



Article

BRECCIA, G.^{1,2*}

BISIO, M.B.²

PICARDI, L.^{1,3}

NESTARES, G.^{1,2}

A RAPID PHENOTYPING METHOD FOR IMAZAMOX RESISTANCE IN WHEAT

Um Método Rápido para Fenotipagem da Resistência à Imazamox em Trigo

ABSTRACT - The availability of imidazolinone (IMI) resistant cultivars has provided an effective option for weed control in wheat production systems. IMI herbicides control several weeds, including *Avena fatua* and *Lolium multiflorum*, which are the most frequent grass weeds in wheat crops of the Argentine Pampas. The aim of this study was to develop a soil-less method that allows rapid phenotyping of IMI resistance in wheat. Nine wheat cultivars differing in IMI resistance were evaluated through a between-paper germination method. Herbicide concentrations required to reduce shoot and root length by 50% for resistant cultivars were > 75 fold that of the susceptible cultivars. The response of resistant and susceptible commercial cultivars was assessed in the between-paper and top-paper methods at 100 mM imazamox as discriminating dose. The Z'-factor was calculated for evaluation of the quality of the screening methods. Both germination methods showed Z'-factors > 0 indicating that the assays were appropriate but the between-paper method allowed to save space in the growth chamber. The germination methods were useful for distinguishing between susceptible and resistant plants carrying at least one resistance gene. The rapid, simple and cost-effective method described in the present study could be a potential tool when selecting for IMI resistance in wheat in breeding programs.

Keywords: imidazolinone, herbicide resistance, germination test, *Triticum aestivum* L.

RESUMO - A disponibilidade de cultivares resistentes à imidazolinona (IMI) proporcionou uma opção eficaz para o controle de plantas daninhas em sistemas de produção de trigo. Os herbicidas IMI controlam várias plantas daninhas, incluindo *Avena fatua* e *Lolium multiflorum*, as gramíneas mais frequentes nas culturas de trigo nos Pampas argentinos. O objetivo deste trabalho foi desenvolver um método sem solo que permita a fenotipagem rápida de resistência ao IMI em trigo. Avaliaram-se nove cultivares de trigo, diferentes na resistência ao IMI, por meio de um método de germinação entre folhas de papel. As concentrações de herbicidas necessárias para reduzir o comprimento da parte aérea e da raiz em 50% para cultivares resistentes foram > 75 vezes que as dos cultivares suscetíveis. A resposta de cultivares comerciais resistentes e suscetíveis, avaliada nos métodos entre folhas de papel e papel, foi superior a 100 mM de imazamox como dose discriminante. O fator Z' foi calculado para a avaliação da qualidade dos métodos de triagem. Ambos os métodos de germinação mostraram Z'-fatores > 0, indicando que foram apropriados, porém o método entre folhas de papel permitiu economizar espaço na câmara de crescimento. Os testes de germinação foram úteis para distinguir entre plantas suscetíveis e resistentes portadoras de pelo menos um gene de resistência. O método rápido, simples e econômico descrito no presente estudo pode ser uma ferramenta importante para selecionar trigo resistente ao IMI em programas de melhoramento.

Palavras-chave: imidazolinona, resistência a herbicidas, teste de germinação, *Triticum aestivum* L.

* Corresponding author:

<breccia@iicar-conicet.gob.ar>

Received: May 10, 2017

Approved: August 7, 2017

Planta Daninha 2018; v36:e018179638

Copyright: This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided that the original author and source are credited.



¹ IICAR-UNR-CONICET, Zavalla, Argentina; ² Cátedra de Genética-FCA-UNR, Zavalla, Argentina; ³ CIUNR, Zavalla, Argentina.

INTRODUCTION

Imidazolinone (IMI) herbicides control weeds by inhibiting the enzyme acetohydroxyacid synthase (AHAS), which is involved in the biosynthesis of the branched-chain amino acids valine, leucine, and isoleucine (Duggleby et al., 2008). These herbicides control a wide spectrum of grass and broadleaf weeds at low application rates (Tan et al., 2005).

