Agronomy Research **17**(1), 22–32, 2019
https://doi.org/10.15159/AR.19.003

Effects of experimental warming on peroxidase, nitrate reductase and glutamine synthetase activities in wheat

L. Argentel-Martínez[1,2], J. Garatuza-Payán[1,3], T. Arredondo[4] and E.A. Yépez[1,3,*]

[1]Instituto Tecnológico de Sonora, 5 de febrero, 818 Sur, Col. Centro, Cd. Obregón, Sonora, México, CP: 85000
[2]Instituto Tecnológico del Valle del Yaqui, Calle 600, Block 611, Bácum, San Ignacio Río Muerto, Sonora, México, CP: 85275
[3]Laboratorio Nacional de Geoquímica y Mineralogía, Sede Regional Sur de Sonora, 5 de febrero, 818 Sur, Col. Centro, Cd. Obregón, Sonora, México, CP: 85000
[4]Instituto Potosino de Investigación Científica y Tecnológica. Camino a la Presa San José 2055, Col. Lomas, Sección 4, San Luis Potosí, México, CP: 78216
[*]Corresponding author: enrico.yepez@itson.edu.mx

Abstract. Given the effects of climate change and its significant consequences on plant productivity, it is necessary to evaluate the enzymatic responses of the most important crops such as wheat (*Triticum durum* L.). We examined the response of foliar peroxidase activity, nitrate reductase, and glutamine synthetase to experimental increments of temperature (+2 °C) under field conditions following conventional agricultural practices. Foliar samples, in both control and warming treatments were taken during growth, tillering and flowering phenophases to test the peroxidase activity. Similarly, nitrate reductase, glutamine synthetase activities, and glutamine content were measured during the heading phenophase. Due to the effects of experimental warming, peroxidase activity significantly increased. The nitrate reductase activity was also higher in the warming treatment, suggesting a high nitrogen metabolism efficiency. Whereas, the increase observed in the glutamine synthetase activity, and consequently the glutamine content, evidenced a biochemical signal of an early senescence due to the effect of warming.

Key words: *Triticum*, warming, nitrogen metabolism, Yaqui Valley.

INTRODUCTION

Warming is one of the most stressing factors for plant productivity (Asseng et al., 2017). Every year, a significant amount of land is abandoned because species do not tolerate heat-stress conditions and, when they show some tolerance, their genetic potential is not fully expressed (Argentel-Martínez et al., 2017). Warming is a common stress in agricultural ecosystems that is often ignored by producers (Siebers et al., 2017) but it has a significant impact on agricultural yield reductions (Xin et al., 2016).

The most common warming effects on plant physiology, consist in modifications of growth patterns and affectations to phenology, however all physiological process are closely tied to cellular metabolism (Ramani et al., 2017; Garatuza-Payan et al., 2018). A

single heat wave can alter the activity of the enzymatic system that modulates both, the synthesis and the degradation processes of macromolecules needed for plant yield (Mphande, et al., 2016). Under such stressing conditions, plants might turn on various enzymatic and non-enzymatic mechanisms for tissue and organ protection, to guarantee seed formation, as a final phase of the biological cycle (Baxter et al., 2014).

For example, the peroxidase participates in the antioxidative defense system, when reactive oxygen species are synthesized following stress. The importance of the study of nitrate reductase and glutamine synthetase activities lies on the variation of nitrogen use efficiency (NUE), mainly at the most critical wheat phenophases such as heading and flowering, when considerable amounts of nitrogen are necessary to ensure a good fertilization and seed formation (Majláth et al., 2016). The N required for the synthesis of proteins that accumulate in wheat pollen comes mainly from remobilization of N previously assimilated and accumulated into the leaves. N remobilization occurs through the phloem (and occurs actively) mostly in the form of aminoacids (Zhang & Du, 2016). In this way, N remobilization efficiency from leaves becomes the main factor of grain protein concentration at the end of the crop cycle. This process is affected by abiotic stresses such as heat. Despite of the importance of this theme, there are not abundant reports about its regulation. It is known that remobilization processes are regulated by internal factors (biochemical and molecular), with an important role in nitrogen metabolism, highlighting the role of glutamine synthethase (GS) family (Tian et al., 2016), and by external factors (abiotic stresses) such as heat, drought and salinity (Hoffman et al., 2016).

