

The Effect of Salicylic Acid and 20 Substituted Molecules on Alleviating Metolachlor Herbicide Injury in Rice (*Oryza sativa*)

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Abstract: Salicylic acid (SA) is an endogenous plant hormone that has a wide range of pharmacological effects. Studies have indicated that SA has herbicide safening activity. In this study, the herbicide safening activity of SA and 20 substituted molecules were tested on agar-cultured rice. Biological assay results indicated that SA and substituted SA had a low inhibitory effect on the growth of rice seedlings (*Oryza sativa*), and partially alleviated the effects of metolachlor toxicity. Moreover, at 0.25 mg L⁻¹, the safening effect of compounds **l** and **u** lessened the effects of metolachlor phytotoxicity on plant height and fresh weight when compared to the effects of the control, fenclorim. The effects of metolachlor toxicity were reduced on root length due to the safening effects of compounds **l**, **n**, and **u**; these effects were greater than those of fenclorim. These compounds could facilitate the development of novel herbicide safeners.

Keywords: SA; substituted SA; metolachlor; herbicide safeners; rice

1. Introduction

Herbicides are commonly used to control weeds, and guarantee the normal growth and development of grain crops to meet rapidly growing demands [1]. Chloramides (e.g., metolachlor, acetochlor, and pretilachlor) are among the most commonly used herbicide types. However, chloramides negatively affect the yield and growth of crops such as sweet potato, sorghum, wheat, soybean, corn, and rice [2–6]. Technologies that can combat the poor selectivity of herbicides include selective conventional herbicides and herbicide safeners [7,8]. Herbicide safeners are the most direct and cost-effective of these methods [9]. When used with specific herbicides, safeners can reduce the toxicity and improve the selectivity of herbicides [9,10].

A common mechanism of herbicide detoxification by safeners, of which there are many, was derived from the theory of glutathione conjugation. According to this theory, herbicide safeners induce the expression of glutathione S-transferases (GSTs) in plants, which increases the rate of conversion of GST-bound herbicide into a non-toxic intermediate compound, thus preventing toxicity towards crops [11,12]. The first commercialized herbicide safener, 1,8-naphthalic anhydride (NA), was discovered in 1978 by Hoffman et al. and was used to protect corn from thiocarbamate herbicide damage [13–16]. Synthetic herbicide safeners such as fenclorim, dichlormid, flurazole, fenchlorazole, and dymrone have since been commercialized for crop protection [17–20]. However, some commercially available herbicide safeners (e.g., dichlormid, benoxacor, and furilazole) are toxic to both aquatic organisms and mammals [21]. The environmental toxicity of these compounds has led to a greater search for highly efficient, highly active, and environmentally friendlier herbicide safeners, representing a new direction for the development of pesticides.

Salicylic acid (SA) is an endogenous plant hormone that plays an important role in the regulation and control of plant growth and developmental processes [22], such as seed germination, stomatal movement, photoperiodic responses, organ differentiation, and senescence mediation [23–25]. SA is also involved in the regulation of starvation-induced flowering in duckweed (*Lemna paucicostata*) [26], which has led to the use of SA as an inhibitor of ethylene release in sliced flowers and to preserve fruit [27–29]. Numerous studies have shown that SA is an important signal molecule for plant disease resistance [30–33], and that plants' SA concentration is closely related to overall disease resistance. SA induces plants such as tobacco, cucumber, soybean, beet, rice, and corn to produce and accumulate protein, which can convey resistance to fungi, bacteria, viruses, and other harmful microorganisms [34–39].

Few studies describe the effectiveness of SA and substituted SAs as herbicide safeners, and no research has been undertaken into the effects of SA and substituted SA on alleviating chloramide herbicide injury in rice (*Oryza sativa*) [40]. Hence, the purpose of this study was to explore the herbicide safener activity of SA and SA derivatives substituted with electron donating, withdrawing, or neutral groups (20 groups labeled respectively in Figure 1a–u). The structure–activity relationship (SAR) of these compounds was also studied. These were tested against a metolachlor (M) herbicide using agar culture methodology, as reported in our previous articles [41,42]. Fenclorim (F), a commercial herbicide safener, was used as the positive control [43]. Several substituted SA compounds were associated with a strong effect on rice seedling height and were used at lower concentrations for additional tests. This study identified a novel and highly-active herbicide safener, and lays the foundation for the development of novel herbicide safeners.

