Research Article

Cellular Responses Observed Following Contamination by the Pathogen of Task Halo "*Pyrenophoratrirtici-repentis*" or Heavy Metal "Copper" in Durum Wheat (*Triticum durum Desf*)

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ABSTRACT

The plant, like any living organism, is influenced throughout its life by the climatic and non-climatic conditions of the environment. These conditions will ensure either an environment favorable to growth and development or subject to abiotic or biotic stresses that will disrupt its metabolism. The essential objective of our study is to understand the adaptive strategies of the plant with respect to biotic and abiotic stress caused by the pathogen of a cryptogamic disease "the halo spot" or a heavy metal: The copper. In this context, a sampling of the infested plants (*Triticum durum*, Desf., VarSimeto) was carried out to assess its toxicity according to the severity of the contamination, which is determined according to the number of tasks on the leaves (Uncontaminated, low, medium, and severe contamination) for biotic stress and as a function of increasing concentrations of Copper sulphate (0, 200, 400, 800 µM) for metal stress. Copper is a heavy metal necessary for the development of plants, since it is one of the nutrients essential to the functioning of certain vital cycles. Beyond a certain threshold, it becomes toxic and causes biochemical changes. The results obtained show a toxic effect, which is manifested by a strong increase in the level of proteins and of malondialdehyde (MDA) and a decrease in the lipid and glutathione (GSH) levels. On the other hand, a very significant induction of the enzymatic activities Ascorbate-peroxydase (APX) and glutathion S-transferase (GST) intervening in the system of defense of the plant. All these results show the presence of an oxidative stress generated by both types of stress.

Keywords: *Triticum durum* Desf, *Pyrenophoratrirtici-repentis*, Copper sulphate, abiotic and biotic stress, Oxidative Burst, biomarkers.

INTRODUCTION

Plants are constantly confronted with different types of stress affecting their growth and consequently their development. The more quickly a plant is able to put in place rapid and appropriate defense mechanisms, the less stress will be on plant health (Rémus-Borel, 2007). Most diseases cultivated plants are caused by microscopic fungi, which destroy much of the world's crops each year (Nasraoui, 2006). The current diseases situation in Algeria in the durum wheat fields was characterized by predominance of brown rust (*Puccinia recondita*) and the halo spot (*Pyrenophoratritici-repentis*) (Benbelkacem and Bendif, 2010).Recognition of these diseases as well as their means of control remain important tools to better control these constraints and improve productivity thereafter (Aouali and Douici-Khalfi, 2009).Like animals, plants are able to recognize non-self and modified self, as well as to induce defense mechanisms in response to pathogen attacks (Nürnberger and Kemmerling, 2009).There are two types of plant defense; Constitutive and inducible. The former may be of biochemical or morphological origin (Gravot, 2009), it is present at all times in the development of the plant. It is constituted by a set of physical structures or chemical barriers that counter the penetration and development of bio-aggressors (Klarzynsk and Fritig, 2001) .Le deuxième type de défense regroupe un ensemble de défenses induites; La perception de l'agent pathogène va conduire à l'activation d'une cascade de signalisation intracellulaire (Bollerand Felix, 2009).Cellular signaling events early induced following recognition of the microorganism are more generally studied via elicitors (Zhao et *al.*, 2005, Hofius et *al.*, 2007).Among these events figures the production of phytohormones such as salicylic acid and, above all, changes in the permeability of the plasma membrane manifested by influxes of Ca^{2+}, efflux of K^{+} and anions (Garcia-Brugger et *al.*, 2006) .These ion streams can act upstream of other cellular events, in particular production of oxygen-reactive species (ROS), such as the superoxide anion $^{\circ}O_2^{-}$ and H_2O_2, as well as a phosphorylation cascade Involving protein kinases (Sbartai et *al.*, 2015). All of these reactions will lead to reprogramming of the expression of genes, more particularly of the defense genes, in order to set up adapted defense responses (Dubreuil, 2010).In addition to aggressions caused by living organisms, plants are exposed to environmental changes induced in most cases by human activities. Trace elements of metal are considered among the elements most dangerous for living organisms and especially for plants that are directly exposed to these elements whose toxic potential is undeniable. Exposure to heavy metal toxicity has become a major limiting factor in the growth and yield of crops, affecting the sustainability of agricultural production and

