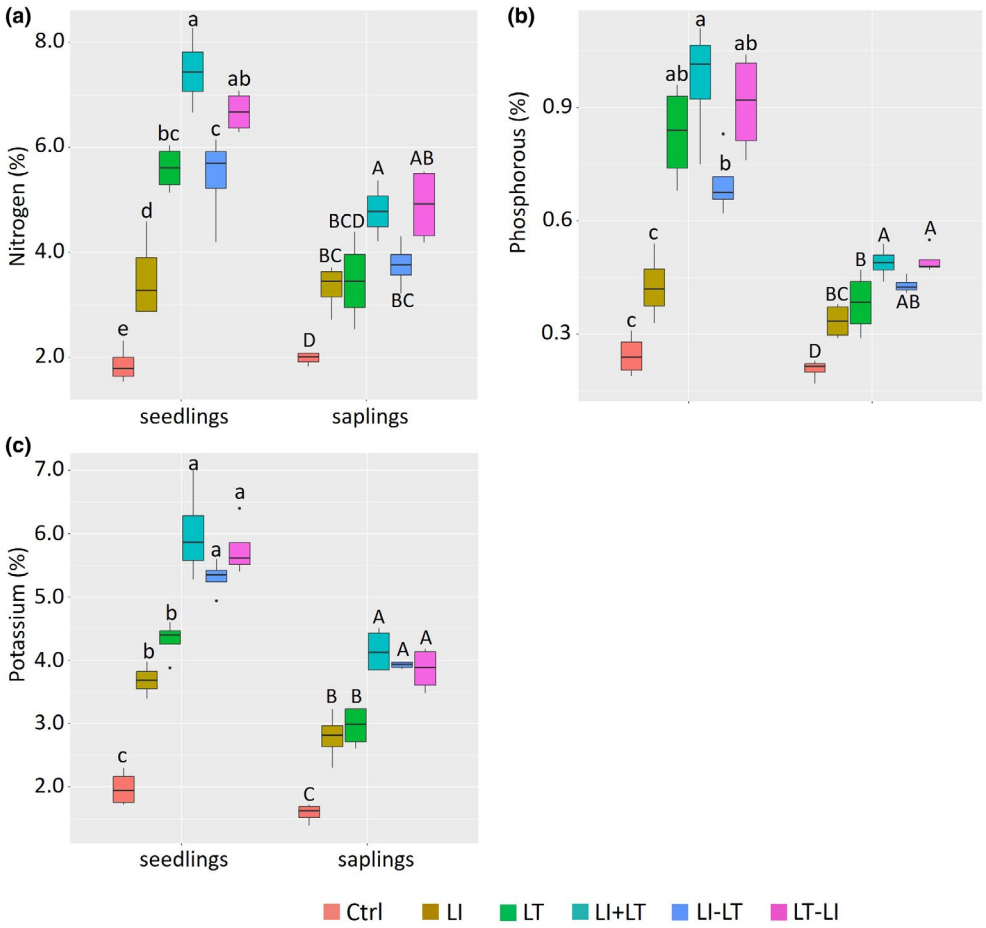


FIGURE 3 Macronutrient allocation in *Melaleuca quinquenervia* seedlings and saplings in response to *Lophodiplosis trifida* and *L. indentata* herbivory and their interactions. (a) Nitrogen; (b) Phosphorous and (c) Potassium. Different lowercase and uppercase letters on adjacent boxes indicate significant differences ($p < 0.05$) in nutrient concentration among treatments. CTRL—Control, LI—*Lophodiplosis indentata* alone, LT—*L. trifida* alone, LI+LT—*L. indentata* and *L. trifida* released together, LI–LT—*L. indentata* released prior to *L. trifida*, LT–LI—*L. trifida* released prior to *L. indentata*. Boxplots are presented which indicate the interquartile range (box), the median (line within the box) and the range of the data.

(LT or LI) in saplings (Figure 2). There was a reduction in height when herbivores were released together (LI + LT), indicating an additive interaction (Figure 2a). LI herbivory when released alone did not increase the number of tips produced but when LT was released after LI (LI–LT), a significant increase in the number of tips was recorded in saplings (Figure 2d).

