

Communication

Natural Salicylaldehyde for Fungal and Pre- and Post-Emergent Weed Control

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Featured Application: The herbicidal and fungicidal system developed in this study can be used by the agricultural industry for sustainable crop production by lowering the pesticide burden in fields and promoting byproduct recycling.

Abstract: A sustainable, alternative weed control strategy is developed using salicylaldehyde (SA; 2-hydroxybenzaldehyde) as an active ingredient. SA is a natural, redox-active small molecule listed as a Generally Recognized As Safe food additive by the European Food Safety Authority and the United States Food and Drug Administration. The repurposing of SA determined that SA possesses both pre- and post-emergent herbicidal, fumigant activity, where the emitted SA from the source completely prevented the germination of plant seeds and/or the growth of the germinated plants. As a proof-of-concept, we developed agricultural byproducts (tree nutshell particles) as SA delivery vehicles to the soil, thus helping the growers' sustainable byproduct recycling program, necessary for carbon sequestration. In plate assays, SA emitted from the nutshell vehicles (0.15 to 1.6 M) completely prevented the germination of six invasive or native weed seeds (monocots, dicots). In Magenta vessel assays, SA emitted from the nutshell vehicles (0.8 to 1.6 M) not only prevented the germination (pre-emergent) of *Lagurus ovatus* (Bunny Tails Grass) seeds but also inhibited the growth (post-emergent) of the germinated weeds. We determined further that soil covering (soil pasteurization) could be one of the practices to effectively deliver SA to the soil, whereby 1.6 M of SA emitted from the nutshell vehicles prevented the germination of the *L. ovatus* seeds maintained in soil trays covered with plastic tarp at 22 °C, while 0.8 M SA allowed partial (15%) germination of the weed seeds. Of note, SA also possesses an intrinsic antifungal activity that overcomes the tolerance of the stress signaling mutants of filamentous fungal pathogens (*Aspergillus fumigatus*, *Penicillium expansum*) to the phenylpyrrole fungicide fludioxonil. Environmental degradation data available in the public database indicate that, once released to the environment, SA will be broken down in the air by sunlight or microorganisms and, thus, is not built up in aquatic organisms. Altogether, SA can serve as a safe, potent pesticide (herbicidal, fungicidal) ingredient that promotes sustainable crop production by lowering the pesticide burden in fields.

Keywords: agricultural byproducts; fludioxonil; fungal pathogens; herbicides; natural compounds; pre-emergent; post-emergent; salicylaldehyde; soil pasteurization; weeds



Citation: Kim, J.H.; Chan, K.L. Natural Salicylaldehyde for Fungal and Pre- and Post-Emergent Weed Control. *Appl. Sci.* **2022**, *12*, 3749. <https://doi.org/10.3390/app12083749>

Academic Editors: Sara Vitalini and Marcello Iriti

Received: 19 March 2022

Accepted: 7 April 2022

Published: 8 April 2022

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1. Introduction

The timely control of weeds in crop fields is an important task for the agricultural industry. Uncontrolled weed growth engenders diverse flora in crop fields, which not only compete with crops for water and nutrients, especially nitrogen, but also host harmful pests (e.g., fungi, bacteria, insects, nematodes) that damage/contaminate crops (Figure S1a). Transmission of pests from the weeds (viz., field reservoir) can trigger pest outbreaks in the environment. For example, a recent study on *Fusarium* in French maize fields determined

that termination of herbicide usage during crop cultivation triggered unexpected increases in mycotoxin contamination in the grains, with near maximum threshold levels for ni-valenol [1]. The study identified that the uncontrolled weeds in the crop fields functioned as *Fusarium* reservoirs/spillovers, thus highlighting the importance of adequate weed control practices for assuring public food safety as well as food security. Therefore, the rapid elimination of weeds via safe, cost-effective methods will positively impact the safety, quality, and competitiveness of agricultural commodities.

However, there are emerging issues with the current uses of conventional herbicides, including: (1) the increasing negative perceptions of the public towards conventional herbicides such as glyphosate (*N*-(phosphonomethyl) glycine), a broad-spectrum post-emergent herbicide [2], (2) the development of weed resistance to commercial herbicides [3], (3) the European Union's (EU's) new policy in lowering maximum residue limits against paraquat (pulmonary toxicity) and glufosinate (reprotoxicity) (European Food Safety Authority (EFSA) [4–6]), and (4) the cancellation of the registration of toxic seed-disinfecting fungicides such as Ferbam (iron tris (dimethyldithiocarbamate)) and Ziram (zinc dimethyldithiocarbamate) (Pest Management Regulatory Agency [7]). Moreover, certain herbicides and fungicides, such as glyphosate and azoles (tebuconazole, propiconazole), respectively, trigger severe toxicity to non-target organisms, including bees, when they are applied in combination [8,9]. Therefore, the development of a new, sustainable weed control system or formulation that can be applied as safe alternatives to conventional, toxic herbicides is continually needed.

Despite this need, the development of entirely new herbicides is a very expensive and time-consuming process. While there has been a steady increase in the numbers of herbicide-resistant weeds in the fields, no new herbicide modes of action have been identified or introduced to the market in 30 years [10]. We investigate a fast pesticide screening system to expedite the identification of new, safe pesticide alternatives or intervention strategies. One of the approaches is drug/chemical repurposing [11], which is the repositioning of already marketed drugs or chemicals previously developed for treating human diseases/pathogens or as food additives to treat new types of problems such as weed invasion. The advantage of the repurposing approach is that the mechanism of action, cellular targets, toxicity profile, or safety of the repurposed drugs/chemicals have already been identified and documented, thus accelerating the regulatory approval following the repurposing. We previously identified via repurposing that salicylaldehyde (SA; 2-hydroxybenzaldehyde) exerted a potent anti-aflatoxigenic and antifungal activity as a fumigant [12,13]. SA is a natural, redox-active small molecule listed as a Generally Recognized As Safe agent by the EFSA [14] and the United States Food and Drug Administration (FDA) [15] that has been used in the drug/food industries as an intermediate for pharmaceuticals or a food-flavoring agent.

Meanwhile, agricultural industries produce an excess of byproducts/biomass of crops annually. For example, tree nuts (almonds, walnuts, pistachios) are the largest commodity produced in California, United States [16], which results in the excess production of agricultural byproducts such as nutshells and hulls each year. Considering tree nuts also capture and store a huge amount of carbon over their life cycle, effective utilization of the byproducts is one of the key components to reducing carbon emissions (thus, carbon sequestration). Therefore, finding effective ways to manage agricultural byproducts is of high importance [17,18]. Currently, orchard recycling, namely, byproduct incorporation back into the orchard soil, is one of the compelling alternative methods to managing agricultural byproducts.

