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Effect of Hydrogen Peroxide Pretreatment on Physiological and Biochemical Variables during Germination of Alfalfa Seeds

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ABSTRACT

Background: Hydrogen peroxide is reactive oxygen species that plays role in plant response to biotic and abiotic stress. The pretreatment with hydrogen peroxide can confer an adaptive capacity for the plants in unpredictable environments. The alfalfa (*Medicago sativa* L.) is the legume more utilized in animal feeding in the world. Moreover, the alfalfa sprouts are known for the phytochemicals that promote health with antioxidant properties.

Methods: This work aimed to determine the effect of hydrogen peroxide in the pretreatment process of alfalfa seeds on variables as total germination, speed of germination, activity and antioxidant enzymes. The alfalfa seeds were soaked for 12 h in the next treatments 0, 98, 294, 490, 784, 980 mM of hydrogen peroxide.

Result: The results showed that total germination was higher with the hydrogen peroxide than with water except 980 mM. The results of the present research indicated that hydrogen peroxide had physiological and biochemical effects on the germination processes of alfalfa.

Key words: Antioxidant enzyme, Germination, Hydrogen peroxide, *Medicago sativa* L.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) has been called the queen of forages because all livestock species can consume it and tolerate water deficit (Wenxu *et al*., 2020). Furthermore, as a sprout, it can also be consumed by humans and provide health benefits for the consumer (Zieliński *et al*., 2007). The production of sprouts for consumption as human food has been an activity to increase the volume and nutritional quality of grains and seeds. Furthermore, the amount of antinutritional elements decreases (Abellán *et al*., 2019).

For its part, the use of elicitors aims to interact with biochemical pathways to modify the production of secondary metabolites, in this case, in plants (Aguirre-Becerra *et al*., 2021). In plants, one of the practices to ensure their survival, persistence and competitiveness (Thakur *et al*., 2018), as well as to improve nutritional qualities and has been the controlled application of different types of elicitors to influence secondary metabolism (Parola-Contreras *et al*., 2020). Elicitors such as jasmonic acid (JA) (Ho *et al*., 2020), salicylic acid (SA) and the exogenous application of hydrogen peroxide (H_2O_2) are responsible for the induction of reactive oxygen species (ROS) whose accumulation triggers the uptake action that involves enzymes such as Superoxide dismutase (SOD), Catalase (CAT) and products of Phenylammoniumlyase (PAL) that mitigate the effects of stress and positively regulate the homeostasis of the plant (Jahan, 2020).

Within the group of elicitors, H_2O_2 stands out as a pristine role in the conservation and development of the plant through the phenomenon of apoptosis. It has very little reactivity with most organic molecules, diffuses easily through cell membranes and reaches relatively distant locations from where it was generated. There is growing evidence that H_2O_2 plays an essential role in plant defense responses (Kuniak and Urbanek, 2000).

In alfalfa seeds, H_2O_2 has been applied sequentially in combination with heat to inactivate *Salmonella typhimurium*; collaterally, they reported that the germination percentage of the seeds increased (Hong and Kang, 2016). Subsequently, added vacuum packaging was in the sequential treatment and they reported that the inactivation of *S. typhimurium* and the germination capacity of the seed were not affected (Hong *et al*., 2019). However, the reports

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are not clear about the effect of H_2O_2 alone has on germination speed and total germination. Alfalfa seeds are exposed to stress factors that can affect them; such factors can be related to humidity, fertility, salinity, or pest and pathogen attack (Huihui *et al*., 2017). H_2O_2 was reported to affect the increase in seed germination rate in pea seeds (Barba-Espin *et al*., 2010) and cereal plants such as wheat (Ishibashi *et al*., 2008). Therefore, the objective of this work was to determine the application to effect of alfalfa seeds of H_2O_2 only in the variables of germination speed and total germination, as well as in variables associated with the nutraceutical properties of the sprouts.

