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First report of a wide distribution of glyphosate-resistant compact brome (*Bromus madritensis*) in the Iberian Peninsula: confirmation and field management

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Abstract

Glyphosate resistance is spreading in Spain and Portugal due to excessive herbicide use, in both annual and perennial crops. Compact brome (*Bromus madritensis* L.) is increasing in frequency in these different cropping systems when under conservation agriculture, particularly when glyphosate fails to control it. Fourteen populations from different areas in the Iberian Peninsula were confirmed as being *B. madritensis* using simple sequence repeat markers and clearly separated from the closely related species red brome (*Bromus rubens* L.) and ripgut brome (*Bromus diandrus* Roth). Six *B. madritensis* populations were classified as resistant, according to both their shikimic acid accumulation levels and their resistance factors based on LD_{50} or GR_{50} (values between 4 and 8). Populations with higher resistance factors also showed lower shikimic acid concentrations. Moreover, these resistant populations were able to survive the minimum registered dose for glyphosate in Spain in perennial crops (1,080 g ae ha^{-1}, five populations) or in arable crops before seeding for annual weeds (540 g ae ha^{-1}, one population), under both greenhouse and field conditions. The trials carried out in a glyphosate-resistant field during 2 consecutive years showed that acceptable control (≥90%) was only consistently achieved 90 d after application for preemergence treatment with flazasulfuron in a tank mix with glyphosate, while control with postemergence treatments, such as propaquizafop plus glyphosate, was below 80%. This research describes the first herbicide-resistance report for the weed species *B. madritensis*, confirming the presence of glyphosate-resistant populations mainly in perennial cropping systems but also in winter cereals from Spain. Due to the limited chemical tools to manage these populations, there is an urgent need for farmers to implement integrated weed management strategies.

Introduction

The genus *Bromus* is found within tribe Bromeae, subfamily Pooideae within the Poaceae family (Pavlick 1995). *Bromus* can be divided into several sections, of which *Bromus*, *Ceratochloa*, and *Genea* (Smith 1980) stand as harboring important weed species. For example, sect. *Ceratochloa* includes important perennial weed species such as rescuegrass (*Bromus catharticus* Vahl), while the most relevant self-pollinating annual weed species occur in sect. *Genea*, such as ripgut brome (*Bromus diandrus* Roth) (Oja and Laarmann 2002). The *Bromus* genus is globally distributed and well known to be taxonomically complex (Smith 1980), particularly within sect. *Genea*, due to hybridization, morphological variations, and plasticity (Fortune et al. 2008). The most important *Bromus* weed species worldwide are found in this section: *B. diandrus*, compact brome (*Bromus madritensis* L.), red brome (*Bromus rubens* L.), poverty brome (*Bromus sterilis* L.), and cheatgrass (*Bromus tectorum* L.) (Borger et al. 2021; Davies et al. 2019; Vázquez-García et al. 2021a).

Bromus madritensis [syn.: *B. madritensis* L. ssp. *madritensis*, *Anisantha madritensis* (L.) Nevski] is native to the Mediterranean region in southern Europe (Tutin et al. 1980). It is accepted as being a separate species from the related *B. rubens* (Oja 2002; Tutin et al. 1980), although some taxonomists recognize both as two subspecies of *B. madritensis*. Compared with the brush-like condensed and erect panicles of *B. rubens* or the very loose flexible and nodding panicles of *B. diandrus*, the panicles of *B. madritensis* exhibit intermediate characters (Castroviejo et al. 1993). Therefore, *B. madritensis* is often confounded with one of the other two related *Bromus* species, particularly *B. rubens*. The literature often fails to distinguish between *B. madritensis* sensu stricto and *B. rubens* in areas where their distributions overlap, whether in their native range or in regions colonized by both species, such as North America (Horn et al. 2017). In fact, to best of our knowledge, there have been no studies focused on *B. madritensis* as a weed, its management, or its herbicide resistance.

The presence of *B. madritensis* is increasing in several cropping systems. The implementation of conservation tillage together with low diversity of crop rotations would explain its spread throughout Europe, North America, and Australia (Borger et al. 2021; Recasens et al. 2016). Conservation tillage increases crop water-use efficiency and moisture availability in rainfed Mediterranean regions, along with reducing costs and improving soil health (Borger et al. 2021; García et al. 2014). For these reasons, farmers have been implementing no-till techniques, such as direct drilling or cover crops, in the last decades in several perennial crops, but also in arable crops (Vázquez-García et al. 2020a). Due to the lack of soil disturbance in no-till cropping systems, weeds present at preseeding are usually managed with glyphosate, the sole nonselective herbicide available and the most applied herbicide in Europe. Moreover, there are few selective herbicides for *Bromus* species in cereals, most of them being nonselective for barley (*Hordeum vulgare* L.) (Royo-Esnal et al. 2018).

Glyphosate is the most used herbicide in the world (Duke and Powles 2008). Its mode of action (MOA) is the inhibition of the chloroplast enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; Group 9 HRAC/WSSA), which catalyzes the synthesis of phenylalanine, tryptophan, and tyrosine, essential amino acids for plants (Boocock and Coggins 1983; Steinrücken and Amrhein 1980). At present, 56 glyphosate-resistant species are already reported globally (Heap 2022). Most of these cases occur in agricultural systems in which genetically modified crops tolerant to glyphosate (GMOs) are cultivated, but also in non-cropped areas or perennial crops with high selection pressures (Beckie 2011). In Europe, GMOs are banned; therefore, glyphosate is usually applied in perennial crops, mostly under the row, and in annual crops at preseeding (Collavo and Sattin 2012, 2014).

