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Changes in affinin contents in *Heliopsis longipes* (chilcuague) after a controlled elicitation strategy under greenhouse conditions

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ABSTRACT

Heliopsis longipes (H. longipes) is an endemic plant of the Sierra Gorda, Mexico, whose root crude extract is used as an analgesic, anti-inflammatory and anti-ulcerative. Affinin is the main substance that provides these properties. Affinin concentrations have been seen to vary depending on the time of the year in which it is collected. Controlled cultivation of *H. longipes* may reduce the synthesis of affinin but using elicitation technique it might be possible to increase and standardize the biosynthesis of this specialized metabolite. The aim of this research was to determine the changes in the affinin contents in *H. longipes* roots after controlled elicitation using salicylic acid and hydrogen peroxide as elicitors under greenhouse conditions. After a period of acclimation of 150 days for chilcuague plants, both elicitors were foliarly sprayed for two weeks. Besides affinin contents, changes on the biomass production, catalase, superoxide dismutase, phenylalanine ammonia-lyase and valine decarboxylase enzymatic activities in *H. longipes* were evaluated. The results showed that low doses of both elicitors significantly increased affinin contents in roots in comparison with controls without affecting morphological variables. This increase in affinin was correlated with concomitant increases in enzymatic activities related to stress responses as catalase (CAT), superoxide dismutase (SOD), phenylalanine ammonia-lyase (PAL), as well as the increase in valine decarboxylase activity (VDC), an enzyme related with biosynthesis of aliphatic alkamides as affinin. Thus, controlled elicitation might be an interesting strategy to increase affinin contents in chilcuague cultivated under greenhouse conditions.

1. Introduction

Heliopsis longipes is an endemic plant from Sierra Gorda in Central Mexico used in alternative medicine. *H. longipes* is commonly known as chilcuague, golden root, pelitre, and Aztec root (Cilia-López et al., 2014). The roots of this species are consumed as culinary condiment and flavouring alcoholic beverages, and also are used to health toothache, respiratory diseases and muscular aches (Ríos, 2012). The leaves of *H. longipes* are rich in sterols, terpenes and flavonoids and in the roots are sugars, flavonoids, terpenes and alkamides (García-Chávez et al., 2004). One of these alkamides is affinin, that is synthesized in the roots, and displays properties as analgesic, antimutagenic, anaesthetic, anticarcinogenic, anti-inflammatory, antinociceptive, arthritic and vasodilator (Arriaga-alba et al., 2013; Castro-Ruiz et al., 2017; Escobedo-Martínez et al., 2017). Affinin has also shown biological activities such as antimicrobial, bacteriostatic, fungicide and larvicide (Hernández-Morales et al., 2015; Molina-Torres et al., 2004; Salgado-Garciglia et al., 2008).

The extraction of this compound involves collecting the wild plant material in huge quantities thus reducing their native population (Aguilar et al., 2016; Castro-Ruiz et al., 2017). Wild medicinal plants have a wide genetic diversity useful for developing more productive, nutritious and disease resistant crops. However, the conservation status of wild plants in their environment and their availability for their pharmacological, construction, nutritional, culinary, industrial and medicinal uses are still under development (Lubbe and Verpoorte, 2011). To safeguard the genetic diversity of wild species as *H. longipes* in their natural habitat, strategies such as sustainable crops in greenhouses that can provide biomass and quality in specialized metabolites should be evaluated (Barata et al., 2016; Jamwal et al., 2018).

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Controlled elicitation is a method that uses eustressors in a dose and periodical applications to raise phytochemical contents without negative effects on plant performance (Vazquez-Hernandez et al. 2019a). An eustressor is a stress factor of biological (also called "elicitors"), physical or chemical nature that in adequate dose, trigger the immune system of the plants (Vázquez-Hernández et al., 2019a). Controlled elicitation using salicylic acid and hydrogen peroxide has been applied in several plant species to increase specialized metabolites demonstrating effectiveness *in vivo* and *in vitro* (Arriaga-Madrid et al., 2017; Ávila-Juárez et al., 2017; Cardenas-Manríquez et al., 2016; Mejía-Teniente et al., 2013; Vargas-Hernández et al., 2016; Vazquez-Hernandez et al., 2019b). Thus, the aim of this research was to determine the changes in the affinin contents in *H. longipes* roots after controlled elicitation using salicylic acid and hydrogen peroxide as elicitors under greenhouse conditions.

