

## Article

# Rice By-Products Reduce Seed and Seedlings Survival of *Echinochloa crus-galli*, *Leptochloa chinensis* and *Fimbristylis miliacea*

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**Abstract:** Extracting allelochemicals from rice (*Oryza sativa*) straw and use for weed management is more sustainable than burning and reduces herbicide dependence. Water soluble compounds were extracted from shoots and roots of OM 5930, generating both a crystallized by-product and liquid extract. Crystallized product was applied to soil with pre-germinated barnyardgrass (*Echinochloa crus-galli* L. Beauv), red sprangletop (*Leptochloa chinensis* L. Nees), and grass-like fimbry (*Fimbristylis miliacea* L. Vahl) seeds. As little as 9.4 g per pot (1 ton ha<sup>-1</sup> biomass) reduced survival of all species, with the order of sensitivity barnyardgrass (BG) < red sprangletop (RS) < grasslike-fimbry (GF). Increased rates or time of exposure (3 to 42 days after treatment; DAT) resulted in a stepwise reduction in seed survival. Using liquid extract, 5.33 g pot<sup>-1</sup> (3 tons ha<sup>-1</sup> biomass) reduced BG survival by 49.8%, while 2.67 g pot<sup>-1</sup> reduced survival of RS and GF by 49.7 and 54.3%, respectively at 42 DAT. A rate of 8 g pot<sup>-1</sup> reduced survival of BG seedlings by 78.3% but was lethal to RS and GF seedlings. The most abundant allelochemicals present were ergosterol peroxide, *p*-coumaric acid, and salicylic acid. OM 5930 rice is a promising variety for extraction of allelopathic compounds and application for extended herbicidal activity.

**Keywords:** allelochemicals; barnyardgrass; bioherbicide; grass-like fimbry; red sprangletop



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## 1. Introduction

Weeds are one of the largest impediments to optimizing rice production [1]. Specifically, grass species such as barnyardgrass (BG; *Echinochloa crus-galli* L. Beauv), red sprangletop (RS; *Leptochloa chinensis* L. Nees), and grass-like fimbry (GF; *Fimbristylis miliacea* L. Vahl) are widespread, competitive species in rice fields. Rice yields can be reduced by 50–70% following season-long competition of each species with rice [1–3]. Barnyardgrass has growth and morphological characteristics like rice, making timely removal difficult. Another factor contributing to the competitiveness of BG is that plants can remove 60–80% of the available nitrogen in the soil [4]. As a result, BG established in an area as little as 40 cm<sup>2</sup> reduced rice yields 27% [5]. According to Chin (2001), rice yield decreased 50% following season-long competition with as few as 15 BG m<sup>-2</sup> [1]. The competitiveness of both BG and RS partially results because they are C4 plants; more efficient photosynthesis increases overall biomass. A density of 2–6 plants m<sup>-2</sup> of BG or RS reduced rice yield from 14–44% [6]. Additionally, both species are also intermediate hosts for a number of other rice pests such as leaf blight (*Nephotettix* spp.), rice black bug (*Scotinophara latiscula*), and leaf-folder (*Cnaphalocrocis medinalis*) [7]. Unlike BG or RS, GF is a dominant species in rice fields because of prolific seed production. Plants germinate continuously during the rice growing

season [8], constantly posing a competitive threat if left unchecked. GF competition with rice reduced yields up to 10% [9].

Continuous use of herbicides to remove weeds in rice has negative impacts on the environment as well as human health, in addition to facilitating selection of herbicide-resistant weeds. Currently, BG and RS are the two most important herbicide-resistant weed species in rice. Juliano et al. (2010) confirmed BG resistance to both butachlor and propanil in the Philippines [10]. Resistance to herbicides inhibiting acetolactate synthase (ALS) and quinclorac has also been confirmed [11,12]. Rahman et al. (2010) identified an SB2 strain of RS resistant to propanil and cyhalofopbutyl, at 5500 and 800 mL ai ha<sup>-1</sup>, respectively [13]. Schaedler et al. (2015) identified ALS resistance in a biotype of GF from Brazil [14].

An alternative to continued dependence on synthetic herbicides for weed management is adoption of plant varieties that exhibit allelopathy [15]. Allelopathy is the natural production and release of secondary metabolites that exhibit positive or negative effects on neighboring plants [16]. Production of allelochemicals as secondary metabolites is beneficial to plants in several ways: serve to deter invasion by pathogens, feeding by insects, and improving plant tolerance to abiotic stresses [17]. However, allelochemicals in plants may also directly interfere with physiological functions of weeds such as germination, root and shoot growth, or disruption of symbiotic relationships [18]. Several studies have identified rice straw as a mulch that can suppress weed emergence. Abouziena and Radwan (2014) determined rice straw suppressed weed biomass in onion by 51% [19]. Rahman et al. (2010) found that rice straw reduced weed biomass from 50–70% in no-till wheat [13]. However, leaving rice straw following harvest may detrimentally impact nutrient availability and soil moisture, and complicate establishment of subsequent crops [20]. Environmentally, this significantly contributes to emitted gases and solids that reduce air quality [21] and contribute to climate change [22]. With increased concern of rising carbon dioxide levels, identification of new uses of rice straw is needed.