Although imidazolinone-resistant (IMI-R) plants have been produced by both transgenic and non-transgenic mechanisms, all of the IMI-R crops currently sold were developed by using non-transgenic methods such as mutagenesis and introgression of resistance genes from wild populations. IMI-R crops including maize, canola, lentil, rice, wheat, and sunflower have become available on a commercial basis in different countries in North and South America, Europe, and Australia since the introduction of IMI-R maize in 1992 (Shaner et al., 2012).

Bread wheat (*Triticum aestivum* L.) is grown on more than four million hectares of land in Argentina. Weed management is an important concern for wheat producers. Most frequent weeds species in wheat crops are prostrate knotweed (*Polygonum aviculare*), wild oat (*Avena fatua*), wild buckwheat (*Polygonum convolvulus*) and Italian ryegrass (*Lolium multiflorum*) (Scursoni et al., 2014). IMI-R wheat cultivars provide an alternative for a weed control system that includes the application of imazamox. This herbicide provides a broad-spectrum and post-emergence weed control. On other hand, the availability of IMI-R wheat cultivars could avoid the injury to wheat observed in normal crop rotational sequence after IMI herbicide application to IMI-R sunflower (Giménez et al., 2014).

In wheat, IMI resistance genes were obtained by seed mutagenesis (Newhouse et al., 1992; Pozniak and Hucl, 2004; Jimenez et al., 2016). IMI-R wheat cultivars have been marketed under the trade name 'Clearfield'(CL). It was suggested that two resistance genes (*AhasL-D1* and *AhasL-B1*) were adequate for providing high levels of field resistance to the IMI herbicides (Pozniak and Hucl, 2004; Pozniak et al., 2004). The basis of resistance is due to the presence of an altered form of AHAS that is resistant to inhibition by IMI herbicides (Pozniak et al., 2004) but the presence of additional resistance mechanisms such as herbicide metabolism were also suggested for some cultivars (Jimenez et al., 2016).

Protocols for screening resistance to herbicides with different sites of action have been developed for several crop and weed species (Beckie et al., 2000). For many soil-less bioassays, seeds or seedlings are placed on filter paper or agar treated with a specific rate of herbicide. This kind of methods makes it possible to evaluate a large number of samples in less time than it would take to conduct the classical whole-plant assay (Burgos, 2015).

Conventional phenotyping methods for IMI resistance involve treating two-to four-leaf plants and selecting the plants that survive. A soil-less method that provides a fast and inexpensive alternative to field screening could be useful for screening large numbers of samples. Therefore, the objective of this study was to develop a seedling method that allows rapid phenotyping of IMI resistance in wheat.

MATERIALS AND METHODS

Plant material and growth conditions

Six commercial Argentinian wheat cultivars were evaluated in this study: three imidazolinone resistant (IMI-R) cultivars (Klein Titanio CL, Buck 55 CL2 and Baguette 560 CL) and three susceptible (IMI-S) cultivars (Klein Serpiente, Buck SY 300 and Baguette 9). Three IMI-R cultivars homozygous for a single resistant gene were also analyzed: Fidel FS-4 (Newhouse et al., 1992), BW755 and TealIMI 11A (Pozniak and Hucl, 2004).

Immediately before each germination test, the wheat seeds were soaked in 4% sodium hypochlorite solution for five minutes and rinsed three times with distilled water. The seeds were incubated in a growth chamber under controlled conditions of temperature, photoperiod and light intensity (23 ± 2 °C, 16 h light and $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ respectively) for five days.

