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Effects of Light and Autotoxicity on the Reproduction of *Bidens pilosa* L.: From Laboratory to the Field

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Abstract: In Taiwan, the good agricultural practices for *Bidens pilosa* L. (Cobbler's pegs) had been established due to its pharmacology application. However, the reproduction of this species that may cause phytotoxicity to the subsequent crops has not been investigated extensively. We hypothesized that both the phytotoxicity and canopy shading conditions that were altered by agricultural practices might affect its seed reproduction. Three experiments from laboratory, pot and field were conducted under different light treatments and residue application rates to evaluate the light requirement and phytotoxicity on the germination of Cobbler's pegs. The results showed that the germination in the laboratory, dark treatments was higher than that of light treatment while it was inhibited in the darkness in the pot experiments (24% of the light treatments). Moreover, some seeds in the pot experiments germinated in the far-red light (FR) pretreated dark treatments. This observation indicated that the germination response of the investigated plant might be a very low fluence response (VLFR). Results also showed that the autotoxicity enhanced the germination reduction in the FR pretreated dark treatment while increasing the residues buried in the field. Accordingly, both autotoxicity and canopy shading may inhibit the reproduction of Cobbler's pegs, but the application method needs further study.

Keywords: autotoxicity; far-red light pretreatment; phytotoxicity; plant extracts; very low fluence response (VLFR)

1. Introduction

Bidens pilosa L. (Cobbler's pegs), originally from South America [1], is a member of the Asteraceae family [2]. Seeds of this pant are black and ribbed with barbed awns that facilitate the dispersal through animals. It is widely distributed across almost all over the subtropical and tropical regions [3,4] and can be classified into six varieties based on their morphological characteristics [4]. Cobbler's pegs has been reported to be highly invasive and may cause damage to many crops in China [5,6], Japan [7,8], Taiwan [9–11] and Thailand [12,13]. However, it was also an edible herb with medicinal applications in many countries. For example, it was a leaf vegetable as well as a traditional medicinal herb in many African countries such as Sierra Leone, Liberia, Côte d'Ivoire, Nigeria, Cameroon, Kenya, Tanzania, etc. [14]. In Taiwan, three varieties of Cobbler's pegs, i.e., *B. pilosa* var. *minor*, *B. pilosa* var. *pilosa* and *B. pilosa* var. *radiata*, are the important ingredients of traditional herb tea and the common wild vegetables consumed by indigenous Amis tribes [11,15–17].

Hsueh et al. [18] reported that Cobbler's pegs, as an edible and medicinal herb with phytotoxicity, possessed the potential to be introduced into the crop rotation system to enhance weed control.

Furthermore, the Taiwan Good Agricultural Practices proposed a Cobbler's pegs growing protocol due to its pharmacology application [19]. However, due to the characteristics of invasion, the effects of its natural reproduction on the subsequent crops require additional considerations before introducing this species into the crop rotation system. Studies indicated that this species can reproduce both sexually and vegetatively through seeds and cuttings, respectively [20]. In addition, the seed production of this species was found to be stimulated if the clipping interval was longer than eight weeks [8]. Therefore, for a continuous harvest crop such as Cobbler's pegs, abundant seeds may accumulate in the soil and become an important propagator at the end of production.

Seeds of most species were reported to be capable of responding well to suitable environmental signals, especially to light, for establishing seedlings in the subsequent life stage [21]. Although the germination responses to light varied depending on the species composition of the seed bank [22–24], soil disturbance by plowing or harrowing in the daytime could stimulate more weed plant emergence than that in the night time [25]. Surprisingly, as a highly invasive weed, seeds of Cobbler's pegs were reported to be able to germinate normally in the darkness as well as under the white light [26,27]; however, most of the results were obtained from the well-controlled conditions, and maybe different in the seminatural or natural conditions such as pots or fields.

The responses of phytochromes to light can be divided into low fluence response (LFR), very low fluence response (VLFR) and high irradiance response (HIR) according to the light fluence requirements of seed germination [21]. For the LFR, germination in the darkness revealed that the far-red light (FR) absorbing-form of phytochromes (P_{fr}) was present in the seeds before they were moved into the darkness (such as buried by plowing practices). Meanwhile, the germination responses can be inverted by a FR pulse (i.e., P_{fr} is photoconverted to the red light (R) absorbing-form of phytochromes (P_r) in the FR). For the VLFR, seeds could germinate with a very low level of P_{fr} (<2%) that could be induced by a very low fluence of R (moonlight, green light or a millisecond exposure to sunlight) or by a FR pulse (due to the overlap of absorbing wavelength between P_r and P_{fr}) [21,22,28].

In addition to the changes in the light environment, phytotoxicity is another factor that may affect the Cobbler's pegs germination in the field. Rashid et al. [29] found that phenolics contained in the litter of *Pueraria montana* Lour. exerted phytotoxic effects on the germination and radicle growth of Cobbler's pegs. El-Gawad et al. [30] showed that the phenolics and alkaloids released by the *Plantago major* L. and *Plantago lagopus* L., respectively, also inhibited the germination and radicle growth of Cobbler's pegs. Cobbler's pegs had been recognized as a plant that could release phytotoxins of phenolics from the leaves, branches and roots. Phenolics released from this plant had been reported to inhibit the germination and radicle growth of *Echinochloa crus-galli* L. and *Raphanus sativus* L. [7]. In addition to phenolics, the phenylheptatriyne (PHT) contained in the leaf was phototoxic to bacteria, yeast, fungi [31] and weeds [32] when illuminated with a long-wave ultraviolet light or cool white fluorescent light.

As a result, it was hypothesized that both the phytotoxicity of Cobbler's pegs residue and canopy shading conditions that were altered by agricultural practices might affect its seed reproduction in the field. To test the hypothesis, different light conditions coupled with Cobbler's pegs residues or residue extracts were employed in the experiments of the laboratory, pots and field to investigate the influence of the controlled, seminatural and natural conditions, respectively. Moreover, the results from the laboratory to the field may also provide evidence to distinguish the phytotoxicity/autotoxicity of this plant from environmental factors. In this study, five light treatments, i.e., white light, darkness, FR, FR pretreatment followed by changing to white light or darkness, were performed for seeds exposure to light, darkness (buried in the soil), canopy shade, canopy shade followed by the exposure to light (seeds exposure to light after plowing) and canopy shade followed by the exposure to the darkness (seeds buried in the soil after plowing), respectively. Different concentrations of aqueous residue extract and residue were also applied.

2. Materials and Methods

In the present study, three experiments, i.e., laboratory, pot and field, were conducted to evaluate the effects of light and autotoxicity/phytotoxicity on the reproduction of *Bidens pilosa* L. (Cobbler's pegs). For the laboratory experiment, three different light factors (light requirement, far-red light (FR) pretreatment and FR irradiation) coupled with phytotoxic treatment (aqueous residue extract) were conducted to investigate the germination potential of Cobbler's pegs in the controlled conditions. For the pot experiment, the factors of light requirement and FR irradiation coupled with phytotoxic treatment (residue) were conducted in seminatural conditions. Moreover, to obtain more experimental evidence to confirm the light response type of Cobbler's pegs phytochrome, the effects of different FR pretreatment time on the germination instead of the interaction between FR pretreatment (in single pretreatment time) and phytotoxicity in the pot experiments were investigated. For the field experiment, due to the field conditions may be affected, only FR pretreatment coupled with phytotoxic treatment (residue) were conducted to test the germination potential in the natural conditions.

