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Protocols for robust herbicide resistance testing in different weed species

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Abstract:	Robust protocols to test putative herbicide resistant weed populations at whole plant level are essential to confirm the resistance status. The presented protocols, based on whole-plant bioassays performed in a greenhouse, can be readily adapted to a wide range of weed species and herbicides through appropriate variants. Seed samples from plants that survived a field herbicide treatment are collected and stored dry at low temperature until used. Germination methods differ according to weed species and seed dormancy type. Seedlings at similar growth stage are transplanted and maintained in the greenhouse under appropriate conditions until plants have reached the right growth stage for herbicide treatment. Accuracy is required to prepare the herbicide solution to avoid unverifiable mistakes. Other critical steps such as the application volume and spray speed are also evaluated. The advantages of this protocol, compared to others based on whole plant bioassays using one herbicide dose, are related to the higher reliability and the possibility of inferring the resistance level. Quicker and less expensive in vivo or in vitro diagnostic screening tests have been proposed (Petri dish bioassays, spectrophotometric tests), but they provide only qualitative information and their widespread use is hindered by the laborious set-up that some species may require. For routine resistance testing, the proposed whole plant bioassay can be applied at only one herbicide dose, so reducing the costs.
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Dear Editor,

We are pleased to submit the revised version of the manuscript “**Protocols for robust herbicide resistance testing in different weed species**” by Panozzo et al. for possible publication in the Journal of Visualized Experiments.

The manuscript has been thoroughly checked again by a professional English speaker.

Figure legends have been improved.

We hope the revised version meets your expectations and look forward to receiving the outcome of the reviewing process.

Yours Sincerely,

Maurizio Sattin

TITLE:

Protocols for robust herbicide resistance testing in different weed species

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KEYWORDS:

Weed science, resistant biotypes, monitoring, seed germination, weed control, herbicide efficacy, herbicide treatment, resistance level.

SHORT ABSTRACT:

A robust and flexible approach to confirm herbicide resistance in weed populations is presented. This protocol allows the herbicide resistance levels to be inferred and applied to a wide range of weed species and herbicides with minor adaptations.

LONG ABSTRACT:

Robust protocols to test putative herbicide resistant weed populations at whole plant level are essential to confirm the resistance status. The presented protocols, based on whole-plant bioassays performed in a greenhouse, can be readily adapted to a wide

range of weed species and herbicides through appropriate variants. Seed samples from plants that survived a field herbicide treatment are collected and stored dry at low temperature until used. Germination methods differ according to weed species and seed dormancy type. Seedlings at similar growth stage are transplanted and maintained in the greenhouse under appropriate conditions until plants have reached the right growth stage for herbicide treatment. Accuracy is required to prepare the herbicide solution to avoid unverifiable mistakes. Other critical steps such as the application volume and spray speed are also evaluated. The advantages of this protocol, compared to others based on whole plant bioassays using one herbicide dose, are related to the higher reliability and the possibility of inferring the resistance level. Quicker and less expensive *in vivo* or *in vitro* diagnostic screening tests have been proposed (Petri dish bioassays, spectrophotometric tests), but they provide only qualitative information and their widespread use is hindered by the laborious set-up that some species may require. For routine resistance testing, the proposed whole plant bioassay can be applied at only one herbicide dose, so reducing the costs.

INTRODUCTION:

Herbicides are the most extensively used weed control measure, accounting for up to 50% of the global plant protection market ¹. They are relatively cheap tools, avoid labor-intensive and time-consuming soil cultivation practices, and ultimately result in cost-effective, safe and profitable food production ². However, the great phenological and genetic variability present in many weed species, together with an over-reliance on herbicide use, frequently results in the selection of herbicide-resistant weed populations. The introduction of selective herbicides with a very specific metabolic target ³⁻⁵ has dramatically increased the number of resistance cases over the years. To date, 240 weed species (140 dicots and 100 monocots) worldwide have evolved resistance to different herbicide Sites of Action (SoA) ⁴. This is a major concern for weed management and more in general for sustainable crop production.

Early detection of resistance, based on reliable tests, frequently performed in a greenhouse, is a key step to manage herbicide resistant weeds. Different approaches have been developed according to the aims, required level of accuracy, time and resources available, as well as the weed species considered ⁶⁻¹². However, when confirmation of the resistance status of a new weed biotype is required (i.e. a group of individuals that share several physiological characteristics, including the ability to survive one or more herbicides belonging to a particular group used at a dose that would normally control them), a robust whole plant bioassay needs to be performed in a controlled environment ^{4, 11}.

A biotype is seldom resistant to just one herbicide. Each biotype is therefore characterized by a certain resistance pattern, i.e. number and type of SoA of the herbicides it is resistant to, and by a given resistance level to each herbicide ¹³. The early and reliable determination of the pattern of cross or multiple resistance ^{5, 14} is important for field resistance management.

It is worth mentioning that herbicide resistance has nothing to do with the natural tolerance that some weed species exhibit towards some herbicides, e.g. dicot species vs. ACCase-inhibiting herbicides, monocot species vs. 2,4-D, *Equisetum arvense* vs. glyphosate.

This paper presents a robust approach for testing putative herbicide resistant biotypes sampled in fields where poor control by herbicide(s) had been reported. Relevant variants to the standard protocols in relation to the weed species involved are presented. The advantages over alternative techniques/protocols based on either whole plant bioassays using only one herbicide dose ¹⁵, or treating seeds in Petri dishes ⁸ are related to the higher reliability and the possibility of inferring the resistance level because of the inclusion of two herbicide doses in the experiments. However, for routine resistance testing, the same methods can be applied at only one herbicide dose, so reducing the costs.

As well as allowing confirmation of the resistance status, the information obtained can be used for both optimizing the following research steps and/or devising sound resistance management strategies.

