Induction of oxidative stress in wheat (*Triticum aestivum* L) following infection by the pathogen of task halo (*Pyrenophora tririci-repentis*)

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**Abstract**

Any plant infected by a microorganism can develop a disease. But often, she is able to resist him naturally, thanks to the activation of defense mechanisms. Our main objective is to study the induced defense mechanism in a variety of soft wheat (HD 1222) following infection by the pathogen of halo task *Pyrenophora tririci-repentis*. For this, a sample of infested plants was performed according to the severity of contamination (not contaminated, low, medium and high). The results obtained show a fluctuation of chlorophylls rates with a decrease in protein levels as well as a highly significant increase in proline. Finally, the determination of certain biomarkers shown that catalase increases with the degree of infestation, as well as for the rate of malondialdehyde (MDA) leaving suggest a possible lipid peroxidation.

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Introduction
During their lifecycle, plants are facing various stresses, abiotic in nature (drought, cold, heavy metals) and biotic (microorganisms, insects). They have developed adaptive mechanisms vis-à-vis these environmental factors. Thus, most plants are resistant to most pathogens with which they are in contact. They have a natural resistance to biotic stress through preformed barriers and induced mechanisms (Mauch-Mani and Métraux, 1998). Plants do not have a mobile and adaptive immune system such as animals, but they have developed in their evolution, innate immunity at the level of their cells, as well as systemic signals produced at the site of infection and capable of migrating in the plant (Dangl and Jones, 2001). The microorganisms are generally unable to cross the external protective barriers of plants which are a passive defense: the cuticle, which covers the aerial organs and pecto-cellulosic wall rigid envelope surrounding plant cells. However, some attackers circumvent these mechanical barriers and enter the plants through the natural pores called stomata, through accidental injury or destruction of protective barriers. If the agent has the weapons to invade a plant, or the plant does not detect it, the invader benefited. As against the plant can confining the aggressor on the site attack through the implementation of active defenses triggered by the presence of the pathogen. The work of recent years have established the events leading to the induction of resistance in plants (Benhamou; 1996) which can be divided into three major steps: recognition between the two partners by issuing a signal; perception and signal transduction of recognition; and finally the expression of defense genes in response to these signals (Nürnberger and Kemmerling, 2009). Thus, it was clearly shown that the earliest events occurring within minutes of contact with the pathogen, included a selective increase in the permeability of the plasma membrane which leads to Ca2+ flux, K+, Cl− and H+ thereby resulting in membrane depolarization (McDowell and Dangl, 2001; Bolwell and Wojtaszek, 1997; Garcia-Brugger et al., 2006), an intense production of active forms of oxygen (ROS) (Torres et al., 2006), such as the superoxide anion O2− or H2O2 and the hydroxyl radical OH−. Also, gene activation as for the synthesis of G proteins that is associated with membrane receptors (Ponchet et al., 1999; Dixon et al., 1999). These proteins are also involved in signal transduction and induction of defense reactions of the plant (Zhao et al., 2005). There are also production phytohormones like jasmonic acid (JA), salicylic acid (SA), ethylene (ET) and abscisic acid (ABA), the first three being mainly produced during biotic and the last in abiotic stress (Walley et al., 2007; Fujita et al., 2006). These secondary metabolites are sending signals to the nucleus to activate kinase cascades, regulatory enzymes. These can then cause the activation of transcription factors (TF) that they activate the transcription of specific genes in response to stress. Lipid peroxidation has a special place in the hypersensitive response HR. It is the source of many metabolites involved in the defense response of the plant (Jalloul et al., 2002). Some assume that oxidative metabolism might also be involved in the implementation phase of the hypersensitive cell death (Parent et al., 2008). Many researchers have focused on understanding the mechanisms by which plants were running in the HR, the disruption of cell membranes appears to be one of the earliest events (Bennett et al., 1996). This disruption of membranes was associated with the massive hydro peroxides of polyunsaturated fatty acids and the production of FAO (or Reactive Oxygen Species ROS) (Jalloul et al., 2002). It is in this context that our study was conducted to better understand the defense mechanisms of a variety of wheat by the determination of certain biomarkers involved in the antioxidant system due to microbial attack by the pathogen haloed task: Pyrenophora tritici-repentis in the region of El-Tarf (eastern Algeria) known for its humid climate (90%) and rainy (800mm) favoring the onset of fungal diseases.

