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Morpho-Biochemical Responses of Brassica Coenospecies to Glyphosate Exposure at Pre- and Post-Emergence Stages

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Abstract: Crop wild relatives (CWRs) belonging to the Brassicaceae family possess extensive genetic diversity and have frequently been utilized in the enhancement of cultivated *Brassica* species. However, their tolerance to glyphosate, a widely used herbicide, has remained unknown. Our study examined the glyphosate response of 20 genotypes from the Brassicaceae family, which included genotypes within the U triangle and their wild relatives. We evaluated their behaviour based on morpho-biochemical responses, specifically focusing on the traits of germination percentage, root length, and survival percentage. By calculating the mean membership function value (MFV) for each genotype's response to these traits, we classified them into three distinct groups: susceptible, moderately tolerant, and tolerant. Among these genotypes, *Brassica rapa* (NRCPB rapa 8) demonstrated tolerance to glyphosate, as indicated by their mean MFV value of 0.68. Moderate tolerance to glyphosate was observed in *Brassica juncea* (Pusa Jaikisan) with a mean MFV of 0.52. Conversely, *Diplotaxis catholica*, *Diplotaxis muralis*, and *Enarthrocarpus lyratus* were susceptible, with mean MFV values of 0.37, 0.35, and 0.34, respectively. These findings revealed varying levels of response to glyphosate among these genotypes, with some displaying significant tolerance. The study provides valuable insights into the herbicide tolerance of Brassica CWRs and emphasizes the potential use of phenotypic and biochemical markers in evaluating herbicide tolerance.

Keywords: herbicide tolerance; glyphosate; crop wild relative; *Brassica*

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1. Introduction

Glyphosate is a widely used herbicide that targets a broad range of vegetation. It was first introduced in 1974 and used for controlling weeds in plantation crops, no-tillage systems, and non-agricultural areas [1]. Glyphosate is a highly water-soluble herbicide that quickly penetrates into the plant leaves and is transported symplastically throughout the plant through the phloem stream [2]. In the shikimate pathway, the herbicide inhibits a specific enzyme EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) [3]. This enzyme is vital for converting PEP (phosphoenol pyruvate) and shikimate 3-phosphate into EPSP and inorganic phosphate. Inhibition of EPSPS disrupts the biosynthesis of aromatic amino acids, like phenylalanine, tyrosine, and tryptophan, in the shikimate pathway. It is estimated that ~60% of a plant's dry weight comprises molecules produced through the shikimate pathway [4]. Additionally, plants in the Brassicaceae family, which include crucifers, use

aromatic amino acids to make aromatic glucosinolates, a group of bioactive compounds that give pungency to mustard oil, cabbage, horseradish, etc. [5–7]. Therefore, glyphosate exposure can cause a chain reaction that affects the production of aromatic amino acids and their derivatives. The widespread use of glyphosate in the farming system has led to the emergence of glyphosate-resistant weeds [8,9]. This is classified as either target-site or non-target-site resistance [9,10].

Target-site resistance in glyphosate-resistant weeds is caused by mutations in the EPSPS gene, specifically at the amino acid positions of threonine, alanine, and proline [11]. These mutations prevent the herbicide from binding to the target location, reducing its effectiveness on the EPSPS enzyme [11,12]. Non-target site resistance mechanisms decrease the amount and speed of herbicide accumulation at the target location [10]. This type of resistance can be caused by a variety of factors, such as decreased herbicide penetration into the plant, reduced uptake or translocation, increased sequestration or metabolism of the herbicide, or a combination of these mechanisms. These factors can decrease herbicide efficacy, resulting in non-target site resistance [13]. Previously, the evolution of glyphosate resistance in plants was rare [14] due to various reasons, such as the limited metabolism of glyphosate in plants, short half-life in the environment, and unique biochemical characteristics of the herbicide. Additionally, engineering glyphosate resistance in crops requires complex molecular modifications [15]. However, some plant species and biotypes within the species possess natural resistance to glyphosate. This variation in resistance levels among different plant species and biotypes is a significant factor in the evolution of glyphosate resistance [16].

Managing Orobanche, an achlorophyllous holoparasite, *Orobanche aegyptiaca*, poses significant challenges due to its underground location, close physical attachment to the host plant, prolific seed production, and the long viability of seeds in the soil over multiple years. Studies conducted in India by Punia [17,18] have demonstrated the efficacy of glyphosate sprays in mitigating Orobanche infestations in mustard fields. Recently, glyphosate resistance transgenic lines in *Brassica juncea* variety Varuna were developed using three genes, i.e., cp4 esps, gox, and gat, for their potential use in agriculture [19].

Several studies have focused on evaluating herbicide tolerance in various crop species and weeds based on physiological and biochemical responses. For instance, in a recent study, the effect of broad-spectrum herbicide weedlock was investigated on *Ageratum conyzoides* L., *Eleusine indica* (L.) Gaertn, *Zea mays* L., and *Amaranthus gangeticus* L., which were treated at a concentration of 672.75 L ha^{-1} at different time points [20]. They observed significant reductions in chlorophyll content and inhibition of photosynthesis, which was highest at 24 h post-treatment. In addition, phytotoxic stress was indicated by a rise in the production of malondialdehyde (MDA), proline, and other antioxidant enzymes in the plants treated with weedlock herbicide. In another study, Juan et al. [21] assessed the responses of the 2,4-D herbicide-tolerant biotype of *Brassica rapa* using different formulations of 2,4-D and other auxin herbicides. Their findings elucidated the dose required to achieve 50% inhibition of survival for different formulations.

Here, we investigated the behaviour of 20 genotypes from the Brassicaceae family to glyphosate in order to identify potential tolerant species. Morphological data analysis and biochemical assays were carried out at pre- and post-emergence stages to assess the tolerance across the genotypes. Identifying potential herbicide tolerance in genotypes of the Brassicaceae family paves the way for developing herbicide-resistant genotypes in cultivated species through a crop breeding program.