However, since agricultural byproducts are commonly contaminated with environmental fungal and bacterial pathogens, it is critical to ensure that byproducts do not harm soil health or integrity by passing along pathogens, especially those resistant to conventional antibiotics/fungicides or producing mycotoxins, during field application (orchard recycling). Of note, the fumigant SA emitted from the delivery vehicles not only inhibited the production of aflatoxins B and G (AFB1, AFB2, AFG1, AFG2) by *Aspergillus flavus* and

Aspergillus parasiticus (Figure S2) but also prevented the growth of fungal pathogens [12,13]. Fungal mutants lacking genes in the antioxidant system (such as superoxide dismutase and glutathione reductase) were highly susceptible to the treatment, indicating that SA interferes with cellular redox homeostasis in fungi [12,13]. The intrinsic anti-aflatoxigenic and antifungal activity of SA as a fumigant are well-suited for sanitation practices in crop fields, especially during agricultural byproduct recycling in tree nut orchards.

In this proof-of-concept investigation, the following was performed: (1) repurposing the natural food additive SA as a pre- and post-emergent weed control agent, (2) developing tree nutshell particles as SA delivery vehicles for use in orchards, thus integrating into the growers' sustainable byproduct recycling program, and (3) determining soil covering/pasteurization as one of the optimum practices to effectively deliver SA to the soil.

2. Materials and Methods

2.1. Testing the Level of Fungal Contamination on the Surfaces of Weed Seeds or Nutshell Particles

A representative bioassay to determine the level of fungal contamination on the surfaces of weed seeds was performed by placing *Schizachyrium scoparium* (Little Bluestem) or *Lagurus ovatus* (Bunny Tails Grass) seeds (Table 1) on potato dextrose agar plate (PDA; 100 mm × 15 mm) (Corning Inc.-Life Sciences, Tewksbury, MA, USA). A total of 10 seeds per species were placed on each PDA (in duplicate). Petri plates were sealed with Parafilm (Bemis Associates Inc., Shirley, MA, USA), covered with aluminum foil to maintain a dark condition, and kept at room temperature (22 °C) for 3 to 5 days. The level of fungal contamination was monitored during seed germination.

Table 1. List of fungi and weed seeds used in this study.

Fungi	Characteristics	References/Source
<i>Aspergillus fumigatus</i> AF293	Human pathogen (aspergillosis), Reference clinical strain	The University of Texas, MD Andersen Cancer Center, Houston, TX, USA [19]
<i>A. fumigatus sakAΔ</i>	Human pathogen (aspergillosis), MAPK mutant derived from AF293	The University of Texas, MD Andersen Cancer Center, Houston, TX, USA [19]
<i>A. fumigatus mpkCΔ</i>	Human pathogen (aspergillosis), MAPK mutant derived from AF293	The University of Texas, MD Andersen Cancer Center, Houston, TX, USA [20]
<i>Aspergillus flavus</i> 3357	Plant pathogen (aflatoxigenic), Human pathogen (aspergillosis), Reference aflatoxigenic strain used for genome sequencing	National Center for Agricultural Utilization and Research, USDA-ARS, Peoria, IL, USA
<i>Penicillium expansum</i> W1	Plant pathogen (Patulin-producing, Parental strain)	Washington State University, Wenatchee, WA, USA [21]
<i>P. expansum</i> FR2	Plant pathogen, Fludioxonil resistant mutant derived from <i>P. expansum</i> W1	Washington State University, Wenatchee, WA, USA [21]
<i>Penicillium expansum</i> W2	Plant pathogen (Patulin-producing, Parental strain)	Washington State University, Wenatchee, WA, USA [21]
<i>P. expansum</i> FR3	Plant pathogen, Fludioxonil resistant mutant derived from <i>P. expansum</i> W2	Washington State University, Wenatchee, WA, USA [21]
Plant Seeds	Characteristics	References/Source
<i>Brassica rapa</i> var. <i>pekinensis</i> (dicot)	Chinese cabbage; field weed	Plant nursery, Oakland, CA, USA
<i>Centaurea solstitialis</i> (dicot) (Yellow starthistle)	California invasive weed	USDA-ARS, Albany, CA, USA
<i>Salsola tragus</i> (dicot) (Russian thistle)	California invasive weed	USDA-ARS, Albany, CA, USA
<i>Genista monspessulana</i> (dicot) (French broom)	California invasive weed	USDA-ARS, Albany, CA, USA
<i>Lagurus ovatus</i> (monocot) (Bunny Tails Grass)	Ornamental grass	Plant nursery, Berkeley, CA, USA
<i>Schizachyrium scoparium</i> (monocot) (Little Bluestem)	USA native grass	Plant nursery, Berkeley, CA, USA

To test the level of fungal contamination on the surface of walnut shell particles (6/10 mesh) (Kramer Industries, Inc., Piscataway, NJ, USA), a total of 0.3 g of walnut shell particles were placed at the center of the PDA plate (100 mm × 15 mm), sealed with Parafilm, and the level of fungal contamination in duplicate plates was monitored at 22, 28, and 35 °C, respectively, for 3 to 5 days.

2.2. Optimization of Nutshell Particles as Salicylaldehyde (SA) Delivery Vehicles

Walnut shell particles (6/10 mesh) were used as the delivery vehicles for salicylaldehyde (SA) (Sigma Aldrich Co., St. Louis, MO, USA). We reasoned that the newly developed system not only sanitizes raw byproducts per se after SA permeation but also functions as SA delivery vehicles for emission for pathogen and weed control in orchards.

The surface of the PDA medium (in duplicate) was spread with *A. flavus* 3357 spores (1×10^5 CFU/mL) and allowed to air dry. At the center of each plate, 0.3 g of walnut shell particles w/o or w/ SA (1.2 M) saturation was placed; then, the plate was sealed with Parafilm. Fungal germination/growth was monitored for up to 10 days. SA was dissolved in dimethyl sulfoxide (DMSO; AMRESCO Co., Solon, OH, USA) before application.

2.3. Overcoming Fludioxonil Tolerance of Mitogen-Activated Protein Kinase (MAPK) Mutants of Fungi

The capability of SA to overcome fludioxonil tolerance of mitogen-activated protein kinase (MAPK) mutants of *Aspergillus fumigatus* (*sakAΔ*, *mpkCΔ*) or *Penicillium expansum* (FR2, FR3) was examined in PDA. Petri plates (in triplicate) were prepared with PDA containing fludioxonil (50 μM) (Sigma Aldrich Co., St. Louis, MO, USA) or DMSO only (control). SA (0.09 M for *A. fumigatus*; 0.12 M for *P. expansum*) was applied to qualitative filter paper (Grade 1, 2.5 cm diameter) (Cytiva Co., Marlborough, MA, USA). The saturated filters were transferred onto the lower panel of each PDA plate; then, fungal spores (1×10^3 CFU) were spotted onto the upper panel. The Parafilm-sealed, inoculated plates were incubated at 35 or 28 °C for *A. fumigatus* or *P. expansum*, respectively. SA was delivered to the target fungi as a fumigant. Compounds were dissolved in DMSO before application. Fungal growth (radial growth) was monitored for 3 to 5 days.

