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Short Communication

Innovative *in vitro* protocol for the screening of acetolactate synthase (ALS) herbicide resistance in Indian mustard (*Brassica juncea* L)

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Abstract

Development of herbicide resistance is one of the essential traits for the development of high yielding varieties. The Indian mustard plants show high sensitivity towards pre and post emergence herbicides, thus limiting the effective use of herbicides in the mustard crop. The acetolactate synthase (ALS) class herbicides are widely used in many cropping systems worldwide because of the availability of wide range of herbicides (sulfonylurea, metsulfuron, imidazolinone, triazolopyrimidine, pyrimidinyl (thio) benzoate, sulfonyl-aminocarbonyl-triazolinone). Consequently, it is crucial to address the development of ALS class herbicide resistance in Indian mustard. The important mechanisms for developing herbicide resistance in crops are hybridization, mutation, transgenics and genome editing. Crucial to these approaches is testing and selection for herbicide tolerance or resistance. However, conducting field screenings for herbicide resistance poses challenges due to its resource-intensive and time-consuming nature, compounded by the difficulty of off-season screenings, which restrict the progression of herbicide resistance development programs. Consequently, we have devised an *in vitro* screening protocol specifically for the ALS class herbicide (metsulfuron), facilitating efficient and timely identification of herbicide resistance traits in Indian mustard.

Keywords: *Indian mustard, ALS herbicide, herbicide resistance, in vitro screening, protocol*

Introduction

The global utilization of herbicides in crop production is on the rise. Emerging nations are quickly adopting herbicide solutions, dealing with manual weeding labor shortages and the need to increase crop yields (Gianessi, 2013). Enhanced weed management through herbicides holds significant promise for substantially boosting crop yields in the foreseeable future. Moreover, the optimum use of herbicides fosters the proper utilization of fertilizers, consequently enhancing the crop yield. In case of Indian mustard, some reports are available for the application of pre-emergence herbicides *viz* atrazine, diuron, isoproturon, oxyfluorfen, metolachlor, pendimethalin and fluchloralin (Yaduraju, 2000; Yadav, 2004; Chauhan *et al.,* 2005). However, higher doses produced toxic effects on mustard plants leading to yield losses (Sarkar *et al.,* 2005; Vercampt, 2017). Also, due to phytotoxic effects on mustard plants, no post-emergence herbicide is recommended in mustard crop in India. Studies showed that the application of post-emergence herbicide reduces the weed population but decreased plant height and seed yield due to its phytotoxic effects (Awan *et al.,* 2006; Bharat *et al.,* 2022). Therefore, development of herbicide resistant Indian mustard varieties is becoming important to increase its production.

Herbicides intended to block the enzyme acetolactate synthase (ALS) are among the most widely used class of herbicides globally (Tranel and Wright, 2002). There are many herbicides belonging to this category including sulfonylurea, metsulfuron, imidazolinone, triazolopyrimidine, pyrimidinyl (thio) benzoate, sulfonylaminocarbonyl-triazolinone, targeting ALS enzyme with different mode of action. Therefore, development of ALS herbicide-resistant Indian mustard genotypes is crucial for bolstering overall edible oil production. By creating mustard varieties resilient to ALS herbicides, farmers can enhance crop yield and ensure sustainable cultivation practices. This resistance enables efficient weed control, contributing to improved mustard crop performance and increased agricultural output.

The herbicide resistant trait can be developed in crops by using different methods *viz* hybridization, mutation breeding, transgenics and genome editing (Lombardo *et al.,* 2016; Hussain *et al.,* 2021). Screening for herbicide resistance/tolerance is an essential activity of these methods. However, the field screening for herbicide resistance has its limitations that it is resource and time consuming and off season screening is very difficult, limiting the entire herbicide resistance development program. Therefore, we have developed an *in vitro* screening protocol for the ALS class herbicide metsulfuron for effective and timely screening for herbicide resistance traits in Indian mustard [*Brassica juncea* L. (Czern. and Coss.)].

Material and Methods

Plant material

The experimental material consisted of two popular high-yielding cultivars DRMRIJ-31 and NRCHB-101 of Indian mustard (*B. juncea*) and one newly developed herbicide resistant line DRMRHR-2-1.