2.1. Seeds and Plant Materials

B. pilosa var. *radiata* Sch. Bip. was grown in the experimental field of Taitung District of Agriculture Research and Extension Station (Taitung DARES, located at 22°44′52″ N, 121°8′59″ E), Taiwan. Seeds were harvested when the capitula were fully expanded and stored in a paper bag at room temperature. The average mass of an air-dried seed was 1.05 ± 0.32 mg. The plant materials of all aerial parts, harvested at the bloom stage, were dried in a shaded greenhouse at ambient temperature (30−35 °C) for 15 days, then shattered into small pieces (<2 cm) with an electric cutter, mixed thoroughly, and stored in a refrigerated compartment at −20 °C before use.

2.2. Laboratory Experiment: The Effects of Light and Residue Extract on Cobbler Pegs in the Controlled Conditions

2.2.1. Light Treatments

In a rotation system involving Cobbler's pegs, the fate of ripened seeds of the investigated species can be expected to have five different scenarios: (1) moving to an open field by animals (exposed to white light); (2) being buried in the soil (in the darkness); (3) being shaded by the canopy, but changing to light afterward due to external disturbance such as mowing; and (4) being shaded by the canopy, but changed to the darkness afterward due to external disturbance such as plowing; and (5) falling on the soil surface and being shaded by the canopy (exposed to the light of low red/far-red ratio (R/FR). Accordingly, the following light treatments were designed to mimic the conditions in the aforementioned scenarios: (1) incubated in white light (LED, 400–750 nm, 6500 k) with a 12-h (h) photoperiod (W_L); (2) incubated in the continuous darkness by wrapping with two layers of aluminum foil (D_L); (3) pretreated in the FR (LED, 730 nm) for 3 h then incubated in the white light with a 12-h photoperiod (FR-W_L); (4) pretreated in the FR for 3 h then incubated in the continuous darkness (FR-D_L); and (5) incubated in FR with a 12-h photoperiod (FR_L) (Table 1). The intensities of the white LED, and far-red LED light (VitaStar, Taiwan LED Lights Online, Tainan, Taiwan) measured with a light meter (LI-250A, LI-COR, Inc., Lincoln, NE, USA) were 149 and 0.71 μmol m^{-2} s^{-1}, respectively. The R/FR ratios of white LED and far-red LED light determined with an R/FR sensor (SKR 110, Skye Instruments, Ltd., Powys, UK) were 11 and 0.01, respectively.

Among these five light treatments, W_L and D_L treatments were used to evaluate the light requirement of Cobbler's pegs germination, FR-W_L and FR-D_L treatments were used to evaluate the effects of FR pretreatment on germination, while the FR_L treatment was used to evaluate the effects of FR irradiation on germination.

Table 1. The experimental conditions of the laboratory experiment.

Light Factor	Phytotoxic Treatment [1]	Light Treatment [2]	Type of Light [3]	Hour(s) of Far-Red Light (FR) Pretreatment	Photoperiod	Incubation Days
Incubation temperature 25 °C						
Light requirement	Residue extract	W_L	White LED	0	12/12	5
		D_L	Darkness	0	Continuous	5
FR pretreatment	Residue extract	FR-W_L	White LED	3	12/12	5
		FR-D_L	Darkness	3	Continuous	5
FR irradiation	Residue extract	FR_L	Far-red LED White LED	0	12/12 12/12	14 [4] 7

[1] Four concentrations of residue extract, i.e., 0, 0.02, 0.05 and 0.1 g dry weight (DW) mL^{-1}, were used in all light treatments. [2] Light treatments were (1) incubated in the white light with a 12-h (h) photoperiod (W_L); (2) incubated in the continuous darkness by wrapped with two layers of aluminum foil (D_L); (3) pretreated in the FR for 3 h then incubated in the white light with a 12-h photoperiod (FR-W_L); (4) pretreated in the FR for 3 h then incubated in the continuous darkness (FR-D_L); (5) incubated in FR with a 12-h photoperiod (FR_L). Subscript L denoted treatments that belong to the laboratory experiment. [3] The light intensities of white light (LED, 400–750 nm, 6500 k) and FR (LED, 730 nm) were 149 μmol m^{-2} s^{-1} and 0.71 μmol m^{-2} s^{-1}, respectively. The red/far-red ratios (R/FR) of white LED, and far-red LED light were 11 and 0.01, respectively. [4] Due to no seed germinated in the first 5 days, incubation continuous for 14 days (under FR irradiation) and then changed to white light for 7 days.

2.2.2. Residue Extract Preparation

Residues of Cobbler's pegs were shattered into small pieces (<2 cm) with an electric cutter, and then soaked in distilled water at 1:10 ratio (*w*/*v*) and shaken by an oscillating vibrator in the darkness for 24 h at room temperature (25–30 °C). Residue extracts were collected by filtering through cheesecloth and centrifuged at 3000 rpm for 30 min. To reduce the influence of microbial activities on the phytotoxins, the supernatant was vacuum-filtered through a 0.2 μm membrane filter (Advantec, Toyo Roshi Kaisha, Ltd., Japan) and diluted with distilled water to the concentrations of 0.1, 0.05 and 0.02 g DW mL^{-1} for further application. All the water extracts were prepared one day before use.

2.2.3. Germination Condition

Seeds were presterilized with 0.5% sodium hypochlorite (NaOCl) for 10 min and washed with distilled water 5 times before germination assay [10]. Two layers of filter paper (Advantec No.1, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) were placed in a 9 cm-diameter Petri dish and moistened with 5 mL distilled water or residue extracts. Each Petri dish contained 30 seeds and was sealed with parafilm and placed under different light treatments at 25 °C for 5 days. This experiment was conducted with a completely randomized design with 5 replicates for each treatment. Seeds were considered germinated when the radicle could be observed visually. Germination percentage was inspected daily under green light, which was obtained by wrapping the fluorescent lamp with 4 layers of green cellophane. The wavelength of maximum absorbance of the green light was 522 nm, which was measured by a portable spectrometer (Jaz Modular, Ocean Optics, Inc., Dunedin, FL, USA). The germination experiment of FR_L treatment was continued for 14 days due to the lack of seed germination in the 5-day incubation, and the light source was changed to white LED for the following 7 days (i.e., a total duration of germination in FR_L treatment was 21 days). The germination percentage of FR_L was investigated 5 and 7 days after changing the light source to white LED.

2.2.4. Radicle Length Measurement

After 5 days of incubation, all the germinated seeds in each treatment were carefully pulled out from the filter paper with the help of a tweezer and an appropriate amount of water. After this, seeds with radicle longer than 2 mm were measured by an electronic vernier caliper (TESA-CAL IP67, TESA Tech., Renens, Switzerland) with an accuracy of 0.01 mm. Radicle length was measured immediately to prevent dehydration, which may result from snapping.

2.3. Pot Experiment: The Effects of Light and Residue on Cobbler Pegs in the Seminatural Conditions

The pot experiment with soil substrate was conducted to obtain the germination responses to different light factors and Cobbler's pegs residue in a seminatural condition. Soils used in the pot experiments were collected from the experimental field of Taitung DARES and the initial conditions were: pH = 6.35, EC = 0.07 dS cm^{-1}, P_2O_5 = 116.01 mg kg^{-1}, K_2O = 95.49 mg kg^{-1} and organic matter = 3.57%. Collected soils were used, placed in 3-inch plastic pots and mixed with ground residues (0 or 2 g pot^{-1}). Each pot contained 30 seeds on the soil surface and was incubated under the white light (W_P), darkness (D_P) and FR (FR_P) (Table 2). W_P and D_P treatments were used to evaluate the effects of light requirement and residue on the germination, while FR_P treatment was used to evaluate the effects of FR irradiation and residue on the germination. The treatments of W_P, D_P and FR_P were conducted with a completely randomized design with 4 replicates. Furthermore, to obtain more pot experimental evidence to confirm the light response type of Cobbler's pegs phytochrome, seed germination responses to the different time length of FR pretreatment followed by incubation under light or darkness were evaluated. Thirty seeds per pot (3-inch plastic pot) were sowed on the soil surface and pretreated (i.e., imbibed and incubated) under FR for 0, 3 or 24 h. After FR pretreatment, one-half of the pots of each pretreatment were incubated under the white light (12-h photoperiod), while the other half was incubated in the darkness (wrapped with double aluminum foil) (Table 2). The FR pretreatments were conducted with a completely randomized design with 6 replicates. The pot experiment was conducted in the growth chamber at 25 °C (Table 2). Seed germination percentages were investigated after 7 days of incubation.