In the Yaqui Valley, Sonora, one of the main wheat production sites of México and across the world, increments of about 2 °C are expected in the next 50 years (Navarro-Estupiñan et al., 2018), likely bringing profound effects on wheat yields as suggested by modelling synthesis studies (Mendoza-Ochoa et al., 2018). An important step for the adoption of agronomical strategies to adapt to climate change consist in carrying detailed studies about the enzymes involved in plant protection and nitrogen metabolism. Such information will allow to explain the variability of physiological and agronomic responses to climate change scenarios in commercial varieties. In this context, the present study aimed to evaluate the peroxidase, nitrate reductase and glutamine synthetase activity in the commercial crystalline wheat variety CIRNO C2008, under experimental warming conditions in the field, in the Yaqui Valley, Sonora, Mexico.

MATERIALS AND METHODS

Experimental area location

The experiment was carried out during the crop cycle of December 2016 to April 2017, under field conditions, at the Technology Transfer Experimental Center (CETT-910) of the Instituto Tecnológico de Sonora (ITSON), located at 27°22'0.4"N and 109°54'50.6"W (UTM: 607393.24 m E; 3027508.34 m N). During the cropping cycle mean daily temperatures were in the range of 8° to 24 °C with the lowest temperatures occurring during December and January. Accumulated rainfall was less than 25 mm with two events occurring in February. The wind speed did not exceed 3 m s^{-1}. The experiment was established on a vertisol soil with 25 years of crop management. Soil characteristics previous to sowing are shown in Table 1.

Table 1. Soil characteristics and mineral composition

pH	M.O (%)	CE (dS m^{-1})	Cations (meq L^{-1})				Anions (meq L^{-1})			
			K	Na	Ca	Mg	SO_4	CO_3	HCO_3	Cl
7.1	1.7	1.96	1.13	8.7	7.8	5.23	4.59	0.85	3.34	5.7

Treatments and temperature control

Two treatments were established: T1: increase of 2 °C with respect to the ambient temperature of the crop canopy (Warming treatment); and T2: ambient temperature of the crop canopy (Control treatment) as described previously by Garatuza-Payan et al. (2018). Shortly, treatments were distributed following a completely randomized experimental arrangement. To increase the crop canopy temperature, a T-FACE system designed by Kimball (2015) was mounted. Six 1000 W thermal radiators were used per plot (model FTE-1000, 245 mm long x 60 mm wide, built by Mor Electric Company Heating Association Inc. Comstock Park, MI, EEU.). The radiators were deployed on five triangular equilateral structures of 5.2 m on each side (Fig. 1). Two radiators were installed on each side of the triangular structures forming a regular hexagon that effectively raised the temperature in a 3 m diameter circle on each plot. To control the temperature, infrared temperature sensors were installed (ITSR Apogee Instruments Inc., Logan, UT, USA) in both control and warmed plots. The ITSR sensors were coupled to a data logger (CR1000 Campbell Sci, Inc. Logan, UT, USA) that send a voltage signal to an interface (MAI-05V, Avatar Instruments) which, in turn, translates the signal from volts to milliamps into a dimmer (Attenuator A1P-24-30-S05, Avatar Instruments). This dimmer controlled the current delivered to heaters, so that the amount of emitted heat increases or decreases depending on the temperature difference between the warmed and control plots, through the proportional integrative and derivative routine described by Kimball (2015).

Figure 1. Aerial image of de experimental site where triangular structures were placed containing six thermal radiators each one.

Plant material and Agronomic management

CIRNO C2008 wheat variety was used as experimental model, which is classified as a crystalline or hard wheat (*Triticum durum* L.). It originated from a segregating selection of interbreeding SOOTY-9 / RASCON-37 // CAMAYO, carried out in the International Center for the Improvement of Maize and Wheat (CIMMyT). This variety was released for crop extension since 2008 (Figueroa-López et al., 2010) and still maintains genetic stability of yield components (Argentel-Martínez et al., 2018).

