applied sciences

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Allelopathic Potential of Phenolic Compounds in *Secale Cereale* Cultivars and Its Relationship with Seeding Density

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Received: 12 June 2019; Accepted: 26 July 2019; Published: 30 July 2019

Abstract: In this study, we investigated the allelopathic effect of *Secale cereale* cultivars on different weeds that grow in the cultivated fields of *Perilla* frutescens. Two *S. cereale* cultivars, Paldong and Singhi, were used to test the allelopathic effect on in vitro grown *Digitaria ciliaris*, *Chenopodium album*, *Amaranthus lividus*, *Portulaca oleracea*, *Pinellia ternata* and *Commelina communis*. The results indicated that *S. cereale* extracts affect callus growth of weeds in terms of fresh weight and percentage of growth inhibition. The inhibitory effects of both *S. cereale* cultivars combined with grass cover extracts were higher than using grass weeds alone. Concentrations of all identified phenolic compounds were significantly higher in the leaves extracts of Paldong compared to Singhi. Particularly, syringic acid in leaves extract of the Paldong cultivar were 12.87-fold higher than in the Singhi cultivar. The other predominant phenolic compounds such as salicylic acid, *p*-coumaric acid, vanillic acid, and *p*-hydroxybenzoic acids were 3.30, 4.63, 3.11, and 1.28 times higher, respectively, in the leaves extracts of Paldong compared to Singhi. Principal component analysis (PCA) results indicated that the composition of phenolic compounds was significantly related to cultivar types and plant parts used. In addition, biomass increase caused increased weed inhibitory capacity of *S. cereale* both in tillage and no-tillage regimes. These results suggest that the biomass of cover crops negatively influenced weed density.

Keywords: allelopathic properties; cultivars types; cover crop; weed inhibition; callus growth

1. Introduction

Weeds compete with cultivated crops for space, light, water, and nutrition, and they have a negative impact on the health and yield of growing plants [1–4]. A biological control of weeds offers more advantages over mechanical methods and chemical herbicides, as it is more environmentally friendly and favorable for conserving biodiversity. Weed control in cultivating land using mechanical methods is not effective as it lacks durability, is labor-intensive, and expensive [5]. Excessive use of chemical herbicides has numerous negative impacts on human health and also increases the accumulation of chemical pollutants in the environment [6]. Consequently, the use of herbicides in cultivated fields not only causes environmental pollution but also triggers severe health issues in human beings, including cancer, birth defects, and neurological problems, due to toxic residual contents of herbicides [7]. Globally, over 307 biotypes from 183 plant species have acquired herbicide resistance, which is a major challenge in the agriculture sector [8]. Thus, there is an increasing interest in finding a potential and novel technique to control weeds to overcome the impact of chemical herbicides on human health and

agricultural products. Allelopathy has been considered as a more environmentally friendly technique to control weeds as it effectively suppresses their germination and growth by restricting nutrient uptake [9,10]. Allelopathic plants release various phytochemicals from different plant parts at different ratios [11,12]. Release of these chemicals into the soil either results in toxic effects on neighboring plants, or the chemicals are transformed into new toxic compounds by soil microbes [13]. Several factors, including temperature, irradiation, draught, disease, nutrition, insects, competition, and cultivar type, influence the degree of allelopathy [14–17]. The release of allelochemicals from different plant parts into the soil varies among different cultivars [18–21].

The application of cover crops has several beneficial effects on the agroecosystem, as it enhances soil fertility by improving the soil moisture and organic matter content [22,23]. Planting cover crops not only enhances microbial activity but also protects the soil from erosion during heavy rain and builds up soil fertility, which often results in higher yields [24,25]. Moreover, some cover crops, such as rye (*Secale cereale*), fix atmospheric nitrogen, prevent NO_3 leaching [26,27], and inhibit the growth of weeds [28], which reduces the need of herbicides [29]. Cover plant cultivation is considered the best management practice (BMP) in the agroecosystem, as it distributes the nutrition from the soil to the roots and leaves and prevents nutrient runoff to streams and rivers. After termination of the cover crops, their roots decompose and release organic matter into the soil, thereby enhancing soil fertility and soil quality [11,30–33]. Cover crops are often coupled with no-tillage farming (not disturbing the soil). Recently, crop cultivation using cover crop based no-tillage farming has gained attention as an innovative practice to mitigate environmental pollution [34] and to protect soil microbes [35]. No-tillage farming stores more carbon in the soil in the form of plant residue and improves the soil structure by maintaining size distribution of aggregates, pores, and voids [36]. Tillage farming, on the other hand, results in a higher root penetration, easier weed control, and a higher yield [37], and it is considered a promising technique for improving soil quality and sustaining crop production [38,39].

S. cereale is widely used as a cover plant for its rapid and reliable growth in a wide range of climatic conditions and cultivated crop fields [40]. The high survival rate during winter and the extensive rooting system of *S. cereale* facilitates reduction of soil erosion, retention of soil nutrients, nitrogen fixation, carbon sequestration, and inhibition and suppression of weeds in the cultivating land [40]. Moreover, the residual components of this plant in the soil effectively inhibit seed germination and seed dormancy of weeds [41] and mainly attribute to the exudation of phenolic compounds into the soil, including glycosylated benzoxazinone, 2-(2,4-dihydroxy-1,4-benzoxazin-3-one)-beta-D-glucopyranose (DIBOA-glucoside), benzoxazinoids compounds [42], 2-hydroxy-1,4-benzoxazine-3(2H)-one (HBOA), the 2-benzoxazolinone (BOA), and 2-amino-3H-phenoxazin-3-one (phenoxazinone APO) [43,44]. Previous studies on the allelopathic properties of *S. cereale* have led to the identification of important allelochemicals. However, most of these studies were performed using a single cultivar. It is possible that there are still other allelochemicals present in the several unexplored cultivars. Variation in the allelopathic properties, phenolic compound profiles, and abilities to suppress weeds have been documented for many plant species [45–49]. Previous studies have shown that allelochemical composition, concentration, and allopathic properties vary significantly among the cultivars [32,50–52]. However, the effects of different cultivars on weed control have not been documented yet. In this study, we focused on the comparative allelopathic potential of two *S. cereale* cultivars: Paldong and Singhi.

The main objective of the present study was to explore the possible effects of varying seeding density, high plant density (HPD), and low plant density (LPD), both in tillage and no-tillage systems, on weed growth in field conditions. Furthermore, we documented the major allelochemicals present in the two cultivars of *S. cereale* leaves extracts. This study provides insight into the mechanism underlying inhibition of weed callus growth by *S. cereale* leaf extracts. Additionally, correlation between the phenolic compound exudates of cover crop and fresh weight of weed callus was assessed. To the best of our knowledge, the present study is the first report on the allelopathic effects of *S. cereale* extracts on callus growth and seed germination of different weed species growing in *P.* frutescens fields.