Characterization of imazamox resistance through between-paper germination test

A germination test was conducted according to the between-paper germination method (ISTA, 2014). Two folded paper towels (25 x 22.5 cm, Scott®, Argentina) were imbibed in 15 mL of different concentrations of commercial imazamox (70% imazamox acid, dispersible granules, Trigosol®, BASF, Argentina): 0 – 0.1 – 0.316 – 1 – 3.16 – 10 μ M for IMI-S cultivars and 0 – 10 – 31.6 – 100 – 316 – 1000 μ M for IMI-R cultivars. Twenty seeds were placed equidistantly between two moist paper towels which were rolled up and placed in polyethylene bags. These were sealed and incubated in an upright position. After five days, shoot length, total root length and longest root length of each seedling were measured through image analysis. Shoots and roots from the seedlings were imaged with a HP Scanjet G3010 flatbed scanner. TIFF images (300 dpi) were analyzed by using Image J (Schneider et al., 2012) and RootNav (Pound et al., 2013) software. Four repetitions were made for each cultivar and herbicide concentration.

The data were analyzed by nonlinear regression, using the package *drc* (Ritz et al., 2015) within R software (R Core Team, 2016). Dose-response curves were adjusted to a log-logistic model of three parameters:

$$y = d / [1 + (x / e)^b]$$

where e (also known as GR50) denotes the herbicide dose (x) that inhibited plant response (y) by 50%; d reflects the response upper limit and b denotes the relative slope around e (Seefeldt et al., 1995). The response (lower limit) was set equal to zero. Data from similar type of cultivars were pooled when their curves did not differ (Ritz et al., 2015). Comparisons between cultivars in terms of GR50 values were carried out by using approximate t-tests (*compParm* function of the package *drc*).

Quality estimation of the top-paper and between-paper germination tests

Seeds of commercial Argentinian cultivars were germinated with 100 μ M imazamox using between-paper and top-paper methods. For the top-paper method, 20 seeds were germinated on top of a moistened paper towel placed at the bottom of a rectangular polypropylene vessel (107 x 94 x 96 mm). Four repetitions were made for each cultivar and germination method. After five days of incubation, the shoot length and the longest root length of each seedling were measured.

The Z' -factor was used to evaluate the quality of the germination tests (Zhang et al., 1999). The Z' -factor was calculated for each variable and germination method. The IMI-R and IMI-S cultivars were considered as the positive and negative controls:

$$Z' = 1 - [(3 \sigma \text{IMI-R} + 3 \sigma \text{IMI-S}) / (| \mu \text{IMI-R} - \mu \text{IMI-S} |)]$$

where σ IMI-R and σ IMI-S are the standard deviations and μ IMI-R and μ IMI-S are the means of the response variable for IMI-R and IMI-S cultivars. The Z' -factor ranges from negative infinity to 1, and a high value (> 0.5) defines an effective method, a low value (> 0) an acceptable method and a negative value (< 0) an ineffective method.

RESULTS AND DISCUSSION

Characterization of imazamox resistance through the between-paper germination test

The development of an efficient method for herbicide resistance detection can be determined only by preliminary experimentation with known resistant and susceptible genotypes (Beckie et al., 2000). In this study, the response to imazamox was evaluated in six IMI-R and three IMI-S wheat cultivars. Figure 1 shows dose-response curves for shoot and root growth of seedlings obtained by the between-paper germination test. Herbicide concentrations required to reduce shoot and root growth by 50% (GR50) were statistically different between IMI-R and IMI-S cultivars (Table 1). Estimates of GR50 for shoot, total root and longest root length of IMI-R cultivars were > 99 -, > 75 - and > 101 -fold that of the IMI-S cultivars on average, respectively.

Table 1 - Parameters estimates and standard errors of the log-logistic equation describing the response of wheat seedlings to the herbicide imazamox in the between-paper germination test