Table 2. The experimental conditions of the pot experiment.

Conditions of The Growth Chamber						
Light Factor	**Phytotoxic Treatment** [1]	**Light Treatment** [2]	**Type of Light** [3]	**Hour(s) of FR Pretreatment**	**Photoperiod**	**Incubation Days**
Incubation temperature 25 °C						
Light requirement	Residue	W_P	White LED	0	12/12	7
		D_P	Darkness	0	Continuous	7
Different FR pretreatment time	N/A	FR0-W_P	White LED	0	12/12	7
		FR0-D_P	Darkness	0	Continuous	7
		FR3-W_P	White LED	3	12/12	7
		FR3-D_P	Darkness	3	Continuous	7
		FR24-W_P	White LED	24	12/12	7
		FR24-D_P	Darkness	24	Continuous	7
FR irradiation	Residue	FR_P	Far-red LED	0	12/12	7
Conditions of The Soil						
pH	**EC (dS cm^{-1})**	**P_2O_5 (mg kg^{-1})**		**K_2O (mg kg^{-1})**	**Organic Matter (%)**	
6.35	0.07	116.01		95.49	3.57	

[1] Two residue application rates, i.e., 0 and 2 g pot^{-1}, were used in the light factors of light requirement and FR irradiation. No residue was used in the treatments of different FR pretreatment time. [2] W, D and FR were defined as the incubation light environments of white light, darkness and FR, respectively; FR0, FR3 and FR24 were defined as 0-, 3- and 24-h FR pretreatment, respectively; and subscript P denoted treatments that belong to the pot experiment. [3] The light intensities of white light (LED, 400–750 nm, 6500 k) and FR (LED, 730 nm) were 149 μmol m^{-2} s^{-1} and 0.71 μmol m^{-2} s^{-1}, respectively. The red/far-red ratios (R/FR) of white LED, and far-red LED light were 11 and 0.01, respectively.

2.4. Field Experiment: The Effects of FR "Pretreatment" and Residues on Cobbler Pegs in the Natural Conditions

The field experiment was conducted in Taitung DARES to investigate the response of Cobbler's pegs seed germination to different plant residues after FR or dark pretreatment. The experimental field was divided into 8 plots (4.8 m in length and 3 m in width). All the plots were plowed, and 4 of them were sowed with 20 g Cobbler's pegs seeds; as the control treatment, the weeds in the soil bank were allowed to germinate and grow in the other 4 plots. All the aboveground biomass of the experimental plots was weighted and incorporated into the soil 3 months after the experiments took place.

To prevent the interference of soil seed bank, 100 Cobbler's pegs seeds per polyester gauze bag were pretreated (imbibed and incubated) under FR or darkness for 3 h and then buried in the field (about 1 cm in depth) at night. For each pretreatment (FR and darkness), half of the bags were buried in the plots that incorporated with Cobbler's pegs residues (FRB_F and DB_F for FR and dark pretreatment, respectively) and the other half of the bags were placed in the plots that incorporated with weed residues (FRW_F and DW_F for FR and dark pretreatment, respectively). Each plot contained 3 seed bags (Figure 1). The field experiment was conducted with a randomized design with 4 replicates. Seed germination was quantified after 7-day incubation.

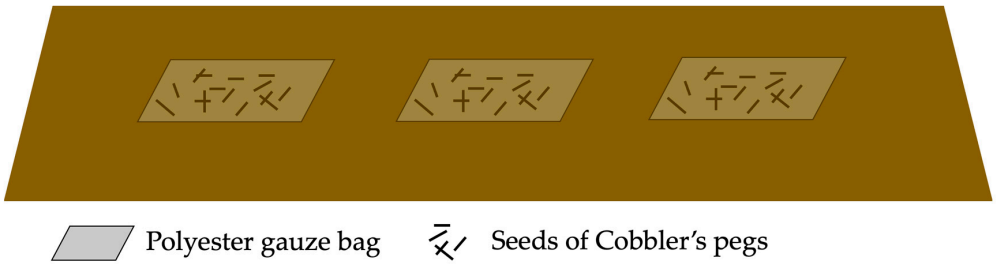

Figure 1. A diagram to illustrate the field placement of the seed bag of *Bidens pilosa* L. (Cobbler's pegs). The seed bag of Cobbler's pegs was buried in the soil about 1 cm in depth. The germination percentage of each bag was calculated after 7 days of field incubation.

2.5. Statistical Analysis

Prior to proceeding to ANOVA, the Levene test was conducted to test the homogeneity of variance of the data in this study. Data in each experiment were subjected to the analyses of variances (ANOVA) with Fisher's least significant difference (LSD) test (at 5% level of significance) by the SAS software (SAS Enterprise Guide 7.1, SAS Institute, Inc., Cary, NC, USA). Germination percentage data were arcsine-square-root transformed prior to subsequent analysis [33]. However, data of germination percentage and radicle length in D_L and W_L treatment of the laboratory experiment, respectively, and data of germination percentage in the field experiment were analyzed by Kruskal–Wallis nonparametric rank test and Dunn's post hoc comparison test (IBM SPSS Statistics V25, IBM Crop., Armonk, NY, USA) since these data did not pass the Levene test.

3. Results

3.1. The Effects of Light and Residue Extract on Bidens pilosa L. (Cobbers Pegs) in the Controlled Conditions

3.1.1. Light Requirement of Germination

For the residue extract of 0 g DW mL^{-1}, the highest seed germination percentage was found in the D_L treatment, followed by FR-W_L, W_L, FR-D_L and FR_L treatments. The results of the laboratory experiment showed that no light requirement was found in the germination of Cobbler's pegs (Figure 2A,B). For the effects of residue extract, the results indicated that seed germination showed more affected by residue extract than by light treatments. Except for the extract of 0.02 g DW mL^{-1} in W_L treatment, the germination was decreased as the extract concentration increased and was almost inhibited at the highest concentration (0.1 g DW mL^{-1}).