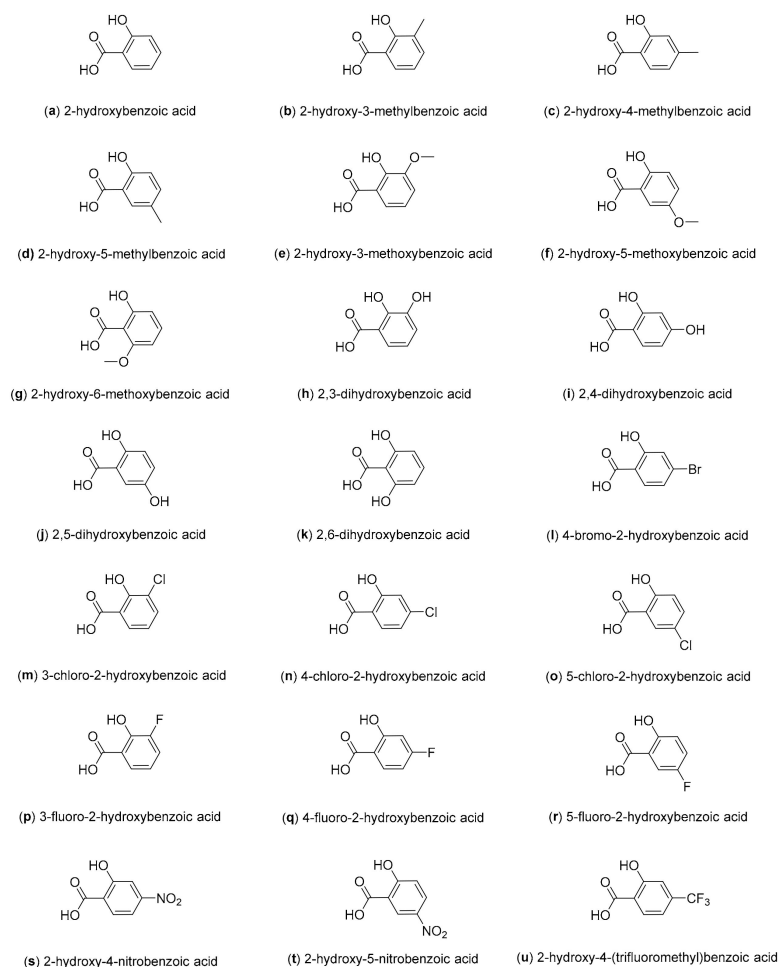


Figure 1. Salicylic acid and 20 substituted molecules.

2. Materials and Methods

2.1. General Materials

SA and 20 substituted SA compounds (Figure 1a–u) at 97.8–99.7% purity were purchased from Shanghai Macklin Biochemical Co., Ltd. (Shanghai, China). Metolachlor (emulsifiable concentrate, 960 g L⁻¹), F (purity 98%), and acetone for herbicide safener activity tests were purchased from Sinopharm Group Co., Ltd. (Shanghai, China). Acetone was used after drying and further purification steps. Rice seeds (*Oryza sativa* L. ssp. Indica var. xiang early 45#) for this experiment were purchased from Yuan Longping High-Tech Agriculture Co., Ltd. (Changsha, China). Agar strips were obtained from Xi'an Da Feng Harvest Biotechnology Co., Ltd. (Xi'an, China).

2.2. Growth Conditions

Rice seeds (*Oryza sativa*, xiang early 45#) were germinated according to a reported method [41]. Rice seeds that exhibited a full grain and uniform size were deemed “high quality” grains, and were sterilized using 5% sodium hypochlorite solution, then washed using deionized water. The sterilized rice seeds were then soaked in deionized water for 24 h and germinated for a further 36 h at 28 °C in a growth cabinet in the dark.

The phytotoxicity of compounds **a–u** on rice and the herbicide safening activities of SA and 20 substituted SA compounds (**a–u**) were evaluated by agar culture methodology under laboratory conditions according to a previous method [42]. A 12 g agar strip was added to 3 L of deionized water at 100 °C. The mixture was then constantly stirred over heat until the agar strip had completely dissolved. The mixture was then diluted to 4 L with deionized water to produce a 0.3% agar medium solution. A standard solution containing **M** (0.96 µg µL⁻¹) was also prepared by diluting 0.1 ml emulsifiable concentrate (960 g L⁻¹) in 99.9 ml deionized water. Once the agar medium had cooled to 45 °C, 200 ml was added to three 250 ml plastic boxes then cooled until solidified (negative control). A volume of 88.75 µL **M** standard solution was mixed with 1.2 L 0.3% agar medium solution to obtain agar medium with 0.25 µM **M**. A volume of 200 ml 0.25 µM **M** agar medium at 45 °C was then added to 250 ml plastic boxes and cooled until solidified. Using the same method, solidified agar media (without metolachlor) containing compounds **a–u** (8 mg L⁻¹) were prepared to evaluate the phytotoxicity of compounds **a–u** on rice.