LI herbivory resulted in a reduction in basal stem diameter in saplings, but this effect was attenuated when LT was released after LI (LI–LT) (Figure 2b). As for the tips' growth, release of LI after LT (LT–LI) marginally reduced the effect of LT. The observed increase in the number of tips following herbivory may suggest a positive interaction between LT and MQ. However, this proliferation is unlikely to represent an adaptive plant response. Rather, it is more plausibly interpreted as a forced growth in response to LT herbivore, a characteristic effect of gall-inducing arthropods on their host plants.

Nutrients accumulated in saplings were identical for LT and LI herbivory (nitrogen: $F_{5, 18} = 14.93$, $p < 0.01$; phosphorous: $F_{5, 18} = 21.49$, $p << 0.01$; potassium: $F_{5, 18} = 43.13$, $p < 0.01$). There was a greater accumulation of macronutrients (e.g. nitrogen, phosphorous, potassium) when LI and LT released together (LI+LT) or one after another (LI–LT or LT–LI) suggesting increased response possibly due to the additive effects of LI and LT (Table 1; Figure 3). Regarding micronutrients, additive interaction was noticed for calcium, sodium, chromium, sulphur and zinc, and the interaction was independent for all other elements (Figure 4; Tables S5–S7).

Tissues in which nutrients accumulated or depleted (roots, shoots, LI galls or LT galls) did not change due to the interaction (Figures S1–S7). Tissue-wise analyses revealed that saplings accumulated nitrogen, phosphorus, potassium, and sulphur only in the gall tissues (in LT and LI galls, respectively for LT and LI herbivory) not in root or shoot tissues (Table 1; Tables S5–S7). Chromium was depleted only in root tissues.

4 | DISCUSSION

We demonstrated that herbivory by *L. indentata* and *L. trifida* suppressed the growth of *M. quinquenervia* seedlings and saplings under