2.4. Antifungal Synergism between SA and Mild Heat (42 °C)

Antifungal synergism between SA and mild heat (42 °C) was tested against the aflatoxin-producing *A. flavus* 3357. The surface of the PDA medium (in triplicate) was spread with *A. flavus* spores (1×10^5 CFU/mL) and allowed to air-dry. Qualitative filter paper (Grade 1, 2.5 cm diameter) saturated w/0.06 M SA or DMSO only (control) was placed at the center of each plate, and the fungal germination/growth was monitored for 3 to 5 days at 35 and 42 °C (triplicate), respectively. SA was dissolved in DMSO before application.

2.5. Pre- and Post-Emergent Herbicidal Activity of SA

2.5.1. Petri Plate Assay

The pre-emergent herbicidal activity of SA was tested against *L. ovatus*, *Brassica rapa* var. *pekinensis*, or other invasive weeds (*S. scoparium*, *Centaurea solstitialis*, *Salsola tragus*, *Genista monspessulana*) (see Table 1). First, plant seeds were germinated in Petri plates (100 mm × 15 mm), where 10 seeds per plate (in duplicate) were placed on a Murashige and Skoog basal salt mixture medium (MS medium; 0.5×) (Sigma Aldrich Co., St. Louis, MO, USA). SA concentrations tested were: 0.05 to 0.50 M (saturated on 0.3 g/plate walnut shell particles) for *Brassica rapa* var. *pekinensis* (0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50 M) and 0.05 to 1.6 M for other weed seeds, respectively. The level of seed germination/growth was monitored for 10 days at 22 °C (12 h light/12 h dark cycle).

2.5.2. Magenta Vessel Assay

The pre- and post-emergent herbicidal activity of SA was compared further in Magenta GA-7 vessels (Magenta LLC, Lockport, IL, USA) using *L. ovatus* seeds (in duplicate). For

the pre-emergent herbicidal activity of SA, 10 seeds of *L. ovatus* were placed per vessel supplemented with MS medium (0.5×). The culture vessels were supplied with 0.3 g of walnut shell particles w/o or w/ SA (0.8 or 1.6 M) saturation.

For the post-emergent herbicidal activity of SA, the *L. ovatus* seeds were germinated in Magenta vessels filled with MS medium (0.5×) (in duplicate), where 10 seeds per vessel were placed on the surface of agar (w/o walnut shell particles in any of the culture boxes). After 5 days of seed germination, the culture vessels were supplied with 0.3 g of walnut shell particles w/o or w/ SA (0.8 or 1.6 M) saturation, and the germinated seeds were cultured further for up to 10 days for determining the post-emergent herbicidal activity of SA.

2.6. SA Herbicidal Efficacy in Soil

Pre-emergent herbicidal activity of SA was tested further in soil (garden soil purchased from a local store, Berkeley, CA, USA). Four plastic trays (22 cm × 50 cm × 15 cm) were half-filled with soil, and forty *L. ovatus* seeds were sown (~1 cm deep) in two rows per tray. SA preparation for each tray was: (1) No SA w/o nutshells (control), (2) No SA w/ nutshells (10 g) (control), (3) SA 0.8 M w/ nutshells (10 g), and (4) SA 1.6 M w/ nutshells (10 g). To mimic the soil pasteurization practice, trays were covered with a plastic layer and then were incubated in the dark (22 °C) for seed germination. After 7 days of incubation, the plastic covers were removed from the trays and the trays were shifted to a light (12 h)/dark (12 h) cycle at 22 °C. The germination/growth of *L. ovatus* seeds were monitored for 4 more days (up to 11 days from sowing to monitoring).

2.7. Statistical Analysis

Statistical analysis (Student's *t*-test) was performed based on "Statistics to use" [22], where $p < 0.05$ was considered significant.

3. Results and Discussion

3.1. Agricultural Byproducts (Walnut Shell Particles) as SA Delivery Vehicles: Intrinsic Antifungal Activity of SA as a Fumigant

Initially, walnut shell particles were investigated as sustainable delivery vehicles for SA. Since nutshells are commonly contaminated with environmental microbial pathogens (Figure S1b), caution should be exercised during the recycling of tree nut byproducts. For the optimization of SA delivery, SA was tested as a fumigant at 1.2 M to target the aflatoxin-producing *A. flavus* 3357.

As shown in Figure 1, SA at 1.2 M, emitted from the delivery vehicles, completely prevented the growth of *A. flavus* 3357 on the culture plate, while none of the nutshell particles showed an indication of microbial contamination on the surface. It can be concluded that walnut shell particles could serve as safe, effective delivery vehicles for emission of SA, which possesses an intrinsic antifungal activity.

Tree nutshells (e.g., almond, hazelnut, pistachio, walnut shells) have been investigated extensively for use in various industrial processes/practices. While tree nut biomass, such as walnut shells, has been converted to solid biofuel through torrefaction [23], tree nutshells have also been tested as economic biosorbents, such as: (1) toxic heavy metal removal from contaminated waters by nutshells [24], (2) rhodamine B cationic dye elimination from contaminated aqueous solutions with acrylic-acid-modified walnut shells [25], (3) amine-functionalized walnut shells as a novel adsorbent for the removal of the pollutants PO_4^{3-} and NO_3^- ; the mechanism of the adsorption was determined as electrostatic interactions and hydrogen bonding [26]. Although walnut shell-derived cellulose nanocrystals possess the potential as effective nanocarriers in the food and drug delivery industries [27], studies exploring the use of walnut shells as drug/chemical delivery vehicles are currently very scarce.