PROTOCOL TEXT:

1. Seed sampling and storage

1.1) Monitor cultivated fields for unjustified poor herbicide performance, i.e. not due to adverse climatic conditions or low quality herbicide treatments.

1.2) Collect a seed sample from one species at a time and assign a unique code.

Mature seeds are usually collected before crop harvesting from plants that had survived the herbicide treatment(s). Timely monitoring is needed if seeds are shed by the mother plant when mature.

1.3) Fill in a form for each sample indicating the assigned unique code, name of species, collection date, GPS coordinates, municipality, farmer's name, field size, infestation level, crop, herbicide(s) used during the season and historical records of the field.

1.4) Collect seeds from at least 30 randomly selected plants that should be representative of the field infestation. The seed sample should contain at least 5,000 mature seeds. If an obligate outcrossing weed species (e.g. *Lolium* spp. or *Amaranthus* spp.) is considered, the number of plants can be reduced to 10-15, keeping the total number of collected seeds around 5,000 ¹¹.

1.5) Subsample the field when patches of weeds are scattered over large areas (more than a hectare) because different herbicide resistant biotypes could have been selected.

1.6) Store seeds in unsealed paper bags labeled with the unique code assigned.

1.7) Allow moisture to evaporate but do not expose seeds to high temperature (i.e. avoid leaving them in a car under the sun) or to extreme temperature fluctuations to avoid induction of secondary dormancy.

1.8) Clean (remove chaff, de-hull seeds, etc.) and store them at ambient temperature in a dry room. After the first resistance tests have been conducted, seeds can be stored for long periods of time in a cold room at 4 °C, preferably in vacuum sealed plastic bags. In this way seeds preserve their viability for a significantly longer time.

2. Seed dormancy breaking

NOTE: Seed dormancy provides a flexible and efficient mechanism that enables weeds to adapt and persist in agro-ecosystems. To break dormancy and allow seed germination, different protocols have to be used depending on weed species, i.e. the type of dormancy¹⁶.

There are three main ways to remove dormancy:

2.1) Vernalization

NOTE: To obtain simultaneous germination and seedling emergence, a period of seed vernalization ranging from a few days to a week is required to remove physiological dormancy from many species: e.g. *Amaranthus retroflexus*, *Chenopodium album*, *Lolium* spp., *Avena fatua*, *Polygonum persicaria*, *Phalaris paradoxa*¹⁷⁻¹⁹. A longer period of up to 15 days is required for *Papaver rhoeas*, *Cyperus difformis* and *Ammania coccinea* and up to 30 days for *Schoenoplectus mucronatus*²⁰.

2.1.1) Put some distilled water in plastic dishes. Cut two layers of filter paper and soak them in the water, remove any excess. Place the air-dried seeds on the paper. Transfer the plastic dishes to a fridge at 4 °C for the required period of time.

2.2) Scarification

NOTE: Some weed species are more recalcitrant to germination than others due to mechanical dormancy, i.e. seed coat characteristics, and necessitate the use of a chemical scarification using sulfuric acid to germinate²¹.

2.2.1) Prepare a beaker with concentrated sulfuric acid (95-98%). Prepare a beaker full of water. Put the seeds in an envelope of non-woven fabric.

2.2.2) Dunk e.g. *Echinochloa* spp. or *Sorghum halepense* seeds for 20 min or 5 min, respectively, in concentrated sulfuric acid.

2.2.3) Take the envelope out of the beaker using a pair of tweezers and put it in the beaker full of water. Open the envelope, put the seeds in a small colander and rinse them thoroughly under running water.

Variant: *A. plantago-aquatica* needs a different protocol^{22, 23}:

2.2.4) Soak the seeds for 2 min in chloroform. Rinse the seeds with deionized water and dry them with absorbent paper. Dunk the seeds in 80% sulfuric acid for 5 min.

2.2.5) Put the seeds in a small colander and rinse them thoroughly under running water.

2.3) Post-harvest seed maturation

NOTE: Seeds of other weed species do not germinate at all for a few months after maturity, regardless of the method used to break dormancy.

2.3.1) Store the seeds for a period of at least 3-4 months at room temperature and low humidity and then follow the above protocols for dormancy breaking (e.g. *Oryza sativa* var. *sylvatica* or *P. rhoeas*).

3. Seed germination

3.1) Place seeds to be germinated in plastic dishes containing 0.6% (m/v) agar with 0.1% potassium nitrate (KNO_3) added:

3.1.1) Prepare a solution of agar at 0.6% + 0.1% KNO_3 using deionized water. Dissolve the agar in a microwave oven.

3.1.2) Pour the agar solution into plastic dishes. Cool the substrate and then put in the seeds.

NOTE: Variant: Soil can also be used as a substrate in plastic dishes. This practice is particularly efficient for *Echinochloa* spp., *S. halepense* and *Lolium* spp.

3.2) Place plastic dishes in a germination cabinet for about a week with light and temperature conditions depending on the optimum conditions for each weed species. For most winter species, the temperature range is 15/25 °C night/day and 12 h photoperiod with neon tubes providing a Photosynthetic Photon Flux Density (PPFD) of 15-30 $\mu\text{mol m}^{-2} \text{s}^{-1}$. For many summer species, the temperature range is 15/30 °C night/day.

NOTE: Variant: Some species, such as *S. halepense*, need a heat treatment. Therefore after the scarification, seeds of *S. halepense* are subjected to the following conditions: cycles of 4 h at 45 °C and 20 h at 24 °C for three days in the germination cabinet, and then three days in normal conditions.

4. Seedling transplanting and growth

4.1) Transplanting, instead of direct sowing, allows a uniform stand of plants at the same growth stage to be obtained, which is an important pre-condition to optimize the performance of the herbicide treatment.

4.1.1) Transplant fifteen to twenty seedlings into plastic trays (325 x 265 x 95 mm) filled with a standard potting mix (60% silty loam soil, 15% sand, 15% agriperlite and 10% peat – by volume).

