



## Article

# Nitrogen Influence to the Independent Invasion and the Co-Invasion of *Solidago canadensis* and *Conyza canadensis* via Intensified Allelopathy

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**Abstract:** The allelopathy of alien plants is critical to their successful invasion. In nature, two alien plants can co-invade the same habitat. Changes in the forms of nitrogen may have the potential to alter the invasion process of alien plants by causing alterations in their allelopathy. This study aimed to evaluate the individual and combined allelopathy of two alien plants from the *Asteraceae* family, *Solidago canadensis* L. and *Conyza canadensis* (L.) Cronq. This study examined the effects of aqueous leaf extracts of the two alien plants with different nitrogen forms (NO<sub>3</sub>–N, NH<sub>4</sub>–N, and CO(NH<sub>2</sub>)<sub>2</sub>–N, and the mixed nitrogen forms at a 1:1:1 ratio) on the seed germination and seedling growth of the horticultural *Asteraceae* species *Lactuca sativa* L. using a germination bioassay. The allelopathy of the two alien plants significantly reduced the seed germination and seedling growth of *L. sativa*. Extracts from *S. canadensis* produced stronger allelopathy on the seed germination and seedling growth of *L. sativa* compared with those from *C. canadensis*. The mixture of extracts from the two alien plants produced an antagonistic effect when compared with the effects of extracts from each plant species. The addition of nitrogen intensified the allelopathy of the two alien plants on the seed germination and seedling growth of *L. sativa*. The degree of influence of nitrogen on the individual and combined allelopathy of the two alien plants was similar. Thus, nitrogen deposition may facilitate the independent invasion and co-invasion of the two alien plants via intensified allelopathy.

**Keywords:** alien plant; allelochemicals; aqueous leaf extracts; nitrogen deposition; *Lactuca sativa* L.



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## 1. Introduction

The distribution of alien plants in the world is increasing; more importantly, these alien plants are causing serious adverse ecological impacts in their newly colonized habitats [1–3]. In particular, one of the main factors of the successful invasion of alien plants is that they are often highly allelopathic to adjacent species [4–6]. In addition, numerous studies also verified that many alien plants can significantly reduce the ability of adjacent species.

This is particularly true for seed germination and seedling growth using allelopathy and subsequently facilitating their invasion process [4–6]. Moreover, the numerous habitats in China enhance the likelihood of colonization triggered by alien plants [7–9]. In addition, the *Asteraceae* contains the highest number of alien plants that are active in China. A total of 92 alien plants in the *Asteraceae* currently grow in China. They comprise approximately 17.86% of the total species number of alien plants at the family level in China [8,9].

The successful invasion of one alien plant can create favorable conditions, such as an enhanced level of available nutrients in the occupied habitats that can increase the probability of the successful invasion of another alien plant in the same habitat [1,10,11]. Thus,

the co-invasion of two or more alien plants may occur in some natural habitats [3,12,13]. More importantly, changes in the species number of alien plants are likely to primarily modify their allelopathy on the seed germination and seedling growth of adjacent species by alterations in the secreted amounts and chemical components of allelochemicals secreted from their leaves and/or roots.

With the progressively growing intensity and frequency of human activities, such as the use of fossil energy for industrial development and the use of nitrogenous fertilizers for crop production, the level of atmospheric nitrogen deposition has gradually increased in recent years [14–16]. In addition, East Asia, particularly China, is currently one of the three regions with the most serious levels of pollution from the deposition of nitrogen [14–16]. Moreover, the natural nitrogen deposition involves several nitrogen forms ( $\text{NH}_4\text{-N}$ ,  $\text{NO}_3\text{-N}$ , and  $\text{CO}(\text{NH}_2)_2\text{-N}$ ).

The relative concentration of particular nitrogen forms in the natural nitrogen deposition could change in the future primarily due to the progressively increased intensity and frequency of human activities [15,17,18]. However, the changes in the forms of nitrogen deposition may have the potential to alter the invasion process of alien plants by causing changes in their allelopathy on the seed germination and seedling growth of adjacent species [19–21].

Previous studies have also confirmed that nitrogen can alter the amounts secreted and the chemical components of the secondary metabolites [22,23]. Thus, it is important to delineate the effects of nitrogen deposition on the seed germination and seedling growth of adjacent species by examining the combined allelopathy that is mediated by two *Asteraceae* alien plants that can co-invade the same habitat. However, research on this topic, particularly the effects of different nitrogen forms, is currently limited.

The objective of the current study is to evaluate the individual and combined allelopathy of Canada goldenrod (*Solidago canadensis* L.) and horseweed (*Conyza canadensis* (L.) Cronq.) on the seed germination and seedling growth of the horticultural *Asteraceae* species lettuce (*Lactuca sativa* L.) with and without inorganic and organic forms of nitrogen that commonly occur with atmospheric nitrogen deposition. Canadian goldenrod and horseweed, which are native to North America, often co-invade the same habitat (e.g., farmland, wasteland, roadsides, industrial district, municipal land, residential area, urban green space, and garden scenic areas) in South Jiangsu, China (Figure S1) [24].

Moreover, the two alien plants are native to North America [7–9]. There are currently 137 alien plants in China that originated in North America [7–9]. These species comprise approximately 21.5% of the total alien plants that have been introduced to China [7–9]. The two alien plants may share a similar evolutionary history in China [7–9]. More importantly, the two alien plants have dramatically changed the structure and function of native ecosystems and have been listed as two of the most aggressive and deleterious alien plants in China [2,24,25].

In addition, the allelopathy of the two alien plants has been listed as one of the most vital factors for the success of their invasion [26–28]. Moreover, as one of the more common cultivated vegetables in the areas invaded by the two alien plants and also affected by atmospheric nitrogen deposition, lettuce is commonly used in bioassays for allelopathy due to its sensitivity to external environment stresses [26–28]. Moreover, the two alien plants and lettuce all belong to the *Asteraceae* family, which comprises the largest number of alien plants in China at the family level [8,9].

For this study, the following hypotheses were proposed: (1) a mixture of extracts from the two alien plants can cause synergistic effects on the seed germination and seedling growth of the lettuce compared with the individual extracts; (2) the addition of nitrogen can decrease the allelopathy of the two alien plants on the seed germination and seedling growth of lettuce; (3) the degree of influence of  $\text{CO}(\text{NH}_2)_2\text{-N}$  on the allelopathy of the two alien plants may be higher than those of  $\text{NO}_3\text{-N}$  and  $\text{NH}_4\text{-N}$ , and the degree of influence of the mixture of the three nitrogen forms on the allelopathy of the two alien plants may be higher than those of the single nitrogen forms; and (4) the degree of influence

of nitrogen on the combined allelopathy of the two alien plants may be higher than on their independent allelopathy.

## 2. Materials and Methods

### 2.1. Preparation of Aqueous Leaf Extracts of the Two Alien Plants

Mature leaves of the two alien plants were collected from a wasteland site in Zhenjiang (32.21° N, 119.52° E), South Jiangsu, China on 9 September 2020. The leaves of the two alien plants were cleaned slightly and thoroughly air-dried at nearly 25 °C to obtain a constant weight. The air-dried leaves were soaked in sterile distilled water at approximately 25 °C to produce aqueous leaf extracts of 20 g L<sup>-1</sup> [29,30] to simulate invasive conditions using sterile distilled water as the control.

The aqueous extracts were assumed to contain putative allelochemicals that are released into the soil in invaded areas. Sterile distilled water was used as a no-extract control. In addition to the control and the extracts from the individual alien plants, a 1:1 v/v mixture of Canada goldenrod and horseweed aqueous leaf extracts (extract mixture) was also prepared for evaluation. The aqueous leaf extracts were maintained at 4 °C for one week at the longest.