threatening food security. La toxicité des métaux lourds retarde la croissance des plantes en marginalisant les fonctions cellulaires des protéines, des lipides et des composants élémentaires des membranes des chloroplastes (Sharma et *al.*, 2003 ; Scoccinati et *al.*, 2006 ; Sbartai et *al.,*2012).Among these metals, copper is considered to be a trace element necessary for metabolic functions and plant development (Burkhead et *al.*, 2009).It exists in multiple forms redox thanks to its ability to exchange electrons from its orbit. This metal is a cofactor in the electron transport chain in mitochondria and chloroplast (Palmer and Guerinot, 2009).It is also defined as a Potentially Toxic Trace Element, as any element essential to plant development, it can induce symptoms of toxicity (reduction of biomass, inhibition of root growth, chlorosis, loss of chloroplast integrity, Etc.), at exposures higher than its cellular homeostasis. Its toxicity comes first from its contribution to the production of ROS such as superoxides ($^{\circ}O_2^-$), hydroxyl radicals ($HO^{\bullet}$) and hydrogen peroxide (H_2O_2) (Marschner, 2011, Azzizi et *al.*, 2016).

It is in this context that our study was carried out in order to better understand the defense mechanisms in a variety of durum wheat with respect to an oxidative burst generated by different stresses (biotic or abiotic) througth the determination of certain Biomarkers involved in the plant defense system.

MATERIAL AND METHODS

A part of the experiment was carried in the collective agricultural operation in HallalaAissa, Echatt (city of EL-Tarf) located in the north-east of Algeria known for its humid climate associated with a strong rainfall favoring the development of cryptogamic diseases. The pathogen responsible for the disease is: *Pyrenophora trirticirepentis*. It manifests as oval necrotic spots on the leaves (Boulal H et *al.*, 2007).And the second part at the Pedagogical Laboratory of Plant Physiology of the Department of Biology, BadjiMokhtar University Annaba.The experimental material used in our work is hard wheat Variety; Simeto. It is a variety of Italian origin, with thin straw, and medium grains half elongated. It has an average percentage of germination and a high yield.

Experimental Protocol

Concerning the biotic stress (cryptogamic disease) in the field, we randomly selected control plants (zero-spot equivalents), poorly affected plants (≈15% spots), moderately affected plants (≈30% spots) and (> 50% of the infested leaf). For abiotic stress (metal stress: Cu) in vitro, the selected durum wheat seeds are disinfected with 3% bleach for 30 minutes and then rinsed thoroughly with distilled water.To facilitate and accelerate the germination process, the seeds are vernalized for 24 hours, then germinated in petri dishes covered with filter paper soaked in distilled water. At the 2-3 leaf stage, plants are subjected to different concentrations of copper sulphate (0, 200, 400, and 800 µmol) for one week. Irrigation is provided on average three (03) times a week for all treatments.

Analytical techniques

Protein Determination

The proteins are assayed by colorimetry according to the method of Bradford, (1976) which consists in measuring the concentration of proteins in solution by spectroscopic analysis of 0.1 g of ground plant leaf with 10 ml of distilled water. After filtration, 0.2 ml of the supernatant is taken and 2 ml of BBC (Bradford reagent) are added. The principle of the method is based on the fixation of the Comassie Blue on the proteins at the level of basic residues and aromatics, the presence of proteins is revealed by a blue coloring. The Absorbance is read at the spectrophotometer at a wavelength of 595 nm.

Dosage of lipids

Total lipids are assayed according to the methods (Goldsworthy et *al.*, 1972). Each sample consisting of 0.5 g of fresh leaf material is macerated in 10 ml TCA (20%). 1 ml of the extract is removed and centrifuged at 5000 t / 10 min. The supernatant is poured and the pellet containing the lipids is kept. To the latter, 1 ml of the Ether / Chloroform mixture (1/ 1) is added, followed by a second centrifugation of 5000 t / 10 min, which gives two phases: a pellet and a supernatant. 100 µl of the supernatant to which 1 ml of sulfuric acid is added are placed in tubes in a water bath at 100 ° C for 10 minutes. After cooling, 200 µl of the extract is taken, to which is added 2.5 ml of the 85% sulfo- phospho- vanillinic mixture. The spectrophotometric reading is carried out at a wavelength of 530 nm.

Glutathione (GSH)

The enzyme extract is homogenized in a Tris / EDTA solution and undergoes deproteinization with 0.25% sulfo- salycilic acid. After centrifugation at 2000 g for 10 minutes, the supernatant is used for the spectrophotometric assay with the DTNB reagent at 0.01 M to 412 nm. The concentrations of GSH are measured by the method of (Weckbecker and Cory, 1988) and expressed in µM / mg of proteins.

