Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE60764R1
Full Title:	Repeatable stair-step assay to access the allelopathic potential of weedy rice (Oryza sativa ssp.)
Section/Category:	JoVE Biology
Keywords:	weed control; rice; sustainable; organic farming; allelopathy; weed interference
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
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TITLE:

Repeatable stair-step assay to access the allelopathic potential of weedy rice (*Oryza sativa* ssp.)

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KEYWORDS:

allelopathy, competition, weed management, biochemical compounds, plant-plant interactions, competitive ability

SUMMARY:

Allelopathy has shown promise as a useful supplemental weed control strategy in cropping systems. To determine the allelopathic potential of a desired plant specimen, a stair-step screening method is provided.

ABSTRACT:

Weed competition contributes significantly to yield losses in cropping systems worldwide. The evolution of resistance in many weed species to continuously applied herbicides has presented the need for additional management methods. Allelopathy is a physiological process that some plant species possess that provide the plant with an advantage over its neighbors. Allelopathic crop varieties would be equipped with the ability to suppress the growth of surrounding competitors, thus reducing potential yield loss due to weed interference. This paper focuses on the construction and operation of a stair-step assay used for the screening of the allelopathic potential of a donor species (*Oryza sativa*) against a receiver weed species (*Echinochloa crus-galli*) in a greenhouse setting. The structure described in this paper serves as a stand for the plant samples and incorporates a timed watering system for the accumulation and distribution of allelochemicals. Allelochemicals produced by the plant roots are allowed to flow downward through a series of four pots separately into a collection tank and recycled back to the top plant through electric pumps. This method of screening provides an avenue for the allelochemicals from the donor plant to reach receiver plants without any resource competition, thus allowing quantitative measurement of the allelopathic potential of the selected donor plant. The allelopathic potential is measurable through the height reduction of the receiver plants.

Preliminary screening data for the effectiveness of this method demonstrated height reduction in the receiver species, barnyardgrass (*E. crus-galli*), and thus the presence of allelopathic residues from the donor plant, weedy rice (*Oryza sativa*).

INTRODUCTION:

Allelopathy is a natural and complex phenomenon that has been the focus of many plant scientists in the past few decades. The mechanisms relating to allelopathy for use in crops have been the subject of much research since the 1930s, when Molisch observed that a plant has a direct or indirect effect on a neighboring plant through the production and secretion of chemical compounds into the environment[1]. Allelopathy is the production of secondary metabolites that have inhibitory effects on the growth and germination of some plant species. Released allopathic chemical compounds help provide the donor plants with a competitive advantage by adding phytotoxins to the environment around them[2]. Many factors contribute to the allelopathic activity. It is selective in its effectiveness and varies between varieties, environmental conditions, growth stage, stress, environment, and nutrient availability[3].

In recent years, allelopathy has been highlighted in research as a possible supplement to the constant and growing weed control crisis. With the growing global population, the demand for sustainable food and fiber production has increased[4]. Weed control is one of the biggest threats to production faced by agronomists[5,6]. Traditional weed control methods focus on mechanical, chemical, and cultural practices. The continuous usage of herbicides, while effective, useful, and efficient, has promoted the evolution of resistant weed populations at an alarmingly fast pace[7]. Genetic engineering and breeding practices have been used effectively to give crops competitive advantages over weeds by designing them to withstand chemical applications that their neighbors cannot survive[7,8]. Although effective, these technologies are not always sustainable and sometimes pose outcrossing concerns[9]. Supplemental weed management practices need to be introduced if the goal of increasing food production is to be met[10]. Allelopathy shows excellent promise as a new defense tool for crops to improve their quality and outlive their competitors[1,7].

Allelochemicals are often secondary products, and because their production is highly influenced by environmental factors, the specific compounds associated with plant suppression can be difficult to identify[3]. Production factors include genetics and the joint action of secondary metabolites that may act synergistically[11,12]. It is challenging to separate allelopathic activity from the competition that naturally exists within crop-weed interactions, and due to this, when screening for allelopathy there must be a standard set of outcomes that qualify the assay as valid and repeatable. Below is a set of criteria that qualifies findings of allelopathy as outlined by Olofsdotter et al.[12] 1) One plant must demonstrate suppression of another plant in a pattern; 2) The chemicals that are released into the environment in bioactive amounts must be produced by the donor plant; 3) The chemicals produced must be transportable to the receiver plant; 4) Some mechanism of uptake must be present in the receiver plant; 6) The pattern of inhibition observed must have no other exclusive explanation (e.g, competition for resources)[12].

In an effort to overcome the barrier between the lack of knowledge of the mechanisms supporting allelopathy and variety development, phenotypic traits associated with allelopathic

varieties can be identified and selected for further research and use. Some plants known to have allelopathic qualities are rye, sorghum, rice, sunflower, rapeseed, and wheat[13]. During the early observations of allelopathy in crops, due to distinguished borders of weed growth in field experiments, it was proposed that chemicals were involved rather than competition for resources[14]. However, most studies were field experiments that made it impossible to eliminate competition as a factor[14]. Competition elimination efforts gave way to lab and greenhouse experiments in attempts to prove and quantify allelopathic activity in rice and other crops. Field and greenhouse methods to screen plants for allelopathy demonstrate that allelopathic tendencies are present in both growing conditions[11,15]. Some critics believe that laboratory screenings may only hold limited value due to the lack of natural conditions, which may affect the results[15].