4.1.2) Identify each tray with a barcode including all information for the unique identification: population code, herbicide being tested, replicate number and progressive tray number.

4.1.3) Place trays in a heated greenhouse and water plants as needed to maintain the substrate at or near field capacity.

NOTE: The growth temperature varies depending on the weed species. Often tests are done during autumn/winter/spring, so light is supplemented using 400 W metal-halide lamps, which provide a PPFD of about 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and a 12 h photoperiod^{24, 19}. Summer weed species with C_4 photosynthetic cycle usually require higher light intensity and therefore tests are done in late spring-summer or the supplemented light intensity is about 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with a 14 h photoperiod.

4.2) A different protocol is used for some weed species infesting paddy rice, e.g. *A. plantago-aquatica*, *S. mucronatus* and *C. diffinis*²².

4.2.1) Transplant the seedlings into polystyrene trays with 24 round cells (55 mm diameter, 64 mm deep) filled with 60% silty loam soil, 30% sand and 10% peat (by volume).

4.2.2) Set the trays in 12 cm deep plastic containers filled with water and battened down by screwed stainless steel rods to prevent them floating (Figure 1).

4.2.3) Maintain water level in the containers at 1-2 cm below the level of the soil surface and add 1.5 g of copper sulfate to each container (which contains 10-12 liters of water) to avoid the proliferation of algae.

5. Herbicide treatments

5.1) Treatments with pre-emergence herbicides:

5.1.1) After about three days in the germination cabinet as described in section 3, transplant the germinating seeds into plastic trays containing the substrate described above and cover with a layer of soil (about 1 cm). This is a critical step in order to ensure that seedlings will not emerge due to the herbicide effect rather than an excessive burial depth.

5.1.2) Take the substrate to field capacity by placing the trays, which have a few holes at the bottom, on saucers filled with water.

5.1.3) One day after transplanting, treat the trays with the pre-emergence herbicide ²⁵.

5.1.4) Keep the substrate at or near field capacity by adding water as needed both from above and below by capillarity from the saucer. This procedure favors the permanence of the herbicide at the proper depth (i.e. where the germinating seeds are) for a good treatment efficacy.

5.2) Treatments with post-emergence herbicides:

5.2.1) Spray plants when they reach the 2-3 leaf stage (i.e. growth stage 12-13 of the Extended BBCH growth scale ²⁶).

5.2.2) Starting from the day after the treatment, set the irrigation system according to the water requirements of the weed species and the season (e.g. for *Echinochloa* spp. it delivers water for 3 min 4 times per day, at regular intervals from 9 am to 9 pm). Water is distributed using an automatic sprinkler irrigation system.

NOTE: Variant: glyphosate is applied at plant stage BBCH 14–21.

5.3) Herbicide preparation and distribution.

NOTE: All herbicides (pre- and post-emergence) are applied as commercial formulations with recommended surfactants at two doses, recommended field dose (1x) and three times that (3x).

5.3.1) If needed, prepare the surfactant solution in bulk according to the label instructions; the final concentration is usually expressed as a percentage of the final volume (e.g. 0.3%) or as volume to be distributed per unit area (e.g. 1 L ha⁻¹).

5.3.2) Use the surfactant solution as solvent for the herbicide (solute) solution in order to keep the right concentration of the active ingredient. Prepare the most concentrated herbicide solution first (3x). Calculate the quantity of commercial product to be dissolved in the surfactant solution (or in deionized water if a surfactant is not necessary) using the following equation:

$$\text{Dose}_{\text{herb}} = [(\text{Dose}_{\text{field}} \times \text{Dose}_{\text{max}}) \times V_{\text{fin}}] / V_{\text{del}}$$

Where: $\text{Dose}_{\text{herb}}$ = herbicide dose (mL), $\text{Dose}_{\text{field}}$ = herbicide field dose (mL ha⁻¹), Dose_{max} = maximum dose delivered, V_{fin} = final volume of the solution (L), V_{del} = volume delivered by the bench sprayer (L ha⁻¹).

5.3.3) Dilute (2:1, v/v) the herbicide solution 3x to prepare the less concentrated one (1x). This procedure reduces the chance of making mistakes when weighing or pipetting the herbicides. Herbicide solution concentration is expressed as volume to be distributed per unit area (L ha⁻¹).

5.3.4) Start the sequence of treatment with the lower herbicide dose (1x). In this way there is no need to wash the spraying cabinet between two treatments with the same herbicide.

5.3.5) Distribute the herbicide solution using a precision bench sprayer delivering 300 L ha⁻¹ (±1%), at a pressure of 215 kPa, and a speed of 0.75 m s⁻¹, with a boom equipped with three flat-fan (extended range) hydraulic nozzles.

5.3.6) Wash the spraying cabinet twice when the herbicide is changed using bleach 1% (v/v) and then rinse.

NOTE: Particular attention has to be paid when highly biological herbicides, such the sulfonylureas sulfometuron or flazasulfuron, are used. In the latter case do one washing with a bleach solution and another with ammonia (2.5% v/v), followed by a careful rinse with water. Variant: glyphosate is applied with a spray volume of 200 L ha⁻¹ ²⁷.

6. Collection and analysis of the data

6.1) Through a barcode reader, which automatically identifies each tray, record the number of plants that survived the treatment as well as the Visual Estimated Biomass (VEB). Plants are assessed as being dead if they show no active growth regardless of color or other appearance.

6.1.1) Make the assessment three or four weeks after treatment (WAT) depending on the herbicides tested (e.g. three WAT for ACCase inhibitors and four WAT for ALS inhibitors or glyphosate).

6.1.2) Evaluate the general treatment efficacy by including a susceptible population (check S) in all experiments, i.e. a population collected in a site which was never or seldom treated with herbicides.

