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Glyphosate resistant ryegrass competitive ability

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ABSTRACT: Herbicide resistant plants may present changes in their physiology and aggressiveness. In order to evaluate the competitive differences of glyphosate resistant ryegrass, in a first experiment, resistant and susceptible biotypes grew in intra and interbiotypic competition in different environmental conditions (check, irradiance and water deficit). In a second experiment, these biotypes grew together in different proportions in the presence of wheat. The development of plants was evaluated in the first experiment by analyzing the number of leaves, height and dry biomass accumulated until flowering. In the second, the culture (tiller, nutrients and estimated productivity) and the ryegrass plants (quantitative and qualitative analysis) were analyzed through graphs and indices of grouping and competitiveness. Resistant ryegrass plants developed better in both conditions and experiments. In wheat, the presence of the potassium, sulfur and zinc nutrients and the final yield of plants in the coexistence with greater proportions of resistant plants were higher reduced. By the substitutive analysis, resistant ryegrass presented higher clustering coefficient and greater competitiveness than the susceptible biotype.

Key words: competition; herbicide resistance; *Lolium multiflorum*; *Triticum aestivum*; weeds

Habilidade competitiva do azevém resistente ao glyphosate

RESUMO: Plantas daninhas resistentes à herbicidas podem apresentar alterações na sua fisiologia e agressividade. A fim de avaliar as diferenças competitivas do azevém resistente à glyphosate, em um primeiro experimento, plantas resistentes e suscetíveis cresceram em convivência intra e interbiotípica, submetidas a diferentes condições ambientais (controle, restrição de irradiância e restrição hídrica). Em um segundo experimento, essas plantas cresceram juntas em diferentes proporções na presença do trigo. Avaliou-se, no primeiro experimento, o número de folhas, altura e biomassa seca acumulada por ocasião do florescimento de plantas. No segundo, analisaram-se a cultura (perfilhos, nutrientes e produtividade estimada) e as plantas de azevém (análise quantitativa e qualitativa), por meio de gráficos, índices de agrupamento e competitividade. Plantas de azevém resistente se desenvolveram melhor em ambas as condições e experimentos. No trigo, reduziu-se em maior grau o acúmulo dos nutrientes potássio, enxofre e zinco e a produtividade final de plantas na convivência com maiores proporções de plantas resistentes. Pela análise substitutiva, o azevém resistente apresentou maior coeficiente de agrupamento e maior competitividade do que o azevém suscetível.

Palavras-chave: competição; resistência à herbicidas; *Lolium multiflorum*; *Triticum aestivum*; plantas daninhas

Introduction

The presence of weeds causes productivity losses in agricultural crops by reducing on average 20% of the total food products produced worldwide (Soltani et al., 2016). Herbicides are the most method applied to control these weeds, among which the most used in the world and in Brazil is glyphosate. The repetitive and indiscriminate application of this herbicide however, has resulted in the selection of 45 resistant weed species in the last years, eight of which are in Brazil (Heap, 2018).

Among the selected species, ryegrass (*Lolium multiflorum* Lam.) interferes in agricultural crops by competing for nutrients, water, light and still by exerting an allelopathic effect (Moraes et al., 2011) and reducing grain yield (Paula et al. 2011) of crops. In recent years, glyphosate-resistant ryegrass has caused problems in wheat, soybean, eucalyptus fields and orchards in southern Brazil (Roman et al., 2004; Agostinetto et al., 2017a). In wheat, the presence of ryegrass plants can reduce the final grain yield by an average of 60 to 80% (Agostinetto et al., 2008).

With the evolution of resistant populations, the adaptive value of different alleles can be accompanied by adaptation costs in plants. These effects may be positive, negative or neutral, depending on the environment and mechanism of resistance that the plant presents (Délye et al., 2013; Mariani et al., 2016). Studies with glyphosate-resistant ryegrass plants report a greater adaptive advantage of the susceptible genotype, indicating "fitness" penalties in resistant genotypes (Vargas et al., 2005). However, these works were conducted with specific coexistence only between ryegrass plants. In the field, it is known that these populations will coexist with crops of interest, and this may alter these effects because such coexistence is a stressor. Furthermore, other stressing conditions such as different environmental conditions can modify the ecological adaptability of a plant.

