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Articles

Enzymatic activity in *Sorghum bicolor* by micro-nano encapsulated microbial metabolites and plant extracts

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Abstract

Chemical herbicides for weed control represent a current problem, since their indiscriminate use causes the emergence of resistant weed populations, in addition to affecting the environment and human health. Therefore, secondary metabolites of microorganisms and plant extracts in micro-nano encapsulated formulations emerge as a possible alternative to the use of chemically synthesized herbicides. Therefore, elucidating their mechanism of action is necessary to understand the biochemical changes they induce in plants and to develop weed control strategies. The objective of the research was to determine the activity of the antioxidant enzymes phenylalanine ammonia lyase, peroxidase, and superoxide dismutase in Sorghum bicolor plants treated with secondary metabolites of *Alternaria* sp. The secondary metabolites of microorganisms and a plant extract of *Solanum rostratum*, alone and formulated in micro-nano encapsulated formulations based on the biopolymer's alginate and chitosan. The study was carried out during the month of June 2024, for this, *S. bicolor* y plants were used and the activity of the enzymes was determined for 0, 3, 6, 12, 24 and 48 h. It was observed that the formulations loaded with the *Solanum rostratum* plant extract and the secondary metabolites of microorganisms were those that induced the highest enzymatic activity at different times, reaching 0.36 and 0.34 U mol^{-1} respectively in the case of PAL, 4.7 and 4.3 U mol^{-1} with the peroxidase enzyme and 7.3 and 6.5 U mol^{-1} with super oxide dismutase. It is concluded that the secondary metabolites of microorganisms and the *Solanum rostratum* plant extract formulated in micro-nano encapsulates have potential as agents that can modify biochemical processes in plants.

Keywords:

bioherbicides, metabolites, nanotechnology, plant extracts.

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Introduction

Weeds compete with crops for nutrients, water, sunlight, and space, so controlling them is of utmost importance. Chemical herbicides are the most used option by producers. Nonetheless, their indiscriminate use has generated the appearance of resistant weeds, such as the species *Amaranthus palmeri*, *Bromus sterilis*, *Digitaria sanguinalis*, *Panicum dichotomiflorum*, *Echinochloa crus-galli* (Ofosu *et al*., 2023), so it is necessary to develop alternatives to reduce the negative effects of chemical herbicides, such as environmental pollution, impact on pollinators, and effects on human health (Van Bruggen *et al*., 2021).

Thus, bioherbicides based on microbial secondary metabolites (MSMs) and plant extracts (PEs) emerge as an option for weed control, being a sustainable alternative that does not generate the appearance of resistant weeds (Anwar *et al*., 2021).

Interest in the development of bioherbicides has been increasing and several authors have reported allelopathic activity by MSMs and PEs. In this sense, fungi imperfecti, such as *Alternaria* sp., are characterized by producing phytotoxins, which can be used for the development of bioherbicides (Kausar *et al*., 2022); regarding PEs, herbicide compounds that inhibit plant germination and development have been identified in the species *Solanum rostratum* (Tucuch-Pérez *et al*., 2023).

Although MSMs and PEs are an option for weed control, their herbicidal activity can be improved when formulated in biopolymer-based micro-/nano-encapsulates (NPs), as the active ingredient is protected and its degradation by factors, such as light, temperature, humidity, and radiation, is prevented (Tucuch-Pérez *et al*., 2023).

In plants, enzymatic activity increases to counteract the effects caused by herbicides, such as the production of reactive oxygen species (ROS) that generate oxidative damage, which induces increased activity of antioxidant enzymes, such as superoxide dismutase (SOD) and peroxidase (POD), as well as enzymes such as phenylalanine ammonia lyase (PAL), which is crucial in the phenylpropanoid pathway for the biosynthesis of phenolic compounds, lignin, and flavonoids that reduce stress in plants (Yin *et al*., 2008; Caverzan *et al*., 2019).

Therefore, detecting and quantifying enzymatic activity in plants emerges as an option to elucidate and understand the effect of bioherbicides on weeds. Thus, the objective of this study was to determine the enzymatic activity of the enzymes PAL, POD, and SOD in plants of *Sorghum bicolor* as a model plant, treated with NPs loaded with ASMs and a plant extract of *S. rostratum* (SRPE).

Materials and methods

Obtaining the extract from the seeds of *Solanum rostratum* and the strain of *Alternaria* sp.