6.1.3) Express plant survival as percentage of the number of treated plants, counted just before the herbicide treatment, and calculate the standard error (S.E.) per mean value (mean value of the two replicates).

6.1.4) The VEB is obtained through a visual comparison of plant biomass between treated and not-treated check of the same population^{25, 28}. A score, ranging from 10 for plants not affected by the herbicide (compared with the not-treated check) to 0 when the plants are clearly dead, is given to each treated tray.

6.2) Ascribe populations to four categories based on the results obtained from treatments with two herbicide doses: S when less than 5% of plants survived the herbicide dose 1x, SR when survivors ranged from 5% to 20% at herbicide dose 1x, R when more than 20% of plants survived the herbicide dose 1x and RR when survivors are more than 20% at herbicide dose 1x and more than 10% at herbicide dose 3x¹⁷.

REPRESENTATIVE RESULTS:

To assess the resistance status of a putative resistant population, it is fundamental to include a susceptible check in the assay in order to verify the herbicide efficacy. The results of a screening test conducted on *P. rhoeas* populations, a weed infesting wheat fields, are reported in **Figure 2**, where the efficacy of four post-emergence herbicides on a susceptible check (09-36) and on the suspected resistant one (10-91) are presented. Population 09-36 was completely controlled by the ALS inhibitor iodosulfuron while only one plant survived dose 1x of the other two ALS herbicides tested, florasulam and tribenuron-methyl (**Figure 2**). Instead, around 60% of the plants of population 10-91 survived both herbicide doses of iodosulfuron and tribenuron-methyl and around 50% survived the 1x dose of florasulam. These results confirm that population 10-91 is highly resistant (RR) to iodosulfuron and tribenuron-methyl and resistant (R) to florasulam. A different response was observed with 2,4-D, a herbicide having a different SoA (i.e. it mimics endogenous auxin), widely used to control dicot weeds in wheat. Only 33% of plants of the S check were killed with this herbicide at dose 1x and the VEB value was > 20% (**Figure 2**). 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. An example of good control of the susceptible check is reported in **Figure 3**. The *Echinochloa* spp. population 07-16L was completely controlled by all herbicides at the recommended field dose (1x). In this case, it is possible to state that population 08-42 is highly cross-resistant to all ALS inhibitors tested, i.e. azimsulfuron, bispyribac-Na, imazamox and penoxsulam. The not-treated check of both populations is reported on the left. These plants are used to calculate the VEB; the amount of biomass is visually estimated tray by tray giving a score of 10 to the not-treated check and then assigning a score from 0, for the replicate without any green plant tissue, to 10 when the biomass is comparable to the not-treated check (**Figure 3**).

Another example of output is reported in **Figure 4**, where plant survival of *Lolium* spp. to glyphosate is shown. The populations tested were collected in wheat-based cropping systems where glyphosate is exerting different selection pressure (i.e. occasionally or 1-2 treatments per year or 3-more times per year). Plants were sprayed at early tillering stage (BBCH 14-21) using two doses of glyphosate: 480 and 1440 g a.e. ha⁻¹, which represent the minimum and maximum recommended field dose for annual weeds (i.e. therophytes) at that growth stage. Data were collected four weeks after treatment. Based on both experiments, seven of the tested populations had a survival rate of 80% or more (populations 343, 383, 384, 403, 509, 512 and 537) at the lowest dose applied but only 50% of plants of populations 403 and 509 survived the highest glyphosate dose. One population had a survival rate of around

40% at 1x dose, whereas just a few plants of population 509 survived the lowest dose and population 508 was fully controlled at any dose. In summary, these experiments showed different levels of resistance to glyphosate that often reflected the field history of herbicide usage. The level of glyphosate resistance was higher for the populations that had been more intensely treated: i.e. the number of field applications per year and number of years of selection pressure was higher.

The protocol described for one herbicide (**Figure 4**) can be applied to numerous others having different SoA; in this way the resistance pattern of one or more populations can be determined. An example of resistance pattern variability of *Echinochloa* spp. populations is reported in **Table 1**. Historical records of herbicide use and crop management obtained from the farmer indicated that ALS-inhibiting herbicides were the selecting agent (i.e. penoxsulam or imazamox). The resistance test was therefore performed with three ALS-inhibiting herbicides (azimsulfuron, penoxsulam and imazamox) belonging to different chemical families, and one herbicide having another SoA, the ACCase-inhibiting herbicide profoxydim. The susceptible check (07-16L) was completely controlled by all herbicides tested (**Table 1**). Three resistance patterns were detected: thirteen populations resulted as being resistant only to ALS inhibitors, four populations resulted as being resistant only to the ACCase inhibitor profoxydim, and three populations showed a multiple resistance pattern to both the ACCase inhibitor profoxydim and ALS inhibitors. Within each resistance pattern it is possible to distinguish different biotypes, e.g. four populations resistant to ALS inhibitors survived only treatments with the sulfonylurea azimsulfuron while two of the multi-resistant populations survived only treatment with the ALS inhibitor azimsulfuron but were quite controlled by penoxsulam and imazamox.

TABLES AND FIGURES:

Figure 1: Example of *C. difformis*, a weed species infesting paddy rice, experiment set-up.

Polystyrene trays are put into plastic containers and blocked by screwed stainless steel rods to prevent them floating. Water is maintained at 1-2 cm below the level of the soil surface to mimic paddy rice conditions. Photograph was taken four weeks after the treatment.

Figure 2: Response of two *P. rhoeas* populations to post-emergence herbicides.

Effect of iodosulfuron, tribenuron, florasulam and 2,4-D tested at the recommended field dose (1x) and at three times that (3x) on plant survival (blue bars) and visual estimation biomass - VEB - (red bars) of the susceptible check (09-36) and of a resistant population (10-91). The assessment was done four weeks after the herbicide treatment. Plant survival and VEB are expressed as percentage of the number of treated plants and the VEB of the not-treated checks (%). The horizontal line at 20% of plant survival represents the discriminating threshold between resistant and susceptible populations when plants are treated at dose 1x. Vertical bars represent standard errors calculated on the mean value of the two replicates.