In Spain, glyphosate resistance has been reported in seven grass weed species (Torra et al. 2022), such as rigid ryegrass (*Lolium rigidum* Gaudin) and Italian ryegrass [*Lolium perenne L.* ssp. *multiflorum* (Lam.) Husnot] (Fernández et al. 2016), johnsongrass [*Sorghum halepense* (L.) Pers.] (Vázquez-García et al. 2020b), barnyardgrass [*Echinochloa crus-galli* (L.) P. Beauv.] (Vázquez-García et al. 2021b), and false barley (*Hordeum murinum* L.) (Vázquez-García et al. 2020a). Regarding the genus *Bromus* in Spain, glyphosate resistance has recently been reported in *B. rubens* from several perennial crops (Vázquez-García et al. 2021a), and some other cases have been reported throughout the world: namely, *B. diandrus* and *B. rubens* from Australia, *B. tectorum* from Canada, *B. sterilis* from the United Kingdom, and *B. catharticus* from Argentina (Davies et al. 2019; Heap 2022). Among those, resistance levels were well established and/or resistance mechanisms were revealed only in *B. diandrus*, *B. sterilis*, and *B. catharticus*, with overexpression of EPSPS and non–target site mechanisms conferring glyphosate resistance in *B. diandrus* and *B. catharticus*, respectively (Davies et al. 2019; Malone et al. 2016; Yanniccari et al. 2021). On the other hand, irrespective of the resistance mechanism to glyphosate or the weed species, resistant plants accumulate significantly less shikimate than susceptible plants, because EPSPS is not or is less inhibited than in susceptible plants (Sammons and Gaines 2014). Therefore, determination of shikimate acid accumulation (SAA) levels can be employed to rapidly screen for glyphosate-resistant biotypes (Shaner et al. 2005).

Up to 2018, the unique herbicide-resistance case for *B. madritensis* reported worldwide was, in fact, the glyphosate-resistant populations studied and described in depth in the present research, and they remain the only reported case in this species (Heap 2022). From 2017, failures in the field regarding *B. madritensis* control with glyphosate were reported by farmers in Spain, mainly in perennial crops such as olive (*Olea europaea* L.), almond [*Prunus dulcis* (Mill.) D.A. Webb], or citrus (*Citrus* spp.), but also a few cases in winter cereals under direct drilling. The glyphosate resistance of these populations could have been selected due to a long history of selection pressure with this herbicide. Because the number of herbicides available for *Bromus* management is limited in all these cropping systems, this continued selection pressure from glyphosate is very threatening.

Due to the intermediate characters of *B. madritensis* between *B. rubens* and *B. diandrus*, and the taxonomic complexity of the *Bromus* genus, particularly sect. *Genea*, the aims of this research were: (1) discriminate several species of the genus *Bromus* using molecular markers to confirm that the studied populations from Spain belong to *B. madritensis*; (2) check for glyphosate resistance in these *B. madritensis* populations using rapid SAA screening and dose–response experiments; and (3) test in field trials the efficacy of alternative herbicides with different MOAs to manage them.

Materials and Methods

Samples Collection

In summer 2018, 12 suspected populations of *B. madritensis* were harvested from different locations on the Iberian Peninsula. Two populations (labeled Bms8 and BmS14) were collected from sites never treated with herbicides (Portugal and Spain, respectively). The remaining populations were collected from fields with different cropping systems and herbicide application histories (Table 1). Samples were placed in paper bags and stored in a cold chamber (4 C) until needed (June 2018 to January 2019).

Germination and Growth Conditions

The 14 *B. madritensis* populations were scarified manually. Seeds were put in trays (196 by 147 by 27 mm) pre-filled with peat moss and were placed in a greenhouse (University of Cordoba) at 28/18 C day/night temperature, and a 12/12-h photoperiod under 350 μmol m^{-2} s^{-1} photosynthetic photon flux density. After 5 d, emerged seedlings were sown in individual pots with sand:peat moss (1:1) substrate. Plants were grown in the greenhouse under the conditions described earlier and fertilized and watered as needed.

Characterization by Simple Sequence Repeat Markers

Bromus populations from six different species were used in this trial. The samples were collected in different sites (Table 2). Seeds were germinated in June 2018 and grown as described earlier. Nineteen populations were characterized using simple sequence repeat (SSR) markers (Ramakrishnan et al. 2002). Ten plants at the BBCH-13 stage were collected from each population for DNA isolation. For each plant, 100 mg of tissue was taken and placed in an Eppendorf tube (2 ml) containing four steel balls. Samples were frozen in liquid nitrogen after collection. DNA

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Table 1. Population information: code assigned, location (province, country), application history, and coordinates of *Bromus madritensis* used in this investigation.