Materials and methods
Plant materials
The experimental material used in our work is a variety of Wheat: Triticum aestivum L.(HD 1220).

Sampling method

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For our experiment, we chose the area Chbaïta Mokhtar (City of El-Tarf, Algerian East) and because of its humid and rainy climate. From the site selected, a plot of 2 hectares has been identified. We took five randomly 1m observation points, each following a prescribed route. Each square we collected 5 plants or 25 plants in total by the plot. The degree of infestation of each disease is determined by a table that has several levels of concentration of spots on the leaves, so simply divide the cereal leaf into 5 parts. Each part is then compared with that of the table. The average gives us the degree of infection of the plant. Each plant receives a score corresponding to its infestation status for each disease and the note of the plot is the average of the scores for 25 plants (Bovey, 1972). Depending on the evaluation of the degree of infestation, we conducted a 2nd sample (3 repetitions) to the chosen land:
- A control plant (0 stain)
- A weakly reached plant (15%)
- Moderately affected plant (~30%)
- A strongly affected plant (more than 50% of the infested leaf).

**Analytical Techniques**

**Determination of total protein**

The proteins are assayed by colorimetry according to the method of Bradford; 1976. The principle of the method is based on the attachment of an acid dye (Coomassie blue) on the proteins at basic and aromatic residues, this attachment causes a transfer of its color changes from red to blue. This color change is measured at a wavelength of 595 nm by spectrophotometer (JENWAY 3600) using Bovine Serum Albumin (BSA) as standard.

**Determination of chlorophylls**

The extraction of the chlorophylls is carried out according to the method of Holden, 1975, which consists of a maceration of the plant in acetone. Sample processing is as follows: 1g of the plant leaf cut into small pieces and ground in a mortar with 20 ml of 80% acetone and about 100 mg of calcium bicarbonate (CaCO₃). After the total grinding, the solution is then filtered and black boxes in order to avoid oxidation of chlorophyll by light. Reading is done at both wavelengths 645nm and 663nm, after calibration of the instrument with 80% acetone control solution.

**Determination of proline**

Proline was assayed according to the method of Troll and Lindsley (1955), modified by Monneveux and Nemmar (1986). 100mg fresh material is weighed to which is added 2 ml of 40% methanol. The assembly is heated in a water bath at 85 °C for 1h. Extract 1ml removing at which 1ml of acetic acid is added, 1 ml of the mixture containing (120 ml distilled water, 80 ml orthophosphoric acid, 300 ml of acetic acid) and 25 mg of ninhydrin. The assembly is placed in a water bath for 30 minutes at 100 ° C. After appearance of a pink color, we added 5ml of toluene. The mixture is stirred and allowed to settle. Then there is a separation into two phases: a lower aqueous phase and an upper organic phase which contains proline. The collected organic phase was placed into clean tubes containing Na₂SO₄, anhydride. Reading the optical density (OD) is at 525 nm after calibration of the instrument by the white.

**Determination of malondialdehyde (MDA)**

Lipid peroxidation was estimated by changing the content of malondialdehyde (MDA) determined according to the method described by (Alia et al., 1995). The homogenization of the plant tissue in trichloroacetic acid (TCA) 5% to 10 ml to 1 g of plant tissue is followed by centrifugation for 15 min at 12 000 g. The supernatant was added an equal volume of thiobarbituric acid (TBA) in the 0.5% (TCA) 20%. The mixture was incubated at 100 ° C for 25 min. The absorbance of the supernatant obtained after centrifugation at 10 000g for 5 min and read at 532 nm. MDA concentration is calculated using the extinction coefficient 155 mm⁻¹ cm⁻¹.

