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Application sequences of pyroxsulam and pyrethroids in wheat: effects on phytotoxicity and glutathione S-transferase enzyme activity

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Abstract: The use of pesticide mixtures is a common agricultural practice that can lead to antagonistic, synergistic, or additive effects. The objective of this research was to evaluate the effects of different application sequences and intervals of the herbicides pyroxsulam and pyrethroid insecticides on wheat, with a focus on phytotoxicity, plant biometrics, total protein levels, and the catalytic activity of the antioxidant enzyme glutathione S-transferase (GSTs). Two experiments were conducted using the herbicide pyroxsulam and the insecticides deltamethrin (5 g a.i. $\bullet ha^{-1}$) and acetamiprid + fenpropathrin (30 g a.i.$\bullet ha^{-1}$ + 45 g a.i. $\bullet ha^{-1}$). The insecticides were applied either alone or in combination with the herbicide (18 g a.i.$\bullet ha^{-1}$) in different sequences (insecticide–herbicide or herbicide–insecticide) and at varying intervals (tank mixture, three, and seven days). Each insecticide was evaluated separately in a completely randomized design comprising seven treatments with four replications. Phytotoxicity symptoms were visually assessed at 7, 14, 21, and 28 days after application, while plant growth parameters, GSTs activity, and leaf protein content were measured at 24, 48, and 72 h postapplication. When applied at specific intervals and sequences, the combined application of the insecticides deltamethrin or fenpropathrin + acetamiprid with the herbicide pyroxsulam induces only slight phytotoxic effects. However, the combination of fenpropathrin + acetamiprid significantly affects plant height or biomass. Both combinations decreased protein levels and elevated GSTs activity. These findings highlight the importance of selecting appropriate application strategies to minimize crop stress.

Keywords: catalytic activity; phytotoxicity; selectivity; *Triticum aestivum*.; detoxification.
Abbreviations: GST**,** glutathione S-transferase; GSH, reduced glutathione (GSH); CDNB, 1-chloro-2,4-dinitrobenzene.

Introduction

During the wheat growth cycle, various biotic factors can compromise crops, requiring proper management to prevent yield losses. Among these factors, pest infestations pose a significant threat, causing direct damage and transmitting diseases. To mitigate these losses, pest management strategies are employed, with insecticide application remaining the most widely used approach (Balci et al., 2019). Among the commercially available options, pyrethroids stand out for their mode of action on the insect central nervous system, offering broad-spectrum efficacy against multiple pests. Pyrethroids currently represent more than one-third of the global insecticide market, with the synthetic pyrethroid market valued at approximately US$ 3.7 billion in 2023 and projected to reach US$ 5.6 billion by 2032 (Ruberti et al., 2024).

Another major challenge in wheat production is weed competition, which significantly reduces crop productivity by competing for essential resources such as light, water, and nutrients. This issue is particularly critical during the early stages of wheat development (45–50 days after emergence), when wheat is most vulnerable to competitive stress. Chemical control, particularly through the use of herbicides, remains the predominant management strategy. However, ensuring herbicide selectivity is crucial to avoid phytotoxic effects and safeguard crop health (Bari et al., 2020).

Among the available herbicides, pyroxsulam is commonly applied in the early postemergence stage of wheat, where it inhibits the acetolactate synthase (ALS) enzyme, which is responsible for the biosynthesis of the essential amino acids isoleucine, leucine, and valine. This herbicide has demonstrated high efficacy in controlling various weed species at reduced doses while maintaining selectivity for several crops (Zobiole et al., 2018).

Herbicide metabolism is a key determinant of selectivity in target crops (Mueller et al., 1990; Matzenbacher et al., 2015), a process primarily mediated by a group of transferase enzymes. These enzymes facilitate herbicide detoxification by transforming the active compounds into more water-soluble, less toxic forms. Among them, glutathione S-transferase (GSTs) plays a central role not only in the conjugation of xenobiotics but also in protecting plant tissues from oxidative damage, thereby mitigating herbicide-induced phytotoxicity (Borah et al., 2017).

