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## Research Article

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# Defense strategies of invasive plant during flowering and growth stages

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**Abstract:** Studying the defense mechanisms of invasive plants is crucial for understanding their successful invasion. The Enemy Release Hypothesis suggests that invasions thrive due to a lack of specialized enemies, allowing invasive plants to allocate resources typically reserved for defense toward growth and reproduction, enhancing their competitive advantage. However, this hypothesis does not fully explain how invasive plants cope with threats from generalist enemies. This study focuses on the invasive plant *Sphagneticola trilobata*, investigating its defensive levels during vegetative and flowering stages through field surveys of pest feeding patterns, seed germination experiments with local plants, and assessments of changes in secondary metabolite concentrations after mechanical leaf damage. Our findings reveal significant differences in defensive strategies between these two phases and

insights into trade-offs among growth, reproduction, and chemical defenses. Field survey results demonstrated a negative correlation between flower production and leaf tunnels caused by herbivorous pests, suggesting that *S. trilobata* increases its defensive investment during flowering to mitigate herbivory. Conversely, a positive correlation was observed between flower heads and pest-inflicted tunnels on nearby native plants. Seed germination experiments indicated that leaves of *S. trilobata* in the vegetative stage significantly inhibited the germination of local plant seeds compared to those during flowering, demonstrating stronger allelopathic effects in the vegetative phase. Analysis of secondary metabolite concentrations revealed higher levels of defense-related compounds (flavonoids, total phenols) and tolerance indicators (DPPH) during flowering. After mechanical damage, these compounds increased, while soluble sugars decreased. Our research suggests that *S. trilobata* actively enhances its defenses during flowering without sacrificing growth and reproduction. This indicates that effective defense strategies may support the successful growth and reproduction of invasive plants, challenging the idea of a trade-off between defense and growth/reproduction.

**Key words:** invasive plant, plant defense, reproductive, allelopathy, trade-offs

## Introduction

As a prominent aspect of global change, biological invasions have garnered extensive attention worldwide (Colberg et al. 2024; Rodrigues et al. 2024). Ecologists have put forth various hypotheses to elucidate the mechanisms driving biological invasions. The "Enemy Release Hypothesis" (ERH) posits that invasive plants entering new territories are released from the pressure of native predators, as they escape from specialized predators that regulate their populations in their native habitats (Agrawal et al. 2005; Joshi, Vrieling 2005). Consequently, invasive plants reallocate resources originally designated for defense against herbivorous insects towards their own growth and reproduction, facilitating rapid proliferation and spread in the invaded regions (Genton et al. 2005; Ivison et al. 2023; Lucero et al. 2020). Moreover, Blossey

and Notzold (1995) introduced the more inclusive "Evolution of Increased Competitive Ability Hypothesis" (EICA), which suggests that in invaded environments, invasive species undergo rapid evolutionary shifts in resource allocation from defense to growth and reproduction, thereby gaining a heightened competitive edge (Blossey, Notzold 1995; Montesinos et al. 2019).

However, some studies have indicated that certain invasive plants, such as *Triadica sebifera*, *Eupatorium odoratum*, *Mikania micrantha*, and *Sphagneticola trilobata*, do not diminish their investment in defense-related traits (Carrillo et al. 2012; Huang et al. 2010; Xie et al. 2010; Zhai et al. 2024). Despite the absence of specialized predators in novel habitats, invasive plants remain susceptible to predation by generalist predators (Kambo, Kotanen 2014; Lin et al. 2021). Research has revealed that native pests like aphids and small moths in China can prey on the invasive plant *M. micrantha* (Diaz et al. 2015; Wang et al. 2019), and *Laelia coenosa* inflicts damage on the invasive plant *Spartina alterniflora* (Ju et al. 2016). Hence, when confronted with the threat posed by generalist insects, invasive plants may uphold their defense mechanisms. An increasing number of studies indicate that invasive plants shift from specificity-based defenses in their native range to broad-spectrum defenses in their invaded habitats (Sun et al. 2023; Yin et al. 2023). The premise of the enemy release hypothesis often requires that invasive species possess specialized enemies that can regulate their population size (Crawley 1989). In most cases, invasive plants still face threats from herbivorous pests in the invaded area. Colautti et al. argued that invasive species are influenced by local herbivorous insects to a degree that is no less than that of native species (Colautti et al. 2004). Therefore, the idea that invasive plants enhance their defenses in invaded areas has been increasingly supported by empirical evidence (Zhai et al. 2024).

In addition to bolstering their defense mechanisms, invasive plants also exert a potent allelopathic influence in the invaded areas, significantly impacting the growth and reproduction of native plant species. Allelopathy in plants is recognized as a manifestation of their capacity to enhance competitiveness within their environment (Pan et al. 2023; Yuan et al. 2013). Callaway et al. (2004) introduced the "Novel

Weapons Hypothesis," which posits that invasive species can gain a competitive edge by releasing chemicals through allelopathy, thereby inhibiting the germination of native plant seeds and impeding plant growth (Callaway, Ridenour 2004). It has become widely acknowledged among plant ecologists that invasive plants commonly exhibit robust allelopathic traits (Al Musalami et al. 2023; Callaway, Ridenour 2004; Hierro, Callaway 2003; Zhao et al. 2008).

Plants' defensive responses to external damages, in addition to constitutive defences (e.g. toughness, thickness and dense cell walls) (Rolón et al. 2024), often involve increasing or altering the levels of secondary metabolites to adapt to different damages (Hauri et al. 2024). However, during their life history, plants do not exhibit consistent defensive responses to external environments at different growth stages (Jacobsen 2022). Research has demonstrated significant variations in the content of secondary metabolites in plants between the flowering and growing stages (Çirak et al. 2007). A study on *Perilla frutescens* leaves revealed notable fluctuations in phenolic acids and flavonoids across different harvest periods, with the phenolic acid content of *P. frutescens* reaching its lowest point during the flowering stage (Chen et al. 2022; Kang, Lee 2011). This observation implies that plants may diminish their allocation of resources to secondary metabolites during the flowering stage to prioritize investment in reproductive organs. However, investigations have also indicated that the total polyphenol content and total antioxidant capacity of *Hypericum perforatum* stems and leaves peak during the flowering and peak flowering stages (Çirak et al. 2007). From a plant's perspective, investing in defense mechanisms can offer enhanced protection and benefits for their reproductive behavior; otherwise, plant defense efforts would be futile (Lemoine et al. 2017; Wolfe et al. 2004). Vila et al. found that although invasive plants lack natural enemies, the invasive plant does not reproduce in large quantities, based on a comparison of herbivorous insect feeding on native European and introduced North American populations of *Hypericum perforatum* (St John's Wort) (Vilà et al. 2005). Therefore, there may not be a trade-off relationship between the defenses and reproduction of invasive plants. Nonetheless, there remains a gap in research regarding the disparities

in defense characteristics of invasive plants at various developmental stages. Understanding the investment strategies for defense during the flowering and growing stages of plants could provide deeper insights into the regulatory mechanisms of invasive plant defense strategies, shedding light on the defense mechanisms employed by invasive plants.