### 2.2. Preparation of Nitrogen Solution with Different Nitrogen Forms

Three solutions based on different forms of nitrogen were prepared separately and in a mixture of the three forms using KNO<sub>3</sub> (NO<sub>3</sub>-N; AR, ≥ 99%; Aladdin®, Shanghai, China), NH<sub>4</sub>Cl (NH<sub>4</sub>-N; GR, ≥ 99.8%; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China), CO(NH<sub>2</sub>)<sub>2</sub> (CO(NH<sub>2</sub>)<sub>2</sub>-N; BC, ≥ 99.5%; Sangon Biotech Co., Ltd., Shanghai, China).

The ratio of the three nitrogen forms in the mixed nitrogen forms was established at 1:1:1 to simulate the actual average ratio in the forms found in atmospheric nitrogen deposits [17,18,31]. The nitrogen in the solutions was established at 0.1 g N L<sup>-1</sup>. The concentration imitated the approximate level of atmospheric N deposition at the study site (i.e., Zhenjiang, Jiangsu, China) [19,20]. Sterile distilled water lacking any nitrogen was used as the control.

### 2.3. Germination Bioassay of Lettuce

The germination bioassay of lettuce included 20 treatment combinations in triplicate with all the individual and combined treatments of aqueous leaf extracts of the two alien plants. They included the four forms, no-extract control, leaf water extract of Canadian goldenrod, leaf water extract of horseweed, and extract mixture. The nitrogen treatment included five types in addition to the control: NO<sub>3</sub>-N, NH<sub>4</sub>-N, and CO(NH<sub>2</sub>)<sub>2</sub>-N, and the mixed nitrogen forms at a 1:1:1 ratio.

The lettuce bioassay was conducted using 9 cm diameter Petri dishes [27,29]. Seeds of the lettuce cultivar Xiangsihong were obtained from Keguanguang Seed Co., Ltd., (Chongqing, China). They were sterilized using NaClO (concentration: 1%; active Cl<sup>-</sup> concentration: 7~8%; free NaOH concentration: ≥5.2%; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) for approximately 15 min and rinsed with sterile distilled water for nearly 30 min.

Thirty lettuce seeds of uniform size were placed on two layers of 9 cm filter paper in each Petri dish. The seeds were incubated in a growth chamber for 8 days from 28 September to 5 October 2020, at approximately 25 °C 12 h of light per day (27.5 μmol m<sup>-2</sup> s<sup>-1</sup>). A volume of 4 mL per Petri dish was added on the first day to moisten the filter paper and seeds, and 1 mL was added per Petri dish on the second day. For the duration of the experiment, 0.5 mL per Petri dish was added from day 3 to day 7.

The seeds were considered to have germinated when the radicle had emerged, and the number of the normal germinated lettuce seeds was counted each day [27,29]. After 8 days of incubation, 10 lettuce seedlings per Petri dish (i.e., 30 lettuce seedlings per treatment) were randomly selected to evaluate the indicators. The indicators assessed included the germination percentage, germination potential, germination index, germination rate index, germination vigor index, promptness index, seedling height, radicle length, leaf dimensions

(including leaf length and width), green leaf area (the area of leaf photosynthesis), seedling biomass (including fresh weight and dry weight), and moisture content.

The stress intensity of all treatments on lettuce growth was measured using the stress intensity index. The determination methods of lettuce indicators are shown in Table S1.

#### 2.4. Statistical Analysis

Differences in the values of the lettuce indicators among the different treatment combinations were analyzed using a one-way analysis of variance (ANOVA) and means separation was performed using Tukey's test. Two-way ANOVA was used to estimate the effects of the form of nitrogen solution, and the type of aqueous leaf extracts, and their interactions on the measured variances. In addition, the Partial Eta-squared ( $\eta^2$ ) values were calculated to determine the effect size of each factor for use in the two-way ANOVA.

### 3. Results

#### 3.1. The Effects of the Individual and Combined Aqueous Leaf Extracts of the Two Alien Plants

The leaf water extract of Canadian goldenrod significantly reduced almost all of the seed germination indicators, radicle length (55.08% lower), and seedling fresh weight (27.83% lower) of the lettuce compared with the control ( $p < 0.05$ ; Figures 1A–F and 2B,F). The leaf water extract of horseweed significantly reduced the germination index (33.51% lower), germination rate index (35.83% lower), germination vigor index (42.68% lower), promptness index (20.42% lower), and radicle length (80.96% lower) of the lettuce compared with the control ( $p < 0.05$ ; Figures 1C–F and 2B).

The extract mixture significantly reduced the germination index (32.37% lower), germination rate index (33.14% lower), germination vigor index (29.71% lower), and radicle length (54.02% lower) of the lettuce but significantly increased the leaf length (22.09% higher) of the lettuce compared with the control ( $p < 0.05$ ; Figures 1C–E and 2C).

All the seed germination indicators and the leaf length (23.65% and 32.27% lower, respectively) of lettuce following treatment with the leaf water extract of Canadian goldenrod were significantly lower than those treated with the leaf water extract of horseweed and the extract mixture ( $p < 0.05$ ; Figures 1A–F and 2C).

Similarly, the green leaf area (41.95% lower) and seedling fresh weight (30.83% lower) of lettuce following treatment with the leaf water extract of Canadian goldenrod were significantly lower than those following treatment with the extract mixture ( $p < 0.05$ ; Figure 2E,F). In addition, the seedling dry weight (22.22% lower) of lettuce following treatment with the leaf water extract of Canadian goldenrod was significantly lower than that following treatment with the leaf water extract of horseweed ( $p < 0.05$ ; Figure 2G).

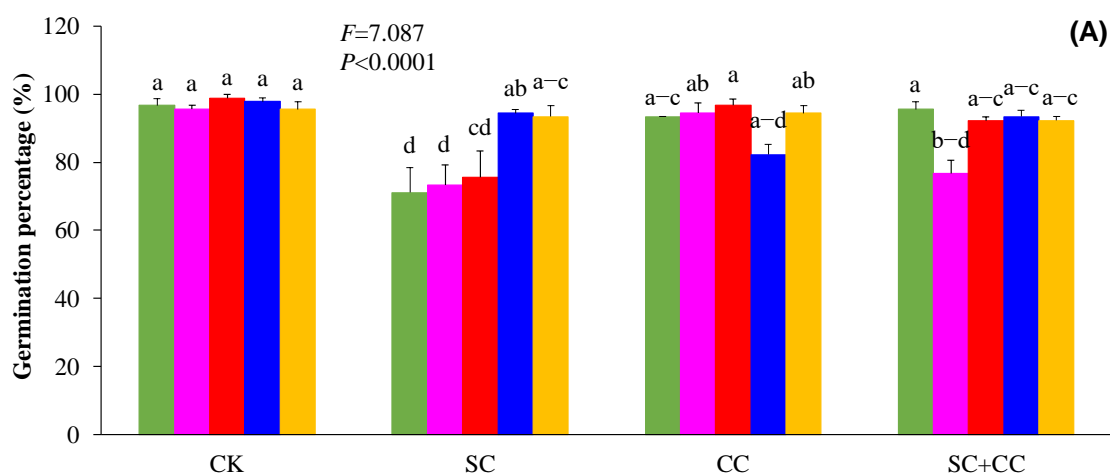


Figure 1. Cont.