2. Materials and methods

2.1. Plant material and growth conditions

Wild plants of *H. longipes* were collected in Xichú, Guanajuato (Central Mexico). *H. longipes* is growing in the undergrowth of *Quercus-Pinus* forest, at an altitude of 1334 m above sea level located in "Rancho de Beltrán" of the municipality of Xichú, Gto., Mexico. The climate recorded in this area is temperate, temperate sub-humid with rains in summer, the average annual temperature is 18–20 °C, and 600–800 mm of annual average rainfall. The lithology in the soil where chilcuague plants were collected is mostly igneous (71.43 %), and sedimentary (28.57 %), with soil type lithosol, feozem and luvisol, moderately acidic and rich in organic matter (a layer of leaf litter 15–40 cm deep) according to a previous report (Cilia-López et al., 2014).

The biological material was placed in plastic bags of 30 Kg with peat moss, compost and tezontle (1:2:1). After transplant, the seedlings were kept in a greenhouse of 16 m^2 for five months for acclimation; plants with homogeneous height were used. Weather conditions during acclimation and experimentations were 28 °C, photoperiod of 14 h light/10 h dark and relative humidity of 43 %. Plants were watered with the Steiner nutritive solution at 50 % (Mejía-Teniente et al., 2013). Plant morphological variables determined were basal stem width (W) in mm (measured with digital Vernier Multitoyo Absolute), stem height (H) in mm (measured with a graduated ruler) and root length (L) in mm (measured with a graduated ruler) (See Fig. 1 for a timeline visualization of the experimentation carried out in this study).

2.2. Elicitation treatments

After an acclimatization period of 150 days within a greenhouse (Fig.1), the elicitors SA (5 mM and 10 mM) and H_2O_2 (200 mM and 400 mM) were foliarly applied by spraying to drop point onto the plants. The application of both elicitors was carried out weekly, for 2 weeks. The control plants were treated with distilled water (Vazquez-Hernandez et al., 2019b). The concentrations of the elicitors evaluated were: 5 and 10 mM for salicylic acid SA (J. T. Baker), and 200 mM and 400 mM for H_2O_2 (Golden Bell) (Mejía-Teniente et al., 2013; Vazquez-Hernandez et al., 2019b). Both elicitors were applied at day 150 and 157 after transplanting (Fig. 1). Plant samples (leaves, stems and roots) were collected 2 h post-elicitation in each case and immediately frozen in liquid nitrogen and storage at −80 °C until further analyses (Mejía-Teniente et al., 2013).

2.3. Enzyme activity assays

2.3.1. Samples preparation for enzymatic assays

The *H. longipes* root dried samples (0.3 g) from individual plants were homogenized with 1 mL of cold extraction buffer and the slurry were centrifuged at 13,000 rpm for 20 min at 4 °C. The supernatants were used to determine the enzyme activities. Protein concentration in enzymatic extracts was determined according to the method reported by Bradford (1976), using bovine serum albumin as standard (Sigma-Aldrich, St. Louis, Missouri, USA)

2.3.2. Superoxide dismutase (SOD) activity assay

The activity of superoxide dismutase (SOD; EC 1.15.1.1) was assessed spectrophotometrically by the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) according to the method described by Hayat et al. (2018). The reaction was carried out by adding 1.5 mL 50 mM potassium phosphate buffer (pH 7.8), 0.3 mL 0.1 mM EDTA, 0.3 mL 0.13 M methionine, 0.3 mL 0.75 mM NBT, 0.3 mL 0.02 mM riboflavin, 0.05 mL enzymatic extract and 0.25 mL distilled water. The mixture was exposed to fluorescent light (86.86 μmol/m^2·s) for 20 min. Subsequently, the solution absorbance was measured at 560 nm. One unit of SOD inhibit reduction of NBT by 50 % at pH 7.8 and 25 °C. The SOD activity was expressed as U/mg protein.