Agar media were also prepared containing 0.25 µM metolachlor, substituted SAs (compounds **a–u**), and the positive control **F**. Compounds **a–u** and **F** (each 2.4 g) were dissolved in separate 8 ml volumes of acetone, then each was diluted to 100 ml using deionized water containing 0.5% Tween 80 to obtain 24 µg µL⁻¹ standard solutions. A volume of 40 µL of each standard solution was added to separate volumes of 1.2 L 0.25 µM metolachlor agar medium to produce agar media containing 8 mg L⁻¹ of each compound. A volume of 200 ml of each agar medium was poured and set in three 250 ml plastic boxes.

Uniformly germinated rice seedlings were collected before shoot emergence and planted in each box containing 0.3% agar medium. Agar media were as follows: 0.25 µM **M**; 8 mg L⁻¹ of compounds **a–u**; 8 mg L⁻¹ **F** with 0.25 µM **M**; 8 mg L⁻¹ compounds **a–u** with 0.25 µM **M**; and no additional compounds. Seedlings were collected and planted according to previous methods [41,42]. Fifty seedlings were added to each plastic box and incubated for 14 h at 30 °C under a grow light (intensity 110–130 µE m⁻² s⁻¹), followed by a 10 h dark photoperiod at 25 °C. After 7 days, indexes related to herbicide safener activity (plant height, root length, and fresh weight) of the seedlings in each box were measured. The phytotoxicity indexes of compounds **a–u** on rice and their herbicide safening indexes used for SAR analysis were calculated according to the following equations:

$$\text{plant height relative value} = \frac{\text{plant height under each treatment}}{\text{the average height of untreated control plants}} \times 100\%$$

$$\text{root length relative value} = \frac{\text{root length under each treatment}}{\text{the average root length of the untreated control}} \times 100\%$$

$$\text{fresh weight relative value} = \frac{\text{fresh weight under each treatment}}{\text{the average fresh weight of the untreated control}} \times 100\%$$

Compounds deemed to have high herbicide safener activities on the basis of plant height were screened for herbicide safener activities in subsequent experiments at lower concentrations. All experiments were performed in triplicate boxes.

2.3. Data Analysis

All data for SA and 20 substituted SA compounds (a–u) were analyzed by a one-way analysis of variance using SPSS 22.0 software. The relative mean value of plant height, root length, and fresh weight relative value were determined using an analysis of variance (ANOVA). The differences were compared via Duncan's range test at a significance level of $p < 0.05$.

3. Results and Discussion

3.1. Safening Effects of SA and Substituted SA on Rice Growth

The phytotoxicity of M, SA, and substituted SA on rice plant height, root length, and fresh weight are shown in Table 1. In the presence of 0.25 μM M, plant height, root length, and fresh weight were 44.87%, 46.35%, and 68.20% of those of the non-treated control (no additional compounds in agar media), respectively, which suggests that the growth rate of seedlings was suppressed in the presence of M. In the presence of SA or substituted SAs, plant height, root length, and fresh weight were 88.59–98.15%, 80.84–98.17%, and 87.32–98.40% of those of the non-treated control. Results suggest that in general, SA or substituted SAs did not inhibit the growth of rice seedlings to the same extent as those treated with M.

Table 1. Phytotoxicity of 8 mg L^{−1} salicylic acid (SA) and substituted SAs on rice plant height, root length, and fresh weight.