Rice by-products from allelopathic rice varieties may be potential utilization to control some invasive weeds. Indirectly, allelochemicals may reduce pathogen infestations or insect damage, and possibly diminish crop sensitivity to environmental stress [8]. Khanh et al. (2009) investigated the allelopathic potential of 73 different rice varieties on BG under laboratory, greenhouse, and field conditions [23]. Chau et al. (2008) assessed the allelopathy of 19 of the most grown rice varieties in the Mekong Delta [24]. Several of the top rice accessions reduced the root growth of cress (*Lepidium sativum* L.), kale (*Brassica oleracea*), and weedy rice (*Oryza sativa*) by 75.8, 76.8, and 75.4% (OM 5900); 70.9, 82.0, and 91.0% (OM 5930); 62.7, 87.3, and 87.4% (OM 4498); and 52.0, 85.3, and 79.0% (OM 3536), respectively. These preliminary results demonstrated that eight rice varieties exhibit plant growth inhibitory potential and may contain allelochemicals that inhibit shoot and root length growth of kale, cress, and weedy rice.

Further studies by Thi et al. (2014) found that OM 5930 and OM 3536 varieties were likely to contain many of the most promising allelochemicals. It is because the remaining 11 extracted fractions from OM 5930 rice varieties and 26 extracted fractions from OM 3536 were determined as promising allelochemicals (Figure S1) [25]. Thereafter, several allelopathic substances have been isolated from the OM 5930 rice cultivar; predominantly among them, *N-trans*-cinnamoyltyramine which inhibits the shoot and root growth of BG and RS at concentrations as low as 2.4 µM [25]. The recent report by Thi et al., 2020 reveals that twenty allelochemicals were semi-quantified and seven of them were detected predominantly and five was putatively confirmed in OM 5930 as salicylic acid, vanillic acid, *p*-coumaric acid, 2,4-dimethoxybenzoic acid, and cinnamic acid [26]. These compounds posed the average EC<sub>50</sub> value of 1.24 mM and were active to inhibit the growth of mustard green (*Brassica juncea*) at concentrations greater than 0.5 mM. The results indicated that OM 5930 may use as promising rice cultivar in weed biological control for rice production. However, the chemical basis of OM 5930 allelopathy may not be fully understood. In addition, testing the allelopathic activity of OM 5930 has been only done in the laboratory condition. Therefore, the objective of this greenhouse study was to crystallize extracts from

OM 5930 rice and examine their efficacy on the growth and development of BG, RS, and GF seedlings.

## 2. Materials and Methods

### 2.1. Rice Seeds

Seeds for OM 5930 rice variety were harvested in March 2019 at the Cuu Long Delta Rice Research Institute (CLRRI), Vietnam and stored in a  $-20\text{ }^{\circ}\text{C}$  freezer until use. OM 5930 is a promising rice variety for production in the Mekong Delta. This variety has several desirable characteristics, including a maturity time of 95–100 days, forms a long rice grain (7.0–7.3 mm), yields from 5.0–7.0 tons  $\text{ha}^{-1}$ , the weight of 1000 seeds is about 26.0 g, the rice grain is elongated, the amylose content is form 22.0–22.5%. Plants are also highly resistant to brown planthopper (BPH) with partial resistance to blast (level 3) (CLRRI, 2008. Internal circulation document).

### 2.2. Weed Seeds

Seed from BG, RS, and GF were collected at maturity from experimental fields at CLRRI and dried in an incubator (Forced Convection Laboratory Incubators, Esco Isotherm) at  $50\text{ }^{\circ}\text{C}$  for 16 h, maintained at room temperature ( $25 \pm 1\text{ }^{\circ}\text{C}$ ) for 1 h, followed by storage at  $4\text{ }^{\circ}\text{C}$  until used for greenhouse experiments.

### 2.3. Crystallized Rice by-Products

Dormancy of rice seeds was broken at  $40\text{ }^{\circ}\text{C}$  for 2 days in an incubator, soaked in distilled water for 24–48 h, then incubated at  $32\text{--}35\text{ }^{\circ}\text{C}$  for an additional 24 h. Next, 40 g of germinated rice seeds were sown in rectangle sentiment tanks ( $L \times W \times D = 250 \times 200 \times 120\text{ cm}$ ), which were filled with alluvial soil (Section 2.5) to 75% of tank capacity. Rice was fertilized (Petro Vietnam Ca Mau Fertilizer Joint Stock Company, Camau, Vietnam) with the recommended dosage per hectare (N-P-K; 85-40-30) according to the Ministry of Agriculture and Rural Development, Vietnam, and was applied using standard recommendation practices for each development stage of rice. Pots were watered daily to maintain the water level 3 cm above the soil surface. At 60 days after sowing, rice plants reached the reproductive stage and total biomass (leaves, roots, and shoots) was optimum. Plants were harvested, washed carefully under tap water, and dried under vacuum condition at  $50\text{ }^{\circ}\text{C}$  for 72 h. Dry biomass was ground into fine powder using a standard mill machine (Retsch Cutting Mill SM100, final fineness from 0.25–20 mm, 1500 W, Haan, Germany), and placed in distilled water (50:50,  $v/v$ ). The aqueous rice extract was filtered through filter paper (No. 3, Whatman) to collect the rice supernatants and evaporated to dryness using a vacuum rotary evaporator at  $60\text{ }^{\circ}\text{C}$ , 300 rounds  $\text{min}^{-1}$  to collect crystallized solids. Collected residues were placed at room temperature  $\sim 4\text{ h}$  for stabilization, then placed in 0.5 kg plastic bags, labeled, and stored at  $-20\text{ }^{\circ}\text{C}$  for later use.