Cultivar	GR50 (μM)	<i>b</i> (%control μM^{-1})
Shoot length		
Klein Titanio CL	835.8 \pm 98.8 a	2.0 \pm 0.6
Buck 55 CL2	371.0 \pm 69.1 b	1.0 \pm 0.2
Baguette560 CL	338.0 \pm 56.7 b	1.0 \pm 0.2
IMI-R single-gene cultivars	384.1 \pm 28.4 b	1.6 \pm 0.2
Susceptible cultivars	3.4 \pm 0.3 c	1.3 \pm 0.1
Total root length		
Klein Titanio CL	1078.5 \pm 126.6 a	3.3 \pm 3.8
Buck 55 CL2	938.9 \pm 169.1 a	1.3 \pm 0.4
Baguette560 CL	426.7 \pm 67.4 b	1.2 \pm 0.2
IMI- R single-gene cultivars	449.1 \pm 36.0 b	1.7 \pm 0.2
Klein Serpiente	9.4 \pm 1.9 c	1.1 \pm 0.2
SY 300	2.3 \pm 0.5 e	1.4 \pm 0.4
Baguette 9	5.2 \pm 1.1 d	1.1 \pm 0.3
Longest root length		
Klein Titanio CL	824.8 \pm 131.2 a	1.4 \pm 0.3
Buck 55 CL2	1293.1 \pm 327.8 a	1.1 \pm 0.3
Baguette560 CL	400.1 \pm 62.0 b	1.2 \pm 0.2
IMI- R single-gene cultivars	490.3 \pm 44.8 b	1.2 \pm 0.1
Klein Serpiente	2.2 \pm 0.4 d	1.2 \pm 0.2
SY 300	5.6 \pm 0.9 c	1.5 \pm 0.3
Baguette 9	4.0 \pm 0.9 c	0.9 \pm 0.2

A log-logistic model: $y = d / [1 + (x / e)^b]$ with upper limit (*d*) fixed at 100% was adjusted. Parameter *e* (also known as GR50) denotes the herbicide dose (*x*) that inhibited shoot or root length (*y*) by 50% and *b* denotes the relative slope around *e*. Data from similar type of cultivars were pooled when their curves did not differ ($p > 0.05$). Different letters after GR50 estimations within the same column indicate significant differences at $p < 0.05$. Three imidazolinone resistant (IMI-R) Argentinian commercial cultivars (Klein Titanio CL, Buck 55 CL2, Baguette 560 CL), three susceptible cultivars (Klein Serpiente, SY 300, Baguette 9), and three IMI-R cultivars with a single resistant gene (Fidel FS-4, BW755, TealIMI 11A) were analyzed

Seedling tests have been developed for several species to determine either coleoptile length or root length as growth parameters in order to discriminate between resistant and susceptible biotypes. Shoot or root length was used as an indicator for resistance to AHAS inhibitors in weeds (Kuk et al., 2008; Xu et al., 2010) and crops (Vega et al., 2009; Roso et al., 2010).

Herbicide response did not differ among IMI-R cultivars with a single resistant gene (Table 1). Similarly, a previous work showed that crop injury and yield in response to imazamox applications slightly differed in cultivars with a single resistant gene (Ball and Peterson, 2007).