In a previous study, we conducted experiments simulating insect feeding by creating holes in leaves to analyze the content of defense-related metabolites in the invasive plants *S. trilobata*, *M. micrantha*, and *Ipomoea cairica*. Our findings revealed that compared to native plants, invasive plants exhibited higher levels of metabolites both before and after leaf damage (such as total phenols, flavonoids, jasmonic acid, tannins, and total antioxidant capacity (TAC)). This study suggests that invasive plants maintain active defense strategies in invaded area (Zhai et al. 2024). Building upon this research, we focused on the common invasive plant *S. trilobata* in Guangdong Province to explore the defense strategies of invasive plants during their growth and flowering stages, thereby uncovering the trade-offs between defense and growth and reproduction in invasive plants during the invasion process. We aimed to delve into the defense strategies of invasive plants at different stages through a three-fold experimental approach.

1) Variation in the defense strategies of invasive plants across different growth stages may influence the extent of leaf damage caused by phytophagous pests and alter the feeding patterns of these pests on local plants surrounding invasive species. Our previous study demonstrated that the presence of invasive plants significantly altered the distribution pattern of phytophagous pests on neighboring native plants (Zhai et al. 2024). Building on this foundation, we further examined the number of flowers and degree of leaf damage in three invasive plants (*S. trilobata*, *Bidens alba*, and *I. cairica*) in various vegetation habitats. We also investigated leaf damage on native plants surrounding invasive species and analyzed the correlation between the number of flowers on invasive plants and the leaf damage on both invasive and native plants. This investigation sought to validate potential differences in the defense mechanisms against phytophagous pests between the growth and reproductive stages

of invasive plants.

2) By comparing the allelopathic effects of leaf extracts from *S. trilobata* during the flowering and growing stages on the germination of local crop seeds, we aimed to verify variations in *S. trilobata*'s investment in plant competition at different stages.

3) Through an analysis of secondary metabolite contents in the leaves of *S. trilobata* during its growing and flowering stages, as well as a comparison of the response of secondary metabolites in these two stages to leaf damage, we aimed to elucidate the underlying mechanisms of defense regulation in invasive plants at different developmental stages.

## **Materials and methods**

### **Leaf holes distribution investigation of invasive plants and surrounding native plants in different habitats**

To enhance the universal significance of our field survey results, we selected three invasive plants commonly found in South China as subjects for investigation: *S. trilobata*, *B. alba*, and *I. cairica*. *S. trilobata* is a perennial creeping vine belonging to the Asteraceae family, characterized by its opposite leaves and yellow flower heads. It has the ability to bloom year-round, with peak flowering typically occurring from June to September. Native to Tropical America, *S. trilobata* was introduced to Hong Kong in the 1980s as an ornamental ground-cover plant (Wu et al. 2013). Since then, it has spread rapidly and now widely distributed throughout Southern China (Qi et al. 2014; Zhang et al. 2024). This species exhibits strong allelopathic properties, with terpenoids released into the environment having a significant inhibitory effect on the seed germination and seedling growth of native plants, posing a considerable threat to native species diversity (Liu et al. 2015; Ullah et al. 2021). The other two invasive plants, *B. alba* and *I. cairica*, share habitats similar to those of *S. trilobata* (Zhai et al. 2024). *B. alba* is an annual upright herb of the Asteraceae family, while *I. cairica* is a perennial twining vine from the Convolvulaceae family. These invasive species are frequently found together in various disturbed habitats across Guangdong Province,

including forest edges, agricultural margins, orchards, forest parks, roadsides, pond banks, and wastelands where crops are no longer cultivated (Supp. Figure 1A, 1B). The impact of these invasive species on the local environment has garnered the attention of ecologists (Chen, Peng 2018; Wang et al. 2016; Wang et al. 2020). Although *M. micrantha* is also relatively common in Guangdong Province (Fang et al. 2021; Jia et al. 2022), its flowering period does not coincide with that of the other three plants, making it challenging to conduct comparative research using simultaneous sampling.

To investigate the effects of these three invasive plants on herbivorous pests during the flowering and growing stages, we are specially targeting four sites in Guangdong Province, each containing all three invasive plants, in order to minimize environmental variability and reduce potential errors. The sites including a forest park, an orchard, cropland, and wasteland (Supp. Table 1) (Supp. Figure 1C). The growth of invasive plants at these sites was not disturbed, and no pesticides or herbicides were applied by farmers in the orchards and croplands. Within each of the four habitats, we designated six quadrats measuring  $1\text{m} \times 1\text{m}$  for each invasive species. Among the four sites, we established  $1\text{m} \times 1\text{m}$  native plant quadrats at distances of 1m, 2m, and 3m around the six *S. trilobata* quadrat locations. For each distance, there were four replicate quadrats of native plants, resulting in a total of  $4 \times 6 \times 4 \times 3 = 288$  native plant quadrats. The native plant species present in each quadrat and the number of holes observed in each species were documented (Supp. Figure 1D, 1E).

### **Experiment on the impact of the leaf extract from invasive plant *S. trilobata* on native plant seed germination**

To investigate the allelopathic effects of invasive plants on the seed germination of native species, we utilized *S. trilobata* as our study subject. The plant materials were sourced from the invasive plant seedling cultivation and propagation area at South China Normal University. The *S. trilobata* specimens in this cultivation area were

derived from the same wild seedling through branch cuttings and grew naturally without any human intervention. Leaves of *S. trilobata* were collected separately during the flowering and growing stages to prepare leaf extracts for the allelopathy experiment on seed germination. For this study, we chose seeds of local crops lettuce (*Lactuca sativa*) and pakchoi (*Brassica rapa* var. *chinensis*). Although these two vegetable crops are not native to China, they have been widely cultivated as domesticated crops. We used these two common crops as materials to verify the broad-spectrum allelopathic effects of the invasive plant *S. trilobata* on other plants.

Initially, leaves of *S. trilobata* from both the flowering and growing stages were harvested, followed by washing them with distilled water. Subsequently, the water on the leaf surface was allowed to air-dry naturally, after which the leaves were cut into small pieces measuring approximately 1 cm. A quantity of 200 g of leaves was then accurately weighed using a balance and placed into clean glass conical flasks. To ensure that the leaves were fully immersed in the liquid, 1000 ml of distilled water was added. An oscillator was employed to maintain a constant temperature of 25 °C while the mixture was oscillated for 48 hours. Upon completion of the soaking process, the residue was filtered through sterile gauze to acquire a mother liquor with a concentration of 200 g/l. Subsequently, the mother liquor was diluted in a gradient manner to generate leaching solutions with concentrations of 10 g/l, 50 g/l, 100 g/l, and 200 g/l. Following appropriate sealing, the samples were stored in a refrigerator at 4 °C until required for further experimentation. Distilled water served as the blank control in the experiment. The seeds of lettuce and pakchoi were carefully chosen for their optimal quality and uniform size. They were then subjected to a 30-minute disinfection process by soaking in a 1% sodium hypochlorite solution. Following this, the seeds were rinsed thoroughly three to four times with sterile water. Subsequently, the cleaned seeds were placed on dry sterile gauze to facilitate seed germination.

The seed germination experiments were carried out using the culture dish filter paper germination method, as described in previous studies (Kalske et al. 2023; Rafikova, Veselkin 2022). Initially, the culture dish, filter paper, tweezers, and other experimental equipment were autoclaved for 15 minutes to ensure sterility.