GSTs participate in a wide range of intracellular processes, including primary and secondary metabolism, stress response, herbicide detoxification, and defense against ozone, heavy metals, xenobiotics, and microbial infections. Additionally, they influence key processes of seedling development, such as hypocotyl elongation and anthocyanin accumulation (Estévez and Hernández 2020).

The combined application of the herbicide pyroxsulam with pyrethroid insecticides may lead to phytotoxic effects on wheat GSTs, as pyrethroids have been reported to inhibit GSTs catalytic activity (Ribeiro et al., 2022). Pesticide mixtures can lead to synergistic, additive, or antagonistic effects, potentially compromising herbicide selectivity and increasing crop phytotoxicity (Gazziero 2015).

Despite the widespread use of pesticide combinations, research on their effects, particularly their impact on enzymatic activity, such as that of GSTs, remains limited across different organisms (Islam et al., 2019). Therefore, the objective of this study was to investigate the effects of different application sequences and intervals of the herbicide pyroxsulam in combination with pyrethroid insecticides on wheat, with a particular emphasis on phytotoxicity, plant growth parameters, total protein content, and the activity of GSTs.

Results

Phytotoxicity and plant growth parameters

The results obtained from visual assessments of phytotoxicity at different evaluation times are presented in Table 3 for the insecticide deltamethrin and Table 4 for the insecticide acetamiprid + fenpropathrin.

In the initial evaluations at 7 and 14 DAA, no significant differences were observed between the treatments and the control. However, at 21 DAA, the mixture of deltamethrin and pyroxsulam had the highest phytotoxicity, reaching 7.5%. By 28 DAA, this mixture continued to exhibit the highest phytotoxicity (10.5%), along with the treatment in which deltamethrin was applied 7 days before pyroxsulam (7.5%).

With respect to the evaluations of plant height and dry mass in wheat plants treated with deltamethrin, no significant differences were observed among the treatments (Table 3). Therefore, the phytotoxicity observed at 28 DAA did not result in a reduction in these variables.

For the insecticides fenpropathrin + acetamiprid and the herbicide pyroxsulam, no differences were detected between the treatments at 7 and 14 DAA (Table 3). However, once again, when the herbicide was applied after the insecticide, that is, when the fenpropathrin + acetamiprid treatment was applied 7 days before pyroxsulam, it resulted in greater phytotoxicity, reaching 12.5%. Despite the significant differences, the phytotoxicity observed for both insecticides can be considered low.

With respect to plant height and dry biomass, a significant difference was observed for the FA + pyroxsulam treatment at a 7-day interval, with values of 24.62 cm and 0.89 g, respectively, which differed from those of the other treatments (Table 3).

Table 1. Chemical parameters for assessing the fertility of a dystrophic Red Latosol sample (0–20 cm).

Red Latosol distrofic													
P	O.M	pH	K	Ca	Mg	H+Al	Al	BS	CEC	V	Clay	Sand	Silt
mg/dm^3	g/dm^3	$CaCl_2$	mmol/dm^3				%			%		g kg^{-1}	
15	24	5.1	2.5	28	12	40	0.4	42.5	82.5	52	660	150	190

OM, organic matter; BS, sum of bases; CEC, cation exchange capacity; V%, base saturation %.

In the treatments involving the insecticide fenpropathrin + acetamiprid, no significant difference was observed compared with the control with respect to total proteins at 24 and 72 h. However, at 48 h after application, the control had a greater concentration than did the treatment where the herbicide was applied before the insecticide with a 7-day interval, with a reduction of 43.7% compared with the control.