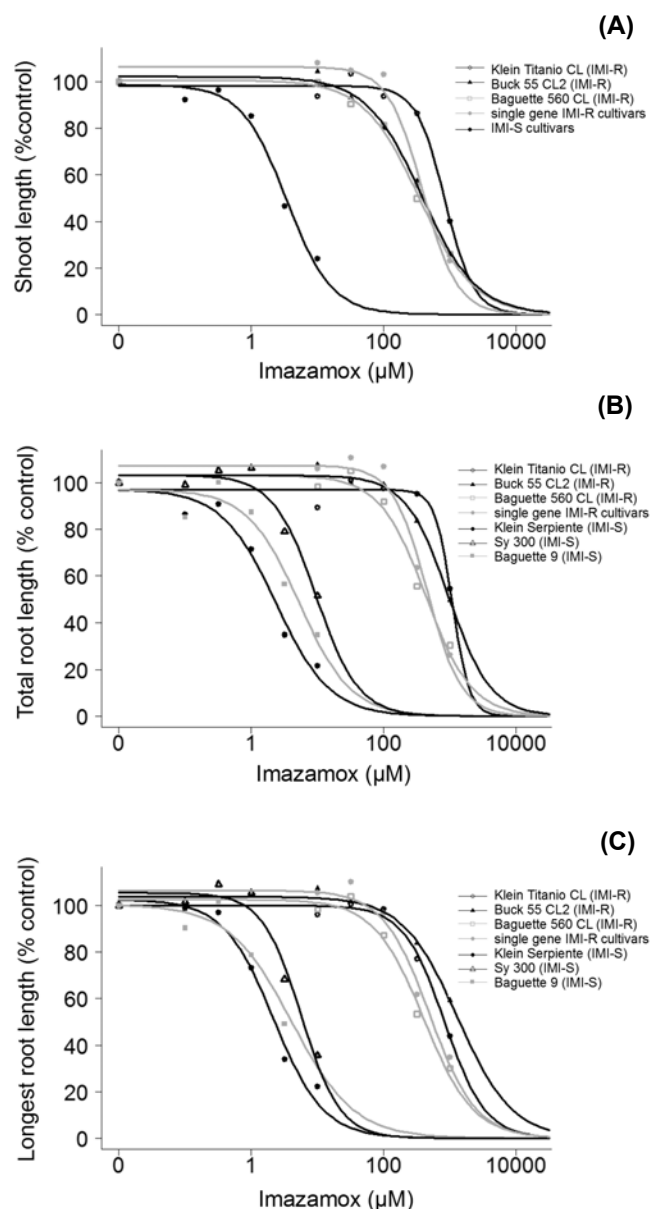
IMI-R cultivars with a single resistant gene showed lower GR50 values than some commercial IMI-R cultivars that stacked two resistance genes. Several works demonstrated that IMI resistance was affected by number of resistance genes, growth habit and other factors (Pozniak et al., 2004; Jimenez et al., 2016). The differences among cultivars could also be explained by

their different genetic backgrounds. The expression of the IMI resistance trait at germination level could be affected by biological factors such as growth rate and vigor. Rainbolt et al. (2005) evaluated the response to imazamox by an *in vivo* enzymatic assay and also found differences among IMI-R cultivars.

Considering that no differences were found between single gene IMI-R cultivars and one commercial IMI-R cultivar, this method could not be recommended for discriminating plants with different number of resistance genes. The usefulness of the method is limited to distinguishing between IMI-S and IMI-R plants carrying at least one resistance gene.

Quality estimation of the top-paper and between-paper germination tests

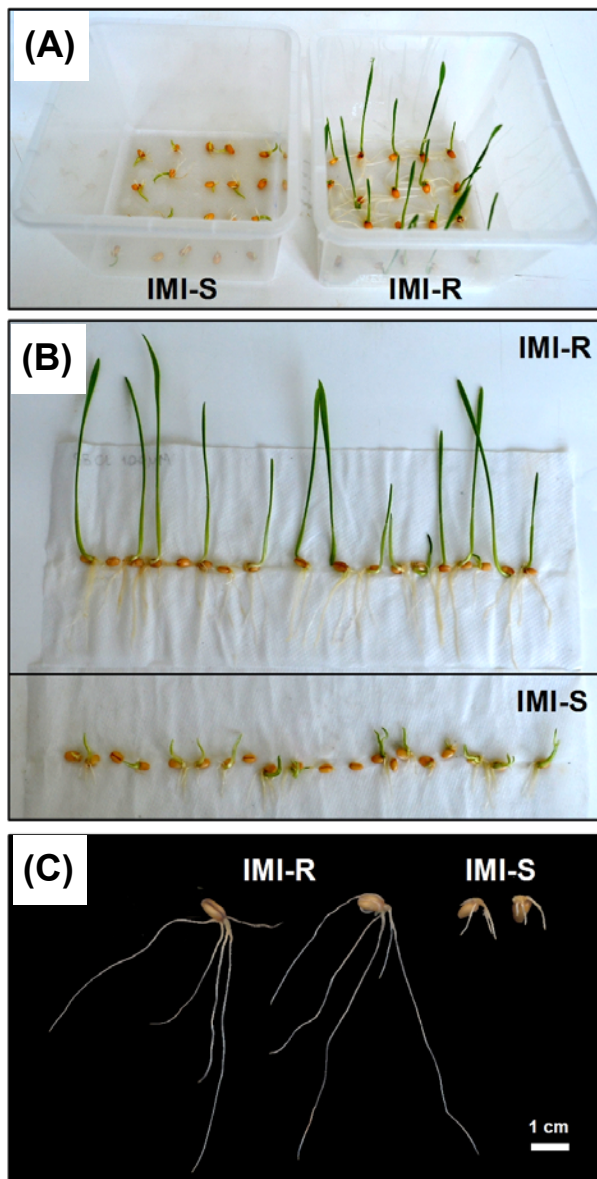
The largest difference in shoot and root length between IMI-R and IMI-S cultivars was found at 100 μM imazamox (Figure 1). Therefore, this concentration was selected to be used as a discriminating dose for quality estimation of the germination tests.