Code	Location	Crop	History	Coordinates
Bmr1	Cordoba, Spain	Citrus	<5 yr glyphosate	37.7492240°N, 5.1672640°W
Bmr2	Sevilla, Spain	Olive	>5 yr glyphosate	37.537909°N, 5.068860°W
Bmr3	Cordoba, Spain	Olive	>3 yr mix[a]	37.513054°N, 4.841346°W
BmR4	Sevilla, Spain	Olive	10 yr mix[a]	37.536481°N, 4.9594725°W
BmR5	Sevilla, Spain	Olive	10 yr glyphosate	37.539152°N, 4.963558°W
BmR6	Cordoba, Spain	Olive	10 yr glyphosate	37.511713°N, 4.838902°W
BmRR7	Cordoba, Spain	Olive	10 yr mix[a]	37.518257°N, 4.841909°W
Bms8	Beja, Portugal	Road	0	38.009263°N, 7.710739°W
Bms9	Beja, Portugal	Almond	3 yr glyphosate	38.056871°N, 7.954293°W
BmRR10	Malaga, Spain	Olive	10 yr glyphosate	36.999412°N, 4.930857°W
Bmr11	Malaga, Spain	Olive	10 yr mix[a]	37.536937°N, 4.973989°W
Bmr12	Malaga, Spain	Olive	<10 yr mix[a]	37.008075°N, 4.645182°W
BmR13	Lleida, Spain	Cereals	>10 yr glyphosate	41.855000°N, 1.127500°E
BmS14	Lleida, Spain	No crop	0	41.628056°N, 0.595278°E

[a]Farmers indicated preemergence use of flazasulfuron (acetolactate synthase inhibitor) or oxyfluorfen (protoporphyrinogen oxidase inhibitor). In addition, application of auxin mimics was sometimes reported.

Table 2. Locations of the species used in molecular characterization.

Species	Coordinates
Bromus rubens	37.916582°N, 4.719029°W
Bromus sterilis	37.918523°N, 4.723713°W
Bromus diandrus	37.916643°N, 4.725164°W
Bromus madritensis	Shown in Table 1
Bromus catharticus	37.901390°N, 4.780186°W
Bromus tectorum	38.284321°N, 4.343085°W

isolation, primer labeling, and PCR conditions were as described by Vázquez-García et al. (2021a). The PCR products were sent to Animal Breeding Consulting S.L. (ABC S.L., University of Cordoba, Spain) for analysis. Results were analyzed using Genotyper software (v. 3.7, Applied Biosystems, Waltham, MA, USA). The fragment size of the alleles was determined using a DNA standard (400HD-ROX) for each SSR primer. Alleles were scored as present or absent (1 or 0) for each marker/species, and a binary data matrix was created. Jaccard's similarity coefficient was used to calculate genetic distances between populations. The unweighted pair group method with arithmetic mean was employed to determine the grouping of the *Bromus* species. Finally, a dendrogram was generated with the NTSYS program (Rohlf 1998).

Shikimic Acid Trial

Ten plants at BBCH-13 stage from the 14 *B. madritensis* populations were selected for shikimic acid extraction (SAE). This trial was divided into three steps: (1) The first and second leaves of each plant were cut into 5-mm pieces and pooled (~50 mg). (2) Samples were placed into Eppendorf tubes (1.5 ml) containing 990 ml of monoammonium phosphate plus 10 μl of glyphosate (100 μM) for 24 h with the photoperiod described in section "Germination and Growth Conditions." (3) The SAE was undertaken according to Vázquez-García et al. (2021b). Six technical replicates with/without herbicide were used in a completely randomized test. Obtained data were transformed and reported as milligrams of shikimic acid per gram of fresh tissue. A histogram was generated to discriminate high and low shikimate–accumulating populations.

Whole-Plant Bioassay

A whole-plant bioassay was performed in 2019 to determine the response of *B. madritensis* populations to glyphosate. Glyphosate (480 g ae L^{-1}, Roundup Ultimate®) was applied on plants at BBCH-13 to BBCH-14 stages. For each population, eight doses ranging from 31.25 to 1,500 g ha^{-1} were applied to 10 plants per dose, with 10 additional plants maintained without herbicide treatment acting as controls. At 21 d after herbicide application (DAHA), plant growth and mortality were evaluated. To determine the 50% growth inhibition rate (GR_{50}), plants were cut at ground level, and fresh weight was recorded. The 50% death rate (LD_{50}) was evaluated based on plant survival (binary data: 1 = live, 0 = dead) in each population. All fresh weight data were converted to percentage of weight compared with the weight of untreated plants. The experiment was repeated once.

Field Trials

The field management of *B. madritensis* was performed on the farm where population BmR4 was collected, in Cordoba, Spain, during seasons 2019 to 2020 and 2020 to 2021 (Figure 1). Five herbicide treatments (plus an untreated control) (Table 3) were applied in a total of 24 plots of 20 m^2 in a completely randomized block design with four replicates. Two treatments were applied in November (time A, TA) at preemergence or early emergence of the weed. The other three treatments were applied in December (time B, TB), at postemergence stages of the weed (BBCH-13 to BBCH-14 stages) in separate plots. The herbicide rates are shown in Table 3. The application was performed with a Pulvexper (Pulvexper, Ramerupt, France) backpack sprayer equipped with four flat-fan nozzles TeeJet® 11002 (TeeJet Technologies GmbH, Schorndorf, Germany), at a spraying pressure of 200 kPa and calibrated to deliver a volume of 200 L ha^{-1}. Finally, *B. madritensis* control was evaluated at 15, 30, 60, and 90 DAHA. The visual estimation of plant damage/death was performed by the same person for all evaluations. Control ratings were expressed on a 0 (no control) to 100 (all plants dead) scale.

Data Statistical Analysis

For the whole-plant trials, because no significant interaction and statistical differences were obtained between the two replications (ANOVA), pooled data were fit to three-parameter nonlinear regression analysis to determine the GR_{50} and LD_{50}:

$$Y = c + (d - c)/[1 + (x/g)b] \qquad [1]$$

where Y is the weight or the survival, c (set to zero) and d parameters are the lower and upper asymptotes, b is the slope of the curve, g is the herbicide dose to inhibit the growth of or kill 50% of the

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Table 3. Herbicides and rates applied on farm with *Bromus madritensis* resistant to glyphosate.