GSTs activity

The results of the GSTs activity at 24, 48, and 72 h after treatment with deltamethrin and fenpropathrin + acetamiprid are presented in Table 5. For the insecticide deltamethrin, at 24 and 48 h, the treatments involving the tank mixture of insecticide and herbicide and the 7-day interval between deltamethrin and herbicide application resulted in higher GSTs activity than the control. After 72 h of treatment, the control still presented lower GSTs activity than did the other treatments. The combinations of herbicide and insecticide applied at 3-day intervals, the tank mixture, and the herbicide applied alone presented higher values than did the control.

In the combination of fenpropathrin + acetamiprid with the herbicide pyroxsulam, at the 24-hour evaluation interval, only the treatment with the insecticide-herbicide sequence applied with a 3-day interval was significantly different from the control, with a higher value. At the 48-hour interval, no significant difference was observed between the treatments and the control. However, at 72 h, three treatments differed from the control: the tank mixture, the insecticide applied before the

Table 2. Treatments used in the experiments combined pyrethroid insecticides and the herbicide pyroxsulam and were applied at different intervals and sequences.

Treatments	Application sequence and interval
Deltametrina	Without herbicide
Deltametrina	Insecticide → Herbicide Pyroxsulam – 0 days (Tank mix)
Deltametrina	Insecticide → Herbicide Pyroxsulam – 3 days
Deltametrina	Insecticide → Herbicide Pyroxsulam – 7 days
Deltametrina	Herbicide Pyroxsulam → Insecticide - 0 days (Tank mix)
Deltametrina	Herbicide Pyroxsulam → Insecticide - 3 days
Deltametrina	Herbicide Pyroxsulam → Insecticide - 4 days
Acetamiprido + Fenpropatrina	Without herbicide
Acetamiprido + Fenpropatrina	Insecticide → Herbicide Pyroxsulam – 0 days (Tank mix)
Acetamiprido + Fenpropatrina	Insecticide → Herbicide Pyroxsulam – 3 days
Acetamiprido + Fenpropatrina	Insecticide → Herbicide Pyroxsulam – 7 days
Acetamiprido + Fenpropatrina	Herbicide Pyroxsulam → Insecticide – 0 days (Tank mix)
Acetamiprido + Fenpropatrina	Herbicide Pyroxsulam → Insecticide – 3 days
Acetamiprido + Fenpropatrina	Herbicide Pyroxsulam → Insecticide – 7 days
Herbicide Pyroxsulam	Without insecticide
Control	_

Table 3. Phytotoxicity in wheat plants evaluated at 28 DAA of the insecticides deltamethrin and FA alone or in combination with the herbicide pyroxsulam at different intervals and sequences of application and height and dry mass of the aerial parts of the wheat plants evaluated at 28 DAA.

	Phytotoxicity (%) at 28 DAA			
Treatments	**Sequence of applications**	**Interval between applications (days)**		
		0	**3**	**7**
Deltametrina	Alone	6.25 aA	6.25 aA	6.25 bA
Deltametrina	Insecticide → Herbicide	7.50 aA	2.50 aB	7.50 bA
Deltametrina	Herbicide → Insecticide	7.50 aA	3.75 aA	3.75 cA
FA	Alone	2.50 bA	2.50 aA	2.50 cA
FA	Insecticide → Herbicide	0.00 bC	6.25 aB	12.50 Aa
FA	Herbicide → Insecticide	0.00 bB	3.75 aA	6.25 bA
Herbicide	Alone	0.00 bA	0.00 aA	0.00 cA
Control		0.00 bA	0.00 aA	0.00 cA
F	F(A)=7.00**; F(B) = 2.93ns; F(AxB)=2.58**			
CV	94.24%			
Height (cm)				
Treatments	**Sequence of applications**	**Interval between applications (days)**		
		0	**3**	**7**
Deltametrina	Alone	29.37 aA	29.37 aA	29.37 bA
Deltametrina	Insecticide → Herbicide	29.12 aA	30.00 aA	29.12 bA
Deltametrina	Herbicide → Insecticide	29.12 aA	31.12 aA	33.12 aA
FA	Alone	32.00 aA	32.00 aA	32.00 aA
FA	Insecticide → Herbicide	34.00 aA	29.87 aA	24.62 bB
FA	Herbicide → Insecticide	34.00 aA	30.00 aA	28.12 bB
Herbicide	Alone	30.87 aA	30.87 aA	30.87 aA
Control		32.00 aA	32.00 aA	32.00 aA
F	F(A)=1.55^{ns}; F(B)=1.70^{ns}; F(AxB)= 1.93*			
CV	9.95%			
Dry mass (g)				
Treatments	**Sequence of applications**	**Interval between applications (days)**		
		0	**3**	**7**
Deltametrina	Alone	1.21 a	1.21 a	1.21 a
Deltametrina	Insecticide → Herbicide	1.32 a	1.19 a	1.28 a
Deltametrina	Herbicide → Insecticide	1.32 a	1.29 a	1.26 a
FA	Alone	1.28 a	1.28 a	1.28 a
FA	Insecticide → Herbicide	1.11 a	1.08 a	0.89 b
FA	Herbicide → Insecticide	1.11 a	1.12 a	1.25 a
Herbicide	Alone	1.03 a	1.03 a	1.03 b
Control		1.30 a	1.30 a	1.30 a
F	F(A)=3.57*; F(B)=0.13^{ns};F(AxB)=0.33^{ns}			
CV	16.93%			