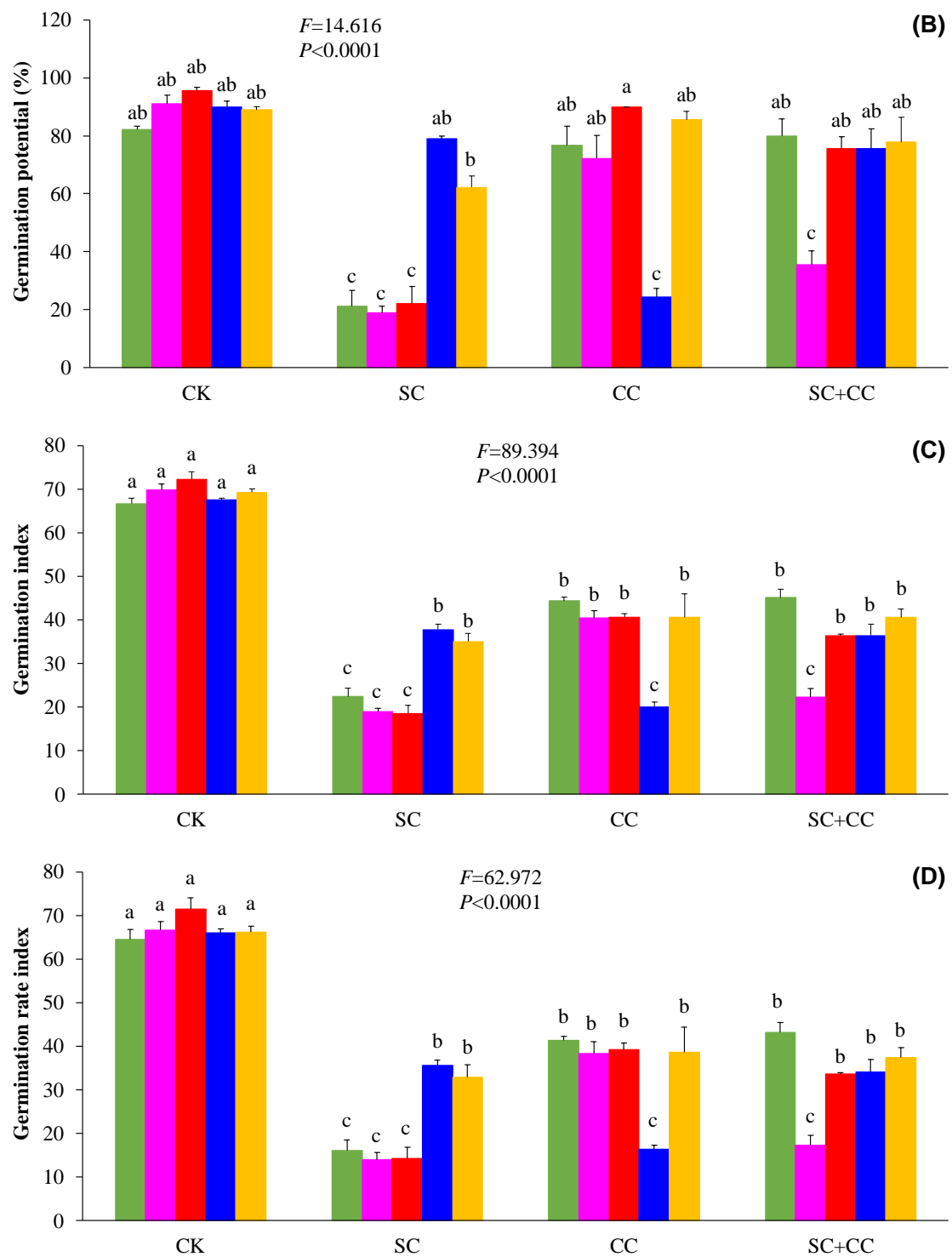
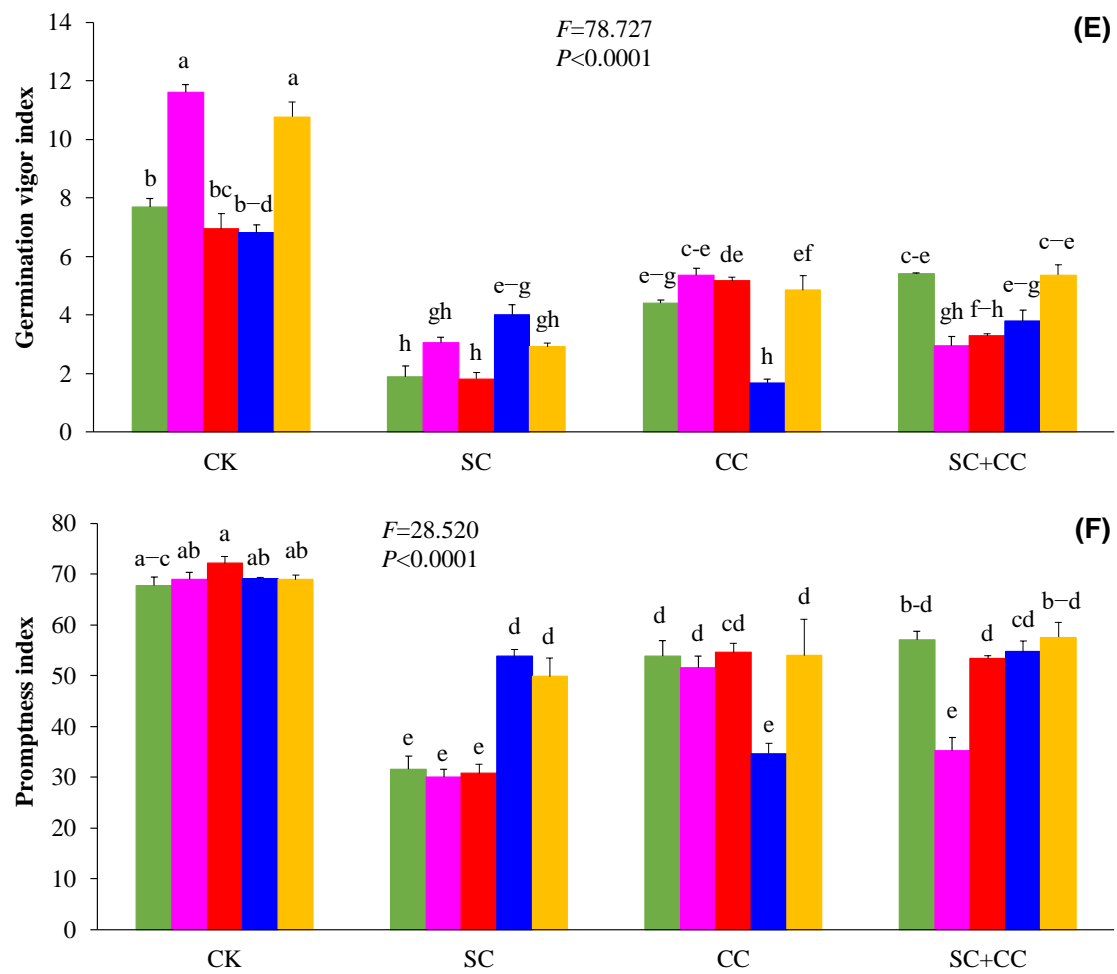
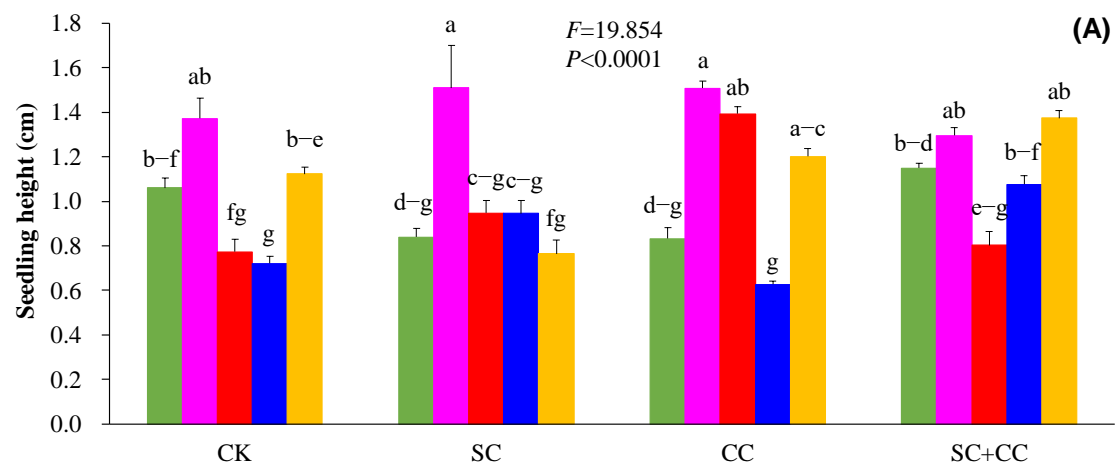


Figure 1. Cont.