The SRPE was provided by the company GreenCorp biorganiks de México, whereas the strain of *Alternaria* sp. was provided by the Mycology and Biotechnology Laboratory of the Department of Parasitology of the Antonio Narro Autonomous Agrarian University (UAAAN), for its acronym in Spanish, which is identified in the strain collection with the code UAAA#3.

Production of secondary metabolites of *Alternaria* sp.

ASMs were produced using a liquid culture medium based on potato infusion 400 g L^{-1}, yeast extract 7.5 g L^{-1}, peptone 2 g L^{-1}, dextrose 15 g L^{-1}, $MgSO_4$ 0.5 g L^{-1} and $FeSO_4 7H_2O$ 1 g L^{-1}. In a flask, the medium was inoculated with a 5 mm explant with seven days of growth. The flask was placed in stirring at 120 rpm for seven days at a temperature of 28 °C. The biomass was separated and the fermented obtained was centrifuged at 6 000 rpm and filtered with a 0.2 µm Millipore filter (Todero *et al*., 2018).

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Characterization of secondary metabolites of *Alternaria* sp. and phytochemical compounds present in *Solanum rostratum* seed extract by HPLC-MS

The characterization of the ASMs and compounds present in the SRPE was performed in an HPLC system with autosampler, a ternary pump, a PDA detector, and a liquid chromatograph-ion trap mass spectrometer equipped with an electrospray ion source (Agilent 6520B Q-TOF). Five microliters of the sample were injected into 200 mg L^{-1} in a Denali C18 column; the oven temperature was kept at 30 °C. The eluents used were formic acid (0.2% v/v) and acetonitrile (3-50%).

The gradient used was as follows: initial, 3% B; 0-5 min, 9% B linear; 5-15 min, 16% B linear; 15-45 min, 50% B linear. Subsequently, the column was washed and reconditioned; the flow rate was kept at 0.2 ml min^{-1} and the elution was controlled at 245, 280, 320, and 550 nm. All the effluent was injected into the source of the mass spectrophotometer, without splitting it. The data were processed using the MS Workstation software (Ascacio-Valdés *et al*., 2016).

Production of micro-nano-encapsulates loaded with secondary metabolites of *Alternaria* sp. and extract of *Solanum rostratum*

The NPs were produced using a $CaCl_2$ solution which was added with 3.75 ml of sodium alginate solution through a system composed of a peristaltic pump under constant and vigorous stirring. Subsequently, 12.5 ml of chitosan was added to the solution of $CaCl_2$ and sodium alginate, and it was kept in constant stirring for 90 min. This was done in the presence of ASMs and SRPE at 100% concentration (Tucuch-Pérez *et al*., 2023).

Characterization of micro-nano-encapsulates loaded with secondary metabolites of *Alternaria* sp. and extract of *Solanum rostratum*

The size was determined by dynamic light scattering (DLS). The zeta potential (mV) was measured through the Colloid Metriz ZETA-Check system, and the pH was determined with a potentiometer. The encapsulation efficiency was determined using the technique proposed by Taban *et al*. (2021).

The absorbance of the metabolites and extract was measured and then the NPs were centrifuged, followed by a measurement of the absorbance of the supernatant. The encapsulation efficiency (EE) was calculated using the following formula:

$$\%EE = \left(\frac{T0\text{-}S0}{T0}\right)100$$

Where: T0 is the absorbance of the plant extract and S0 represents the absorbance of the supernatant of the NPs loaded with the ASMs and SRPE at 100% concentration.

Evaluation of enzymatic activity in *Sorghum bicolor* test plants

The plants used were plants of *S. bicolor* Var. Sudan of 20 days of development, and the substrate used was a mixture of sterile perlite, soil and peat moss (1:1:1); the plants were kept in a greenhouse free of pests and diseases. The treatments were applied by foliar spraying. The treatments were: T1= NPs loaded with ASMs, T2= NPs loaded with SRPE, T3= ASMs, T4= SRPE, T5= Unloaded NPs and T6= absolute control. Sampling was performed at 0, 3, 6, 12, 24 and 48 h after spraying (González-Gallegos *et al*., 2015).

Enzyme extraction

One gram of the plant tissue was macerated, then a 0.1 M sodium tetraborate buffer solution (pH 8.8) was used for PAL extraction (Rodríguez-Pedroso *et al*., 2006), a 0.05 M pH 6 phosphate buffer to extract POD (Yedidia *et al*., 1999) and a 0.05 M pH 8.8 phosphate buffer for SOD (Romero-Tejeda et al., 2015); finally, the samples were centrifuged at 10 000 rpm at 4 °C and the supernatant was taken.