2.3.3. Catalase (CAT) activity assay

The activity of catalase (CAT; EC 1.11.1.6) was assessed spectrophotometrically by measuring the rate of H_2O_2 decrease at 240 nm according to the method described by Afiyanti and Chen (2014). The reaction was carried out by adding 950 μL 50 mM potassium phosphate

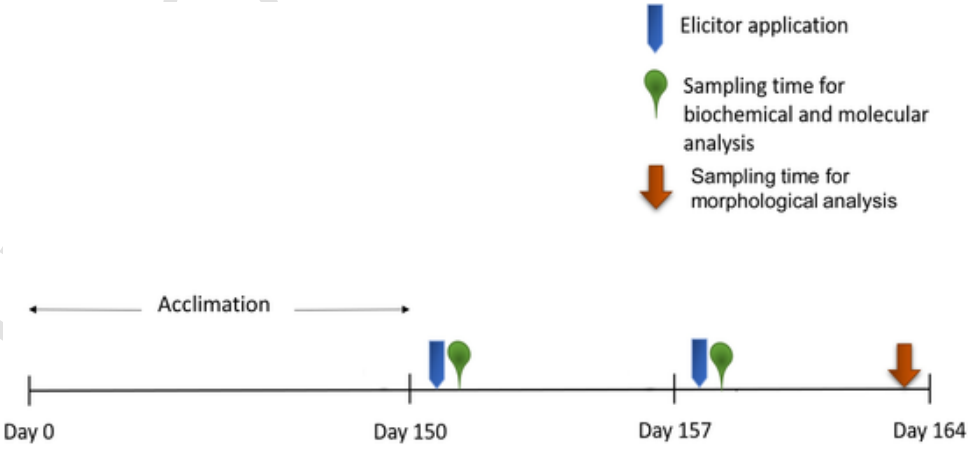

Fig. 1. Time line of the experiments carried out during this research.

buffer (pH 8.0), 50 μL enzymatic extract and 100 μL 100 mM H_2O_2. The change in absorbance at 240 nm was measured for 1 min and used to determine the rate of decomposition of H_2O_2 by CAT. One unit of CAT decompose 1 μmol of H_2O_2 per min at pH 8.0 and 25 °C. The CAT activity was expressed as U/mg protein.

2.3.4. Phenylalanine ammonia-lyase (PAL) activity

The activity of phenylalanine ammonia-lyase (PAL; EC 4.3.1.5) was assessed spectrophotometrically according to the method described by Toscano et al. (2018) with slight modifications. L-Phenylalanine was used as the substrate, and the released cinnamic acid was quantified by absorbance at 290 nm. The reaction by adding 230 μL 0.1 M borate buffer (pH 8.8 containing 10 mM L-phenylalanine) and 20 μL enzymatic extract was carried out. The mixture was incubated at 40 °C for 1 h and the reaction was stopped by the addition of 50 μL 1 N HCl. Subsequently, the solution absorbance was measured at 290 nm. One unit of PAL release 1 μmol of cinnamic acid per min at pH 8.8 and 40 °C. The PAL activity was expressed as U/mg protein.

2.3.5. Valine decarboxylase (VDC) activity assay

The activity of valine decarboxylase (VDC; EC 4.1.1.14) was assessed spectrophotometrically according to the method described by Cortez-Espinoza et al. (2011). L-Valine was used as the substrate, and the released isobutylamine was quantified by absorbance at 550 nm. The reaction was carried out by adding 200 μL 50 mM potassium phosphate buffer (pH 7.5 containing 1 mM pyridoxal phosphate and 20 mM L-valine) and 40 μL enzymatic extract. The mixture was incubated at 42 °C for 1 h and the reaction was stopped by the addition of 15 μL acetone. Subsequently, the solution absorbance was measured at 550 nm. One unit of VDC release 1 μmol of isobutylamine per min at pH 7.5 and 42 °C. The VDC activity was expressed as U/mg protein.

2.4. Samples processing for affinin determinations

The *H. longipes* root extracts for affinin determinations were prepared as follows: root samples from individual plants were freeze drying for three days, ground to a fine powder homogenized. Sample (10 mg) was macerated with 1 mL ethanol (reagent grade), sonicated for 15 min, centrifugated at 10,000 rpm for 10 min at 4 °C; and recovered the supernatant. The extracts were concentrated in a Savant™ SpeedVac™ at 4 °C for two days. Finally, the samples were resuspended in 1 mL ethanol (HPLC grade), the solutions were filtrated in acrodisc of 0.45 μm pore mesh and 25 mm diameter (Agilent Technologies) (Aguilar et al., 2016; Bae et al., 2010).