**Determination of catalase activity (CAT)**

The spectrophotometric assay of catalase (CAT) activity is performed according to the method of (Cakmak and Horst, 1991). The decrease of absorbance is recorded for three minutes for a 240
nm wavelength and an extinction coefficient $\varepsilon = 39400$ linear molar $M^{-1} \cdot cm^{-1}$. For a final volume of 3 ml, the reaction mixture contains: 100 $\mu$l of enzyme extract, 50 $\mu$l of $H_2O_2$ and 0.3% phosphate buffer 2850$\mu$l (50 m Mole, $pH = 7.2$). The reaction is initiated by the addition of hydrogen peroxide. The catalase activity is expressed as nano moles / min / mg protein.

**Statistic study**
The statistical test performed in this study is the analysis of variance at a controlled (ANOVA) and depending on the degree of infestation of wheat leaves by the agent of the haloed task (Pierre Dagnelie, 1975).

**Table 1. Chlorophylls rate observed in infected wheat leaves.**

<table>
<thead>
<tr>
<th>Infection</th>
<th>Chl a</th>
<th>Chl b</th>
<th>A+B</th>
<th>A/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>26.69 ±1.03</td>
<td>33.44 ±1.17</td>
<td>60.73</td>
<td>0.79</td>
</tr>
<tr>
<td>Low</td>
<td>27.60 ±2.65</td>
<td>35.43 ±8.35</td>
<td>63.04</td>
<td>0.77</td>
</tr>
<tr>
<td>Average</td>
<td>23.96 ±2.64</td>
<td>23.92 ±11.52</td>
<td>47.89</td>
<td>1.00</td>
</tr>
<tr>
<td>Serious</td>
<td>21.84 ±1.69</td>
<td>15.46 ±8.75</td>
<td>37.30</td>
<td>1.41</td>
</tr>
</tbody>
</table>

**The total protein**
Figure 1 outlines a very highly significant ($p < 0.0001$) of the total protein in the degree of infestation compared to control plants to reach a minimum value of 15.72 mg / g FM equivalent to a loss of 70% in heavily infested leaves.

![Fig. 1. Total protein observed in infected wheat leaves.](image1.png)

**Proline levels**
According to Figure 2, there is a highly significant increase ($P \leq 0.01$) of proline levels depending on the degree of infestation of the plants with a maximum observed at moderately affected leaves where it reaches a value of 9.51 mcg / mg FM compared to control sheets for which its value is 0.5 mcg / mg FM.

![Fig. 2. Proline levels observed in infected wheat leaves.](image2.png)

**Malondialdehyde (MDA)**
The rate of malondialdehyde (MDA) obtained from different samples of wheat is shown in Figure 3. It is noted that the MDA levels tends to increase in a highly significant manner ($P \leq 0.01$) and that depending on the degree of infestation of wheat.
leaves. This rate peaked at $1.73 \mu$mole / mg P in leaves highly contaminated compared to controls.

**Catalase activity (CAT)**

Monitoring of catalase activity (Figure 4) shows highly significant induction of it and at the first sign of infestation of wheat leaves. This activity is $0.03 \mu$mol / min / mg protein in control leaves and reaches a maximum of $0.7 \mu$mol / min / mg of protein in heavily infested leaves.

![Figure 3. MDA levels observed in infected wheat leaves.](image)

**Discussion**

Plants are constantly exposed to pests, insects, potentially pathogenic microorganisms and herbivores. Their survival is associated with a multitude of strategies to deter herbivores or limit the harmful microorganisms. Although defense strategies vary depending on the nature of bio-aggressor, there are common characteristics such as the "oxidative burst", which corresponds to an early accumulation, rapid and substantial ROS at the invasion site (Garcia-Brugger, 2006; Wojtaszek, 1997). The major products in this ROS biotic stress response peak are superoxide, hydrogen peroxide, and hydroxyl radical. These are essential for transduction of the original signal major physiological changes, such as the membrane polarity, acidification of the intracellular pH, or activation of enzymes such as kinases or phospholipases. All these defense responses are usually accompanied by necrotic lesions at the site of infection, which reduce the penetration of the aggressor and represent local resistance mechanisms defined under the term hypersensitivity or HR (Holub, 1994). In our work, we focused on some biochemical parameters that can inform us about the state of stress of infected wheat leaves.