Time[a]	Commercial name[b]	Active ingredient	Rates
			g ai/ae ha^{-1}
	Untreated	—	—
TA	Terafit® + Roundup Ultimate®	Flazasulfuron + glyphosate	50 + 1,080
	Musketeer® + Roundup Ultimate®	Diflufenican + iodosulfuron + Glyphosate	150 + 10 + 1,080
TB	Roundup Ultimate®	Glyphosate	1,080
	Roundup Ultimate®	Glyphosate	1,800
	Roundup Ultimate® + Agil®	Glyphosate + propaquizafop	1,080 + 150

[a]Application dates: TA = first year: November 15, 2019; second year: November 20, 2020. TB = first year: December 16, 2019; second year = December 18, 2020.
[b]Manufacturers: Terafit (Syngenta, Spain); Roundup Ultimate (Monsanto, Spain); Musketeer (Bayer CropScience, Spain); Agil (ADAMA, Spain).

Figure 1. Location of the plots (Cordoba, Spain) where *Bromus madritensis* control was evaluated.

population, and x is the herbicide rate. The analysis was performed using the DRC package (Ritz et al. 2015) in the R software program (v. 4.1.3).

The resistance factor (RF) was then estimated based on the GR_{50} and LD_{50} values according to the following equation:

$$RF = (GR_{50} \text{ or } LD_{50}R/GR_{50} \text{ or } LD_{50}\ S) \quad [2]$$

where "R" is the resistant population and "S" is the susceptible population. In addition, a box plot was performed to visually group populations (arbitrarily based on the GR_{50} RF).

Once it had been established that normality and homoscedasticity requirements were met, shikimic acid and field management assays were submitted to one-way and two-way ANOVA, respectively, followed by a multiple mean comparison using Tukey's test to separate the different groups (at $P < 0.05$). All ANOVAs were performed with Statistix 10 (Analytical Software, Tallahassee, FL, USA).

Results and Discussion

Bromus Species Characterization

The dendrogram obtained using the similarity matrix and Jaccard's coefficient revealed five groups. In Group I, two species were characterized, *B. sterilis* and *B. rubens*, named I.I and I.II, respectively. Group II corresponds to *B. diandrus* alone. The 14 populations of *B. madritensis* were included in Group III. However, these populations can be separated in three subgroups; the Andalusian populations (III.I), the Portuguese populations (III.II), and the populations from Lleida (III.III). *Bromus catharticus* and *B. tectorum* were separated into Groups IV and V, respectively (Figure 2).

Bromus madritensis sensu lato, including *B. rubens*, forms a group of morphologically close taxa with erect and small lemmas and more or less contracted inflorescences. These characters separate the *B. madritensis* complex from other species in sect. *Genea* (Oja 2002). Molecular markers such as SSRs or others have been widely used in the assessment of genetic diversity in weeds, both within and between related species (O'Hanlon et al. 2000); in the present research, seven SSR markers (Ramakrishnan et al. 2002) were used to discriminate among six *Bromus* species (Table 2). These SSRs were transferable to the six considered species and polymorphic. Using estimated genetic distances, the six species were clearly differentiated (Figure 2), such as *B. catharticus* sect. *Ceratochloa*, but also the five species of sect. *Genea*, like *B. madritensis*, *B. rubens*, and *B. diandrus*, which are often confounded. Moreover, within the 14 *B. madritensis* populations included, the constructed dendrogram was able to discriminate among them according to their geographic areas (Portugal, Andalusia in the south of Spain, or Catalonia in northeastern Spain), but not according to their glyphosate-resistance status

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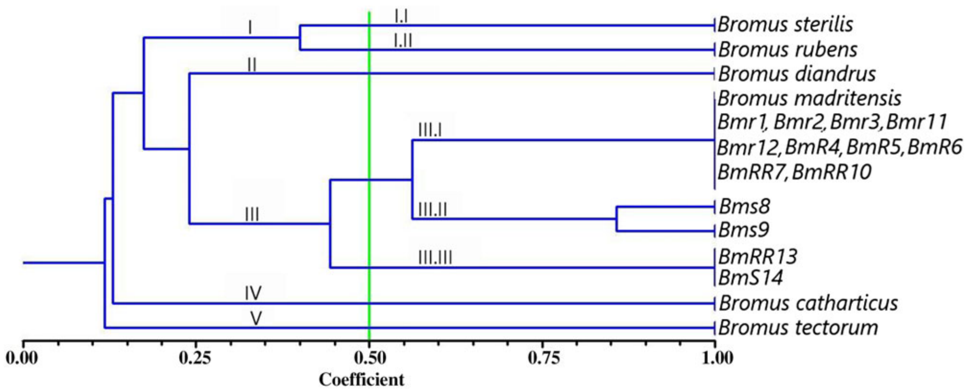

Figure 2. Dendrogram of the six considered *Bromus* species. The 14 populations studied in this work are included within *Bromus madritensis* (Bm).