3. Quantification of affinin in *H. longipes* extracts by HPLC-DAD

A standardized method developed in our lab was used for quantification of affinin. This analysis was performed using a Waters HPLC system (Millipore Corp., Waters Chromatography Division, Milford, MA, USA), composed of a 600E multi solvent delivery system and a 2998 PDA detector. The column used was ZORBAX ECLIPSE XDB-C8 (4.5 × 150 mm, 5 um particle size, 120 Å pore size). An isocratic system was achieved where mobile phase composition was 50 % of 1% acetic acid in HPLC-grade water and 50 % HPLC-grade acetonitrile at a flow rate of 1 mL/ min. The injection volume was 20 μL, and the total analysis time was 8.5 min. Affinin content was obtained using a calibration curve of pure affinin (15–90 μg/mL). Five points were obtained, each one corresponding to the mean of three replicates. The areas under the curves (Y) obtained for each point were plotted *versus* the respective concentrations used (X) and a linear correlation established as $Y = aX + b$ (Bae et al., 2010; Aguilar et al., 2016).

3.1. Statistical analysis

Statistical analyses were carried out with GraphPad Prism 6.0 program (GraphPad Software, San Diego, CA). A completely randomized experimental design was used to evaluate the effect of the foliar application of both elicitors on affinin content and morphological and enzymatic activities studies. The arrangement of the experiment was 5 treatments with an experimental unit of 12 plants for triplicate. Data were subjected to analysis of variance (ANOVA) and the differences between means were compared using Tukey´s multiple comparisons test ($P = 0.05$).

4. Results and discussion

4.1. Effects of elicitation on plant morphology

After two weeks of elicitation (day 164 according to Fig. 1), *Heliopsis longipes* plants were evaluated in morphological variables (Table 1). In regards to stem width and root length, a significant increase was observed with 10 mM SA (1.46-fold); the rest of the treatments did not show differences with control. Moreover, no significant changes were observed for plant height with all the treatments evaluated in comparison with control (Table 1). Additionally, on one hand, the typical morphological aspect of *H. longipes* after elicitation at day 164 after transplanting showed that treatment with H_2O_2 400 mM, caused a significant reduction in plant performance (3.1-fold in comparison with control); on the other hand, SA 10 mM displayed a significant increase of (1.02-fold) in root length and (2.0-fold) in plant performance (Fig. 2 and Table 1). These results suggest that treatment with SA 10 mM displayed a significant improvement in morphological variables and plant performance (similar as biostimulant activity) under the conditions evaluated in this study for *H. longipes* plants. To our knowledge, these data are the first obtained about the effects of controlled elicitation strategies on performance of *H. longipes* under greenhouse conditions. In similar studies using SA as elicitor in other plant species, Gorni et al. (2016) found a significant increase in biomass accumulation in *Achillea millefolium* L. plants at 0.5 mM SA. Also, exogenous applications of SA at (0.25, 0.5 and 1 mM) resulted in biomass accumulation in marigold plants (*Calendula officinalis* L.) (Pacheco et al., 2013). Likewise, it had been suggested that exogenous applications of SA influenced growth plant over photosynthesis, altering water relations, stomatal regulation and nutrient uptake (War et al., 2011). Moreover, it is likely that SA it is inducing changes in the balance of phytohormones (i.e gibberellins, auxins, *etc*) within chilcuague plants permitting the biostimulant phenotype observed, in similar way as suggested in recent reports (Vazquez-Hernandez et al., 2019b).

Table 1
Effect of elicitation on morphological variables in *H. longipes* cultivated during 160 days after transplanting under greenhouse conditions.

Treatment	W [a] (mm)	H [b](mm)	L [c] (mm)
Control	0.207 ± 0.04 [a]	73.50 ± 28.9 [a]	191.68 ± 1.7 [b]
200 mM H_2O_2	0.257 ± 0.14 [ab]	56.25 ± 20.8 [a]	73.666 ± 6.6 [ab]
400 mM H_2O_2	0.227 ± 0.03 [ab]	37.50 ± 8.5 [a]	56.000 ± 7.9 [a]
5 mM SA	0.400 ± 0.09 [b]	58.50 ± 39.1 [a]	191.75 ± 1.1 [b]
10 mM SA	0.403 ± 0.05 [b]	58.48 ± 29.1 [a]	194.68 ± 3.7 [c]

Different letters in each column means significant difference according to the ANOVA with Tukey's multiple comparisons test ($P = 0.05$).
[a] W:stem width.
[b] H: plant height.
[c] L: root length.