2. Materials and Methods

2.1. Experimental Material

Twenty (20) genotypes of the *Brassica* species, including 15 wild species and five U triangle species, were used in this study. The details of the wild species were described previously by Kashyap et al. [22]. Prior to the treatment, the seeds of the genotypes were cleaned and surface-sterilized with sodium hypochlorite 1% (*w*/*v*) for 10 min, rinsed twice

with distilled water, and air-dried before planting. For the glyphosate treatment, Roundup Monsanto (Bayer, India) (41% glyphosate), a commercial source of glyphosate, was used to prepare a 100 mg L^{-1} solution.

2.2. Germination Test (Pre-Emergence)

A germination test was used to determine herbicide resistance among different genotypes with slight modifications [23]. The experiments were conducted using polystyrene Petri dishes (90 mm diameter) containing a single sheet of germination paper, with 3 technical replicates per treatment. A glyphosate solution of 100 mg L^{-1} was applied to saturate the germination sheets, and 30 seeds of each genotype were placed in a separate Petri dish for germination. In another set, seeds grown in Petri dishes with germination paper saturated with distilled water were considered a control. Seeds were initially kept in the dark for two days at 25 ± 1 °C, then transferred to a growth chamber set at a temperature of 25 ± 1 °C having 16/8 h light/dark cycle. The germination percentage and root and shoot length of treated and control seedlings were measured on the 6th d after treatment in replicated manner. Samples for biochemical assays were also collected on the same day. The germination percentage was calculated as follows:

The total number of seeds germinated to the total number of seeds placed is known as the germination percentage. It was calculated using the formula:

$$\text{Germination percentage (GP)} = \frac{\text{no. of seeds germinated}}{\text{total number of seeds placed}} \times 100$$

2.3. Spray Test (Post-Emergence)

In a separate experiment, spray tests on 10 days old *Brassica* seedlings were conducted in a greenhouse. The surface sterilized seeds were sown directly in plastic pots (6.5 cm in height and 7.5 cm in diameter) containing a growth medium of equal parts garden soil and cocopeat. Each pot contained 30 seeds, and each genotype was sown in a replicated manner in triplicate. The plants were grown at a temperature of 25 ± 1 °C 16/8 with an 8 h light/dark cycle and watered as needed, simulating normal field conditions when glyphosate is applied. After 10 days, they were sprayed with a 100 mg L^{-1} glyphosate solution using an indoor sprayer. Visual mortality ratings were recorded daily after treatment, and samples for biochemical assay were collected along with untreated control from all genotypes after 6 days of treatment in a replicated manner. The survival percentage was calculated as:

The total number of plants per pot was recorded before and after 6 days of the glyphosate application. Survival percentage was calculated using the formula:

$$\text{Survival Percentage (SP)} = \frac{\text{no. of seedlings survived}}{\text{total number of seedlings}} \times 100$$

2.4. Biochemical Assay: Ascorbate Peroxidase (APX)

The activity of ascorbate peroxidase (APX) was measured using the protocol described by Chen & Asada [24] using the assay buffer consisting of 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM ascorbic acid, and 0.1 mM hydrogen peroxide. One ml of enzyme extract was added to the buffer. The absorbance of the reaction mixture was then measured at 290 nm using a UV-VIS spectrophotometer (Evolution 300 UV-VIS, Thermoscientific, Crawley, UK). The decrease in absorbance at 290 nm was used to determine the oxidation of ascorbic acid [24].

2.5. Protein Estimation

For protein estimation, 1 g of tissue was crushed in a chilled buffer containing 50 mM phosphate buffer (pH 7.8), 1 mM DTT (dithiothreitol), 2 mM EDTA (ethylenediaminetetraacetic acid), 1 mM PMSF (phenylmethylsulfonyl fluoride), 10% w/v PVPP-40 (polyvinyl polypyrrolidone), and 0.5% (v/v) TritonX-100. The homogenate was then centrifuged

for 20 min at 12,000 rpm, and the supernatant was used for further analysis. Total protein was determined using Bradford protein assay, with BSA (bovine serum albumin) as the standard.

2.6. Tolerance Index and Membership Function Value (MFV)

The tolerance index (TI) was calculated based on shoot length (SL), root length (RL), and the biochemical parameters of the genotypes under controlled and treatment conditions according to [25,26]. The following equation was used for the calculation:

$$\mathrm{TI}ij = \frac{X_s^{ij}}{X_{ns}^{ij}}$$

where TIij is the tolerance index of the trait (j) for the genotype (i), and X_s^{ij} and X_{ns}^{ij} are the values of the trait (j) for the genotypes (i) obtained under stressed (s) and non-stressed conditions (ns), respectively.

The stress tolerance index was derived by calculating the membership function value (MFV) using the following equations [25].

If a trait is positively correlated with tolerance, then

$$\mathrm{U}ij = \frac{\mathrm{TI}_{\ ij} - \mathrm{TI}_{j\ min}}{\mathrm{TI}_{j\ max} - \mathrm{TI}_{j\ min}}$$

If a trait is negatively correlated with tolerance, then

$$\mathrm{U}ij = 1 - \frac{\mathrm{TI}_{\ ij} - \mathrm{TI}_{j\ min}}{\mathrm{TI}_{j\ max} - \mathrm{TI}_{j\ min}}$$

where Uij is the MFV of the trait (j) for genotype and (i) for tolerance; $TI_{j\ min}$ and $\mathrm{TI}_{j\ max}$ are the minimum and maximum values, respectively, for the tolerance index (TIij) for the trait, and (j) is the tolerance index for genotype (i). Then, the mean value of the MFV obtained from different traits was calculated, and the genotype's tolerance was determined according to the average mean MFV values [25].

2.7. Statistical Analysis

The post hoc Duncan's multiple range test (DMRT) was used to compare the treatment mean values, with significance at $p < 0.05$, and was performed using SAS. Multivariate cluster analysis of various genotypes was performed with SPSS 20.0 based on Ward's algorithm, and principal component analysis (PCA) was performed with R version 4.2.3.