MATERIALS AND METHODS

The present investigation was carried out at the Amazcala Campus of the Autonomous University of Querétaro. Alfalfa seeds (variety San Miguel, California) were used for the study. The experiments were carried out using 0, 98, 294, 490, 785, 980 mM hydrogen peroxide treatments, with 160 seeds per treatment. Alfalfa seeds were immersed in H_2O_2 solutions and monitored overnight for 12 h.

Total germination

To measure the effectiveness of the application of H_2O_2 to the seeds, we determined the value of the variable according to the following equation (Chiapusio *et al*., 1997).

$$G_T = \frac{N_T * 100}{N}$$

Where,

G_T : Total germination, N_T : Proportión of germinated sedes by each treatment for last measurement; N: Number of total seeds used in the bioassay by each treatment.

Speed of germination

We determined the value of this variable by using the following equation (Wardle *et al*. 1991, modified by Chiapusio *et al*., 1997).

$$S = N_1*1 + (N_2 - N_1) *1/2 + (N_3 - N_2) * 1/3 + (N_4 - N_3) * 1/4 + ...(N_n - N_{n-1}) * 1/n$$

Where,

N_1, N_2, N_3, N_n: Proportion of germinated seeds obtained the first (1), second (2), third (3)….n days.

Evaluation of antioxidant activity

Seeding samples (0.03 g) were extracted with 10 mL methanol for 24 h. The antioxidant activity was evaluated using the radical 1,1-diphenil-2-pycrylhydrazil ($DPPH^{\bullet}$) assay reported by (Fukumoto and Mazza, 2000) and the monocation radical 2,2-azinobis-(3-ethylbenzothiazoline-6-sulphonic) acid ($ABTS^{\bullet+}$) method, as described by Nenadis *et al*., (2004). The antioxidant activity was expressed as Trolox Antioxidant Capacity (TEAC) using a calibration curve of Trolox. The Trolox concentration curve ranged from 50 to 800 µM.

Evaluation of antioxidant enzyme activities

Catalase activity assay (CAT)

The protein content for all the enzymes was measured according to the Bradford´s method (1976) with the same extracted that was utilized in antioxidant activity using bovine serum albumin as standard for protein concentration (Sigma-Aldrich, St. Louis Missouri, USA). Total CAT activity was monitored spectrophotometrically according to Aebi (1984). The change in absorbance at 240 nm was measured for 6 min and used to determine the rate of decomposition of H_2O_2 by CAT (µmole H_2O_2 consumed min^{-1}. mg $protein^{-1}$). Each treatment was repeated at least three times.

Superoxide dismutase activity assay (SOD)

Total SOD activity was estimated by the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT) (Gao, 2006). The absorbance was measured at 560 nm. The data were expressed as U/mg protein.

Phenylalanine ammonia-lyase (PAL)

The PAL activity was assessed according to Mozzetti *et al*., (1995). The absorbance was read at 290 nm. PAL activity was calculated as the mmol of cinnamic acid per g of tissue produced under the specific conditions and expressed as U/mg protein content.

Statistical analysis

The experimental assays used to obtain all results were repeated at least three times, under the same conditions. The data were analyzed using statistical software Statgraphics Centurion XVI.II with an analysis of variance (ANOVA) to discriminate significant differences (defined as $p \leq 0.99$).

RESULTS AND DISCUSSION

Total germination and germination speed

We determinate the total germination considering the count of the alfalfa sprouts at four days; the failed seeds were not considered. As shown in Fig 1 Panel I, all treatments exceeded the control except the treatment with a higher concentration of hydrogen peroxide, that is, with 980 mM. The other treatments were statistically the same and showed germination close to 90%.

In Fig 1, Panel II shows that, as in the total germination variable, the germination speed was higher in the treatments with 98 mM, 294 mM, 490 mM and 784 mM hydrogen peroxide in the treatments with 0 mM and 980 mM. This suggests that the speed of germination can influence the success of the event itself.