The proposed method for screening allelopathic potential in plants provides adequate resources and space and eliminates resource competition with the use of a stair-step structure[11,17]. The method was adapted and modified from previous experiments exploring allelopathy in turfgrass and barley[17,18]. These studies found that a similar system was able to produce accurate results on the allelopathic potential of a target plant while removing any doubts that the observations could be attributed to natural competition. The stair-step method creates a circulatory system where a nutrient solution from a reservoir can cycle through each plant to an incubation tray through a few steps. An electric pump then recycles the solution along with any allelochemicals produced[18]. A method such as this is efficient in both time, space, and resources. It also provides similar field conditions for the plants and eliminates any resource competition. The methods and tools used for screening are easily manipulated to fit the desired study goals, conditions, and specific species. The objective of this study is to confirm weedy rice allelopathy through height suppression measurements on barnyardgrass with the use of the stair-step method.

PROTOCOL:

1. Stand construction

NOTE: Measurements for the wood are listed as thickness (cm) x width (cm) x length (m).

1.1. Cut wood into appropriate sizes and amounts as follows: five 10.16 cm x 5.08 cm x 0.91 m wooden pieces, three 10.16 cm x 5.08 cm x 0.76 m wooden pieces, three 10.16 cm x 5.08 cm x 0.61 m wooden pieces, five 10.16 cm x 5.08 cm x 0.46 m wooden pieces, three 10.16 cm x 5.08 cm x 0.3 m wooden pieces, and three 10.16 cm x 5.08 cm x 0.15 m wooden pieces.

1.2. For the tallest level, stand one 2.44 m board across two 0.91 m pieces on each end at the edge and drill two screws vertically into each of the 0.91 pieces. Screw one more 0.91 m piece 1.22 m from each end for support, and place a 2.44 m board across the back of the 0.91 m stands and screw into place for support.

NOTE: The eight 3.175 cm x 15.24 cm x 2.44 m are kept as is and uncut to serve as the benchtop for each bench level.

1.3. Repeat step 1.2 for the next bench level with the 0.76 m pieces.

1.4. Repeat step 1.2 for the next bench with the 0.61 m pieces down to the sixth bench at 0.15 m.

NOTE: No supporting 2.44 m board is needed for benches 3–6. The final stand has six benches with three vertical supports each, one on each end and one in the middle.

1.5. Line benches in descending height order with the overhanging lip facing the backside touching the bench above it, allowing for a gap between levels.

1.6. Line a 0.91 cm board on each of the bottom edges of the benches along the ground and screw the benches in place.

1.7. Screw a 0.46 m board horizontally for support on the tallest three benches on each side of the structure 0.61 m from the ground.

1.8. Screw three corner braces onto the front-facing ends and center of the tallest bench.

1.9. Screw one 2.54 cm x 5.08 cm x 20.32 cm wooden piece across the braces 2.54 cm from the base of the bench.

NOTE: Make one 0.91 m by 0.91 m by 2.44 m structure. Refer to **Figure 1** for the final base product. Dimensions are subject to change with the experimental needs. The structure described was designed to fit 15.24 cm pots. The heights between benches were designed to fit the pots and plant material used in this experiment in order to maintain a steady flow of allelochemicals and solution from one pot to another down the benches by gravity.

[Figure 1]

2. System assembly

2.1. Remove the cap from a 1 L soda bottle and spray paint with black paint.

NOTE: The soda bottles will serve as a reservoir at the top of the system for one column. The paint provides a block for the light, decreasing or preventing algae growth.

2.2. At the bottom of each soda bottle, drill a small hole, just large enough to embed a 0.35 cm inner diameter (ID), 0.64 cm outer diameter (OD), 5.08 cm long plastic PVC tube.

2.3. Smear a layer of silicone waterproof sealant around the edge of the hole after insertion to prevent any leaks. Let it dry completely.

2.4. Repeat steps 2.2 and 2.3 on each of the plastic dishes used to hold the pots.

NOTE: Four dishes will be needed for one column.

2.5. Remove the lid and spray paint the outside of 2.27 L plastic canisters with black paint. These canisters will serve as the collection tanks at the base of each column.

2.6. Drill a small hole in the upper backside of the canister.

NOTE: The supplies listed in steps 2.1–2.6 make one column. The number of columns is subject to the number of samples needed for the experiment desired. Two columns are needed for one sample. All dimensions are subject to change depending on the experimental needs.

2.7. After the supplies have been prepared and dried, place the soda bottle on the highest bench so that the PVC tube is hanging over the rim facing the stairs.

2.8. Just below the soda bottle on the next bench, place one plastic dish with its tube hanging over the rim of the bench.

2.9. Repeat step 2.8 for the next two benches.

2.10. Place the canister on the bottom bench with the hole facing the back.

2.11. Connect the canister with the dish above it by stringing the tube from the dish through the hole in the back of the canister.

2.12. Smear waterproof sealant around the edge of the canister where the tube runs through to prevent leaks.

2.13. Place a 21 W 1,000 L/hr submersible electric pump inside the bottom canister.

2.14. Connect a 1.07 m long, 1.27 cm ID, 1.59 cm OD PVC tube to the nozzle of the electric pump.

2.15. String the tube through the gap between the benches and over the back of the soda bottle at the top of the system.

2.16. Plug the pump into a digital timer and set the timer setting as needed.

NOTE: The timer was set to run for 1 min every 3 h throughout the entire experiment. The selected timing allowed for the maximum amount of liquid in the collection tank to be cycled and allowed for approximately 10 min of flow each time the pump was turned on while avoiding flooding and spillovers.