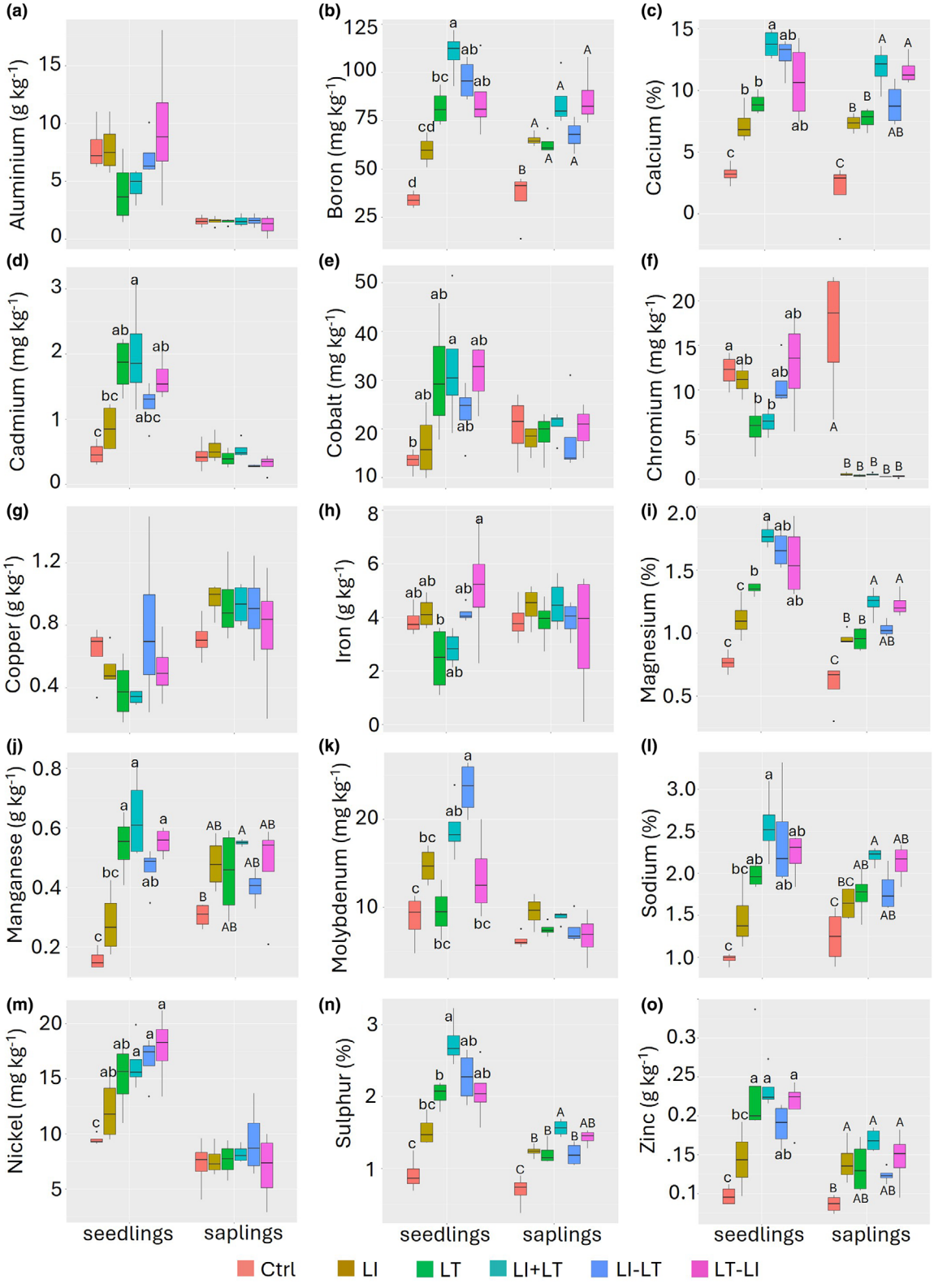


FIGURE 4 Micronutrient allocation in *Melaleuca quinquenervia* seedlings and saplings in response to *Lophodiplosis trifida* and *L. indentata* herbivory and their interactions. (a) Aluminium; (b) Boron; (c) Calcium; (d) Cadmium; (e) Cobalt; (f) Chromium; (g) Copper; (h) Iron; (i) Magnesium; (j) Manganese; (k) Molybdenum; (l) Sodium; (m) Mickel; (n) Sulphur and (o) Zinc. Different lowercase and uppercase letters on adjacent boxes indicate significant differences ($p < 0.05$) in nutrient concentration among treatments. CTRL—Control, LI—*Lophodiplosis indentata* alone, LT—*L. trifida* alone, LI+LT—*L. indentata* and *L. trifida* released together, LI–LT—*L. indentata* released prior to *L. trifida*, LT–LI—*L. trifida* released prior to *L. indentata*. Boxplots are presented which indicate the interquartile range (box), the median (line within the box) and the range of the data.

controlled glasshouse conditions. *Lophodiplosis trifida* exerted a strong negative effect on *M. quinquenervia*, whereas the effect of *L. indentata* was comparatively weaker. The interaction between *L. indentata* and *L. trifida*, mediated by *M. quinquenervia*, appeared either additive or independent. Herbivory by *L. trifida* was unaffected by its interaction with *L. indentata*, signalling an independent effect of each of these gall insects with the plant, while the addition of *L. trifida* enhanced the effect of *L. indentata*, suggesting an additive effect.

Herbivory effects were different between plant stages. In general, seedlings suffered greater growth reduction than saplings. Growth of saplings was more resilient to herbivory, although basal stem diameter was reduced because of the impacts of *L. indentata*. Interestingly, this reduction was attenuated when *L. trifida* was released, suggesting an antagonistic interaction between these two herbivores. While individual herbivore treatments did not significantly affect sapling height, the simultaneous release of *L. indentata* and *L. trifida* resulted in a significant height reduction. *Lophodiplosis trifida* also induced an increase in the number of tips in saplings, and this effect was independent when *L. indentata* was released subsequently. While *L. indentata* did not affect the number of tips, the release of *L. trifida* subsequently increased the number of tips produced, further supporting the additive impacts of these herbivores.

Despite the differential phenotypic effects of herbivory, nutrient accumulation or depletion was evident for both herbivores and this response of *M. quinquenervia* to herbivory was similar among the combined treatments irrespective of the order in which the herbivores were released (i.e. LI+LT, LI−LT and LT−LI). This suggests that *L. indentata* and *L. trifida* possibly induced additive effects, but this was perhaps subsequently compensated for by *M. quinquenervia*, particularly in response to herbivory by *L. indentata*. These findings highlight the complex and context-dependent nature of herbivore interactions and provide insights into the potential for additive or independent effects in biocontrol strategies targeting *M. quinquenervia*. The mechanistic basis and implications of these interactions on biocontrol of *M. quinquenervia* are discussed further below.