Our results regarding the total germination of the alfalfa seed were similar to other plants where the application of exogenous hydrogen peroxide at low concentrations increased the germination rate and the seeds germinated faster in the case of cucumber (Li *et al*., 2016), *Solanum Lycopersicum* (Nazir *et al*., 2019b). In general, the application

of hydrogen peroxide stimulated the growth and development of plants under stress, as reported by Nazir *et al*., 2020.

Evaluation of antioxidant activity

We determined the antioxidant activity using the methodologies to estimate ABTS and DPPH (Fig 2). Samples were collected 96 hours later for germination. Regarding the ABTS indicator, the treatment with the highest activity was 98 mM H_2O_2 with statistical difference compared to the control and other treatments. The concentration of H_2O_2 980 mM was the treatment with the lowest antioxidant activity. On the contrary, in DPPH, all treatments had a higher percentage compared to the control (0 mM). The most active treatment was 294 mM H_2O_2, the next was 98 mM H_2O_2.

Our results regarding antioxidants, it is necessary to remember that alfalfa contains flavonoids, saponins, vitamins, minerals, organic acid and polysaccharides (Li *et al*., 2016) and these substances possess bioactivity as an antioxidant, anti-inflammatory, anticancer and improve immune function (Gatouillat *et al*., 2014). On the other hand, flavonoids derived from alfalfa have exhibited extreme DPPH antioxidant activity (Chen *et al*., 2016) and help maintenance of the production. Ruminal immunity and fermentation in cattle (Zhan *et al*., 2017). In this context, our results showed the trend of a higher antioxidant activity ABTS and DPPH in the treatments with exogenous application of H_2O_2 over the control during germination (Fig 2) and coincide with the results in sprouts of lentils provoked with hydrogen peroxide at 20 and 200 mM (Swieca, 2015) where an increase in antioxidant capacity is also shown. However, in all treatments with exogenous H_2O_2, there is a higher DPPH antioxidant activity in germination (Fig 2) than the control, with hydrogen peroxide being 294 mM, the highest activity in germination. This suggests that the stressor (hydrogen peroxide) is within the range of eustress or beneficial stress for the alfalfa plant (Vargas-Hernández *et al*., 2017).

Evaluation of Antioxidant enzyme activities

Regarding SOD (Fig 3) the highest activity was induced by treatments with 490 and 784 mM of H_2O_2, these results presented statistically significant differences concerning the control (0 mM, H_2O_2). All treatments with H_2O_2 had more

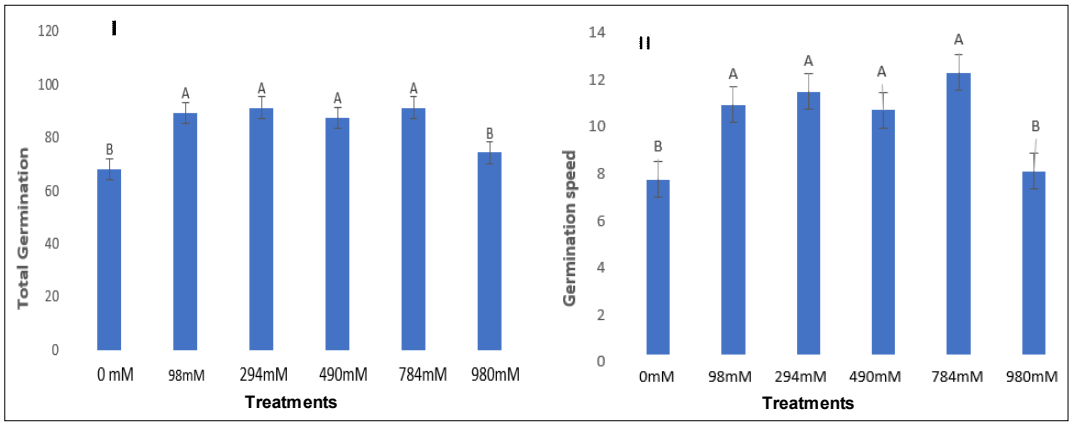

Fig 1: Panel I. Total germination of alfalfa seeds at four days. Panel II. The germination speed estimated with the results of four days, in both cases with different concentrations of H_2O_2; 98, 294, 490, 784, 980 mM. Data are presented as the mean; A, B, represent the statistical difference (p=0.99).