Estimated dose-response curves and means are shown for shoot length (A), total root length (B) and longest root length (C). Three imidazolinone resistant (IMI-R) and three susceptible (IMI-S) Argentinian commercial cultivars, and three IMI-R cultivars with a single resistant gene were analyzed. Data from similar type of cultivars were pooled when their curves did not differ.

Figure 1 - Response of wheat seedlings to the herbicide imazamox in the between-paper germination test.

Beckie et al. (2011) evaluated wheat seeds by a soil-less method that used a top-paper method for screening of imazamox resistance. In order to compare both germination methods, six commercial cultivars were evaluated by using the top-paper and between-paper methods in the presence of 100 μ M imazamox (Figure 2). The responses of IMI-R and IMI-S cultivars were similar for both germination methods (Figure 3).



Between-paper (A) and top-paper (B) germination tests at 100 μ M imazamox. Scanned roots of IMI-R and IMI-S plants treated with 100 μ M imazamox (C). IMI-R and IMI-S are imidazolinone resistant and susceptible Argentinian commercial cultivars, respectively.

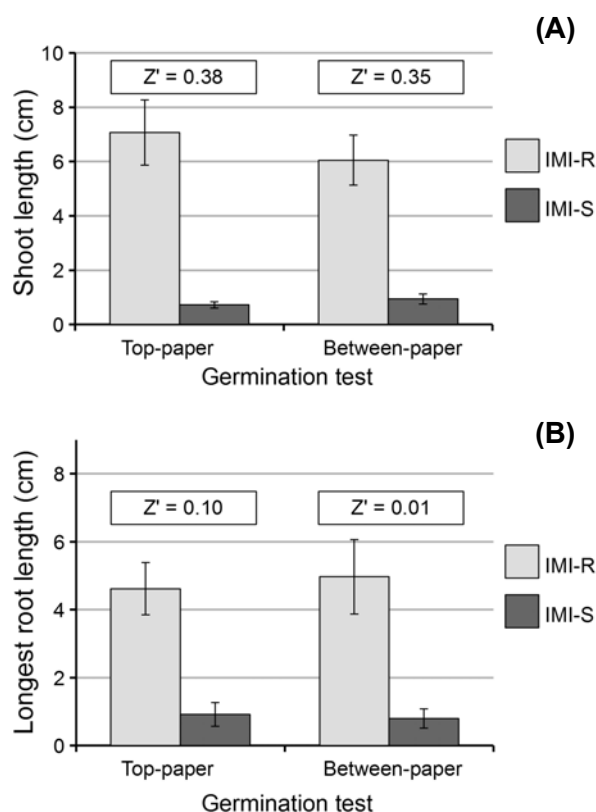
Figure 2 - Wheat germination test at 100 μ M imazamox.