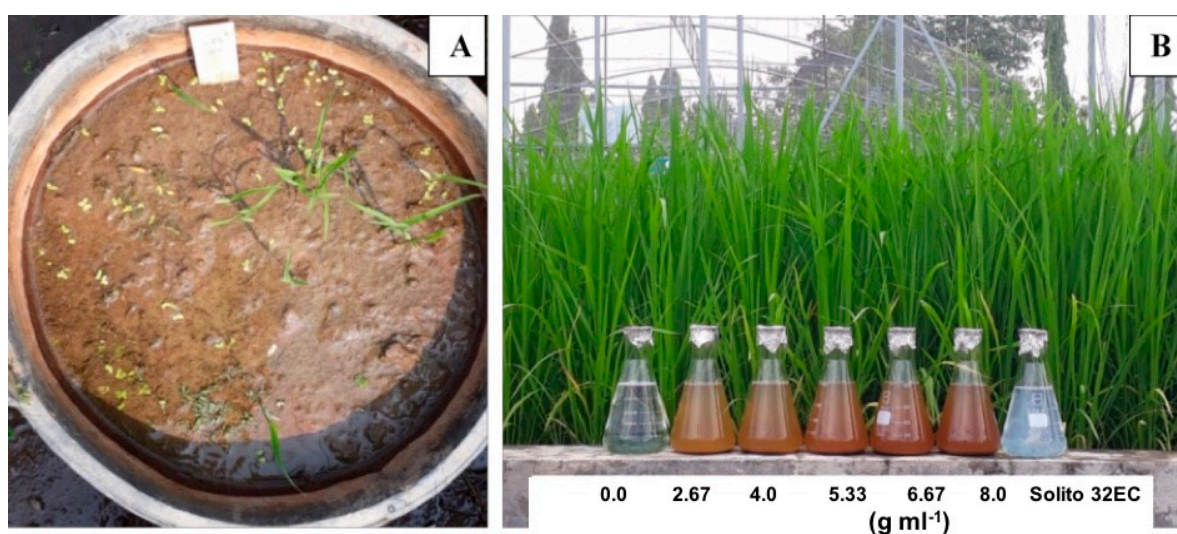
### 2.4. Solubilization of Crystallized Rice by-Products (Rice Extract)

Crystallized rice by-products were completely dissolved in 1 L of distilled water to generate various concentrations: 2.67, 4.0, 5.33, 6.67, and  $8.0\text{ g L}^{-1}$  (Table 1 and Figure 1B). Each concentration represented a treatment and corresponded to 9.40, 14.1, 18.84, 23.55, and 28.26 g of crystallized rice  $\text{pot}^{-1}$ . To enhance solubility, a rotary shaker (Multi Shaker NB-101MT, Jeju, Korea) was set at 120 rounds  $\text{min}^{-1}$  for 3 h. The samples were then incubated for 24 h at room temperature ( $25 \pm 1\text{ }^{\circ}\text{C}$ ) and passed through filter paper (90 mm diameter) to collect the rice extract.

**Table 1.** Crystallized rice by-products and corresponding rice extract derived from mature rice biomass. By-products and rice extract used for grass weed suppression under greenhouse conditions.

Treatment	Crystallized by-Product (g pot <sup>-1</sup> )	Rice Extract (g L <sup>-1</sup> )	Rice Biomass (Tons ha <sup>-1</sup> )
1	9.40	2.67	1
2	14.13	4.00	2
3	18.84	5.33	3
4	23.55	6.67	4
5	28.26	8.00	5
NC	0.0	0.0	-
PC		Solito 320 EC *	-

Note: NC: Negative control; PC: Positive control. \* Active ingredients are pretilachlor (300 g ai L<sup>-1</sup>) + pyriben-zoxim (20 g ai L<sup>-1</sup>).



**Figure 1.** Photographs depicting experimental set up in the greenhouse. (A) Individual pot with pregerminated weed seed, one day before treatment; (B) rice extract at different concentrations (g mL<sup>-1</sup>), water (0.0; negative control), and Solito (positive control).

### 2.5. Soil Preparation

Alluvial soil was collected from a representative rice field at CLRRRI and place under sunlight until dry. Soil was then pulverized to a uniform consistency, not sterilized to maintain microbial activity, and placed in polyethylene pots (20 × 30 cm (height × width) with a total of 5.2 kg of dried soil per pot.

### 2.6. Rice Materials and Chemicals Standards for HPLC Qualification

Standard samples were provided by Energy Chemicals (China). The standard samples included salicylic acid, cinnamic acid, 2,4-dimethoxybenzoic acid, 2,4-dihydroxybenzoic acid, benzoic acid, p-coumaric acid, coumarin, ergosterol peroxide, and vanillic acid. The purity of the standard sample exceeded 98% as determined by high performance liquid chromatography analysis (HPLC). Acetonitrile and methanol for HPLC were supplied by Merck KGaA (Darmstadt, Germany). The purified water used was filtered through the Milli-Q filtration system (Millipore, Bedford, MA, USA). Analytical rice samples were extracted with 20% MeOH solution after fractionation using the chromatographic columns of liquid/liquid partition, Silicagel column, Sephadex LH-20 column, and C18 Sep-Pak cartridge [26].

### 2.7. HPLC/UV-VIS Conditions

An Agilent 1260 HPLC system equipped with a quadrupole pump G1311C, automatic sample pump G2260A, column thermostat G1316A, probe DAD G1315D, and XDB-C18



(150 mm × 4.6 mm, 5 µm; Agilent) column with guard column head guard (3.9 mm × 20 mm, C18, 5 µm) were used. The elution solvent system consisted of methanol (solution A) and water + 0.1% formic acid (solution B). The analytical system was eluted with a flow rate of 0.5 mL min<sup>-1</sup>, with a scanning UV resolution of 200 to 400 nm. A spectral chromatographic program was run on specialized software by Agilent.