Compound	Safening Effect (% of Non-Treated Control)		
	Plant Height	Root Length	Fresh Weight
M	44.87 ± 0.35	46.35 ± 0.71	68.20 ± 0.78
a	97.28 ± 0.48	88.40 ± 1.52	95.90 ± 0.53
b	90.45 ± 0.98	80.94 ± 1.23	91.80 ± 1.62
c	95.14 ± 0.37	91.79 ± 0.72	90.91 ± 0.81
d	91.01 ± 0.65	90.79 ± 0.83	96.03 ± 0.42
e	91.77 ± 0.60	92.13 ± 0.98	92.12 ± 1.06
f	92.75 ± 0.51	90.98 ± 1.26	91.21 ± 1.16
g	93.29 ± 0.45	92.67 ± 1.73	93.20 ± 0.45
h	96.19 ± 0.39	90.69 ± 0.64	93.72 ± 0.85
i	93.53 ± 0.63	91.92 ± 0.76	98.40 ± 0.56
j	95.39 ± 1.22	86.98 ± 0.82	92.91 ± 0.41
k	97.49 ± 0.96	93.70 ± 0.72	96.61 ± 0.95
l	98.15 ± 0.43	95.48 ± 0.10	90.50 ± 0.31
m	90.44 ± 0.05	94.65 ± 0.55	87.32 ± 2.19
n	94.00 ± 0.12	81.31 ± 0.78	90.54 ± 0.70
o	96.22 ± 0.46	88.69 ± 1.22	87.31 ± 0.73
p	92.78 ± 0.45	98.17 ± 1.21	91.51 ± 0.65
q	95.01 ± 0.80	88.22 ± 0.72	89.88 ± 0.55
r	94.27 ± 0.68	84.76 ± 0.55	88.03 ± 0.83
s	88.59 ± 0.50	94.56 ± 0.72	97.45 ± 1.56
t	92.87 ± 1.17	87.65 ± 1.85	94.15 ± 1.18
u	95.76 ± 1.15	91.34 ± 0.52	92.74 ± 0.82

Table 2 shows the effects of 8 mg L⁻¹ SA and substituted SA and 0.25 µM M on the heights, root lengths, and fresh weight of rice seedlings. The plant height relative values under combined treatment with compounds a–u and M ranged from 36.55%–91.10%. However, plant height relative values under combined treatment with M and compounds h, k, or o were 38.44%, 36.55%, and 40.66%, respectively, which were significantly lower than that of M. Results suggest that except for compounds h, k, and o, substituted SAs (compounds a–g, i, j, l–n, and p–u) alleviated the negative effect of M on rice plant height to some extent. The relative plant height values when treated with compounds (l, n, r, and u) and M were 88.45%, 91.10%, 84.57%, and 87.45%, respectively, which were similar to the relative plant height value when using F (88.42%), indicating l, n, r, and u showed good safening activity by reducing the effects of phytotoxicity on plant height.

Table 2. Safening effect of 8 mg L⁻¹ fenclorim (F), SA, and substituted SAs (a–u) on the height, root length, and fresh weight of 0.25 µM metolachlor (M)-treated rice seedlings.

Comd.	Safening Effect (% of Non-Treated Control)		
	Plant Height	Root Length	Fresh Weight
M ¹	44.87 ± 0.35	46.35 ± 0.71	68.20 ± 0.78
F + M	88.42 ± 0.28	73.93 ± 0.42	91.45 ± 0.79
a + M	47.98 ± 0.61	81.80 ± 0.36	82.44 ± 0.97
b + M	72.90 ± 0.81	86.59 ± 0.93	90.56 ± 0.55
c + M	69.37 ± 0.73	84.59 ± 0.89	87.30 ± 0.80
d + M	70.51 ± 0.12	80.97 ± 0.99	92.16 ± 0.77
e + M	53.24 ± 0.56	67.76 ± 0.68	85.55 ± 0.51
f + M	63.01 ± 0.35	83.74 ± 0.72	88.21 ± 0.79
g + M	60.80 ± 0.39	89.34 ± 0.76	87.90 ± 0.38
h + M	38.44 ± 0.45	51.53 ± 0.95	81.00 ± 0.36
i + M	46.55 ± 0.35	80.72 ± 0.65	77.14 ± 0.90
j + M	47.06 ± 0.98	82.58 ± 0.85	86.82 ± 0.62
k + M	36.55 ± 0.32	61.12 ± 0.92	85.15 ± 0.08
l + M	88.45 ± 0.12	71.11 ± 0.91	93.41 ± 0.95
m + M	81.25 ± 0.49	83.31 ± 0.22	93.09 ± 0.69
n + M	91.10 ± 0.37	71.17 ± 0.54	89.42 ± 0.58
o + M	40.66 ± 0.28	60.54 ± 0.56	71.41 ± 0.77
p + M	64.60 ± 0.54	83.00 ± 0.73	82.14 ± 0.64
q + M	53.82 ± 0.46	63.27 ± 0.36	65.92 ± 0.45
r + M	84.57 ± 0.28	90.23 ± 0.67	93.46 ± 0.21
s + M	47.95 ± 0.31	71.65 ± 0.42	78.69 ± 0.95
t + M	45.79 ± 0.23	71.77 ± 0.92	81.81 ± 0.41
u + M	87.45 ± 0.28	89.01 ± 0.31	89.47 ± 0.10

¹ M: 0.25 µM metolachlor; F + M: 8 mg L⁻¹ fenclorim and 0.25 µM metolachlor; a–u + M: compounds a–u and 0.25 µM metolachlor. All experiments were performed in triplicate.