The Z' -factor provides a useful tool for comparison and evaluation of the quality of screening methods (Zhang et al., 1999). Z' -factor values were > 0 for both germination tests, which indicates that the methods were acceptable. The difference of the IMI-R and IMI-S means was greater for shoot length with a higher Z' -factor value (Figure 3). These results suggested that this variable would be preferable for herbicide resistance selection. Other studies demonstrated that shoot length was a sensitive parameter in response to different herbicide doses and had a strong correlation with the response to plant-sprayed methods in wheat and other cereals (Escorial et al., 2001; Loureiro et al., 2001; Roso et al., 2010).

The visual inspections of root growth and shoot growth were useful parameters to distinguish between IMI-S and IMI-R cultivars. IMI-S seedlings could be identified by a reduced growth of roots and shoots, which in all cases were shorter than 1 cm (Figures 2, 3). Plants obtained from these methods could be easily transferred to soil to conclude their cycle in the greenhouse. The between-paper method occupied less space in the growth chamber and is more appropriate for screening many specimens.

Plant breeders are continuing to develop IMI-R wheat varieties adapted to different production regions. The between-paper germination test has the potential to be scaled-up for screening large numbers of cultivars or segregating populations. This soil-less method could accelerate selection for IMI resistance in backcrossing programs to develop new resistant wheat lines. This study only evaluates three types of cultivars: susceptible, homozygous for a single resistant gene and homozygous for two resistant genes; thus, further studies including other genotypic alternatives, especially those in heterozygous state, should be conducted to validate this protocol as a diagnostic screening test for assisting breeding programs.

Another important application of the method is the selection for IMI-R candidates after mutagenic treatments when screening for a new source of resistance. On other hand, the method presented here could be used in gene flow studies and in seed quality testing to assess wheat resistance to the herbicide imazamox.



IMI-R and IMI-S are imidazolinone resistant and susceptible Argentinian commercial cultivars, respectively. Vertical bars represent standard deviation of the mean.

Figure 3 - Quality of the germination tests at 100 μ M imazamox estimated by Z' factor determination.

ACKNOWLEDGEMENTS

We are grateful to seed companies Criadero Klein S.A., Buck Semillas S.A., Nidera Semillas S.A. and BASF Argentina S.A. for providing seed material and the commercial herbicide. The financial support from Agencia Nacional de Promoción Científica y Tecnológica y el Fondo Nacional de Promoción Científica y Tecnológica (PICT-2015-1325) is also acknowledged. Special thanks are given to PhD María Laura Mayor for detailed language edition.

REFERENCES

- Ball DA, Peterson CJ. Herbicide tolerance in imidazolinone-resistant wheat for weed management in the Pacific Northwest U.S.A. In: Buck HT, Nisi JE, Salomón N editors. *Wheat production in stressed environments*. Dordrecht: Springer; 2007. p.243-50.
- Beckie HJ, Heap IM, Smeda RJ, Hall LM. Screening for herbicide resistance in weeds. *Weed Technol.* 2000;14(2):428-45.
- Beckie HJ, Warwick SI, Sauder CA, Hall LM, Harker KN, Lozinski C. Pollen-mediated gene flow in commercial fields of spring wheat in Western Canada. *Crop Sci.* 2011;51:306-13.
- Burgos NR. Whole-plant and seed bioassays for resistance confirmation. *Weed Sci.* 2015;63:152-65
- Duggleby RG, McCourt J, Guddat LW. Structure and mechanism of inhibition of plant acetohydroxyacid synthase. *Plant Physiol Biochem.* 2008;46:309-24.
- Escorial MC, Sixto H, Gracia-Baudin JM, Chueca MC. A rapid method to determine cereal plant response to glyphosate. *Weed Technol.* 2001;15:697-702.