#### 2.8. Exposure of Pre-Germinated Weed Seed to OM 5930 Crystallized Rice by-Product

Crystallized rice by-product was uniformly spread on the soil surface in the experimental pots at 5 different doses; 9.40, 14.13, 18.84, 23.55, and 28.26 g pot<sup>-1</sup> (Table 1), which was equivalent to 1.0, 2.0, 3.0, 4.0, and 5.0 tons ha<sup>-1</sup> of rice straw biomass, respectively. Additional treatments included no rice by-product (negative control) [27]. After addition of the by-products, the soil was saturated with approximately 3 cm of water for 24 h. Water was allowed to evaporate until soil remained moist. Weed seeds of each species were soaked in water for 48 h, followed by incubation at room temperature for 24 h for 2–4 days, depending on weed species (usually 2 days for GF, 3 days for RS, and 4 days for BG). Basing on incubation time, the time for taking enough imbibed/pre-germinated weed species were determined to conduct the experiments. Following incubation, 20 seeds of each imbibed/pre-germinated weed species were randomly spread on the treated soil surface. It was noted when each experiment was initiated for each species such that data were collected at the proper time. Pots were watered twice daily, with surviving seedlings recorded at 3, 7, 14, and 42 days after treatment (DAT). Treatments were set up as a completely randomized design with three replications; the experiment was repeated.

#### 2.9. Foliar Application of OM 5930 Rice Extract on Barnyardgrass, Red Sprangletop, and Grass-Like Fimbray Seedlings

Pots containing moistened soil were prepared as described above. Under greenhouse conditions, 20 pre-germinated seeds of each species were distributed on the soil surface. Emerged seedlings were watered as needed until plants reached 2 or 3 fully expanded leaves (Figure 1A). At this point, rice extracts as described above were applied as a foliar spray at different doses: 2.67, 4.0, 5.33, 6.67, and 8.0 g ml<sup>-1</sup>, which was equivalent to 1.0-, 2.0-, 3.0-, 4.0-, and 5.0-tons rice biomass ha<sup>-1</sup> (Table 1 and Figure 1B). To improve spray adhesion to plant foliage, 1.25 mL of 10% Alkyl polyglycosides (APG) [28] in distilled water was added as a surfactant to rice extracts. Two additional treatments included use of Solito 320EC (Pretilachlor 300g/L + Pyribenzoxim 20g/L EC) as positive control, and water with surfactant as a negative control. The experiment was established as a completely randomized design with three replications and repeated. To assess treatment efficacy, the number of surviving seedlings of each species was recorded at 3, 7, 14, and 42 DAT.

#### 2.10. Sample Preparation for Analysis

Analytical samples in Section 2.6 were diluted in certain ratios depending on the concentrations of the sample solution and in accordance with the analytical running conditions of the HPLC system [26]. All standard sample solutions and test samples were filtered through a 0.21 µm membrane filter before analysis.

#### 2.11. Validate Analytical Method

Stock solutions were generated by dissolving 20 mg of each chemical standard in 1.0 mL of methanol, resulting in concentrations of 20,000 ppm. A calibration curve was constructed using serial dilutions of stock solutions with 100% methanol. Reference solutions of standards at different concentrations were analyzed by HPLC/UV-VIS systems. Standard curves were regressed linearly using  $y = ax + b$ , where  $y$  and  $x$  correspond to the ratio of the substance (area of substance) and concentration of the substance, respectively.

### 2.12. Optimization of Chromatographic Conditions

The HPLC chromatographic procedure conditions were selected based on requirements to obtain the best resolution between peaks with the shortest retention time. The effect of the solvent system composition on the separation has been specifically defined to optimize the chromatographic conditions. Methanol as a solvent was used because rice by-product exhibited the greatest solubility, giving results in the shape of standard peaks with the best resolution. All the calibrators are relatively high polar substances, so the polarization of the solvent system is suitably calibrated for the best retention times. All standards are separated completely over a period of 32 min. The peaks in the chromatographic spectrum of a test sample solution are determined by comparing their retention times with the corresponding standard samples. To obtain the most appropriate sensitivity, the UV spectra of the standard samples are recorded for comparison.

### 2.13. Data Analysis

Inhibition levels (IL) of crystallized rice by-products and rice extracts on the growth and development of weeds were calculated using Abbott's formula [29], which is determined as follows:

$$IL (\%) = [(C - T)/C] \times 100,$$

where C is the number of surviving plants in the untreated control while T stands for the number of surviving plants in a treatment.

Statistical analyses were performed using SPSS software ver. 22.0 (IBM, Armonk, NY, USA). Because there was no main effect for experiment or interaction between experiment and treatments, data for crystallized rice by-product and rice extract were combined over experiments prior to the ANOVA. Means were separated using Duncan's multiple range test with  $p = 0.05$ .