Dosage of malondialdehyde (MDA)

Lipid peroxidation is estimated by the evolution of the MDA content according to the method of Draper and Hadley (1990).The homogenization of the plant tissue in trichloroacetic acid (TCA 5%) at a rate of 10 ml per 1 g of plant tissue is followed by centrifugation for 15 min at 12,000 g, the supernatant is added to an equal volume of thiobarbituric acid (TBA) at 0.5% in TCA at 20%, and then the mixture is incubated for 30 min at 100 ° C.The absorbance of the supernatant obtained after centrifugation at 10000 g for 5 min is read at 532 nm. The concentration of MDA is calculated using its molar extinction coefficient = 155 mM -1 cm -1.

Assays Ascorbate- Peroxidase Activity (APX)

The spectrophotometric determination of the ascorbate-peroxidase activity is carried out according to the protocol adopted by Nakano and Azada (1987).The final reaction volume of 3 ml contains 100 µl of enzyme extract, 50 µl of 0.3% H2O2 and 2850 µl of NaK-Ascorbate phosphate buffer (50 mMNaK, and 0.5 mMascorbate, pH = 7.2). The calibration of the apparatus is carried out in the absence of the enzymatic extract. The reading is carried out at 290 nm (spectrophotometer GeneSys 8) for 1 min and this for a molar linear extinction coefficient ε = 2800 M -1 cm -1. The APX activity is expressed in nmol / min / mg protein.

Determination of Glutathione S-Transferase Activity (GST)

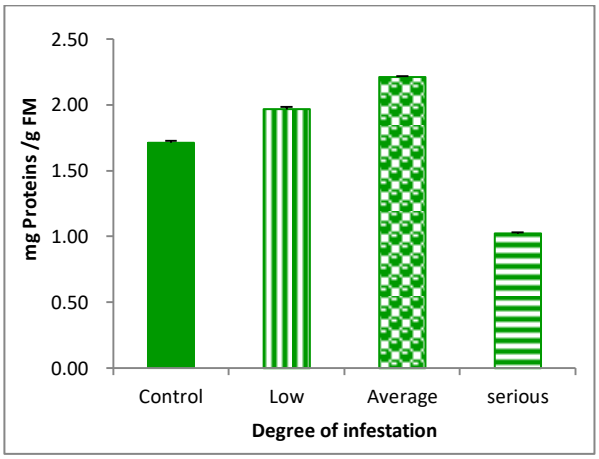
The determination of glutathione S-transferase is carried out by the method of (Habig et *al.*, 1974). The samples are homogenized in a phosphate buffer at pH 6.5 and 100 mM and centrifuged at 9000 g for 30 min.The method consists in reacting the GSTs on a mixture of CDNB (20 mM) -GSH (100 mM), the variation in the optical density due to the appearance of the CDNB-GSH complex is measured every 15 seconds for 1 minute at 340 nm. Concentrations of GST are expressed in nmol / min / mg protein.

Statistic study

The results obtained are expressed by the mean more or minus standard deviation (m ± sd).The averages in the same series were compared with each other using the ANOVA statistical test according to the degree of infestation of wheat leaves and / or as a function of increasing concentrations of Copper sulphate with a threshold of significance (P).

RESULTS

Effects of the halo spot and copper sulphate on protein levels



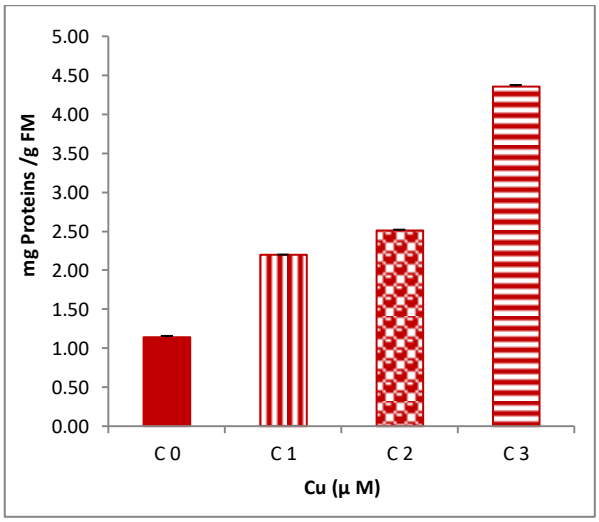


Figure 1: Effects of the halo spot (a) and copper (b) on protein levels

Figure 1 shows the variation in the level of proteins as a function of wheat leaf infestation by the pathogen of the halo spot (a) or by copper (b), showing a very highly significant increase in (1.96mg / g MF, 2.20mg / g MF), shows a very highly significant increase in the amount of proteins in weakly and moderately infested leaves (respectively 1.96mg / g MF, 2.20mg / g MF), whereas this rate decreases for severely infested leaves compared to the control leaves to reach 1.023mg / g MF equivalent to 40% (Figure 1.a).Similarly, a significant increase ($p \leq 0.001$) in the amount of total protein is observed as a function of the increasing concentrations of copper compared to the control leaves (Figure 1.b). The highest value (4.35 mg / g MF) is observed for the highest concentration (800 µmol) equivalent to five times (≈ 5) the control value (1.14 mg / g MF).