Preliminary structure–activity relationship analysis of SA and substituted SA compounds was performed. When the substituent group was an electron donor, plant height relative values were as follows: methyl (b–d, 69.37%–72.90%) > methoxy (e–g, 53.24%–63.01%) > phenolic hydroxy (h–k, 36.55%–47.06%), and 3-methyl (b, 72.90%) > 3-methoxy (e, 53.24%) > 3-phenolic hydroxy (h, 36.55%). When the substituent group was an electron-accepting group, plant height relative values were as follows: bromo (l, 88.45%) > trifluoromethyl (u, 87.45%) > fluoro (p–r, 53.82%–84.57%) > nitryl (s–t, 45.79%–47.95%), and 4-chloro (n, 91.10%) > 4-bromo (l, 88.45%) > 4-trifluoromethyl (u, 87.45%) > 4-fluoro (q, 53.82%) > 4-nitryl (s, 47.95%).

The relative values for plant root lengths of samples treated with compounds a–u ranged from 51.53% to 90.23%. This suggests that compounds a–u may have reduced the toxicity from metolachlor in rice roots. The herbicide safening effects of compounds a–d, f–g, i, j, m, p, r, and u were determined by their effects on M-treated rice seedling root length; the root lengths of seedlings treated with these compounds were greater than those treated with F. The relative plant root lengths of M-treated rice

were greatest in the presence of compound **r** (90.23%), followed by compound **g** (89.34%). Preliminary structure–activity relationship analysis indicated that when the substituent group was an electron donor, safener activity on root length led to the following pattern: 3-methyl (**b**, 81.80%) > 3-methoxy (**e**, 67.76%) > 3-phenolic hydroxyl (**h**, 51.53%), and 4-methyl (**c**, 84.59%) > 4-phenolic hydroxyl (**i**, 80.72%). When the substituent group was an electron-accepting group, relative root lengths showed the following pattern: 4-trifluoromethyl (**u**, 89.01%) > 4-nitryl (**s**, 71.65%) > 4-chloro (**n**, 71.17%) > 4-bromo (**l**, 71.11%) > 4-fluoro (**q**, 63.27%), and 5-fluoro (**r**, 90.23%) > 5-nitryl (**t**, 71.77%) > 5-chloro (**o**, 60.54%).

The relative fresh weight values of plants under combined treatment of 0.25 μM **M** and compounds **a–u** ranged from 65.92% to 93.46%. With the exception of compound **q** (65.92%, 4-fluoro substituent), all substituted SAs (**a–p** and **r–u**) reduced the toxicity of metolachlor on rice fresh weight by varying degrees. In the presence of **M**, compound **r** was associated with the highest plant root relative value (93.46%) compared to the non-treated control, followed by compound **l** (93.41%) and compound **m** (93.09%). Preliminary structure–activity relationship analysis indicated that when the substituent group was an electron donor, the trend in relative fresh weight was as follows: 3-methoxy (**e**, 85.55%) > 3-methyl (**b**, 82.44%) > 3-phenolic hydroxyl (**h**, 81.00%), and 4-methyl (**c**, 87.30%) > 4-phenolic hydroxyl (**i**, 77.14%). When the substituent group was an electron-accepting group, the following trend in relative fresh weight was observed: 4-bromo (**l**, 93.41%) > 4-trifluoromethyl (**u**, 89.47%) > 4-chloro (**n**, 89.42%) > 4-nitryl (**s**, 78.69%) > 4-fluoro (**q**, 65.92%), and 5-fluoro (**r**, 93.46%) > 5-nitryl (**t**, 81.81%) > 5-chloro (**o**, 71.41%).

The following observations and hypotheses were made on the basis of the above analysis: (1) In general, there was no significant difference in herbicide safener activity between electron donor and electron-accepting groups on the phenoxy ring. (2) Methyl substituents generally had greater safener activities than methoxy substituents and hydroxyl substituents on the basis of plant height results, whereas among all electron-withdrawing groups at the para-position, chlorine atoms were associated with the strongest safener activity. (3) Fluorine atoms at position 5 on the phenyl ring may have increased the safener activity on root length. (4) The safener activity on rice fresh weight showed a similar pattern to that of plant height (methyl substituents > methoxy substituents and hydroxyl substituents), with the exception of compounds with a 5-methoxy group. Insertion of an electron-accepting group (bromine atom) at the para-position may exert a strong protective effect on fresh weight when grown in the presence of metolachlor.