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Determination of PAL enzyme activity

Nine hundred microliters of L-phenylalanine were used as a substrate, the enzyme extract was added, and it was incubated at 40 °C for 30 min. The reaction was stopped with 5 N HCl. Finally, the samples were placed on ice and 5 ml of distilled water was added, the reading was at 290 nm. Activity was reported as a unit of enzymatic activity, defined as the production of 1 µmol of trans-Cinnamic acid per minute^{-1} (Rodríguez-Pedroso *et al*., 2006).

Determination of POD enzyme activity

A reaction mixture was prepared with the enzymatic extract, 0.2% phenol red and sodium citrate (50 nM pH 4.2). The reaction was started with H_2O_2 and stopped 3 min later with 2 N NaOH. The absorbance was 610 nm. The unit of enzymatic activity consisted of the production of 1 µmol of oxidized phenol red per minute^{-1} (Yedidia *et al*., 1999).

Determination of SOD enzyme activity

It was determined by adding 400 µl of the enzyme extract and 30 µl of riboflavin (4.4 mg ml^{-1}) to a reaction mixture with nitro blue tetrazolium (NBT) (1.41 mg ml^{-1}) and 0.1% Triton X-100. The mixture was stirred and illuminated with fluorescent light of 20 watts for 7 min, making the reading at an absorbance of 560 nm. The unit of enzymatic activity was equal to the amount of supernatant that photoinhibits 50% of the formation of nitro blue tetrazolium formazan (Romero-Tejeda *et al*., 2015).

Statistical analysis

The design used was a completely randomized design with six replications per treatment; the comparison of means was performed through analysis of variance and the Tukey means comparison test (*p*# 0.05) with the statistical analysis system computer program, version 9.0.

Results and discussion

Identification of secondary metabolites of *Alternaria* sp. and phytochemical compounds present in *Solanum rostratum* extract by HPLC-MS

Several metabolites produced by *Alternaria* sp. were identified, such as scopoletin, caffeic acid 4-O-glucoside, gallic acid 3-O-gallate, p-HPEA-EA, and 3,7-Dimethylquercetin. On the other hand, phytochemical compounds, such as caffeic 4-O-glucoside, protocatechuic acid 4-O-glucoside, resveratrol 3-O-glucoside, sinensetin, ferulic acid 4-O-glucoside, tetramethylscutellarein, and isorhamnetin 3-O-glucoside 7-O-rhamnoside, were detected in the seed extract of *S. rostratum* (Table 1).

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Table 1. Secondary metabolites of *Alternaria* sp. and phytochemical compounds of *Solanum rostratum* extract identified by HPLC-MS.

Extract	Retention time (min)	Mass	Compound	Family
Metabolites of *Alternaria* sp.	5.515	190.8	Scopoletin	Hydroxycoumarin
	6.358	341.6	Caffeic acid 4-O-glucoside	Hydroxycinnamic acids
	7.056	322.8	Gallic acid 3-O-gallate	Hydroxybenzoic acids
	8.813	361.7	p-HPEA-EA	Tyrosols
	19.074	328.8	3,7-Dimethylquercetin	Methoxyflavonols
Extract of *Solanum rostratum*	6.552	341	Caffeic acid 4-O-glucoside	Hydroxycinnamic acids
	17.96	389.9	Resveratrol 3-O-glucosideo	Stilbenes
	18.707	314.8	Protocatechuic acid 4-O-glucoside	Hydroxybenzoic acids
	28.397	370.8	Sinensetin	Methoxyflavones
	31.085	354.7	Ferulic acid 4-O-glucoside	Methoxycinnamic acids
	33.77	622.8	Isorhamnetin 3-O-glucoside 7-O-rhamnoside	Methoxyflavonols

Caffeic acid, detected in ASMs and SRPE, has the ability to increase ROS levels in plants, triggering oxidative stress, which structurally damages plant cells (Tucuch-Pérez *et al*., 2023). On the other hand, resveratrol and sinensetin have also been reported as compounds with allelopathic activity on plants, and it has been elucidated that they affect plants by inhibiting enzymes and certain metabolic pathways (Husic *et al*., 2023).

Finally, flavonoids, such as isorhamnetin3-O-glucoside 7-O-rhamnoside, and 3,7-dimethylquercetin, have been reported as compounds that affect plant development by inhibiting aerial and root growth, causing a decrease in the amount of biomass produced by plants (Balah *et al*., 2020; Fernández-Aparicio *et al*., 2021).