Figure 3: Visual results of a screening on two *Echinochloa* spp. populations.

The susceptible check, 07-16L (S), and the resistant population, 08-42, were tested with four ALS inhibitors (reported on the right) at two doses, 1x and 3x, (reported at the bottom). For the S check only results of dose 1x are reported, because all plants were already controlled at that dose. Three examples of VEB score are reported in red: 0 = all plants dead, 10 = all plants survived and biomass is comparable to the not-

treated (NT) check (reported on the left), 5 = biomass is about half of that in a tray of the not-treated check.

Figure 4: Percentage of plant survival for ten *Lolium* spp. populations tested with glyphosate.

Plant survival recorded in two experiments (blue bars and orange bars for exp. I and II, respectively). Data are expressed as a percentage (%) of the number of treated plants. Two susceptible checks were fully controlled at dose 1x and are therefore not reported in the graph. Two doses were tested, the minimum ($1x = 480 \text{ g a.e. ha}^{-1}$) and maximum ($3x = 1440 \text{ g a.e. ha}^{-1}$) doses reported on the product label. The horizontal line at 20% of plant survival represents the discriminating threshold between resistant and susceptible populations when plants were treated at dose 1x. Vertical bars represent standard errors calculated on the mean value of the two replicates.

Table 1: Resistance status of twenty one populations of *Echinochloa* spp.

Susceptible check (07-16L) is reported in bold. Resistance levels are reported for each of the four herbicides tested (one ACCase inhibitor, proflumetsulfuron, and three ALS inhibitors, azimsulfuron, penoxsulam and imazamox) according to four categories: S = less than 5% of plants survived the herbicide dose 1x, SR = plant survival ranged from 5% to 20% at herbicide dose 1x, R = more than 20% of plants survived the herbicide dose 1x, RR = plant survival was more than 20% at herbicide dose 1x and more than 10% at dose 3x. Different resistance patterns are highlighted: red = resistance only to ACCase inhibitor, yellow = resistance only to ALS inhibitor(s), orange = resistance to the ACCase inhibitor and to at least one ALS inhibitor.

DISCUSSION:

Several steps within the protocols are critical for a successful assessment of herbicide resistance in a population: 1) seeds should be collected when mature from plants that had survived the herbicide treatment(s). Maturation of the seeds on the mother plant is crucial to avoid difficulties in seed germination later; 2) the proper storage of seeds is recommended to avoid proliferation of molds that would prevent germination; 3) seedlings should be treated at the right growth stage, as reported on the label of the herbicide package. Care must be taken so that plants to be treated have reached approximately the same growth stage; 4) the herbicide solutions should be prepared and handled with accuracy so that plants are sprayed with the correct concentration of active ingredient therefore avoiding unverifiable mistakes; 5) after each herbicide treatment it is recommended to thoroughly cleanse the spraying cabinet and glassware used to prepare the solutions to avoid contamination in the following herbicide treatment, especially when highly biologically active herbicides are involved.

The protocols presented herein can be readily adapted to a wide range of weed species with the necessary modifications according to species and herbicides of interest. In particular, methods to break seed dormancy and for germination are steps that should be reconsidered for each new weed species (see sections 2 and 3). Spraying equipment sometimes needs adjustments when different herbicides are used, e.g. glyphosate requires different settings of the spraying cabinet (see section 5.3) and plants are treated at a later growth stage than with most herbicides.

The time and space required to perform these protocols can be a limiting factor and may not be suitable for routine testing. However, to limit the costs, only one herbicide dose may be used. In this way information can still be obtained on whether the

population is resistant. A potential limitation of the approach is related to the fact that no resistant checks are included in the experiments. In fact, due to the large number of biotypes evaluated (i.e. different species and herbicides involved), many checks should be included in each experiment, so increasing the costs.

However, the advantages over alternative techniques/protocols based on whole plant bioassays using only one herbicide dose¹⁵ are related to the higher reliability and the possibility of inferring the resistance level. Quicker and less expensive diagnostic screening tests have also been devised, *in vivo* or *in vitro* (e.g. Petri dish bioassays⁸, spectrophotometric tests on herbicide target enzyme²⁹). However, they only provide qualitative information and require some preliminary work, sometimes laborious, to identify the herbicide dose for discriminating between resistant and susceptible plants. The *in vitro* assays also need to be adapted according to the active ingredient used.

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DISCLOSURES:

The authors declare that they have no competing financial interests.