Fig. 2. Typical morphological aspect at day 164 post-transplanting of *H. longipes* treated with H_2O_2 and SA. A) 400 mM H_2O_2, B) 200 mM H_2O_2, C) 10 mM SA and D) Control (distilled water).

4.2. Effect of elicitation on enzyme activity of SOD, CAT, PAL and VDC

Elicitors such as SA and H_2O_2 might increase antioxidative enzymes involved in plant defence against stress (POD, CAT, SOD, PAL) (Shanker and Venkateswarlu, 2012; War et al., 2011). The enzymatic measurement of the 5 mM SA and 400 mM H_2O_2 concentrations were omitted from the study since 5 mM SA had no better responses in *H. longipes* in comparison with 10 mM SA treatment, and 400 mM significantly affected in a negative manner the morphology and biomass of the plant in the leaf area and in the root length (Table 1 and Fig. 2). In general, the results displayed that both 10 mM SA and H_2O_2 200 mM treatments increased the enzyme activities of SOD, PAL, CAT and VDC in comparison with controls depending on the time in which the elicitation was carried out (Figs. 3–6). Significant high activity of SOD was observed in *H. longipes* plants treated with 10 mM SA (30.37-fold) in the first (day 150) and second (day 157) applications (12.83-fold), but not when using H_2O_2 200 mM (Fig. 3). During their

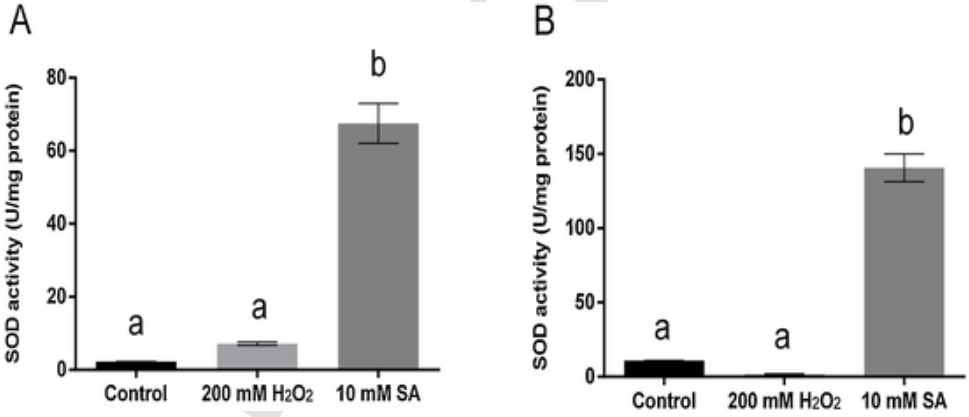

Fig. 3. Superoxide dismutase (SOD) specific activity in *H. longipes* as a result of two applications of H_2O_2 200 mM and 10 mM SA: A) First application (day 150), B) Second application after eight days (day 157). Different letters indicates significant difference according to ANOVA and Tukey´s test (P = 0.05).

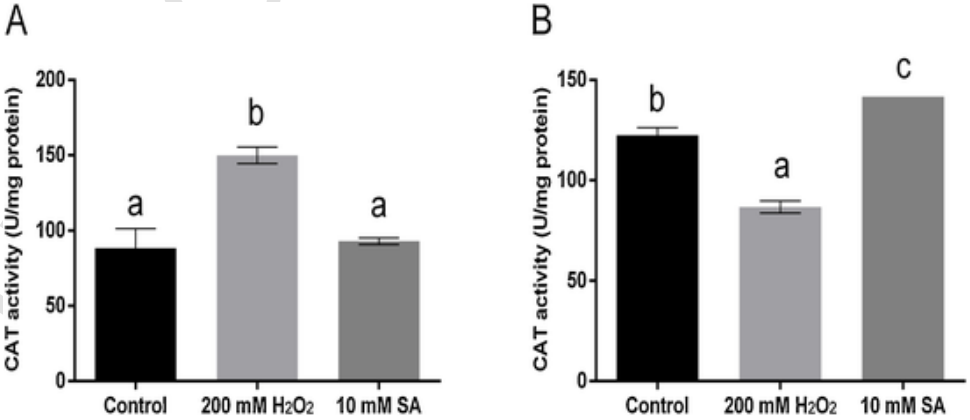

Fig. 4. Catalase (CAT) specific activity in *H. longipes* as a result of two applications of H_2O_2 200 mM and 10 mM SA: A) First application (day 150), B) Second application (day 157). Different letters indicates significant difference according to ANOVA and Tukey´s test (P = 0.05).