3. Results

3.1. Pre-Emergence and Post-Emergence Effects of Glyphosate

To analyse the pre- and post-emergence tolerance of the genotypes to glyphosate, phenotypic characters such as germination percentage, shoot length, root length and survival percentage were recorded 6 days after the treatment. A significant variation in glyphosate tolerance levels was observed among the various wild relatives and U triangle species of Brassica, with decreases ranging from 33% to 90% in germination percentage, 22% to 86% in shoot length, 65% to 93% in root length, and 20% to 63% in survival percentage.

These genotypes exhibited differences in traits and were ranked between 1 to 20 for each trait based on the percentage decrease calculated by comparing the trait studied under exposure to glyphosate with the control (Figure 1 and Table 1). The lowest decrease in germination percentage was observed in *Crambe abyssinica* (EC694145) (33.5%) and *Crambe abyssinica* (EC400058) (36.6%), and these two genotypes ranked as 1 and 2 in terms of higher tolerance, respectively. Besides these genotypes, a decrease in germination percentage was observed in *B. rapa* (NRCPB rapa 8) (39.7%), *B. nigra* (EC472708) (40%), and *B. carinata* (PC-6) (43.3%), which were ranked as 3–5, respectively (Table 1).

The maximum decrease in germination percentage was observed in *Diplotaxis muralis* (90%), *Diplotaxis catholica* (85%), and *Enarthrocarpus lyratus* (72.9%), which ranked as 18–20, respectively (Table 1).

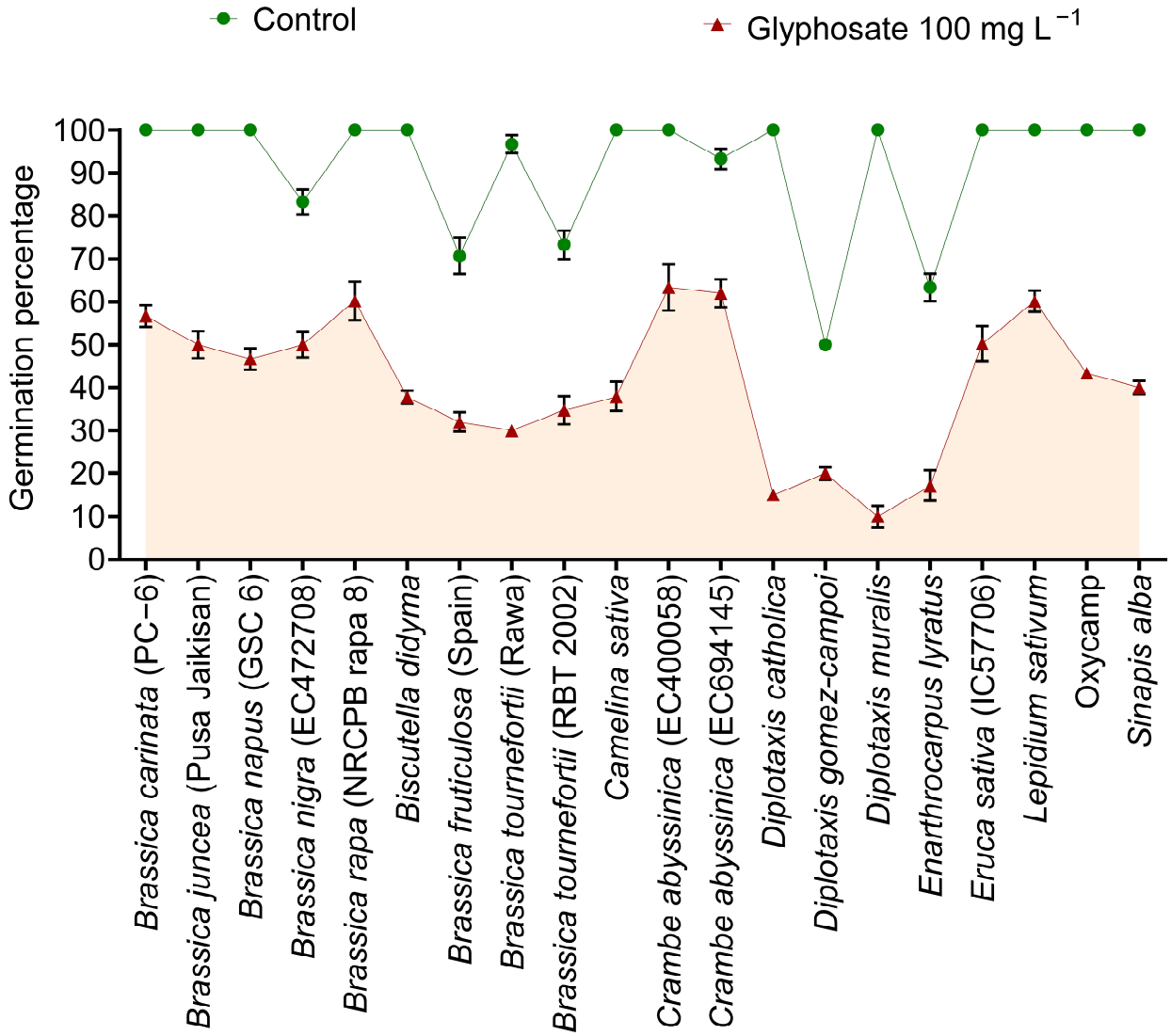

Figure 1. Effect of glyphosate (100 mg L^{-1}) on seed germination.