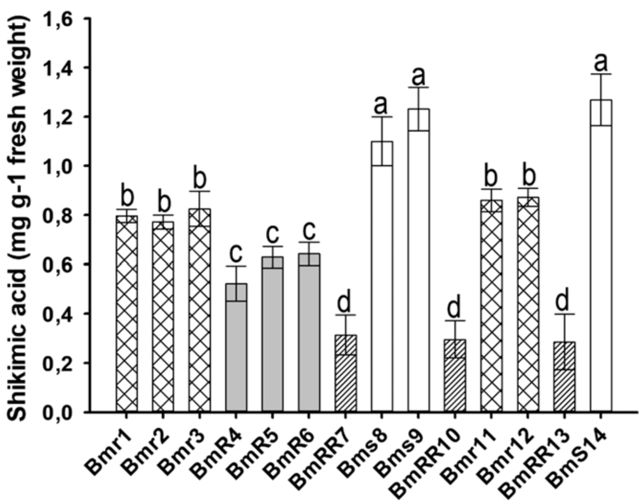

Figure 3. Shikimic-acid accumulation in 14 *Bromus madritensis* populations from Spain and Portugal. Different letters denote significant differences between means. Bars represent the standard error of the mean ($n = 6$).

(susceptible or resistant). Overall, we confirmed that the 14 populations belong to *B. madritensis* and that they are genetically different from the other five *Bromus* species.

Glyphosate Resistance

The response of the 14 *B. madritensis* populations to glyphosate was diverse, with different levels of SAA (Figure 3). Populations Bms8, Bms9, and BmS14 accumulated significantly more shikimic acid (>1,000 μg g^{-1}) than the rest, rendering putative susceptibility. Among the remaining 11 populations, six had 600 μg g^{-1} or lower levels of SAA, clearly pointing to glyphosate resistance, while five had around 800 μg g^{-1}, with a low resistance profile. Populations BmRR7, BmRR10, and BmRR13 showed the lowest SAA levels (around 300 μg g^{-1}).

Different resistance levels were obtained for the 14 *B. madritensis* populations (Figure 4; Table 4). Significatively the most susceptible population was BmS14, with GR_{50} and LD_{50} values of 144 and 291 g ae ha^{-1}, respectively (Supplementary Tables 2s and 3s). Populations Bms8 and Bms9 could also be designated susceptible (Group I in Figure 4), with RFs from 1.1 to 1.4, depending on the parameter analyzed (Supplementary Table 1s; Supplementary Figure 1s). This group of susceptible populations is well defined, as LD_{50} values were lower than the lowest recommended rate used for glyphosate. Six populations had resistance factors above 4, both for survival and fresh weight data, and could be classified as resistant, with LD_{50} values always above 1,080 g ae ha^{-1}, the minimum registered rate in Spain for perennial crops (five populations) (Figure 5). Among these, two subgroups of populations with different resistance levels could be defined: (1) BmRR7, BmRR10, and BmRR13 were highly resistant, with GR_{50} and LD_{50} values ranging between 757 to 2,153 g ae ha^{-1} and RFs between 5.3 and 8.0 (Table 4; Group IV in Figure 4); and (2) BmR4, BmR5, and BmR6, with GR_{50} and LD_{50} values between 593 and 1,398 g ae ha^{-1} and RFs below 5 but above 3 (Table 4; Group III in Figure 4). Finally, the remaining five populations showed RFs below 3 (Table 4), and could not be clearly designated resistant. In this group, GR_{50} and LD_{50} values were also lower compared with those of the resistant populations (Groups III and IV in Figure 4). Group II with very low resistance levels had LD_{50} values ranging between the Spanish minimum registered rate in perennial crops (1,080 g ae ha^{-1}) and those for Australia or the United Kingdom (Figure 5), which correspond to minimum label doses in Spain in arable crops in pre-sowing for annual weeds (540 g ae ha^{-1}).

Dose–response trials confirmed glyphosate resistance in at least six *B. madritensis* populations from Spain, with resistance levels 4- to 8-fold those of the susceptible populations (Table 4; Figure 4). SAA experiments also confirmed resistance to glyphosate in these populations, which showed the lowest levels compared with susceptible populations (more than 1,000 μg g^{-1} of shikimic acid) (Figure 3). Nevertheless, shikimic acid accumulated to some level in both susceptible and resistant plants, indicating that glyphosate was inhibiting the EPSPS enzyme, although at significantly different extents among populations (Figure 3). SAA is widely used to screen for glyphosate resistance (Shaner et al. 2005). Accordingly, those populations with higher LD_{50} and GR_{50} values also showed lower levels of SAA. Overall, considering the RFs (both for LD_{50} and GR_{50}) and SAA, two groups of glyphosate-resistant *B. madritensis* populations could be established, one with moderate resistance levels and one with higher resistance levels, each having three populations (Table 4; Figures 3 and 4). In the first group, RFs ranged between 4 and 5 with around 600 μg g^{-1} of shikimic acid, while in the second group, RFs ranged between 5 and 8 with around 300 μg g^{-1} of shikimic acid. It has been proposed that different SAA patterns between susceptible and resistant plants can provide information about the target site–based resistance mechanism to glyphosate (Adu-Yeboah et al. 2020). In the case of the three populations with higher glyphosate resistance, SAA was also lower, suggesting a mechanism related to the