4.1 | Mechanistic explanation for the effects and responses observed

The greater negative impact of *L. trifida* relative to *L. indentata* may be attributed to the specific *M. quinquenervia* tissues targeted by each herbivore; the larvae of *L. trifida* penetrate the actively growing tissue, forming galls on young shoots in contrast to the leaf galls of *L. indentata* (Purcell et al., 2007; Wright & Center, 2008). The shoot galls caused by *L. trifida* distort apical growth and disrupt vascular function, thereby impairing the translocation of water and nutrients (Poorter et al., 2012). Changes in nutrient dynamics across root, shoot, and gall tissues further suggest that *L. trifida* imposes significant whole plant stress, limiting the compensatory ability of *M. quinquenervia*. In contrast, the effects of *L. indentata* appear to be at least partially compensated. Although our study spanned three generations of recurrent herbivore damage, the observed phenotypic responses are likely conservative estimates and may underestimate long-term or cumulative effects, particularly in perennial species (Doak, 1992; Strauss, 1991). Evidence for such sustained herbivore-driven effect is well documented in *M. quinquenervia* (Martin et al., 2019; Tipping et al., 2009; Tipping et al., 2012; Tipping et al., 2021).

The differential ontogenetic response to herbivory is consistent with previous studies. *Melaleuca quinquenervia* seedlings suffer substantial growth reduction following herbivory (Franks et al., 2006; Purcell et al., 2007; Tipping et al., 2021). In contrast, mature plants typically exhibit reduced sensitivity to herbivory, possibly due to their greater capacity for resource storage and compensatory growth (Niinemets, 2010; Price, 1991; Zangerl & Bazzaz, 1992). The interaction between *L. trifida* and *L. indentata* did not modify the stage-specific growth responses. However, additive effects were evident in some physiological parameters, as indicated by increased macronutrients accumulation in the combined treatments than in the individual treatments. It is plausible that the herbivore densities used in our study were below the threshold required to elicit pronounced phenotypic responses in saplings. Further exploration of different density combinations of insects may be necessary to fully characterize the demographic and physiological consequences of herbivory in saplings; such studies are planned for the evaluation of these gall insects under field conditions now they are released or approved for release (LI) to manage *M. quinquenervia*.

4.2 | Response of *M. quinquenervia* to herbivores and their interaction: Implications for biocontrol

Our results suggest that nutrient reallocation, a form of compensatory response, may explain the lack of additive effects observed in some parameters, as well as the relatively lower impacts of *L. indentata* compared with *L. trifida*. While such compensation may represent an adaptive response, it could also entail indirect costs. For instance, reallocating nutrients towards compensatory growth may deplete finite reserves that would otherwise support other life-history traits not recorded in this study (e.g. reproduction), as evidenced in other systems (Hartnett & Abrahamson, 1979; Wise & Sacchi, 1996). These costs were likely undetectable in our mesocosm experiments due to non-limiting nutrient conditions. Further, such herbivore-induced trade-offs could affect the adaptive response of *M. quinquenervia* to the negative effects from other regulating forces such as biotic resistance from co-occurring species and herbivory from other biocontrol agents. Detecting these indirect costs will require long-term studies in the field, particularly given the perennial nature of *M. quinquenervia*.

The only possible antagonistic effect observed was the reduced impact of *L. indentata* on the basal stem diameter in saplings when *L. trifida* was released. Such an antagonistic interaction could

potentially reduce the collective ability of multiple herbivores in reducing *M. quinquenervia* growth. Nevertheless, compensatory nutrient accumulation observed in saplings suggests a potential trade-off, reinforcing the need for further studies examining the role of other ecological forces (e.g. competition from co-occurring plants; Figure 1) and how herbivory may facilitate them indirectly. An earlier study by Franks et al. (2006) found independent effects of *O. vitiosa* and *B. melaluecae*, with a greater effect from the latter. Conversely, *O. vitiosa* and a rust fungus, *Puccinia psidii* influenced each other's life cycle and possibly antagonized each other's effects with both organisms competing for the newly expanding foliar tissues for colonization, reproduction and survival (Rayamajhi et al., 2006). Our results, in addition to supporting results of these studies, provide nutrient allocation and compensation as a plausible explanation for the differential plant responses to herbivore interactions.