2. Materials and Methods

2.1. Chemicals

The chemicals and solvents used in the present study were of analytical grade and supplied by commercial providers. Methanol was obtained from J. T. Baker (Phillipsburg, NJ, USA). Standard compounds for the estimation of phenolic compounds and saponins were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

2.2. Plant Materials

The seeds of *Digitaria ciliaris*, *Chenopodium album*, *Amaranthus lividus*, *Portulaca oleracea*, *Commelina communis*, *Pinellia ternata,* and *Secale cereale* used in this study were supplied by the Department of Applied Plant Science, Kangwon National University, South Korea.

2.3. Experimental Site and Preparation of Cover Crops

The two *S. cereale* cultivars Paldong and Singhi were grown for 70 d in an experimental field of Kangwon National University, South Korea (37°52′09.53″ N; 127°44′42.82″ E; average altitude of 122 m) in the years 2014, 2015, and 2016. An experimental trial was performed in a completely randomized block design. Each cultivar of *S. cereale* was sown in a separate plot. The growing plants in the plots were irrigated every two days. Three replicas were retained for each treatment.

2.4. Preparation of Plant Extracts

The leaves of *S. cereale* cultivars were collected from the experimental field to assess the composition and concentration of the allelochemical compounds. The collected leaf samples were dried at room temperature (25 °C) for 24 h. In brief, 10 g of ground dried powder samples from each cultivar was dissolved in 80% ethanol using 100 mL conical flasks by continuous shaking (40 rpm) at room temperature (25 °C) for 20 h. Thereafter, the solution was filtered through a No. 1 Whatman filter to remove the debris. The obtained filtrate was evaporated using a rotary evaporator (Eyela, SB-1300, Shanghai Eyela Co. Ltd., Shanghai, China) at 42 °C. The dry filtrate residues were re-dissolved in 80% high performance liquid chromatography (HPLC)-grade ethanol (0.01 mL) for phenolic compound analysis by using the HPLC method.

2.5. Regeneration and Subculture of Callus

Initially, the seeds of weeds were sterilized by sodium hypochlorite (6%) for 30 min, rinsed with distilled water at least six times, and dried on sterilized paper towels for 1 h. Sterilized seeds were germinated in Murashige and Skoog (MS) medium. Young leaves of in vitro grown plantlets of *C. album*, *A. lividus*, *D. ciliaris*, *P. oleracea*, *P. ternata,* and *C. communis* were used for callus induction using MS medium. MS medium was prepared by mixing 3% (w/v) sucrose with 1 mgL^{-1} 2,4-dichloroohenoxyacetic acid (2,4-D). All media used were adjusted to a pH value of 5.8 with 1 N NaOH and HCl solution. The medium was gelled by using 0.8% plant agar before autoclaving at 1.1 kg/cm^2 for 15 min. Sterilized medium (20 mL) was dispensed in Petri dishes (10 × 15 cm) and used for callus induction. Young leaves from the in vitro grown plants were cut across the midrib and immediately placed in the callus induction medium. Regenerated calli were proliferated further by weekly subculturing in the MS medium supplemented with 1 mgL^{-1} 2,4-D.

2.6. Evaluation of Allelopathic Effects of Plant Extract on Callus Growth

Calli induced from the leaf section of different weeds were cultured in MS medium supplemented with 1 mgL^{-1} 2,4-D. Fresh and healthy calli (50 mg) from each plant sample were soaked in different concentration of *S. cereale* leaf extracts (0.1%, 5%, and 10% w/v). Six to eight calli were grown in each Petri dish containing supplemented MS medium and plant extracts. The Petri dishes were incubated

in a culture room for four weeks, and three replications were performed for each treatment. Plant extracts were replaced by distilled water as the control treatment.

2.7. Effect of Allelopathic Compounds on Callus Growth

To assess the effect of allelopathic compounds on callus growth, identified phenolic compounds including salicylic acid, *p*-coumaric acid, vanillic acid, *p*-hydroxybenzoic acid, ferulic acid, and syringic acid at concentrations of 10^{-2}, 10^{-3}, and 10^{-5} M were prepared. These compounds were mixed with MS medium supplemented with 1 mgL^{-1} 2,4-D, upon which six to eight young and fresh calli were placed and maintained at 25 ± 1 °C for 40 d. The fresh weight of the calli was recorded after 40 d.

2.8. Identification and Quantification of Phenolic Compound Analysis by HPLC

Identification and quantification of phenolic compounds from different accessions were carried out using an HPLC. The dried solutions were re-dissolved with 80% methanol (10 mL) to obtain a solution of 50 μgmL^{-1} and filtered through a 0.45 µm filter unit (TITAN syringe filter nylon membrane), before being injected into the HPLC system. The quantities of the individual phenolic compounds from each sample were analyzed by an HPLC (Shimadzu Instruments CO., LTD, Kyoto, Japan) equipped with a pump (LC-10ADVP, Shimadzu, Japan), a detector model SPD-M10A, and a diode array detector (280 nm), following the method described by Ghimire et al. [53]. Chromatographic separation of phenolic compounds was carried out using an analytical HPLC column (YMC-Pack ODS-AM-303, 5 um, 250 × 4.6 mm I.D). The injection volume was 20 µL with a flow rate of 1 mL min^{-1} and a wavelength of 280 nm. The mobile phase consisted of solvent A (0.1% glacial acetic acid adjusted in water) and solvent B (0.1% glacial acetic acid in acetonitrile). The gradient elution program was applied as follows: solvent B was elevated to 8%–10% (2 min), 10%–30% B (27 min), 30%–90% B (50 min), 90%–100% B (52 min), and 100% of B (57 min). Calibration curves of phenolic compounds were obtained from standard compounds at different concentrations (10, 50, and 100 ppm). Quantification and identification of the polyphenols were measured by matching their retention times with authentic standard phenolic compounds.

2.9. Tillage and No-Tillage Systems

The tillage plots were ploughed to a depth of 15 cm once a year in spring to incorporate the crop residues into the soil. The cover crops were sown mechanically into the 12 × 8 m cultivation plots at 2–3 cm depth during the growth period. The seeds of the cover crop were sown separately in two split plots at a density of 8 kg ha^{-1} and 16 kg h^{-1} on 2 May in 2014, 2015, and 2016. It was planted at a spacing of 20 cm between the rows. After 10 weeks, the fully grown cover crops were harvested. The harvested *S. cereale* biomass was chopped and mixed with soil. Experiments were performed and replicated three times for each treatment. The total number of weeds and the fresh weight of the weeds were measured for all the treated plots. Plots without tillage and cover crops were considered the controls plots. The total number of weeds that emerged in the control plots were counted and collected independently at the same time as the tillage plots. No-tillage plots were maintained to compare the contribution of the tillage by covering the plot by cover plants. The experiment of no-tillage was conducted in two split block design. No ploughing was maintained throughout the season in the no-tillage system. Seeds of cover crops were sown directly in the no-tillage plot without disturbing soil using no-till seeder. Mineral fertilizers containing nitrogen and phosphorus were applied to all the plots at growing seedlings of cover crop at 110 kg/ha as urea and 40.5 kg/ha as calcium superphosphate. All the plots were regularly irrigated. The cover crop straw was chopped and spread uniformly on the soil surface in the no-tillage system. After the preparation of tillage and no-tillage plots, the seeds of *P. frutescens* were planted in two seeding rates: high population density (HPD, 40 plants m^{-2}) and low population density (LPD, 20 plants m^{-2}).