Subsequently, 10 milliliters of water extracts obtained from the leaves of *S. trilobata* at both the flowering and growing stages, each at four different concentrations (10, 50, 100, and 200 g/l), were pipetted into separate culture dishes containing sterile filter paper at the bottom. In the control group, 10 ml of distilled water was added. Each treatment was replicated five times, with twenty test seeds evenly distributed in each culture dish. The prepared samples were then placed in a constant-temperature incubator set at 25 °C. Throughout the experiment, 3 milliliters of the leaching solution or distilled water were added daily, and the number of germinated seeds per day was meticulously recorded over a period of one week. A seed was considered germinated when the radicle emerged by at least 1 mm through the seed coat, following the criteria outlined by Khatri et al. (Khatri et al. 2024).

Four indicators were employed to assess the allelopathic impact on seed germination caused by *S. trilobata* at different growth stages. The calculation formula for these indicators, utilized to evaluate germination capacity, was adapted from the research methodology outlined by Williamson and Richardson (Williamson, Richardson 1988):

- 1)  $GP = \text{germinated seed number on the 3rd day} / \text{total seed number} \times 100\%$ .
- 2)  $GR = \text{germinated seed number on the 7rd day} / \text{total seed number} \times 100\%$ .
- 3)  $GS = \sum Gt / Dt$  ( $Gt$  is the number of seeds germinating on  $t$  day, and  $Dt$  is germination days).
- 4)  $RI = 1 - C/T$  ( $T \geq C$ ) or  $RI = T/C - 1$  ( $T < C$ ).

Where  $GP$ ,  $GR$  and  $GS$  respectively represent germination potential, germination rate, and germination speed,  $C$  is the control value,  $T$  is the treatment value, and  $RI$  is the allelopathy intensity.  $RI > 0$  indicates a promoting effect on seed germination, and  $RI < 0$  indicates a suppressing effect on seed germination. The absolute value of the  $RI$  represents the strength of the allelopathic effects.

### **Analysis of secondary metabolites in leaves of invasive plant *S. trilobata* during the growing and flowering stages**

Quantifying the extent of pest feeding on leaves under natural conditions can be

challenging. In this study, we replicated pest feeding on invasive plant leaves by mechanically puncturing them in the field. We investigated the variations in secondary metabolite indicators of leaves before and after perforation during the flowering and vegetative growing stages of *S. trilobata*. Leaf samples were collected from the four previously mentioned habitats. Within each habitat, five distinct sampling points were chosen for each invasive plant species. At each sampling point, leaves from plants at both the growing and flowering stages were gathered. To simulate insect feeding on leaves, the 4th–5th leaf position from the top of the branch was punctured using a coring tool. Two circular holes with a diameter of 6 mm were created on either side of the main leaf vein (Supp. Figure 1F). After a time interval of 24 hours (Zhai et al. 2024), leaf samples were retrieved from the punctured leaves, while leaves from the same position without any punctures were utilized as controls. Subsequently, all collected leaves were rapidly frozen in liquid nitrogen and stored in a -80°C ultra-low temperature refrigerator before being transported back to the laboratory for measuring the content of secondary metabolites.

The resistance-related indicators of *S. trilobata* encompass flavonoids, total phenols, total antioxidant capacity (TAC), tannins, and soluble sugars as growth-related indicators. These resistance and growth-related indicators were analyzed utilizing a UV-2450 ultraviolet spectrophotometer. Following sample extraction, a standard curve was constructed for the absorbance values of each indicator. The flavonoid content was determined using aluminum salt (aluminum chloride) color spectrophotometry (Heimler et al. 2005), while the total phenol content was measured based on the Folin Denis method (Ainsworth, Gillespie 2007). The condensed tannin content was quantified using the vanillin hydrochloric acid method (Makarova et al. 2021), and the soluble sugar content was assessed through modified anthrone sulfuric acid colorimetry (Pernía et al. 2019). Additionally, the TAC was evaluated using the DPPH radical scavenging reaction method (Saha et al. 2008). The absorbance values of each index at specific wavelengths (765 nm for total phenols, 510 nm for TAC and condensed tannins, and 620 nm for soluble sugars) were recorded using the UV-2450 ultraviolet spectrophotometer. Subsequently, the flavonoid content, total phenol

content, total antioxidant capacity, condensed tannin content, and soluble sugar content of the samples were calculated separately based on the standard curve equations correlating absorbance values with the respective indicators.

Plant growth regulators, such as jasmonic acid (JA), gibberellins (GA), and auxins (IAA), were quantified utilizing enzyme-linked immunosorbent assay (ELISA) kits procured from Shenzhen Zike Biotechnology Co., Ltd, located in Shenzhen, China. The absorbance (OD value) of each sample was determined at a wavelength of 450 nm employing a multifunction microplate reader. Subsequently, the concentrations of the different growth-regulating substances present in the samples were computed.

### **Data analysis**

For the field survey data analysis, we utilized the number of flowers of invasive plants as the independent variable, while the number of holes on the leaves of invasive plants and neighboring native plants were considered as dependent variables. A linear regression analysis was conducted to investigate the relationship between the number of flowers of invasive plants and leaf holes.

In the allelopathy experiment on seed germination conducted with invasive plants, we used one-way ANOVA to analyze the effect of *Chrysanthemum coronarium* leaves on the crop seed germination index (as the dependent variable) under two different periods (as the independent variable). In the experiment involving invasive plants and the analysis of secondary metabolites in leaves, a two-factor variance analysis was employed to examine differences in metabolic indicators between leaves pre- and post-punching, as well as between leaves during the flowering and growing stages. The Tukey method was utilized for post-hoc testing, with the significance level set at  $\alpha = 0.05$  for the analysis of variance. Prior to the analysis, the data underwent square-root transformation to enhance normality. All statistical analyses were performed using SPSS® V22.0, and graphical representations were generated using the Origin® V2022 software.

Using Origin 2022 software, Principal Component Analysis (PCA) was applied to assess the variance explained by different explanatory variables of the secondary metabolites. The arrows represent the explanatory variables, and their lengths indicate the strength of the correlation. PERMANOVA (Permutational Multivariate Analysis of Variance), also known as Adonis, was performed using the Adonis function of the online software (<https://www.omicshare.com/tools/>) to evaluate the inter-group differences in the content of secondary metabolites in the leaves during the flowering and growing stages.

## Results

### **The impact of *S. trilobata* at different stages on the distribution of leaf holes in native plants**

The summary of the survey results from four different habitats in the wild shows that, although the three invasive plant species have different numbers of flowers per unit area, the number of herbivorous insects feeding on the leaves of the three invasive plants exhibits a significant negative correlation with the number of flowers ( $n=24$ ,  $p<0.05$ ) (Figure 1A). This observation indicates that as the number of flowers on invasive plants increases, there is a corresponding decrease in the number of leaf holes caused by leaf feeding. Additionally, the trend of a decrease in the number of leaf holes with the increase in flower numbers on the leaves of the three invasive plants is not consistent across different habitats (Supp. Figure 2). These findings suggest that in the four habitats, the more flowers the three invasive plant species produce, the less herbivorous pest feeding occurs on their leaves. This also indicates that invasive plants have a certain antifeedant effect on herbivorous pests.