**Figure 1.** The values (means and SE;  $n = 30$ ) of the seed germination indicators (A, germination percentage; B, germination potential; C, germination index; D, germination rate index; E, germination vigor index; F, promptness index) of lettuce (green bars, control; pink bars,  $\text{NO}_3\text{-N}$ ; red bars,  $\text{NH}_4\text{-N}$ ; blue bars,  $\text{CO}(\text{NH}_2)_2\text{-N}$ ; orange bars, the mixed nitrogen forms). Bars with different letters indicate statistically significant differences ( $p < 0.05$ ). Abbreviations: CK, control; SC, leaf water extract of *S. canadensis*; CC, leaf water extract of *C. canadensis*; SC+CC, the extract mixture of *S. canadensis* and *C. canadensis* in equal proportions.



**Figure 2.** Cont.

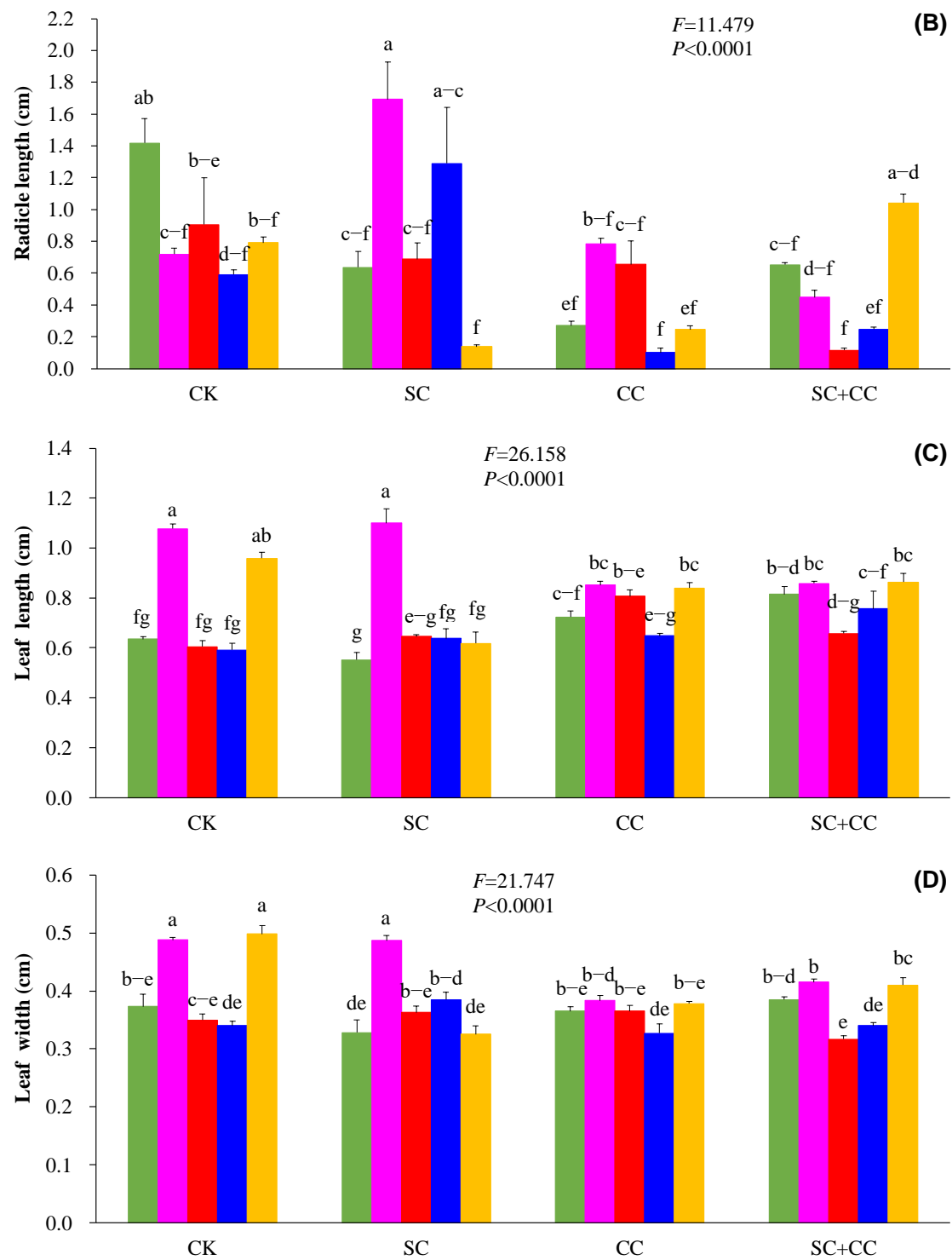


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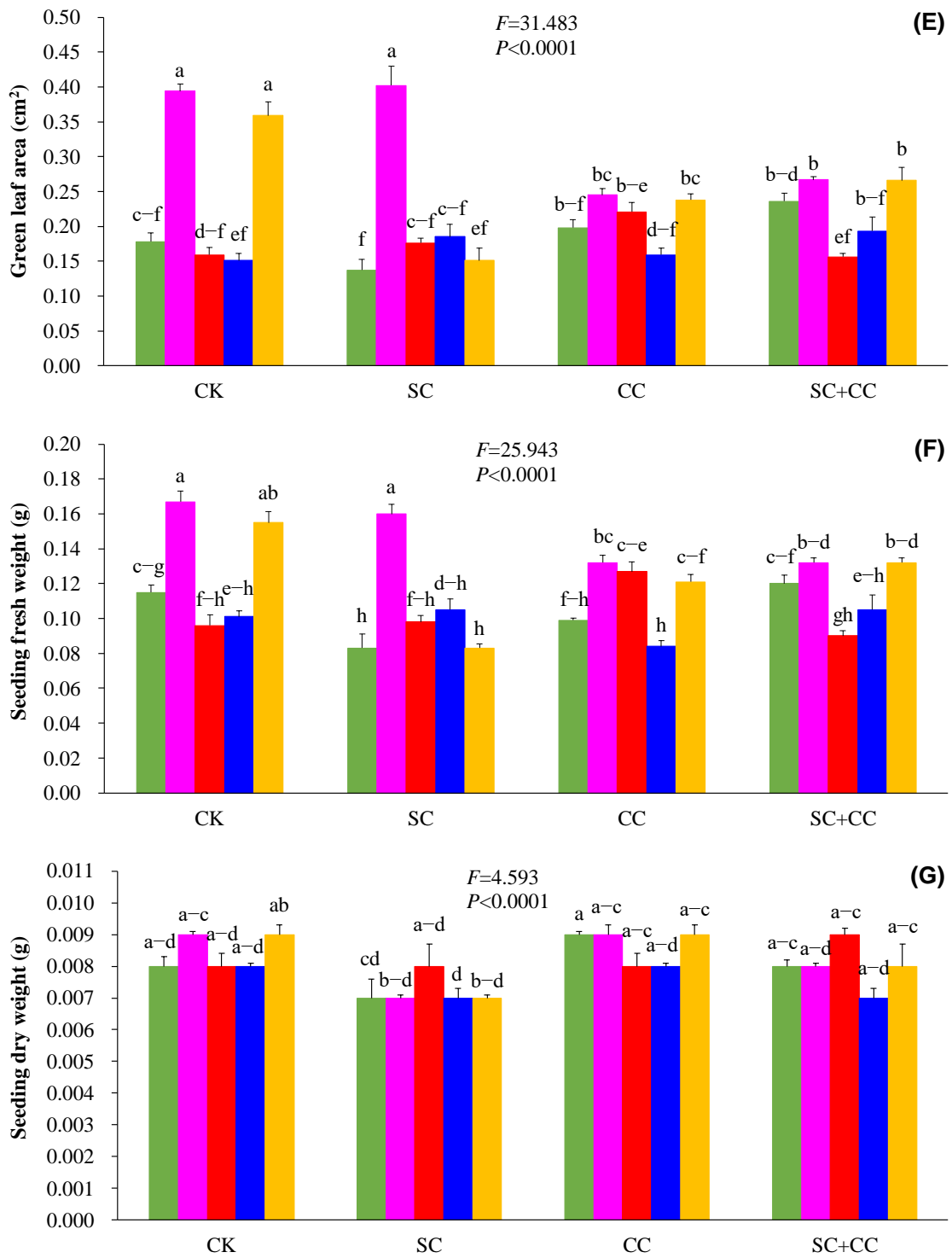
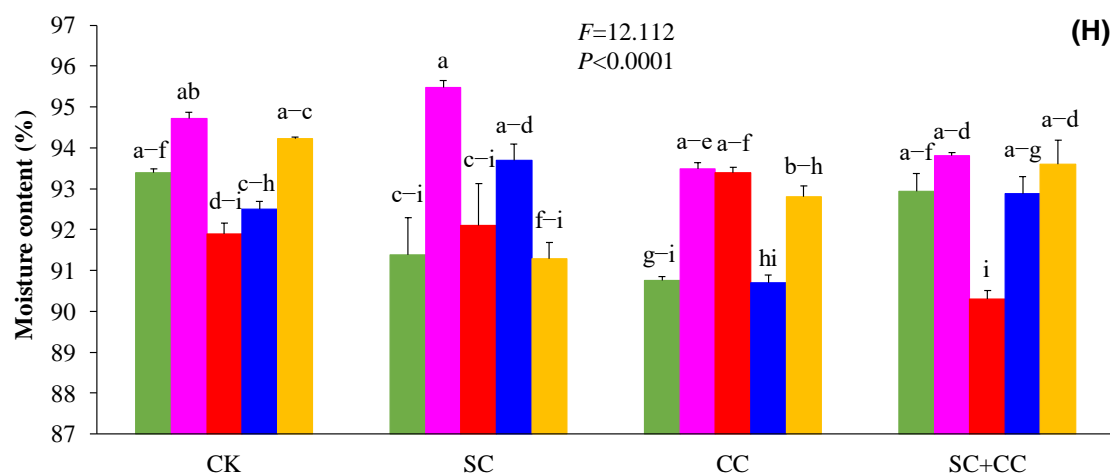