2.10. Statistical Analysis

Each experiment was performed in triplicate. The data obtained from the experiments were expressed as mean ± standard deviations. Quantitative data were statistically analyzed using a one-way analysis of variance (ANOVA). Significant differences between the obtained data were determined by using Duncan's multiple range test at $p < 0.05$ (SPSS ver. 20.0, SPSS Inc., Chicago, IL, USA). Correlations among the different variables were obtained by calculating Pearson's correlation coefficient using SPSS software version 20.0. Principal component analysis (PCA) of quantitative morphological traits was performed using SPSS software ver. 20.0

3. Results

3.1. Effect of Leaf Extracts of Secale cereale on Fresh Weights of Callus

Our results showed a significant inhibitory effect of ray extract on callus growth of different weed species (Table 1, Figure 1). Leaf extracts at all the applied concentrations in this study inhibited the callus growth of *D. ciliaris*, *C. album*, *A. lividus*, *P. ternata*, *P. oleracea*, and *C. communis*. Among the different concentrations of extracts used, 10% recorded the higher inhibition in callus growth of *D. ciliaris*, *P. ternata*, *C. album*, and *A. lividus.* In the case of *P. oleracea* and *C. communis* callus, a concentration of 5% leaf extract showed the highest inhibitory effect.

In this study, six standard phenolic compounds were analyzed to measure their impact in the callus growth of *C. album*, *P. oleracea*, and *P. ternata* (Table 2). All phenolic compounds inhibited the callus growth in terms of fresh weight. Among all standard compounds, syringic acid with a concentration of 10^{-2} M showed the highest inhibitory effect to the callus induction in *C. album* and *P. ternata*. Comparatively, ferulic acid at a concentration of 10^{-2} M inhibited callus growth of *P. oleracea* more than other reference phenolic compounds. The salicylic acid at a concentration of 10^{-2} M had the higher inhibitory effect on callus growth of *P. ternata*, as represented by the higher fresh weight. No significant difference in the fresh callus weight of *C. album* was observed under the treatment of syringic acid at concentrations of 10^{-5} and 10^{-3} M. Similar results were obtained when *p*-coumaric acid at concentrations of 10^{-3} and 10^{-2} M were used on the callus of *C. album*.

Table 1. Effect of *S. cereale* (Paldong cultivars) extracts on the fresh weight of callus.

Treatment	Concentration (%)	Callus Fresh Weight (mg)					
		D. ciliaris	*C. album*	*A. lividus*	*P. oleracea*	*C. communis*	*P. ternata*
Control		230.50 ± 10.12 ^{d}	1130.00 ± 15.50 ^{d}	250.00 ± 5.00 ^{c}	1120.00 ± 15.90 ^{d}	1130.00 ± 11.50 ^{c}	1620.00 ± 10.00 ^{d}
S. cereale (Paldong cultivars)	0.1	218.50 ± 5.00 ^{c}	599.50 ± 7.50 ^{c}	375.00 ± 4.50 ^{d}	806.40 ± 8.50 ^{c}	1175.00 ± 12.50 ^{d}	1267.00 ± 18.00 ^{c}
	5	65.00 ± 4.50 ^{b}	279.40 ± 4.50 ^{b}	125.00 ± 3.55 ^{b}	203.80 ± 5.00 ^{a}	128.80 ± 2.00 ^{a}	234.70 ± 3.00 ^{b}
	10	60.00 ± 6.50 ^{a}	101.70 ± 3.50 ^{a}	63.00 ± 2.00 ^{a}	407.70 ± 5.00 ^{b}	496.10 ± 4.50 ^{b}	120.20 ± 3.00 ^{a}

Experimental data are expressed as mean ± standard deviation ($n = 3$). Data with the same letter in a column are not significantly different, as determined by Duncan's multiple range test ($p < 0.05$).

Table 2. Effect of the standard phenolic compound on the fresh weight of callus.

Chemical	Concentration (M)	*Portulaca oleracea*		*Chenopodium album*		*Pinellia ternata*	
		Fresh Weight (mg)	Fresh Weight (%)	Fresh Weight (mg)	Fresh Weight (%)	Fresh Weight (mg)	Fresh Weight (%)
Salicylic acid	10^{-5}	140.10 ± 4.00 ^{o}	19.80 ± 0.90 ^{o}	26.90 ± 2.50 ^{h}	18.53 ± 1.50 ^{h}	160.20 ± 5.50 ^{o}	64.08 ± 1.50 ^{o}
	10^{-3}	30.50 ± 2.00 ^{h}	4.31 ± 0.50 ^{h}	11.70 ± 1.00 ^{e}	8.06 ± 0.50 ^{e}	83.30 ± 2.00 ^{k}	33.32 ± 1.00 ^{k}
	10^{-2}	7.20 ± 0.50 ^{d}	1.01 ± 0.10 ^{d}	5.70 ± 0.20 ^{b}	3.92 ± 0.20 ^{b}	40.20 ± 1.50 ^{g}	16.08± 1.00 ^{g}
Syringic acid	10^{-5}	193.30 ± 5.90 ^{q}	27.31 ± 2.00 ^{q}	45.90 ± 2.00 ^{j}	31.61 ± 0.40 ^{j}	187.00 ± 11.00 ^{q}	74.80 ± 4.00 ^{q}
	10^{-3}	48.20 ± 2.50 ^{j}	6.81 ± 0.50 ^{j}	45.60 ± 4.00 ^{j}	31.40 ± 0.20 ^{j}	42.30 ± 2.00 ^{h}	16.92 ± 1.50 ^{h}
	10^{-2}	4.40 ± 0.50 ^{b}	0.62 ± 0.01 ^{b}	3.70 ± 0.20 ^{a}	2.55 ± 0.10 ^{a}	3.80 ± 0.50 ^{a}	1.52 ± 0.01 ^{a}
Ferulic acid	10^{-5}	286.30 ± 10.50 ^{r}	40.44 ± 0.50 ^{r}	82.40 ± 4.00 ^{l}	56.75 ± 3.00 ^{l}	190.00 ± 10.00 ^{r}	76.00 ± 4.00 ^{r}
	10^{-3}	45.80 ± 2.00 ^{i}	6.47 ± 1.00 ^{i}	94.70 ± 5.00 ^{n}	65.22 ± 2.00 ^{n}	84.20 ± 3.00 ^{l}	33.68 ± 2.00 ^{l}
	10^{-2}	4.10 ± 0.30 ^{a}	0.57 ± 0.01 ^{a}	8.60 ± 0.50 ^{c}	5.92 ± 0.30 ^{c}	15.30 ± 1.50 ^{c}	6.12 ± 0.10 ^{c}
Vanillic acid	10^{-5}	93.40 ± 3.50 ^{m}	13.19 ± 1.00 ^{m}	128.50 ± 2.00 ^{o}	88.49 ± 4.00 ^{o}	166.00 ± 3.00 ^{P}	66.40 ±2.00 ^{P}
	10^{-3}	90.70 ± 4.50 ^{l}	12.81 ± 1.00 ^{l}	41.80 ± 1.00 ^{i}	28.79 ± 2.00 ^{i}	82.00 ± 2.00 ^{j}	32.80 ± 0.20 ^{j}
	10^{-2}	6.20 ± 1.00 ^{c}	0.88 ± 0.10 ^{c}	10.60 ± 0.50 ^{d}	7.30 ± 0.50 ^{d}	5.40 ± 0.40 ^{b}	2.16± 0.10 ^{b}