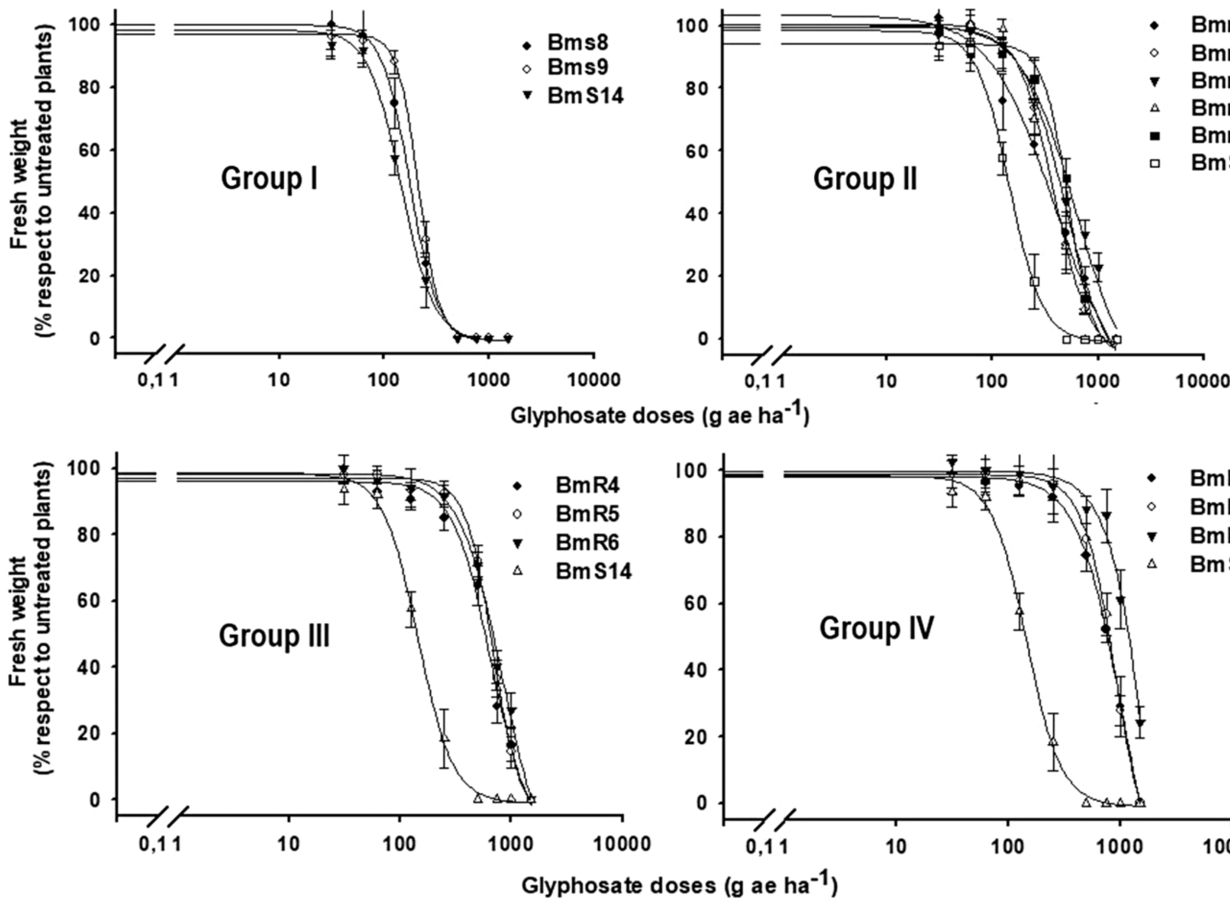

Figure 4. Response to glyphosate of eight resistant and three susceptible (Bms8, Bms9, and BmS14) populations of *Bromus madritensis*. Populations are grouped according to resistance levels: Group I, susceptible; Group II, low resistance; Group III, medium resistance; Group IV, high resistance. Bars represent the standard error of the mean ($n = 20$). Note that the most susceptible population (BmS14) is plotted in all the graphs for a better comparison.

EPSPS enzyme. Finally, there was a third group of *B. madritensis* populations with low levels of resistance (Group II in Figure 4), which could not be fully designated glyphosate resistant, with RFs below 4 (Table 4) and around 800 μg g^{-1} of shikimic acid (Figure 3). Interestingly, while glyphosate had been applied for at least 10 yr in the resistant populations, in this Group II of low-resistance populations, the herbicide had not been applied for more than 5 yr. Conservation agriculture and no-till practices are spreading in different crops of the Iberian Peninsula; thus more glyphosate applications can be expected. Moreover, higher glyphosate selection pressure can be predicted in those fields under no-till for more years. Previous studies also reported different levels of glyphosate resistance between populations with different intensities of glyphosate selection pressure in crops of the study area (Vázquez-García et al. 2020a, 2021a, 2021b).

The minimum registered rates for glyphosate in Spain are 1,080 g ae ha^{-1} and 540 g ae ha^{-1} in perennial crops and arable fields, respectively, and are usually the rates that are applied. The former rate is 2-fold higher than the label doses for glyphosate in countries like United Kingdom or Australia (Davies et al. 2019; Malone et al. 2016). The six *B. madritensis* populations (one from winter cereals) classified as resistant had LD_{50} values above the registered rate in Spain, as shown in Figure 5. It is accepted that a classification of "resistant" requires that the resistant population must survive the herbicide label dose under normal field conditions (HRAC 2022; Heap 2022), which was in-field corroborated for one of the populations (Figures 6 and 7). On the other hand, five populations could not be fully designated resistant, with LD_{50} values between registered doses in Spain and those of the United Kingdom and Australia (Figure 5). Thus, these populations might have been classified as resistant according to the recommended field rates of those countries. Consequently, as registered doses can vary between countries, one must be cautious when referring to a resistant population from an agronomic perspective by the LD_{50} value (HRAC 2022; Heap 2022).