Several studies report an increase or decrease in total proteins regardless of the type of stress (Abdellatef and Tran, 2016). Seventeen (17) families of PR proteins (Patogenesis-Related) have been identified in a number of plant species (Sels et *al.*, 2008) as a result of various stresses (TuzunandSomanchi, 2006).In our study, the proportional increase in protein levels observed as a function of increasing concentrations of the pathogen and metal in wheat leaves confirms this hypothesis and highlights their major roles in the plant defense system both to the two stress (biotic and abiotic).This increase is due, on the one hand, to the activation of the genes as for the synthesis of the proteins of the PR type and the G proteins which are associated with membrane receptors as well as the proteins of the secondary metabolism (Ponchet et *al.*, 2000) And on the other hand to the induction of the response of hypersensitivity RH which is often observed locally (Pedley and Martin, 2005).This activation is related to the triggering of phosphorylation as well as protein kinases (MAPKs) involved in signal transduction and induction of plant defense reactions (Zhao and Davis 2005).On the other hand, a decrease in

the total protein content is observed in severely infested wheat leaves. This decrease is attributed to the oxidative burst generated as a result of the interaction between the parasite and the host plant where a strong ROS accumulation is observed. Their action leads to direct lesions of biological molecules by oxidation such as the oxidation of proteins which has been reported by several authors (Parent et *al.*, 2008, Van Loon et *al.*, 2008, Sbartai et *al.*, 2015).In addition, necrotic lesions located at the fungus penetration sites observed in severely infested wheat leaves demonstrate that the hypersensitivity (HR) response is stimulated thereby limiting pathogen development by reducing access to nutrients (Dubreuil, 2010) and consequently the activation of programmed cell death (PCD) hence the reduction in the level of proteins.

Effects of halo stain and copper sulphate on lipid levels

The systemic lipid signal is among the first events involved in the defensive response and in the signal transmission mechanisms. Signaling by phospholipids is an important component in the signalitic pathways in Eukaryote. It plays a major role in plant growth and development as well as in systemic response to environmental stresses, including attack by pathogens (Song and Goodman 2002, Profotovà et *al.*, 2006, Akram, 2008).

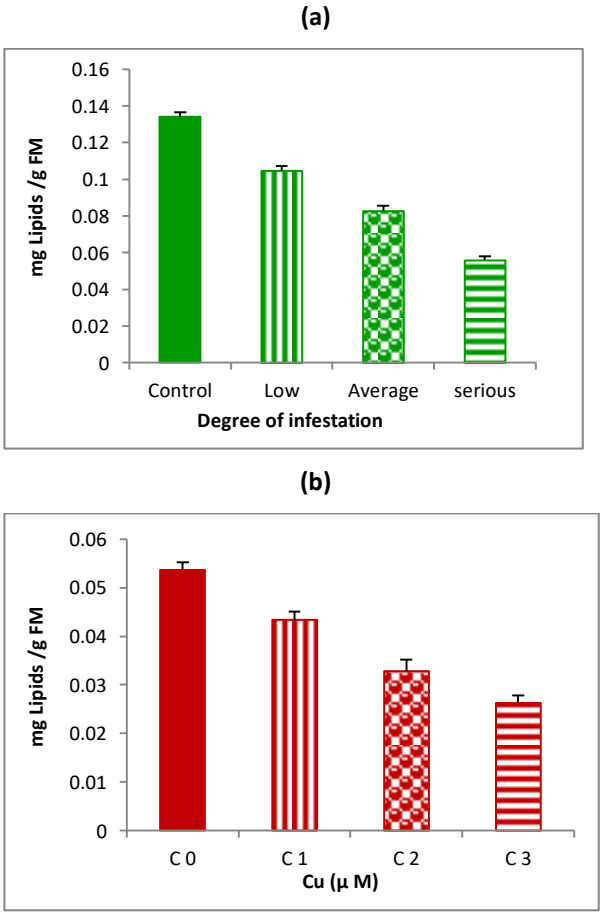


Figure 2: Effects of the halo spot (a) and copper (b) on lipid levels

In our study, lipid assay revealed a very highly significant reduction in its content in wheat leaves contaminated with *Pyrenophoratrirtici- repentis* (Figure 2.a).The higher the degree of infestation by the fungus, we observe a decrease in this rate wich reaches the lowest value (0.055 mg / g MF) in the highest degree of infestation (severely affected). This reduction is equivalent to 58% compared to the control.Similarly, a significant ($p \leq 0.001$) decrease in lipid content was observed in a dose-dependent manner in wheat leaves compared to control leaves (Figure 2.b). This decrease reaches its maximum (0.026 mg / g MF) at the highest concentration equals 50% of the control leaves value (0.053 mg / g MF).