![Figure 4. Catalase activity recorded in infected wheat leaves.](image)

Photosynthesis is the physico-chemical process by which photosynthetic organisms use light energy to synthesize organic compounds. However, this process essential to a photoautotrophic life is also a producer of reactive oxygen species. The first source of ROS is at photosystem II (PSII) at the reaction center. The absorption of a photon by chlorophyll a, increases energy and quickly releasing an electron, which is then transferred to a chain of electron carriers in the thylakoid membrane (Telfer et al., 1994). Variations in the photochemistry of photosystem II (PS II) can be explained by the change in the availability of the substrate. The decrease in the amount of chlorophyll found in our study shows the stress state of the plant and this decline could be explained by the existence of oxidative damage and the electrochemical potential disruptions. Our results confirm those of Singh et al., (2004) who report frequent degeneration of the amount of chlorophyll and carotenoids in plants exposed to different concentrations of heavy metals. Proteins undergo direct changes under the action of oxygen free radicals with consequences including the amino acid oxidation and the formation of cross-links between proteins. Other indirect effects may be due to the reaction with carbonyl compounds from the glyco-oxidation and lipid peroxidation (Bargnoux et al., 2009). In parallel, proteins can be modified directly by these reactive oxygen species leading to the oxidation of amino acids. For example, amino acids having a thiol (cysteine, methionine) will give sulfoxides and able to form disulfide bridges (Stadtman and Levine, 2003). Direct oxidation of amino acids may also generate carbonyl compounds.
The proteins can also be modified indirectly by reactive carbonyl compounds resulting from the oxidation and glyco-lipid peroxidation. These compounds can then react with the lysine and arginine residues of proteins to form two product groups the AGE (advanced glycation end products), and ALE (advanced lipoxidation end products) (Weiss et al., 2000). Among the precursors of carbohydrate origin of carbonyl compounds, particularly include 3-deoxyglucosone, the Darabinose, the glyoxal which will form AGEs such as pentosidine and carboxymethyllysine (CML). Lipid peroxidation can lead to the formation of malonaldehyde and 4-hydroxynonenal that will form FTAs (MDA and HNE-lysine-lysine) (Miyata et al., 2000). The oxidation of proteins and certain amino acids, glycoxidation and lipo-oxidation may form carbonyl compounds whose increase is called "carbonyl stress". Indeed, the decrease in protein levels recorded in our study suggests an alteration of basal metabolism. Assuming that any kind of stress causes release of free radicals in the organism (Aurousseau, 2000) alteration of cellular components occurs when the intensity of these phenomena increases abnormally. All cell components may be affected, lipids, proteins and so the membranes together, carbohydrates and DNA (Meneghini, 1997). The resulting modified proteins are generally affected in their function or completely inactivated. The cell must be able to remove the oxidized protein. On the other hand, and as suggested by Huang and Fwu (1993) and Schelling et al., (1995), the typical effects of radical attacks occur at the cellular level by lipid peroxidation and protein. The oxidative attack proteins involved many changes from simple oxidation of an amino acid to the fragmentation of polypeptide chains (Ghezzi et al., 2003; Moller et al., 2007) where the reduction in their rates. In addition to modifications of their amino acid, proteins can undergo alterations of their electric charges, the inter or intra molecular cross links by training including bi-tyrosine bridge and fragmentation of the polypeptide chains in the case of strong attack. The proteins can also be modified by secondary mechanisms resulting from the reaction of the reactive oxygen species with other cellular components such as carbohydrates and lipids. These reactions involve the oxidation and the fixation on carbohydrate proteins (glycation and glycoxidation reactions) or aldehydes from lipid peroxidation, as forming adducts lysine, arginine, histidine or cysteine of the proteins (Ramel et al., 2009). The resulting modified proteins are generally affected in their function or completely inactivated. The cell must be able to remove the oxidized protein. The degradation of oxidized proteins is provided mainly by the proteasome complex proteolytic Multicatalytic (Coux et al., 1996) and by the proteases that function downstream of the proteasome (Polge et al., 2009). Also, the accumulation of proline, following