The sowing was carried out with a SUB-24 drill on December 8, 2016, in a vertisol soil (Bockheim et al., 2014), forming three rows for each furrow, using a seed density of 170 kg ha^{-1}. Background fertilization was carried out applying 250 kg ha^{-1} of urea + 100 kg ha^{-1} of monoammonium phosphate (MAP), 11-52-00. Three irrigations were applied with an average water depth of 14 cm and irrigation intervals of 25 days. Two additional nitrogen applications of 50 kg ha^{-1} of urea were applied before the second and third irrigations (growth and heading phenophases). A slight presence of aphids (*Schizaphis graminum*) was observed during tillering phenophase using the Muralla Max (ia Imidacloprid + Betaciflutrin) pesticide for control, at a dose of 0.20 L ha^{-1}. Weed control was done manually before irrigations.

Peroxidase activity (POD)

Peroxidase activity was measured according to the continuous method similar to the one described by Maehly & Chance (1954). The enzyme extract was obtained with a sample of 0.25 g of leaf fragments sampled from 15 plants per repetition at each treatment, which were homogenized in a mortar, with 5 mL of Tris-HCl buffer pH 7.4. The homogenate was centrifuged at 12,900 rpm during 15 min and the supernatant was used to determine POD activity. The oxidative substrate was made of Guaiacol (2-Methoxyphenol, Catechol monomethyl ether, Pyrocatechol monomethyl ether) (0.5 mL) and hydrogen peroxide at 30% (0.1 mL). The POD extract (0.1 mL) was mixed with the oxidative substrate (0.25 mL) to measure absorbance at 470 nm. This reaction was used to define enzymatic activity units (UEA) where one unit was the change of 0.01 in the absorbance reading for one minute and is reported by per gram of fresh weight. This assay was done during growth, tillering and heading phenophases, when 51% of plants showed similar characteristics.

Nitrate reductase activity (NR)

The Jaworsky (1971) methodology was used for NR measurement during the heading phenophase. In a final volume of 2 mL of the reagents, 0.8 mL of phosphate buffer (100 mM of K_2HPO_4 / KH_2PO_4, pH 7.5), 0.2 mL of 100 mM KNO_3, 0.2 mL of 10 mM cysteine, 0.2 mL of 2 mM NADH, were mixed together with 0.6 mL of enzymatic extract (see above). The NR activity was stopped by the addition of 0.1 mL of 1 M zinc acetate after 30-min incubation of samples in the dark at 30 °C. Subsequently, to eliminate the precipitate formed after the addition of zinc acetate, the samples were centrifuged at 12,000 rpm for 15 min. The NR activity data were expressed in (μmol NO_2^- g FW^{-1} h^{-1}).

Activity of glutamine synthetase (GS)

The extraction of the GS was carried out following the method of O'Neal & Joy (1973) during heading phenophase. For this, an amount of 0.5 g of fresh plant material (a sample from fragments of 15 plants by repetition at each treatment) was homogenized with 5 mL of 0.2 M HEPES buffer, pH 7.9. The homogenate was centrifuged at 16,000 rpm for 20 min, and the obtained supernatant was used to measure GS activity. The enzymatic activity was determined by the method described by Slawky & Rodier (1988). The extracted material was centrifuged at 11,000 rpm, for 10 min., then a 50 µL sample was taken from the resulting supernatant to quantify the inorganic phosphorus (Pi) from the enzymatic use of ATP which was determined using the colorimetric method of vanadomolydophosphoric (Hogue et al., 1970), at a wavelength of 430 nm and against a standard curve of K_2HPO_4 (5–100 µM) and it was expressed in µmol GS s^{-1} g^{-1} FW. To determinate glutamate concentrations (mg g^{-1} FW), a blank per omission of the enzymatic extract and glutamate was used, where the corresponding volumes were replaced by the HEPES buffer.

Statistical analysis

For POD activity data comparison, we implemented a two way analysis of variance using a factorial arrangement, based on a linear model of fixed effects, taking as sources of variation the phenophases (growth, tillering and heading) and treatments (Warming and Control) (Fisher, 1937). Treatment means were compared by the Tukey multiple comparison test for $p < 0.01$ (Tukey, 1960). Statistical indicators: coefficient of determination without adjustment (R^2) was determined for the isolated factors and the phenophase-treatment interaction. The coefficient of variation (CV) was also determined.