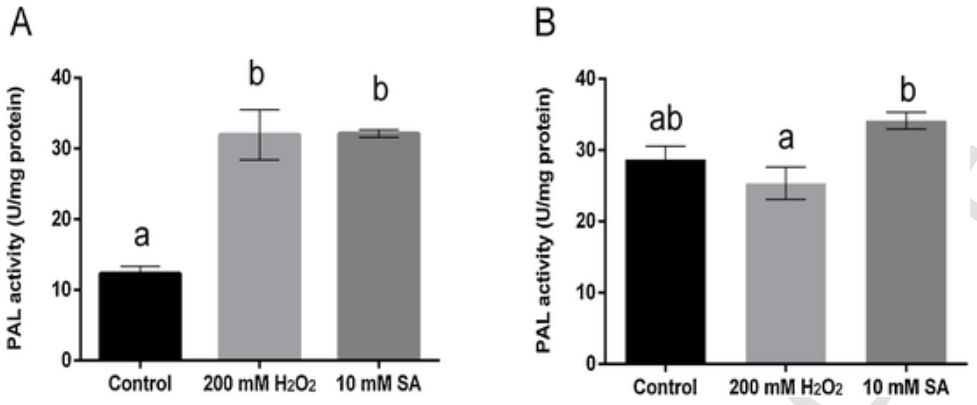

Fig. 5. Phenylalanine ammonia lyase (PAL) specific activity in *H. longipes* as a result of H_2O_2 200 mM and 10 mM SA: A) First application (day 150), B) Second application (day 157). Different letters indicates significant difference according to ANOVA and Tukey´s test (P = 0.05).

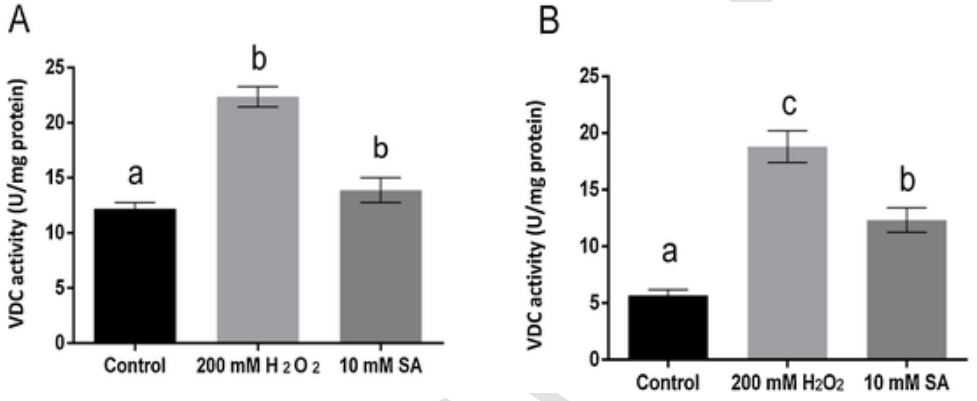

Fig. 6. Valine decarboxilase (VDC) specific activity in *H. longipes* as a result of two applications of H_2O_2 200 mM and 10 mM SA: A) First application (day 150), B) Second application (day 157). Different letters indicates significant difference according to ANOVA and Tukey´s test (P = 0.05).

growth plants are in balance of antioxidants and pro-oxidants, any disproportion may trigger oxidative stress leading destruction at cellular levels (Czégény et al., 2016). SOD is the most important enzyme against reactive oxygen species (ROS), its function is disrupting the superoxide radical (O^{-}_2) into H_2O_2 and O_2 (Hernández-Barrera et al., 2015). A possible explanation might be that the plant blocked the SOD enzyme by detecting exogenous H_2O_2 in both applications as suggested Price et al. (1994). They used tobacco (*Nicotiana plumbaginifolia* line MAQ2.4) and seedlings were grown *in vitro*. Hydrogen peroxide was added at 10 mM, and they observed a 45 and 67 % reduction in SOD activity after 1 h and 4 h, also it was observed that exogenous application of H_2O_2 induced $[Ca^{2+}]_{CYT}$ mobilizations, that might inhibit the SOD activity (Price et al., 1994). Based on the present work and the studies on rice (*Oryza sativa* L. cv. Yuexiangnian), maize (*Zea mays* L. cv. Yuedan 1) and mung bean (*Vigna radiate* (Linn.) Wilczek Sulv 1) on the inhibition of SOD activity by H_2O_2 at 1–15 mM, the variation of this phenomenon depended on the organs, plant age, species and likely the concentration of H_2O_2 used in the experiments (Cheng and Song, 2006).