CV: coefficient of variation; Factor A: treatments; Factor B: application timings. Means followed by the same lowercase letter in a column and uppercase letter in a row are not significantly different according to the Scott–Knott test at the 5% significance level.

Table 4. The total protein content was standardized at 24, 48, and 72 h on the basis of the fresh mass of each wheat sample subjected to the insecticides deltamethrin and fenpropathrin+acetamiprid, applied alone or in combination with the herbicide pyroxsulam, under different application intervals and sequences.

Standardized total proteins (mg mL^{-1}) - 24 h				
Treatments	**Sequence of applications**	**Interval between applications (days)**		
		0	**3**	**7**
Deltametrina	Alone	0.030 b	0.030 b	0.030 b
Deltametrina	Insecticide → Herbicide	0.054 a	0.058 a	0.071 a
Deltametrina	Herbicide → Insecticide	0.054 a	0.047 a	0.044 b
FA	Alone	0.025 b	0.025 b	0.025 b
FA	Insecticide → Herbicide	0.059 a	0.062 a	0.054 a
FA	Herbicide → Insecticide	0.059 a	0.060 a	0.072 b
Herbicide	Alone	0.049 a	0.049 a	0.049 a
Control		0.026 b	0.026 b	0.026 b
F	F(A)=10.13**; F(B)=0.11^{ns};F(AxB)=0.31^{ns}			
CV	26.63%			
Standardized total proteins (mg mL^{-1}) - 48 h				
Treatments	**Sequence of applications**	**Interval between applications (days)**		
		0	**3**	**7**
Deltametrina	Alone	0.024 bA	0.024 bA	0.024 cA
Deltametrina	Insecticide → Herbicide	0.057 aB	0.057 aB	0.101 aA
Deltametrina	Herbicide → Insecticide	0.057 aA	0.053 aA	0.060 bA
FA	Alone	0.028 bA	0.028 bA	0.028 cA
FA	Insecticide → Herbicide	0.054 aA	0.055 aA	0.060 bA
FA	Herbicide → Insecticide	0.054 aA	0.058 aA	0.049 bA
Herbicide	Alone	0.044 aA	0.044 aA	0.044 bA
Control		0.022 bA	0.022 bA	0.022 cA
F	F(A)=25.33*;F(B)=2.4^{ns};F(AxB)=2.20*			
CV	19.54%			
Standardized total proteins (mg mL^{-1}) - 72 h				
Treatments	**Sequence of applications**	**Interval between applications (days)**		
		0	**3**	**7**
Deltametrina	Alone	0.036 bA	0.036 bA	0.036 bA
Deltametrina	Insecticide → Herbicide	0.077 aA	0.071 aA	0.051 bA
Deltametrina	Herbicide → Insecticide	0.077 aA	0.078 Aa	0.045 Bb
FA	Alone	0.038 bA	0.038 bA	0.038 bA
FA	Insecticide → Herbicide	0.087 aA	0.078 aA	0.054 bB
FA	Herbicide → Insecticide	0.087 aA	0.052 bB	0.032 bB
Herbicide	Alone	0.076 aA	0.076 aA	0.076 aA
Control		0.039 bA	0.039 bA	0.039 bA
F	F(A)=16.73**;F(B)=13.90**;F(AxB)=2.66**			
CV	17.27%			