The shoot growth was less sensitive to glyphosate compared to the root growth in seedlings, which can be used as an identification mark for glyphosate-tolerant genotypes. Data were expressed as a percentage reduction of the shoot and root length based on the untreated control for the respective genotypes. The lowest decrease in shoot length was obtained for *B. nigra* (EC472708) (22.1%), *Diplotaxis muralis* (26.9%), and *Crambe abyssinica* (EC694145) (35.8%) (ranked as 1 to 3) (Tables 1 and 2). In contrast, the highest decrease in shoot length was found for *Camelina sativa* (75%), *Brassica fruticulosa* (Spain) (76%), and *Eruca sativa* (IC57706) (86.3%) (ranked as 18 to 20). On the other hand, *Diplotaxis catholica* (65.71%), *Diplotaxis muralis* (71%), and *Brassica tournefortii* (RBT 2002) (75.6%) (ranked as 1 to 3) showed the lowest change in root length, indicating that these genotypes exhibited the higher tolerance to glyphosate (Table 1). Based on inhibition of root length, the most glyphosate susceptible genotypes were Eruca sativa (IC57706) (93.1%), *Crambe abyssinica* (EC694145) (93.2%), and *B. napus* (GSC 6) (93.7%) (ranked as 18 to 20) (Tables 1 and 2).

Table 1. Ranking of genotypes based on the percentage decrease in shoot length (SL), root length (RL), and germination percentage (GP) in along with visuals scoring of survival percentage (SP) after 6 days of pre- and post-emergence exposure of 100 mg L^{-1} glyphosate.

S. No.	Genotypes	% Decrease			SP
		SL	RL	GP	
1	*Brassica carinata* (PC-6)	47.5 [7]	92.0 [17]	43.3 [5]	56.6 [2]
2	*Brassica juncea* (Pusa Jaikisan)	60.1 [11]	90.0 [14]	50.0 [7]	50.0 [8]
3	*Brassica napus* (GSC 6)	63.3 [14]	93.7 [20]	53.3 [9]	46.6 [10]
4	*Brassica nigra* (EC472708)	22.1 [1]	89.1 [10]	40.0 [4]	40.0 [12]
5	*Brassica rapa* (NRCPB rapa 8)	67.8 [16]	89.8 [12]	39.7 [3]	63.2 [1]
6	*Biscutella didyma*	70.4 [17]	77.5 [6]	62.1 [16]	35.8 [15]
7	*Brassica fruticulosa* (Spain)	76.0 [19]	76.0 [4]	54.7 [10]	21.4 [18]
8	*Brassica tournefortii* (Rawa)	50.9 [8]	80.4 [8]	68.9 [17]	40.0 [11]
9	*Brassica tournefortii* (RBT 2002)	61.5 [13]	75.6 [3]	52.5 [8]	20.0 [19]
10	*Camelina sativa*	75.0 [18]	78.0 [7]	62.0 [15]	35.9 [14]
11	*Crambe abyssinica* (EC400058)	65.8 [15]	91.3 [15]	36.6 [2]	51.0 [6]
12	*Crambe abyssinica* (EC694145)	35.8 [3]	93.2 [19]	33.5 [1]	52.0 [5]
13	*Diplotaxis catholica*	53.2 [10]	65.7 [1]	85.0 [19]	20.0 [20]
14	*Diplotaxis muralis*	26.9 [2]	71.0 [2]	90.0 [20]	30.0 [16]
15	*Diplotaxis gomez-campoi*	42.6 [6]	76.9 [5]	60.0 [12]	28.0 [17]
16	*Enarthrocarpus lyratus*	36.1 [4]	85.1 [9]	72.9 [18]	37.1 [13]
17	*Eruca sativa* (IC57706)	86.3 [20]	93.1 [18]	49.7 [6]	53.2 [4]
18	*Lepidium sativum*	39.8 [5]	89.8 [13]	43.3 [14]	46.6 [9]
19	Oxycamp	52.2 [9]	91.6 [16]	56.6 [11]	53.3 [3]
20	*Sinapis alba*	60.5 [12]	89.8 [11]	60.0 [13]	50.0 [7]

Note: The numbers in parentheses indicate the genotype ranking between 1 to 20 for each trait based on the percentage decrease. The percentage decrease was calculated by comparing the trait under exposure to glyphosate with the control. The table represents the average values from three independent experiments.

Table 2. Root and shoot length of wild and U triangle species of Brassica germinated under 100 mg L^{-1} glyphosate. Different letters denote a significant difference at $p < 0.05$ based on the least significant difference (LSD) test.