3.2. Safening Effect of Compounds **l**, **n**, **r**, and **u** in Rice at Lower Concentrations

The herbicide safener activity of SA and substituted SA compounds **l**, **n**, **r**, and **u** was further explored using concentrations of 0.25 mg L^{-1} , 0.5 mg L^{-1} , 1 mg L^{-1} , 2 mg L^{-1} , and 4 mg L^{-1} . These compounds were chosen because height recovery rates of seedlings treated with **M** were similar to those of seedlings treated with **F**. Herbicide safener effects of compounds **l**, **r**, **n**, and **u** on **M**-treated rice seedling height, root length, and fresh weight are illustrated in Figure 2, Figure 3, Figure 4 and Tables S1–S3 (in Supplementary Materials).

As shown in Figure 2, relative plant height values at different treatment concentrations (0.25–4 mg L^{-1}) were also described; ranges were 66.39–94.51% (**l**), 52.88–95.23% (**n**), 54.74–98.69% (**r**), and 59.05–91.84% (**u**).

When the concentration of **F** was 0.25–4 mg L^{-1} , relative plant height values of **M**-treated rice ranged from 54.76% to 84.39%. At concentrations of 2 mg L^{-1} and 4 mg L^{-1} of compounds **l**, **r**, **n**, and **u**, the relative plant height value of **M**-treated rice was higher than that of rice treated with the same concentration of **F**. At concentrations of 0.5 mg L^{-1} of compounds **l**, **r**, and **u**, the relative plant heights were 68.26%–69.85%, which were greater than that of **F** (63.26%). In the presence of 0.25 mg L^{-1} compound **l**, the relative height of **M**-treated rice was 66.39%, which was greater than that of those treated with compounds **r**, **n**, and **u**, and significantly greater than those treated with **F** (54.76%).

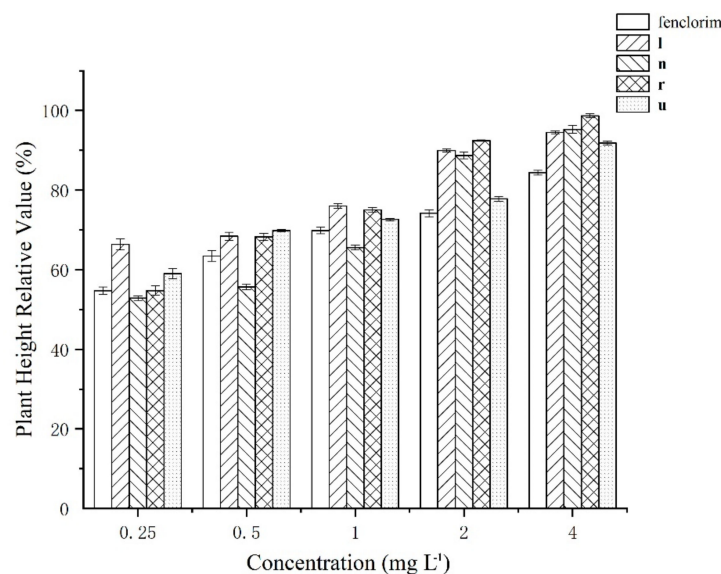


Figure 2. Plant height relative values of 0.25 μ M metolachlor (**M**)-treated rice plants treated with compounds **1**, **n**, **r**, and **u** at 0.25 mg L⁻¹, 0.5 mg L⁻¹, 1 mg L⁻¹, 2 mg L⁻¹, and 4 mg L⁻¹.