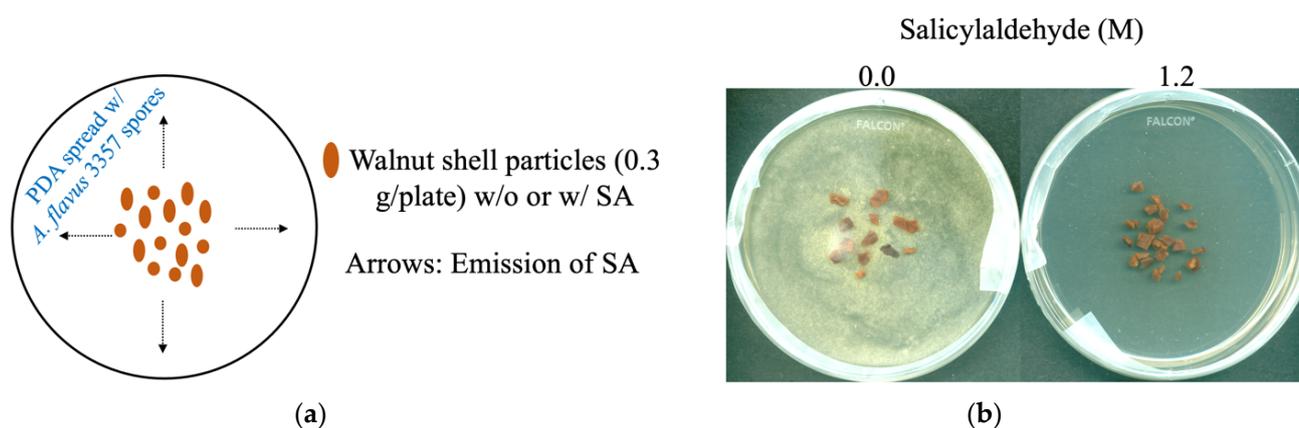


Figure 1. (a) Diagram showing the fumigant activity of salicylaldehyde (SA) as an antifungal agent, which enables remote delivery of SA to the target sites; (b) SA at 1.2 M emitted from the delivery vehicles (walnut shell particles, 0.3 g/plate) completely inhibited the growth of the aflatoxin-producing *A. flavus* 3357.

3.2. Overcoming Fludioxonil Tolerance of Mitogen-Activated Protein Kinase (MAPK) Mutants of *Aspergillus fumigatus* (*sakA* Δ , *mpkC* Δ) or *Penicillium expansum* (FR2, FR3) by SA

Chemo-sensitization was previously developed as a new antifungal formulation strategy, whereby co-application of a second compound (chemo-sensitizer; natural or synthetic) with a commercial antifungal agent (fungicides, drugs), both at sub-inhibitory concentrations, greatly enhances antifungal efficacy of the treatment [28]. A chemo-sensitizer causes the target fungi to become more susceptible to the treatment by modulating the pathogen's defense system, such as antioxidant or cell wall integrity systems, to the agents. Therefore, chemo-sensitization could contribute to the development of a new antifungal formulation, wherein the chemo-sensitizers serve as potent "adjuvants" enhancing the antifungal efficacy of commercial fungicides or drugs co-applied.

The potential of SA as a chemo-sensitizer was investigated to overcome the fludioxonil (phenylpyrrole fungicide) tolerance of *A. fumigatus* and *P. expansum*. Fludioxonil was chosen as a model fungicide for SA chemo-sensitization because its current application to the tree nut orchards has increased public concern over the toxicity fludioxonil may trigger in humans and non-target organisms, including aquatic organisms [29,30]. Therefore, the chemo-sensitization capacity of SA to fludioxonil will enhance the antifungal efficacy of the treatment, lowering the doses of the fungicide required for the effective control of fungal pathogens.

The fungicidal effect of fludioxonil is exerted via the fungal osmotic/oxidative stress signaling system, namely, the mitogen-activated protein kinase (MAPK) pathway [31]. Fludioxonil disrupts fungal growth by triggering unusual, excessive stimulation of the osmotic/oxidative stress signaling MAPK system, thus causing energy drain [31]. This MAPK pathway is responsive to osmotic/oxidative cues and, hence, protects the wild-type fungal cells from environmental osmotic/oxidative stressors. In contrast, fungi having mutations in the MAPK system escape from fludioxonil toxicity, which results in the development of fludioxonil tolerance in the fields [31].

The wild-type and MAPK mutants, namely, *sakA* Δ and *mpkC* Δ strains of *A. fumigatus* and FR2 and FR3 of *P. expansum* (see Table 1), were examined in this investigation, where SA was remotely applied (from the SA-saturated filter placed on each plate) to the target fungi. As shown in Figure 2, the growth of wild-type *P. expansum* (W2) was completely inhibited by fludioxonil (50 μ M), while the MAPK mutant (FR3) exhibited tolerance to the fungicide. However, the combined application of fludioxonil (50 μ M) and SA (0.12 M) completely prevented the germination of FR3, thus overcoming the fludioxonil tolerance of the mutant. Similar results were also observed with other MAPK mutant strains (*A. fumigatus* (*sakA* Δ , *mpkC* Δ), *P. expansum* (FR2); figure data not shown).

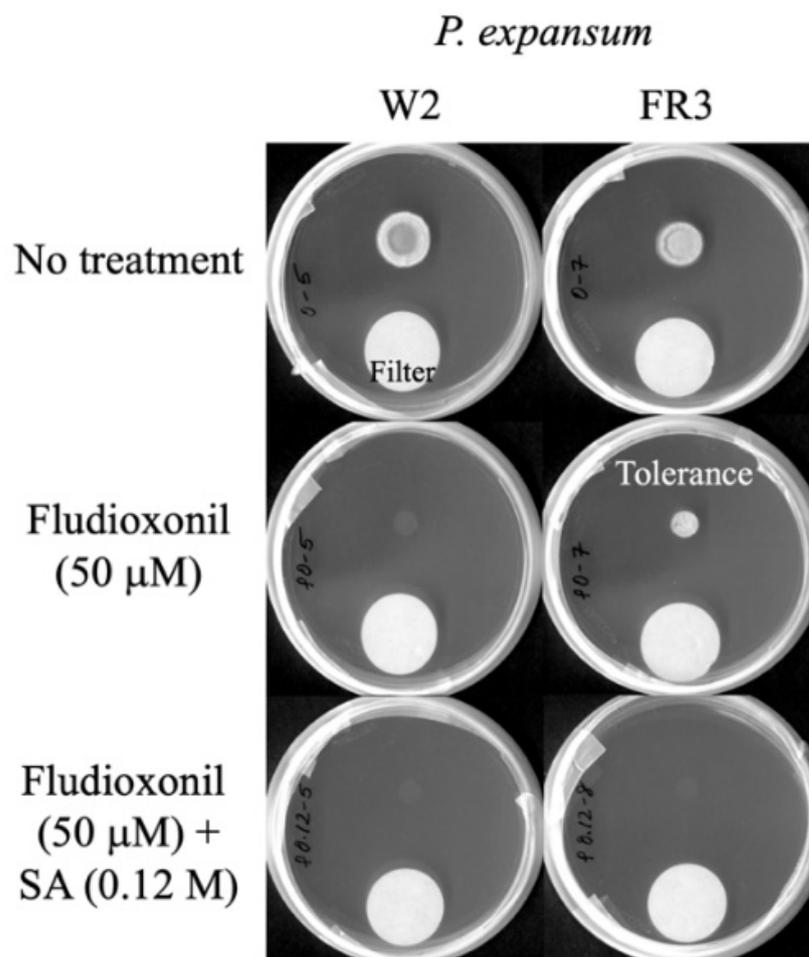


Figure 2. A representative bioassay showing the adjuvant activity of SA fumigant that overcomes fludioxonil tolerance of mitogen-activated protein kinase (MAPK) mutants of *Penicillium expansum* (FR3). W2, *P. expansum* parental strain. The upper panel of each plate shows fungal growth (radial growth) or no growth (empty); the lower panel of each plate shows the filter (2.5 cm) w/o (DMSO only; no treatment control) or w/ SA (0.12 M).