## 3. Results

### 3.1. Response of Pre-Germinated Weed Seed to OM 5930 Crystallized Rice by-Product

Plant response to crystallized rice by-product varied between weed species, with GF as the most sensitive and BG least sensitive (Table 2). Grass-like fymbr survival was reduced by 40.5% at the lowest dose (9.4 g pot<sup>-1</sup>) in as little as 3 days after treatment with rice by-product. This same level of by-product required 7 d for RS to exhibit a similar effect (42.8% reduced survival) and reduced survival of BG did not exceed 32.1% at 42 DAT. As the rate of rice by-product increased, inhibition level increased, but differential sensitivity between species remained. Rice by-product at all rates killed germinating GF seeds by 42 DAT. For RS, rice by-products were lethal at 18.84 g pot<sup>-1</sup> and higher, but 9.4 g pot<sup>-1</sup> only reduced BG survival by 46.8%. The maximum inhibition level in BG was 67.9% at 28.26 g pot<sup>-1</sup>. For all species, there was a step-wise inhibition level increasing with increasing rice by-product. For BG, RS, and GF at 3 DAT, the inhibition level ranged from 6.7–40%, 30.0–63%, and 40.5–94%, respectively from the lowest (9.4 g pot<sup>-1</sup>) to the highest rate (28.26 g pot<sup>-1</sup>). However, it should be noted that all species exhibited a response to rice by-product, indicating a broad-spectrum effect on the grasses examined in this study. While differential species response and a rate response to rice by-product was anticipated, seed survival was strongly influenced by the length of time pre-germinated seeds were exposed to treatments. Barnyardgrass inhibition level increased from 6.7 to 32.1% at the lowest rate (9.4 g pot<sup>-1</sup>) and 40 to 67.9% at the highest rate (28.26 g pot<sup>-1</sup>) from 3 to 42 DAT (Table 2). Similarly, RS inhibition level increased from 30.3 to 46.8% at the lowest rate (9.4 g pot<sup>-1</sup>) and 63 to 100% at the highest rate (28.26 g pot<sup>-1</sup>) from 3 to 42 DAT. For GF, inhibition level increased from 40.5 to 100% at the lowest rate (9.4 g pot<sup>-1</sup>) and 94 to 100% at the highest rate (28.26 g pot<sup>-1</sup>) from 3 to 42 DAT. No rate was lethal to all BG seedlings by 42 DAT, while a minimum of 18.84 and 9.4 g pot<sup>-1</sup> were lethal to all RS and GF seedlings, respectively. These results indicate that the allelochemicals causing the weed inhibition in the crystallized rice by-product were not quickly degraded under natural conditions, and in fact exhibited residual activity.

**Table 2.** Effect of OM 5930 rice crystallized by-product on the survival of pre-germinated barnyardgrass, red sprangletop, and grass-like fimbry seed.

Crystallized Rice (g pot <sup>-1</sup> )	Inhibition Level (% of the Control)											
	Barnyardgrass				Red Sprangletop				Grass-Like Fimbry			
	Days after Treatment (DAT)											
	3	7	14	42	3	7	14	42	3	7	14	42
9.4	6.7d *	11.7 d	12.3 d	32.1 d	30.3 c	42.8 e	46.1 d	46.8 b	40.5 d	50.9 c	60.2 d	100.0
14.13	13.3 c	20.0 c	26.3 c	43.4 c	41.5 b	60.5 d	67.7 c	91.7 a	69.5 c	75.4 b	80.6 c	100.0
18.84	23.3 b	30.0 b	35.1 b	56.5 b	44.9 b	72.6 c	81.9 b	100.0 a	83.8 b	90.7 a	94.5 b	100.0
23.55	33.3 a	41.7 a	45.6 a	62.2 ab	59.6 a	83.2 b	85.7 b	100.0 a	89.8 a	94.9 a	97.2 ab	100.0
28.26	40.0 a	46.7 a	52.6 a	67.9 a	63.0 a	91.7 a	94.9 a	100.0 a	94.0 a	96.6 a	98.1 a	100.0
F	**	**	**	**	**	**	**	**	**	**	**	ns
CV (%)	8.3	6.2	7.1	4.7	2.6	2.5	2.1	5.9	1.6	2.3	1.1	0.1

\* Similar letters within a column are not significantly different using Duncan's multiple range at  $p = 0.05$ . \*\* Indicates a significant difference at 1%.

### 3.2. Response of Weed Seedlings to Foliar Applications of OM 5930 Rice Extract

Similar to crystallized rice by-product, rice extract increased inhibition level with increasing rates (Table 3). At 3 DAT, BG inhibition level increased from 3.3 to 21.7% by 2.67 to 8 g L<sup>-1</sup> rice extract, with corresponding rates increasing inhibition level of RS by 13.7 to 72.5% and GF by 15 to 81.7%. Species response to rice extract showed GF as the most sensitive species and BG as the least sensitive. The maximum increase in inhibition level were observed at 42 DAT, with rice extracts of 5.33 g L<sup>-1</sup> or higher lethal to GF. For RS, 8 g L<sup>-1</sup> was necessary to increase inhibition level by 100%. The maximum reduction in survival of BG seedlings was 78.3% at 8 g L<sup>-1</sup>.

**Table 3.** Effect of OM 5930 rice extract on the survival of barnyardgrass, red sprangletop, and grass-like fimbry seedlings from 3 to 42 days after treatment.