The investigation into the distribution of leaf holes on native plants neighboring invasive plant *S. trilobata* revealed intriguing patterns across the four habitats. It was observed that the distribution of leaf holes in native plant quadrats positively correlated with the number of flowers of *S. trilobata* (Figure 1B). Moreover, The number of leaf holes in native plant quadrats located 2 or 3 meters away from *S.*

trilobata was significantly higher than in those located 1 meter away ( $n=96$ ,  $p<0.05$ ), indicating that as the distance from the invasive plant *S. trilobata* increases, the incidence of leaf damage on native plants also rises (Figure 1C). This suggests that the presence of invasive plant *S. trilobata* reduces the herbivory rates on local plants in 1m away, but the likelihood of local plants suffering from herbivory increases when they are located 2-3 meters away from *S. trilobata*. Moreover, in different habitats and at different distances, the number of insect holes on native plants surrounding the invasive plant *S. trilobata* also varies, indicating that the deterrent effect of invasive plants on herbivorous pests differs across habitats. (Supp. Figure 3).

### **The impact of leaf extract from *S. trilobata* at different growth stages on the seed germination of native plants**

After being immersed in the aqueous extract of *S. trilobata*, the seeds of two local crops, lettuce and pakchoi, exhibited a significant inhibition in germination. As the concentration of *S. trilobata* in the germination solution increased, both crops displayed notable differences in germination rate (GR) and germination speed (GS) compared to water control group (Table 1). Upon reaching a concentration of 50 g/L of *S. trilobata*, both crops demonstrated a marked decrease in germination potential (GP) and germination speed (GS) (Figure 2A, 2B). It is worth noting that regardless of whether *S. trilobata* was in the flowering or growing stage, when the concentration of *S. trilobata* leaves was increased to 200 g/L, the germination rate of both crop seeds dropped to zero. Therefore, we did not include this data in the statistical analysis and plotting.

**Table 1** After performing a one-way ANOVA, the significant differences in seed germination indices (germination rate (GR) and germination speed (GS)) of lettuce (*L. sativa*) and pakchoi (*B. rapa*) seeds treated with different concentrations of extracts from *S. trilobata* during the flowering and growing stages, compared with the control treatment using water. Growing 10, 50, and 100, as well as Flowering 10, 50, and 100, represent the concentrations of *S. trilobata* leaves during the growing and flowering stages in the seed cultivation solution. At a concentration of 200 g/L, no seeds germinated, resulting in a germination rate of 0%. Consequently, the data is excluded from statistical analysis. Post-hoc multiple comparisons results using Turkey's Test. Asterisk (\*) indicates significant differences,  $p < 0.05$ , ns means no significance.

| Germination Indices | Concentrations | Water | Flowering10 | Flowering50 | Flowering100 |
|---------------------|----------------|-------|-------------|-------------|--------------|
| GR of lettuce       | Water          | ns    | ns          | *           | *            |
|                     | Growing10      | *     | ns          | ns          | ns           |
|                     | Growing50      | *     | ns          | *           | ns           |
|                     | Growing100     | *     | ns          | ns          | *            |
| GS of lettuce       | Water          | ns    | ns          | *           | *            |
|                     | Growing10      | *     | ns          | ns          | ns           |
|                     | Growing50      | *     | ns          | *           | ns           |
|                     | Growing100     | *     | ns          | ns          | *            |
| GR of pakchoi       | Water          | ns    | *           | *           | *            |
|                     | Growing10      | *     | ns          | ns          | ns           |
|                     | Growing50      | *     | ns          | *           | ns           |
|                     | Growing100     | ns    | *           | *           | *            |
| GS of pakchoi       | Water          | ns    | *           | ns          | *            |
|                     | Growing10      | *     | ns          | ns          | ns           |
|                     | Growing50      | *     | ns          | *           | ns           |
|                     | Growing100     | *     | ns          | ns          | ns           |

Furthermore, the daily germination rate (GR) of the two crop seeds exhibited variations under the influence of two types of leaves from *S. trilobata* (Figure 2C, 2D). In the control groups, the germination rate (GR) of lettuce seeds reached 100% on the second day (Figure 2D). However, under the 50 g/L and 100 g/L treatments, the maximum germination rate of lettuce seeds was achieved after a delay of 7 days, while that of pakchoi seeds was attained after a delay of 3 days (Figure 2C). Additionally, the germination speed (GS) of the two crop seeds indicated that the higher the concentration of the *S. trilobata* solution, the significantly slower the

germination speed (GS) of the seeds, markedly lower than that of the control group utilizing water (Figure 3B).

Comparatively, the leaf extract of *S. trilobata* during the growing stage, at the same concentration, demonstrates a more robust inhibitory effect on the germination of local crop seeds than the extract from the flowering stage, resulting in a lower average germination rate (GR) and germination speed (GS). With increasing concentrations, this inhibitory effect on seed germination escalates, leading to a more significant disparity between the two stages. Notably, at a concentration of 200 g/L, the extract solutions from both stages can entirely impede the seed germination of seeds from both crops. This highlights that the invasive plant *S. trilobata* exerts a more potent allelopathic impact on seed germination during the growing stage compared to the flowering stage.

The allelopathic sensitivity index (RI) of the extract from *S. trilobata* during the flowering and growing stages on the germination rate (GR) and germination speed (GS) of two crop seeds was negative, indicating that *S. trilobata* has an inhibitory effect on the germination of crop seeds, and the lower the value, the stronger the inhibitory effect. (Table 2). The total RIs for the GR of the two crops affected by *S. trilobata* at the flowering stage were calculated at -3.15, which was higher than that the sum of RIs for GS (-4.89). Similarly, the combined RIs for the GR of the two crops influenced by *S. trilobata* at the growing stage were -4, which was higher than the sum of RIs for GS (-5.61) (Table 2). These findings suggest that the allelopathic impact of *S. trilobata* on the germination of crop seeds primarily occurs through a reduction in GS, consequently delaying the germination time of the seeds. Our results illustrated that the total RI (the sum of RIs for GR and GS of the two crop seeds) of *S. trilobata* at the growing stage amounted to -9.61, while the total RI of *S. trilobata* at the flowering stage was -8.04 (Table 2), indicating that the allelopathic effect of *S. trilobata* during the growing stage on crop seeds is more potent compared to that during the flowering stage.

**Table 2.** Allelopathic sensitivity indexes (RI) of different concentration aqueous extracts from *S. trilobata* leaves during flowering and growing stages on germination rates of lettuce (*L. sativa*) and pakchoi (*B. rapa*) seeds.

| RI       | Stage     | Seeds   | Concentrations of <i>S. trilobata</i> (g/L) |       |       |       |     | Sum   |
|----------|-----------|---------|---------------------------------------------|-------|-------|-------|-----|-------|
|          |           |         | 0                                           | 10    | 50    | 100   | 200 |       |
| RI of GR | Flowering | lettuce | 0                                           | 0     | -0.07 | -0.68 | -1  | -3.15 |
|          |           | pakchoi | 0                                           | 0     | 0     | -0.4  | -1  |       |
|          | Growing   | lettuce | 0                                           | 0     | -0.21 | -0.93 | -1  | -4.00 |
|          |           | pakchoi | 0                                           | 0     | -0.16 | -0.7  | -1  |       |
| RI of GS | Flowering | lettuce | 0                                           | -0.18 | -0.63 | -0.95 | -1  | -4.89 |
|          |           | pakchoi | 0                                           | -0.05 | -0.31 | -0.77 | -1  |       |
|          | Growing   | lettuce | 0                                           | -0.24 | -0.78 | -1    | -1  | -5.61 |
|          |           | pakchoi | 0                                           | -0.11 | -0.55 | -0.93 | -1  |       |

### Changes in secondary metabolite indicators in leaves of different stage of *S. trilobata* before and after leaf punching in different habitats

In *S. trilobata*, the levels of secondary metabolites in the leaves showed significant variation between the growing and flowering stages. Defense-related substances such as flavonoids, total phenols, and total antioxidant capacity (TAC, measured by DPPH) were higher during the flowering stage compared to the growing stage ( $p < 0.001$ ) (Table 3) (Figure 3). However, tannin levels were elevated during the growing stage (Figure 3). These results suggest that the allocation of defense substances in *S. trilobata* varies across developmental stages.