3. Planting

3.1. Sterilize all the rice seeds needed by rinsing in 70% ethanol for 30 s, soaking in 5% bleach for 20 min, and rinsing 6x with distilled water.

3.2. Pregerminate the sterilized rice seeds in Petri dishes lined with filter paper filled with 5 mL of distilled water in a growth chamber set at 25 °C.

3.3. After the seeds germinate, line the bottom of each pot with two large coffee filters by placing them inside the pots in their natural cupped form.

3.4. Fill each pot to the top of the filter (approximately 75% of the pot) with autoclaved, washed, and screened specially graded quartz sand. Dampen the sand with distilled water by pouring water over the top of the sand or by placing pots in planting trays filled just slightly with distilled water to allow the pots to soak up the water and remain damp. Transplant six pregerminated donor plant seedlings into sand, evenly spaced.

3.5. Cover the seedlings with sand.

3.6. Let the seedlings establish for 3 weeks.

NOTE: The sand dries very quickly. Therefore, placing pots in trays is an efficient watering technique. Changing water out constantly will help prevent mold.

3.7. Pregerminate the receiver plant seedlings (*E. crus-galli*) in Petri dishes 3 weeks after planting donor plants by lining the bottom of the dish with filter paper and along with 5 mL of distilled water. Place the dishes in a growth chamber at 25 °C for 3–5 days.

3.8. Prepare the pots as described in steps 3.1–3.2.

3.9. After the seedlings germinate, transplant three seedlings into the prepared pots and cover with sand.

NOTE: The experiment begins one day after treatment (DAT), or the day that the receiver plant seedlings emerge and are transplanted and placed in the system.

4. Sample placement

4.1. Place four pots of one accession of donor plants in the four dishes of column 1, a single pot per row. Column 1 consists of donor plants only.

4.2. Place two pots of the same accession of donor plants in the dishes of column 2 on the first and third row of the column.

4.3. Place two pots of receiver plants in the dishes of column 2 on the second and fourth row in the column.

4.4. For each replication, ensure that only one row of receiver plants is added. Two columns, the first consisting of donor plants only and the second alternating donors and receivers, make one treatment (**Figure 2**).

[Figure 2]

4.5. Repeat steps 4.1–4.4 for each treatment or donor plant accession (**Figure 3**).

[Figure 3]

NOTE: Each replication requires one column of receiver plant samples to serve as a control for one replication. Treatments were replicated 3x in a randomized complete block design.

5. Operation

5.1. On DAT 1, fill the collection tank at the bottom of each column with half-strength Hoagland solution[17] in distilled water, approximately 1,500 mL.

5.2. Set the timers to run as desired in the auto off setting.

5.3. Cover the collection tanks with black plastic to limit light exposure and evaporation.

5.4. Fill the tanks every 2 days with 500 mL of Hoagland's solution to keep the system flowing constantly.

5.5. Maintain the greenhouse temperatures at 28 °C during the day and 24 °C at night respectively with a 16/8 h split and humidity at 53%.

6. Data collection

6.1. Measure and record the heights of each plant in the stair-step system on DAT 1 and once every week up to DAT 21 by placing a ruler at the base of each plant and observing the tallest leaf stand.

6.2. Measure and record the chlorophyll levels of each plant on DAT 7 and 14 using the chlorophyll content meter.

6.3. On the last day of the experiment (i.e., DAT 21) label one paper bag for each pot.

6.4. Cut plant samples at the base and place in separate bags.

6.5. Place all samples in an oven dryer set at 60 °C for 48 h[16].

6.6. Remove dried samples and empty contents individually onto a scale and record the weight in grams.

7. Data analysis

7.1. Calculate the allelopathic potential of the donor plants based on the percent inhibition of the receiver plant using this equation:

$$\text{height reduction (\%)} = [\text{height of control (cm)} - \text{height of treated (cm)}] \times 100$$

7.2. Calculate the donor plant height reduction as a check for any reverse effect the receiver plant may have on the target plants.

7.3. Analyze accessions as the fixed effect while replications and runs are the random effects[18].

7.4. Analyze the data using a general linear model with mean values separated using Fisher's protected least significant difference at or below a 0.05 probability level in a statistical software (e.g., JMP 14).

7.5. Visualize the correlation among the original variables using principle component analysis of by uploading data.

7.5.1. Select the **Analyze** tab in the toolbar, select **Fit Y by X**. Under columns, **Highlight** the response (i.e., percent height reduction) then click **Y, response** to specifythe factor being observed for Y, (i.e., percent height reduction). For the X factor, **Hightlight** accession and click **X, factor**, then select **OK**.

7.5.2. Select the **red down arrow** on the **Oneway Analysis** bar, select **Means/ANOVA**. Again select the **down arrow** on the **Oneway Analysis bar** and highlight **compare means** then select **each pair, student's T**.