Preparation of herbicide dilution

A stock solution of 1 g/L of herbicide (metsulfuron) was prepared in double distill water (ddH_2O) and filter sterilized with 0.2 micron filters to remove any contamination. This stock solution was subsequently used to prepare a series of ten dilutions with concentrations of 0.12 mg/L, 0.25 mg/L, 0.62 mg/L, 1.25 mg/L, 1.87 mg/L, 2.5 mg/L, 5.0 mg/L, 12.5 mg/L, 25 mg/L, and 50 mg/L. These specific concentrations were defined as the treatments used in this study. The prepared dilutions were stored at room temperature in brown bottles until further use.

Seed sterilization

Mature and healthy seeds of all three samples were used for sterilization. Around 250 mg seeds were weighed and placed in conical flask. Seeds were initially washed with ddH_2O after which they were subjected for 5 min treatment in 70 % ethyl alcohol with continuous stirring. The solution was removed and seeds were again washed thrice with ddH_2O. After washing seeds were let to dry for 30 minutes or till complete removal of moisture.

Basal media preparation and seed inoculation

To prepare basal growth media for the germination of seeds, Murashige and Skoog (MS) medium (Himedia, India) was used supplemented with 30 g/L sucrose and 8 g/L agar. After preparation, media was autoclaved and different dilutions of herbicide were added. Media was poured in plastic phyta jars and placed in light at 25°±2 °C for two days. After two days incubation of media, 16 sterilized seeds per jar were inoculated for each concentration in 3 replications. Inoculated seeds were placed in again similar environment for 10 days for further germination.

Germination percentage and seedling growth

To estimate the effect of herbicide on seed, germination percentage was calculated by using following equation (Li, 2008).

$$\text{Germination percentage (G \%)} = n / N \times 100$$

Where, n is the number of germinated seed at the tenth day; N is the number of total seeds.

All sprouted seeds were considered as germinated either the resulting seedlings were showed normal or stunted growth. The seedling growth was estimated by measuring the height of seedling at ten days post inoculation. The data analysis was conducted using a two-way ANOVA, utilizing MS Excel (Microsoft Office Professional Plus). This statistical method was chosen to evaluate the impact of two independent variables and their interaction on the dependent variable.

Results and Discussion

For effective and precise screening for resistance against herbicide (metsulfuron), an inovative *in vitro* screening protocol was developed. The *in vitro* screening for ALS class herbicide 'metsulfuron' was standardized on two high yielding Indian mustard varieties *viz* DRMRIJ-31 and NRCHB-101 at difference concentrations of the ALS class herbicide (metsulfuron). Ten different concentrations (0.12, 0.25, 0.62, 1.25, 1.87, 2.5, 5.0, 12.5, 25 and 50 mg/L) were used to study the effect of herbicide on the germination and growth of these two varieties. The germination percent for both the varieties showed no significant difference for different doses of the herbicide (Fig. 1A), however, the growth of both the genotypes was hampered with increasing doses of herbicide (Table 1). The growth of the seedlings was assessed by measuring its height, which showed a negative correlation with the increasing doses of the herbicide (Fig. 1B). The rate of germination was found similar in both the genotypes against ten different concentrations of the herbicide. The stored food (amino acids) in th endosperm of the seeds may cause the non lethal effect of the herbicide on the seed germination. However, for seedling growth, branched-chain amino acids (valine, leucine, and isoleucine) were unavailable due to inactivation of acetolactate synthase enzyme (crucial in branched-chain amino acid biosynthesis) by the herbicide. In absence of these branched-chain amino acids, the growth of the mustard seedlings were checked. Zargar *et al.* (2021) also showed the lethal effect of metsulfuron on wild mustard in combination with other ALS class herbicides.

The mean seedling height for DRMRIJ-31 and NRCHB101 in control was 8.16 and 8.62 cm respectively. With increasing herbicide doses, a linear reduction in seedling height was observed. No seedling growth was observed at 25 and 50 mg/L doses of herbicide, marking its maximum lethal effect. Therefore, the 25 mg/L concentration was used for further screening. The seedling growth of herbicide resistant genotype DRMRHR-2-1 was normal whereas susceptible cultivars showed poor growth at 25 mg/L dose fo the herbicide. Similarly, Koch *et al.,* (2012) have developed in vitro protocol for screening of herbicide (imazapyr) tolerant sugercan.