drought conditions (Vasquez-Robinet et al., 2008), to salt stress (Banu et al., 2008) or heavy metals (Dinakar et al., 2008), is related to its protective role compliant solute involved in the stabilization of membranes and proteins (Ashraf and Foolad, 2007). But it has also been shown that proline could be involved in the reduction of the amount of free radicals (Okuma et al., 2000; Okuma et al., 2004). This reinforces our findings that showed an increase of proline levels in infected wheat leaves. To the extent of oxidative stress is generally performed by monitoring changes in enzyme activity levels (CAT, SOD ... etc) and molecular levels (GSH, MDA) involved in antioxidant defense (Torres et al., 2008). There are, in biotic stress situation, many enzymes capable of producing H2O2 such as pH-dependent peroxidases cell wall (Bolwell; 1996; Bestwick et al., 1997), which operate only in the presence of reducing molecules such as cysteine and glutathione. Under normal conditions, peroxidases able to dispel the H2O2,Or, during alkalinization of the apoplast characteristic of infection of a pathogen, they can become producing OH radicals (Salzer et al., 1996). The various ROS produced during this oxidative peak are involved in plant defense mechanisms, either directly by action of their high toxicity on pathogens or by activation of numerous metabolic pathways. These answers and help to strengthen the natural mechanical barrier plant cells, altering the pecto-cellulosic wall by various deposits (glycoproteins, polysaccharides and lignin) to limit the spread and...
development of the pathogen by the synthesis of antibiotics plant (phytoalexins) and synthesize defense proteins (PR), such as chitinases (Lamb and Dixon; 1997). In our work increased catalase activity in wheat leaves infected with the pathogen of halo task was observed, this is due to the fact that catalase is considered an enzyme having a quick and clear response contamination by xenobiotics (Wenning et al., 1988). Indeed, this is an important enzyme in the face protection oxidative stress in all aerobic organisms. It catalyzes, extremely rapidly, the disproportionation of hydrogen peroxide into oxygen and water, thereby protecting cells from oxidizing effects (Sanchez-Casas and Klessig, 1994). The increase in ROS in response to biotic and abiotic stresses in transgenic plants private catalase, revealed the importance of these enzymes in plant tolerance to oxidative stress (Willekens et al.; 1997). On the other hand, high levels of MDA observed in our study may be due to the generation of toxic oxygen free radicals (Chaoui et al., 1997). In addition Oscar et al., (1997) reported a significant decrease in polyunsaturated fatty acids in tomato plants after 17 days of exposure to Cd and Chrome. They noted a decrease of linoleic acid (18: 2) and linolenic acid (18: 3) and an increase in the palmitic acid (16: 0). Thus the increase in MDA levels could be due to peroxidation linoleic and linolenic acids. The oxidation in the formation of the derived lipid bilayer, such as 4-hydroxy-2-nonenal, or malondialdehyde phytoprostanes, lead to disturbances of the micro-architecture of the membranes, alters their permeability and can act with the amine functions lipids, proteins and DNA, as well as the thiol groups of proteins. Indeed, these lipid peroxidation products are reactive electrophilic species (RES) which can bind covalently to proteins and thus damage (Farmer and Davoine 2007; Mueller et al., 2008). Lipid peroxidation in forming aldehydes leads to destruction of structures, inhibits cellular functions and potentially accelerating senescence cells (Reich and Amundson 1985, Dann and Pell; 1989). Peroxidation reaction ends when two lipid radicals meet, or when the lipid moiety interacts with a fat-soluble antioxidant, alpha-tocopherol or vitamin E for example. Singlet oxygen, it, is added directly to the double bonds of fatty acids, leading to the formation of hydroperoxides, which can decompose into free radicals and initiate chain reactions (Dix and Aikens; 1993).

Finally, and having examined all the experimental data obtained throughout the study, it appears that the halo is a task that fungal disease attacking the wheat leaves cause considerable damage resulting at the cellular level by producing of reactive oxygen species responsible for an alteration of the basal metabolism, a lipid peroxidation as well as oxidation of proteins. Our results allowed us to conclude that such a stress face plants will deploy a battery of responses, a defense arsenal, through the activation of their detoxification mechanisms to fight, survive and in some cases s’ acclimate to this new setting.

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