The behaviour of CAT activity is shown in Fig. 4. CAT activity was significantly increased at 200 mM H_2O_2 in the first application (day 150). After the second application, CAT increased significantly in 10 mM SA and significantly diminished in 200 mM H_2O_2 (Fig. 3). Koc et al. (2015) did not find CAT enzymatic activity in none of the extracts evaluated in *O. acanthium*, *C. acanthoides*, *C. Arvense*, and *C. solstitialis* wild belonging to Asteraceae family. These differences may be due to the plants were wild, and did not receive additional stress, which is motive that CAT enzyme did not present activity (Caprioli et al., 2017). CAT transform H_2O_2 to H_2O and O_2, also it has been studied CAT as the essential enzyme used for the elimination of H_2O_2 within the cells. CAT enzyme decrease oxidative stress, its activity depends on period time or the high dose of the exposure at stress as well as plant age (Biczak et al., 2017). Otherwise, Mejía-Teniente et al. (2013) found that CAT activity rose with elicitation, its activity was directly proportional to the H_2O_2 production by elicitation in chilli peppers. Moreover, that study also showed that chilli peppers displayed high CAT activity 4 h after application with 0.1 mM SA and 8 h after application with 6 mM H_2O_2. In a second application, CAT activity was significantly higher 1 d after at 6.7- and 10-mM SA, and at 14 mM H_2O_2 in *C. annum* L. var. Don Benito. These results agree with some of the responses obtained in the present work with *H. longipes*, strongly suggesting that plant age is important in order to be responsive to an elicitation treatment, and also this response seems to be depended on the elicitor. In some cases when plants had low CAT activity and the protection against H_2O_2 was compromised, plants activate another enzyme that also break down H_2O_2 in presence of ascorbic acid and phenolic compounds as peroxidase (POD) activity to overcome the situation (Biczak et al., 2017).

PAL is a crucial enzyme of phenylpropanoid metabolism, up-regulation of this activity correlated with the increase of phenylpropanoids used as one of the main biochemical arsenal of plant defence against environmental stresses (Mejía-Teniente et al., 2013). The effect of the H_2O_2 200 mM and SA 10 mM on *H. longipes* is shown in the Fig. 5. Significant increase in PAL activity was observed in the first application with 200 mM H_2O_2 and 10 mM SA, whereas in the second application significant differences were detected only with 10 mM SA (Fig. 5). Mejía-Teniente et al. (2013) found significant increment after the application of elicitors SA, H_2O_2 and chitosan (QN) and five days later found the higher increment of PAL activity. Taking together these re-

sults regarding the enzyme activities evaluated related to plant stress responses, suggest that the response will depend on the elicitor used, as well as the plant age under the conditions studied in the present work.

VDC activity in *H. longipes* after elicitation is shown in Fig. 6. On one hand, the first application induced significant VDC activity at 200 mM H_2O_2 and 10 mM SA in comparison with control (Fig. 6A). On the other hand, in the second application a similar result was displayed showing significant increases in both elicitation treatments in comparison with control (Fig. 6B). Controlled elicitation increased in all of the treatments evaluated the VDC activity (Fig. 6). The reason to evaluate effects of controlled elicitation on VDC in *H. longipes* in this work was based on the fact that, it has been suggested that VDC activity might be a key enzyme in the channelling of the pathway of synthesis of aliphatic alkamides such as affinin (Cortez-Espinosa et al., 2011). Thus, whether a stress factor (elicitor/eustressor) increase VDC activity, it would be probable that also an increase in affinin contents might be detected within the plants.

4.3. Concentration of affinin in H. Longipes

In this study, it was observed that affinin concentrations increased significantly with treatments 10 mM SA and 200 mM H_2O_2 in comparison with control root plants in both the first and second applications (Fig. 7). These results completely agree with the hypothesis of rise in VDC activity also increment the affinin concentration as suggested by the asseveration of Cortes-Espinoza et al. (2011) regarding that VDC is a key enzyme channelling the biosynthesis of aliphatic alkamides as affinin. Thus, this is the first experimental demonstration that the VDC activity might be directly related with affinin contents in roots of *H. longipes*. This result also indicates the possibility of using VDC activity as biochemical indicator of affinin contents in *H. longipes* roots. More detailed studies throughout the whole plant cycle using controlled elicitation or not, should be carried out in order to fortify this asseveration.