S. No.	Genotypes	Control		Treatment	
		Root Length	Shoot Length	Root Length	Shoot Length
1	*Brassica carinata* (PC-6)	6.2 ± 0.21^{F}	4.0 ± 0.25^{I}	0.5 ± 0.10^{B}	2.1 ± 0.75^{G}
2	*Brassica juncea* (Pusa Jaikisan)	9.0 ± 2.9^{F}	4.6 ± 0.53^{I}	0.9 ± 0.44^{B}	1.8 ± 1.12^{FG}
3	*Brassica napus* (GSC 6)	8.5 ± 2.48^{F}	5.3 ± 0.9^{I}	0.5 ± 0.06^{B}	1.9 ± 0.21^{G}
4	*Brassica nigra* (EC472708)	4 ± 1.82^{F}	3.7 ± 1.00^{I}	0.4 ± 0.15^{B}	2.9 ± 0.23^{G}
5	*Brassica rapa* (NRCPB rapa 8)	3.9 ± 1.48^{F}	3.7 ± 0.06^{HI}	0.4 ± 0.10^{B}	1.2 ± 0.36^{FG}
6	*Biscutella didyma*	1.9 ± 0.21^{EF}	2.3 ± 0.32^{FGH}	0.4 ± 0.15^{B}	0.7 ± 0.26^{DEFG}
7	*Brassica fruticulosa* (Spain)	1.6 ± 0.49^{A}	3.0 ± 0.31^{A}	0.4 ± 0.10^{A}	0.7 ± 0.06^{A}
8	*Brassica tournefortii* (Rawa)	1.3 ± 0.21^{F}	3.6 ± 0.87^{GHI}	0.2 ± 0.15^{B}	1.8 ± 0.26^{EFG}
9	*Brassica tournefortii* (RBT 2002)	1.3 ± 0.06^{A}	3.4 ± 0.25^{A}	0.3 ± 0.06^{B}	1.3 ± 0.47^{B}
10	*Camelina sativa*	1.6 ± 0.15^{B}	2.8 ± 0.26^{AB}	0.3 ± 0.06^{B}	0.7 ± 0.1^{BC}
11	*Crambe abyssinica* (EC400058)	5.8 ± 1.51^{CDE}	5.6 ± 0.25^{EFG}	0.5 ± 0.17^{B}	1.9 ± 0.25^{CDE}
12	*Crambe abyssinica* (EC694145)	4.4 ± 0.64^{CDE}	5.8 ± 0.98^{EFG}	0.3 ± 00^{B}	3.7 ± 0.47^{DEF}
13	*Diplotaxis catholica*	1.1 ± 0.15^{B}	2.0 ± 0.35^{ABC}	0.4 ± 0.10^{B}	0.9 ± 0.06^{BC}
14	*Diplotaxis muralis*	1.2 ± 0.15^{BC}	2.1 ± 0.10^{BCDE}	0.3 ± 0.15^{B}	1.5 ± 0.21^{CDE}
15	*Diplotaxis gomez-campoi*	1.3 ± 0.1^{CB}	2.5 ± 0.30^{ABCD}	0.3 ± 0.10^{B}	1.4 ± 0.12^{CD}
16	*Enarthrocarpus lyratus*	2.4 ± 1.01^{BCD}	2.4 ± 0.66^{CDE}	0.3 ± 0.06^{B}	1.5 ± 0.32^{CDE}
17	*Eruca sativa* (IC57706)	6.3 ± 1.0^{DEF}	5.87 ± 0.32^{EFG}	0.4 ± 0.06^{B}	0.8 ± 0.1^{DEF}
18	*Lepidium sativum*	4.6 ± 1.04^{BCD}	4.1 ± 0.20^{DEF}	0.4 ± 0.31^{B}	2.4 ± 0.4^{CDE}
19	Oxycamp	6 ± 1.80^{F}	5.1 ± 1.04^{FGHI}	0.5 ± 0.10^{B}	2.4 ± 0.42^{EFG}
20	*Sinapis alba*	3.93 ± 0.21^{CDE}	4.73 ± 0.75^{DEF}	0.4 ± 0.17^{B}	1.87 ± 0.4^{CDE}

Plant survival was assessed 6 days after the herbicide treatment as previously described in terms of survival percentage. On the basis of survivability, the genotypes showing the maximum survival rate withstanding glyphosate (100 mg L^{-1}) were *B. rapa* (NRCPB rapa 8) (63.2%), *B. carinata* (PC-6) (56.6%), and Oxycamp (53.3%) and accordingly ranked 1–3. Meanwhile, genotypes *Diplotaxis catholica* (20%), *Brassica tournefortii* (RBT 2002) (20%) and *Brassica fruticulosa* (Spain) (21.4%) ranked 18–20 (Figure 2 and Table 1).

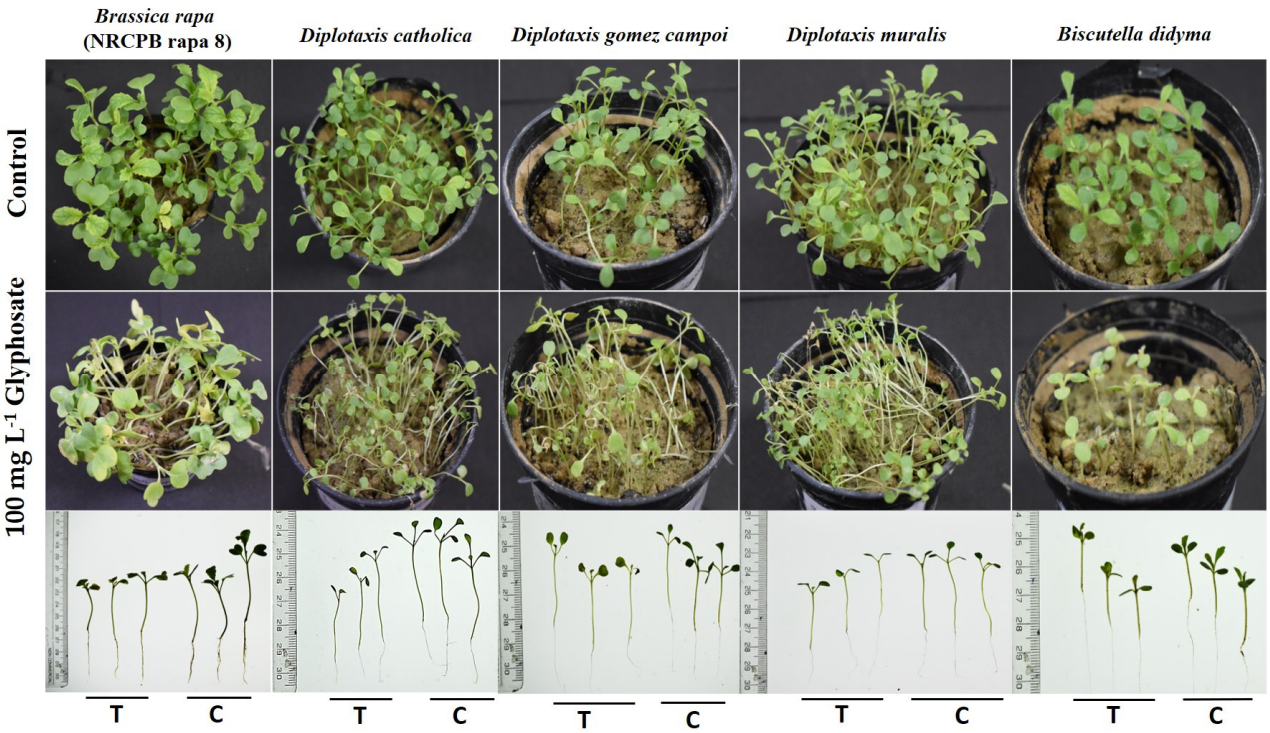

Figure 2. Effect of 100 mg L^{-1} of glyphosate at post-emergence exposure stage. Members of the 3 clusters based on membership function value are represented. *B. rapa* (NRCPB rapa 8) (tolerant, cluster 3), *D. catholica* (susceptible, cluster 2), *D. gomez-campoi* (moderately tolerant, cluster 1), *D. muralis* (susceptible, cluster 2) and *Biscutella didyma* (moderately tolerant, cluster 1). C and T represent control and treatment, respectively.