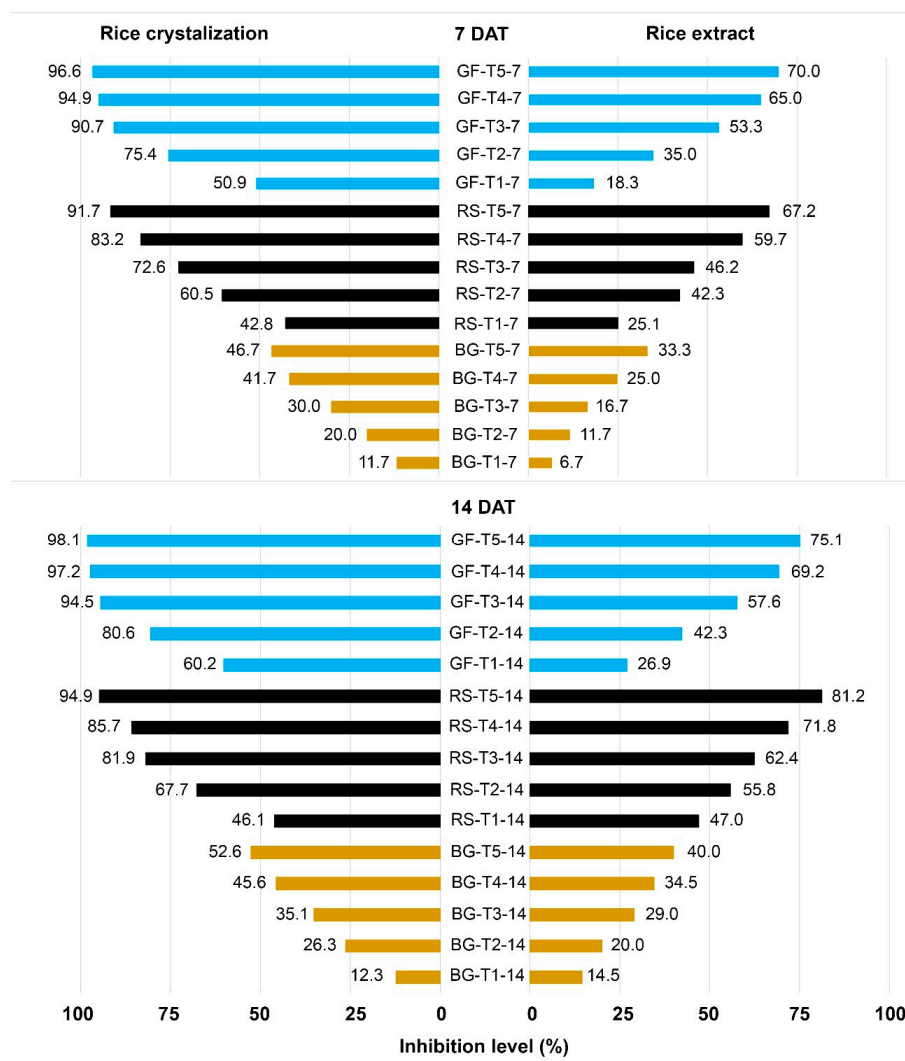
Inhibition Level (% of the Control)												
Rice Extract (g L <sup>-1</sup> )	Barnyardgrass				Red Sprangletop				Grass-Like Fimbry			
	Days after Treatment (DAT)											
	3	7	14	42	3	7	14	42	3	7	14	42
2.67	3.3 e *	6.7 f	14.5 d	25.8 e	13.7 e	25.1 e	47.0 e	49.7 d	15.0 f	18.3 f	26.9 e	54.3 b
4.0	6.7 d	11.7 e	20.0 d	37.9 d	31.0 d	42.3 d	55.8 de	56.7 c	30.0 e	35.0 e	42.3 d	94.2 a
5.33	10.0 cd	16.7 d	29.0 c	49.8 c	41.3 c	46.2 d	62.4 cd	79.9 b	45.0 d	53.3 d	57.6 c	100.0 a
6.67	15.0 bc	25.0 c	34.5 bc	64.2 b	46.5 c	59.7 c	71.8 bc	93.6 a	55.0 c	65.0 c	69.2 b	100.0 a
8.0	21.7 ab	33.3 b	40.0 b	78.3 b	58.5 b	67.2 b	81.2 b	100.0 a	61.7 b	70.0 b	75.1 b	100.0 a
Solito 320 EC	23.3 a	45.0 a	90.8 a	100.0 a	72.5 a	98.0 a	100.0 a	100.0 a	81.7 a	93.3 a	100.0 a	100.0 a
F	**	**	**	**	**	**	**	**	**	**	**	**
CV (%)	13.4	7.6	6.2	6.6	6.3	3.3	4.7	2.3	3.2	1.4	2.7	1.7

\* Similar letters within a column are not significantly different using Duncan's multiple range at  $p = 0.05$ . \*\* Indicates a significant difference at 1%.

The activity of rice extract increased over time on all species (Table 3). For BG, inhibition level decreased by 22.5% at the lowest rate and 56.6% at the highest rate between 3 and 42 DAT. Over this same time, RS and GF inhibition level increased from 36 to 39.3% at the lowest rate and 38.3 to 41.5% at the highest rate. As a postemergence application, rice extract resulted in rapid activity (3 DAT), indicating some level of contact activity. However, for each weed species, survival ability was reduced more as time after treatment

increased, indicating that over a 42-day period, seedlings did not appear to be recovered. The allelochemicals contained in both the crystallized rice by-product and solubilized in rice extract retained their herbicidal properties over the duration of this study.

Application of the commercial herbicide Solito to seedlings of each species resulted with 23.3 to 81.7% reduction in survival at 3 DAT (Table 3). Initial species sensitivity from least to greatest was BG > RS > GF. However, Solito was lethal to all RS and GF seedlings at 14 DAT, with no surviving BG seedlings between 14 and 42 DAT. Application of Solito controlled almost all of the seedlings at 7 DAT (93.3% for GF; 98.0% for RS) and 14 DAT (90.8% for BG) (Table 3; Figure 2).



**Figure 2.** Comparative effect of OM 5930 crystallized by-product and OM 5930 rice extract (Inhibition level-% of the control) on grass-like fimbry—GF (blue columns), red sprandletop—RS (black columns), and banyardgrass—BG (orange columns) at 7 and 14 days after treatment (DAT). Rice crystallization treatments ( $\text{g pot}^{-1}$ ) include T1: 9.4; T2: 14.13; T3: 18.84; T4: 23.55; and T5: 28.26. Rice extract treatments ( $\text{g L}^{-1}$ ) include T1: 2.67; T2: 4.0; T3: 5.33; T4: 6.67; and T5: 8.0. The T1 through T5 treatments for both crystallized rice by-product and rice extract correspond with 1-, 2-, 3-, 4-, and 5-tons  $\text{ha}^{-1}$  of mature rice biomass.