Table 2. *Cont.*

Chemical	Concentration (M)	*Portulaca oleracea*		*Chenopodium album*		*Pinellia ternata*	
		Fresh Weight (mg)	Fresh Weight (%)	Fresh Weight (mg)	Fresh Weight (%)	Fresh Weight (mg)	Fresh Weight (%)
p-Coumaric acid	10^{-5}	95.60 ± 5.00 ^{n}	13.51 ± 1.00 ^{n}	82.50 ± 5.00 ^{l}	56.82 ± 3.00 ^{l}	85.50 ± 3.00 ^{m}	34.20 ± 2.00 ^{m}
	10^{-3}	23.20 ± 2.00 ^{g}	3.28 ± 0.50 ^{g}	14.30 ± 1.00 ^{f}	9.85 ± 0.80 ^{f}	32.10 ± 2.00 ^{f}	12.84 ± 1.00 ^{f}
	10^{-2}	21.80 ± 2.00 ^{e}	3.07 ± 0.50 ^{e}	14.10 ± 0.80 ^{f}	9.71 ± 1.00 ^{f}	20.50 ± 2.00 ^{d}	8.20 ± 0.50 ^{d}
p-Hydroxybenzoic acid	10^{-5}	183.50 ± 6.80 ^{P}	25.92 ± 2.50 ^{P}	87.50 ± 2.00 ^{m}	60.26 ± 3.00 ^{m}	102.30 ± 6.50 ^{n}	40.92 ± 3.00 ^{n}
	10^{-3}	82.20 ± 3.90 ^{k}	11.61 ± 1.00 ^{k}	78.70 ± 3.00 ^{k}	54.20 ± 4.00 ^{k}	53.50 ± 2.00 ^{i}	21.40 ± 1.50 ^{i}
	10^{-2}	22.60 ± 2.00 ^{f}	3.19 ± 0.50 ^{f}	20.00 ± 1.00 ^{g}	13.77 ± 1.00 ^{g}	21.30 ± 1.00 ^{e}	8.52 ± 0.50 ^{e}
Control	-	707.8 ± 15.60 ^{s}	-	145.2 ± 5.00 ^{P}		250.0 ± 8.00 ^{s}	

Experimental data are expressed as mean ± standard deviation (n = 3). Data having the same letter in a column are not significantly different, as determined by Duncan's multiple range test ($p < 0.05$).

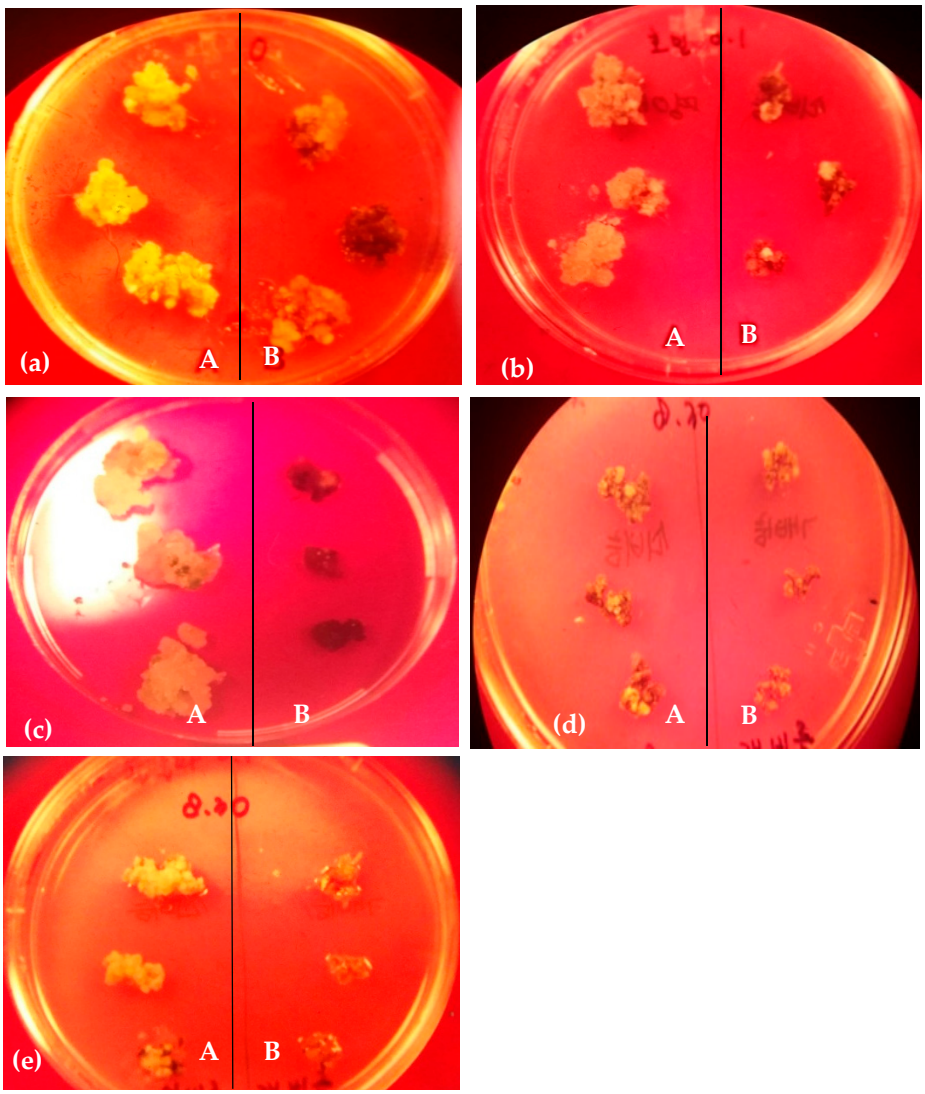

Figure 1. Effect of *Secale cereale* (Paldong cultivars) extract on callus growth of weeds. (**a**) Callus growth in the absence of *S. cereale* extract: (A) Callus of *Chenopodium Album*, (B) callus of *Portulaca oleracea*. (**b**) Callus growth in the Murashige and Skoog (MS) medium supplemented with 0.1% *S. cereale* extract: (A) callus of *C. album*, (B) callus of *P. oleracea*. (**c**) Callus growth of *C. communis* (A) in the absence of *S. cereale* extract and (B) in 5% *S. cereale* extract. (**d**) Callus growth of *Digitaria ciliaris* (A) in the absence of *S. cereale* extract and (B) in 0.1% *S. cereale* extract. (**e**) Callus growth of *Pinellia ternata* (A) in the absence of *S. cereale* extract and (B) 0.1% *S. cereale* extract.