- Giménez JP, Istilart MC, Giménez DO, Yannicari ME. Evaluación de la residualidad de imidazolinonas sobre la implantación de trigo, avena y alfalfa/ryegrass luego del cultivo de girasol imi-tolerante. In: Forján HJ, López Z, Domingo Yaguez JC editores. Actualización técnica en cultivos de cosecha fina 2013/14 Actualización técnica en cultivos de cosecha fina 2013/14. Tres Arroyos: Ediciones INTA; 2014. p.119-23.
- The International Seed Testing Association - ISTA. The germination test. In: International rules for seed testing. Bassersdorf: The International Seed Testing Association; 2014.
- Jimenez F, Rojano-Delgado AM, Fernández PT, Rodríguez-Suárez C, Atienza SG, De Prado R. Physiological, biochemical and molecular characterization of an induced mutation conferring imidazolinone resistance in wheat. *Physiol Plant*. 2016;158:2-10.
- Kuk YI, Burgos NR, Shivrain VK. Natural tolerance to imazethapyr in red rice (*Oryza sativa*). *Weed Sci*. 2008;56:1-11.
- Loureiro I, Avendaño N, Escorial MC, García-Baudin JM, Chueca MC. . Método rápido de evaluación del efecto del herbicida dalapon en cereales. *Invest Agr Prod Prot Veg*. 2001;16(3):463-9.
- Newhouse KE, Smith WA, Starrett MA, Schaefer TJ, Singh BK. Tolerance to imidazolinone herbicides in wheat. *Plant Physiol*. 1992;100:882-6.
- Pound MP, Pound MP, French AP, Atkinson JA, Wells DM, Bennett MJ, Pridmore T. RootNav: navigating images of complex root architectures. *Plant Physiol*. 2013;162:1802-14.
- Pozniak CJ, Hucl PJ. Genetic analysis of imidazolinone resistance in mutation-derived lines of common wheat. *Crop Sci*. 2004;44:23-30.
- Pozniak CJ, Birk IT, O'Donoghue LS, Menard C, Hucl PJ, Singh BK. Physiological and molecular characterization of mutation-derived imidazolinone resistance in spring wheat. *Crop Sci*. 2004;44:1434-43.
- R Core Team. R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2016.
- Rainbolt CR, Thill DC, Zemetra RS, Shaner DL. Imidazolinone-resistant wheat acetolactate synthase in vivo response to imazamox. *Weed Technol*. 2005;19:539-48.
- Ritz C, Baty F, Streibig JC, Gerhard D. Dose-response analysis using R. *PLoS One*. 2015;10:e0146021.
- Roso AC, Merotto JR, Delatorre CA. Bioensaios para diagnóstico da resistência aos herbicidas imidazolinonas em arroz. *Planta Daninha* 2010;28:411-9.
- Schneider C, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 2012;9:671-5.
- Scursoni JA, Gigón R, Martín AN, Vigna M, Leguizamón ES, Istilart C et al. Changes in weed communities of spring wheat crops of Buenos Aires Province of Argentina. *Weed Sci*. 2014;62:51-62.
- Seefeldt SS, Jensen JE, Fuerst EP. Log-logistic analysis of herbicide dose-response relationships. *Weed Technol*. 1995;9:218-27.
- Shaner DL, Stidham M, Singh B, Tan S. Imidazolinone herbicides. In: Krämer W, Schirmer U, Jeschke P, Witschel M editors. *Modern crop protection compounds. Acetohydroxyacid synthase inhibitors (AHAS/ALS)*. 2nd. ed. Weinheim: Wiley-VCH Verlag GmbH & Co. KGaA; 2012. p.88-98.
- Tan S, Evans RR, Dahmer ML, Singh BK, Shaner DL. Imidazolinone-tolerant crops: history, current status and future. *Pest Manag Sci*. 2005;61(3):246-57.
- Vega T, Breccia G, Nestares G, Mayor ML, Zorzoli R, Picardi L. Soil-less bioassays for early screening for resistance to imazapyr in sunflower (*Helianthus annuus* L.). *Pest Manag Sci*. 2009;65(9):991-5.
- Xu X, Wang GQ, Chen SL, Fan CQ, Li BH. Confirmation of flixweed (*Descurainia sophia*) resistance to tribenuron-methyl using three different assay methods. *Weed Sci*. 2010;58:56-60.
- Zhang JH, Chung TD, Oldenburg KR. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J Biomol Screen*. 1999;4:67-73.