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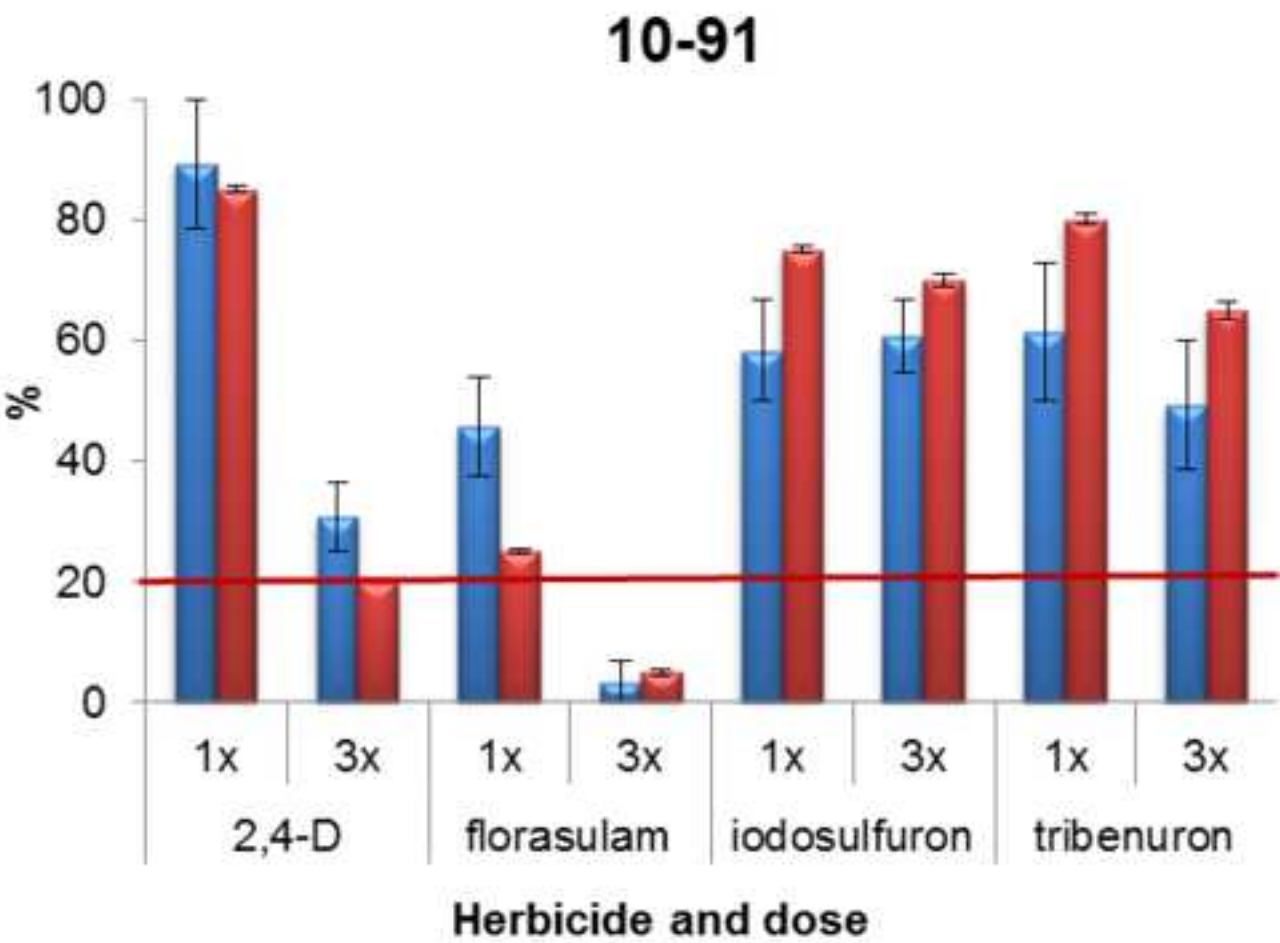
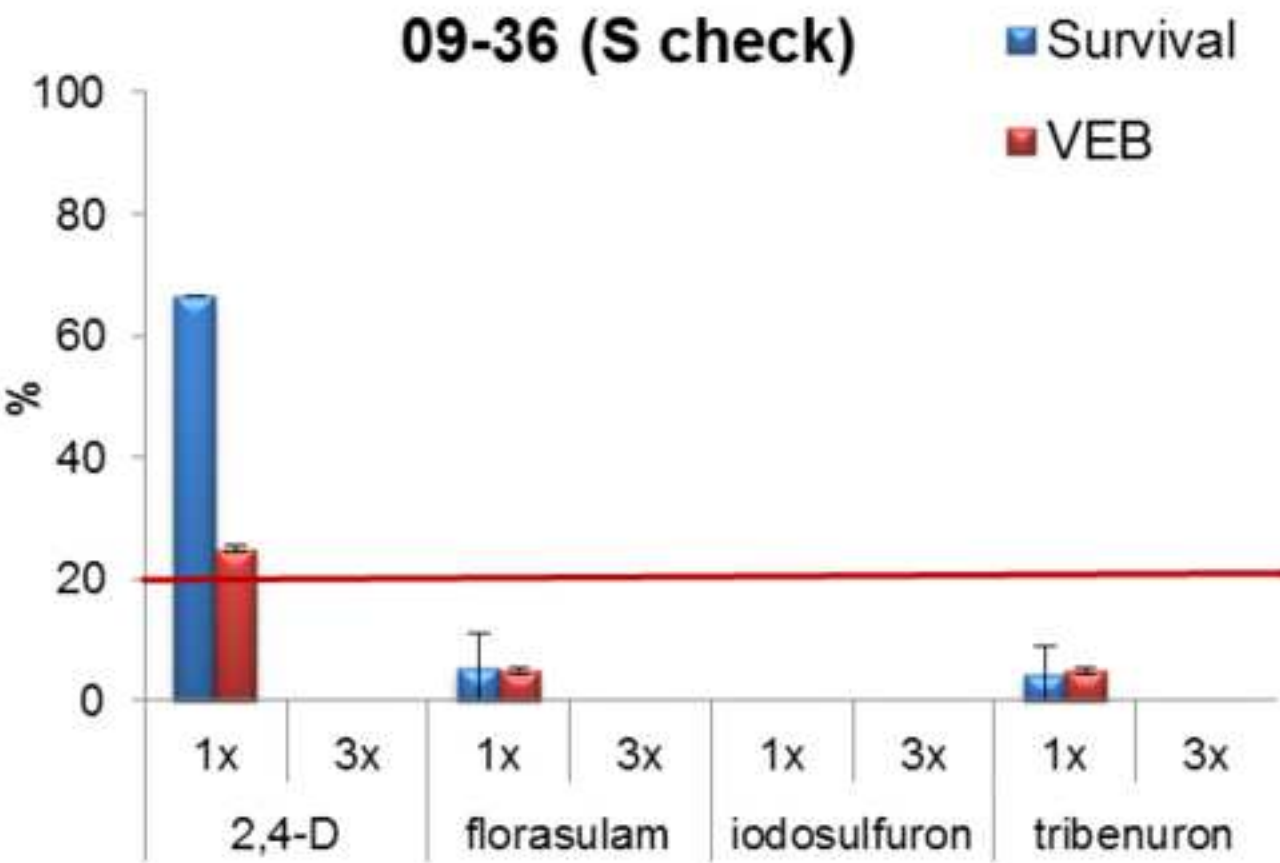