In this sense, studies on weed competitiveness, such as replacement series experiments allow the development of management strategies because they define characteristics that confer greater competitive ability among resistant and susceptible plants (Cousens, 1991). The objective of this work was to establish and compare the adaptive values of glyphosate-resistant ryegrass genotypes in the presence of wheat, to identify the elements of this competition and verify the competitive dominance of genotypes in different environmental conditions of growth.

Material and Methods

Plant material

Two experiments were installed and conducted under semi-controlled conditions. Ryegrass (*Lolium multiflorum* Lam.) seeds were collected in Rio Grande do Sul, Brazil, from at least 40 plants in places with repeated application of glyphosate and places without application of the herbicide; the resistant population presented 7.5 times more tolerance to glyphosate than the susceptible plants (Barroso et al., 2018). Because *L. multiflorum* is a non-cosmopolitan plant occurring exclusively in southern Brazil, differences arising from genetic background of the genotypes is minimized. The susceptible genotype was collected in the city of Ijuí (28°23'18.72" S, 53°55'13.75" W) and the resistant weed in the city of Três de Maio (27°47'02.77" S, 54°14'05.06" W). The wheat used in the competition experiment was the cultivar BRS264.

Environmental effects and biotypic competition

In a first experiment, resistant (R) and susceptible (S) ryegrass genotypes were submitted to intra and inter-biotype coexistence under different growing conditions (normal growth under semi-controlled conditions; growth under lower light intensity; and growth under water restriction). Light restriction was obtained through the use of artificial shadow on the vessels reducing on average 64.5% of the total irradiance transmitted during the day (radiation measured with the aid of a photosynthetic active radiation meter - LP 80, Decagon Services, Pullman, USA). Water restriction was obtained through to three cycles of water deficiency that began when ryegrass plants presented on average 15 leaves. Soil moisture for this condition was maintained with the aid of tensiometers (HidroFarm, Falker®, Porto Alegre, Brazil) installed in the soil in the normal and stressful conditions, and followed at a daily basis. The soil initial moisture content was the same as before water deficit (30%). In the stress treatment, soil moisture was reduced to 8.2% in the first cycle of stress, 10.2% in the second and 11% in the third cycle. The average moisture content throughout the studied period in the soil without stress was 29%, and in the soil with stress, 16.2%. These values were adopted based on the manufacturer's recommendation and according to the soil and crop used.

In each of the above conditions, the genotypes were seeded in 10 L pots to obtain the following treatments: 16 ryegrass R plants m^2 (T1); 8 ryegrass R plants m^2 and 8 ryegrass S plants m^2 (T2); and 16 ryegrass S plants m^2 (T3). The vessels were filled with Dark Red Latosol with the following characteristics: pH: 5.1; OM: 60g dm^{-3} P- (res.): 72 mg dm^{-3}; K, Ca, Mg, H + Al, SB, T and V (%): 7.9; 22; 10; 28; 39.7; 67.3 mmolc dm^{-3} and 59%, respectively; and clay, silt, fine sand and coarse sand contents: 37; 8; 18 and 36%, respectively. Plant health was maintained by applying deltamethrin to control caterpillars, and irrigation whenever visually necessary except in the treatment with water deficit.

At 90 days after sowing, when the plants began to bloom, the aerial parts of the ryegrass plants were cut close to the soil and were evaluated for height (greater leaf extension), number of leaves and dry biomass that was obtained after drying the greenhouse plant material in a forced air circulation oven at 60°C for 72 h. The experiment was set up in a completely randomized design, in a 3 x 3 factorial scheme, where three environmental conditions and three treatments of coexistence between plants were observed, with four replications. The data were submitted to analysis of variance by the F test. When significant results were found, the data were compared by the Tukey test at 5% of probability ($p \leq 0.05$).