Our results contradict the literature, which assumes that the mobile signal for SAR may be a lipid molecule (Durrant and Dong 2004; Mosher et *al.*, 2006) whereas we find ourselves in a situation where this rate decreases gradually proving that this resistance (SAR) is already triggered (observed accumulation of proteins) by using different mechanisms of defenses with respect to both stresses either by the production of ROS thus inducing an oxidative stress which leads to a lipid peroxidation following an oxidative attack by singlet oxygen or the hydroxyl radical either by the involvement of other signaling pathways such as jasmonic acid (JA) and methyl-jasmonate acid (MEJA) which are Signal molecules derived from fatty acids and their biosynthesis involves enzymes such as lipase, lipoxygenase (LOX), etc..

Effects of the halo spot and copper sulphate on the GSH level

As for the assay of stress biomarkers, glutathione (GSH) is considered among the most important since it intervenes in the antioxidant defense system of the plant.The results obtained concerning the effect of contamination by the pathogen on the level of glutathione (Figure 3.a) show its decrease ($P \leq 0.000$) depending on the different degrees of infestation compared to the control. All GSH values were lower than that of the control (0.0679 mmol / min / mg Prot) and the lowest value (0.0276 nmol / min / mg Prot) was observed in severely infested wheat leaves.The same variations are observed in the leaves treated with the increasing concentrations of copper (Figure 3.b) ie a very significant decrease in the glutathione level.This decrease reached its maximum (0.0334 mmol / mnM / mg Prot) at the highest concentration of the treatment compared to the control leaves (0.0743 mmol / min / mg Prot) and which is equivalent to 50% reduction.

Based on the fact that any stress generates an oxidative stress causing a breakdown of the redox equilibrium which is at the origin of an oxidative damage which can be regulated by glutathione which participates in the reduction of the compounds resulting from the lipid peroxidation (LOOH) Or hydrogen peroxide (H_2O_2) (Parent et *al.*, 2008).

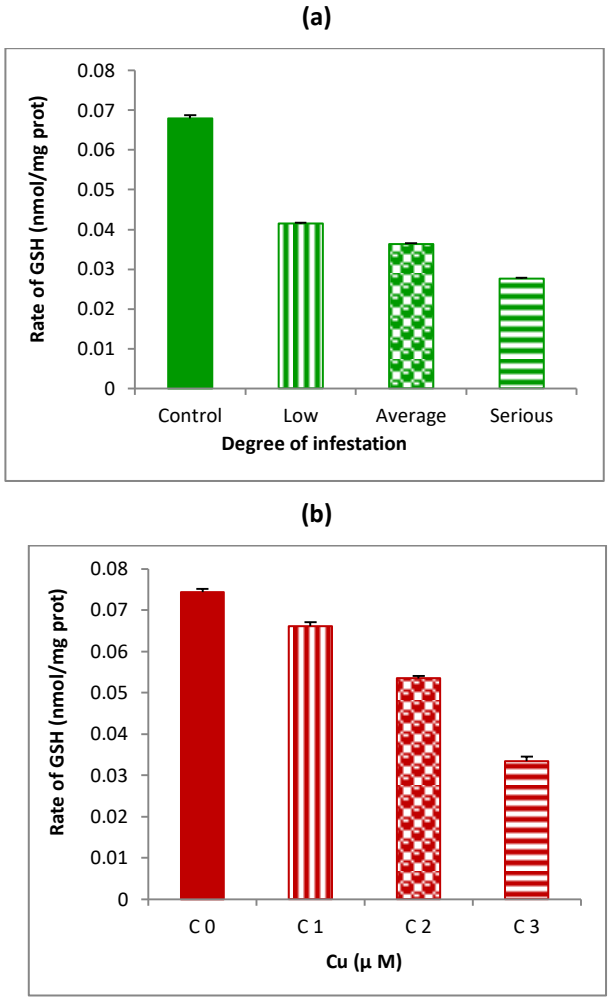


Figure 3: Effects of the halo spot (a) and copper (b) on GSH level

Our results contradict this hypothesis where we observe a decrease in the level of glutathione in all the wheat plants contaminated by the fungus or by copper proving that this biomarker stress is not solicited in these two cases Since all the measured values are lower than the control values.