**Table 3** Two-Way ANOVA analysis of secondary metabolites in *S. trilobata* leaves during the growing and flowering stages before and after artificial leaf damage (df = 1).

| Factors       | Index         | MS        | F       | p     |
|---------------|---------------|-----------|---------|-------|
| Stage         | Flavonoid     | 15225.64  | 23.727  | 0.000 |
|               | Total phenols | 259.39    | 197.386 | 0.000 |
|               | Tannin        | 118801.07 | 14.094  | 0.000 |
|               | DPPH          | 7045.24   | 135.917 | 0.000 |
|               | sugar         | 7.49      | 26.997  | 0.000 |
|               | JA            | 5.65      | 33.340  | 0.000 |
|               | GA            | 0.26      | 0.460   | 0.501 |
|               | IAA           | 0.00      | 0.635   | 0.430 |
| Damage        | Flavonoid     | 16625.21  | 25.908  | 0.000 |
|               | Total phenols | 82.05     | 62.435  | 0.000 |
|               | Tannin        | 245680.22 | 29.147  | 0.000 |
|               | DPPH          | 3588.04   | 69.221  | 0.000 |
|               | sugar         | 65.91     | 237.710 | 0.000 |
|               | JA            | 4.00      | 23.585  | 0.000 |
|               | GA            | 5.38      | 9.492   | 0.003 |
|               | IAA           | 0.00      | 1.377   | 0.247 |
| Stage× Damage | Flavonoid     | 3427.97   | 5.342   | 0.025 |
|               | Total phenols | 13.87     | 10.557  | 0.002 |
|               | Tannin        | 13790.66  | 1.636   | 0.207 |
|               | DPPH          | 86.75     | 1.674   | 0.202 |
|               | sugar         | 3.47      | 12.516  | 0.001 |
|               | JA            | 0.61      | 3.588   | 0.064 |
|               | GA            | 0.77      | 1.358   | 0.250 |
|               | IAA           | 0.00      | 0.003   | 0.958 |

Mechanical leaf punching led to significant changes in the content of flavonoids, phenols, tannins, and DPPH (Figure 3). Compared to control groups, punching resulted in increased levels of these compounds. Notably, after punching during the flowering stage, the concentrations of flavonoids, phenols, and DPPH remained higher than in the growing stage, while tannin levels were higher in the growing stage (Figure 3).

Soluble sugar content decreased significantly 24 hours after leaf punching, with a more pronounced reduction in the flowering stage, affecting leaf palatability. Jasmonic acid (JA), a key defense signaling molecule, was higher during the flowering stage before punching, and increased further after leaf damage, particularly

in the flowering stage (Figure 3).

Before leaf punching, there were no significant differences in the levels of growth regulators gibberellic acid (GA) and indole-3-acetic acid (IAA) between the two stages (Table 3). However, post-punching, GA content decreased, especially in the growing stage, while IAA levels remained stable (Figure 3). These findings indicate that leaf damage alters the distribution of growth regulators and defense-related compounds in *S. trilobata* at different developmental stages.

PCA analysis was conducted to perform dimensionality reduction on the variables of multiple secondary metabolites in the leaves. The results indicated that, prior to mechanical punching, *S. trilobata* exhibited different allocation strategies for secondary metabolites between the growing and flowering stages. During the growing stage, the leaves were more focused on the distribution of tannins, soluble sugars, and gibberellic acid (GA), whereas in the flowering stage, there was a greater emphasis on enhancing defense-related indicators, such as total phenols, jasmonic acid, DPPH, and flavonoids (Figure 4A). After mechanical punching of the leaves, the patterns of secondary metabolites in the flowering and growing stage leaves of *S. trilobata* did not align with those before punching. Compared to the growing stage leaves, the flowering stage leaves showed a significant increase in total phenols, flavonoids, jasmonic acid (JA), and DPPH. In contrast, the growing stage leaves exhibited a higher allocation of tannins and soluble sugars (Figure 4B). PCA analysis further confirmed that the leaves of *S. trilobata* at different stages exhibit distinct allocation strategies, with the flowering stage maintaining and reinforcing the investment in defense.

## **Discussion**

### **The influence of the growing and flowering stages of invasive plants on insect feeding patterns**

Our previous investigation into three invasive plant species, namely *S. trilobata*, *M. micrantha*, and *B. alba*, revealed that invasive plants have the potential to elevate

defensive compounds that elicit antifeeding effects on herbivorous insects. This phenomenon can prompt herbivorous insects to shift their feeding behavior towards neighboring native plants (Zhai et al. 2024). Building upon this research, it was uncovered that during their growing and flowering stages, *S. trilobata* influences the distribution pattern of native herbivorous insects. Despite lacking specific natural enemies in their non-native habitats, invasive plants remain vulnerable to polyphagous pests such as larvae of *Spodoptera litura*, *Atractomorpha sinensis* Bolivar, and *Anoplophora glabripennis* (Colautti et al. 2004; Fan et al. 2016; Junhirun et al. 2018).

Directly confirming the presence of pests feeding on invasive plants through field surveys presents challenges (Zhai et al. 2024). Hence, the study indirectly evaluated insect feeding on invasive plants by assessing leaf damage. Our findings indicated a significant negative correlation between the number of leaf holes on invasive plants and their flower count. This suggests that invasive plants' leaves during the flowering period are less susceptible to herbivorous pests compared to those in the growing period, and the secondary metabolites in the leaves during the flowering period influence pest feeding. Similarly, Çirak et al. (2007) documented elevated total phenol levels in *Hypericum perforatum* during its flowering stage, aligning with our results (Çirak et al. 2007). This enhanced defense strategy during the flowering stage of invasive plants may be crucial for ensuring reproductive success. By boosting defensive compound content instead of prioritizing reproductive investment directly, plants might achieve greater benefits concerning reproductive fitness (English, Bonsall 2019; Hoque, Avila-Sakar 2015).

Analyzing leaf damage on nearby native plants adjacent to the three invasive species unveiled a substantial positive relationship between leaf damage distribution and the flowering intensity of invasive plants. As invasive plants increase their flowering levels, the incidence of local plant feeding also rises. This finding indirectly supports the notion that invasive plants bolster their defensive prowess during flowering, resulting in herbivorous insects targeting neighboring native plants and impacting herbivorous insect distribution patterns. These outcomes are consistent with

the research findings (Zhai et al. 2024). Moreover, our study proposes that invasive plants fortify their defenses against pests during the reproductive phase, contradicting the enemy release hypothesis (ERH) associated with invasive plants. Recent studies have continued to highlight the robust broad-spectrum defensive capabilities retained by invasive plants (Shi et al. 2023; Sun et al. 2023; Wu et al. 2023).