Biotypic competitive ability

In a second experiment, the ryegrass genotypes (R and S) were submitted to intra and inter-biotype coexistence in different proportions concomitantly to coexistence with wheat plants. Ryegrass density was considered to be fixed per pot and the critical densities of each genotype were not evaluated in this case because in the previous experiment the genotypes did not present significant development differences in intra-genotypic coexistence. The proportions used were: 100: 0%+1, 75:25%+1, 50:50%+1, 25:75%+1 and 0:100%+1 (resistant ryegrass/susceptible ryegrass + wheat crop). The density of 21 m^2 of ryegrass plants and 2.6 m^2 of wheat plants was established per treatment. These densities were arranged in 100-L asbestos cement pots filled with the same soil from the previous experiment, plus fertilization with 30 kg ha^{-1} of nitrogen, 156 kg ha^{-1} of phosphorus and 156 kg ha^{-1} of potassium at the time of sowing. The treatments were established in a completely randomized design with seven replicates.

On the occasion of the flourishing of wheat, leaves were collected for nutritional analysis (second leaf completely expanded below the inflorescence). Macro (nitrogen, phosphorus, potassium, calcium, magnesium, sulfur) and micro (boron, copper, iron, manganese, zinc) nutrient content levels were evaluated in three replicates (each being a mixture of two wheat plants, totalizing six repetitions). At maturity, wheat was harvested and the number of tillers per plant and average estimated yield, in kg ha^{-1}, were evaluated. The nutrient contents were quantified first in the tissues (g or mg kg^{-1}) and, later, multiplied by the mass of the wheat in each treatment; this provided the amount extracted of each element in g or mg per plant, according to Malavolta et al. (1997). Tiller, nutrient and productivity data were submitted to analysis of variance by the F test and, if this was significant, means were compared by the Tukey test at 5% probability ($p \leq 0.05$).

Also in the maturation of wheat, ryegrass plants that were in full bloom, separated by genotype, were cut close to the soil and placed for drying in a forced air circulation oven at 60°C for 72 hours and later, the dry biomass of each genotype per treatment was measured. These dry biomass data of ryegrass were used to evaluate the competitive ability among the genotypes that were competing with wheat.

For analysis of ryegrass biomass data, we used qualitative and quantitative analyses, using graphical analysis following the models proposed by De Wit (1960) and regression curve analysis respectively. In the qualitative analysis, called the conventional method for substitution experiments (Agostinetto et al., 2008), it was necessary to calculate the relative biomass productivity of the aerial part of each treatment and genotype and the total productivity. To obtain the relative (PR) and total (PRT) productivity, the relative productivity formula was used as a function of the density described by Cousens & O'Neil (1993). In the quantitative analysis, the relative grouping indices (K) were calculated to obtain the 50R:50S ratio, indicating the relative dominance of one genotype over the other and the aggressive competitiveness (A). Aggressive competitiveness is the difference between the productivity of species, indicating which of the species made the best use of the resources of the environment. Positive values of A are favorable in this case to the resistant genotype (De Wit, 1960; Cousens, 1991).

Results and Discussion

Environmental effects and biotypic competition

In the analysis of ryegrass genotypes in the first experiment, it was verified that the final height of plants was influenced by the tested genotype and also by the growing condition (Table 1). Glyphosate-resistant plants reached higher heights than the others when in intra-biotype coexistence. Resistant and susceptible plants reached the same height when in coexistence. The susceptible plant in interbyotic competition did not reduce its height, while the resistant plant did. Plants under restricted irradiance showed higher growth, regardless of genotype. This higher value was observed due to plant epinasty, in which the plant stops investing in tillering to invest in longer stalks to try to capture more light energy (Galon et al., 2011).