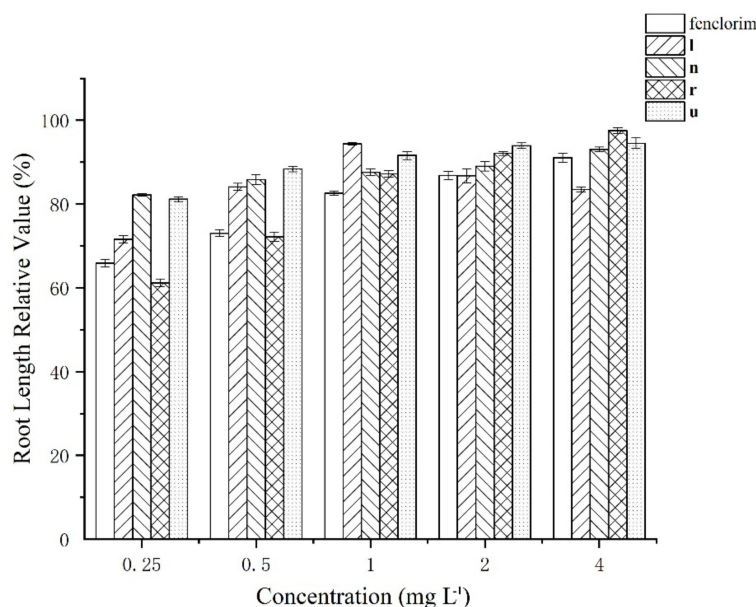


Figure 3. Plant root length relative value of 0.25 μ M metolachlor (**M**)-treated rice seedlings treated with compounds **1**, **n**, **r**, and **u** at 0.25 mg L⁻¹, 0.5 mg L⁻¹, 1 mg L⁻¹, 2 mg L⁻¹, and 4 mg L⁻¹.

Figure 3 shows that for **M**-treated rice in the presence of 0.25–4 mg L⁻¹ compounds **1**, **n**, **r**, and **u**, relative root lengths ranged from 71.57% to 83.48% (**1**), 82.20% to 93.07% (**n**), 61.21% to 97.55% (**r**), and 81.17% to 94.51% (**u**). The relative root lengths of **M**-treated rice in the presence of 0.25–4 mg L⁻¹ **F** ranged from 65.89% to 91.10%. For concentrations of 2 mg L⁻¹ and 4 mg L⁻¹, only the relative root heights of plants treated with compound **1** (86.79% and 83.48%, respectively) were smaller than those treated with compound **F** (91.10% and 86.81%, respectively) in **M**-treated rice. Compounds **1**, **n**, **r**, and **u** (1 mg L⁻¹) were associated with greater rice root length recovery rates than those associated with **F** (82.63%) when in the presence of **M**. At 0.25 mg L⁻¹, the root lengths of plants treated with compounds **1**, **n**, and **u** were 71.57%, 82.20%, and 81.17% that of the non-treated control; these values were greater than that of plants treated with **F** (65.89%), whereas values for compound **r**-treated plants were even less (61.21%).

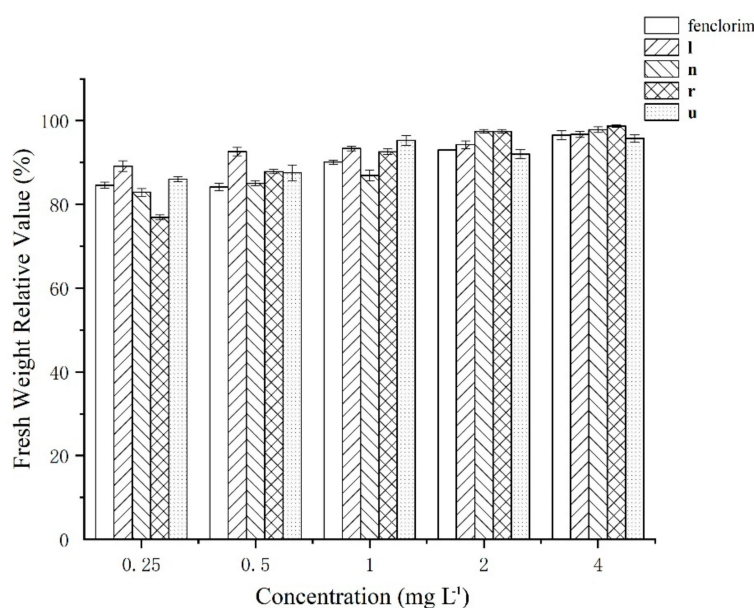


Figure 4. Fresh weight relative value of 0.25 μM metolachlor (**M**)-treated rice seedlings treated with compounds **1**, **n**, **r**, and **u** at 0.25 mg L^{-1} , 0.5 mg L^{-1} , 1 mg L^{-1} , 2 mg L^{-1} , and 4 mg L^{-1} .