5. Conclusions

Based on the aforementioned results, controlled elicitation using SA 10 mM or H_2O_2 200 mM applied as showed in this study are eustressic doses of these elicitors for increasing affinin contents in *H. longipes* grown under the greenhouse conditions evaluated in the present work. Controlled elicitation strategy might increase the industrial potential of *H. longipes* as well as might serve to protect this species of possible extinction based on irrational exploitation for industrial purposes.

Credit autor statement

Ixchel Parola-Contreras: Methodology and conceptualization. **Erik G. Tovar-Perez:** Methodology and checking the manuscript. **Alejandra Rojas-Molina**: Methodlogyand checking the manuscript. **Francisco J. Luna-Vazquez**: Methodology in affinin contents. **Irineo Torres-Pacheco**: Conceptualization ans checking the manuscript. **Rosalia V. Ocampo-Velazquez**: Methodology and checking the manuscript. **Ramon G. Guevara-González:** Conceptualization, checking, writing and editing the manuscript.

7. Uncited references

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

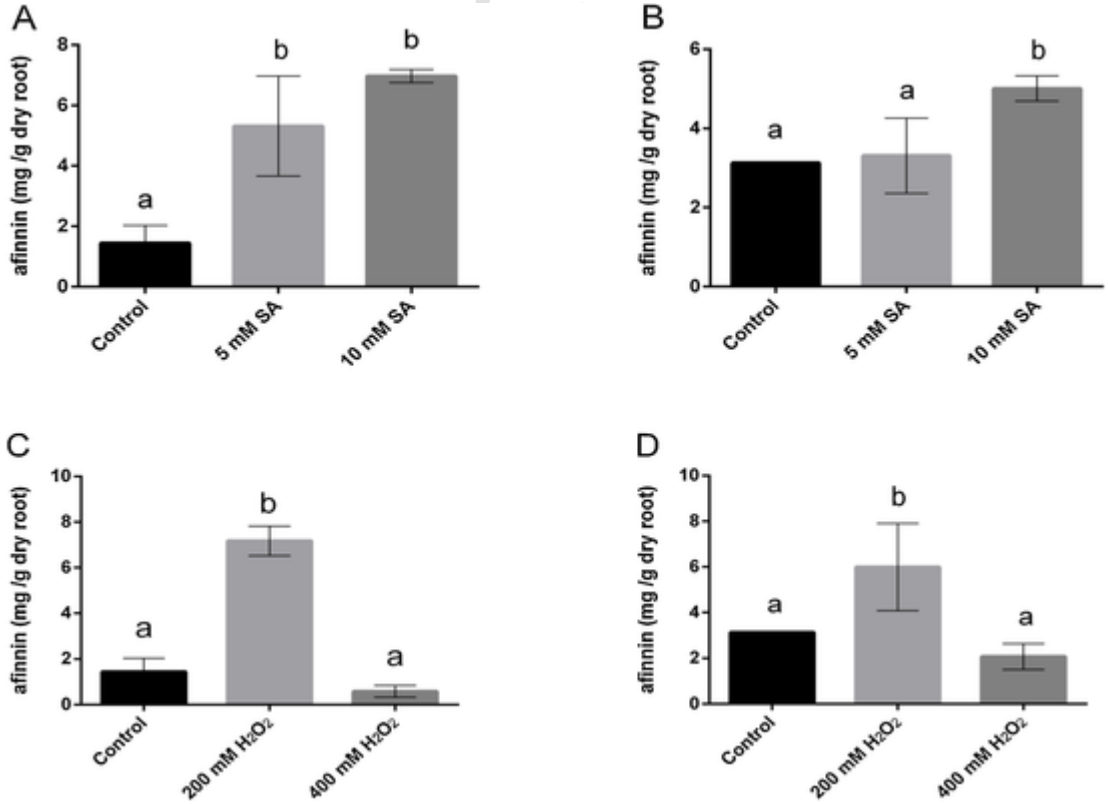

Fig. 7. Affinin contents in *Heliopsis longipes* treated with H_2O_2 or SA at day 150 (panels A and C) or day 157 (panels B and D). Different letters indicates significant difference according to ANOVA and Tukey´s test (P = 0.05).

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