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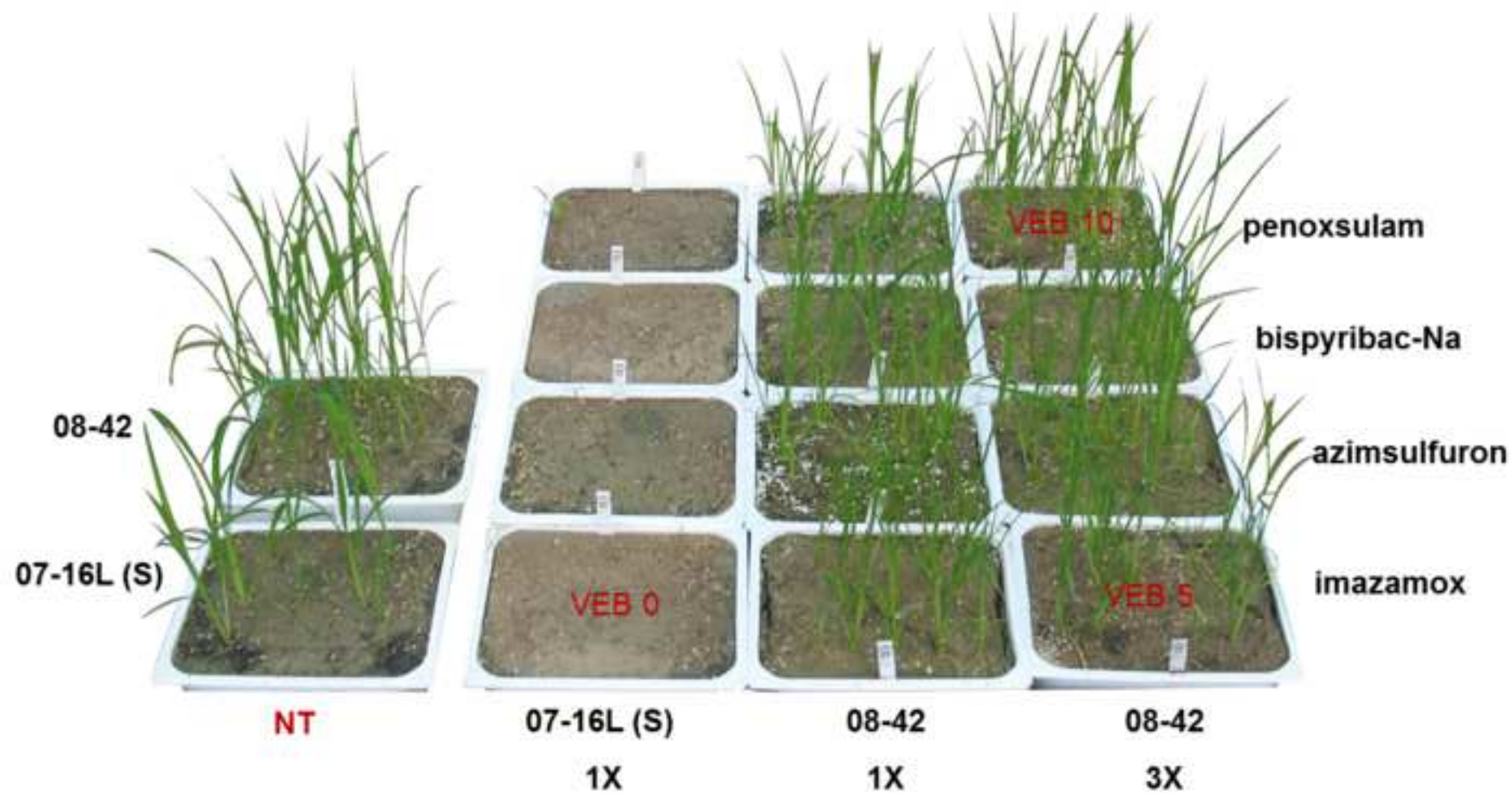
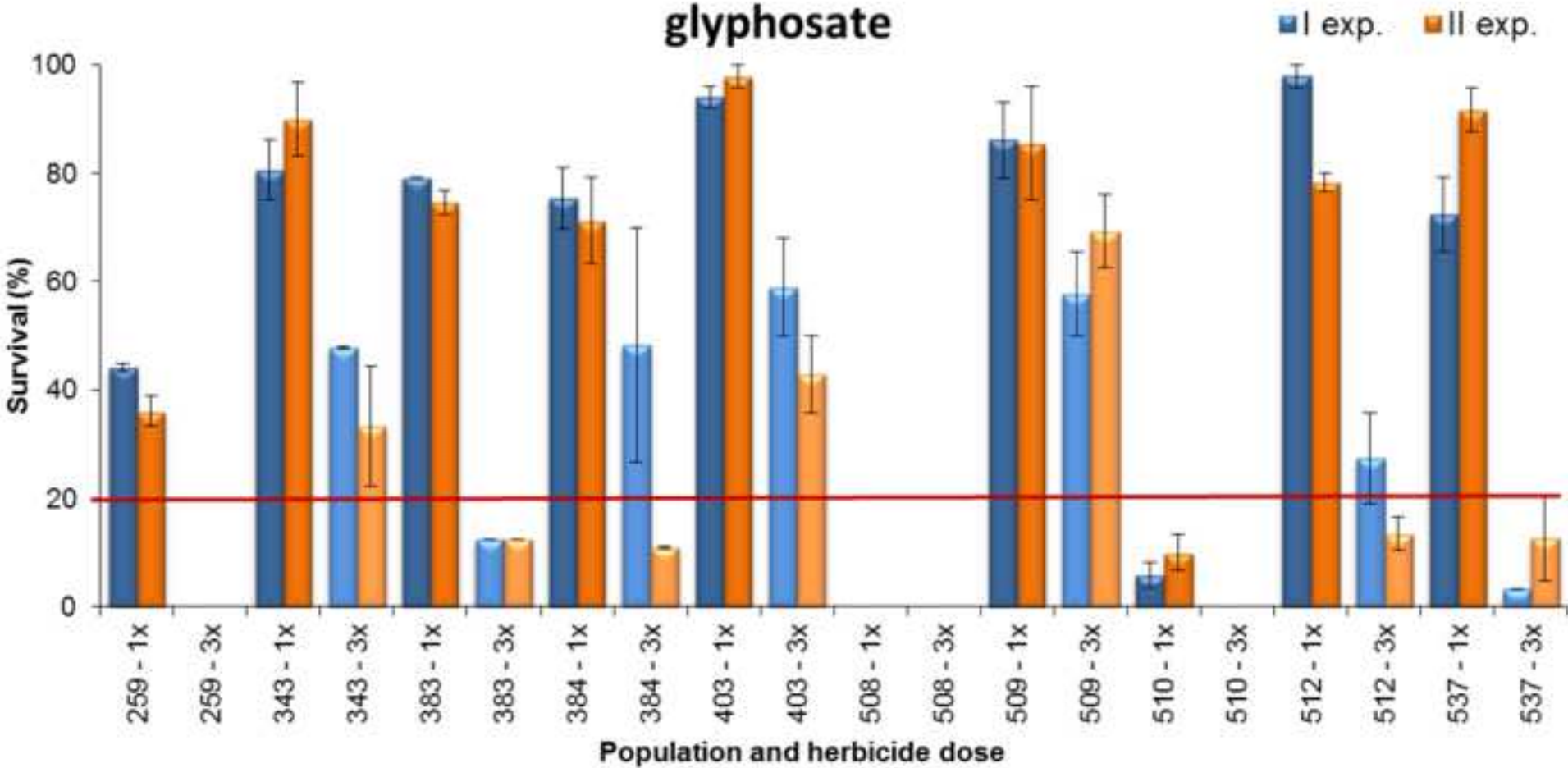