Figure 4 shows the effects of compounds **1**, **n**, **r**, and **u** at 0.25–4 mg L^{-1} on **M**-treated rice seedling relative fresh weights. The values for these were 89.17%–96.73% (**1**), 82.89%–97.85% (**n**), 76.91%–98.72% (**r**), and 86.05%–95.78% (**u**). In the presence of **M**, the concentrations of 0.25–4 mg L^{-1} **F** led to relative fresh weight values ranging from 84.61% to 96.55%. At 2 mg L^{-1} concentrations of compounds, only the relative fresh weight value for 0.25 μM **M**-treated rice seedlings treated with compound **u** (92.03%) was lower than that of **F** (93.01%). At 1 mg L^{-1} , compounds **1**, **r**, and **u** (92.57%–95.53%) were associated with greater relative fresh weights of **M**-treated rice than those treated with **F** (90.13%). At 0.5 mg L^{-1} , all tested compounds **1**, **n**, **r**, and **u** (85.04%–92.26%) were associated with greater rice fresh weight than that of **F** (84.14%). At 0.25 mg L^{-1} , the relative fresh weight associated with compounds **1** and **u** in the presence of **M** was greater than that of **F** (84.61%); compound **1** led to the greatest relative fresh weight of 89.17%. A comparison of the bioassay results of **1**, **n**, **r**, and **u** revealed that **1**, at lower concentrations, had the greatest protective effect against metolachlor phytotoxicity on plant height and fresh weight. Compound **1** also showed good herbicide safener activity through its protective effects on root length at the lowest tested concentrations. These results indicate that **1** could be the best candidate safener against metolachlor phytotoxicity.

Although the application of SA and substituted SAs as herbicide safeners are protected by a patent [40], only the herbicide safener activity of compounds **i** and **q**, which protect maize (*Zea mays*) and soybean (*Glycine max*) from isoxazolone injury and reduce the maize (*Zea mays*) injury from foramsulfuron, were evaluated. The potential herbicide safener activity of these compounds against other herbicides are still unknown, and the SARs of SA and substituted SAs on herbicide safener activity are also unclear. Our research revealed that SA and substituted SA molecules are good potential herbicide safeners against metolachlor compounds in rice. SAR analysis showed that substituted groups on the phenyl ring, such as bromine and chlorine atoms, and a trifluoromethyl moiety, are associated with significant protective effects on rice growth.

Some commercial herbicide safeners have shown a potential environmental risk to aquatic organisms such as fish. For example, benoxacor displayed moderately toxic effect on freshwater fish with an LC_{50} value of 1.4 mg L^{-1} in *Ictalurus punctatus* [40]. On the contrary, the cytotoxicity of SA was very low, with an EC_{50} value of 1200 mg L^{-1} in a topminnow (*Poeciliopsis lucida*) hepatoma cell line (PLHC-1) [44]. SA also has a low acute toxicity in zebrafish (*Danio rerio*) embryos, with an EC_{50} value of 24.585 mg L^{-1} (48h) [45]. In addition, the acute toxicity in zebrafish of the evaluated

substituted SAs (**24b** with EC_{50} value of 44.15 mg L^{-1} , **24c** with EC_{50} value of 31.1 mg L^{-1} , **12b** with EC_{50} value of 44.15 mg L^{-1} , **12c** with EC_{50} value of 48.1 mg L^{-1}) are at a relatively low range (96 h) [46]. Several commercial chloroacetamide herbicide safeners have carcinogenic activity, whereas the SA was reported to have antitumor activity [47,48]. Due to their ecotoxicology and mammalian toxicity, SA and substituted SAs with simple structures are relatively environmentally friendly, and could be used as in the design of new green herbicide safeners. Furthermore, studies on the mechanism of SA and substituted SA molecules for alleviating metolachlor herbicide injury are currently in progress.

4. Conclusions

In summary, the herbicide safener activity of SA and SA substituted with 20 different compounds was tested using agar culture methodology. The results from biological assays indicated that SA and substituted SAs had few inhibitory effects on the growth of rice seedlings, and most alleviated metolachlor toxicity to some extent. Moreover, four of these compounds (**l**, **n**, **r**, and **u**) associated with detoxification were also associated with increased plant height; these were further screened. At 0.25 mg L^{-1} , the safening effect of compounds **l** and **u** lessened the effects of metolachlor phytotoxicity on plant height and fresh weight when compared to the control, fenclorim. Overall, compound **l** showed the best performance. The effects of metolachlor toxicity were reduced on root length due to the safening effects of compounds **l**, **n**, and **u**, which had greater effects than fenclorim. In general, these results suggest that SA and substituted SA molecules are active compounds with high herbicide safener activity, and these have the potential to aid the development of novel herbicide safeners.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/3/317/s1>, Table S1: Herbicide safening effect of compound (**l**, **n**, **r** and **u**) on the rice seedlings treated with metolachlor in plant heights 1, Table S2: Herbicide safening effect of compound (**l**, **n**, **r** and **u**) on the rice seedlings treated with metolachlor in root lengths 1, Table S3: Herbicide safening effect of compound (**l**, **n**, **r** and **u**) on the rice seedlings treated with metolachlor in fresh weight 1.

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