REPRESENTATIVE RESULTS:

Two preliminary screenings using this method were performed on nine weedy rice accessions (B2, S33, B83, S97, S94, B81, B8, B34, B14) and five cultivated rice lines (PI338046, Rex, Rondo, PI312777, CL163). Weedy rice accessions and rice lines were selected based on their performance in previous allelopathic screenings conducted by Shrestha (2018)[18]. The weedy rice seeds were collected from across the state of Arkansas. The rice lines selected are commonly grown lines in the US, some known to express allelopathic activity (e.g., Rondo PI312777) and used as controls in this study[18]. The preliminary data demonstrate the potential of the stair-step method as a means to evaluate the allelopathic potential against barnyardgrass (*E. crus-galli)*. The height of barnyardgrass plants was significantly reduced by the allopathic residues excreted through the rice roots. Competition for resources between weedy rice and barnyardgrass was eliminated, and

all plants were grown in identical conditions. Results demonstrated that the allelopathic activity against barnyardgrass varied among the weedy rice and rice cultivars.

Height measurements recorded at DAT 14 were used to calculate the barnyardgrass height reduction percent. As presented in **Figure 4**, the height reduction was up to 30% in some donor rice accessions with one accession, B81, standing out. Five weedy rice accessions displayed more significant barnyardgrass height reduction than Rondo, the allelopathic rice standard. Weedy rice accessions B8, S33, B14, B97 reduced barnyardgrass height by 25–30%. Weedy rice accession B81 exhibited the most considerable barnyardgrass height reduction by 74%, which was nearly 3x as much as the standard allelopathic rice, Rondo.

[Figure 4]

Height reduction of the weedy and cultivated rice accessions was also recorded to determine if barnyardgrass had any allelopathic activity. From data collected at DAT 14, there was no significant detectable height reduction of weedy rice or rice due to barnyardgrass allelochemicals in the treated column.

Biomass reduction percent from data collected at DAT 21 displayed a range in barnyardgrass biomass reduction percent from 0–86%. Among the weedy rice accessions that reduced barnyardgrass height the most (S33, B97, B14, B8, B81), S33 reduced barnyardgrass biomass by approximately 84% compared to Rondo at 60% (**Figure 5**).

[Figure 5]

Chlorophyll levels of all plant samples were recorded at DAT 7 and 14. Chlorophyll reduction in barnyardgrass samples ranged from 1–14% when exposed to rice root leachates. There was variation among chlorophyll reduction levels among non-allelopathic and allelopathic rice. Of the allelopathic weedy rice accessions, B8 and S33 showed the least chlorophyll reduction (less than 10%). Chlorophyll levels in rice accessions were between 0–30% with variation in levels among non-allelopathic and allelopathic accessions.

FIGURE AND TABLE LEGENDS:

Figure 1: Front view of the wooden base stand. A wooden base serves as the stand for the plant samples. Materials for the system are to be assembled and added depending on the number of samples needed for the experiment. In this study, two stands served as a base for 31 samples.

Figure 2: Placement map. Diagram depicting placements of donor (WR/R) and receiver plants (BYG) in respective positions in the stair-step system. Two columns of the stair-step system with plants in place comprise one treatment. A single column of receiver plants served as a control for one replication (far right), a single column of donor plants as a control for each accession (center), and the treatment column consisted of alternating donor and receiver plants (far left).

Figure 3: Final stair-step structure. The stair-step system assembled with the plants in place. The system contained four rows of plant samples and a collection tank at the bottom for the solution to cycle to the top bottle and downward by gravity through each respective pot.

Figure 4: Receiver plant height reduction data. The height reduction percentages of the receiver plants (*E. crus-galli*) displayed in ascending order when treated with the allelopathic residue from 15 donor plant accessions of *O. sativa* along the X-axis.

Figure 5: Receiver plant biomass reduction data. The biomass reduction percentages of the receiver plants (*E. crus-galli*) displayed in ascending order when treated with the allelopathic residue from 15 donor plant accessions of *O. sativa* along the X-axis.

DISCUSSION:

Exploiting allelopathy may potentially serve as a biological control for weeds that are difficult to manage[1,7,13]. Allelopathy has shown great potential as a possible solution to the weed crisis in rice and serves as an alternative or supplement to chemicals and manual weed control practices[5,13,19]. Identifying allelopathic varieties or accessions of crop species is the first step toward incorporating this technology into weed management strategies. As shown in this study, some accessions of weedy rice and rice (*O. sativa)* exhibit greater suppression of barnyardgrass (*E. crus-galli).* The accessions that performed best in this study are candidates for further research on allelopathy genetics and mechanisms of action.

The stair-step method proved to be a useful screening technique to determine rice allelopathic potential. The methods are not limited to any one donor or recipient plant. A variety of different plants can be screened simultaneously, and the target and recipient can be easily exchanged for accurate results. Susceptibility to allelopathic compounds varies between species[1]. This method can provide a screening of susceptibility of the receiver plant and at the same time determine the allelopathic potential of the donor plant.

It was suggested that more effort is needed to be placed on experiments that mimic field conditions[12]. A multitude of factors contributes to allelopathic activity, such as the environment and genetic background[11,12]. Greenhouse screenings can create a field setting in a controlled environment. Soil is the preferred growth medium as opposed to artificial media such as agar. The sand in this experiment provided a medium that did not alter the nutrients available, allowed the solution to flow cleanly from pot to pot, and limited microbial activity that could have affected the results. Additionally, the temperature can be set at ideal conditions for the desired species. The stair-step method provides a precise way of identifying and measuring the allelopathic activity of a plant species.