In previous studies, the fumigant SA also chemo-sensitized other oxidative stress drugs or fungicides, namely, inhibitors of complex III in the mitochondrial respiratory chain (antimycin A, strobilurin) or itraconazole [12,13]; thus, it is speculated that SA modulates antioxidant defense systems in fungi. In summary, SA could also serve as a potent adjuvant to fludioxonil for the effective control of fungal pathogens. The intrinsic anti-aflatoxicogenic and antifungal activities of SA, especially the capability to overcome fungicide resistance, are highly beneficial characteristics of the compound for fungal pathogen control in the fields.

3.3. Pre- and Post-Emergent Herbicidal Activity of SA

It was determined further whether the fumigant SA could be repurposed as a potent herbicidal reagent. If repurposed successfully, the herbicidal potential of SA will lower the pesticide burden in crop fields by reducing the frequency of both fungicide and herbicide applications. Adoption of this strategy will, therefore, contribute to the Integrated Pest Management (IPM) program in agriculture.

3.3.1. Pre-Emergent Herbicidal Activity of SA

Initially, the pre-emergent herbicidal activity of SA was investigated against *Brassica rapa* var. *pekinensis* seeds on PDA (Petri plates, 100 mm × 15 mm). *Brassica* spp. is listed

as a restricted or prohibited noxious weed seed in the states of Arizona and Michigan, respectively, in the United States [32]. *Brassica* spp. may become weedy or invasive by producing allelopathic chemicals, which prevent the germination of native plants or plants in cultivated fields. Therefore, if not adequately managed, *Brassica* spp. could displace desirable vegetation [32]. Walnut shell particles saturated with SA (concentration: 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40, 0.45, 0.50 M) were placed on the center of PDA (duplicate plates), and seed germination/growth was monitored for 10 days at 22 °C (12 h light/12 h dark cycle). SA emitted from the nutshell vehicles completely inhibited the germination of *Brassica* seeds at as low as 0.15 M of SA (Table 2).

Table 2. Kinetics of pre-emergent herbicidal activity of SA tested against *Brassica rapa* var. *pekinensis* (total 10 seeds placed/plate) determined in duplicated plates. SA delivery vehicles: walnut shell particles 0.3 g/plate.

SA (M)	# of Seeds Germinated	# of Seeds Contaminated w/ Fungi	Level of Nutshell Contamination w/ Fungi ¹
0.00	10, 10	2, 1	+++, +++
0.05	4, 3	1, 0	+++, ++
0.10	1, 0	1, 0	+, -
0.15	0, 0	2, 0	+, +
0.20	0, 0	0, 0	+, -
0.25	0, 0	1, 0	+, -
0.30	0, 0	0, 0	-, -
0.35	0, 0	0, 0	-, -
0.40	0, 0	0, 0	-, -
0.45	0, 0	0, 0	-, -
0.50	0, 0	0, 0	-, -

¹ Level of nutshell particle contamination: +++, High; ++, Medium; +, Low; -, No contamination.

Plant seeds are also commonly contaminated with environmental fungi and other microbes [33,34]. However, the germination/growth of microbial contaminants associated with the surfaces of *Brassica* seeds and nutshell particles was completely prevented w/ SA at or above 0.3 M (Table 2) (Figure 3; representative bioassay w/ 0.0 and 0.3 M of SA).

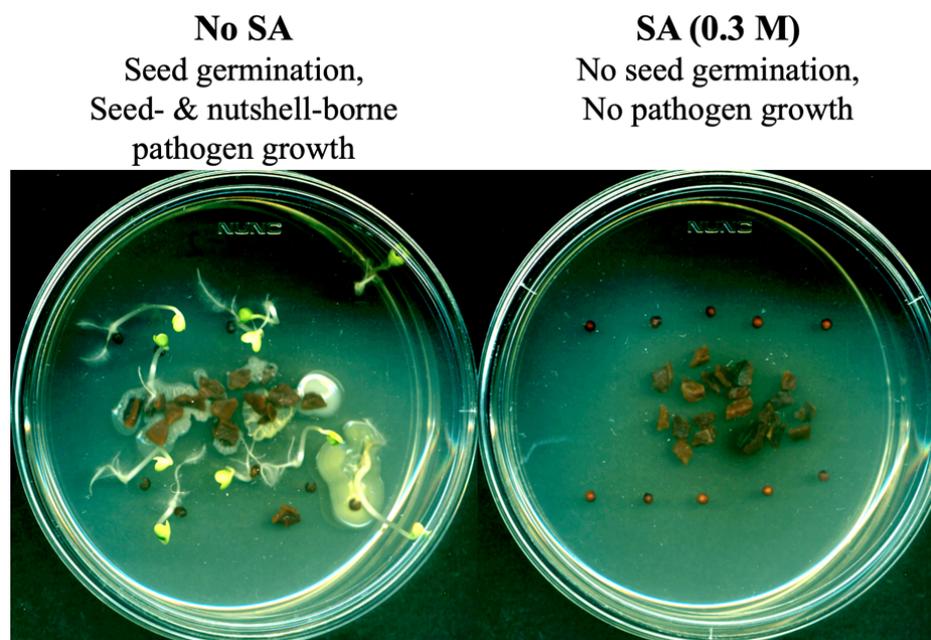


Figure 3. SA (0.3 M) prevented the germination of *Brassica* seeds, where the SA further eliminates seed- and nutshell-borne fungal pathogens.

Similar assays were performed on different weed seeds, including *Centaurea solstitialis*, *L. ovatus*, *Salsola tragus*, *Genista monspessulana*, and *S. scoparium* (see Table 1 for seed characteristics). It was determined that the germination of the seeds as well as that of the fungi associated with the surface of test seeds, was completely prevented at 0.25 to 1.6 M of SA, depending on the types of seeds investigated (Table 3).