The number of leaves per ryegrass plant at the end of the evaluated period was similar in both genotypes and both growing conditions (Table 1). As for dry biomass of plant

Table 1. Height, number of leaves and biomass of resistant ryegrass (R) and susceptible ryegrass (S) plants in intra- (RA or SA) and inter- (RE or SE) genotypic competition under normal growth conditions, with irradiance restriction or water restriction.

Treatments*	Height (cm)		Number of leaves		Biomass (g)	
Genotype						
RA	55.91	a	201.91		9.13	b
SA	49.83	b	235.75		7.90	bc
RE	49.41	b	206.75		11.33	a
SE	44.2	b	223.08		6.61	c
Growth condition						
Control	46.59	b	231.43		10.52	a
Irradiance restriction	55.37	a	211.37		8.76	b
Water restriction	47.56	b	207.81		6.95	c
Fg[1]	10.02	**	2.09	NS	13.02	**
Fc[2]	13.5	**	1.88	NS	13.67	**
Fgxc[3]	1.73	NS	1.42	NS	1.41	NS
CV[4]	10.51		17.11		22.02	

*RA (Resistant in intra-genotypic competition), SA (Susceptible in intra-genotypic competition), RE (Resistant in inter-genotypic competition), SE (Susceptible in inter-genotypic competition). [1]F-values for genotype, [2]Growing condition and [3]"genotype x growing condition" interaction. [4]Coefficient of variation (%). Means followed by the same letter in the column were not significantly different according to the Tukey test (p = 0.05). *, ** Significant at 5% and 1% probability, respectively. NS Not significant shoot, the highest value was observed in resistant plants in association with susceptible plants which consequently obtained the lowest accumulation of dry biomass. Intra-genotypic competition, in this case, resulted in similar effects among genotypes. It is important to note that resistant plants seem to have a higher accumulation of biomass compared to susceptible plants due to the development of leaf structures, regardless of the growing condition. In the different growing conditions, the greatest reduction of ryegrass growth occurred in the situation of water restriction and a lighter reduction in the presence of lower irradiance, what reduced plant growth by 33.9 and 16.7%, respectively. There were no significant interactions between environmental stresses in the development of each genotype, indicating in this case the absence of environmental influence on the resistance mechanisms presented by the species.

Biotypic competitive ability

Of the analyzed nutrients, wheat plants absorbed them in the following order: nitrogen > potassium > calcium > sulfur > phosphorus = magnesium > iron > manganese> zinc > copper = boron. The coexistence of wheat with ryegrass plants altered the total nutrients absorbed and accumulated by the crop in all treatments. regarding macronutrients, the average values per plant of nitrogen, phosphorus and potassium were reduced by 59, 65 and 60%, respectively. In turn, coexistence with ryegrass reduced the accumulation of calcium, magnesium and sulfur by 59, 63 and 60%. Asor micronutrients, reductions were higher for copper (80%), iron (62%), manganese (60%), zinc and boron (57%). For all nutrients, on average, coexistence with 21 ryegrass plants per m² reduced the total nutrient accumulation per wheat plant by 60%.

In most cases, coexistence with different proportions of resistant and susceptible ryegrass did not affect the reduction of the total accumulated by wheat plants, but in some cases, such as in the case of potassium, sulfur and zinc, coexistence in larger proportions with resistant ryegrass plants had a more intense negative effect on the accumulation by wheat plants when compared with the coexistence with susceptible plants (Figures 1 and 2).