On the other hand, Gara et *al.* (2003) reports that the inhibition of glutathione synthesis is due to the production of H_2O_2 and NO following a pathogen attack that disrupts the glutathione / ascorbate cycle. Thus, the oxidized form of glutathione predominates and leads to a modification of the redox state of the cell that participates in the establishment of the RH. These two hypotheses allow us to conclude that the inhibition is not due to the nature of the stress but to the degrees of contamination since the high concentration totally inhibits the glutathione in the leaves of wheat proving that the system in question is surpassed and replaced By a more efficient (enzymatic) antioxidant defense system in response to the oxidative burst generated by these two types of stress. Therefore, glutathione intervenes indirectly in the regulation of the redox equilibrium either by the synthesis of phytochelatins in the case of metallic stress or by conjugation to the elicitor or Copper by GST in their detoxification.

Effects of halo spot and copper sulphate on MDA level

Lipid peroxidation is an oxidation of lipids due in most cases to the presence of oxygen-reactive species (ROS). Monitoring the variation of malondialdehyde (MDA) as a function of the degree of infestation by the pathogen shows that this level increases very significantly according to the degree of infestation of the wheat leaves relative to the control (Figure. 4a).

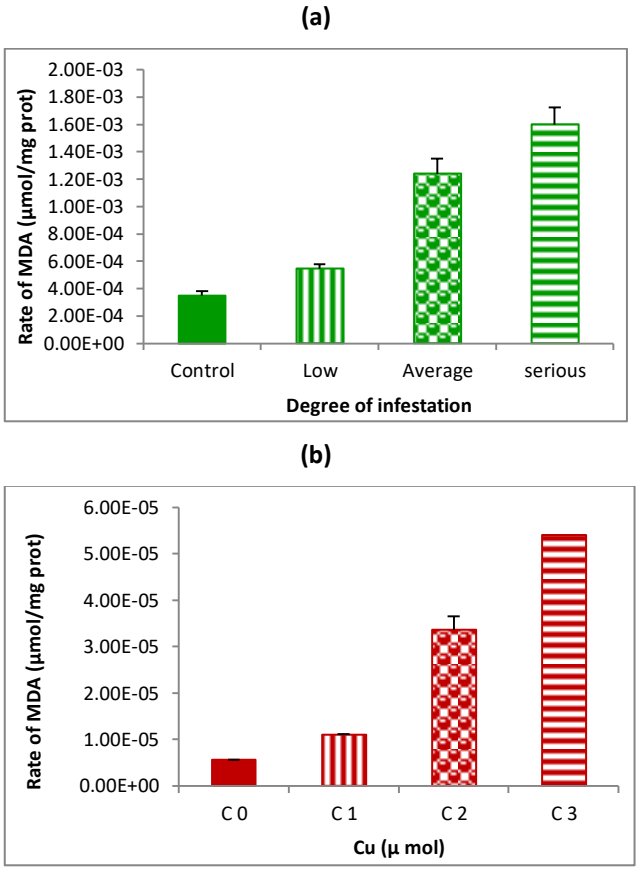


Figure 4: Effects of the halo spot (a) and copper (b) on MDA level

The highest level of MDA was observed in severely infested wheat leaves (0,016 µmol / mg Prot), which is equivalent at five times (≈5) the control value ($3.5.10^{-4}$µmol / mg Prot).Similarly, MDA levels increased significantly (P≤0,000) in dose-dependent ways in wheat leaves subjected to increasing copper concentrations compared to control (Figure 4.b). This variation in the MDA level increased from $5.6.10^{-6}$µmol / mg Prot in the control leaves to two (≈2) times its value ($1.1.10^{-5}$µmol / mg Prot) in the leaves treated with 200µM copper and six (≈6) ($3.36.10^{-5}$µmol / mg Prot) in leaves treated with 400 µM of copper and finally with nine (≈9) times its value ($5.4.10^{-5}$µmol / mg Prot) in the leaves treated with 800 µM Copper.

The high levels of MDA observed in our study are due to lipid oxidation triggered by oxidative stress in response to fungus infestation and / or increased copper levels in wheat leaves. Indeed, in the case of an oxidative burst during the interaction between the parasite and its host plant, the stress is relatively large and its impact extends