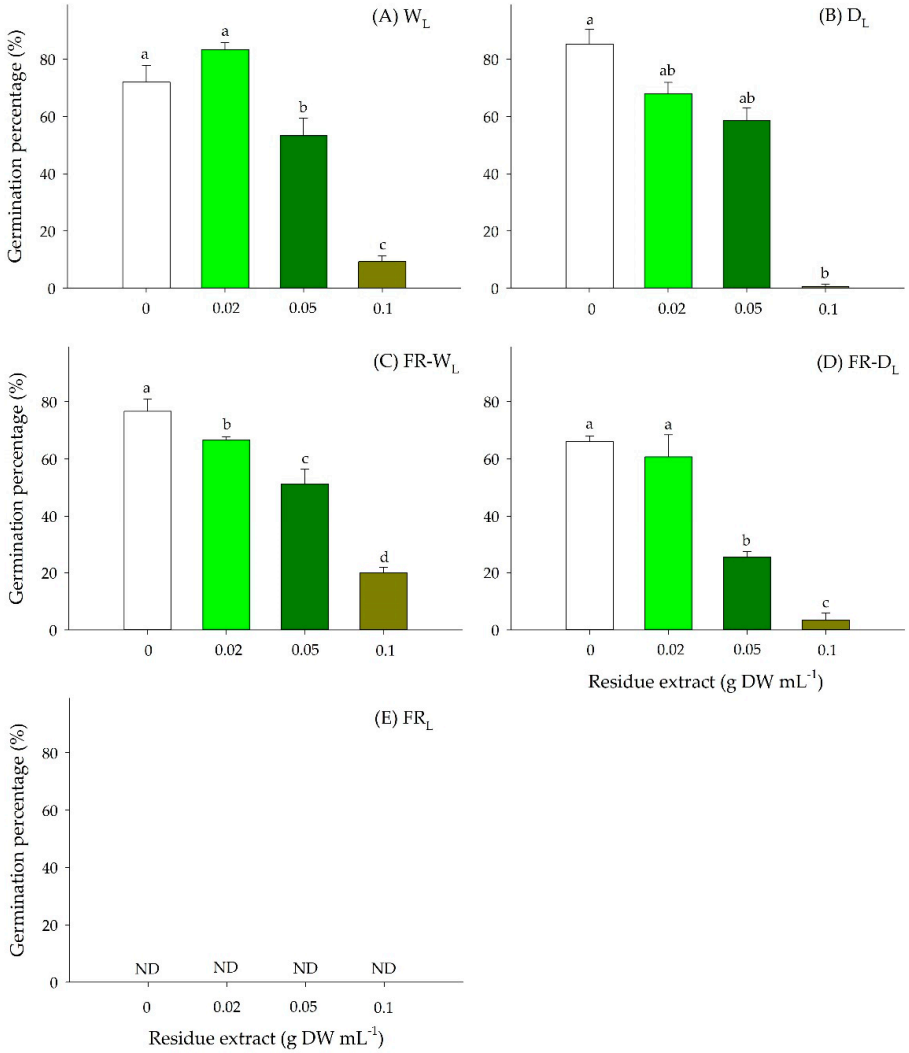

Figure 2. Seed germination percentage (%) of *Bidens pilosa* L. (Cobbler's pegs) in four concentrations of residue extract (0, 0.02, 0.05 or 0.1 g dry weight (DW) mL^{-1}) and under five different light treatments in the laboratory experiment. Light treatments were (**A**) incubated in the white light with a 12-h (h) photoperiod (W_L); (**B**) incubated in the continuous darkness by wrapped with two layers of aluminum foil (D_L); (**C**) pretreated in the far-red light (FR) for 3 h then incubated in the white light with a 12-h photoperiod (FR-W_L); (**D**) pretreated in the FR for 3 h then incubated in the continuous darkness (FR-D_L); (**E**) incubated in FR with a 12-h photoperiod (FR_L). The light intensities of white light (LED, 400–750 nm, 6500 k) and FR (LED, 730 nm) were 149 μmol m^{-2} s^{-1} and 0.71 μmol m^{-2} s^{-1}, respectively. The red/far-red ratios (R/FR) of white and far-red LED light were 11 and 0.01, respectively. Error bars are the standard error ($n = 5$). The same letters in (**A**,**C**,**D**) indicate no significant difference at $p < 0.05$ by Fisher's protected LSD test, while in (**B**) indicate no significant difference at $p < 0.05$ by Dunn's post hoc comparison test. ND in (**E**) means no seed germinated in FR_L treatment during the 5-day incubation.

3.1.2. Responses to FR Pretreatment

Although the germination of FR_L was completely inhibited during the 5-day incubation, a few germinated seeds were observed in the FR pretreatments (i.e., FR-D_L and FR-W_L) (Figure 2C,D). It was noted that the seeds of Cobbler's pegs could germinate not only in the darkness but also in the FR pretreated dark treatments, revealing that the light responses of its phytochromes may be very low fluence response (VLFR). The results also showed that the germination of FR-D_L treatment was more inhibited than other light treatments when extract concentration was higher than 0.05 g DW mL^{-1} (Figure 2D).

3.1.3. Responses to FR Irradiation

In the FR_L treatment, since no seed germinated in the first 5 days under FR irradiation (Figure 2E), we extended the duration of germination bioassay to 14 days and then changed the light source to white LED for another 7 days. It was observed that no seeds germinated in the first 14 days until the light source was changed to white light (Figure 3). During the 7-day white light incubation, the seed germination at the low extract concentrations (<0.02 g DW mL^{-1}) was comparable to that of FR pretreated light treatment (FR_L) but showed more sensitivity at the high extract concentrations (>0.05 g DW mL^{-1}) (Figures 2C and 3). The results indicated that extended FR irradiation, coupled with a high concentration of residue extract might cause detrimental effects on the germination of Cobbler's pegs.

Moreover, it was also observed that both FR irradiation and residue extract inhibited the germination of Cobbler's pegs seed, but in different ways. Generally, FR irradiation only inhibited seed germination but had little effect on the seed viability during the light treatment. However, the residue extract reduced the seed viability as the concentration increased.

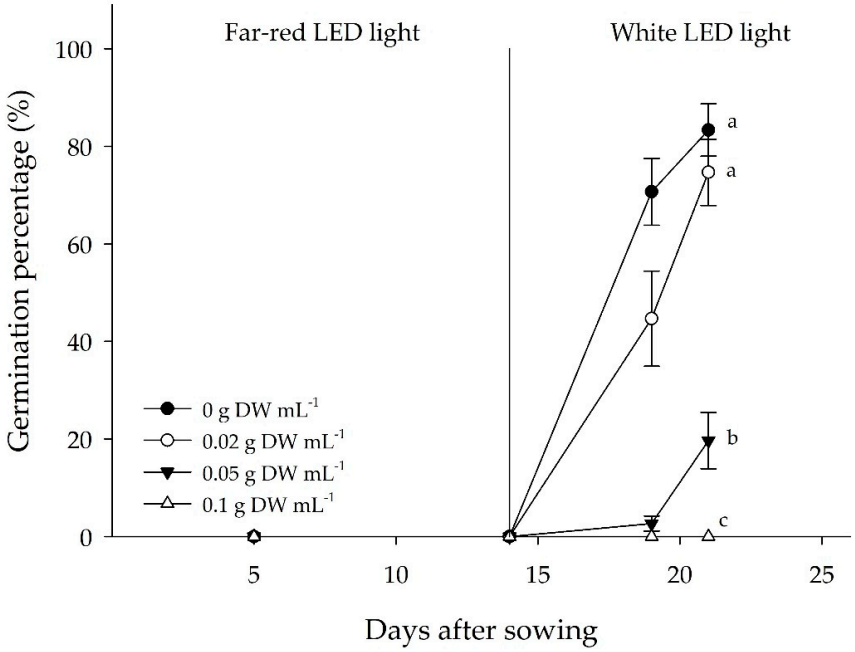

Figure 3. Germination percentage (%) of Cobbler's pegs seeds that incubated in different concentrations of residue extract under FR (LED, 730 nm, 0.71 μmol m^{-2} s^{-1}, 12-h photoperiod, FR_L treatment of the laboratory experiment) for 14 days and then changed the light source to white light (LED, 400–750 nm, 6500 k, 149 μmol m^{-2} s^{-1}, 12-h photoperiod) for the next 7 days. The R/FR of white LED and far-red LED light were 11 and 0.01, respectively. Seeds incubated in the concentration of 0.1 g DW mL^{-1} were not germinated until the end of the experiment. Error bars are the standard error (n = 5). The same letters indicate no significant difference at $p < 0.05$ by Fisher's protected LSD test.