3.2. Identification of Allelochemicals

Phenol compounds in *S. cereale* were analyzed by HPLC. The number of phenol compounds in the analyzed plants was dependent on the plant parts and cultivar used. In total, six active phenolic compounds in the leaf and root of two cultivars of *S. cereale* were identified and quantified (Table 3, Figures 2 and 3). A wide range of quantitative differences in the concentration of the phenolic compounds was observed between the two cultivars. In this study, the concentration of total phenolic compounds in the leaf extracts of Paldong cultivars (4576.50 ± 25.80 μg mL^{-1}) was 2.35-fold higher than in the root extracts (1947.00 ± 15.90 μg mL^{-1}). Similarly, the concentration of total phenolic compounds in the leaf extracts of Singhi cultivars (2467.00 ± 14.40 μg mL^{-1}) was 1.85 times higher than that in the root extracts. The concentration of all identified phenolic compounds was significantly higher in the leaf extracts of Paldong compared to Singhi. In particular, syringic acid in Paldong was 12.87 times higher than in the leaf extract of Singhi. Other predominant phenolic compounds, such as salicylic acid, *p*-coumaric acid, vanillic acid, and *p*-hydroxybenzoic acids, were 3.12, 1.28, 3.30, and 4.62 times higher in the leaf extracts of Paldong, respectively. In contrast, ferulic acid, *p*-coumaric acid, syringic acid, and vanillic acid were higher in the root extracts of the Singhi cultivar. Among all the identified compounds, syringic acid content was 14 times higher in the root extracts of the Singhi cultivars.

Table 3. Identification of phenolic compounds from the different tissues and cultivars of *S. cereale*.

Cultivar	Parts	Phenolic Compound Concentration (μg mL^{-1})						
		Salicylic Acid	*p*-Hydroxybenzoic Acid	Vanillic Acid	Syringic Acid	*p*-Coumaric Acid	Ferulic Acid	Total
Paldong	Leaf	1492.50 ± 12.20 ^{d}	1286.50 ± 12.50 ^{d}	1354.00 ± 15.00 ^{d}	1506.50 ± 10.00 ^{d}	1392.50 ± 15.00 ^{d}	292.00 ± 5.00 ^{d}	7324.00 ± 25.80 ^{d}
	Root	383.00 ± 5.00 ^{b}	437.50 ± 5.00 ^{c}	311.50 ± 6.90 ^{a}	7.00 ± 0.50 ^{a}	752.00 ± 8.80 ^{a}	57.00 ± 2.00 ^{a}	1948.00 ± 15.90 ^{a}
Singhi	Leaf	479.00 ± 4.00 ^{c}	278.20 ± 4.00 ^{a}	410.00 ± 5.00 ^{c}	117.90 ± 5.00 ^{c}	1085.50 ± 15.00 ^{c}	98.500 ± 4.00 ^{b}	2469.10 ± 14.40 ^{c}
	Root	358.50 ± 5.00 ^{a}	351.00 ± 5.00 ^{b}	403.00 ± 4.00 ^{b}	98.00 ± 3.00 ^{b}	1065.00 ± 10.00 ^{b}	162.00 ± 2.50 ^{c}	2437.50 ± 16.50 ^{b}

Experimental data are expressed as mean ± standard deviation ($n = 3$). Data with the same letter in a column are not significantly different, as determined by Duncan's multiple range test ($p < 0.05$).

3.3. Principal Component Analysis (PCA)

To analyze correlation between the cultivars, the obtained data of the major phenolic compounds were subjected to PCA. The PC1 accounted for 94.05% of the total variance, while the PC2 explained 4.94% (Figure 4). The PCA results indicated that phenolic compound's composition was significantly dependent on the cultivar type and plant part. The variables located in the same direction are considered more closely related to each other. The PCA analysis distinctly separated the Paldong and Singhi cultivar. Except for Paldong leaves, all variables from the two cultivars were located on the negative side of the PC1. Samples extracted from the leaf of Paldong cultivars, with a higher concentration of phenolic compounds, showed a close correlation to vanillic acid, syringic acid, salicylic acid, and *p*-hydroxybenzoic acid. Variations in the phenolic compound's composition between the two cultivars can be attributed to the variation in the geographical region of cultivar origin, climatic conditions, and genetic variations. Moreover, variations in the composition of the phenolic compounds can provide useful information for distinguishing the two *S. cereale* cultivars.

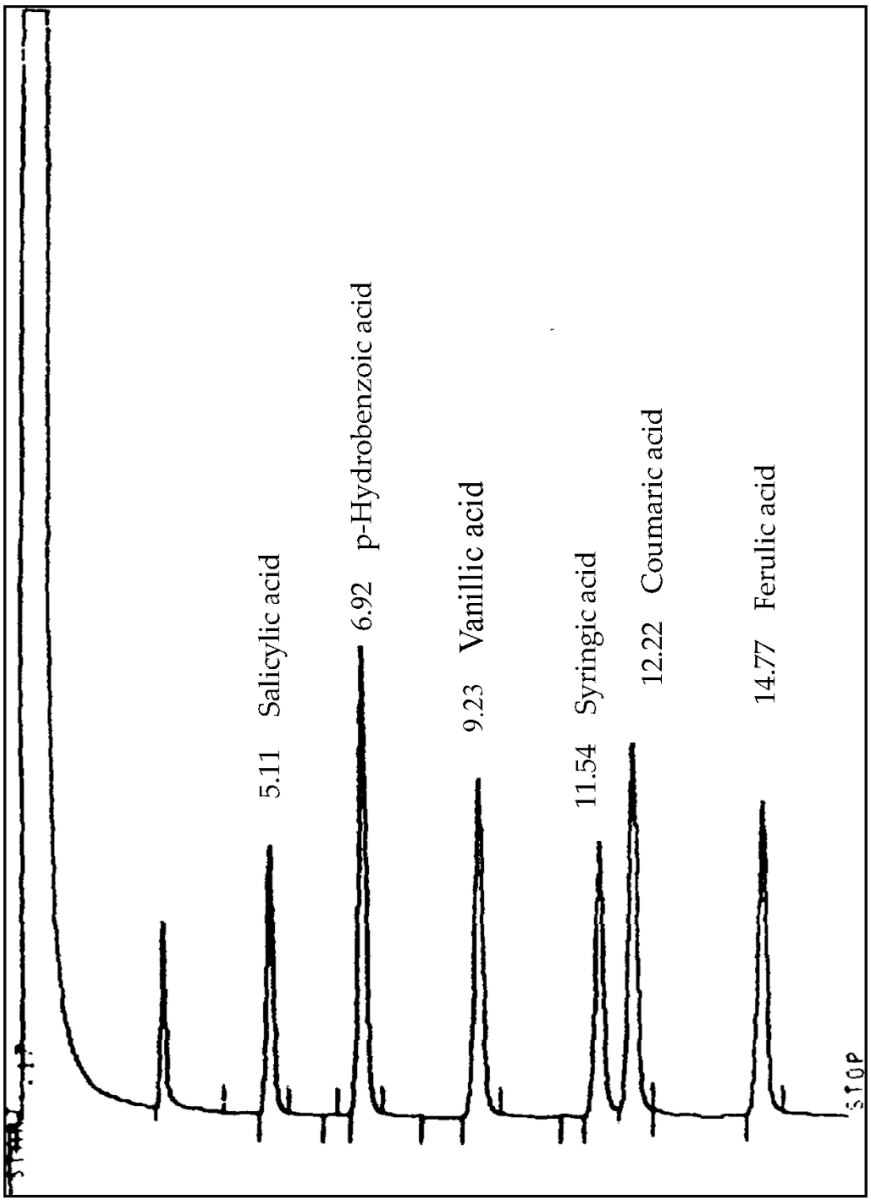

Figure 2. Chromatogram of standard phenolic compounds.