This study confirms and details the first case of herbicide resistance in the weed species *B. madritensis*. The presence of glyphosate-resistant populations was established across different crops and geographic areas in Spain, except for the two Portuguese populations. Of the six *B. madritensis* populations classified as resistant (RFs > 4), five were located in the south of Spain (Cordoba, Sevilla, and Malaga), and one was found in northeastern Spain. Most were found in olive orchards, one came from a citrus grove, and another from winter cereals under no-till. Therefore, this research demonstrates that there is a great threat of glyphosate resistance continuing to evolve in this species and spreading to several cropping systems under conservation agriculture. High glyphosate selection pressures are expected to persist both in perennial crops and cereals at preseeding in the region (Beckie 2011; Collavo and Sattin 2012, 2014).

Table 4. Results of different parameters that define resistance to glyphosate in *Bromus madritensis*.[a]

Population code	*d*	*b*	GR_{50}	RF	*d*	*b*	LD_{50}	RF
			—g ae ha^{-1}—				—g ae ha^{-1}—	
Bmr1	101.23	1.84	301.65 ± 20.07	2.67	96.19	6.83	812.23 ± 12.71	2.79
Bmr2	97.24	2.78	376.93 ± 17.46	2.62	95.28	6.79	802.15 ± 13.17	2.76
Bmr3	98.22	2.68	498.33 ± 21,57	3.37	98.86	8.35	916.80 ± 12.16	3.55
BmR4	94.13	4.69	593.48 ± 13.37	4.12	100.81	4.51	1,091.22 ± 25.76	3.75
BmR5	96.69	4.18	652.54 ± 14.32	4.53	100.91	3.77	1,274.12 ± 30.16	4.38
BmR6	97.14	3.50	666.98 ± 18.06	4.63	100.75	3.65	1,398.60 ± 33.54	4.81
BmRR7	96.67	3.47	757.19 ± 20.01	5.26	100.89	3.16	1,588.23 ± 35.52	5.46
Bms8	99.91	3.29	176.02 ± 5.07	1.22	101.17	2.99	317.52 ± 11.03	1.09
Bms9	97.04	4.67	204.83 ± 5.59	1.42	99.83	3.07	413.32 ± 15.54	1.42
BmRR10	98.21	3.63	787.99 ± 14.81	5.48	100.78	3.18	1,718.60 ± 37.60	5.91
Bmr11	100.43	3.17	342.03 ± 11.95	2.37	97.85	6.85	762.96 ± 13.83	2.62
Bmr12	95.21	3.57	510.19 ± 16.24	3.45	99.53	5.90	844.98 ± 13.57	2.9
BmRR13	97.69	4.07	1,145.95 ± 26.24	7.97	100.58	3.23	2,153.03 ± 43.98	7.4
BmS14	98.13	2.86	143.77 ± 5.55	—	100.58	2.70	290.58 ± 10.85	—

[a] *d* is the upper coefficient; *b* is the slope of the curve; GR_{50} is the 50% growth inhibition rate; LD_{50} is the 50% death rate; and RF the resistance factor.

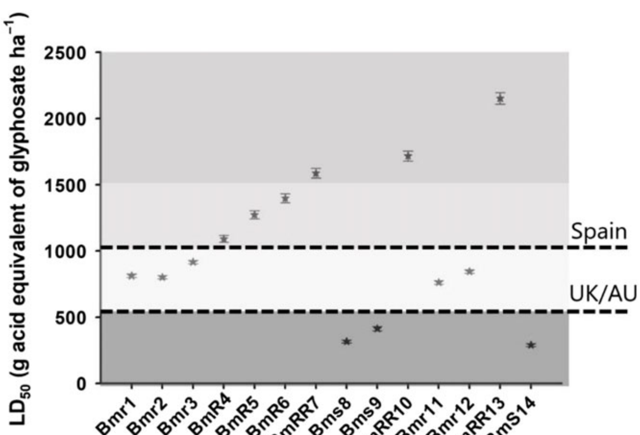

Figure 5. LD_{50} values for the 14 *Bromus madritensis* populations from Spain and Portugal. Dotted lines represent the minimum recommend rate in Spain in perennial crops (1,080 g ae ha^{-1}) and in the United Kingdom and Australia (540 g ae ha^{-1}), respectively, which is also the minimum dose in arable crops before sowing for annual weeds in Spain (the case for BmRR13).

Field Trials

Field trials showed that there are some potential herbicide alternatives to glyphosate for the chemical management of *B. madritensis*, though they were tested in only 1 of the 14 populations. Overall, the control percentage of *B. madritensis* was similar in both years (Figure 6). In TA (preemergence or early emergence), only flazasulfuron plus glyphosate achieved acceptable control levels at 90 DAHA (≥90%), while in TB (postemergence) none of the three herbicide treatments reached 90% of efficacy at 90 DAHA. In both years, the best treatment in TB was glyphosate plus propaquizafop, which controlled almost 100% of *B. diandrus* at 15 and 30 DAHA, but these efficacies dropped below 80% at 90 DAHA. Figure 7 presents images showing the control efficacies reported quantitatively in Figure 6. Note the high efficacy achieved by flazasulfuron plus glyphosate applied at TA, particularly at 90 DAHA.

To the best of our knowledge, there is no information available concerning herbicide efficacies for the control of *B. madritensis*. Moreover, little information is available about the application of gramicides for the control of *Bromus* spp. (Brewster and Spinney 1989; Royo-Esnal et al. 2018; Vázquez-García et al. 2021a).