3.1.4. Response of Radicle Growth to Light and Residue Extract

Except for FR_L, the radicle growth was inhibited more apparently by residue extract than by light treatment. More than 55%, 77% and 82% of radicle growth were reduced by the residue extract concentrations of 0.02, 0.05 and 0.1 g DW mL^{-1}, respectively, as compared to the control (0 g DW mL^{-1}) (Figure 4). The results demonstrated that although low residue extract concentration (0.02 g DW mL^{-1}) had no or slight reduction in the germination, it possessed strong inhibition on the radicle growth. Radicles were observed to exhibit growth retard, and they were even twisted and swollen at the high concentration of residue extract (0.05 and 0.1 g DW mL^{-1}). Furthermore, the radicle growth of D_L

treatment was found more sensitive to residue extract than those of W_L treatment, especially when the concentration is high (i.e., 0.1 g DW mL^{-1}).

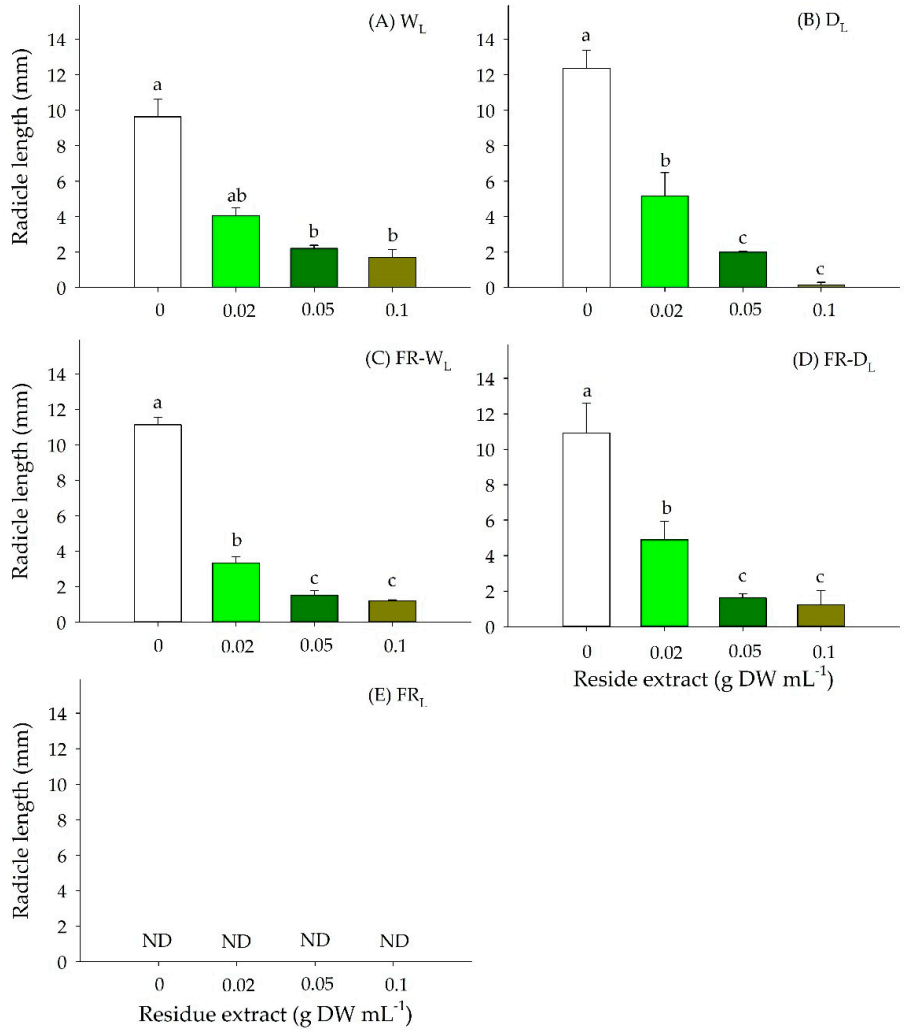

Figure 4. Radicle growth of Cobbler's pegs treated with four concentrations of residue extract (0, 0.02, 0.05 or 0.1 g DW mL^{-1}) and five light treatments: (**A**) incubated in white light with a 12-h photoperiod (W_L); (**B**) incubated in the continuous darkness by wrapped with two layers of aluminum foil (D_L); (**C**) pretreated in the FR for 3 h then incubated in the white light with a 12-h photoperiod (FR-W_L); (**D**) pretreated in the FR for 3 h then incubated in the continuous darkness (FR-D_L); (**E**) incubated in FR with a 12-h photoperiod (FR_L). The light intensities of white light (LED, 400–750 nm, 6500 k) and FR (LED, 730 nm) were 149 μmol m-2 s^{-1} and 0.71 μmol m^{-2} s^{-1}, respectively. The R/FR ratios of white and far-red LED light were 11 and 0.01, respectively. The same letters in (**A**) indicate no significant difference at $p < 0.05$ by Dunn's post hoc comparison test, while in (**B**–**D**) indicate no significant difference at $p < 0.05$ by Fisher's protected LSD test. ND in (**E**) means no seed germinated in FR_L treatment during the 5-day incubation.

In addition, results of the light treatments except for FR_L showed that the radicle growth declined exponentially with an increasing concentration of residue extract ($p < 0.001$, Figure 5). The coefficient of determination, r^2, reached 0.97, illustrating the residue extract had a strong concentration-dependency on radicle growth. It could also be found that the radicle growth was almost inhibited at the concentration of 0.05 g DW mL^{-1}. Overall, the results of the laboratory experiment demonstrated that the radicle growth was more sensitive to the residue extract than the germination did.

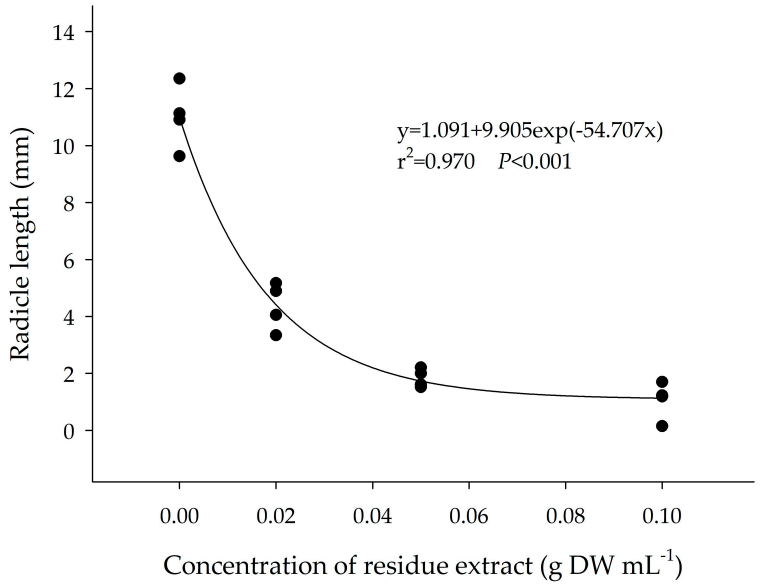

Figure 5. Relationship between the concentration of residue extract and the radicle length of Cobbler's pegs seedlings.

3.2. The Effects of Light and Residue on Cobbler's Pegs in the Seminatural Conditions

3.2.1. Light Requirement of Germination

For the residue application rate of 0 g pot^{-1}, the Cobbler's pegs germination in W_P treatment was significantly higher than that of D_P treatment (Figure 6). It was different from that of the laboratory experiment, in which no germination reduction was found in the dark treatment of 0 g DW mL^{-1} (D_L)as compared to that of the light treatment of 0 g DW mL^{-1} (FR_L) (Figure 2A,B). The different responses between the laboratory and pot experiments demonstrated that the Cobbler's pegs exhibited different light requirements in the controlled and seminatural conditions.