Characterization of micro-nano-encapsulates

The size of NPs loaded with ASMs and SRPE ranged between 258 and 260 nm, whereas unloaded NPs had a size of 158 nm. In relation to the zeta potential, NPs loaded with ASMs had zeta potential of -30 mV, whereas in those loaded with SRPE, it was -29 mV. The chemical stability of the biopolymers was determined by measuring the pH, with values of 4.89 and 4.45 for NPs loaded with ASMs and SRPE, respectively. Finally, the encapsulation efficiency of NPs was 87% for those loaded with ASMs and 81% for those loaded with SRPE (Table 2).

Table 2. Values derived from the evaluation of different variables in formulations of micro-/nano-encapsulates loaded with secondary metabolites of *Alternaria* sp. and *Solanum rostratum* extract.

Variables	Size (nm)	Potential Z (mV)	pH
NPs metabolites of *Alternaria* sp.	258±25	-30±2	4.89
NPs metabolites of *S. rostratum* seed	360±11	-29±3	4.6
NPs without metabolites or extracts	158±16	-30±1	4.45

The results obtained suggest that the size of NPs is influenced by ASMs and SRPE; in this sense, various studies have reported different sizes of NPs loaded with plant extracts, as reported by

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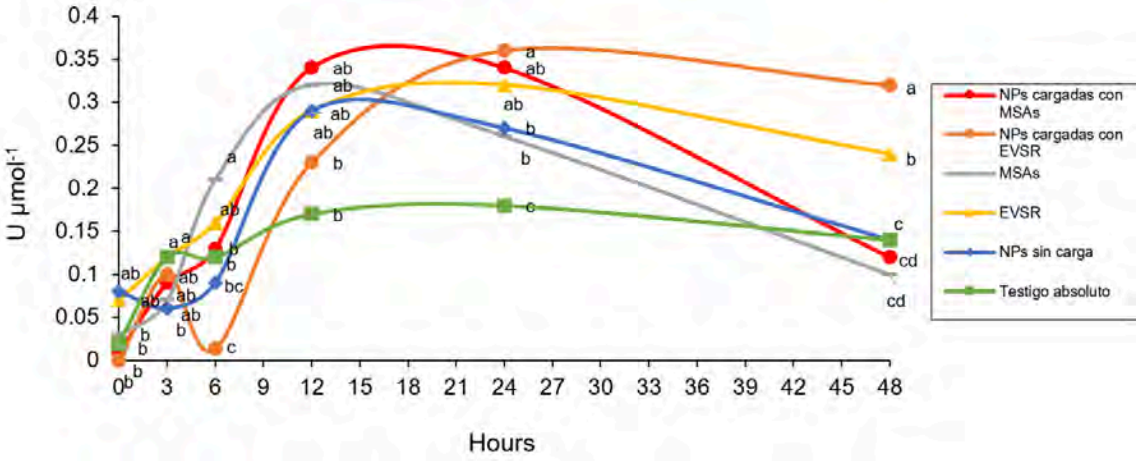
Tucuch-Pérez *et al*. (2023), who produced alginate and chitosan NPs with *S. rostratum* extract with a size of 340 nm; on the other hand, NPs loaded with secondary metabolites of *Bacillus* spp., with a size of 500 nm, have been reported (Ureña-Saborío *et al*., 2017).

Regarding the zeta potential, the negative values observed indicate stability in the NPs, which allows good dispersion and mobility through the plants. Chemical stability is related to the final particle size and encapsulation capacity; in this regard, there reports of chitosan and alginate NPs with pH values between 4.5 and 4.6 (Tucuch-Pérez *et al*., 2023).

Enzymatic activity of PAL, POD, and SOD in *Sorghum bicolor* plants treated with micro-nano-encapsulates

In the first sampling times, the enzymatic activity was low, increasing after 6 h. Subsequently, from 12 h onwards, the amount of the PAL enzyme increased until reaching its maximum peak at 24 h, with no statistical difference observed between the treatments with the best enzymatic activity, which were NPS with SRPE, NPs with ASMs and SRPE with 0.36, 0.34 and 0.32 U mol^{-1} (Figure 1).

Figure 1. PAL activity in *S. bicolor* plants at 0, 3, 6, 12, 24 and 48 h after spraying.