For the NR, GS activities the mean of a total of 6 repetitions from each treatment and its standard deviation were determined, which were compared by a theoretical distribution of probabilities for continuous quantitative variables of t-Student for $p < 0.01$. For all analyzes the statistical package ESTATISTICA, version for Windows (StatSoft, 2014) was used.

RESULTS AND DISCUSSION

Peroxidase activity (POD)

Peroxidase activity increased significantly from the tillering phenophase in the warming (Fig. 2) compared to the control treatment. This result suggests that during the initial growth phenophase the warming treatment did not affect the enzymatic activity. This result could be related to the protective mechanisms of macromolecules reserve to avoid biological oxidations during its mobilization towards the reproductive organs (Yoneyama et al., 2016).

Highly significant differences in POD activity were obtained between the phenophases of warmed plants and such increase was more than double (Fig. 2). The observed increase in control plants during the heading phenophase, denotes the beginning of biological oxidation processes in plants induced by stress (Djanaguiraman et al., 2018). On the other hand, a high POD activity indicates the arrival of adverse conditions that affect cellular dynamics (Rached-Kanouni & Alatou, 2013). The variability found in POD activity was explained up to 61% by the warming effect, while

a 23% was explained due to changes in phenophases. There was also a significant interaction (warming treatments x phenophases, p = 0.05), although its contribution to total POD variability was only 15%. POD plays an important role in the symplastic hydrogen peroxide (H_2O_2) level regulation by its conversion to H_2O together with the regeneration of $NADP^+$ (Kumar et al., 2015). Several studies have shown that POD activity increases during exposure to high temperatures in rice and maize (Xin et al., 2016; Matos-Trujillo et al., 2017).

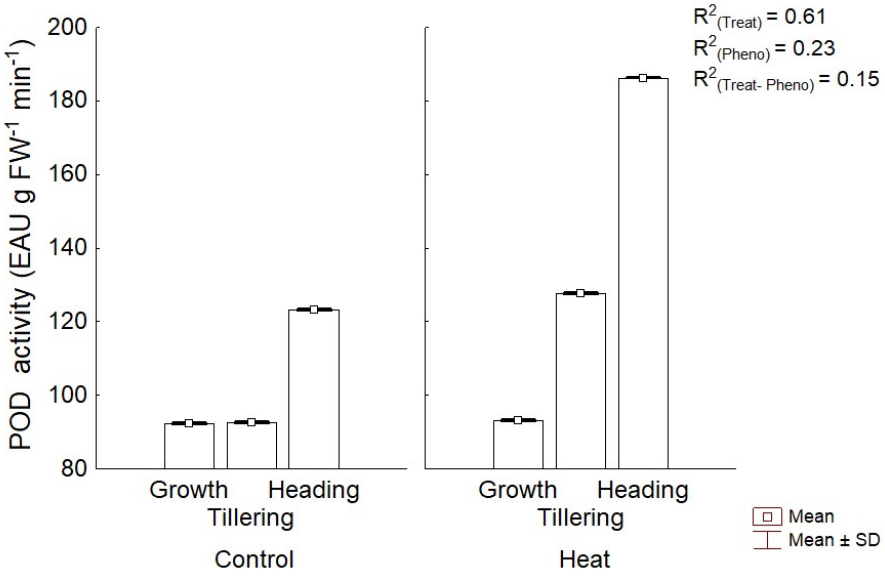

Figure 2. Peroxidase activity during Growth, Tillering and Heading phenophases. R^2: Determination coefficient without adjustment for: Treat (treatments) Pheno (phenophases) and Treat-Pheno (interaction). SD: Standard deviation.

There are also evidences that in corn (*Zea mays*) exposed to drought and saline stress the peroxidase activity increases significantly causing the formation of superoxide radicals (O_2^-) (Mittler, 2017). The superoxide radical and its reduced product $H_2O_2^-$ are potentially toxic when combined with a hydroxyl radical (OH^-), and are also known as reactive oxygen species (ROS) (Zhang et al., 2017; Hasanuzzaman et al., 2018). The plant species that show drought and salinity stress tolerance have an efficient active system (Goyal & Asthir, 2016) that avoid oxidative damage as a function of peroxidase activity (Argentel-Martínez et al., 2017).