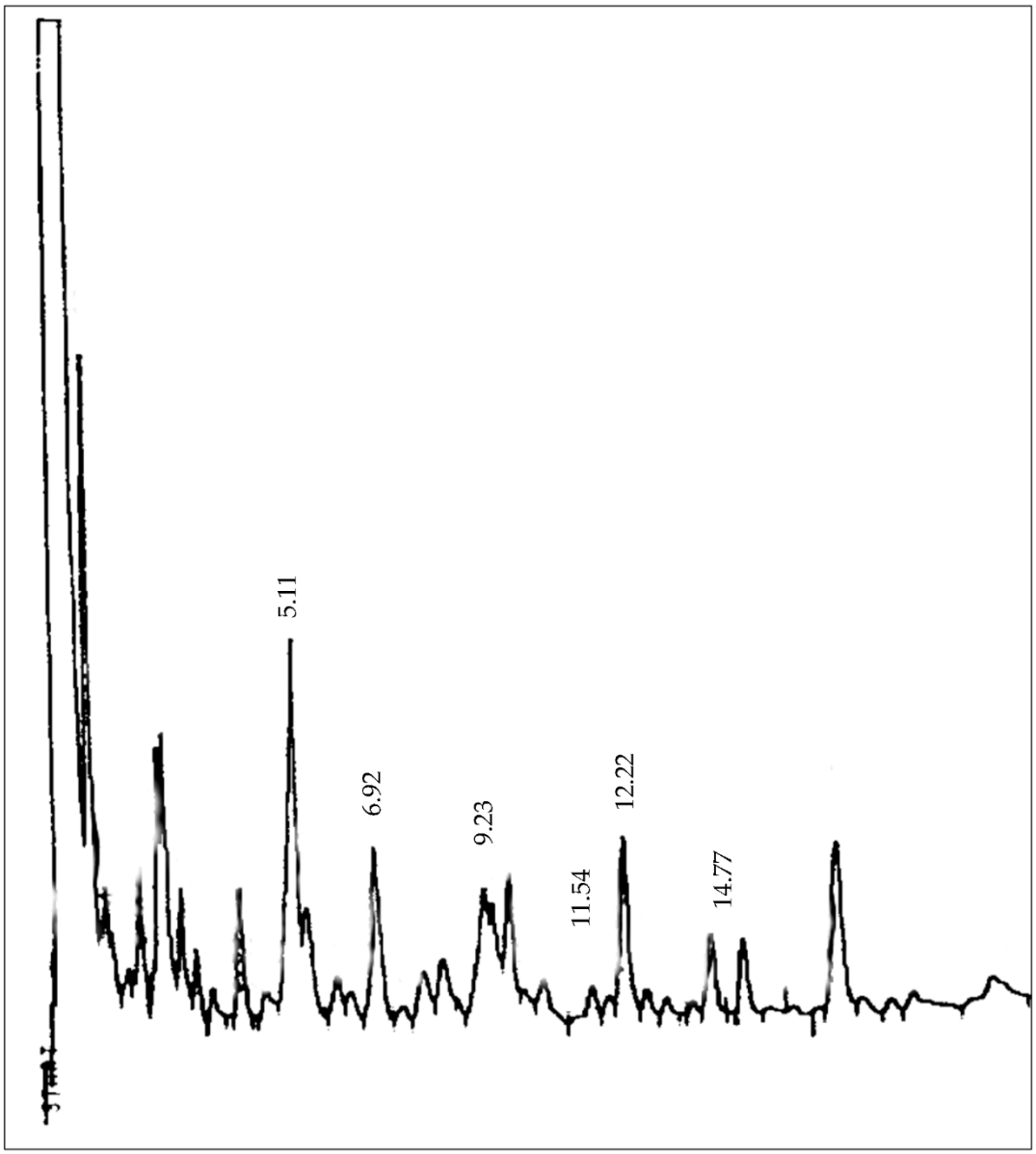

Figure 3. Chromatogram of phenolic compounds identified from Paldong cultivars.

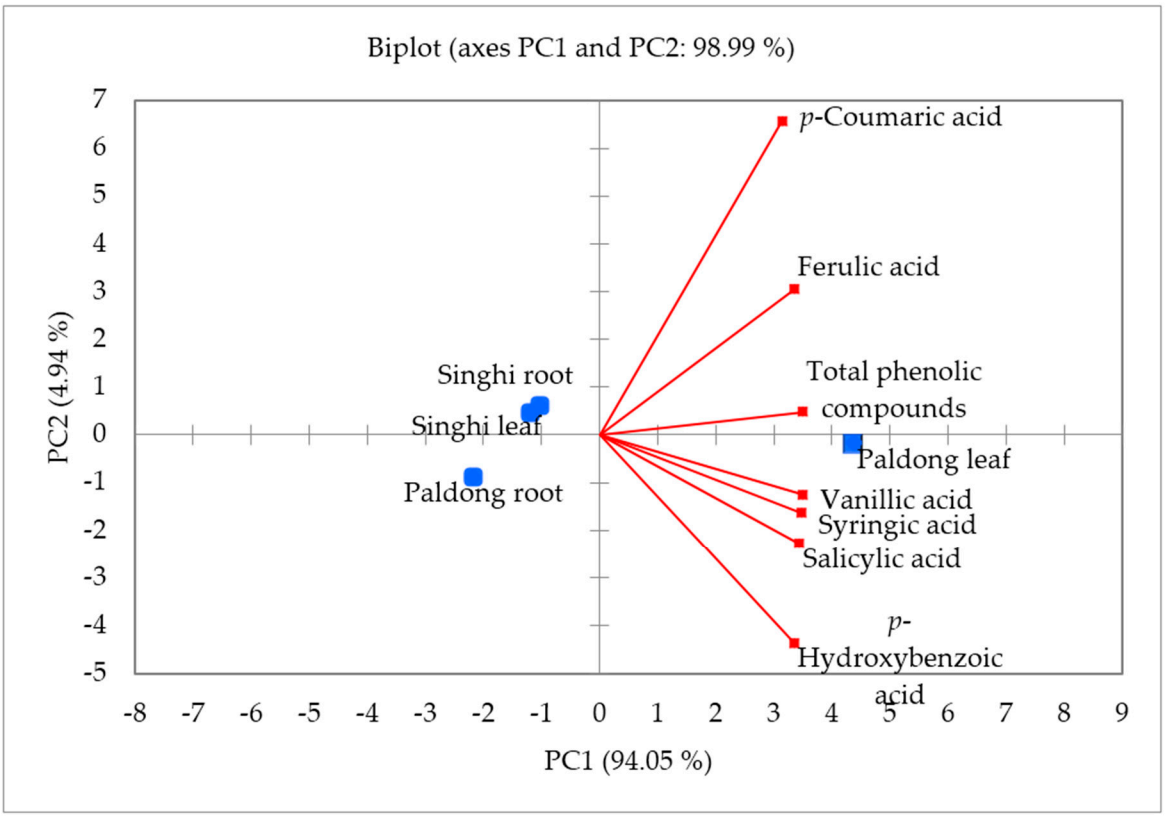

Figure 4. Principal component analysis (PCA) biplot constructed variance, based on the phenolic compounds identified from Paldong cultivars.