3.2. Tolerance Index and Membership Function Value

The membership function value (MFV) calculated based on the tolerance index (TI) was used as an index to screen potentially tolerant genotypes to herbicide stress. The estimated TI and MFV values of the genotypes, based on traits studied under stress conditions, are mentioned in Table S1. In our study, the MFV was the cumulative outcome of the TI of all the traits studied; this includes germination percentage, root length and survival percentage. A maximum mean MFV of 0.68 was recorded in *B. rapa* (NRCPB rapa 8). At the same time, lower values as 0.34, 0.35 and 0.37 were observed in *Enarthrocarpus lyratus*, *Diplotaxis muralis*, and *Diplotaxis catholica*, respectively (Table S1).

3.3. Multivariate Cluster Analysis

Cluster analysis using Ward's algorithm and squared Euclidean distance categorized the 20 genotypes into three groups (Figure 3). This analysis determined cluster-I, cluster-II, and cluster-III members to be glyphosate moderately tolerant, sensitive, and tolerant genotypes, respectively (Figure 3). After the glyphosate exposure pre- and post-emergence, clustering was performed based on the mean MFV calculated using tolerance index data observed for germination percentage, root length, and survival percentage. The results were in good correlation with the phenotypic observations for glyphosate tolerance.

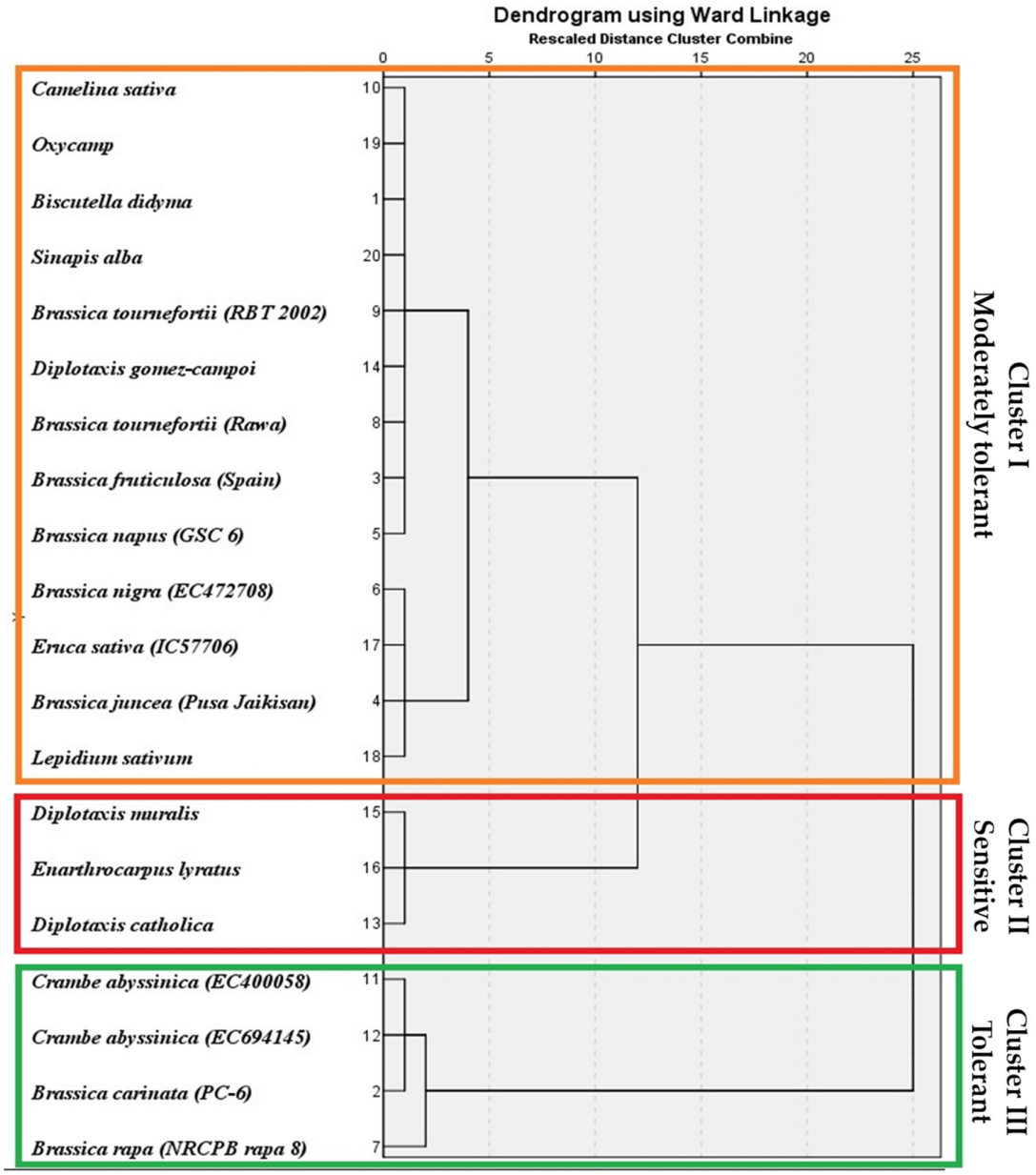

Figure 3. Dendrogram showing clustering of the 20 genotypes of Brassica under study using Ward's linkage. The clustering was performed based on their mean MFV calculated using the tolerance index observed for the trait's germination percentage, root length, and survival percentage.