Additionally, interspecific competition between these two species is unlikely, as they occupy different feeding guilds and appear to cause different impacts on *M. quinquenervia* in terms of nutrient dynamics. As a result, they are unlikely to interfere with each other, even though *L. trifida* may contribute more to *M. quinquenervia* control. This approach is further supported by previous findings showing facilitative interactions among *M. quinquenervia* gall-formers (Raghu et al., 2012). Collectively, the limited potential for interspecific competition, the negative impacts of both *L. trifida* and *L. indentata* on the growth and nutritional physiology of *M. quinquenervia* and the additive or independent interactions between the herbivores, which do not diminish the effectiveness of either species, suggest that both species can be used in combination as biocontrol agents for *M. quinquenervia*. However, *L. trifida* exerts a stronger suppressive effect on the plant's growth and physiology, relative to *L. indentata*. If limited resources necessitated only one agent to be prioritized that is recommended to be *L. trifida*. If both are to be considered, it may be of value to release *L. trifida* before *L. indentata*.

Given the glasshouse context of the experiment, we were unable to include broader biotic interactions, such as direct competition from native plants, habitat context and indirect effects mediated through higher trophic levels (e.g. parasitoids of *Lophodiplosis* spp.; Figure 1). Future field-based evaluations should aim to incorporate these ecological interactions, as they can significantly influence the cumulative impact of multiple herbivores, especially those in the same guild (Luz et al., 2021). Studies involving community-level interactions, particularly in the invaded range where multiple agents have been introduced, are crucial for understanding the long-term trajectories of invasive species management (Holt & Hochberg, 2001; Holt & Bonsall, 2017; Kuebbing & Nuñez, 2015; Soler et al., 2012; Stam et al., 2014).

AUTHOR CONTRIBUTIONS

S. Raghu and Nagalingam Kumaran conceptualized and designed the study with input from Matthew Purcell and Bradley Brown. S. Raghu, Nagalingam Kumaran, and Bradley Brown carried out the laboratory experiments and managed data collection. Nagalingam Kumaran performed statistical analyses and drafted the manuscript, and all authors made substantial contributions to its revision.

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CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

PEER REVIEW

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DATA AVAILABILITY STATEMENT

Data are presented in graphs, tables and as Supporting Information. All data and statistical data analyses results are available from CSIRO's Data Access Portal https://doi.org/10.25919/fxm3-gb49 (Kumaran et al., 2025).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Tissue-wise allocation of nitrogen in *Melaleuca quinquenervia* in response to *Lophodiplosis trifida* and *L. indentata* herbivory and their combined effects.

Figure S2. Tissue-wise allocation of phosphorous in *Melaleuca quinquenervia* in response to *Lophodiplosis trifida* and *L. indentata* herbivory and their combined effects.

Figure S3. Tissue-wise allocation of potassium in *Melaleuca quinquenervia* in response to *Lophodiplosis trifida* and *L. indentata* herbivory and their combined effects.

Figure S4. Allocation of micronutrients in roots of *Melaleuca quinquenervia* seedlings in response to *Lophodiplosis trifida* and *L. indentata* herbivory and their combined effects.

Figure S5. Allocation of micronutrients in shoot tissues of *Melaleuca quinquenervia* seedlings in response to *Lophodiplosis trifida* and *L. indentata* herbivory and their combined effects.

Figure S6. Allocation of micronutrients in LI galls of *Melaleuca quinquenervia* seedlings in response to *Lophodiplosis trifida* and *L. indentata* herbivory and their combined effects.

Figure S7. Allocation of micronutrients in LT galls of *Melaleuca quinquenervia* seedlings in response to *Lophodiplosis trifida* and *L. indentata* herbivory and their combined effects.

Table S1. Summary statistics (two-way ANOVA) of effects of plant stage and herbivory treatments on growth parameters.

Table S2. Summary statistics (MANOVA) of effects of plant stage and herbivory treatments on macronutrients.

Table S3. Effect of herbivory by *Lophodiplosis trifida* and *L. indentata* on each macronutrient of different tissues of seedlings and saplings (univariate ANOVA).

Table S4. Summary statistics (two-way ANOVA) of effect of plant stage and herbivory treatments on macro nutrients (data combined for all tissues).

Table S5. Summary statistics (MANOVA) of effect of plant stage, herbivory treatment and tissue types on micronutrients.

Table S6. Summary statistics of effect of herbivory treatments on each micronutrient in different tissues of seedlings and saplings (univariate ANOVA).

Table S7. Summary statistics of effect of herbivory treatments on each micronutrient in seedlings and saplings (data combined for all tissues) (univariate ANOVA).

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