### **The impact of the allelopathic effects of invasive plant *S. trilobata* on seed germination of native plants**

For invasive plants, allelopathy is regarded as a novel strategy that enhances their competitiveness in the environment (Pan et al. 2023; Uesugi, Kessler 2013). This study utilized water extracts from the leaves of invasive species to treat seeds, simulating the effects of plant leachates on neighboring flora. The results demonstrated that water extracts from the leaves of *S. trilobata* during both flowering and growing stages significantly inhibited the germination of local crop seeds. Compared to the control group treated with clear water, the germination rate of crop seeds was markedly reduced, and germination time was delayed. The inhibitory effect on seed germination exhibited a positive correlation with the concentration of the water extracts. These findings are consistent with the research conducted by Ullah et al. regarding the allelopathy of *S. trilobata* (Ullah et al. 2021).

Our research confirmed that the invasive plant *S. trilobata* can impede the growth of local plants through robust allelopathy during its invasion process, thereby augmenting its competitive advantage in the ecosystem. Since these two crops are widely cultivated around the world, the inhibitory effect of *S. trilobata* may not be specific. Moreover, our findings revealed that the inhibitory effect of *S. trilobata* on local crop seeds is less pronounced during its flowering stage compared to the growing stage. This suggests that during the growing phase, *S. trilobata* may enhance its competitiveness by allocating resources towards allelochemical production, while during the reproductive stage, it might reduce its investment in allelopathy and instead allocate resources to defense mechanisms. Therefore, considering the trade-offs

involved in plant resource allocation, there appears to be a trade-off between investing in reproduction and allelopathy for *S. trilobata*. Our field research supports the notion that *S. trilobata* invests more in defense during the flowering stage than during the growing stage, highlighting a trade-off between allelopathy and plant defense strategies. When *S. trilobata* is in the flowering phase, it amplifies its investment in defensive compounds while diminishing its allelopathic effects. In contrast, invasive plants in the growing stage, requiring more resources and space for growth and expansion, release higher levels of allelochemicals, hindering the regeneration of local plants.

Studies have emphasized the significant role allelopathy plays in the growth and spread of invasive plants (Hierro, Callaway 2003; Xie et al. 2010; Zhao et al. 2008). The allelopathic behavior of invasive plants aligns with the concept of novel weapons, as proposed by many researchers (Callaway, Ridenour 2004; Chen, Peng 2018; Yuan et al. 2013). Furthermore, our research indicates that invasive plants tend to reduce their investment in allelopathy during the flowering stage, suggesting dynamic shifts in resource allocation strategies during different growth phases.

### **Changes in secondary metabolites in the leaves of invasive plant *S. trilobata* during flowering and growing stages after leaf punching**

Researches have explored the defensive responses of plants to insect herbivory (Adedayo et al. 2025; Kato-Noguchi, Kato 2024; Schulz et al. 2025; Zong, Wang 2007). Specialized natural enemies and plants often evolve unique defensive compounds over long-term evolutionary interactions (Sharma et al. 2024). However, in the case of generalist insects, the defensive responses of plants tend to exhibit broader-spectrum characteristics (Gaillard et al. 2018; Volf et al. 2018). Despite this, in quantitative studies of the damage caused by herbivorous insects to plants, mechanical damage is often used as a simulation of insect herbivory (Ali et al. 2024; Gaupels et al. 2016). Mechanical damage to leaves, such as puncturing, triggers plant defense responses similar to those caused by pest feeding (Bustos-Segura et al. 2022;

Ye et al. 2022). The study has shown that such damage induces changes in defense-related compounds in invasive plants, such as *M. micrantha*, *S. trilobata*, and *I. cairica* (Zhai et al. 2024), making it a reliable method to study plant defense mechanisms.

Secondary metabolites like total phenols and flavonoids are crucial for plant resistance and stress tolerance (Wu et al. 2023; Xiao et al. 2019). Jasmonic acid, a key signaling molecule in stress responses, also plays a pivotal role (Xiao et al. 2019). In this study, mechanical damage significantly increased total phenols, flavonoids, and jasmonic acid levels in *S. trilobata* during the flowering stage compared to the growing stage, suggesting enhanced defense investment during reproduction.

Environmental stress often increases reactive oxygen species (ROS) in plants, affecting membrane integrity (Lassouane et al. 2016; Liu, Zhang 2022). To mitigate ROS-induced damage, *S. trilobata* releases antioxidants like DPPH. After leaf punching, the total antioxidant capacity (TAC) of leaves was higher during flowering, indicating sustained defense activity.

To deter herbivory, plants can reduce leaf palatability by decreasing soluble sugar content (Casal-Porras et al. 2021). In this study, although there is a lack of experimental evidence on how changes in the soluble sugar content of *S. trilobata* leaves affect pest palatability, the analysis of the soluble sugar content in the leaves indicates that the soluble sugar levels during the flowering period are lower than those during the growth period after the leaves have been damaged. This suggests that *S. trilobata* does not increase nutrient allocation for reproduction during the flowering period, and also, the decrease in soluble sugar content may, to some extent, reduce the palatability of the leaves.

Analysis of growth hormones revealed significant variations in jasmonic acid, gibberellin (GA), and auxins (IAA) between flowering and growing stages. Jasmonic acid levels increased significantly in flowering-stage leaves after damage, reinforcing its role in activating defense. However, changes in GA and IAA were habitat-specific and not consistently observed, highlighting their variable roles in metabolic processes during growth and reproduction.

These findings demonstrate that *S. trilobata* enhances its defense mechanisms during flowering without compromising growth or reproduction. This aligns with strategies observed in invasive plants, which balance defense and reproduction to maximize fitness (Abhilasha, Joshi 2009; González-Teuber et al. 2017). While the "enemy release hypothesis" (ERH) suggests a trade-off between defense and reproduction in invasive plants (Agrawal et al. 2005; Genton et al. 2005), our study found no reduction in defense investment during flowering. Instead, *S. trilobata* maintains active defenses, as the benefits of these responses likely outweigh their costs (González-Teuber et al. 2017; Hoque, Avila-Sakar 2015).

## Conclusion

Our study provides valuable insights into the defense mechanisms of the invasive plant *S. trilobata*, specifically examining the trade-offs between defense, growth, and reproduction in different developmental stages. Our findings demonstrate that *S. trilobata* strategically enhances its defensive capabilities during the flowering stage, effectively protecting itself from herbivory while simultaneously ensuring reproductive success. In contrast, during the growing stage, this plant prioritizes allelopathy, which serves to inhibit the germination of nearby native seeds and maintain competitive advantages in its environment.