In the case of the POD enzyme, the enzymatic activity was low in the first hours, increasing from 6 h, reaching the highest activity at 48 h, with the treatments of NPs loaded with SRPE and ASMs being the ones that presented the highest amount of the enzyme with 4.7 and 4.3 U mol^{-1}, with a statistical difference between the NPs with SRPE and the unencapsulated treatments and the absolute control (Figure 2).

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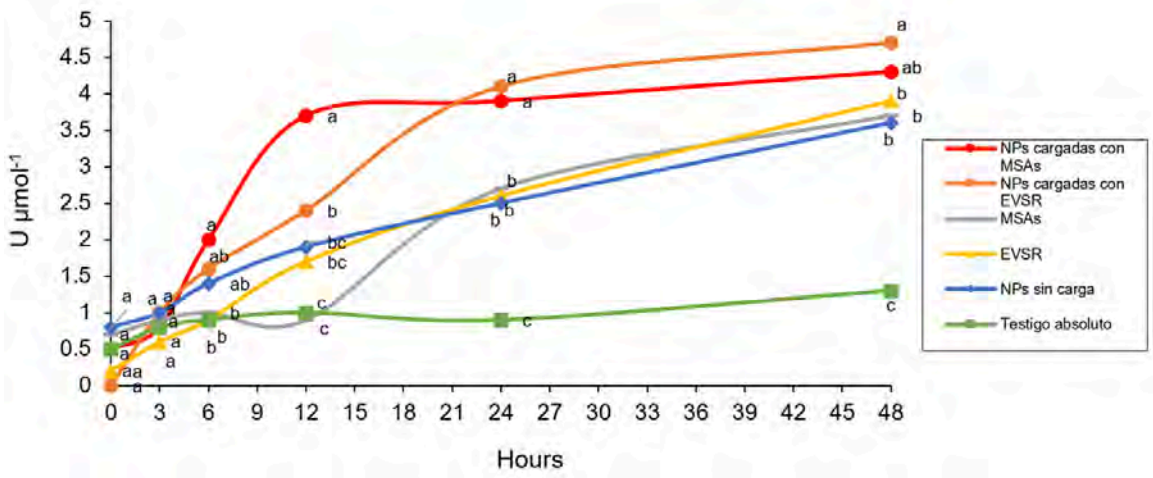
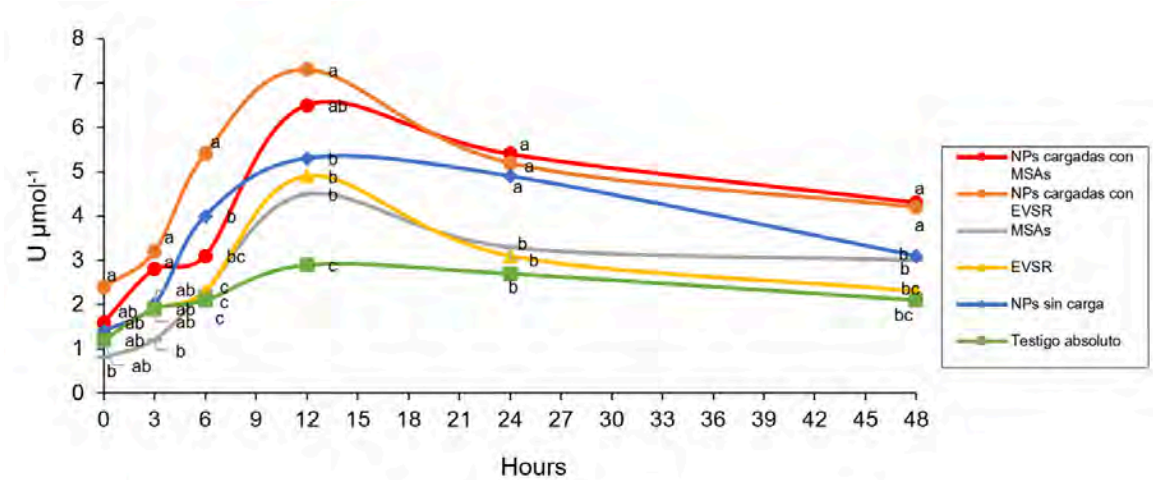
Figure 2. POD activity in *S. bicolor* plants at 0, 3, 6, 12, 24 and 48 h after spraying.



The enzymatic activity of SOD increased after 6 h, reaching the maximum peak of enzymatic activity at 12 h, with a statistical difference between NPs with SRPE and the other treatments, with the treatments corresponding to NPs with SRPE and ASMs presenting the highest activity with 7.3 and 6.5 (Figure 3).