Figure 2. Cont.





**Figure 2.** The values (means and SE;  $n = 30$ ) of the seedling growth indicators (A, seedling height; B, radicle length; C, leaf length; D, leaf width; E, green leaf area; F, seedling fresh weight; G, seedling dry weight; H, moisture content) of lettuce (green bars, control; pink bars,  $\text{NO}_3\text{-N}$ ; red bars,  $\text{NH}_4\text{-N}$ ; blue bars,  $\text{CO}(\text{NH}_2)_2\text{-N}$ ; orange bars, the mixed nitrogen forms). Bars with different letters indicate statistically significant differences ( $p < 0.05$ ). Abbreviations: CK, control; SC, leaf water extract of *S. canadensis*; CC, leaf water extract of *C. canadensis*; SC+CC, the extract mixture of *S. canadensis* and *C. canadensis* in equal proportions.

### 3.2. The Effects of the Individual and Combined Aqueous Leaf Extracts of the Two Alien Plants Treated with Different Types of Nitrogen

All of the combined treatments of the aqueous leaf extracts of the two alien plants regardless of the form and assuming the mean effect of nitrogen treatment significantly reduced the germination index, germination rate index, and germination vigor index of the lettuce compared with the control ( $p < 0.05$ ; Figure 1C–E). Most of the combined treatments of aqueous leaf extracts of the two alien plants regardless of the form and assuming the mean effect of nitrogen treatment significantly reduced the promptness index and radicle length of the lettuce compared with the control ( $p < 0.05$ ; Figures 1F and 2B).

### 3.3. Differences in the Effects of the Two Alien Plants Treated with the Same Type of Nitrogen

The germination percentage (22.36% lower) of lettuce following treatment with the leaf water extract of Canadian goldenrod was significantly lower than that following treatment with the leaf water extract of horseweed treated with  $\text{NO}_3\text{-N}$  ( $p < 0.05$ ; Figure 1A). The germination potential (73.84% and 50.77% lower, respectively), germination index (53.13% and 44.90% lower, respectively), germination rate index (63.48% and 54.99% lower, respectively), germination vigor index (43.09% and 44.74% lower, respectively), and promptness index (% and % lower, respectively) of lettuce following treatment with the leaf water extract of Canadian goldenrod and the extract mixture were significantly lower than those following treatment with the leaf water extract of horseweed treated with  $\text{NO}_3\text{-N}$  ( $p < 0.05$ ; Figure 1B–F).

The radicle length (53.61% and 73.52% higher, respectively) of lettuce following treatment with the leaf water extract of Canadian goldenrod was significantly higher than that following treatment with the leaf water extract of horseweed and the extract mixture treated with  $\text{NO}_3\text{-N}$  ( $p < 0.05$ ; Figure 2B). The leaf dimensions, green leaf area (39.05% and 33.58% higher, respectively), and seedling fresh weight (17.50% and 17.50% higher, respectively) of lettuce following treatment with the leaf water extract of Canadian goldenrod were significantly higher than those following the leaf water extract of horseweed and the extract mixture treated with  $\text{NO}_3\text{-N}$  ( $p < 0.05$ ; Figure 2C–F).

The germination percentage (21.84% lower) of lettuce subjected to the leaf water extract of Canadian goldenrod was significantly lower than that following treatment with the leaf water extract of horseweed treated with  $\text{NH}_4\text{-N}$  ( $p < 0.05$ ; Figure 1A). The germination

potential (21.84% and 18.07% lower, respectively), germination index (54.39% and 49.17% lower, respectively), germination rate index (63.73% and 57.59% lower, respectively), and promptness index (43.66% and 42.34% lower, respectively) of lettuce following subjection to the leaf water extract of Canadian goldenrod were significantly lower than those following subjection to the leaf water extract of horseweed and the extract mixture treated with  $\text{NH}_4\text{-N}$  ( $p < 0.05$ ; Figure 1B–D,F).

The germination vigor index (64.89% and 36.50% lower, respectively), seedling height (32.02% and 42.35% lower, respectively), and seedling fresh weight (22.83% and 29.13% lower, respectively) of lettuce following treatment with the leaf water extract of Canadian goldenrod and the extract mixture were significantly lower than those following treatment with the leaf water extract of horseweed treated with  $\text{NH}_4\text{-N}$  ( $p < 0.05$ ; Figures 1E and 2A,F). The moisture content (3.31% lower) of lettuce that had been subjected to the extract mixture was significantly lower than the seedlings that had been subjected to the leaf water extract of horseweed treated with  $\text{NH}_4\text{-N}$  ( $p < 0.05$ ; Figure 2H).

The germination potential (69.02% higher), germination index (47.11% higher), germination rate index (54.01% higher), germination vigor index (58.09% higher), and promptness index (35.60% higher) of lettuce following treatment with the leaf water extract of Canadian goldenrod and the extract mixture were significantly higher than those subjected to the leaf water extract of horseweed treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  ( $p < 0.05$ ; Figure 1B–F).

The seedling height (12.26% higher) and moisture content (0.88% higher) of lettuce subjected to the extract mixture were significantly higher than those subjected to the leaf water extract of Canadian goldenrod treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  ( $p < 0.05$ ; Figure 2A,H). The radicle length (80.85% higher) of lettuce subjected to the leaf water extract of Canadian goldenrod was significantly higher than that when subjected to the leaf water extract of Canadian goldenrod and the extract mixture treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  ( $p < 0.05$ ; Figure 2B).

The germination vigor index (39.99% and 45.72% lower, respectively), seedling height (36.36% and 44.36% lower, respectively), leaf length (26.43% and 28.39% lower, respectively), green leaf area (36.55% and 43.23% lower, respectively), and seedling fresh weight (31.40% and 37.12% lower, respectively) of lettuce subjected to the leaf water extract of Canadian goldenrod were significantly lower than those subjected to the leaf water extract of horseweed and the extract mixture treated with the mixed nitrogen forms ( $p < 0.05$ ; Figures 1E and 2A,C,E,F).