The observed elevated levels of defense-related compounds, such as flavonoids and total phenols, during flowering refute the notion of a strict trade-off between defense and reproduction. Instead, *S. trilobata* appears to allocate resources adeptly, balancing its defensive strategies with reproductive needs. This challenges traditional paradigms that suggest invasive plants reduce their investment in defense in the absence of specialized enemies. Our research highlights that invasive plants bolster their defense mechanisms during the flowering stage to ensure reproductive success, whereas during the growing stage, they reinforce allelopathy to enhance competitiveness. This study offers a novel perspective on understanding the defense mechanisms of invasive plants throughout the invasion process, underscoring that

enhancing plant defense should be recognized as a crucial factor contributing to the successful invasion of invasive plants and safeguarding their reproductive success.

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**Authors' contribution** GL and XH contributed to the study conception and design; HFY, LZQ, CJC, CJY, WY, SJY, YD and YCL collected the data; GL and HFY analyzed the data; GL and HFY wrote the manuscript. All authors commented on previous versions of the manuscript. All the authors have read and approved the final manuscript.

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**Data availability** The data will be provided upon request on authors.

## **Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

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**Figure 1.** The linear regression relationship between the number of flowers of the invasive plant and the abundance of herbivorous pests on the leaves of the invasive plant and surrounding native plants in the field habitat. **A)** Linear regression between the number of flowers (x-values) and the number of leaf holes (y-values) of three invasive plants collecting from four natural habitats (Wasteland, Cropland, Forests, and Orchards), illustrated with linear regression (n=24). **B)** Linear regression between the number of flowers (x-values) of invasive plant *S. trilobata* and the number of leaf holes (y-values) in native plants within three distances (1m, 2m, and 3m) collecting from four natural habitats (Wasteland, Croplands, Forests, and Orchards), illustrated with linear regression (n=96). The shaded areas represent the 95% confidence intervals (CIs). **C)** Comparison of the number holes by pests on leaves in native plant quadrats at three distances around the invasive plant *S. trilobata* (n=96). Different letters indicate statistically significant differences among treatments ( $p < 0.05$ ), as determined by one-way analysis of variance followed by a post-hoc Tukey test.

**Figure 2.** Effects of leaf extracts from the invasive plant *S. trilobata* during the flowering (F) and growing (G) stages on seed germination of two crops: lettuce (*L. sativa*) and pakchoi (*B. rapa*). **A)** Germination rate after 3 days for both crop seeds treated with extracts from *S. trilobata* leaves at different concentrations during the flowering and growing periods, presented as mean  $\pm$  SE. **B)** Germination speed (GS) of both crop seeds. **C)** Daily germination rate (GR) of lettuce (*L. sativa*) seeds. **D)** Daily germination rate (GR) of pakchoi (*B. rapa*) seeds. Different letters indicate statistically significant differences among treatments ( $p < 0.05$ ), as determined by one-way analysis of variance followed by a post-hoc Tukey test.

**Figure 3.** Variations in defense and resistance-related secondary metabolite indicators after mechanical punching (Damage) and control (CK) of leaves from invasive plant *S. trilobata* during the flowering and growing stages, presented as Mean  $\pm$  SE (n = 20). **A)** Flavonoids, **B)** Total phenols, **C)** Tannins, **D)** DPPH, **E)** Soluble sugars, **F)** Jasmonic acid (JA), **G)** Gibberellin (GA), **H)** Auxin (IAA). Different letters indicate statistically significant differences among the various treatments ( $p < 0.05$ ), determined through one-way analysis of variance followed by a post-hoc Tukey test.

**Figure 4.** The principal component analysis (PCA) of secondary metabolites in the leaves of *S. trilobata* before and after mechanical leaf punching (Damage) and control (CK) during the

flowering and growing stages. **A)** Leaf before punching, **B)** Leaf after punching. FCK (flowering stage without damage), GCK (growing stage without damage), FD (flowering stage with damage), GD (growing stage with damage). The Adonis analysis, showing the intergroup differences in the secondary metabolites of leaves between the flowering and growing stages.