### 3.3. Comparison of OM 5930 Rice by-Products in Crystallized form Versus Soluble Extracts

Use of rice biomass for weed management should consider optimal application timing. A direct comparison of crystallized rice by-product to rice extract showed that germinating grasses were overall more sensitive than seedling grasses (Figure 2). At 7 DAT,



the lowest rice biomass (1 ton ha<sup>-1</sup>) as crystallized by-product (9.4 g pot<sup>-1</sup>) resulted in 32.6, 17.7, and 5% greater seedling inhibition of GF, RS, and BG, respectively than rice extract (2.67 g L<sup>-1</sup>). For each species at increasing amounts of rice biomass, survival of each grass was lower in response to crystallized by-product versus rice extract. Seedling inhibition levels of both bioherbicide forms were greater at 14 DAT than at 7 DAT. Averaged across all rates, reductions in GF, RS, and BG survival were greater by 31.9, 11.6, and 6.8%, respectively for crystallized rice by-product compared to rice extract (Figure 2).

### 3.4. HPLC Identification of Allelochemicals in OM 5930 Rice

From a previous study, twenty allelochemicals were tentatively identified in the OM 5930 rice cultivar by using UHPLC-MS coupled with XCMS online cloud-based metabolomics platform [26]. In the present study, the exact concentrations of eight of the twenty allelochemicals were confirmed by associating their retention times in the rice sample with those of analytical standards using UV-VIS spectral data. Among these, benzoic acid, 2,4-dihydroxybenzoic acid, coumarin, and ergosterol peroxide were newly identified as allelochemicals in rice at concentrations of 0.061, 0.06, 0.017, and 1.1 mg 100 g<sup>-1</sup> fresh rice plants, respectively (Table 4). Classification of the chemicals included coumarin, which is a benzopyrone and considered a lactone; ergosterol peroxide which is a steroid derivative; and the other chemicals are grouped as phenolic acids.

**Table 4.** List of allelochemicals detected in 100 g of OM 5930 by HPLC method.

No.	Allelochemicals	Retention Time (min.)	Purity (%)	Allelochemical Content *	
				In Rice Extract (mg mL <sup>-1</sup> )	In 100 g of Fresh Rice (mg 100 g <sup>-1</sup> )
1.	Salicylic acid **	11.469	98.9	0.7715	5.01
2.	2,4-dimethoxybenzoic acid **	30.058	99.7	0.0161	0.10
3.	2,4-dihydroxybenzoic acid	29.902	98.9	0.0088	0.060
4.	Benzoic acid	34.226	99.0	0.0094	0.061
5.	<i>p</i> -Coumaric acid **	20.269	99.7	0.0245	1.60
6.	Coumarin	27.588	99.5	0.0026	0.017
7.	Vanillic acid **	11.126	99.7	0.0192	0.13
8.	Ergosterol peroxide	32.125	99.8	0.1695	1.10

\* The volume of OM 5930 extract (V = 4.5 mL) corresponds to 69.33 g of fresh rice plants remaining after C18 SPE operation; 1 mL of extract contains 15.41 g of fresh rice plants. \*\* Previously reported in OM 5930 rice [26].

## 4. Discussion

By-products from allelopathic plant species have previously been reported to suppress germination and growth of weed species only when they were mixed into the soil surface wetted with water [30]. As early as 1960, Nielsen et al. (1960) reported that allelopathic plants usually contain water-soluble regulators, thus the experiment should be conducted on a wet-seeded crop system to get better weed inhibitory effect [31]. Putnam and DeFrank (1983) found that water soluble phytotoxins from sorghum (*Sorghum bicolor*) suppressed germination of common purslane (*Portulaca oleracea*) and smooth crabgrass (*Digitaria ischaemum*) by 70 and 98%, respectively [30]. Xuan and Tsuzuki (2002) noted that flatsedge (*Cyperus difformis*) seeds were significantly inhibited when mixed with alfalfa (*Medicago sativa*) powder and watered in the soil, the inhibitory effect was very low when the pots were waterless even still in a mixture of alfalfa powder and soil in the field experiment with 100 g of alfalfa powder m<sup>-2</sup> [32]. Steinsiek et al. (1982) reported that extracts following soaking mature wheat straw in water compared to leaching water through straw resulted in greater inhibition of germination and seedling growth for six weed species [33]. The evidence revealed that the allelochemicals causing the weed inhibition in both crystallized rice by-product and rice extract in this study were gradually released into the soil and affected onto the growth and development of the weed seedlings only just in case the soil was moistened at a certain water level.