The seeds of three genotypes (two susceptible genotypes *i.e.* DRMRIJ-31, NRCHB101 and one resistant mutant DRMRHR-2-1) were in vitro screened at 25 mg/L dose of herbicide. The germination percentage had no

Table 1: Analysis of variance for 11 treatments of herbicide in 2 genotypes of Indian mustard

Source of variation	df	Germination rate (%)	Plant height (cm)
Replication	10	7.04	14.5*
Treatment	1	3.22	0.00
Error	10	2.72	0.02
Analysis of variance for 2 treatments of herbicide in 3 genotypes of Indian mustard			
Replication	1	0.73	46.93
Treatment	2	0.00	8.76
Error	2	2.93	9.7

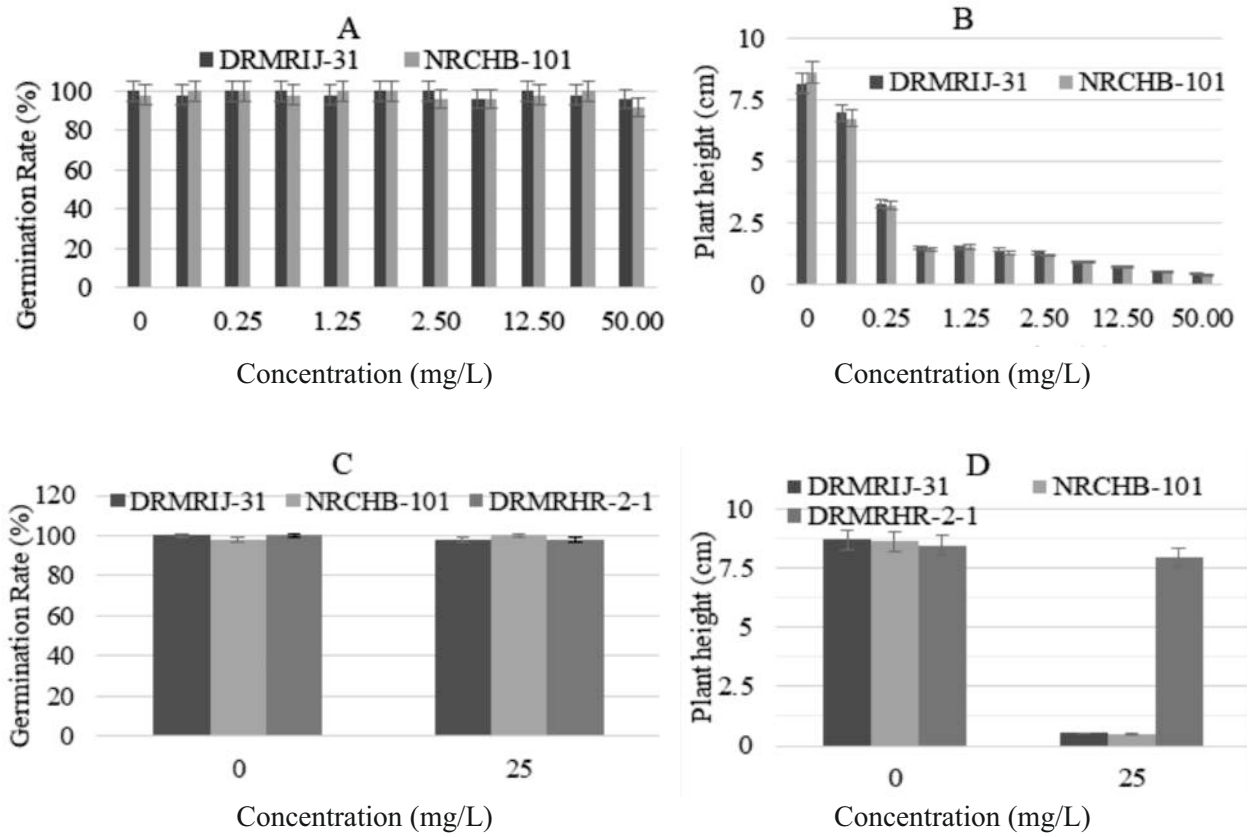

Fig. 1: A) Germination percentage of genotypes DRMRIJ-31 and NRCHB-101 at different doses of herbicide; B) Reduction in seedling height with increasing doses of herbicide; C) Germination percentage of genotypes DRMRIJ-31, NRCHB-101 and DRMRHR-2-1 at 25 mg/L; D) DRMRHR-2-1 genotype showed resistance for the herbicide at 25 mg/L

significant difference among these genotypes and mean seedling height in control was also similar for the three genotypes (DRMRIJ-31, NRCHB101 and DRMRHR-2-1) 8.69, 8.63 and 8.47 cm respectively. However, for seedling height in 25 mg/L herbicide dose, drastic reduction was observed in DRMRIJ-31 (0.53 cm) and NRCHB101 (0.51 cm) (Fig. 1C). The herbicide resistant mutant DRMRHR-2-1 showed significantly higher mean seedling growth (7.69 cm) in comparison to susceptible genotypes.