Figure 1

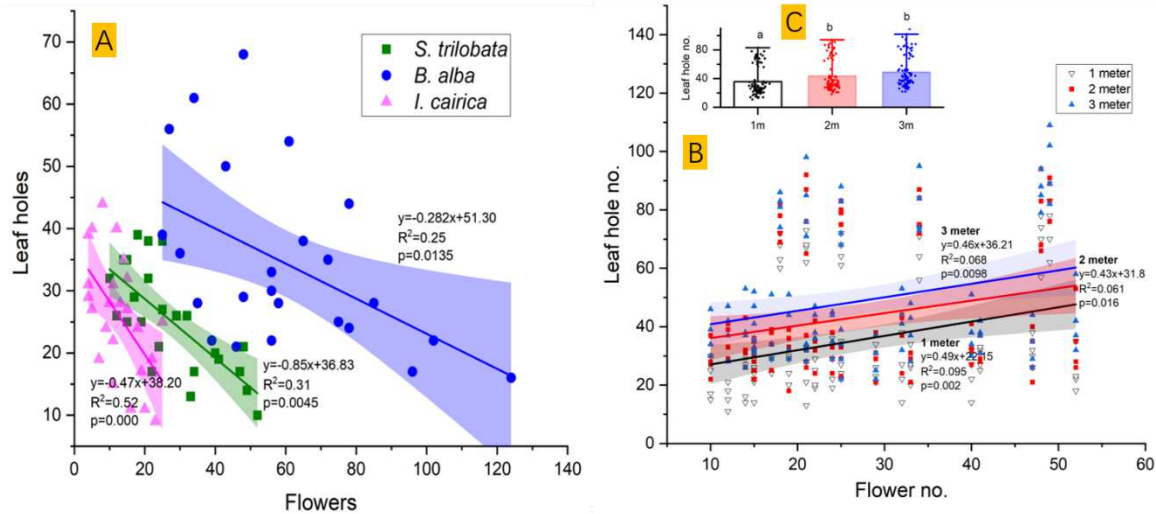


Figure 2

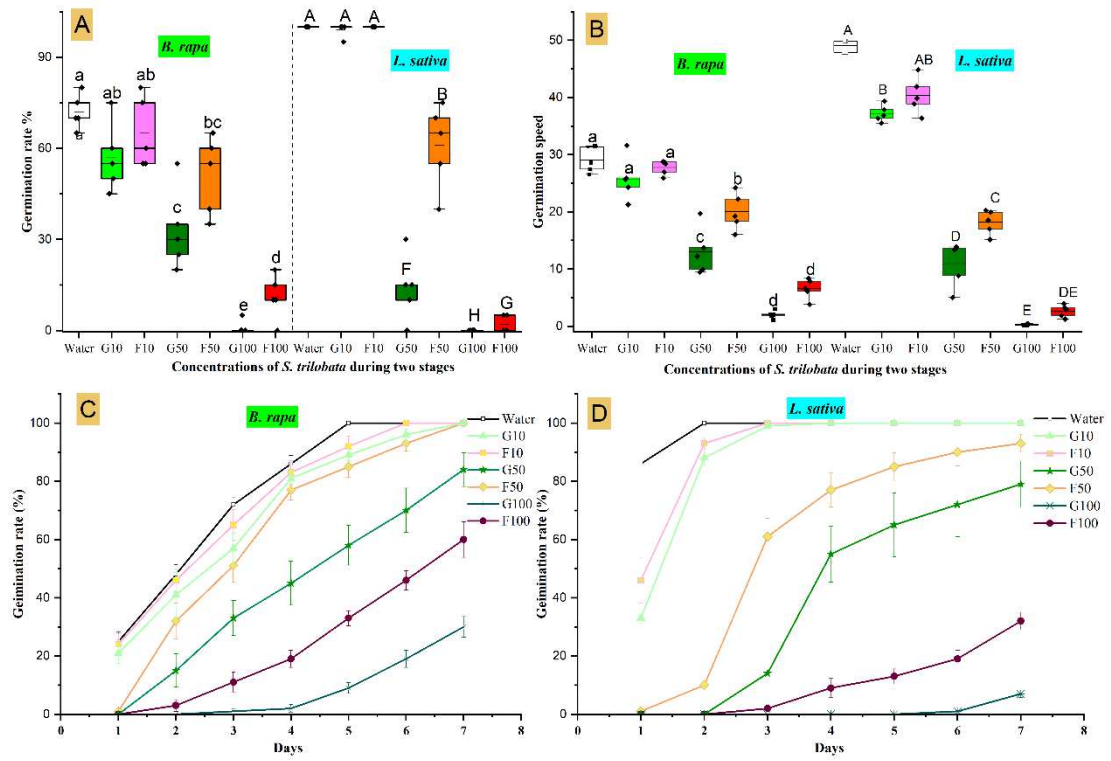


Figure 3

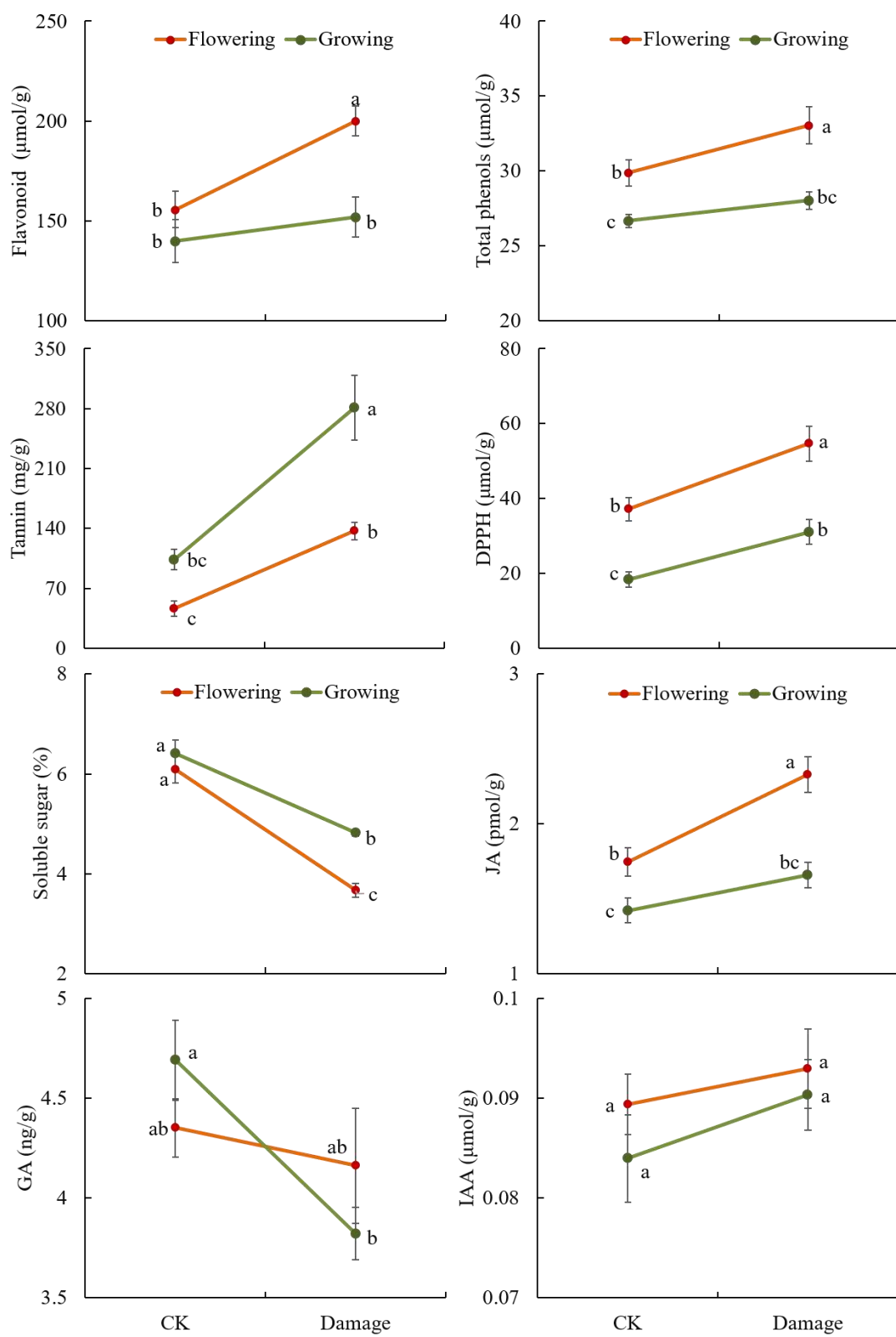
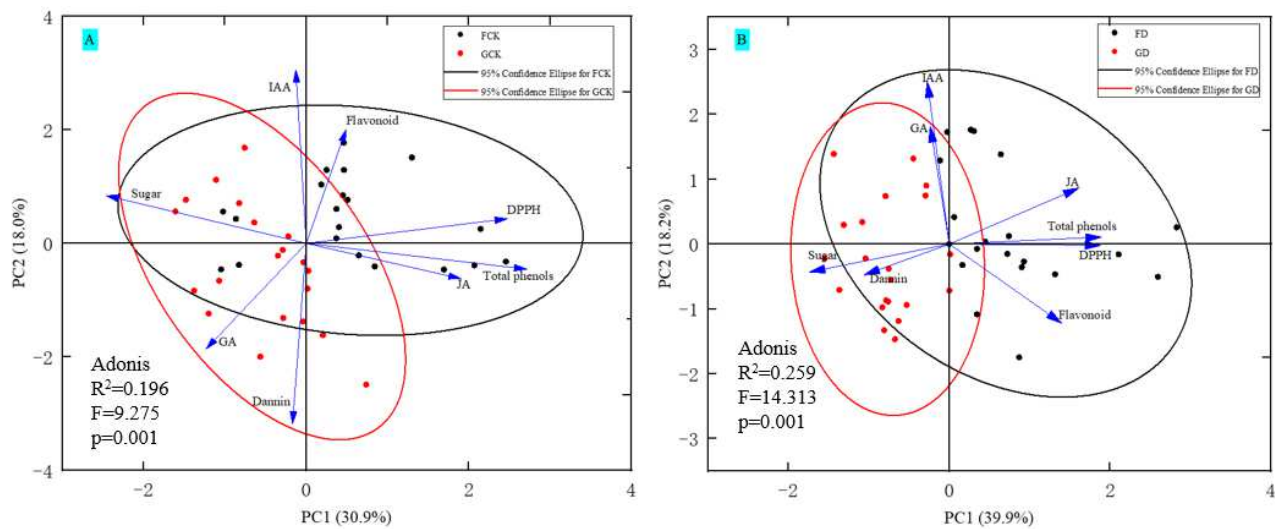


Figure 4



## Supplementary Files

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