Table 3. Pre-emergent herbicidal activity of SA tested against invasive/native weed seeds. Concentrations shown are the minimum inhibitory concentration (MIC) preventing seed germination.

Plants	SA MIC (M)
<i>S. scoparium</i> (Little Bluestem)	0.25
<i>S. tragus</i> (Russian thistle)	0.40
<i>G. monspessulana</i> (French broom)	0.40
<i>L. ovatus</i> (Bunny Tails Grass)	0.80
<i>C. solstitialis</i> (Yellow starthistle)	1.60
Average	0.60 ± 0.54 (<i>p</i> = 0.021) ¹

¹ Student’s *t*-test for paired data (MICs (M) of SA from six plants, including *Brassica rapa* var. *pekinensis*), vs. no treatment control (SA = 0 M).

The pre-emergent herbicidal activity of SA was tested further in Magenta vessels, where 10 seeds of *L. ovatus* were placed per vessel supplemented with MS (0.5×). As shown in Figure 4, the germination of *L. ovatus* seeds exposed to 0.8 to 1.6 M SA vapor was completely inhibited, while those in the control boxes (w/ or w/o walnut shell particles) still germinated.

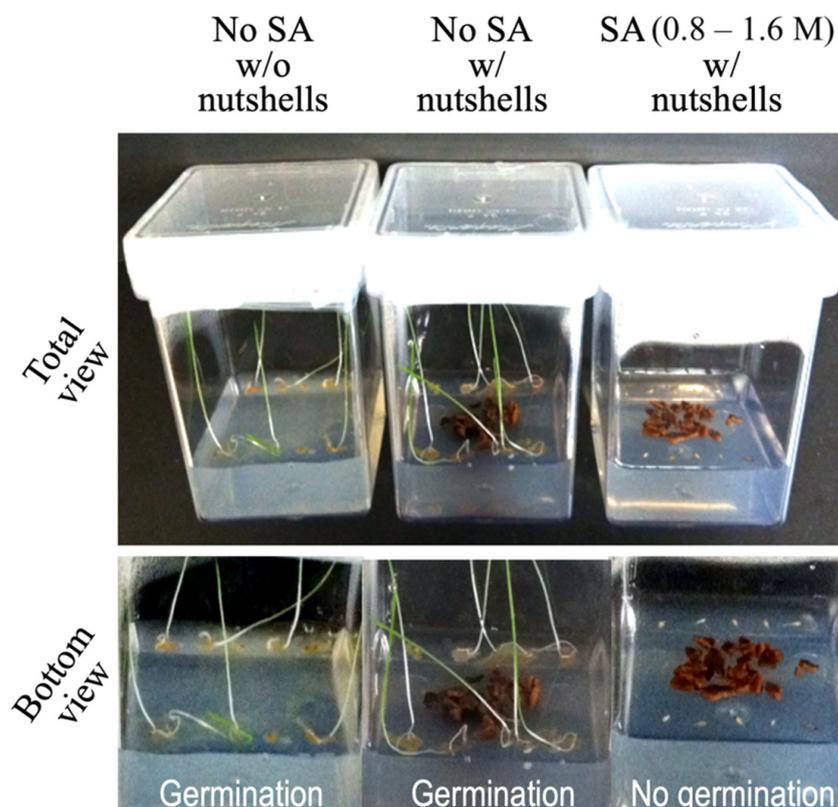


Figure 4. The pre-emergent herbicidal activity of SA tested against *L. ovatus*.

3.3.2. Post-Emergent Herbicidal Activity of SA

The post-emergent herbicidal activity of SA was tested in *L. ovatus* according to the modified protocol described above. Initially, 10 *L. ovatus* seeds were germinated in Magenta vessels without exposure to SA. After 5 days of seed germination/growth, the germinated

weeds (~3 cm of stem length) were provided with 0.3 g nutshell vehicles (w/ or w/o SA, 0.8 to 1.6 M) and were cultivated further for another 10 days. The level of the inhibition of weed growth was evaluated by comparing it to that of the untreated (no SA) weeds. As shown in Figure 5, the emitted SA completely prevented the growth of the germinated weeds at 0.8 to 1.6 M (no further growth, exhibiting wilting), while those without SA (w/ 0.3 g nutshell vehicles) continued to grow further. It can be concluded that SA possesses both pre- and post-emergent herbicidal activity. Interestingly, the fact that SA possessed herbicidal activity against both monocot and dicot plants, as shown above, indicates that SA can be used for broad-spectrum weed control.

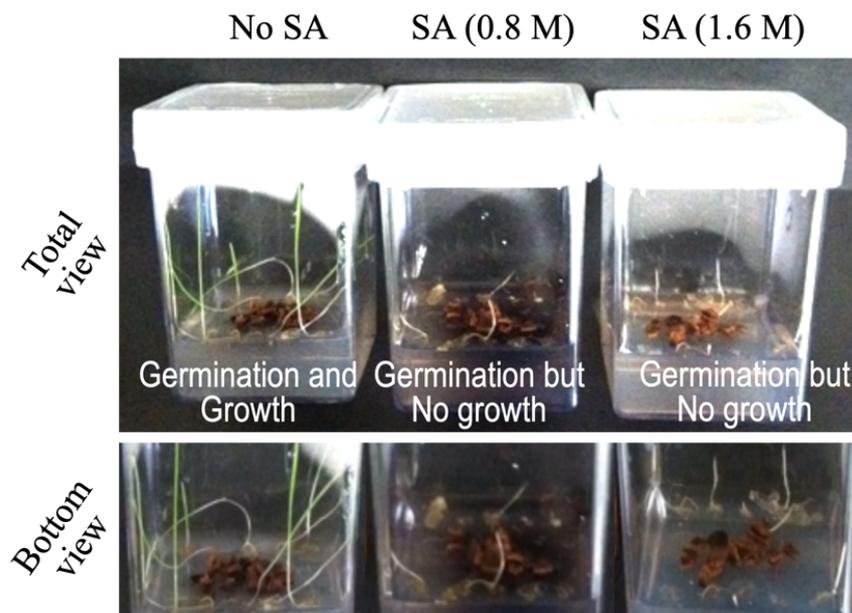


Figure 5. The post-emergent herbicidal activity of SA tested against *L. ovatus*.

3.4. SA Herbicidal Efficacy during Soil Pasteurization

Soil pasteurization (solarization) is a method that uses sunlight to control pests (weeds, microbial pathogens, insects) in crop fields. Soil pasteurization requires: (a) keeping a clear plastic cover in place for at least 4 weeks in the hottest part of the summer and (b) providing enough water to generate steam every day, which effectively kills weed seeds [35,36]. However, although the temperature of the top layers of the solarized soil can reach 60 °C (140 °F) or higher with sunlight, the heat efficiency of soil pasteurization is practically dependent upon the geographic location or weather condition (namely, the amount/days of solar radiation) [36].