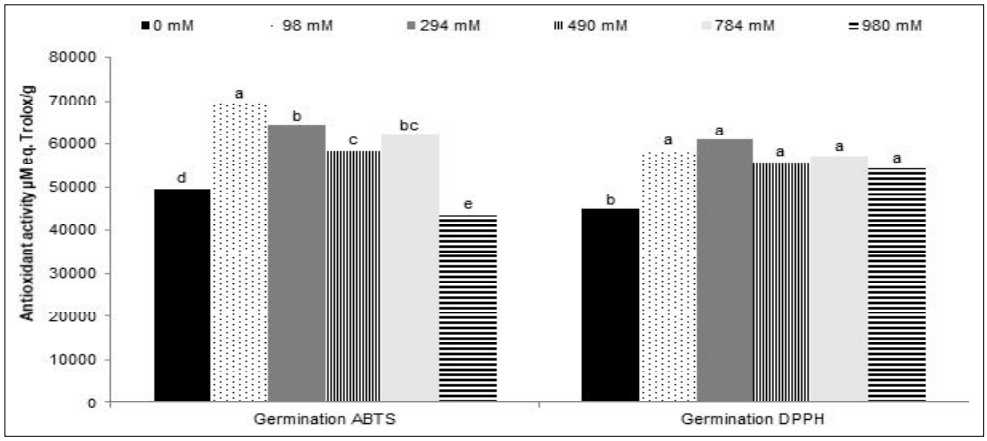

Fig 2: Antioxidant activity (µM equivalents Trolox/g sample) for ABTS and DPPH in germination with different concentration of H_2O_2; 0, 98, 294, 490, 784, 980 mM. Data are presented as the mean, a, b, c, d, e represent the statistical difference (p=0.99).

activity than the control. The activity of antioxidant enzymes was related to tolerance to abiotic stress such as drought, high salinity, adverse conditions that include enzymes such as SOD, POD and CAT (Foyer and Noctor, 2005). Antioxidant enzymes and antioxidant metabolites can modulate ROS such as H_2O_2 under normal conditions (Chen *et al*., 2019). When ROS are overproduced under stress conditions, they can cause cell damage (Xia *et al*., 2020). The high activity of SOD, CAT during the germination phase (490, 784, 980 mM exogenous H_2O_2), suggested that hydrogen peroxide-induced strong defense activity during the oxidation pathway removed high concentrations of H_2O_2 and inhibits accumulation of ROS and protect plants from lipid peroxidation (Wang *et al*., 2009).

Our results are agreement with those obtained in tomatoes (Nazir *et al*., 2019b). The addition of hydrogen peroxide improved the SOD, POX and CAT when tomato plants (*Solanum lycopersicum* L.) were exposed to stress

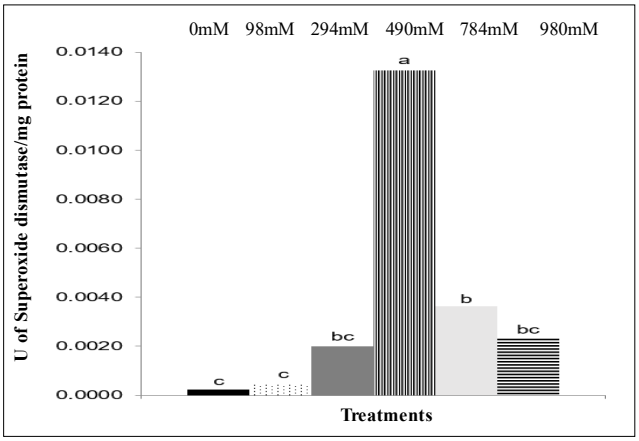

Fig 3: Antioxidant enzyme activity SOD in germination with different concentrations of H_2O_2; 0, 98, 294, 490, 784, 980 mM. Data are presented as the mean, a, b, c represent the statistical difference (p=0.99).