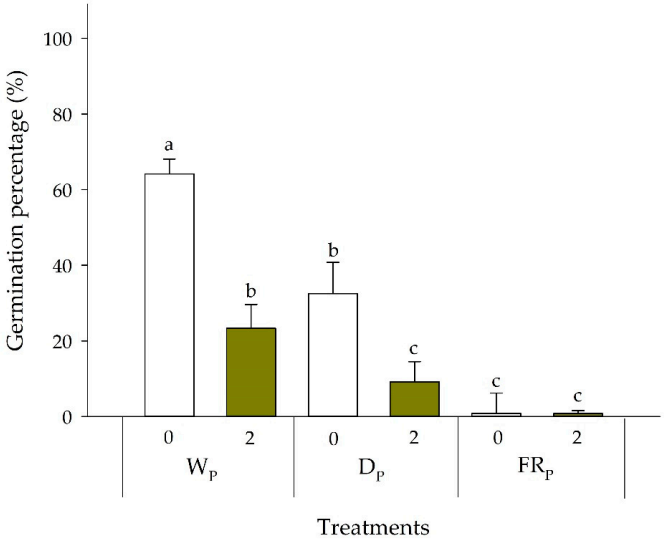

Figure 6. The seed germination percentage of Cobbler's pegs treated with residues of 0 or 2 g DW plot^{-1} under the white light (12-h photoperiod, W_P), darkness (D_P) or FR (12-h photoperiod, FR_P). The light intensities of white light (LED, 400–750 nm, 6500 k) and FR (LED, 730 nm) were 149 μmol m^{-2} s^{-1} and 0.71 μmol m^{-2} s^{-1}. The R/FR ratios of white and far-red LED light were 11 and 0.01, respectively. Error bars are the SE (n = 4). The same letters indicate no significant difference at $p < 0.05$ by Fisher's protected LSD test.

For the effects of residue application rate of 2 g pot^{-1}, significant seed germination reduction was found in both W_P and D_P treatments as compared to that of 0 g pot^{-1}. Although the phytotoxins may be degraded quickly in seminatural conditions, the germination reduction in the pots was observed. It showed that the Cobbler's pegs residues possessed phytotoxicity in the seminatural condition.

3.2.2. Germination Responses to FR Pretreatments

In the experiments for three FR pretreatment time, the germination percentages significantly declined when incubated in the darkness as compared to that incubated in light regardless of the FR irradiation duration (Figure 7). The responses of seed germination to FR pretreatment in the pot experiments differed from that in the laboratory experiment, i.e., FR-W_L and FR-D_L (at extract concentration of 0 g DW mL^{-1}) treatments (Figure 2C,D). In the laboratory experiment, however, no significant difference was observed between FR-D_L and FR-W_L.

The results of different FR pretreatments also indicated that the highest germination percentages of the dark treatment occurred with the 24-h FR pretreatment (FR24-D_P), followed by 0-h FR pretreatment (FR0-D_P) and 3-h FR pretreatment (FR3-D_P). Moreover, there was an increase as the FR pretreatment time increased from 3–24 h. These results also agreed with the aforementioned presumption that the phytochrome light response of Cobbler's pegs might be classified as VLFR since its seed germination may be induced as the FR pretreatment prolonged.

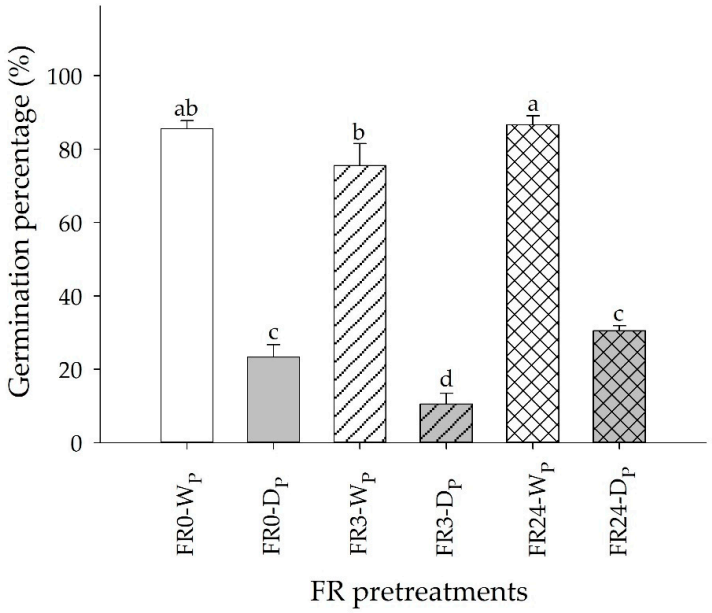

Figure 7. The seed germination response to different FR pretreatment time (0, 3 and 24 h for FR0, FR3 and FR24, respectively) followed by incubation under white light (LED, 400–700 nm, 6500 k, 12-h photoperiod, W) or darkness (D) in a growth chamber. Error bars are the standard error (n = 6). The same letters indicate no significant difference at $p < 0.05$ by Fisher's protected LSD test.

3.2.3. Germination Responses to FR Irradiation

For the FR irradiation treatments (FR_P), the germination was apparently inhibited regardless of the residue application rates (Figure 6). A similar response was also found in FR_L of the laboratory experiment (Figure 2E), which was conducted in the Petri dish, showing the FR irradiation was able to possess strong germination inhibition not only in the controlled but also in the seminatural condition.

3.3. The Effects of Light and Residue on Cobbler's Pegs in the Natural Conditions

3.3.1. Germination Responses to FR Pretreatments

In the field experiment, using the polyester gauze bag prevented Cobbler's pegs seed germination from the interference of seed bank successfully and provided a chance to assess the germination responses to different FR pretreatments and phytotoxicity of Cobbler's pegs residue in the field. The highest germination percentage was found in the treatment that pretreated in the FR and then buried with weed residues (FRW_F, 26.77%), followed by dark pretreatment with weed residues (DW_F, 26.72%), dark pretreatment with Cobbler's pegs residues (DB_F, 24.6%) and FR pretreatment with Cobbler's pegs residues (FRB_F, 21.33%) (Figure 8). The results showed that no difference between dark (DW_F) and FR pretreatment (FRW_F) was found in plots buried without Cobbler's pegs residues. However, compared to the dark pretreatment (DB_F), germination decrease in FR pretreatment (FRB_F) was observed in plots buried with Cobbler's pegs residues.

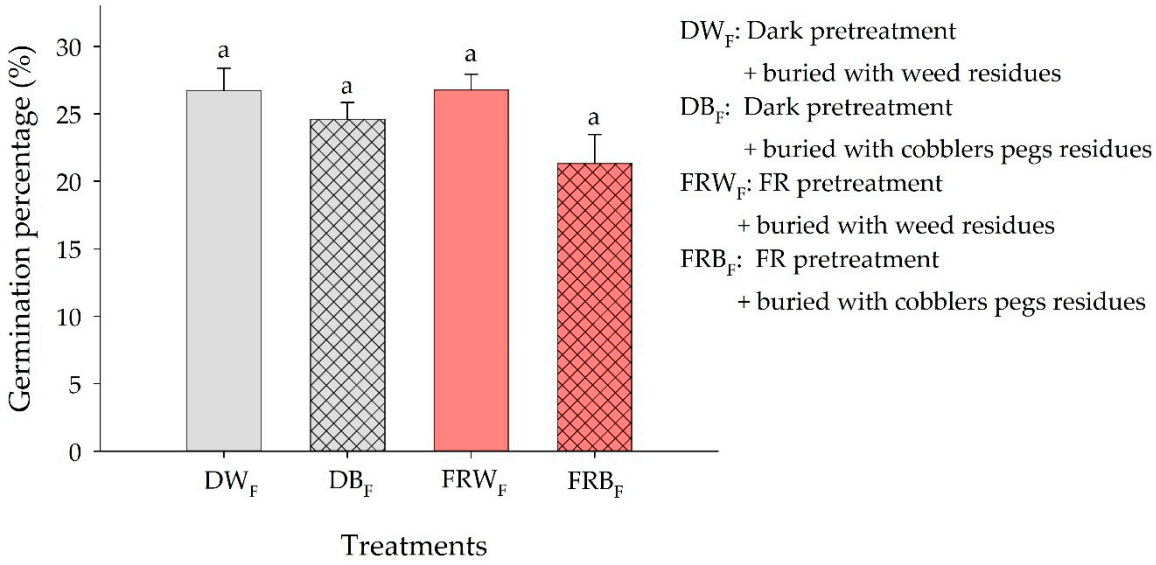

Figure 8. The effects of FR pretreatment and different residues on the Cobbler's pegs seed germination in the field. Error bars are the standard error (n = 12). The same letters indicate no significant difference at $p < 0.05$ Dunn's post hoc comparison test.