Nitrate reductase activity during heading phenophase

The NR activity varied significantly between treatments, showing the highest activity in the warming treatment (Fig. 3). This result demonstrates that increased heat did not negatively affect plant capacity for assimilation and reduction of nitrogen and actually promoted NR activity. A high NR activity in plants indicate faster transformation kinetics of oxidases to reduced forms of nitrogen, therefore, to the formation of proteins. The NR is the first enzyme involved in $N\text{-}NO_3$ assimilation and it is essential for plant growth. In most herbaceous plants, reduction of $N\text{-}NO_3$ occurs in both the root and the shoot (Han et al., 2017). More particularly, $N\text{-}NO_3$ reduction occurs

in the cytosol and the resulting NO_2^- is transported to the interior of chloroplasts, where it is reduced to NH_3 by NR. When there is a supply of N-NH_4^+, and with the participation of α-oxoglutarate, N is directly assimilated into chloroplasts to form glutamate through the activity of glutamine synthetase (GS) which has been localized in both roots and leaves in multiple species of cereals (Tian et al., 2016).

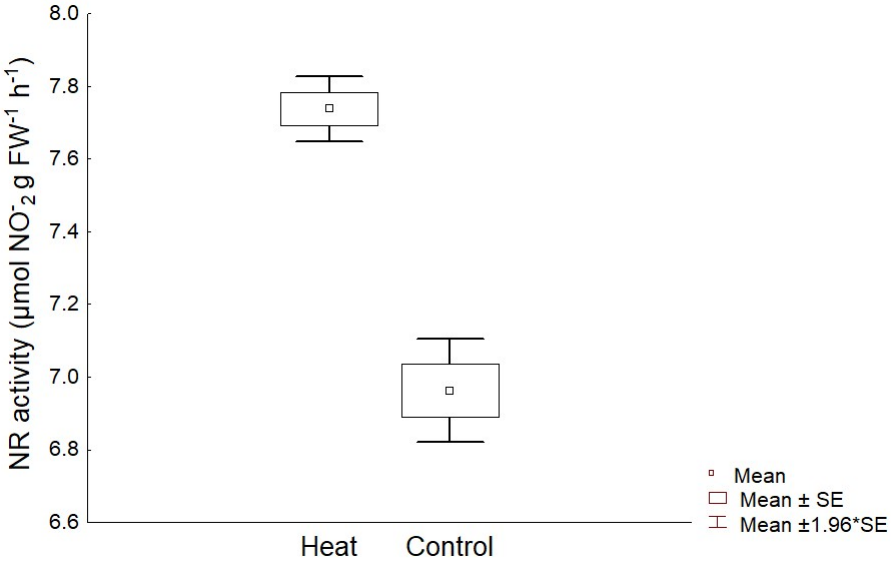

Figure 3. Nitrate reductase activity in wheat in the Control and heat treatments. SE: standard error.

Glutamine synthetase activity in the heading phenophase

The GS activity also significantly increased under warming conditions (Fig. 4, a), and this response led to the increase of glutamine content (Fig. 4, b), a metabolite that is generally the main substrate of remobilization of protein nitrogen during reproductive stage in cereals (Yoneyama, et al., 2016). These results verify that under warming, the observed early senescence occurs through protein metabolic translocation, which suggests a reduction of the biological crop cycle (Zandalinas et al., 2018). The GS activity is directly involved with the ureids and asparagine biosynthesis and with some other reactions of normal cell metabolism. In addition, it may have a close relationship with the self NR synthesis, so the differences in activity of (GS) can be directly or indirectly correlated to the NO_3^- assimilation efficiency of roots (Yang et al., 2017). The increase in GS activity generally forces an imbalance of glutamine / glutamate ratio, also causing a reduction in the glutamate content. Glutamate is the most abundant amino acid in wheat plants phloem during the vegetative stage, while glutamine is the predominant amino acid during reproductive stages, mainly during grain filling. This behavior suggest that GS would act as a repressor of nitrogen compounds transport in vegetative stages and also, as a promoter during reproductive stages. The promoter effect would have a positive consequence in protein accumulation in grains (Kaur & Kaur, 2017). The GS activity is an appropriate parameter to reveal the degradation of reserve protein and the end of the nitrogen assimilation processes in leaves. From this increase in enzymatic activity, the nitrogen mobilization, mainly from amino acid origin, is high and rapid, cascading in foliar senescence (Fernando et al., 2017).