CV: coefficient of variation; Factor A: treatments; Factor B: application timings. Means followed by the same lowercase letter in a column and uppercase letter in a row are not significantly different according to the Scott–Knott test at the 5% significance level.

herbicide with a 3-day interval, and the herbicide applied alone, all of which presented relatively high GST enzyme activity values.

Discussion

The highest phytotoxicity observed in this study was 12.5%, occurring when the fenpropathrin + acetamiprid treatment was applied 7 days before pyroxsulam, which was reflected by slight chlorosis of the wheat leaves. The low phytotoxicity of the herbicide observed in this study was also reported in other studies. Pyroxsulam is a relatively new herbicide that has been widely adopted for controlling broadleaf weeds in wheat fields because of its high efficacy and low application rate. Pyroxsulam binds to the regulatory site of the ALS enzyme, reducing its activity in plants. This inhibition disrupts the synthesis of branched-chain amino acids and, consequently, protein production, leading to growth arrest and eventual plant death (Jursík et al., 2016; Bai et al., 2024). A study on the interactions of the herbicides 2,4-D, bentazon, metosulfuron-methyl, and pyroxsulam, which were isolated or combined with insecticides or fungicides, generally revealed that pyroxsulam had the greatest number of synergistic interactions with insecticides and fungicides, reducing wheat relative tolerance and shoot dry matter (Viecelli et al., 2019).

Galon et al. (2021) tested various herbicides for weed control in wheat and reported that pyroxsulam caused a phytotoxicity of 7.5% at 21 DAA. However, no symptoms of phytotoxicity were observed at 28 or 35 DAA. Similarly, in another study, Galon et al. (2015) reported that the herbicides iodosulfuron, metsulfuron-methyl, 2,4-D, cyhalofop-butyl, penoxsulam, and

Table 5. Glutathione S-transferase (GSTs) activity in wheat at 24, 48, and 72 h after exposure to the insecticides deltamethrin and fenpropathrin+acetamiprid, applied alone or in combination with the herbicide pyroxsulam, under different application intervals and sequences.