Figure 3. SOD activity in *S. bicolor* plants at 0, 3, 6, 12, 24 and 48 h after spraying.



The low initial enzymatic activity of enzymes that was observed in *S. bicolor* may be due to the time it takes for plants to recognize stimuli applied to activate metabolic pathways, which requires a series of previous processes, such as intracellular signaling and gene transcription (Vogt, 2010).

Bioherbicides increase ROS levels due to the stress they cause in plants (Fancy *et al*., 2017; Traxler *et al*., 2023). In this sense, ROS triggers signal transduction pathways in response to stress. However, an excess of ROS generates cell damage, which causes alterations in morphology (Huang *et al*., 2019).

To counteract the effect of ROS, plants developed an enzymatic antioxidant system, which protects them from oxidative stress. This system involves enzymes that catalyze reactions that produce

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compounds that act as antioxidants or enzymes that directly use ROS as a substrate for the production of compounds that detoxify the plant (Grewal *et al*., 2022).

In this sense, the enzymes PAL, POD, and SOD have a key role against oxidative stress caused by bioherbicides. PAL intervenes in the biosynthesis of phenolic compounds by catalyzing phenylalanine into cinnamic acid, initiating the production of phenols with antioxidant properties that can be transformed into lignin, which strengthens plant cell walls (Kumar *et al*., 2024).

Within the ROS, hydrogen peroxide (H_2O_2) oxidizes proteins and lipids; to counteract this effect, POD uses H_2O_2 as a substrate to oxidize phenolic compounds that are used for the polymerization of lignin in cell walls, increasing the resistance of plants (Caverzan *et al*., 2019). Finally, SOD catalyzes the dismutation of superoxide into H_2O_2 and molecular oxygen and serves as a precursor to antioxidant enzymes, such as catalase and POD; thus, these three enzymes neutralize the toxic effects of ROS, protecting plants from the effects induced by bioherbicides (Traxler *et al*., 2023).

Among the metabolites and phytochemical compounds detected, ROS-inducing compounds, such as scopoletin and caffeic acid, were observed, which could induce ROS production in *S. bicolor* plants, with NPs loaded with ASMs and SRPE being the ones that induced oxidative stress, triggering phenoxyl radicals and ROS that affect DNA, lipid peroxidation, and cell division (Tucuch-Pérez *et al*., 2023) due to the fact that NPs improve the efficacy of compounds due to greater penetration into plants and dosed release (Zabot *et al*., 2022).

The use of the aforementioned antioxidant enzymes as biomarkers allows us to infer that the treatments corresponding to NPs loaded with SRPE and ASMs acted on plants, generating greater oxidative stress at the cellular level, causing plants to produce more antioxidant enzymes (Yin *et al*., 2008).

Regarding the above, studies have been carried out to correlate the activity of antioxidant enzymes with the herbicidal activity of weed control products, and the following has been documented: the increase in PAL and POD enzymes in lentil seedlings when treated with imazethapyr (Kumar *et al*., 2024), the increase in the SOD enzyme in rice when treated with bentazon, penoxsulam and Cyhalofop-butyl (Nohatto *et al*., 2016), and the increase of the POD enzyme in *Avena sativa*, *Vicia sativa*, *Raphanus sativus*, and *Lupinus albus* when applying fomesafen and sulfentrazone (Alves *et al*., 2018).

The observed changes in enzymatic activity indicate alteration in the metabolic processes of *S. bicolor* plants when using ASMs and SRPE alone and in NPs as bioherbicides, which were shown to induce ROS, activating the enzymes PAL, POD and SOD, suggesting a defense response by plants to oxidative stress (Sinegovskaya and Dushko *et al*., 2021). These results are presented highlighting the potential of ASMs and SRPE in NPs as agents that can modify biochemical processes in plants.

Conclusion

ASMs and SRPE are presented as an option for the development of bioherbicides due to their content of bioactive compounds with allelopathic activity, which induce the production of ROS, triggering the activation of enzymes, such as PAL, POD and SOD; in addition, the formulation of these compounds in NPs can increase their efficacy. This could be observed in the increased enzymatic activity in plants treated with NPs loaded with ASMs and SRPEs, which suggest that they cause increased oxidative stress, altering plant metabolic processes by increasing the activity of enzymes involved in defense mechanisms against oxidative stress and lipid peroxidation. Thus, the results obtained in the present study demonstrate the potential of ASMs and SRPE as alternatives for weed control, especially when produced in NP formulations.

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