One drawback of the stair-step method is that differences in the nature and amounts of allelochemicals produced by the two plant species may present results that appear as nutrient stress. The use of nutrient additives is essential to ensure adequate conditions. Species differ in their responses to different minerals, and weed species may respond better than a crop to the nutrients provided[20]. Allelopathy is confirmed if there are inhibitory effects even in the presence

of added nutrients[20]. Moreover, the stair-step method is useful only if the plant species in question is allelopathically active through root secretion[16]. Some species do not have active root allelopathic production, because allelochemicals can also be secreted in the form of gas and leachates from aboveground living or dead plant parts or dried tissues[21,22,23]. For this method to successfully demonstrate allelopathic inhibition, the specimen screened must exhibit root allelopathic activity because the system targets chemicals leached through the soil medium.

ACKNOWLEDGMENTS:

Funding for this project was provided by the Special Research Initiative Grant sponsored by the Mississippi Agricultural and Forestry Experiment Station and is based upon work that is supported by the National Institute of Food and Agriculture, U.S. Department of Agriculture, Hatch project under accession number 230060.

DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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Figure 2

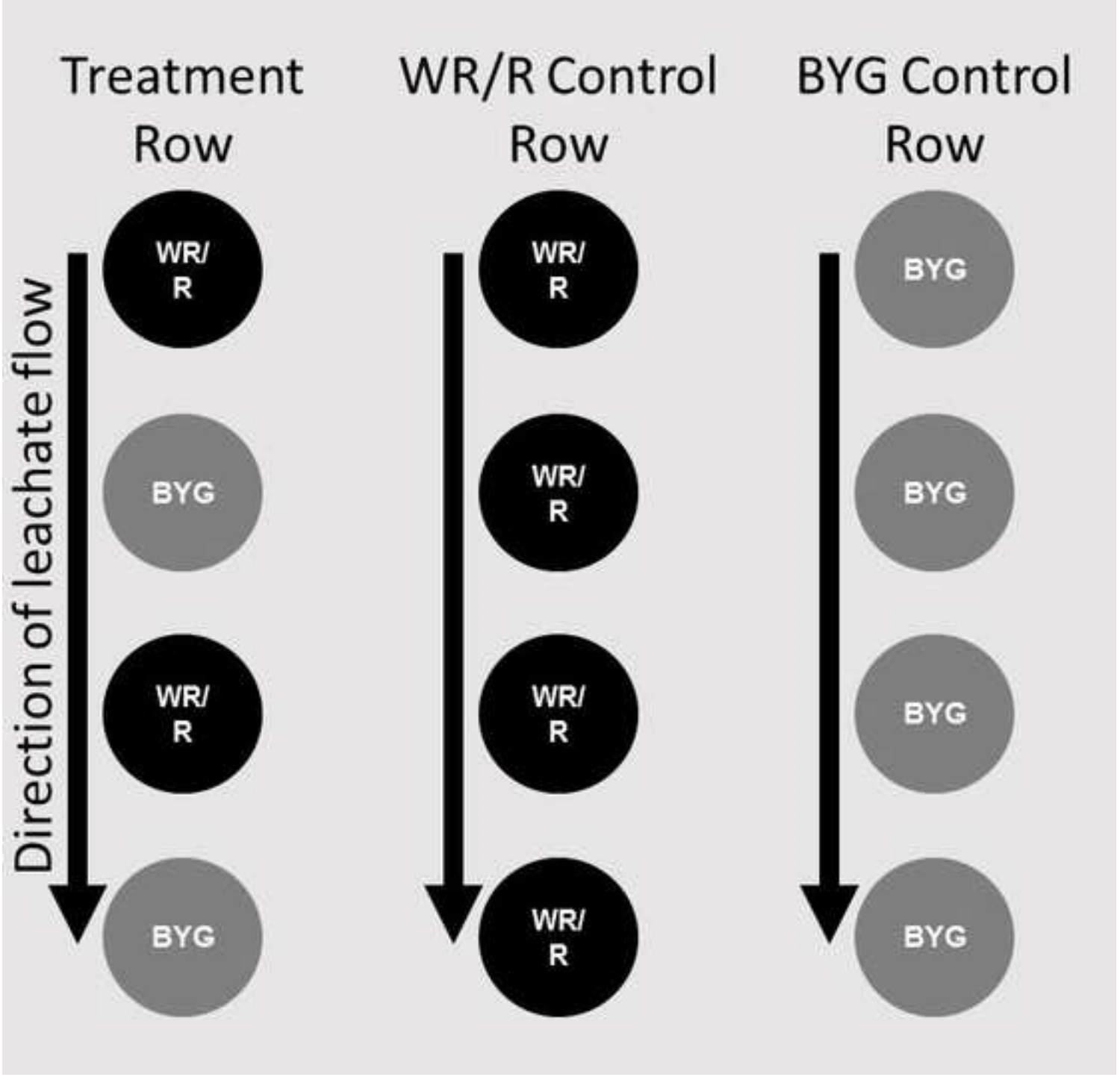

Figure 3

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Figure 4

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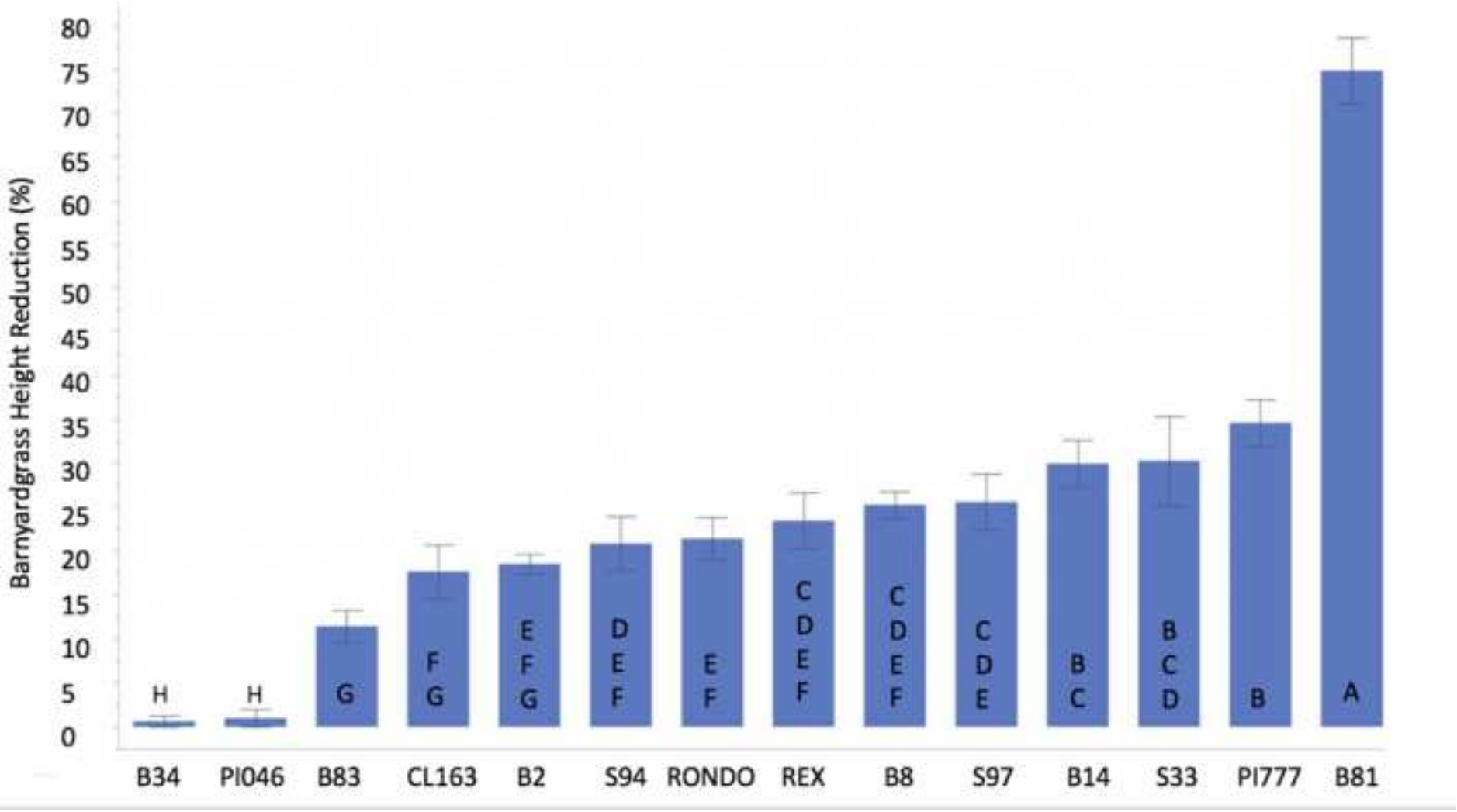

Figure 5

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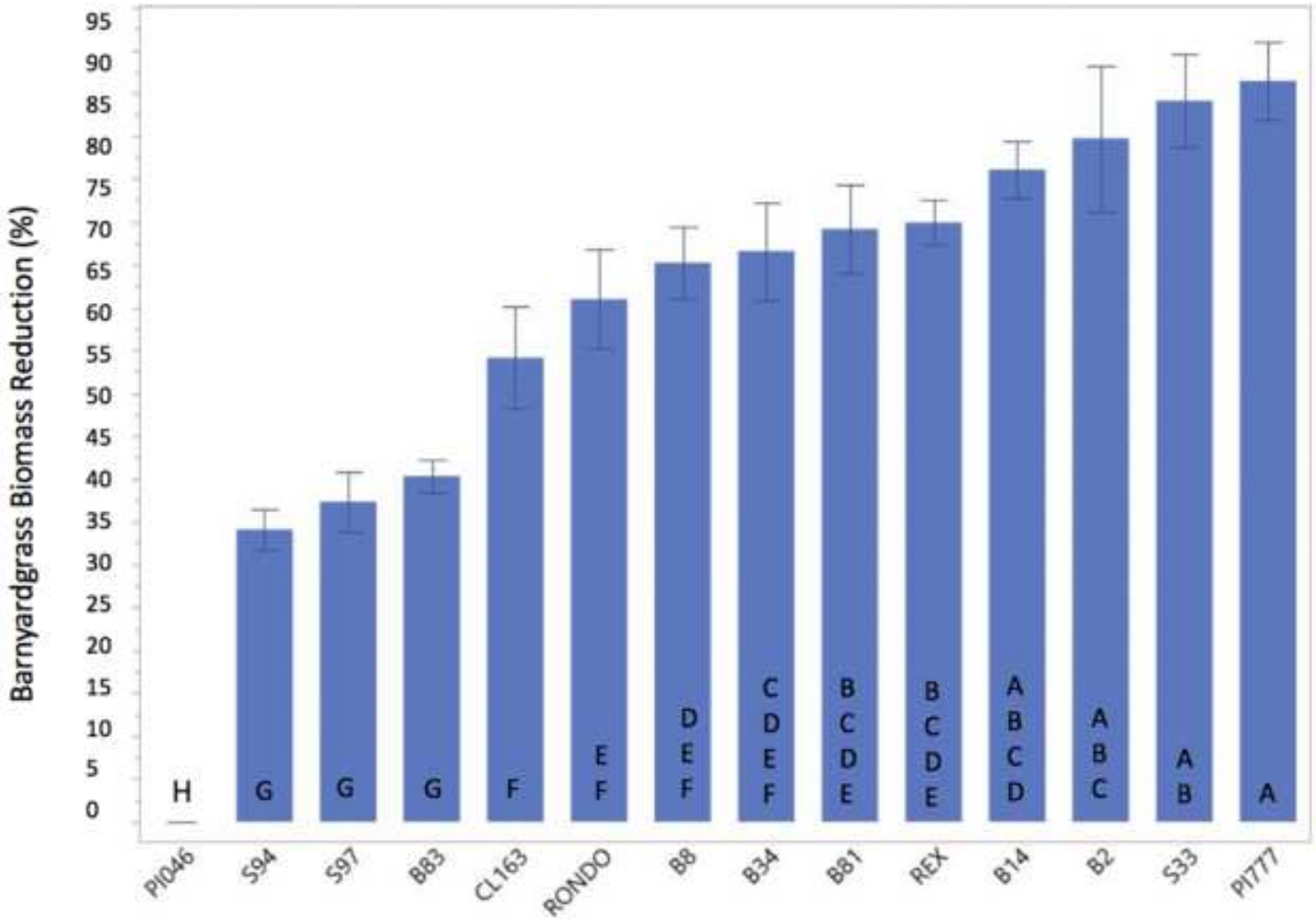