Glutatione S-Transferase (umol min^{-1} mg^{-1} of protein) - 24 h				
Treatments	**Sequence of applications**	**Interval between applications (days)**		
		0	3	7
Deltametrina	Alone	0.55 a	0.55 a	0.55 a
Deltametrina	Insecticide → Herbicide	0.40 a	0.47 a	0.33 b
Deltametrina	Herbicide → Insecticide	0.40 a	0.52 a	0.34 b
FA	Alone	0.62 a	0.62 a	0.62 a
FA	Insecticide → Herbicide	0.42 a	0.41 a	0.38 b
FA	Herbicide → Insecticide	0.42 a	0.53 a	0.25 b
Herbicide	Alone	0.52 a	0.52 a	0.52 a
Control		0.69 a	0.69 a	0.69 a
F	F(A)=6.90**;F(B)=2.16^{ns};F(AxB)=0.53^{ns}			
CV	20.88%			
Glutatione S-Transferase (umol min-1 mg-1 of protein) - 48 h				
Treatments	**Sequence of applications**	**Interval between applications (days)**		
		0	**3**	**7**
Deltametrina	Alone	0.71 a	0.71 a	0.71 a
Deltametrina	Insecticide → Herbicide	0.58 a	0.57 a	0.26 b
Deltametrina	Herbicide → Insecticide	0.58 a	0.60 a	0.27 b
FA	Alone	0.66 a	0.66 a	0.66 a
FA	Insecticide → Herbicide	0.60 a	0.68 a	0.43 b
FA	Herbicide → Insecticide	0.60 a	0.50 a	0.31 b
Herbicide	Alone	0.70 a	0.70 a	0.70 a
Control		0.87 a	0.87 a	0.87 a
F	F(A)=8.79**;F(B)=0.78^{ns};F(AxB)=0.58^{ns}			
CV	13.29%			
Glutatione S-Transferase (umol min^{-1} mg^{-1} of protein) - 72 h				
Treatments	**Sequence of applications**	**Interval between applications (days)**		
		0	**3**	**7**
Deltametrina	Alone	0.68 a	0.68 a	0.68 a
Deltametrina	Insecticide → Herbicide	0.46 b	0.52 b	0.55 a
Deltametrina	Herbicide → Insecticide	0.46 b	0.44 b	0.38 n
FA	Alone	0.42 b	0.42 b	0.42 b
FA	Insecticide → Herbicide	0.43 b	0.50 b	0.47 b
FA	Herbicide → Insecticide	0.43 b	0.53 b	0.58 a
Herbicide	Alone	0.46 b	0.46 b	0.46 a
Control		0.49 b	0.49 b	0.49 a
F	F(A)=3.57*;F(B)=0.13^{ns};F(AxB)=0.33^{ns}			
CV	16.93			

CV: coefficient of variation; Factor A: treatments; Factor B: application timings. Means followed by the same lowercase letter in a column and uppercase letter in a row are not significantly different according to the Scott–Knott test at the 5% significance level.

pyroxsulam, which are applied for weed control in wheat, presented symptoms of phytotoxicity below 10% for the wheat cultivars TBIO Quartzo and TBIO Pioneiro across all evaluations, which aligns with the values reported in the present study. Compared with the control, the herbicide pyroxsulam applied alone resulted in a 94.8% increase in GSTs activity. The pyrethroid insecticides allethrin, cypermethrin, fenpropathrin, and deltamethrin affect GSTs activity by reducing its catalytic function, with deltamethrin exhibiting the highest inhibitory potential (Ribeiro et al., 2022). In contrast, in the present study, enzyme activity increased over time in the combined treatments and with the herbicide applied alone, suggesting that the observed effect may be attributed to the herbicide.

On the other hand, when the activities of the herbicides acetochlor, atrazine, and oxyfluorfen were evaluated in relation to GSTs activity in corn, sorghum, and wheat plants at 24, 48, and 72 h after treatment, higher GSTs activity was observed in the presence of acetochlor, particularly at 48 h after treatment (Cataneo et al., 2008).

GSTs contribute to both herbicide selectivity in crops and herbicide resistance in weeds. Plants rely on GSTs for a range of essential functions, including primary and secondary metabolism, stress tolerance, and cell signaling (Pelon et al., 2023).

Higher expression levels of GSTs genes have been associated with increased enzyme activity and more efficient detoxification of the herbicide metolachlor, which may account for differences in herbicide tolerance among maize cultivars (Liu et al., 2017). In the present study, the wheat leaves subjected to these treatments appeared to possess sufficient GSTs activity to prevent the accumulation of phytotoxic pesticide concentrations in plant tissues. Phytotoxicity, as described by Carvalho et al. (2009), occurs when the protective capacity conferred by selectivity mechanisms is exceeded. When metabolism constitutes the primary mechanism of tolerance, phytotoxic effects may reflect the inherent inability of a species to detoxify a given compound. Moreover, as herbicide metabolism demands substantial energy input, phytotoxic symptoms

can be interpreted as indicators of additional energy expenditure rather than a normal physiological response, potentially leading to significant yield losses.
With respect to GSTs, Cobb and Reade (2011) reported that cultivated plants exhibit greater activity of this enzyme than susceptible weed species do, suggesting that this difference in selectivity may be attributed to GSTs. It has been demonstrated that GSTs can detoxify various classes of herbicides, including triazines, chloroacetamides, thiocarbamates, and diphenyl ethers (Prade et al., 1998). Six different GSTs isoforms have been identified in maize (Shah et al., 1986).