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Species	Population code	profoxydim	azimsulfuron	penoxsulam	imazamox
ECH_CG	07-16L	S	S	S	S
ECH_SP	13-72	SR	R	R	S
ECH_CG	13-125	R	S	S	S
ECH_SP	13-127	S	RR	RR	RR
ECH_SP	13-129	S	SR	R	SR
ECH_SP	13-130	S	RR	RR	RR
ECH_SP	13-132a	SR	RR	R	R
ECH_SP	13-135	SR	RR	RR	RR
ECH_SP	13-136	R	RR	RR	RR
ECH_SP	13-140	R	S	S	S
ECH_SP	13-141	SR	R	SR	S
ECH_SP	13-142	SR	R	SR	SR
ECH_SP	13-143	R	SR	SR	SR
ECH_SP	13-147	SR	R	R	R
ECH_SP	13-148	SR	R	S	R
ECH_SP	13-149	R	R	S	SR
ECH_SP	13-150	R	SR	S	S
ECH_SP	13-151	R	R	SR	SR
ECH_SP	13-153	S	R	R	R
ECH_SP	13-154	S	R	S	S
ECH_SP	13-155	SR	S	S	SR
ECH_SP	13-156b	S	R	SR	SR

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Paper bags	Celcar SAS		
Plastic dishes	ISI plast S.p.A.	SO600	Transparent plastic
Sulfuric acid 95-98%	Sigma-Aldrich	320501	
Non-woven fabric	Carretta Tessitura	Art.TNT17	Weight 17 gr m ²
Chloroform >99.5%	Sigma-Aldrich	C2432	
Agar	Sigma-Aldrich	A1296	
Potassium nitrate >99.0%	Sigma-Aldrich	P8394	
Plastic containers	Giganplast	1875/M	600 x 400 x 110 mm
Plastic trays	Piber plast	G1210A	325 x 265 x 95 mm
Polystyrene trays	Plastisavio	S24	537x328x72 mm, 24 round cells (6x4)
Copper sulfate	Sigma-Aldrich	451657	
Agriperlite	Blu Agroingross sas	AGRI100	
Peat	Blu Agroingross sas	TORBA250	
Germination cabinet	KW	W87R	
Nozzles	Teejet	XR11002-VK, TP11001-VH	The second type of nozzles are used only for glyphosate
Barcode generator	Toshiba TEC	SX4	
Labels with barcode	Felga	TT20200	Stick-in labels with rounded corners
Barcode reader	Cipherlab	8300-L	Portable data terminal
Bench sprayer	-	-	Built in house

HERBICIDES INCLUDED IN THE RESULTS:

Commercial product	Active ingredient	Company	Comments
Altorex	imazamox	BASF	
Azimut	florasulam	Dow AgroSciences	
Biopower		Bayer Crop Science	Surfact to be used with Hussar WG
Dash		BASF	Surfact to be used with Altorex
Granstar	tribenuron-methyl	Dupont	
Gulliver	azimsulfuron	Dupont	
Hussar WG	iodosulfuron	Bayer Crop Science	
Nominee	bispyribac-Na	Bayer Crop Science	

Roundup	glyphosate	Monsanto	
Trend		Dupont	Surfact to be used with Granstar and Gulliver
Viper	penoxsulam	Dow AgroSciences	
Weedone LV4	2,4-D	Isagro	



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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.

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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 V_{fin} 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.