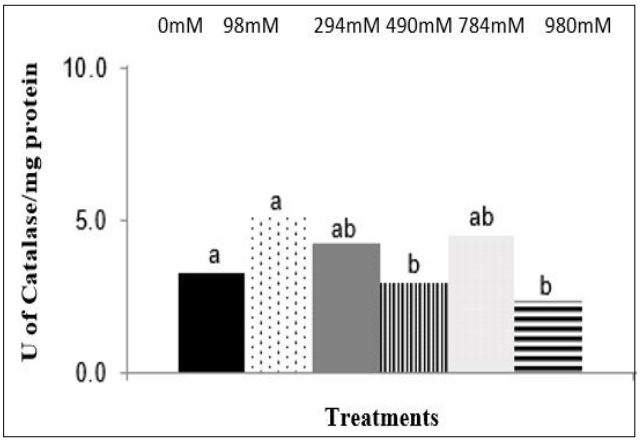

Fig 4: Antioxidant enzyme activity CAT in germination with different concentrations of H_2O_2; 0, 98, 294, 490, 784, 980 mM. Data are presented as the mean, a, b represent the statistical difference (p=0.99).

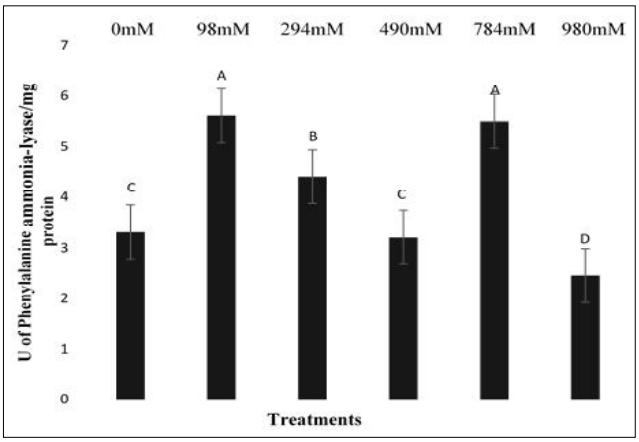

Fig 5: Antioxidant enzyme activity PAL in germination with different concentrations of H_2O_2; 0, 98, 294, 490, 784, 980 mM. Data are presented as the mean; a, b, c, d represent the statistical difference (p=0.99).

by copper and salt (Sathiyaraj *et al*., 2014). Nazir *et al*., 2019b reported that H_2O_2 prevents increased oxidative stress and increased endogenous H_2O_2, generating an increase in antioxidant enzymes SOD, POX, CAT.

Regarding the catalase units per milligram of protein in the germination process (Fig 4), we did not detect a significant statistical difference. However, it might seem that the 98 mM H_2O_2 treatment presented higher activity and it may be a real trend if we accept increasing the Type B error and increasing α value to 0.10. If this were the case, our results obtained of ABTS and DPPH were similar with that reported in other cases (Jahan, 2020).

The PAL enzyme is responsible for synthesizing phenols, their elevation and the increase in antioxidant capacity (Swieca, 2015). In our case, the results showed that the treatments with H_2O_2 except 980 mM had greater amount of PAL enzyme compared to the control (Fig 5) and the same behavior was observed in the antioxidant capacity (Fig 2). Swieca (2015) reported increased PAL and antioxidant capacity in lentil sprouts elicited with hydrogen peroxide (15 mM and 150 mM).

CONCLUSION

The present study results indicated that hydrogen peroxide has physiological and biochemical effects on alfalfa (*Medicago sativa*) germination because total germination and germination speed were increased. The H_2O_2 has the function of elicitor for the production of sprouts of alfalfa seeds. The H_2O_2 is a molecule with the implication in cell growth and proliferation as reported in this experiment. The antioxidant value of sprouts is stimulated and under certain conditions, it accelerates germination and growth.

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