Considering that SA exerted potent fumigant activity, we reasoned that soil covered with a plastic tarp (during soil pasteurization) could improve the herbicidal (also fungicidal) efficacy of SA at a much lower temperature, such as room temperature, during the anti-pest treatment. Therefore, the efficacy of SA was tested by treating weed seeds (*L. ovatus*) with SA in soil trays, which were covered with a plastic tarp and maintained at 22 °C, thus mimicking the treatment under colder or cloudy geographic/weather conditions.

As shown in Figure 6, 1.6 M of SA emitted from the nutshell vehicles completely prevented the germination of the weed seeds, while 0.8 M SA allowed partial germination (6 seeds out of a total of 40 seeds per tray) of the test seeds. Control trays (both w/ or w/o nutshell vehicles; w/o SA) exhibited normal seed germination. It can be concluded that SA could serve as a potent herbicidal agent as a fumigant, especially during soil pasteurization (temperature: 22 °C or above), which, therefore, could effectively complement current soil pasteurization (solarization) practices requiring high temperature in the fields. Of note, the soil testing required a higher concentration of SA (1.6 M) than that tested in Petri plate/Magenta vessels (0.3 to 0.8 M) to achieve complete inhibition of weed germination.

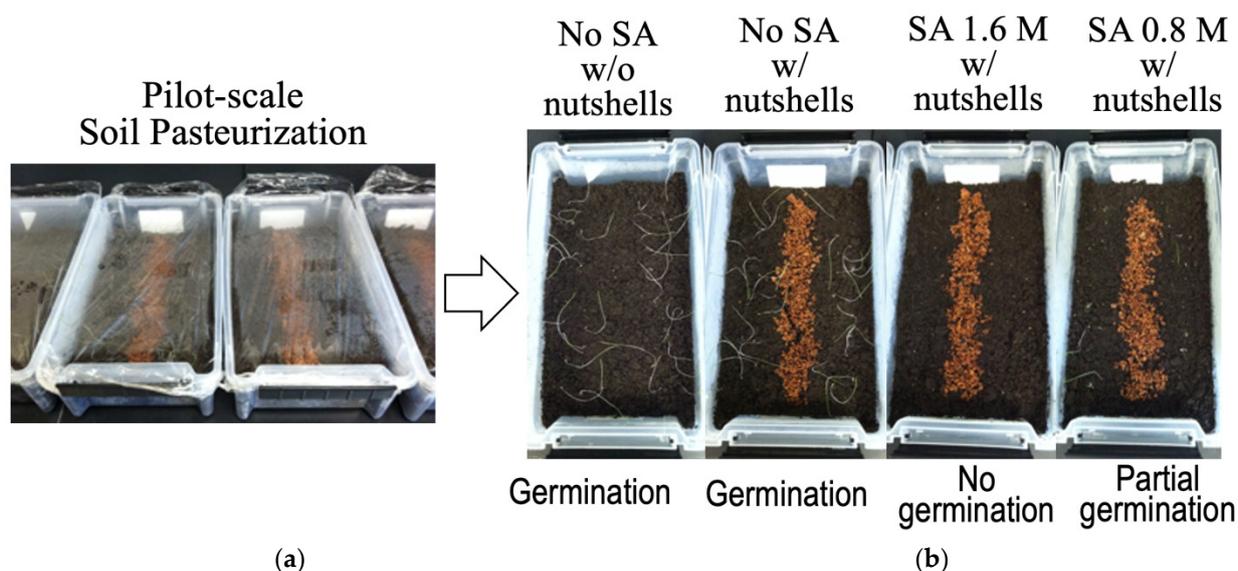


Figure 6. Pre-emergent herbicidal activity of SA (pilot-scale soil pasteurization test): (a) covering of trays (No SA w/o nutshells (Control), No SA w/ nutshells (Control), SA 0.8 M w/ nutshells as SA delivery vehicles, SA 1.6 M w/ nutshells as SA delivery vehicles) after sowing *L. ovatus* seeds; (b) determination of germination/growth of *L. ovatus* seeds after 11 days.

In this proof-of-concept study, we have developed a potent anti-pest system, which used the fumigant compound SA. We repurposed the food additive SA as a pre- and post-emergent weed control agent and developed tree nutshell particles as sustainable SA delivery vehicles for use in orchards. The binary function of SA, namely, the potent herbicidal and fungicidal activity of SA, could lower the pesticide burden in crop fields by reducing the doses and frequency of herbicide and fungicide applications required for effective weed/fungal pathogen control.

SA is a natural constituent identified in many plant species [37]. According to its vapor pressure value (5.93×10^{-1} mm Hg, 25 °C), SA exists mainly as a vapor in the atmosphere; thus, when applied in the fields, SA has high mobility in soil, which then volatilizes from dry soil [38]. Worthy of note, SA is a small molecule lacking “functional groups” that hydrolyze under environmental conditions (between pH 5 to 9) [38]. Hence, hydrolysis of volatilized SA is not supposed to occur in the environment; instead, safe biodegradation and/or photolysis by sunlight are considered an important environmental fate of SA, thus warranting environmental safety [38]. Moreover, the fumigant characteristics of SA have been determined to be “specific” to this molecule compared to other SA derivatives, enabling the effective application of SA, especially during soil pasteurization; other derivatives of SA from the prior study could not be applied as fumigants [39].

While SA modulates antioxidant defense systems in fungi [12,13], the mechanism of herbicidal action of SA has not been investigated thus far. It is speculated that, in nature, the allelopathic activity of SA (namely, SA released from plants into the environment function as inhibitors towards the germination or growth of the neighboring plants) might be one mechanism of herbicidal action. Precise determination of the SA mechanism of action (cellular targets) as a herbicide warrants future investigation.

The derivatives of SA or salicylic acid have previously been developed as herbicides and bioregulators [40]. It was speculated that SA/salicylic acid derivatives might affect plant metabolism or inhibit vegetative and/or generative plant growth, but the mechanisms of herbicidal action of the derivatives were not comprehensively investigated or discussed in the study [40].