Materials and Methods

Study area and genetic material

The experiment was conducted in a greenhouse and in the Agricultural and Environmental Microbiology Laboratory at the Center for Agricultural Sciences of the Federal University of São Carlos, located in Araras, São Paulo State, Brazil. The geographical coordinates are 22°18′ S and 47°23′ W. The average altitude is approximately 600 m.
The wheat cultivar used was TBIO PONTEIRO, sourced from Lagoa Bonita Sementes, with eight seeds sown per pot.

Experimental conditions and treatment application

The experimental units consisted of 5 L polyethylene pots filled with soil classified as Dark Red Latosol (Embrapa, 1999), collected from the 0–20 cm surface layer. The chemical characteristics of the soil are presented in Table 1.
The experiment was conducted with two pyrethroid insecticides: deltamethrin (5 g a.i.•ha^{-1}) and acetamiprid + fenpropathrin (30 g a.i.•ha^{-1} + 45 g a.i.•ha^{-1}) (Table 2). For each insecticide, treatments consisted of applications either alone or in combination with the herbicide pyroxsulam (18 g a.i.•ha^{-1}), applied in different sequences (insecticide before herbicide, herbicide before insecticide, or tank mix) and intervals (0, 3, or 7 days between applications).
The experimental design was a randomized complete block design (RCBD) with four replicates, and the data were analyzed in a two-factor factorial scheme (treatments × sampling times). The first factor (Factor A) consisted of eight treatments: insecticides applied alone; combinations with pyroxsulam at three application intervals (0, 3, and 7 days); herbicide alone; and the untreated control. The second factor (Factor B) corresponded to sampling times at 24, 48, and 72 h after application.
A backpack sprayer pressurized with CO_2 and operating at a pressure of 30 lb•in^2 was used. It was equipped with a boom featuring two fan-type nozzles (XR 110.03) spaced 0.50 m apart, delivering a spray volume of 200 L•ha^{-1}. At the time of application, the meteorological conditions were 25°C, 60% relative humidity, and a wind speed of 3 m•s. The treatments were applied when the wheat plants reached the two fully expanded leaf stages.
The effects of the treatments on the crop were evaluated at 7, 14, 21, and 28 days after application (DAA) on the basis of visual symptoms of phytotoxicity, using a scale from 0% (zero) to 100%, where 0% indicates the absence of any visible damage and 100% represents complete plant death. (Frans 1972).
At 30 DAA, plant height was measured, and one plant per pot was collected to assess the dry biomass of the aerial part. The samples were dried in a forced-air circulation oven at 60°C until a constant dry mass was reached.