3.3.2. Germination Responses to Residue Application

By comparing the relationships between residue application rates and germination, the results further demonstrated that the seed germination was influenced by the residue application rates, but in different ways for the two light pretreatments. Generally, when the residue application rate increased, the germination was decreased in the FR pretreatment but had no clear tendency in that of the dark pretreatment. The different responses indicated that the phytochrome level in the seed might be influenced by the phytotoxins released from the residues (Figure 9).

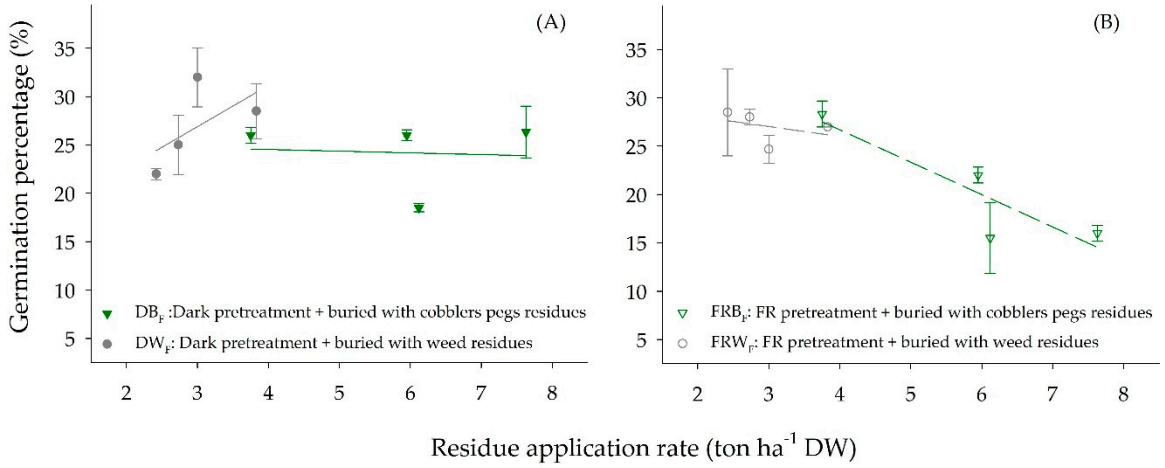

Figure 9. The relationship between the residue application rate and Cobbler's pegs seed germination for (**A**) dark pretreatment and (**B**) FR pretreatment. The Pearson's correlation coefficients of DB_F, DW_F, FRB_F and FRW_F were −0.0736, 0.596, −0.889 and −0.369, respectively.

4. Discussion

4.1. Effects of Light on Seed Germination

4.1.1. Light Requirements of Cobbler's Pegs Germination

In the well-controlled condition (i.e., the laboratory experiment), it was found that the germination percentages of *Bidens pilosa* L. (Cobbler's pegs) in the dark treatment were higher than that of light treatment (Figure 2A,B). However, in the seminatural condition (i.e., the pot experiment), the germination percentage in the dark treatments was significantly lower than that in the light treatments (Figure 6). Previous studies showed that the light responses of Cobbler's pegs germination varied among studies. For example, reports by Fenner [34,35] and Reedy and Singh [36] showed that the seed germination of this species in the dark treatment was only slightly lower than that in the light treatment, whereas Chauhan et al. [37] found that the germination was more significantly stimulated by light in the light/dark regime than that in the darkness. It is generally acknowledged that the light responses of seed germination vary from species to species and even vary among varieties. Species of Cobbler's pegs were reported to contain at least six varieties [4]; however, the varieties used in the aforementioned studies were not clear. In the present study, when the species was considered as a rotation crop in an agricultural system, we chose *B. pilosa* var. *radiata* to evaluate the reproduction potential since it was the most invasive variety of Cobbler's pegs in Taiwan [9]. Hsu and Lin [26] conducted the germination experiments of three of the six varieties, i.e., *B. pilosa* var. *radiata*, *B. pilosa* var. *minor* and *B. pilosa* var. *pilosa*, in the Petri dish and showed that no light requirement was found in *B. pilosa* var. *radiata,* but did in *B. pilosa* var. *minor* and *B. pilosa* var. *pilosa*. The light responses of *B. pilosa* var. *radiata* in the study by Hsu and Lin [26] were similar to our results of the laboratory experiment. Nevertheless, our study further found that the light response of *B. pilosa* var. *radiata* seed germination in the controlled and the seminatural conditions demonstrated inconsistent results between the light and dark treatments (Figures 2 and 6). In other words, the germination responses of *B. pilosa* var. *radiata* seeds in the controlled conditions showed no light requirement but did in the seminatural conditions. The inconsistency observed was hard to ascribe to the different light responses of the dimorphic seeds of Cobbler's pegs [38] or the deep burial inhibition [37] since seeds from the same source were used and placed onto the soil surface in the pots.

4.1.2. The Responses of Germination to Far-Red Light (FR) Pretreatments

From the results of the laboratory and pot experiments, it was found that the germination of Cobbler's pegs was similar in FR pretreated light treatments but different in FR pretreated dark treatments (Figures 2 and 7). Results of the pot experiment showed that although seed germination was low in the FR pretreated dark treatments, about 15−35% of seeds germinated regardless of the length of FR pretreatment time. Moreover, the germination was not inhibited by FR pretreatment for 24 h (h) (FR24-D_P) as compared to that for 0 (FR0-D_P) and 3 h (FR3-D_P) (Figure 7). Similar germination responses were also observed in the field experiment, which showed that more than 20% of seeds germinated in the FR pretreatments (FRW_F and FRB_F) (Figure 8). The seed germination percentage did not reduce with increasing the time length of FR pretreatment, indicating that the light responses of the phytochrome of the investigated plant should be very low fluence response (VLFR). Similar results were also observed in the seeds of *Ruellia tuberosa* L. that had been reported to possess a high germination percentage after a prolonged FR irradiation [39]. However, Fenner [35] pointed out that the imbibed Cobbler's pegs seeds pretreated with low R/FR light by exposing them to leaf-transmitted light for more than 1 h did not germinate in the darkness but could germinate followed by white light irradiation. It was presumed that the light responses of phytochrome in different varieties of Cobbler's pegs might be different. The light response of the variety used by Fenner [35] should be LFR, whereas the light response of *B. pilosa* var. *radiata*, which was used in our study, should be VLFR.