The radicle length (86.85% lower), leaf width (20.73% lower), and moisture content (2.47% lower) of lettuce subjected to the leaf water extract of Canadian goldenrod were significantly lower than those subjected to the extract mixture treated with the mixed nitrogen forms ( $p < 0.05$ ; Figure 2B,D,H).

### 3.4. Differences in the Effects of the Two Alien Plants Treated with Different Types of Nitrogen

The germination percentage (22.36% and 21.43% lower, respectively) of lettuce following treatment with the leaf water extract of Canadian goldenrod treated with  $\text{NO}_3\text{-N}$  was significantly lower than that treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  and the mixed nitrogen forms ( $p < 0.05$ ; Figure 1A).

The germination potential, germination index, germination rate index, and promptness index of lettuce following treatment with the leaf water extract of Canadian goldenrod treated with  $\text{NO}_3\text{-N}$  and  $\text{NH}_4\text{-N}$  were significantly lower than those treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  and the mixed nitrogen forms ( $p < 0.05$ ; Figure 1B–D,F). The germination vigor index (54.58% lower) of lettuce subjected to the leaf water extract of Canadian goldenrod treated with  $\text{NH}_4\text{-N}$  was significantly lower than that treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  ( $p < 0.05$ ; Figure 1E).

The seedling height (37.28%, 37.42%, and 49.34% lower under  $\text{NH}_4\text{-N}$ ,  $\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms, respectively), leaf dimensions, green leaf area (56.22%, 53.98%, and 62.44% lower under  $\text{NH}_4\text{-N}$ ,  $\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms, respectively), and seedling fresh weight (38.75%, 34.38%, and 48.13% lower under  $\text{NH}_4\text{-N}$ ,

$\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms, respectively) of lettuce subjected to the leaf water extract of Canadian goldenrod treated with  $\text{NO}_3\text{-N}$  were significantly lower than those treated with the other three types of nitrogen solutions ( $p < 0.05$ ; Figure 2A,C–F).

The radicle length (59.40% and 91.90% lower, respectively) and moisture content (3.53% and 4.39% lower, respectively) of lettuce subjected to the leaf water extract of Canadian goldenrod treated with  $\text{NO}_3\text{-N}$  were significantly lower than those treated with  $\text{NH}_4\text{-N}$  and the mixed nitrogen forms ( $p < 0.05$ ; Figure 2B,H). Similarly, the radicle length (89.38% lower) and moisture content (2.58% lower) of lettuce subjected to the leaf water extract of Canadian goldenrod treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  were significantly lower than those treated with the mixed nitrogen forms ( $p < 0.05$ ; Figure 2B).

All of the seed germination indicators except the germination percentage, seedling height, and moisture content of lettuce when subjected to the leaf water extract of horseweed treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  were significantly lower than those treated with the other three types of nitrogen solutions ( $p < 0.05$ ; Figures 1B–F and 2A,H). The leaf length (23.94% and 22.86% higher, respectively) of lettuce subjected to the leaf water extract of horseweed treated with  $\text{NO}_3\text{-N}$  and the mixed nitrogen forms was significantly higher than that treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  ( $p < 0.05$ ; Figure 2C).

The green leaf area (14.62% higher) of lettuce when treated with the leaf water extract of horseweed treated with  $\text{NO}_3\text{-N}$  was significantly higher than that treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  ( $p < 0.05$ ; Figure 2E). The moisture content (2.97% and 2.88% higher, respectively) of lettuce subjected to the leaf water extract of horseweed treated with  $\text{NO}_3\text{-N}$  and  $\text{NH}_4\text{-N}$  was significantly higher than that treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  ( $p < 0.05$ ; Figure 2H).

The germination potential (52.94%, 52.94%, and 54.28% lower under  $\text{NH}_4\text{-N}$ ,  $\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms, respectively), germination index (38.87%, 38.82%, and 45.06% lower under  $\text{NH}_4\text{-N}$ ,  $\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms, respectively), germination rate index (48.72%, 49.38%, and 53.97% lower under  $\text{NH}_4\text{-N}$ ,  $\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms, respectively), and promptness index (33.91%, 35.62%, and 38.78% lower under  $\text{NH}_4\text{-N}$ ,  $\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms, respectively) of lettuce when subjected to the extract mixture treated with  $\text{NO}_3\text{-N}$  were significantly lower than those treated with the other three types of nitrogen solutions ( $p < 0.05$ ; Figure 1B–D,F).

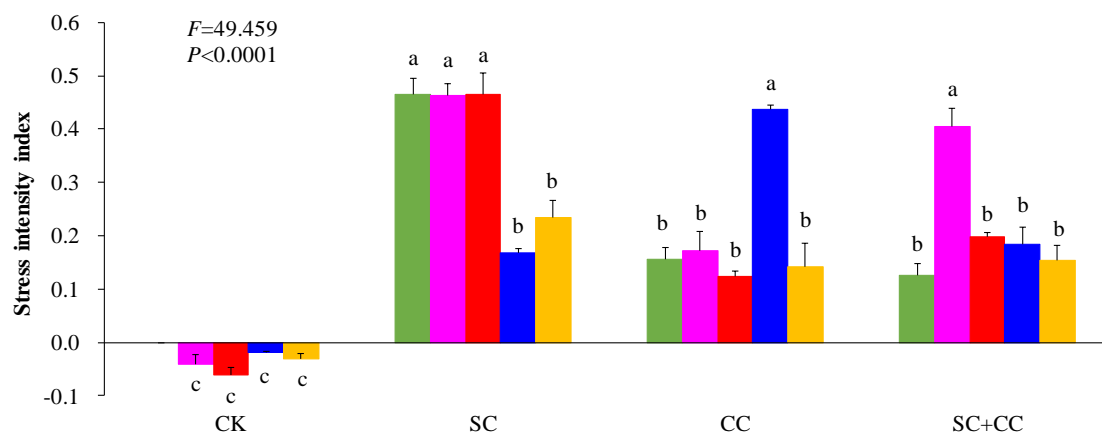
The germination vigor index (44.86% and 38.80% lower, respectively) of lettuce subjected to the extract mixture treated with  $\text{NO}_3\text{-N}$  and  $\text{NH}_4\text{-N}$  was significantly lower than that treated with the mixed nitrogen forms ( $p < 0.05$ ; Figure 1E). The seedling height (37.99% higher), leaf length (23.31% higher), and green leaf area (41.57% higher) of lettuce subjected to the extract mixture treated with  $\text{NO}_3\text{-N}$  and the mixed nitrogen forms were significantly higher than those treated with  $\text{NH}_4\text{-N}$  ( $p < 0.05$ ; Figure 2A,C,E).

The radicle length (5.82% and 21.67% lower, respectively) of lettuce subjected to the extract mixture treated with  $\text{NH}_4\text{-N}$  and  $\text{CO}(\text{NH}_2)_2\text{-N}$  was significantly lower than that treated with the mixed nitrogen forms ( $p < 0.05$ ; Figure 2B). The leaf width and seedling fresh weight of lettuce subjected to the extract mixture treated with  $\text{NO}_3\text{-N}$  and the mixed nitrogen forms were significantly higher than those treated with  $\text{NH}_4\text{-N}$  and  $\text{CO}(\text{NH}_2)_2\text{-N}$  ( $p < 0.05$ ; Figure 2D,F).

The moisture content (3.76%, 2.78%, and 3.53% lower under  $\text{NO}_3\text{-N}$ ,  $\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms, respectively) of lettuce subjected to the extract mixture treated with  $\text{NH}_4\text{-N}$  was significantly lower than that treated with the other three types of nitrogen solutions ( $p < 0.05$ ; Figure 2H).