Evaluation of catalytic activity and total protein content

Samples of the aerial parts of the plants from each treatment were collected in duplicate at 24, 48, and 72 h after the final pesticide application (e.g., herbicide, insecticide, or tank mixture). The samples were weighed, placed in paper bags, and stored in an ultrafreezer at -80°C.
Protein extraction was carried out via previously described methods, with modifications, as adapted from earlier works (Cataneo et al., 2003). The samples were ground with liquid nitrogen via a mortar and pestle and then homogenized in 20 mL of ice-cold Tris-HCl buffer (50 mmol L^{-1}, pH 7.0) containing 20% glycerol (v/v), 1 mmol L^{-1} ascorbic acid, 1 mmol L^{-1} dithiothreitol (DTT), 1 mmol L^{-1} EDTA, 1 mmol L^{-1} reduced glutathione, and 5 mmol L^{-1} $MgCl_2$. The homogenates were transferred to Falcon® centrifuge tubes and centrifuged at 4°C for 12 min at 12,000 × g. The resulting supernatants were collected, transferred to new tubes, and centrifuged again for 15 min at 14,000 × g. The final supernatants were stored at -20°C.
The obtained supernatants were used to determine the total protein content via the Bradford method (1976) and to analyze glutathione S-transferase (GST) enzymatic activity via Hemingway (1998), as described in Brazil (2006). Both analyses were performed spectrophotometrically in 96-well microplates (Corning®) via a Tecan Infinite® 200 PRO plate reader.
For total protein quantification, 10 µL of each sample was pipetted into a microplate in triplicate. The blank consisted of 10 µL of the homogenization buffer used during extraction, which was also performed in triplicate. Then, 300 µL of Bradford reagent (ACS Científica®) was added to each well. After a 4 min incubation, the absorbance was measured at 620 nm. A calibration curve was constructed using known concentrations of bovine serum albumin (BSA) as the standard protein. The results were standardized on the basis of the wet mass of each sample and expressed as mg/mL protein.
GSTs activity was assessed by adding 15 µL of each sample in triplicate, followed by 195 µL of a working solution containing 10 mM reduced glutathione (GSH) in 100 mM potassium phosphate buffer (pH 6.5) and 21 mM CDNB (1-chloro-2,4-dinitrobenzene) in methanol. The working solution was prepared by combining 20 mL of the glutathione solution with 1 mL of CDNB. The reaction started immediately upon adding this solution to the samples. The absorbance was measured at 340 nm at time zero and then at 1 min intervals for 28 min. Absorbance values were corrected by subtracting the blank (15 µL of buffer + 195 µL of the working solution). In the linear phase of the reaction curve, two time points (10 and 20 min) were selected to calculate the absorbance variation per minute (ΔA340•min).
The GSTs enzymatic activity was calculated via the following formula:

The enzymatic activity was calculated via the following formula:
Activity GSTs = ($\Delta A_{340/min}$ × 0.21)/(0.0096 × 1000 × 0.6 × 0.015)
ΔA340/min = absorbance variation between two distinct points divided by time (10 min)
0.21 = final reaction volume (mL)
0.0096 = molar extinction coefficient of the reaction product (μmol•cm^{-1})
0.6 = optical path (height of the reaction volume in the microplate well, in cm)
0.015 = sample volume (mL)
The GSTs activity results were expressed in μmol/min/mg of protein (or nmol/min/mg of protein) by dividing the obtained activity value by the total protein concentration.

Statistical analysis
The data were analyzed via two-factor factorial ANOVA (treatments × sampling times) in a randomized complete block design (RCBD) with four replicates. When a significant interaction was detected, the treatment means were compared via Scott Knott's test at the 5% significance level ($p < 0.05$) via Agroestat Software (Barbosa and Maldonado Jr 2015). In the absence of significant interactions, the main effects were analyzed separately, and unfolding of the isolated factors was performed when appropriate.

Conclusion

The results demonstrate that the combined application of the insecticide deltamethrin with the herbicide pyroxsulam, when applied at specific intervals and sequences, induces only slight phytotoxic effects that do not significantly affect plant height or biomass. However, this treatment was associated by a reduction in protein levels and a marked increase in GSTs enzyme activity, suggesting an induced detoxification response. In contrast, the combination of pyroxsulam with the insecticide fenpropathrin + acetamiprid resulted in mild phytotoxicity with greater physiological impact, including reduced plant height and dry mass, decreased protein content, and elevated GSsT activity. These findings highlight the differential physiological responses of wheat plants to distinct herbicide–insecticide combinations and underscore the importance of selecting appropriate application strategies to minimize crop stress.

Statement of contributions: ALD; PAM, SRCA: prepared and planned the experimental design. ALD; IST; BBRS: carried out the experiments. ALD and BFS conducted the statistical data analysis. ALD, ACSH, and PAM wrote the article.

Conflict of interest
There are no conflicts of interest.

Data availability
The data will be available upon fair request to the corresponding author.

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