3.4. Inhibitory Ability of Different Cultivars of *S. cereale* on Grass and Broadleaf Weeds

A wide range of inhibitory effects of *S. cereale* cultivars on the grass and broadleaf weeds was observed in the *Perilla* field (Table 4). The cover crop was remarkably effective for controlling the weeds. The cover crop inhibited germination of weeds until 50 d after application, and the inhibition rate was above 80%, compared to the control field. As expected, both Paldong and Singhi cultivars significantly inhibited the growth of grass and broad leaf weeds. Comparatively, the inhibitory effect of Paldong cultivars (88.00% ± 5.00%) on grass weeds was higher than Singhi cultivars (84.10% ± 5.00%). A similar trend was observed when Paldong cultivar was used against broadleaf weeds (88.00% ± 6.50%), which was significantly ($p < 0.05$) higher than using Singhi cultivars.

Table 4. Inhibitory ability of different cultivars of *S. cereale* on grass and broadleaf weeds in the *Perilla* field.

Cultivar	Grass Weeds		Broadleaf Weeds			
	Fresh Weight (mg)	Inhibitory Ability (%)	Fresh Weight (mg)	Inhibitory Ability (%)	Total Fresh Weight (mg)	Total Inhibitory Ability (%)
Paldong	51.50 ± 4.00 ^{a}	88.00 ± 5.00 ^{b}	8.50 ± 1.00 ^{a}	89.00 ± 5.00 ^{b}	60.00 ± 4.50 ^{a}	88.00 ± 6.50 ^{b}
Singhi	68.50 ± 4.50 ^{b}	84.10 ± 5.00 ^{a}	10.20 ± 1.80 ^{b}	87.30 ± 6.00 ^{a}	78.50 ± 5.00 ^{b}	85.00 ± 5.00 ^{a}
Control	430.50 ± 15.00 ^{c}		80.00 ± 6.90 ^{c}		510.00 ± 10.70 ^{c}	

Experimental data are expressed as mean ± standard deviation ($n = 3$). Data with the same letter in a column are not significantly different, as determined by Duncan's multiple range test ($p < 0.05$).

3.5. Effect of Seeding Density of *S. cereale* (Paldong cv.), Tillage, and No-Tillage on Weed Control

Variations in the seeding density of cover crops showed a significant effect on the inhibition rate of grass and broadleaf weeds (Table 5). The inhibitory effect of cover crop was lower in the cultivating field with a seeding density of 8 kg ha^{-1}. Tillage cultivating land under an LPD regime showed a lower

inhibitory effect on grass weed types, while tillage field under an HPD system showed inhibitory effect up to 49.10% ± 5.00% on grass weeds. When the number of cover crops planted in the crop field increased to 16 kg ha^{-1}, the inhibitory effect against the grass weeds increased in both tillage and no-tillage systems. Moreover, an increase in the seeding rate of cover crops increased the inhibition of grass weeds, particularly in the no-tillage regime. In the present study, when the seeding density doubled, the tillage and no-tillage systems (both LPD and HPD) showed a higher degree of inhibition in broadleaf weeds (up to 100%).

Table 5. Effect of seeding density of Paldong cv., tillage, and no-tillage on weed control.

Amount of Cover Crops (Kg)	Types of Cultivation	Method of Planting *P. frutescens*	Grass Weeds		Broadleaf Weeds	
			Fresh Weight (mg)	Inhibitory Ability (%)	Fresh Weight (mg)	Inhibitory Ability (%)
8	Tillage	HPD	311.20 ± 10.00 ^{g}	50.92 ± 5.00 ^{b}	13.30 ± 1.00 ^{d}	42.92 ± 3.00 ^{a}
		LPD	355.80 ± 12.50 ^{h}	43.89 ± 3.50 ^{a}	0 ^{a}	100 ^{d}
	No-tillage	HPD	202.10 ± 5.00 ^{a}	68.13 ± 4.99 ^{g}	9.10 ± 1.00 ^{c}	60.94 ± 2.00 ^{b}
		LPD	304.20 ± 7.50 ^{f}	52.03± 3.00 ^{c}	1.90 ± 0.10 ^{b}	91.85 ± 5.00 ^{c}
16	Tillage	HPD	245.70 ± 4.00 ^{c}	61.25 ± 2.50 ^{d}	0 ^{a}	100 ^{d}
		LPD	301.60 ± 7.00 ^{e}	52.44 ± 3.00 ^{c}	0 ^{a}	100 ^{d}
	No-tillage	HPD	206.50 ± 4.00 ^{a}	67.43 ± 4.00 ^{f}	0 ^{a}	100 ^{d}
		LPD	231.90 ± 7.00 ^{b}	63.42 ± 5.00 ^{e}	0 ^{a}	100 ^{d}
Control			634.10 ± 12.00 ^{i}		23.30 ± 3.00 ^{e}	

Experimental data are expressed as mean ± standard deviation (n = 3). Data with the same letter in a column are not significantly different, as determined by Duncan's multiple range test ($p < 0.05$).