Different *in vivo* and *in vitro* screening protocols have been developed for the screening for herbicide resistance in many weed and crops species (Beckie *et al.,* 2000; Panozzo *et al.,* 2015). However, this innovative *in vitro* screening protocol for herbicide resistance is precise, controlled, effective and season independent.

Conclusion

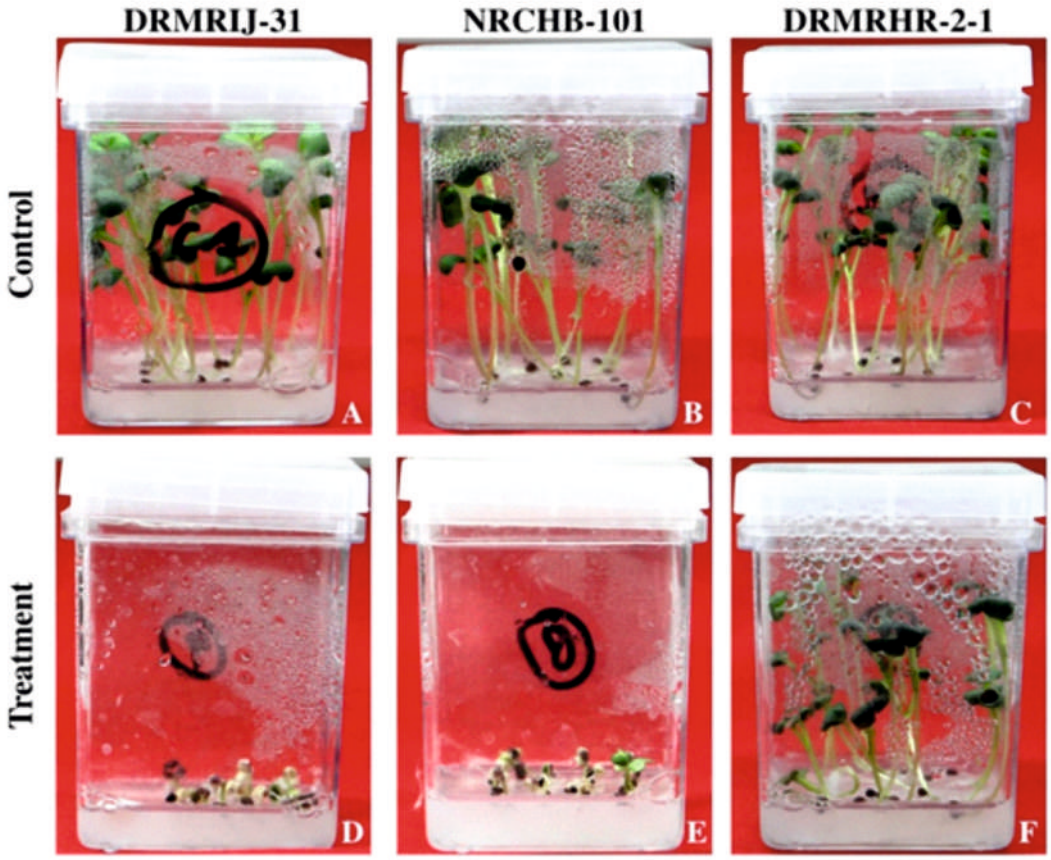

Fig. 2: In vitro screening for herbicide resistance in Indian mustard. Figure 2A, 2B and 2C show the growth of Indian mustard genotypes DRRMIJ-31, NRCHB-101 and DRMRHR-2-1 without the application of herbicide at 10 days post inoculation (DPI). Figure 2D, 2E and 2F show the growth of these genotypes at 10 DPI. The lethal effect of herbicide was revealed in DRRMIJ-31 (D) and NRCHB-101 (F) whereas herbicide resistant genotype DRMRHR-2-1 displayed high level of resistance (F)

A new *in vitro* screening protocol for ALS class herbicide 'metsulfuron' was developed using two Indian mustard varieties. Seedling growth was negatively affected with increasing herbicide doses, with complete inhibition observed at 25 and 50 mg/L. The 25 mg/L concentration was chosen for standard screening, revealing significant reduction in seedling height for susceptible genotypes but normal growth for the herbicide-resistant mutant DRMRHR-2-1. The protocol, deemed precise and effective, provides a controlled, season-independent method for herbicide resistance screening in mustard, contrasting with previous *in vivo* and *in vitro* methods.

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