### 3.5. The Stress Intensity of Different Treatments on the Indicators of Lettuce

The value of the stress intensity index under the four types of nitrogen solution with different nitrogen forms was less than zero (Figure 3). However, the value of the stress intensity index under all the combined treatments of aqueous leaf extracts of the two alien plants regardless of the form and assuming the mean effect of nitrogen treatment was higher than zero (Figure 3).



**Figure 3.** The values (means and SE;  $n = 30$ ) of the stress intensity index of different treatments on the indicators of lettuce (green bars, control; pink bars,  $\text{NO}_3\text{-N}$ ; red bars,  $\text{NH}_4\text{-N}$ ; blue bars,  $\text{CO}(\text{NH}_2)_2\text{-N}$ ; orange bars, the mixed nitrogen forms). Bars with different letters indicate statistically significant differences ( $p < 0.05$ ). Abbreviations: CK, control; SC, leaf water extract of *S. canadensis*; CC, leaf water extract of *C. canadensis*; SC+CC, the extract mixture of *S. canadensis* and *C. canadensis* in equal proportions.

The value of the stress intensity index when treated with the leaf water extract from Canadian goldenrod was significantly higher than that subjected to the leaf water extract of horseweed and the extract mixture treated with the control and  $\text{NH}_4\text{-N}$  ( $p < 0.05$ ; Figure 3). The value of the stress intensity index following treatment with the leaf water extract of Canadian goldenrod and the extract mixture was significantly higher than that after subjection to the leaf water extract of horseweed treated with  $\text{NO}_3\text{-N}$  ( $p < 0.05$ ; Figure 3).

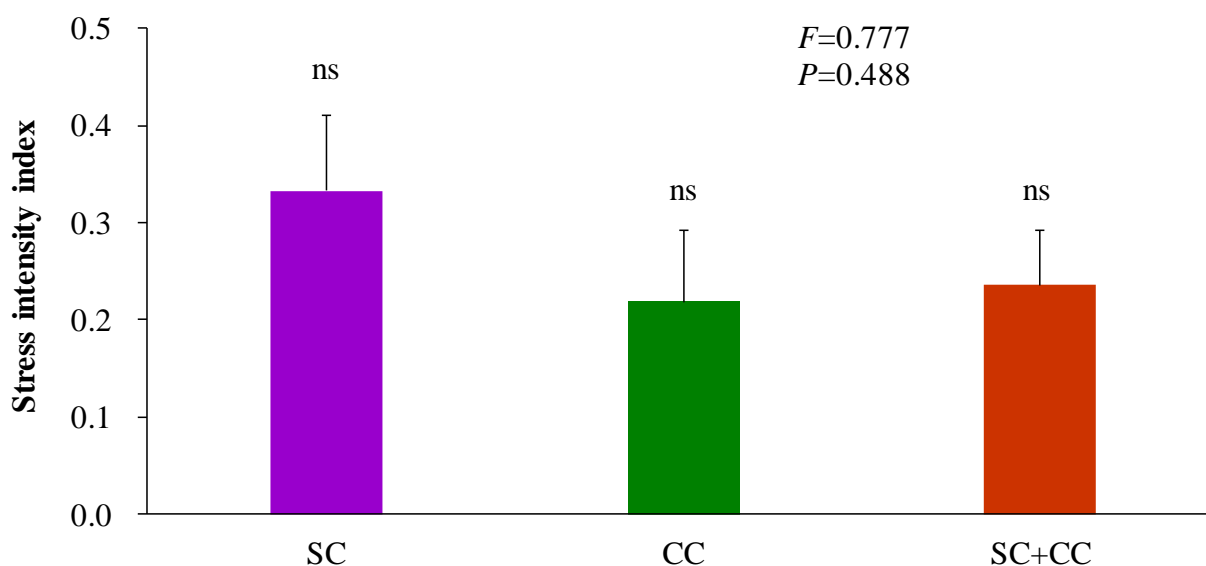
There was no statistically significant difference in the value of the stress intensity index after the seedlings were subjected to the aqueous leaf extracts of the two alien plants with different forms treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  and the mixed nitrogen forms ( $p > 0.05$ ; Figure 3).

The value of the stress intensity index after subjection to the leaf water extract of Canadian goldenrod treated with the control,  $\text{NO}_3\text{-N}$ , and  $\text{NH}_4\text{-N}$  was significantly higher than that treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  and the mixed nitrogen forms ( $p < 0.05$ ; Figure 3). The value of the stress intensity index when subjected to the leaf water extract of horseweed treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  was significantly higher than that treated with the control,  $\text{NO}_3\text{-N}$ ,  $\text{NH}_4\text{-N}$ , and the mixed nitrogen forms ( $p < 0.05$ ; Figure 3). The value of the stress intensity index under the extract mixture treated with  $\text{NO}_3\text{-N}$  was significantly higher than that treated with the control,  $\text{NH}_4\text{-N}$ ,  $\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms ( $p < 0.05$ ; Figure 3).

There was no statistically significant difference in the mean values of the stress intensity index of the aqueous leaf extracts of the two alien plants under the four types of nitrogen solutions that contained different nitrogen forms ( $p > 0.05$ ; Figure 4).

### 3.6. The Effects of the Form of Nitrogen Solution, the Type of Aqueous Leaf Extracts, and Their Interactions on the Indicators of Lettuce

The results of the two-way ANOVA analysis indicated that the form of nitrogen solution significantly affected all indicators of lettuce ( $p < 0.01$ ; Table S2). Similarly, the type of aqueous leaf extracts significantly affected all indicators of lettuce ( $p < 0.05$ ; Table S2). In addition, the interactions of the form of nitrogen solution and the type of aqueous leaf extracts significantly affected all indicators (except for the seedling dry weight) of lettuce ( $p < 0.05$ ; Table S2).



**Figure 4.** The mean values (means and SE;  $n = 4$ ) of the stress intensity index of the aqueous leaf extracts of the two alien plants under the four types of nitrogen solution with different nitrogen forms. “ns” means no statistically significant difference ( $p > 0.05$ ). Abbreviations: CK, control; SC, leaf water extract of *S. canadensis*; CC, leaf water extract of *C. canadensis*; SC+CC, the extract mixture of *S. canadensis* and *C. canadensis* in equal proportions.

#### 4. Discussion

The allelopathy of alien plants has been regarded as one of the essential factors that directly contributes to the success of their invasion [28–30]. Canadian goldenrod and horseweed meet this criterion, which is also critical for the success of their invasion [26,27,29]. Similarly, the allelopathy of the two alien plants significantly reduced the seed germination and seedling growth of lettuce in this study, thus, facilitating the process of their invasion.

The reduced seed germination and seedling growth of lettuce could be due to the allelochemicals that are produced and secreted by the two alien plants. The allelochemicals can act as growth inhibitors and affect plants by decreasing the amounts of cell division, metabolic rate, and nutrient absorption on the plants tested, particularly the seed germination and seedling growth of plants in most cases [4,5,32]. Thus, the results validate the Novel Weapons Hypothesis [33–35].

Although the allelopathy of the two alien plants can significantly decrease the seed germination and seedling growth of lettuce, there were statistically significant differences in the allelopathy of the two alien plants in this study. The leaf water extract of Canadian goldenrod had a stronger allelopathic effect on the seed germination and seedling growth of lettuce than the leaf water extract of horseweed.

Moreover, the leaf water extract of Canadian goldenrod created a higher stress intensity on the seed germination and seedling growth of lettuce than the leaf water extract of horseweed treated with the control and  $\text{NH}_4\text{-N}$ . The major issue could be due to the differences in the amounts of chemicals secreted and the chemical components of the allelochemicals of the two alien plants because of their different secondary metabolism pathways.

The findings could also be due to the higher contents of allelochemicals in Canadian goldenrod leaves than those in the leaves of horseweed. Thus, allelopathy is presumed to be more important in affecting the successful invasion of Canadian goldenrod than that of horseweed [27]. This finding also confirms that Canadian goldenrod allocates more resources into the production and secretion of allelochemicals compared with horseweed.