4.1.3. The Responses of Germination to FR Irradiation

Blue and red light can be markedly reduced by plant canopy than FR can be. As a result, a lower red/far-red ratio was observed under the plant canopy [40]. Fenner [41] indicated that the seeds of Cobbler's pegs, similar to many other tropical weeds, were very sensitive to FR, which could almost completely inhibit their germination. This characteristic makes this species difficult to recruit its seedlings in their parent vegetation. Similar to the previous studies, our results illustrated that the germination of Cobbler's pegs was strongly inhibited in FR irradiation treatments under the controlled (FR_L, Figure 2E) and seminatural (FR_P, Figure 6) conditions. However, it was also observed that the seeds started to germinate normally after changing the light source from FR to white light. It implied that when an agricultural practice, such as harvest, mowing or plowing, was conducted during the flowering period, seeds shaded by the canopy may be dispersed and germinate soon due to the removal of inhibition factor, i.e., FR.

4.2. Other Factors That May Affect the Seed Germination

A variety of factors such as light, substrate and water may affect seed germination [42]. The influence of the germination substrate varied depending on species. Seeds of *Rhexia mariana* var. *interior* (Pennell) Kral and Bostick [43], *Lythrum salicaria* L. and *Epilobium hirsutum* L. [44] germinated at a higher percentage on filter paper than on sand or soil. In the present study, the results of light treatments of the laboratory and pot experiments exerted similar germination responses to germination substrates (i.e., filter paper vs. soil) and relative humidity (i.e., saturated vs. low and dynamic) (Figures 2A and 6). Meanwhile, the germination of FR pretreated treatments of the laboratory and pot experiments also showed a similar conclusion (Figures 2C and 7). The results indicated that in the seminatural conditions, the germination reduction in the dark and FR pretreated dark treatments may not result from the difference of light treatments, germination substrates or the low and dynamic relative humidity. Because the germination in the experiments of light treatments and those of FR pretreated light treatments had no obvious difference.

On the other hand, Hsiao [45] indicated that the germination percentages of stinkweed (*Thlaspi arvense* L.) and wild mustard (*Brassica kaber* (D.C.) Wheeler var. pinnatifida (Stokes) Wheeler) were significantly stimulated by pretreatment with sodium hypochlorite (NaOCl). In the present study, Cobbler's pegs seeds sterilization by NaOCl was performed only in the laboratory to prevent the

germination from the microbial pollution. The same manner was also conducted in the Cobbler's pegs germination study of Hsu and Kao [10] to prevent the influence of fungi. Comparing the germination results of all light and FR pretreated light treatments of the laboratory and pot experiments, no apparent differences were found in these treatments. Therefore, sterilization by NaOCl may have no obvious effect on the germination of Cobbler's pegs.

4.3. Autotoxic/Phytotoxic Effects of Residues on the Seed Germination and Radicle Growth

Many Asteraceae species, such as *Ageratum conyzoides* L. [46], *Helianthus annuus* L. [47], *Parthenium hysterophorus* L. [48] and *Wedelia trilobata* L. [49], possess autotoxicity to regulate their vegetation regeneration and expansion. Several studies indicated that the plant extracts of Cobbler's pegs [7,18,50–52] contained phytotoxins (i.e., phenolics and PHT) that inhibited seed germination and seedling growth of many other species. In the present study, we investigated the autotoxicity of Cobbler's pegs residue from the laboratory to the field and found that the residue or its extract of this plant was able to exert phytotoxicity on its seed germination and radicle growth in the laboratory, pot and field experiments.

4.3.1. Responses of Germination

Previous studies demonstrated that residues of Cobbler's pegs could significantly reduce the sprout reproduction of *Cyperus rotundus* L. in the upland field [18] and inhibited the growth of weeds in the paddy field [12,53]; however, in the present study, this plant exerted strong phytotoxicity on its seed germination in the controlled (Figure 2) and seminatural conditions (Figure 6), but less phytotoxicity in the natural conditions (Figures 8 and 9). The phytotoxicity of donor plants to the target plants depended on the concentration, movement and persistence of phytotoxins in the field condition [54]. Phenolics, the main phytotoxins of this species, were water-soluble and could rapidly be leached and degraded in the field [55,56]. During the experimental field period of the present study, 62 mm of rainfall was recorded [57]. The rainfall may be one of the factors that diminished the phytotoxicity of residues in the field. Although it has been reported that PHT, a phototoxic polyacetylene, could be isolated from the Cobbler's pegs aerial parts [58], our results demonstrated that the phytotoxicity was not stimulated in the light treatments in all experiments.

In the laboratory experiment, it was noted that the germination in the FR-D_L treatment was more inhibited by increasing the concentration of the residue extract than that in the D_L treatment (Figure 2B,D). Moreover, the germination of FR_L also showed more sensitivity to high extract concentration (>0.05 g DW mL^{-1}) than that of the FR pretreated light treatment, i.e., FR-W_L (Figures 2C and 3). Similar responses were found in the FRB_F treatment of the field experiment, and the germination of this treatment was obviously reduced as the residue application rate increased (Figure 9B). Litts et al. [59] indicated that the phytochrome could be degraded by the phenolics contained in the plant. Moreover, Clough and Vierstran [60] reported that the degradation rate of phy A (the phytochrome that mediates VLFR) increased when P_r was transformed to P_{fr} owing to the metabolic instability. As a result, it was supposed that the decreased germination percentages of FR pretreatments might result from the labile P_{fr} breakdown by the phenolics from Cobbler's pegs residues.

4.3.2. Responses of Radicle Growth

The results from the laboratory experiment showed that the residue extracts of Cobbler's pegs significantly inhibited the radicle growth of the germinated seeds in all light treatments except for the FR irradiation treatment (FR_L) (Figure 4). It was also observed that the radicle growth was more sensitive to the residue extracts than germination was. The growth responses of radicles decreased, apparently even treated with the lowest residue extract (0.02 g DW mL^{-1}). Hsu and Kao [10] reported that the radicle growth of Cobbler's pegs was inhibited by its leaf and stem extracts at a low concentration (i.e., 0.001 g DW mL^{-1}). Jiang et al. [44] also found that the fresh leaf and stem extracts of Cobbler's

pegs had a significant autotoxicity on its radicle growth. Studies in the literature also indicated that the radicle growth was more sensitive to phytotoxins than seed germination was [61]. Therefore, the responses of Cobbler's pegs radicle growth to the residue reported in the present study is consistent with the findings in other studies.

4.4. Implications for Agricultural Practices

The present study demonstrated that although Cobbler's pegs seeds possessed a high potential to reproduce when seeds fall on the soil surface, results from the FR pretreated dark treatments and residue application treatments provided some implications. That is to say, in an agricultural system that introduces this species as a rotation crop, they may be controlled by a deep plowing practice and buried seeds with residues into the soils when farmers plan to rotate crops. However, it is recommended to harvest or rotate before the flowering stage even though the highest content of functional compounds was found at the flowering stage [62].

5. Conclusions

In the present study, the results of the laboratory experiment demonstrated that the *Bidens pilosa* L. (Cobbler's pegs) seed germination showed no light requirement but did in the pot experiments. Despite the fact that in the pot experiments, the germination in the dark treatments was significantly lower than that of light treatments, some seeds germinated in the darkness whether pretreated with far-red light (FR) or not. These results indicated that the light response of Cobbler's pegs variety that was used in the present study, i.e., *B. pilosa* var. *radiata*, may be a very low fluence response (VLFR). For the evaluation of autotoxic/phytotoxic effects, Cobbler's pegs residue and its aqueous extracts possessed phytotoxicity on the seed germination in the laboratory, pot and field experiments, despite a number of rainfall events occurred during the experimental field period. Although the P_{fr} level induced by FR enables some seeds germination in the darkness, the level may be reduced due to the phenolics released from residues that were capable of breaking down P_{fr} and thus inhibiting the germination. Lastly, by considering the use of this species, an edible and medicinal herb with phytotoxicity, in a crop rotation system, a deep plowing practice that buried seeds with their residues into the soils is recommended while harvesting at the flowering stage should be prevented.

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