Behind only nitrogen, potassium was the nutrient most accumulated by the wheat crop and its values showed significant differences between the proportions of resistant or susceptible ryegrass, demonstrating that resistant plants, in this case, required higher amounts of this nutrient for their development or, on the other hand, indicating that wheat had the absorption of this nutrient impaired. A similar situation was observed in the case of sulfur (Figure 1). It is known that potassium is not part of any organic component of plants, but acts as an enzymatic activator, being related to changes in the conformations of molecules, increasing active sites for connections with substrates. A greater demand for this nutrient may result from possible mutations in the enzymes responsible for resistance mechanisms in ryegrass plants (EPSPS), or other mechanisms already reported in the literature (Barroso et al., 2018). Sulfur, on the other

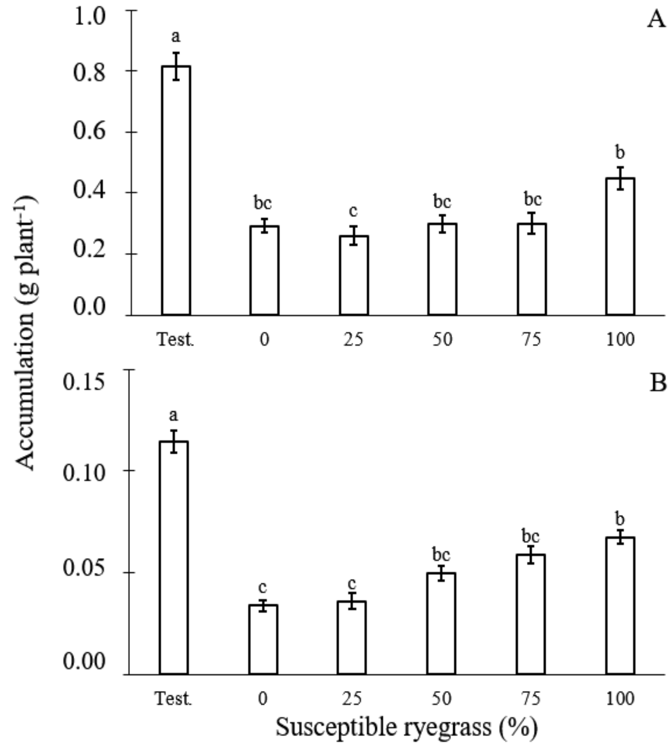

Figure 1. Accumulation of potassium (A) and sulfur (B) (g plant^{-1}) in wheat plants coexisting with different proportions of ryegrass plants resistant to and susceptible to glyphosate. Test: Witness free from interaction.

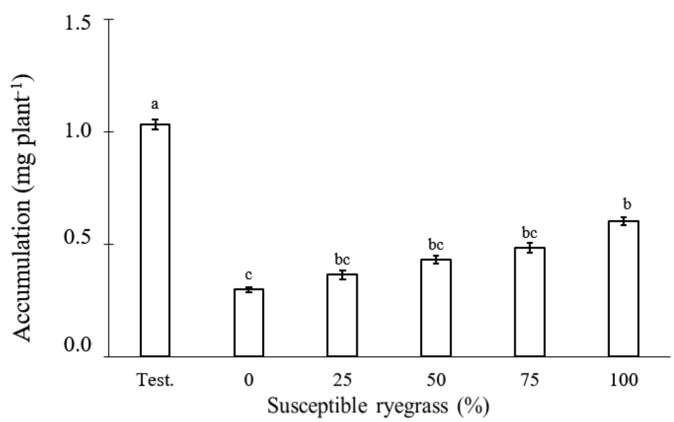

Figure 2. Zinc accumulation (mg plant^{-1}) in wheat plants coexisting with different proportions of ryegrass plants resistant to and susceptible to glyphosate. Test: Witness free from interaction.

hand, participates in the composition of proteins and essential amino acids besides processes such as nitrogen fixation, glutamate synthesis, carbohydrate metabolism and others. The higher growth of resistant ryegrass plants would therefore require larger amounts of this nutrient, thus leading to its unavailability for wheat (Krishnan, 2005).

In the evaluation of the micronutrients, none of them presented differences in their accumulation in response to coexistence with different proportions of ryegrass genotypes, except for zinc (Figure 2).