Two alien plants can co-invade the same habitat in nature [1,3,12]. Thus, the leaves of two alien plants may coexist in the same habitat. However, the mixture of the leaves of two alien plants with different qualities can likely alter their allelopathy primarily owing to changes in the amounts secreted and the chemical components of allelochemicals [27]. In

contradiction to the first hypothesis, the leaf water extract of Canadian goldenrod triggered stronger allelopathy on the seed germination and seedling growth of lettuce than the extract mixture in this study.

In addition, the leaf water extract of Canadian goldenrod generated more intense stress than the extract mixture treated with the control and  $\text{NH}_4\text{-N}$  in this study. However, there was no statistically significant difference in the stress intensity following the subjection to the aqueous leaf extracts of the two alien plants with different forms treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  and the mixed nitrogen forms in this study. Thus, there was an antagonistic effect for the combined allelopathy of the two alien plants compared with their individual allelopathy.

Previous studies have produced similar results [27]. Therefore, the allelopathy of the two alien plants on the seed germination and seedling growth of adjacent species may be critical to their individual invasion, rather than their co-invasion [27]. The main factor that drives the antagonistic interactions of the allelopathy of the two alien plants could be due to the interference of the chemical components of their allelochemicals.

Previous studies also revealed that the antagonism of the combination of two alien plants is primarily owing to their invasional interference [12,13,36]. More importantly, the allelopathy of Canadian goldenrod, rather than that of horseweed, plays a leading role in their co-invasion. The number of species of the alien plants plays an important role in the variation of allelopathy on the seed germination and seedling growth of adjacent species according to the findings of this study.

As the nutrient that is the most intensively required by plant species, nitrogen generally promotes plant growth [14,37,38]. Similarly, the value of the stress intensity index under the four types of nitrogen solution with different nitrogen forms is negative. Thus, a solution of nitrogen with different forms of nitrogen increases the seed germination and seedling growth of lettuce in this study. Moreover, the addition of moderate amounts of nitrogen can alleviate the negative effects of environmental stress on plant growth by the enhancement in resistance under stress [19,37,39].

Thus, it is expected that the addition of nitrogen can reduce the allelopathy of the two alien plants on the seed germination and seedling growth of lettuce. In contrast to the second hypothesis and previous results [19,20,22], the addition of nitrogen intensified the allelopathy of the two alien plants on the seed germination and seedling growth of lettuce in this study. In addition, the value of the stress intensity index under all the combined treatments of aqueous leaf extracts of the two alien plants combined, regardless of the form and assuming the mean effect of nitrogen treatment, is positive in this study.

Thus, the allelopathy of the two alien plants on the seed germination and seedling growth of lettuce will be reinforced under the addition of nitrogen and then facilitate their invasion process. Previous studies also rendered similar conclusions [21,26,40]. The main reason could be due to the induction in acidification by the addition of exogenous nitrogen through the decrease in buffering capacity that can significantly inhibit plant growth, particularly the absorption and utilization of nutrients [26,41,42].

The leaf water extract of Canadian goldenrod treated with  $\text{NO}_3\text{-N}$  and  $\text{NH}_4\text{-N}$  caused more stress to the seed germination and seedling growth of lettuce than those treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  and the mixed nitrogen forms. In contrast, the leaf water extract of horseweed treated with  $\text{CO}(\text{NH}_2)_2\text{-N}$  caused more stress to the seed germination and seedling growth of lettuce than those treated with  $\text{NO}_3\text{-N}$ ,  $\text{NH}_4\text{-N}$ , and the mixed nitrogen forms. The extract mixture treated with  $\text{NO}_3\text{-N}$  induced more stress on the seed germination and seedling growth of lettuce than those treated with  $\text{NH}_4\text{-N}$ ,  $\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms in this study.

Thus, the degree of influence of  $\text{NO}_3\text{-N}$  and  $\text{NH}_4\text{-N}$  on the allelopathy of Canadian goldenrod was significantly higher than that of  $\text{CO}(\text{NH}_2)_2\text{-N}$  and the mixed nitrogen forms, while the degree of influence of  $\text{CO}(\text{NH}_2)_2\text{-N}$  on the allelopathy of horseweed was significantly higher than that of  $\text{NO}_3\text{-N}$ ,  $\text{NH}_4\text{-N}$ , and the mixed nitrogen forms. The

degree of influence of  $\text{NO}_3\text{-N}$  on the combined allelopathy of the two alien plants was significantly higher than that of  $\text{NH}_4\text{-N}$ ,  $\text{CO}(\text{NH}_2)_2\text{-N}$ , and the mixed nitrogen forms.

Thus, the findings do not verify the third hypothesis. The reason could be attributed to the differences in the amounts secreted and chemical components of the allelochemicals of the two alien plants and the degree of influence of nitrogen solution with different nitrogen forms on the varying components in the allelochemicals [20,26]. Moreover, the findings may also be ascribed to the differences in the amount of nitrogen absorbed by lettuce.

However, there was no statistically significant difference in the mean stress intensity on the seed germination and seedling growth of lettuce owing to the aqueous leaf extracts of the two alien plants under the four types of nitrogen solution with different nitrogen forms in this study. Thus, the degree of influence of the deposition of nitrogen on the individual and combined allelopathy of the two alien plants was similar.

Accordingly, the degree of influence of nitrogen deposition on the allelopathy of alien plants does not depend on the number of species of alien plants. Hence, we must reduce the use of fossil energy for industrial development and the use of nitrogenous fertilizers for crop production to decrease the level of nitrogen deposition and then limit the invasion process of alien plants. Thus, the second hypothesis cannot be validated.

The results of this study can create a strong foundation to understand the mechanism driving the co-invasion of the two alien plants under the deposition of nitrogen based on the aspect of allelopathy. This study also provides a solid theoretical guide for the prevention, control, and management of alien plants.

Nevertheless, the experimental design present in this study has certain limits, i.e., the allelochemicals driving the allelopathy of the two alien plants were not tested in this study. As in vivo, plants are under influence of multiple abiotic and biotic factors, which might affect the allelochemicals' activity. Therefore, the study design needs to be further improved—particularly, further studies still need to perform analysis of the allelochemicals driving the allelopathy and the assessment of the effects abiotic and biotic factors on the allelochemical activity, to obtain more comprehensive information about the allelopathy of the two alien plants.

## 5. Conclusions

In summary, the allelopathy of the two alien plants significantly decreased the seed germination and seedling growth of lettuce. Canadian goldenrod generated more intense allelopathy on the seed germination and seedling growth of lettuce compared with that of horseweed. The allelopathy of the two alien plants was stronger in the absence of the other alien plants compared with the effects of the combination of the allelochemicals utilized in this study.

The addition of nitrogen strengthened the allelopathy of the two alien plants for the seed germination and seedling growth of lettuce in most cases. The degree of influence of the deposition of nitrogen on the allelopathy of alien plants did not depend on the number of species of alien plants. Thus, nitrogen deposition can strengthen the allelopathy of the two alien plants on the seed germination and seedling growth of lettuce, which can facilitate the process of their invasion to proceed more aggressively.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/su141911970/s1>, Table S1. The ecological significance, measuring method, and the corresponding references for the measured seed germination and seedling growth indicators of *L. sativa*. Table S2. Two-way ANOVA showing the effect of the main factors: the form of nitrogen solution, and the type of aqueous leaf extracts, and their interactions on the indicators of lettuce. *P* values equal to or less than 0.05 are shown in bold. Figure S1. The co-invasion of *Conyza canadensis* (L.) Cronq. and *Solidago canadensis* L. in the same habitat (by Congyan Wang).

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editing, J.Z., Z.X. (Zhelun Xu), S.Z., Y.Y., Z.X. (Zhongyi Xu), and D.D.; supervision, C.W.; project administration, D.D. and C.W.; funding acquisition, D.D. and C.W. All authors have read and agreed to the published version of the manuscript.

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