3.4. Biochemical Assessment of Ascorbate Peroxidase

The seedlings of all the genotypes germinated in the presence of 100 mg L^{-1} glyphosate were analysed for ascorbate peroxidase enzyme activities after six days of the treatment, taking into account the time when they started to display the differential morphological response but did not die due to the applied stress. Ascorbate peroxidase (APX) activity was measured to study the effect of glyphosate at both pre-and post-emergence. After exposure to glyphosate, the ascorbate peroxidase activity increased in most genotypes, with significant differences between the Brassica CWR and U triangle spp. However, the tolerant genotypes showed a greater increase than the sensitive ones. A significant variation in fold change was observed among various CWRs and U triangle species, ranging from 0.3 to 3.4 in pre- and 0.3 to 4.4 in post-emergence. Fold change in APX activity in other genotypes is represented in Figure 4.

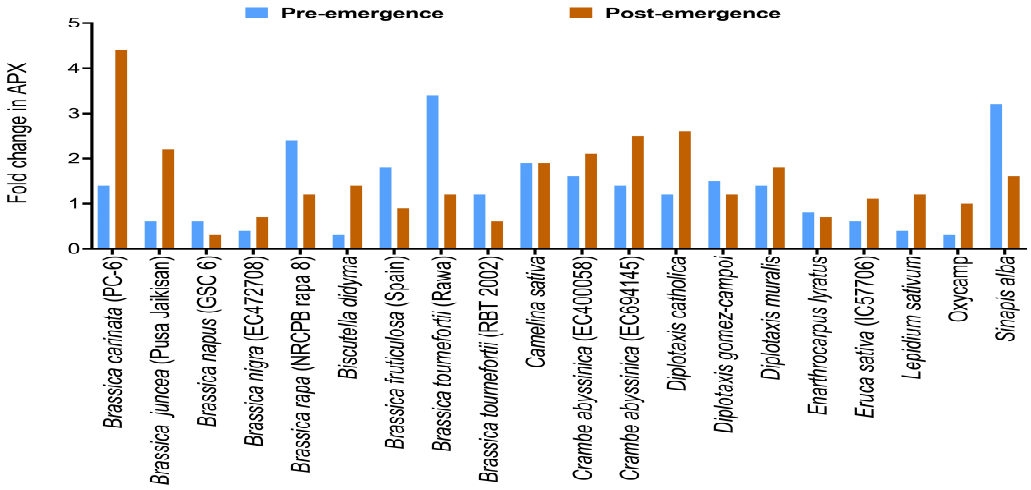

Figure 4. Fold change in APX activity in genotypes in response to 100 mg L^{-1} glyphosate in pre-and post-emergence exposure.

3.5. Principal Component Analysis (PCA)

In addition to cluster analysis, principal component analysis (PCA) was used to identify the superior genotypes among CWR and U triangle species genotypes. The PCA grouped the variables into two principal components that accounted for 48.1% of the total variance in the dataset and had eigenvalues greater than one (Figure 5). The biplot diagram showed that the first principal component (PC1) accounted for 27.8% of the total variance in the data set and had a strong positive correlation with APX activity at the pre- and post-emergence stages. The principal component (PC2) accounted for 20.3% of the total variance in the data set and had a strong positive correlation with the treatment root length (RL). The biplot enables visualization of the relationships between the variables and genotypes, highlighting the importance of each trait in determining glyphosate tolerance.

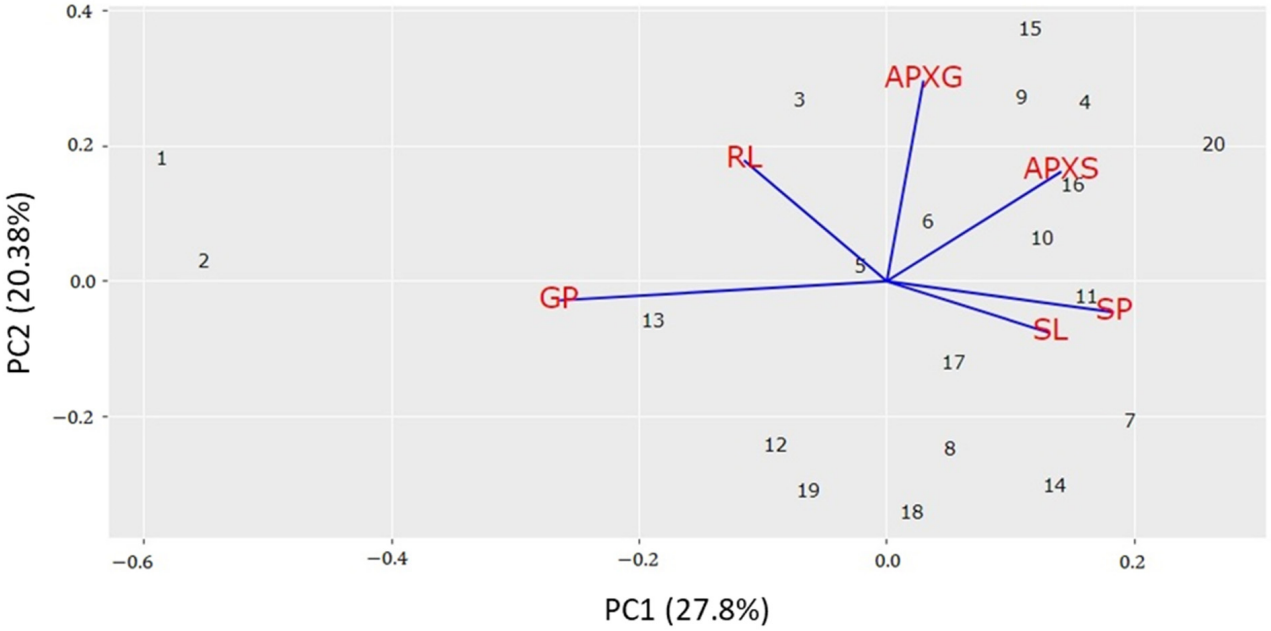

Figure 5. The biplot showing principal component analysis (PCA) to examine the importance of various traits contributing to glyphosate tolerance. The active variables in red used in the analysis were germination percentage (GP), shoot length (SL), root length (RL), survival percentage (SP), and ascorbate peroxidase at pre- and post-emergence (APXG and APXS, respectively).