It is known that zinc is a micronutrient that binds to the amino acid tryptophan, which is in turn required for the synthesis of indoleacetic acid and plant growth, and its synthesis depends on zinc (Skoog, 1940). Furthermore, tryptophan is a direct product of the shikimic acid route, where glyphosate acts. A simple alteration in the EPSPS enzyme, in this case in resistant plants, could reduce tryptophan production and consequently the synthesis of EIA and the growth of plants (what was not observed here). Thus, it can be argued that other mechanisms may be conferring resistance to glyphosate in this ryegrass genotype.

Taking into consideration the number of wheat tillers per plant, it was observed that all proportions of resistant and susceptible ryegrass affected the tillering of the plant, but there were no significant differences as to the different proportions of plants (Table 2). As for productivity, it was verified that the coexistence with ryegrass plants in all situations reduced the values obtained. However, the different proportions of ryegrass plants caused different losses. The highest reduction (78%) occurred when wheat was grown exclusively with resistant ryegrass plants, as the opposite to the situation where wheat grew with susceptible plants, when the reduction was 52% (Table 2). These results corroborate the data observed in the first experiment, in which the resistant genotype obtained better development than the susceptible genotype when growing together.

As observed, wheat plants in competition with resistant ryegrass presented lower amounts of potassium, sulfur and zinc. Thus, these individuals probably showed reduced starch levels and protein synthesis, leading to lower metabolism and growth, resulting in lower productivity.

The graphical analysis of ryegrass plants showed that resistant plants presented advantages in their development in contrast with susceptible plants (Figure 3).

The relative productivity lines showed to be concave, resulting from a loss in the growth of one or both genotypes. However, the line of the resistant genotype showed a higher growth as compared to the susceptible genotype, confirming the previously observed, i.e. its competitive advantage. The total relative productivity, represented by the sum of the yields of the genotypes in the respective proportions also

Table 2. Number of tillers and average yield of wheat estimated in absence (control) or coexisting with different proportions of ryegrass plants resistant (R) and susceptible (S) to glyphosate throughout the crop cycle.

Treatments	Tillers (n. plant^{-1})		Estimated productivity (kg ha^{-1})	
Control	30.85	a	1,151	a
100R:0S[1]	10.28	b	251	c
75R:25S	11.28	b	404	bc
50R:50S	11.42	b	466	bc
25R:75S	11.57	b	485	bc
0R:100S	13.42	b	547	b
F[2]	30.88	**	26.33	**
CV[3]	25.49		26.92	

[1]Proportions of resistant and susceptible ryegrass plants (%) coexisting with wheat, [2]F-value, [3]Variation coefficient (%).Means followed by the same letter in the column were not significantly different according to the Tukey test (p = 0.05). *, ** Significant at 5% and 1% probability, respectively. NS Not significant.

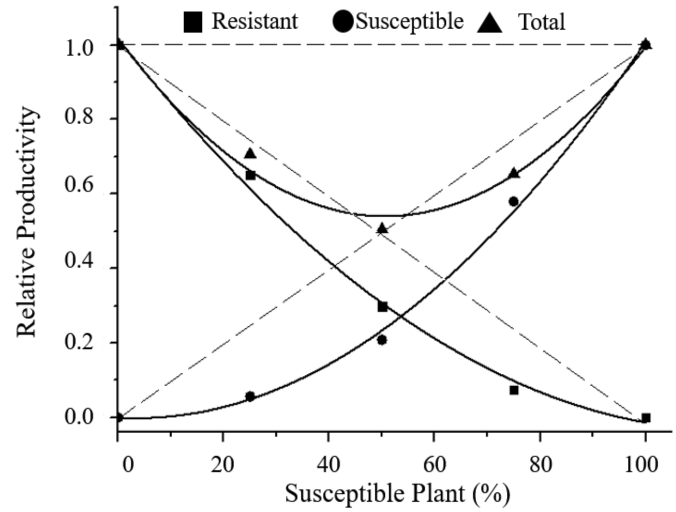

Figure 3. Diagram of the relative dry biomass of ryegrass plants resistant (■) and susceptible (●) to glyphosate and total accumulated total dry biomass (▲).

had a concave shape, indicating negative effects on the growth of both genotypes (Cousens, 1991).