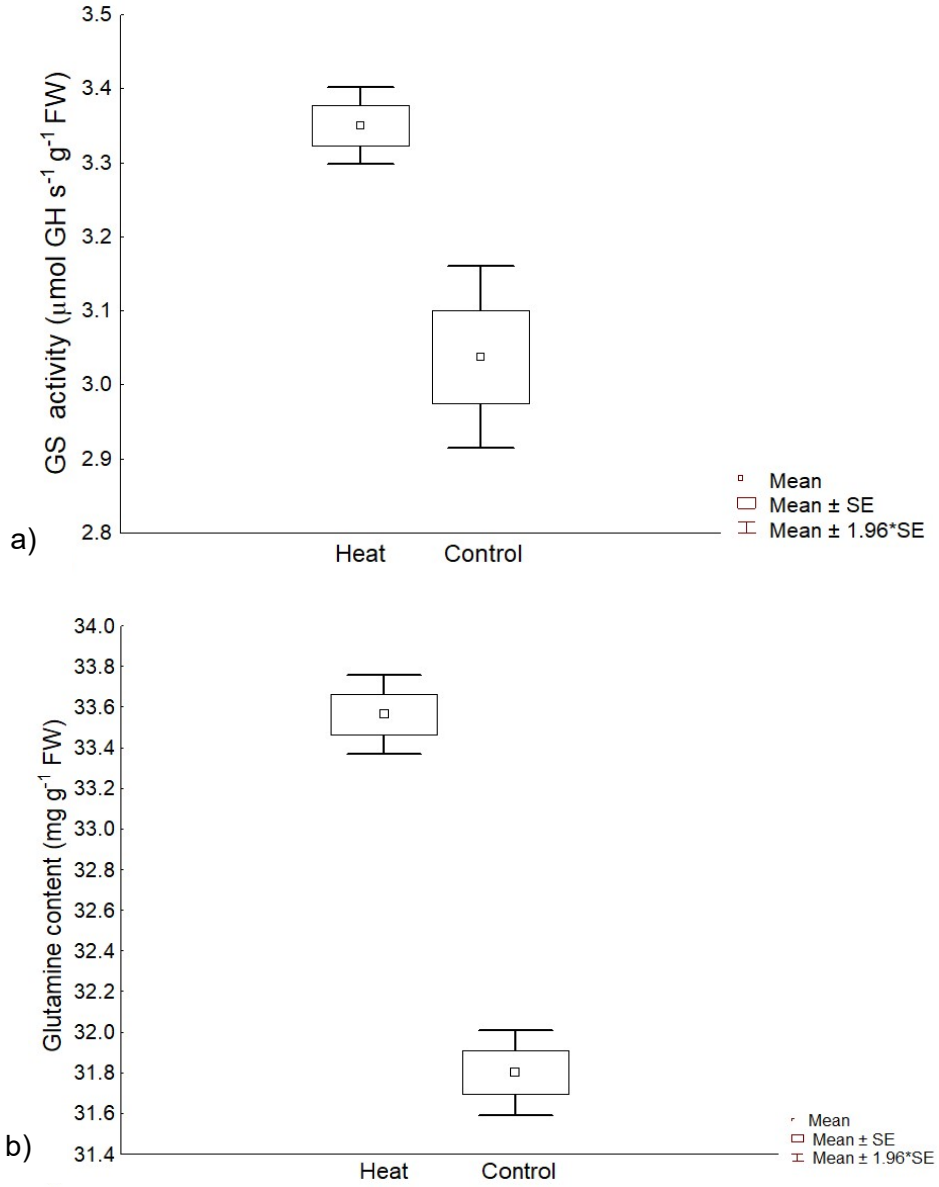

Figure 4. a) Glutamine synthetase activity and b) glutamine content during heading phenophase.

AKNOWLEDGEMENTS. We thank the National Council of Science and Technology Mexico (CONACYT) for funding Projects CB-2013-01: 220788 and CONACYT 662547. Masuly Vega-Puga, Miguel A Rivera-Diaz and Manuel Garibaldi provided invaluable technical support in the temperature manipulation system and crop management. Dr. Bruce A. Kimball provided control algorithms for the warming manipulation system and Dr. Roberto Rodriguez provided advice on enzyme assays.

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