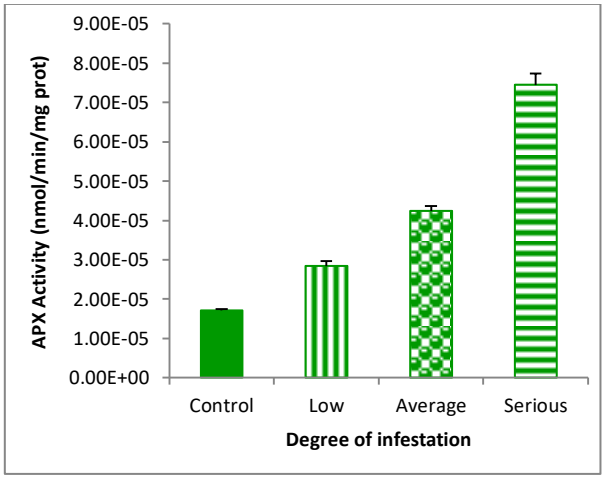
on the cellular scale causing damage both in the parasite and in the plant cell. The excess of ROS causes direct lesions (DNA oxidation, proteins, lipids, carbohydrates) but also secondary damage due to the cytotoxic nature of the metabolites released especially during the oxidation of the lipids (Farmer and Davoine, 2007; Mueller et*al.,* 2008). Our results confirm this hypothesis, which reveal very high levels of MDA equal to five times the value of the control thus proving the magnitude of the stress induced. Similarly, the increase in MDA is attributed to the excess ROS released following the addition of high concentrations of copper in the culture medium. Lin et al., (2005) reports that excess copper in cells increases the H_2O_2 concentration, which can induce oxidative stress, thus disrupting the homeostasis of other elements (Cuypers 2000). These results are in line with our study which determines the maximum value of MDA at nine times the value of the control proving once again the importance of the stress applied.

Effects of halo spot and copper sulphate on APX enzymatic activity

At the same time, the measurement of the APX enzyme activity, responsible for the removal of the oxygenated water (H_2O_2) formed as a result of the applied stress, shows a very significant induction (P≤0.000) of APX activity in wheat leaves Hardened by *Pyrenophoratrirtici-repentis* compared to the control (Figure 5a).

This activity increases with $2.8.10^{-5}$nmole / min / mg Prot (Low infestation), $4.2.10^{-5}$nmole/min/mgProt(Average infestation), to $7.4.10^{-5}$nmole / min / mgProt (severe infestation).For the treatment of wheat plants with copper (Figure 5.b), an increase in the APX activity of the treated leaves was observed as a function of the increasing copper concentrations compared to the control leaves. A peak ($5.6.10^{-4}$nmole / min / mg Prot) was observed in wheat leaves treated with the highest concentration of copper (800 µM).

(a)



(b)

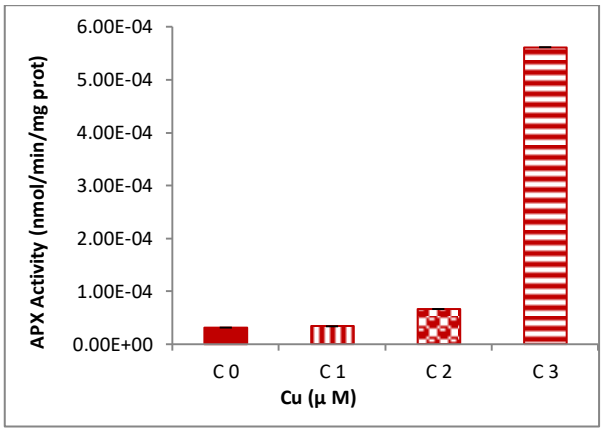


Figure 5: Effects of the halo spot (a) and copper (b) on APXenzymatic activity

Indeed, there are many enzymes capable of producing H_2O_2 in the case of biotic stresses such as pH-dependent cell wall peroxidases (Bolwell, 1996; Bestwick et *al.*, 1997) which function only in the presence of cysteine and Glutathione. Under normal conditions, peroxidases are used to dissipate H_2O_2, whereas during an alkalinization of the apoplast characteristic of the infestation of a pathogen, they can become a producer of OH hydroxyl radicals (Salzer et *al.*, 1996). These ROS are involved in the defense mechanisms of plants, either directly by their high toxicity on pathogens or by activation of numerous metabolic pathways (sbartai et *al.*, 2015) wich explain the induction of APX activity observed in our work in wheat leaves infested with *Pyrenophoratrirtici - repentis*. Similarly, treatment with copper stimulates this activity in wheat leaves with a peak observed for the highest concentration still proving its drastic effect in the cell. Induction of APX activity in response to oxidative stress caused by the presence of the fungus or Cu demonstrates its role in the removal of oxygenated water.

Effects of halo stain and copper sulphate on enzymatic activity Glutathione S-Transferase (GST)

GSTs are known to play an important role in the detoxification of certain molecules by conjugating them to glutathione. The complex, less toxic than the free molecule, can be sequestered in the vacuole or exported to the external environment. Some studies attribute to some particular GSTs a role in the regulation of several enzymes and transcription factors by non-catalytic protein-protein interactions. However, this function does not seem to be linked to any mechanism of detoxification (Calmes, 2011).