4. Discussion

This study demonstrated the inhibitory effect of *S. cereale* extracts on the growth of different weeds both under in vitro and field conditions. An increase in the concentration of *S. cereale* (Paldong cv.) leaf extracts (5%) resulted in an intense inhibitory effect on the biomass of *Commelina communis* callus. Callus growth of *Portulaca oleracea* and *C. album* was more resistant than the callus of *C. communis* to the same amount of leaf extracts. It has been reported that lower inhibitory effects of cover crops are associated with the presence of a strong detoxification mechanism present in the weed species [54]. The growth and development of *Digitaria ciliaris* and *Amaranthus lividus* calli were highly sensitive to all concentrations of leaf extracts, as indicated by a lower amount of biomass and higher inhibition rate. In contrast, a lower concentration of *S. cereale* extracts (0.1%) showed a stimulating effect on callus growth in *C. communis*. These results indicate that the allelopathic effects of *S. cereale* towards weeds are species specific. Moreover, some of the phenolic compounds present in the plant species have been shown to produce both stimulatory and inhibitory effects on callus growth and proliferation [55]. However, in the present study, callus growth and fresh weight were inversely related to the tested phenolic compound's concentration. A similar observation was also recorded by Braga et al. [56], in which a higher concentration of phenolic compounds suppressed the growth of callus and its biomass. The inhibitory effects of these compounds on the biomass of all weed calli were observed in leaf extraction concentrations as low as 10^{-5} M. Previous studies reported a close association between allelopathic properties and phytochemical exudes from plants [57,58]. The allelochemicals released from the plant exudes usually inhibit the growth of plant cells by disrupting the cell membrane and cellular components [56]. Phenolic compounds such as *p*-coumaric acid and its various derivatives were found to inhibit photosynthesis enzymatic activities of glucosephosphate isomerase (PGI), 6-phosphate dehydrogenase (G6P-DH), and aldolase (AID) in the oxidative pentose phosphate pathway (OPPP), causing a detrimental effect on plant growth [59]. It has been suggested that *p*-coumarin developed a waterstress-like situation in plants by accumulating a higher osmolality. Furthermore, *p*-coumarin is known for its inhibitory effect on seed germination and plant growth, and it also causes a deleterious effect on root growth by changing its morphological and physiological structure [60–62]. It has been argued that quercetin can affect the electron transport system (ETS), causing inhibition of substrate

oxidation, thus disrupting the uptake of phosphate [63]. Moreover, some studies have suggested the direct involvement of ROS during allelochemical treatment. For example, polyphenols are responsible for forming superoxide anions [64], hydroxyl radicals formed by donating electrons to molecular oxygen [65], which in turn rapidly change the cellular components, including DNA and proteins, and alter membrane permeability. Some phenolic compounds, such as benzoxazinoids, have been found to have a deleterious effect on nucleic acids and other cellular components such as ribosomes and mitochondrion [66,67]. Similarly, BOA, an important allelochemical from *S. cereale* extract, has been reported to have inhibitory effect on plant growth by enhancing peroxidase activity and regeneration of H_2O_2, and it has also been reported to increase lignin content in the cell by increasing the rigidity of the cell wall [68]. On the other hand, benzoxazolinone is known for its antitoxin activities and effectively blocks lateral root formation in seedlings [67].

The effect of *S. cereale* biomass on the growth of grass and broadleaf weeds were evaluated in conventional tillage and no-tillage systems. A significant difference was observed in the biomass of weeds when the seeding rate of cover crops increased from 8 to 16 kg ha^{-1} in the no-tillage field with the LPD system. The primary reason the increased biomass of cover crop was to enhance the allelopathic effect on weeds. The results of this study support the hypothesis that an increase in the biomass of cover crop enhances the allelopathic effect on grass and broadleaf weeds. In the present study, we observed an increment in the inhibitory effect with the increase in cover crop biomass. Previously, Koger et al. [69] and Uchino et al. [70] also described higher inhibition of weeds by increasing the biomass of cover crops. In the present study, complete inhibition of broadleaf weeds in both high plant density (HPD) and low plant density (LPD) of the cultivating crops (*Perilla*) was found when the amount of cover crops was doubled. However, this change was not observed in the case of grass weeds. Higher resistivity of grass weeds on *S. cereale* biomass has been reported by Norsworthy [71], Tet-Vun and Tsmail [72], and Tabaglio et al. [54]. The results of this study agree with the previous studies, in which the inhibitory effect of *S. cereale* on grass weeds was weaker than on broadleaf weeds [54]. Tabaglio et al. [54] attributed the wide range of variation in the inhibitory effect of *S. cereale* extracts with the variability of allelochemical composition in the cover crops. Inhibitory percentages of weeds by the *S. cereale* cover crops were higher on broadleaf weeds than on grass weeds. Complete inhibition of broadleaf weeds was achieved by increasing the seeding density of cover crop. One of the possible reasons for this could be that broadleaf weeds are highly sensitive to the allelochemicals present in the *S. cereale* extracts compared to grass weeds. This is in agreement with a previous report by Brennan et al. [73]; they found the dry weight of weeds decreased linearly with an increase in seeding rate of cover crops of legumes and oats. However, Buchanan et al. [74] reported that the cover crop density negatively influenced the weed density.

In the present study, we found that, in the *Perilla* cultivating field, the HPD system reduced the weeds (grass and broadleaf) by 40% compared to the LPD. However, the result indicated that the grass weed density remained higher in both HPD and LPD systems. The present study indicated that *S. cereale* contains allelochemicals that can inhibit the growth of weeds selectively, and this varies widely in tillage and no-tillage systems. Therefore, the higher inhibitory properties of *S. cereale* on both grass and broadleaf weeds can be used for mitigating the weed problems in *Perilla* fields as well as for sustainable weed management in organic farms by reducing the use of herbicides.

Different tillage or no-tillage systems affect the soil structure, nutrients, water retention potential, pH, and temperature [75], and it causes significant variations in the germination, growth density, and composition of weeds [76,77]. The result of this study indicated that an increase in the *S. cereale* biomass significantly increased the weed suppression rate in the no-tillage system. It has been reported that the no-tillage system has a better water and soil conservation system and produces more cover crop biomass, which directly influences the weed suppression rate [78]. According to Strudley et al. [79], cover crops in combination with no-tillage further improves the soil structure, and the soil integrity and number of biological pores increased, which ultimately upgrades the soil's hydraulic behavior [80]. Mitchell et al. [81] observed a faster infiltration of applied water in the no-tillage and cover crops system

compared to the tillage and cover crops system. Edwards et al. [82] studied the increased infiltration of water via macropores created by earthworms, which could maintain the continuity of soil pores in the different horizons in the no-tillage and cover crops system [83]. Contrarily, a lower infiltration of water in the tillage system is often caused by the disruption of pore continuity and destruction of the large soil aggregates, which results in particle slaking and pore clogging [80]. An increase in the concentration of phenolic compounds in mulches of cover crops was correlated to a strong inhibitory effect on weeds [84]. Moreover, high levels of phenolic compounds such as *p*-coumaric acid, ferulic acid, vanillic acid, salicylic acid, and *p*-hydrobenzoic acid were reported in the decomposed residue of *S. cereale*, indicating that the presence of a higher biomass in the fields would increase the allelochemical concentration and suppress weed growth [85,86]. However, the presence of these allelochemicals in the soil alone is not sufficient to explain the inhibitory effect of *S. cereale* extracts on weeds tested in the field. Microbial metabolism is an important factor for the degradation or accumulation of allelochemicals in the soil, which influences the allelochemical's effect on the weeds [87,88]. The results indicate that the synergetic effects of these compounds in the presence of other competitive variables are an important factor for weed control. Further research is required to support the evidence of the synergetic effects of the phenolic compounds and the role of microbial metabolism under field conditions.

Author Contributions: B.K.G. contributed by carrying out the experiments and writing the manuscript. C.Y.Y. supervised the experiment. B.G., E.S.S. and I.M.C. contributed by analyzing phenolic compounds and editing of the manuscript.

Funding: This research received no external funding.

Acknowledgments: This work was supported by funding from the KU research professor program.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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