**EDITORIAL COMMENTS:**

1.The manuscript would benefit from copyediting by a native English speaker to avoid typos as well as the errors discussed below:

-Everywhere it says “inferring on the” should just be “inferring the” Done.

-Several mentions of how to avoid “uncontrollable mistakes.”

Typically if you can avoid them, then they are controlled.

“uncontrollable” has been changed to “unverifiable”.

-Check the grammatical tense in 6.1.3. The sentence has been modified.

2. 4.1: should be a note, not a step

Step 2.4.1 does not exist. Perhaps the reviewer means 2.3.1, the sentence has been modified.

3. Please include chemicals and materials in an attached table. For instance, the nozzles (TeeJet, XR11002-VK) should be in the table, not the main text. Also, the surfactant, herbicides, bench sprayer, soils, etc., should be listed in the table.

The Material/equipment table has been updated.

4. There is unnecessary branding in steps 5.3.6, 5.3.7 ("TeeJet"). Done.

5. References: Formatting of journal titles is inconsistent

Journal titles formatting has been changed according to ISI Journal Title Abbreviations.

6. Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

7. Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

8. If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

All figures are original and not previously published.

IMP\* JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information.

Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

All available DOIs are already there.

**REVIEWERS' COMMENTS:**

**REVIEWER #1:**

Manuscript Summary:

This ms is well written, and gives a robust description in how to proceed with the characterization of resistance.

Major Concerns:

N/A

Minor Concerns:

Please consider the points given below to have a better ms.

Additional Comments to Authors:

L41. Comma at the end is right? Addressed.

L54. "Other critical steps of the protocol are also evaluated." Please be more specific: i.e. Other critical steps such the volume of application, spray speed are also evaluated.

The sentence has been modified.

L82. Please consider delete species, a resistant biotype will belong to a species in most cases.

Species has been deleted.

L110. Perhaps the authors would make a comment about the natural tolerance of some species to certain herbicides, such as leguminous species or some members of the Convolvulacea family.

A sentence has been added.

L191. Author of botanical name was given (L.), so please be consistent throughout ms, add author to all botanical names or delete it from all of them. In first time appearance use the full botanical name then the abbreviation of genus can be avoided.

It sounds like a contradiction. However, when the species was mentioned for the first time, the botanical name was reported and then the genus was abbreviated throughout the manuscript.

L204. Please correct sentence, by using soil the fungal infections are limited? Do the authors mean to agar use.?

The sentence has been modified.

L206. S. halepense see comments above. Addressed.

L216. See previous comment (S. halepense). Correct through ms. Addressed.

L227. 15% perlite Addressed.

L231. What does pop. mean? Correct expression. It means population. The sentence has been corrected.

L253. Correct signs. Addressed.

L276. BBCH growth scale. Modified.

L300. by using the follow… Modified with “the following equation.”

L303. switch Vfin and Dosemax to have the same order as formula. Addressed.

L316. Delivering 300 L would not be suitable for all herbicides, pre-emergence herbicides could need more spray volume per ha.

The vast majority of herbicides require 300 L ha-1 both for pre- and post-emergence treatments. According to the labels, all herbicides used require this volume. Only glyphosate is delivered at different volume (200 L ha-1 ) .

L367. the 1x dose of florasulam Addressed.

L379. bispyribac-Na Addressed.

L438. Do the authors mean standard deviation instead of standard error?

No, we calculated the standard errors. We used this because it does not require any information on the data distribution.

L459. Same as above. See above comment.

L503. Check dots at the end of sentence. Addressed.

References, I have noticed that journal names are not consistent through this section. i.e. see 12 (Weed Research) vs 18 (Weed Res.) both are from weed research but are not cited consistently. Please correct all other journal names in this section. All the references have been checked and the journal names shortened as required.

L558. Be consistent with the et al, the next reference has the same number of authors as this one and is not with et al.

The reviewer is right. All reference with more than six authors were consistently changed.

L573. Check this reference as commented above. Addressed.

Fig. 3. Bispyribac-Na The figure has been corrected.

L441. Would be helpful if authors add time between treatment and capture of photo. Addressed.

Fig.4. How can the authors explain this enormous std deviation between exp? Seems to be there is a time, growth stage, application effect in that population.

A population is an heterogeneous entity. That’s why we often observed variability between the replicates.

**REVIEWER #2:**

\_Manuscript Summary:\_

There are many areas where further clarification and revision is necessary, while some statements need additional explanation and more reference to the literature. Strengthening of conclusions' section is also required, while several grey points remain regarding the dormancy breaking procedures, the weed species which are discussed, the unclear picture about populations and biotypes etc

The manuscript does not have a conclusions’ section. We believe that this is the right layout considering that the article deals with methods.

In addition to the previous remarks, several specific comments are shown below and should be addressed one by one, in order to optimize the present manuscript:

Abstract: field herbicide treatments: express it in other way .