According to Fig. 6.a, which represents the effect of the halo spot on the variation of the GST enzymatic activity, a very significant induction of this activity is noted as a function of the degrees of infestation with respect to the control.

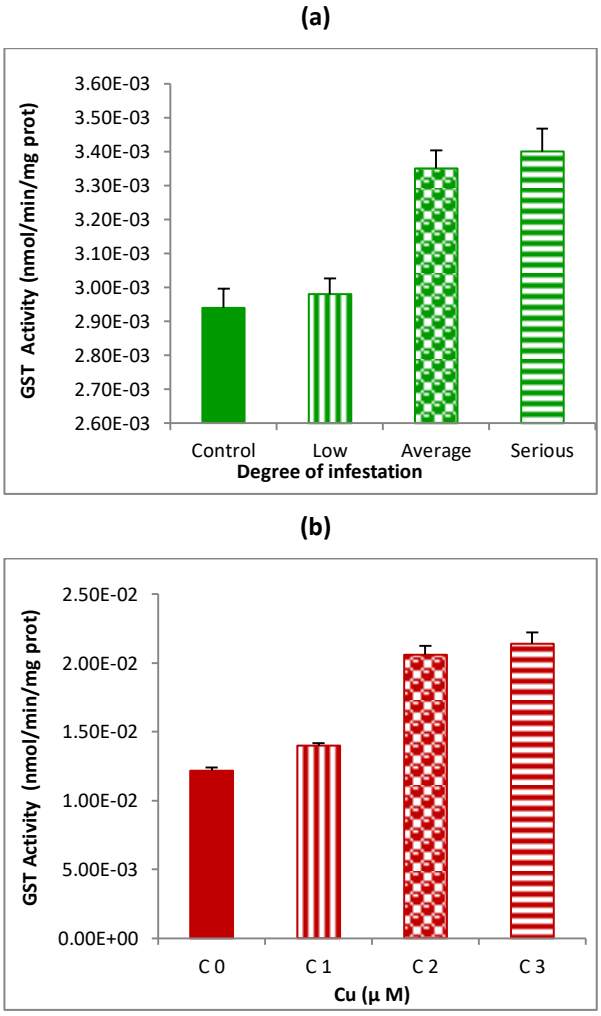


Figure 6: Effects of the halo spot (a) and copper (b) on GST enzymatic activity

This increase is greater in the medium and severely infested leaves which successively reach the values of $3.35.10^{-3}$ and $3.4.10^{-3}$nmole/ min /mgProt. Similarly, a very significant increase ($p \leq 0.001$) in the GST activity of the copper treated leaves was observed compared to that of the control leaves (Fig. 6.b). The highest value (0.0214nmole / min / mgProt) is observed in wheat leaves treated with 800µM Copper. The biochemical analysis of the GST activity shows a stimulation at different treatments and different concentrations indicating a phenomenon of resistance of the cells of Wheat leaves. Indeed, GSH inhibition observed in our study confirms once more that the defense mechanism in question is outdated and replaced by an antioxidant enzyme defense system that performs better GST in response to the oxidative burst generated by the fungus or metal.

CONCLUSION

The great variety of types of stress involves the mobilization of multiple mechanisms of ROS production that are involved in plant defense strategies either directly by action of their high toxicity to the pathogen or metal or by activation many metabolic pathways. They are also signal molecules that rapidly diffuse through the membranes and regulate many defenses genes: chaperone proteins, antioxidant enzymes, ascorbate peroxidase (APX), glutathione-S-transferase (GST), genes linked to pathogenesis (PR) via salicylic acid (SA) or the synthesis of phytoalexins. In the majority of cases, the damage caused by this excess of ROS, if not quickly limited, leads to the death and then the lysis of the cells concerned.

The work carried out shows an important activation of protein biosynthesis, lipid peroxidation represented by the level of MDA and certain enzymatic activities (APX and GST) involved in the antioxidant defense system of durum wheat as well as a drastic decrease in the level of Lipids associated with inhibition of GSH.

All of our results allow us to conclude that during the interaction of *Pyrenophora trirtici-repentis*or copper with leaves of wheat, an oxidative burst is induced that does not depend on the type of stress but on the concentration of the contaminants. It seems that wheat is sensitive to the presence of the fungus and tolerates a little more the presence of copper in the culture medium. This sensitivity was evaluated by the quantification of certain biomarkers, thus proving a greater cellular response in the case of biotic stress compared to abiotic stress.

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