Name of Material/ Equipment	Company
1.25 in by 6 in by 8 ft standard severe weather wood board	Lowe's, Mooresville, NC
2 in by 4 in by 8 ft white wood stud	Lowe's, Mooresville, NC
63 mm (2.5 in) corner braces	Lowe's, Mooresville, NC
Asporto 16 oz Round Black Plastic To Go Box - with Clear Lid, Microwavable – 6.25 in by 6.25 in by 1.75 in - 100 count box	Restaurantware.com, Chicago, IL
ATP vinyl-flex PVC food grade plastic tubing, clear, .125 in id by .25 in od, 100 ft	Amazon, Seattle WA
Ccm-300 chlorophyll content meter	Opti-Sciences, Inc. Hudson, NH
Common 1 in by 2 in by 8 ft pine board	Lowe's, Mooresville, NC
Contractors choice contractor 24-pack 42-gallon black outdoor plastic construction trash bag	Lowe's, Mooresville, NC
EURO POTS	Greenhouse Megastore, Danville, IL
Fisher brand petri dish with clear lid	Fisher Scientific, Waltham, MA
Aexit Ac 220 V-240 V electrical equipment US plug 21 W 1000 L/hr multipurpose submersible pump	Amazon, Seattle WA
Woods 50015 WD outdoor 7 day heavy-duty digital outlet timer	Walmart, Bentonville, AR
GE silicone 2+ 10.1 oz almond silicone caulk	Lowe's, Mooresville, NC
Great Value Distilled Water	Walmart, Bentonville, AR
Great Value White Basket coffee filters 200 count	Walmart, Bentonville, AR
Grip-rite primgaurd plus #9-3 in pollimerdex screws	Lowe's, Mooresville, NC
Hoagland's No. 2 basal salt mixture	aisson Laboratories, INC. Smithfield, U
JMP (14)	SAS Institute Inc. North Carolina State University, NC
Project source flat black spray paint	Lowe's, Mooresville, NC
Project source utility 1.88 in by 165 ft gray duct tape	Lowe's, Mooresville, NC
Rubbermaid 2 qt square food storage canister clear	Walmart, Bentonville, AR

Sealproof unreinforced PVC clear vinyl tubing, food-grade .5 in id by .625 in od, 100 ft	Amazon, Seattle WA
Short Mountain Silica 50 lb Play sand	Lowe's, Mooresville, NC
Steve Spangler's 1 Liter Soda Bottles - 6 Pack - For Science Experiment Use	Amazon, Seattle WA

Catalog Number	Comments/Description
489248	N/A
6005	Cut into appropriate sizes
809449	N/A
RWP0191B	black
B00E6BCV0G	N/A
ccm/300	N/A
1408	N/A
224272	Cut to cover collection tanks
CN-EU	15 cm short black 6 in diameter 4.25 in height 1.37qt volume
FB0857513	N/A
B07MBMYQNT	Nozzle size should fit tubes and can be repaced
565179767	20 settings
48394	Sealant for edges of any attached tubing
565209428	N/A
562723371	Size may vary
323974	N/A
HOP01/50LT	½ strength rate
	N/A
282254	N/A
488070	N/A
555115144	Collection tank discard lid

B07D9CLGV3	Connects to pump
10392	Sand should be purified
UPC 192407667341	Top step tank discard lid

Response to reviewer and editor comments

Editor comments:

All edits associated with the comments are visible in the manuscript with tracked changes on.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Checked
2. Please provide at least 6 keywords or phrases. Added
3. Please reword lines 89-94 as it matches with the previously published literature. Section revised
4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." The protocol has been reviewed and edited accordingly
5. Please ensure you answer the "how" question, i.e., how is the step performed? The protocol has been reviewed and edited accordingly
6. 3.4: What are the pregerminated donor plant seedlings in your experiment? How constantly do you water the seedlings - once in a day? Details added
7. 3.7: What are receiver plants in your experiment? Added
8. 3:10: Please include what are respective positions? 3:10 was removed to avoid confusion as the next section (4) describes in detail the positioning and placement
9. 6: Please include how do you perform data collection steps – how is the height measurement performed, how do you measure and record chlorophyll, etc. Details added
10. 7.3: Please include citation. Added
11. 7.4: We cannot have commercial language in the manuscript. Please use generic terms instead. Please move the commercial term to the table of materials. Please include a click by click instruction of how this is done. Added program to materials table. Instructions for analysis in JMP added
12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Steps have been highlighted in gray; Steps 2, 4, 5, 6, and 7.
13. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of a .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text. Table removed, units revised, and description added
14. Please include some statistical analysis and error bars for the presented results. Added

1. Chopra, N, G Tewari, LM Tewari, B Upreti, N Pandey (2017) Allelopathic Effect of Echinochloa colona L. and Cyperus iria L. Weed Extracts on the Seed Germination and Seedling Growth of Rice and Soybean. Advances in Agriculture 2017:1–5

Response to reviewer and editor comments

Reviewers' comments:
Reviewer #1:

Manuscript Summary:
The paper provides details on how to set up an experiment to estimate whether plants are producing root-exuded phytotoxins that may play a role in allelopathy. The experiment that they describe it not a new idea, but the details provided should be useful in setting such an experiment up.

Major Concerns:
The major issue that I have is that the data provided are not statistically analyzed. This is a requirement of most all journals. Data were analyzed in JMP. Statistical information has been added to the results as more data was recently acquired. New graphs have been presented and results have been revised accordingly.
Something else to consider is that root exudates from one plant species can induce production of allelochemicals by a different plant species. So, the allelochemical production of both species in the first treatment row of Fig. 2 will likely be higher than of the plants in the other rows. This will make interpretation of results problematic unless chemical analyses of likely allelochemicals are done. The design or layout of the experiment has been planned according to previous research. We agree with the statement that there may be additional compound concentrations in the first row with the two species; however, the compounds produced are usually different for each species (based on literature). Some weed extracts such as *E. colona* have been shown to have germination effects on rice depending on plant density[1]. We conducted this experiment with this in mind, as the plant density of the target (*E. crus-galli*) was kept low to avoid high accumulation of these allelochemicals. The barnyardgrass control row has been added in this experiment as a way to check for any effect of the barnyardgrass on the rice in the system. The effect of the barnyardgrass on rice (if any) were noted, and no significant reduction in weedy rice or rice was found. Future research using a modified stair step structure could be conducted to avoid interacting allelochemicals from different species.

Minor Concerns:
Lines:
38 – possesses Revised
41 - ..thus reducing potential yield losses due… Revised
49 - Change precise to quantitative. I do not think that there is any precise method of determining allelopathic potential. Revised
57 - change crop to plant Revised
71 - ref. 5 does not seem to be a good primary reference for this statement Added reference
73 - change have to has Revised
76 - This is a very poor reference for this statement. New reference added
77 - ..and sometimes pose… Revised
82 - Delete The Revised
82-83 - This is not always true. The sentence has been revised, and citations added.
115-117 - Unclear. Rewrite. Revised
336- From where were these accessions obtained? What about the cultivated rice lines? Details

1. Chopra, N, G Tewari, LM Tewari, B Upreti, N Pandey (2017) Allelopathic Effect of Echinochloa colona L. and Cyperus iria L. Weed Extracts on the Seed Germination and Seedling Growth of Rice and Soybean. Advances in Agriculture 2017:1–5

provided
425-435 - With perennials, allelochemicals can be leached from dead litter of shoot tissues.
Additional reference was added to this section
450 -Bertin, not Betin Revised
478 - IRRI, not Irri Revised
483 - Table 1? Mistake removed

1. Chopra, N, G Tewari, LM Tewari, B Upreti, N Pandey (2017) Allelopathic Effect of Echinochloa colona L. and Cyperus iria L. Weed Extracts on the Seed Germination and Seedling Growth of Rice and Soybean. Advances in Agriculture 2017:1–5

Response to reviewer and editor comments

Reviewer #2:

Minor Concerns:

Stand Construction
1) please convert the units of measurement from ft to meters (if it was requested by the journal guidelines please ignore my suggestion); Units converted to metric
2) Is there any specific reason concerning the dimensions and differences in level between the benches (Eg. to allow water movements by gravity etc.) if yes please specify it in a note at the end of the paragraph. Notes added

Planting
1) Please specify or suggest the kind of soil you have used for the experiments (Eg. classic potting soil, silver sand, quartz sand etc.) giving information concerning the physicochemical properties of the material used Specifications added
2) Authors says "Transplant 6 pregerminated donor plant seedlings into sand evenly spaced": do you pregerminated the seeds in petri dishes? Have you done any pre-treatment on seed to synchronize germination and to break dormancy? Do you use a sterilization protocol? Please give all the necessary information. Steps to protocol added

Please add error bars and statistical analysis to your data. Data updated

1. Chopra, N, G Tewari, LM Tewari, B Upreti, N Pandey (2017) Allelopathic Effect of Echinochloa colona L. and Cyperus iria L. Weed Extracts on the Seed Germination and Seedling Growth of Rice and Soybean. Advances in Agriculture 2017:1–5

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Author(s):	Schumaker B. C., Stallworth S., De Castro E., Shrestha S., and Tseng T. M.

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