Meanwhile, a fungal pathogen of *Elytrigia repens* (couch grass; perennial weed) *Ascochyta agropyrina* var. *nana* has been shown to produce natural toxins [41]. One of the toxins characterized is agropyrenol, a substituted SA that causes phytotoxicity in several

weeds, including *Mercurialis annua* (French mercury), *Chenopodium album* (goose foot), and *Setaria viridis* (wild foxtail millet), resulting in necrotic lesions in plants [41]. Additionally, SA is one of the compounds identified in the cattail species *Typha domingensis* [42]. The aqueous extracts of cattail plants, which also contain two phenolic agents (namely, 2-chlorophenol, SA), inhibited the growth of the watermoss *Salvinia*, suggesting SA could function as one of the allelopathic substances released from *T. domingensis* [42]. Recently, the sugar crop “sugar beet” has been determined to be vulnerable to continuous cropping, where allelopathy was one of the crucial factors triggering cropping disorder [43]. The transcriptomics and metabolomics study identified that SA was one of the allelochemicals continuously accumulated in sugar beet root exudates with continuous cropping; it functioned as an autotoxic substance [43]. It was hypothesized in the study that continuous cropping changes the metabolome of sugar beet, which interferes further with the diversity of the rhizosphere microbial community, thus negatively affecting sugar beet growth and quality [43]. However, none of these studies explored the mechanisms of the phytotoxic action of SA or its structural derivatives.

In contrast, SA and its structural derivatives have recently been shown to possess metal chelating activities [44–46]. Of note, certain SA derivatives, such as SA isonicotinoyl hydrazone, exhibited pro-oxidant activity as an iron-chelator, thus increasing the levels of reactive oxygen species (ROS) and modulating the anti-/pro-oxidant balance [44]. It was surmised that, in addition to the intrinsic redox-active nature of SA (see above) [47,48], the pro-oxidant activity exerted by SA or SA derivatives (as metal-chelators) would contribute to the phytotoxicity in the treated plants, thus resulting in disruption of normal germination/growth of weeds. This pro-oxidant effect, along with the modulation of the cellular antioxidant system, as determined in other organisms [12,13], would disrupt the integrity of normal cellular components (such as cellular membranes, lipids, and proteins). Elucidation of the precise mechanisms of SA herbicidal action warrants future in-depth investigation.

Nonetheless, compared to prior research, the SA fumigation developed in our investigation provides several advantages over the state-of-the-art, which include: (i) SA is a safe, natural constituent identified in many plant species, thus maintaining an intact structure (non-synthetic); (ii) compared to the derivatives of SA developed in prior research, SA lacks functional groups that hydrolyze under environmental conditions; hence, SA hydrolysis is not supposed to occur in the environment, warranting environmental safety (FDA-proven safety); (iii) binary functions (herbicidal and antifungal activity) of SA as a fumigant, the unique characteristics of SA, greatly lowers the pesticide burden in orchards, which also enables the sanitation of crop byproducts (SA delivery vehicles); and (iv) the adjuvant capacity of SA towards commercial fungicide fludioxonil overcomes the fludioxonil tolerance of fungi. Notably, in a preliminary bioassay, the fungicidal activity of SA was also greatly enhanced at an elevated temperature (42 °C) (Figure S3), indicating that the antifungal efficacy of SA would be heightened in the solarized soil surface, which can reach to 60 °C or higher with sunlight. Overall, once adopted, the SA fumigant system as a natural herbicide/fungicide will promote the stakeholders’ sustainable agriculture.

In the United States, California leads the nation in the production of agricultural commodities. Tree nuts (almonds, walnuts, and pistachios) are the largest commodity produced in California, representing over USD 9 billion in sales in 2018 [16]. However, there are serious food safety challenges that must be addressed; the tree nuts are commonly contaminated by aflatoxin-producing fungi (*A. flavus*, *A. parasiticus*) and are subsequently susceptible to aflatoxin contamination. Aflatoxins are hepato-/nephrotoxic carcinogens/mutagens deleterious to human and poultry health. Since aflatoxin is a serious threat to food safety and food/crop marketability, with many importing countries, including the EU, imposing limits as low as 4 ppb [49], new intervention strategies to reduce the incidence of this food safety challenge should be developed. As mentioned above, weeds are the field reservoir of important pests, including mycotoxigenic fungi [1]. In California, the eradication of weeds, such as the widespread alkaliweed (*Cressa truxilensis*), along with other invasive weeds, is one of the prominent tasks for the tree nut industry [50]; around 9% of tree nut culture

costs are currently spent on weed control. Hence, the SA herbicidal system can contribute to the cost-effective weed management endeavor in the orchards.

In conclusion, the SA anti-pest system developed in this study could serve as a sustainable, complementary/alternative way to conventional weed and fungal control measures that is applicable to the agricultural industry, including the tree nut industry. The developed method will reduce costs, abate resistance, and alleviate the negative side effects associated with current anti-pest practices. The identification of more practical or improved usage of SA, such as the controlled release of SA to the target sites, as well as translation of the information presented in this study into agricultural practices, warrants future in-depth investigation.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/app12083749/s1>, Figure S1: (a) Weed seed contamination with environmental fungi tested on potato dextrose agar (PDA) plates (28 °C). Fungal growth was observed during germination of tested seeds (*Lagurus ovatus* (Bunny tail grass), *Schizachyrium scoparium* (Little Bluestem)) (10 seeds/plate). Top, top view of test plates; Bottom, bottom view of test plates; (b) Walnut shell contamination with environmental fungi tested on PDA at different temperatures (22, 28, and 35 °C; duplicate plates per each temperature) (walnut shell particles: 0.3 g/plate), Figure S2: Inhibition of the production of aflatoxins (aflatoxin B (AFB) and aflatoxin G (AFG)) by *A. flavus* and *A. parasiticus* via the treatment of SA (adapted from [12]), Figure S3: Enhanced fungicidal activity of SA at an elevated temperature (42 °C). Fungi: *A. flavus* 3357.

Author Contributions: Conceptualization, J.H.K.; methodology, J.H.K.; validation, J.H.K. and K.L.C.; formal analysis, J.H.K.; investigation, J.H.K.; resources, J.H.K. and K.L.C.; data curation, J.H.K. and K.L.C.; writing—original draft preparation, J.H.K.; writing—review and editing, J.H.K. and K.L.C.; visualization, J.H.K. and K.L.C.; supervision, J.H.K.; project administration, J.H.K. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data generated by this study are available in this paper.

Acknowledgments: This research was conducted under USDA-ARS CRIS Project 2030-42000-054-00-D. We thank Guangwei Huang (Associate Director of Food Research and Technology), Drew Wolter (Pest Management Senior Specialist), and Tim Birmingham (Director of Quality Assurance and Industry Services) of the Almond Board of California, Modesto, CA, USA, for their collaboration and helpful discussion. We also thank Patrick J. Moran, Invasive Species and Pollinator Health Research Unit, USDA-ARS, Albany, CA, USA, for providing us with the *Centaurea solstitialis*, *Salsola tragus*, and *Genista monspessulana* seeds.

Conflicts of Interest: The authors declare no conflict of interest.

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