The sentence has been slightly modified.

Abstract: the right growth stage: the suitable or the ideal Abstract closes very bad, needs revision highlight on novelty (in the long version presented later more information is given)

The long abstract has been rewritten according to the suggestion.

L.65: Herbicides are the most extensively used weed control measure:

English revision necessary

This sentence as all the manuscript has been revised for English.

L.75: generally instead of more generally Addressed.

L.78: how is invasion here? Avoid its use (better introduction or establishment)

The sentence has been modified.

L.81: However, when confirmation of the resistance status of new weed species or biotypes is required

Addressed.

L.95: above biotypes, here populations: decide and remain constant

The two terms have different meanings. There can be different biotypes within a weed species according to the number and type of herbicide groups that affect each biotype, and there can be many populations of the same biotype.

L.101: avoid "halving the costs". Authors can use "reduce" Addressed.

L.112-113: Collect mature seeds before crop harvesting from plants that survived herbicide treatment: is this procedure representative for the weed population to be characterized as resistant or not? What about if 95% of weeds had already been killed??

The question is not relevant. The aim is to test whether the plants that survived the treatment are resistant or not. The density of resistant plants is important from the management point of view and it is information that we try to get from the farmers/advisors.

L.137-138: What about storage until the first resistance test? Some weed seeds may lose their germinability even during a very small interval.

Or the seeds of certain species may germinate only after a few months storage (e.g. red rice, *Amaranthus* spp.). It is known that seeds may undergo changes in their germination responses during storage. It is recommended to store dry seeds at low temperature (4 °C) in order to slow the rate of physiological changes. We do use different tricks for different species.

L.154: Is Schoenoplectus mucronatus a typical, widely-known and problematic weed species with serious resistance problem? If no, why is here?

Yes, *Schoenoplectus* spp. is a group of species infesting rice which is often and extensively affected by resistance to ALS inhibitors and other herbicides in Europe as well as North and South America (see the global herbicide resistance database: http://www.weedscience.org/summary/Species.aspx).

We showed it as an example of seed physiological dormancy and the experimental set up for rice weeds differs from the others.

L.169-170: 20 min for Echinochloa seeds with concentrated sulfuric acid seems very high, probably embryo damage will also come

Numerous germination tests at different acid concentrations and for different times were performed (unpublished data) and the protocol presented here is the one that allows the higher germination % to be obtained. No signs of embryo damage were detected while the % of germinated seeds increased significantly.

L.202: Leave to cool before adding the seeds: use a more scientific expression

The sentence has been modified.

L.216-220: Alternating temperature may be beneficial for other weed species, too e.g. Cyperus rotundus

That’s true, *C. rotundus* requires a warm stratification to germinate. The species has been added to the sentence. However, this is a geophyte species with no reports of resistant populations, likely due to its vegetative propagation.

L.288-289: two or three times higher the recommended rate: I think WSSA and most protocols say 2x

As stated in the introduction, the inclusion of 1x and 3x doses in the tests allows to properly test for herbicide resistance as well as to infer on the resistance level. This latter information is very useful for planning more detailed experiments.

L.342-342: probably two replicates are few

For initial resistance testing it is common to use only two replicates. Plus we use a rather high number of plants per replicate in order to be able to take the intra-population variability into account. The robustness of our experiments is demonstrated by the very low number of false-positive cases we have registered in 15 years ( less than 10 in more than 3000 tested populations).

L.348: What about regrowth? How many days to wait for accurate estimation?

As stated in point 6.1, the assessment is made three or four weeks after treatment (WAT) depending on the herbicides tested. A sentence was added where it is stated that “Plants were assessed as being dead if they showed no active growth regardless of color or other appearance.”

L.371: Only 33 % of the plants of the S check was killed

This was cited as a negative example, in fact just after that it is written “The lack of efficacy on the check population does not confirm if population 10-91 is resistant to this herbicide or not. In this case it is recommended to perform the experiment again and if the results are confirmed, to change the S population.”

L.401: avoid the "no." inside the text, use "number"

Addressed.

L.414: three populations characterized as resistant

The sentence highlights that among the populations tested three different resistance patterns were observed: in the first group populations were resistant only to ALS inhibitors, in the second group populations were resistant only to ACCase inhibitors and in the third group populations were resistant to both ALS and ACCase inhibitors.

L.430: maybe "one potentially resistant" than one "suspected resistant"

We still think that “suspected” is more appropriate. However, an alternative could be “putative”.

L.435: studied instead of investigated Addressed.

L.452: Figure legend: of exp. I and II, respectively Addressed.

L.490: with the necessary modifications (instead of variants) Addressed.

L.508: in vivo or in vitro: should be italics Addressed.

L.514-521: this paragraph could be omitted, it does not offer anything Addressed.