4. Discussion

Crop productivity is highly affected by weed infestation worldwide. Developing herbicide-resistant crops and applying herbicides have become an economical method for weed management [27]. The shikimate pathway is important and indispensable in plants, fungi and microbes. The EPSPS enzyme in this pathway is a primary target for developing glyphosate-resistant crops [28]. The halting of the shikimate pathway at EPSPS deregulates the other pathways and accumulates shikimate and its benzoic acid derivatives in a phytotoxic concentration [3]. Efficient germination, which is often highly sensitive to external stresses, is the key to the growth and development of crops [29]. Therefore, seed germination, emergence, and growth of seedlings represent a window of growth phase pivotal for the fitness of the species [30]. In the present study, we attempted to assess glyphosate tolerance in 20 genotypes of the Brassicaceae family, including its wild relatives and U triangle species, based on the effect of the herbicide at pre- and post-emergence stages of glyphosate exposure.

In our study, root length was observed to be a strong indicator of response to glyphosate stress in the studied genotypes; however, as we are dealing with a much more diverse panel of genotypes in this study, we have selected two other traits for the identification of tolerant genotypes, i.e., germination percentage and survival percentage, which showed a significant difference between them. The 20 genotypes were ranked based on the extent to which the trait under study was affected in response to 100 mg L^{-1} glyphosate. A similar approach was also adopted by Jha et al. [31]. The relationship between MFV and TI was further analysed to identify the tolerant genotypes. The membership function of a fuzzy set is a generalization of the indicator function in classical sets; it represents the degree of truth as an extension of valuation [32]. Based upon the TI calculated for traits germination percentage, root length, and shoot length, mean MFV was calculated. According to Rai et al. [26], the higher the TI value, the higher the MFV value. After this, the average MFV(s) value was calculated, concluding which tolerant genotype was identified. Depending on their potential tolerance under glyphosate exposure, genotypes were classified into sensitive, moderately tolerant, and tolerant genotypes. Genotypes identified as tolerant are *B. rapa* (NRCPB rapa 8) (0.68 mean MFV), *B. carinata* (PC-6) (0.59 mean MFV), *Crambe abyssinica* (EC400058) (0.59 mean MFV), and *Crambe abyssinica* (EC694145) (0.59 mean MFV). Our result was in agreement with previous studies, depicting multivariate cluster analysis for salt stress in pearl millet genotypes (Jha et al., 2022) and drought stress in wheat (Singh et al., 2015). Furthermore, PCA analysis was employed to identify key attributes contributing to tolerance to glyphosate.

Glyphosate has been shown to affect seed germination or seedling quality when applied directly to seeds [33,34] or as a pre-harvest application [35,36]. The effect of glyphosate application on seeds and seedlings was studied in two spring wheat varieties, 'Alpowa' and 'Penawawa' [37]. In their study, glyphosate was applied at a concentration of 0.62 or 0.84 kg ae/ha at two different stages of wheat development; a reduction in germination percentage from 2 to 46% was reported, which are consistent with our results. Previous studies on seedling emergence and growth in field peas [38], *Zea mays*, *Glycine max*, and *Sorghum halepense* [39] determined the effects of glyphosate application pre-harvest and at various stages of maturity, revealing that shoot meristematic cell moisture above 40% reduced seedling germination and fresh weight of shoots [40]. Furthermore, the negative impact of glyphosate exposure was observed in a *Zea mays* cultivar, with a decrease of 20.8% in root length [41]. Additionally, in a study on *Pisum sativum*, the phytotoxicity of different doses of glyphosate was evaluated at germination and seedling stages. In their study, the highest decrease of 90% in germination percentage, 14.7% average root length, and 17.6% average shoot length was observed, similar to our study in *Brassica* genotypes [40].

It has been reported that physiological parameters other than root length are much weaker indices of herbicide activity in different plant species measured after 6 days of germination. Inhibition of shoot growth and a lack of seed germination in watergrass, transgenic and non-transgenic soybean using glyphosate were observed by Kohata et al. [42]. We

observed a similar effect of glyphosate exposure on the shoot length, as all the genotypes were recorded with a significant decrease in shoot and root length. Glyphosate significantly reduces acid invertase activity, hydrolysing sucrose to hexose sugars for energy production, ultimately affecting plant growth and maintenance [40].

As a post-emergence herbicide, however, glyphosate was not designed to disrupt seed germination, raising the question of how glyphosate interferes with germination; rather, glyphosate has been shown to induce oxidative stress in plants [43]. We investigated oxidative stress markers in seeds exposed to glyphosate. Reactive oxygen species (ROS) are quite important during germination processes, and germination under stress conditions is related to the seed's ability to cope with ROS accumulation [44]. As shown in Figure 4 the genotypes which are identified to be tolerant to glyphosate showed higher APX activity in pre- and post-emergence. Furthermore, in susceptible genotypes like *D. catholica* and *D. muralis*, the activity of APX activity (H_2O_2-scavengers) increased, and these enzymatic systems failed to prevent H_2O_2 accumulation, which can become toxic and impair the germination process [45] and survivability. H_2O_2 accumulation in embryos can cause oxidative bursts, delaying or decreasing seed germination through the deterioration of cell structures and components such as fatty acids and proteins [46].

5. Conclusions

A total of 20 genotypes of the Brassicaceae family were evaluated for their resistance to glyphosate at pre- and post-emergence stages. Glyphosate-tolerant genotypes showed a lower decline in germination percentage and higher survivability under exposure to glyphosate at pre- and post-emergence stages, respectively. A significant variation in performances at both stages was studied. Based on morphological parameters and survival percentage, the tolerant genotypes were identified, which was supported by statistical analyses, including PCA and MFV. The genotypes identified in the present study can contribute to the breeding of glyphosate-resistant crops.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13071831/s1, Table S1: Tolerance Index (TI) and membership function value (MFV) for trait root length, germination percentage and survival percentage.

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