According to Harper (1997), the reduction in the two relative productivities comes from allelopathy between species (Amini et al., 2009; Moraes et al., 2011) or due to an interaction between shading and nutrient consumption in the soil, what has already been observed for some nutrients. However, changes in the allelopathic behavior of the genotypes are not ruled out. They may occur due to changes in the production of secondary compounds of ryegrass, since this may have altered its shikimic acid pathway and thus the production of amino acids such as phenylalanine and tryptophan, a fact that has not yet been evaluated. In the quantitative analysis, the relative grouping indicated the relative dominance of the resistant genotype. The aggressive competitiveness also highlights the observation. These values corroborate the qualitative analysis and the observed data of interference in the wheat crop (Table 3).

In the literature, the best development of resistant or susceptible ryegrass genotypes is not well defined. It is common for the susceptible genotype to present competitive advantages compared to the resistant one, such as higher chlorophyll and carotenoid content, better physiology and greater development, but the opposite is also recurrent (Vargas et al., 2005; Kaspary et al. al., 2014). According to Oliveira et al (2014), resistant ryegrass plants competing with soybean plants did not present reductions in their development. This also happened among the resistant and susceptible genotypes. In the work of these authors, resistant ryegrass was more competitive with soy than the susceptible

Table 3. Relative grouping index of resistant (KR) and susceptible (KS) genotypes and aggressive competitiveness (A) in the proportion of 50:50% of ryegrass plants.

Resistant – Susceptible	KR	KS	A
50% - 50%	1.43	0.69	0.17

genotype. Moreover, according to Galvan et al. (2011), resistant ryegrass plants showed higher development and, consequently, greater accumulation of total dry biomass. In another case, resistant ryegrass plants showed lower aerial dry mass accumulation and seed production (Yanniccari et al., 2016). From the enzymatic point of view, resistant and susceptible ryegrass plants presented differentiated expression of enzymes linked to oxidative stress under intra and inter-biotypic competition. When the susceptible ryegrass was in competition with resistant ryegrass, the activities of the SOD and CAT enzymes were increased; these enzymes are produced to combat reactive oxygen species that are detrimental to plant development (Apel & Hirt, 2004; Agostinetto et al., 2017b).

Based on these effects, it is inferred that the development of each genotype is strictly linked to the mechanism of resistance present in the plants. Many studies have evaluated the competition between genotypes but the causes of the resistance are not known. It is known that, in general, the reduced translocation of the glyphosate herbicide as well as the overexpression of the EPSPS enzyme, possibly present in the resistant plants tested here, do not reduce the competitive ability of the species; this could justify the non-dominance of susceptible plants over resistant plants (Vila-Aiub et al., 2014; Fernández-Moreno et al., 2017; Barroso et al., 2018). Other characteristics not evaluated in the different genotypes can be different, such as germination and seed production per plant, as observed in other studies. However, as this species comes from cross-fertilization, the exchange of genetic material between plants accumulates mechanisms of resistance in genotypes and increases the genetic variability among them.

From a practical point of view, in this specific case, due to the absence of penalties in the development of competing ryegrass plants, the absence of the glyphosate herbicide will not reduce the resistant population present in the agricultural areas. It is, therefore, recommended that the control of this species be based on other tools, such as cultural practices and mechanical control, instead of chemical methods alone (Oliveira et al., 2014).

Conclusion

Under competition, regardless of the environment, resistant ryegrass plants showed better development compared to susceptible plants. This better development was linked to the greater accumulation of nutrients, among them, potassium, sulfur and zinc. Wheat productivity was more impaired when the crop coexisted with larger proportions of resistant ryegrass plants.

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