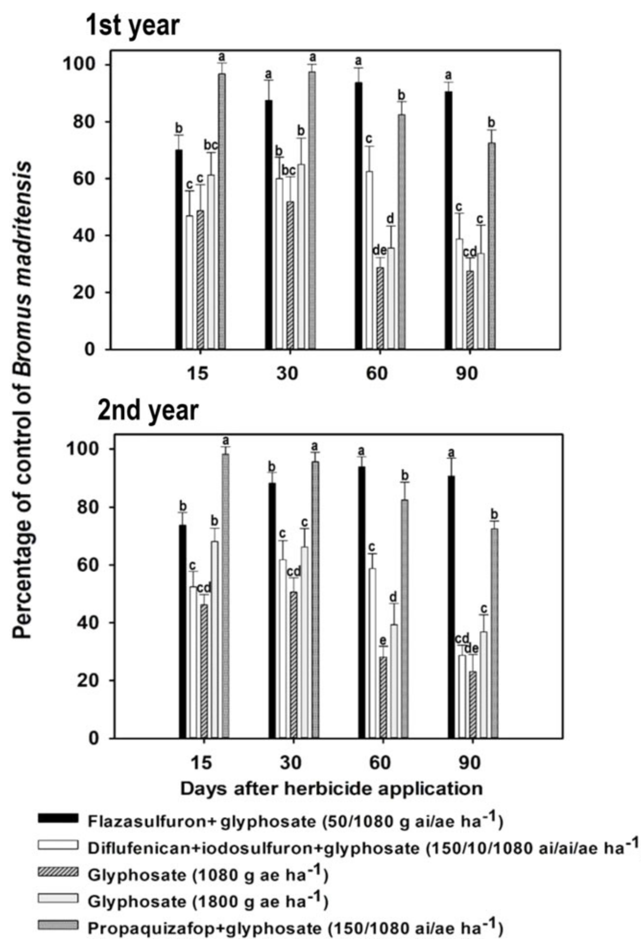

Figure 6. Field efficacies for the control of *Bromus madritensis* in 2019–2020 (top) and 2020–2021 (bottom). Different letters denote significant differences between treatments. Bars represent the standard error of the mean (four plots). The first two treatments were in preemergence–early postemergence (timing TA) (*), while the remaining three were in postemergence (timing TB).

The present study included some herbicide alternatives at pre- or postemergence. The only treatment with efficacies consistently ≥90% in both years was flazasulfuron (acetolactate synthase inhibitor) plus glyphosate preemergence, while the acetyl-CoA carboxylase (ACCase) inhibitor propaquizafop postemergence, also mixed

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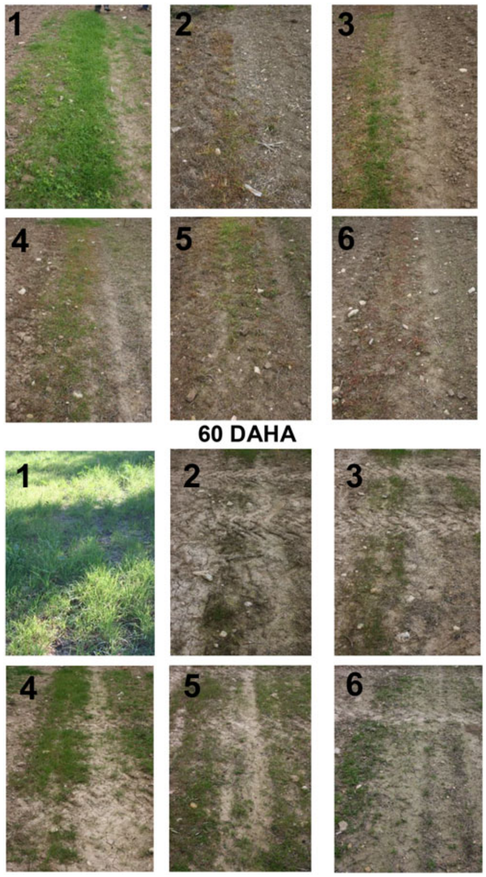

Figure 7. *Bromus madritensis* infestation and visual efficacy images in field treated with different herbicides (alone or mix) 30 and 60 d after herbicide application (DAHA). (1) Untreated; (2) flazasulfuron + glyphosate; (3) diflufenican + iodosulfuron + glyphosate; (4) glyphosate (1,080 g ae ha-1); (5) glyphosate (1,800 g ae ha-1); (6) propaquizafop + glyphosate. Treatments 2 and 3 in preemergence–early postemergence; 4 to 6 in postemergence.

with glyphosate, showed decreasing control levels from >90% to below 80% at 15 to 90 DAHA, respectively. Previous studies showed that flazasulfuron could be a good alternative to manage glyphosate-resistant *B. rubens* populations, although ACCase inhibitors were also effective (Vázquez-García et al. 2021a). Few chemical tools are currently available to control *B. madritensis*, particularly glyphosate-resistant populations, a fact that should encourage farmers to switch their focus to integrated weed management strategies.

The glyphosate-resistant cases reported in Spain and Portugal have escalated rapidly in the last two decades (Torra et al. 2022). This research is the first report of herbicide resistance in *B. madritensis* in six populations that evolved glyphosate resistance in perennial orchards as well as in no-till winter cereals after more than a decade of continuous glyphosate use. The existing nonselective chemical options to control *B. madritensis* in these crops are crucial for farmers, because there are limited (selective) herbicides available. Therefore, to control this and other glyphosate-resistant species already reported in their fields (Torra et al. 2022), growers must employ a diverse integrated approach. The use of crop rotation or nonchemical methods, such as mechanical weeding, among others, is essential to reduce the impact of glyphosate-resistant weeds in the future in these cropping systems.

Supplementary material. To view supplementary material for this article, please visit https://doi.org/10.1017/wsc.2023.9

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