Rice straw has also been reported to suppress grass weeds, with large variations between varieties. Lim et al. (2015) demonstrated that chopped residues of NSIC Rc222 rice at rates as low as 1 ton ha<sup>-1</sup> reduced germination of saramolla grass (*Ischaemum rugosum*) >25%, and that same rate reduced saramolla grass biomass of emerging seedlings by 50% [34]. Chung et al. (2003) screened 114 rice varieties for weed suppression, aqueous extracts of ground rice straw from six varieties, including CUBA 65-v-58 resulted in >30% inhibition of BG germination and/or seedling growth [35]. Jung et al. (2004) conducted bioassays on BG with ground rice leaves, straw, and hulls from 114 varieties, the result demonstrated that Duchunjong variety resulted in the greatest average inhibition of BG response (77.7%), with leaves plus straw as the most potent [36]. Berendji et al. (2008) identified six different phenolic acids from rice hull extracts of 15 rice cultivars, and correlated BG root inhibition with phenolic acid content [37]. Chung et al. (2001) elucidated nine allelochemicals from rice straw extracts of four rice cultivars and determined the phenolic acid such as *p*-hydroxybenzoic acid, coumaric acid (*p*-, *m*-, and *o*); *o*-hydroxyphenylacetic acid; salicylic acid; syringic acid; ferulic acid; and benzoic acid. Among them, *p*-hydroxybenzoic acid resulted in the greatest inhibition of BG germination [38]. Chung et al. (2006) also identified 5 of 99 rice varieties inhibited germination or dry weight accumulation of BG by 50%, with momilactone A and B levels associated with the greatest inhibition [39]. However, very little study has so far discussed about the potentials of rice allelopathic on RS and GF. Results from our research highlighted that crystallized rice by-product as well as liquid rice extract of the OM 5930 rice cultivar resulted in a dose-dependent reduction in survival of not only BG but also RS and GF under greenhouse conditions.

Expression of allelopathy in rice has been attributed to several compounds. Salam et al. (2009) identified 2,9-dihydroxy-4-megastigmen-3-one from BR17 rice, which inhibited growth of seven weed species [40]. Additional studies by Kong et al. (2004) identified a flavone and cyclohexanone with weed suppression properties [41]. Seal et al. (2004) identified 25 compounds from root exudates of different rice varieties, with allelopathic rice varieties producing higher amounts of *trans*-ferulic acid, *p*-hydroxybenzoic acid, and caffeic acid [42]. Previous research with OM 5930 rice identified several plant inhibitory substances such as *N-trans*-cinnamoyltyramine [25], salicylic acid, vanillic acid, *p*-coumaric acid, 2,4-dimethoxybenzoic acid, and cinnamic acid [26]. Some of the eight allelochemicals identified from OM 5930 rice in the present study have reported bioherbicide activity on several plant species [26,38,43,44]. It has also reported that ergosterol peroxide was identified in decaying rice residues [43] and was highly phytotoxic to BG [45]. In this study, three of the compounds in OM 5930 were detected at the highest quantity in mg 100 g<sup>-1</sup> fresh weight included: ergosterol peroxide, 1.1 mg; *p*-coumaric acid, 1.6 mg; and salicylic acid, 5.01 mg. However, Olofsdotter et al. (2002) argued that differential tolerance to the phenolic acids among allelopathic and non-allelopathic rice cultivars suggested a single phenolic acid did not likely explain rice allelopathy [46]. Therefore, the allelopathic activity of OM 5930 rice cultivar may be dependent on a mixture of several secondary metabolites consisting of *N-trans*-cinnamoyltyramine, phenolic acids, and ergosterol peroxide.

Jabran et al. (2015) summarized that planting allelopathic crops for biological suppression of weeds or using residues of allelopathic species as mulches for subsequent desirable crops encompasses the major uses of allelopathic species [47]. Seal and Pratley (2010) determined that several rice varieties exhibited growth inhibitory effects on multiple, unrelated weed species [48]. However, extraction of potent, soluble allelochemicals and application as a residual or directed postemergence bioherbicide holds promise as a new weed management tool for many crops. Results from this research show crystallized by-products from OM 5930 rice contain eight allelochemicals, and its residues continued to exhibit activity for up to 42 days after application. Integration of crystallized rice by-products from OM 5930 rice into weed management practices can reduce dependence on herbicides, lowering selection pressure for resistant weed species. Because rice straw is abundant and often undesirable, utilization of OM 5930 rice straw may serve as a new approach, reducing straw burning which is detrimental to the environment. Future research

should focus on utilization of the crystallized rice by-products from OM 5930 rice cultivar to manage weeds in the field settings.

In summary, OM 5930 in a crystallized formulation exhibited greater inhibitory activity on pre-germinated seed compared to rice extracts applied to emerged seedlings. In both forms, extracts were more suppressive on RS and GF compared to BG. Treatments of 23.55 g of OM 5930 rice crystallization  $\text{pot}^{-1}$  and 6.67 g of rice extract  $\text{L}^{-1}$  are both equivalent to 4 tons of rice biomass  $\text{ha}^{-1}$  in a rice field. However, the BG, RS, and GF plants as pre-germinated seed may be more susceptible to the inhibitory effects of allelochemicals compared to solubilized extracts applied to established seedlings. Comparatively, crystallized by-product versus rice extract at 7 DAT was 40.4, 18.2, and 8.3% more effective on GF, RS, and BG, respectively at an equivalent of 2 tons rice biomass  $\text{ha}^{-1}$ . These differences were equivalent or greater as rates increased from 3 to 5 tons  $\text{ha}^{-1}$ . Both formulations exhibited extended activity (up to 42 DAT), suggesting allelopathic compounds are not simply contact bioherbicides. The existence of six phenolic acids, coumarin, and ergosterol peroxide in the OM 5930 rice cultivar likely explained the strong weed-suppression activity. Therefore, both OM 5930 crystallized by-product and rice extract may potentially be used to control grass weeds, either in subsequent rice crops or possibly in other cropping systems. However, because of the better potential of the crystallized rice by-products from OM 5930 rice cultivar, future research should focus on utilization of this biocide formulation to manage weeds in the field settings.

**Supplementary Materials:** The following are available online at <https://www.mdpi.com/article/10.3390/agronomy11040776/s1>, Figure S1: Isolation scheme of active compounds from the aqueous MeOH extracts of OM 5930 and OM 3536 rice varieties.

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