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The study of biochemical markers resistance in *Melia azedarach* seedlings under heat stress

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ABSTRACT/RESUME

Abstract: *The present study aims at investigating the thermal stress effect on the physiology of ornamental Melia azedarach seedlings to adjust to high temperatures. The heat stress was carried out through raising temperature degrees of 38 °C, 42 °C and 44 ° C for a period of 3 hours. The results showed that proline and soluble sugar accumulated in response to high temperatures, the highest values have been observed at the roots at 44°C where the experimenter recorded a rate of increase ranging from 976.92% to 987.96% respectively. Soluble proteins content quantity in the leaves increased more than that one in the stems and roots. The highest values were observed at 42°C and represented by a percentage of 56.64%. Activities of the antioxidant enzymes; CAT (catalase) and POD (peroxidase) were significantly increased by heat stress at 38°C. Then, the rate of the activity declined with the increase of the temperature. The results obtained from the process made the situation easier and help to identify the possible applicable stress indicators to the urban growing of the Melia plant in order to avoid the problems of premature aging urban trees vitality.*

I. Introduction

Sustainable development of urban forestry faces problematic threats. However, it is increasingly recognized as a vivid sector of production, development and differentiation in management. Ornamental trees are considered as an essential component of the green urban planning [36, 6]. For further reading about the subject matter, there is little research undertaken in the field of integrated physiology for trees in cities compared to their fruit or forest counterparts. In fact, climate changes induce challenges at all levels. It is estimated that by 2030 an increase of 1.1°C and 1.5°C in 2050 in Maghreb cities could prove constrain [45].

Osmotic stresses like salinity heat and drought are estimated to be the essential abiotic factors responsible for urban trees heterogeneous vitality [46]. *Melia azedarach*, a large tree of the Meliaceae family, is native to India. It is now cultivated in most regions of the world, especially in the tropics [50]. It is commonly used in green areas of our cities. This tree is also categorised as roadside trees. It has attracted much attention from scholars as one of the most versatile medical plants, having a wide spectrum of biological activities [39]. It is reported that this plant possess several medicinal properties [40].

In front of this state, it is worth to wonder about the mechanisms and/or lack of adaptation of certain trees that are widely spread in our cities unlike the *Melia azedarach*. A complex range of physiological and biochemical adaptations have developed, that can adapt and cope with various environmental stresses [53]. As a result gained from this experience and the sample of the *Melia azedarach*, it can be generalized by saying that plants have inducible tolerance mechanisms that expand the temperature range for survival during acute temperature stress [21]. Plants develop, also, stress tolerance mechanisms that involve a complex combination of morphological, physiological and

biochemical factors, such as the accumulation of osmoprotectants[37] and the antioxidant defense system for catalases and peroxidases [18, 42].
A global clarification is required of the mechanism in response to high temperature. This could be an ideal strategy to enhance the plants resistance against heat [3]. Therefore, it is necessary to study the plants species' heat stress mechanism and select out tolerant trees species for afforestation in the cities. Our study aim is to determine the heat stress effect using biochemical markers (proline, soluble sugars, proteins, catalase and peroxidase) on the high temperature adjust ability of *Melia azedarach* young plants.

II. Materials and methods

II.1 Analysis by biochemical markers

II.1.1. Plant material: The experiment was carried out since two years ago on the Persian Lilac plants (*Melia azedarach*) supplied by Djebel al Ouahch nursery sector (Constantine, Algeria). The plants were put under semi-controlled conditions in the culture chamber with a temperature of 24°C (day) and 10°C (night) for 16 hours sunlight.

II.1.2. The applied treatment: Five plants were placed in an oven at the following temperatures 38°C, 42°C and 44°C for a period of 3 hours. After each stress, the extraction of markers (proline, soluble sugars and proteins) was carried out on the different organs (leaves, stems and roots) of *Melia azedarach*. While the extraction of catalases and peroxidases were carried out only on the leaves. The obtained results are compared to the control.

II.1.3.The Extraction of biochemical parameters

II.1.3.1. Proline extraction: The method followed is illustrated in that one of [49]. It involves taking 100 mg of fresh material in test tubes containing 2 ml of methanol 40%, the whole are exposed to heat at 85 °C in a water bath for one hour. After cooling, 1 ml of extract to which must be added: 2 ml of acetic acid, 25 mg of ninhydrin, 1 ml of mixture containing:120ml of distilled water, 300ml of acetic acid, 80 ml of orthophosphoric acid. The whole is boiled at 100 ° C for 30 minutes. After cooling, the addition of 5 ml of toluene and vortex stirring makes it possible to distinguish two phases (an upper phase and a lower phase). After recovery of the upper phase, 5mg of sodium sulphate oxide (Na_2 SO_4) are added to dry the extract and remove the moisture. After 48 hours, the optical density of the samples at a wavelength λ = 528 nm is determined spectrophotometrically.

II.1.3.2. The Extraction of soluble sugar: The extraction of total soluble sugars is determined by the phenol method of [16]. The experiment is illustrated as follows: putting 100 mg of plant material in test tubes, 3 ml of ethanol 80% is added to extract the sugars, and then left at room temperature for 48 hours in the darkness. At the time of dosing, the contents are filtered and 20 ml of distilled water are added. In glass tubes, 1 ml of the solution to be analyzed is added beside 1 ml of 5% phenol and then 5 ml of concentrated sulfuric acid at 1.86 N are rapidly added after that the tubes are placed in a water-bath for 15 to 20 minutes at a temperature of 30 ° C. The reading of the optical density is carried out on a wavelength of 490 nm.

II.1.3.3. The Extraction of soluble proteins: The quantification of soluble proteins is carried out by the method of [9]. It is based on the principle that in acidic medium the proteins form complexes with certain organic dyes, most often the azo dyes with sulfonic acid groups which bind to the groups protonated side chains of basic amino acids (Lysine-Arginine-Histidine) and on the free α -NH2 of the polypeptide chain for a given primary structure. The used dye is Coomassie blue G250, by binding to the protein, which is converted from the bramble form to the blue form. This complex has an absorption maximum at 595 nm. The very sensitive coloration can be carried out very quickly and remains stable for 30 minutes.
*Bradford Reagent Preparation
- Dissolve 100 mg of coomassie blue G250 in 50 cm3 of 95% ethanol;
- add 100 cm3 of phosphoric acid H3PO4 at 85%;
- Make up to 1 dm3 with distilled water;
- Keep in the darkness, at laboratory temperature.
To a volume of protein extract (100 µl), 1 ml of Coomassie blue solution is added. The stain is allowed to develop at least in 5 min and at most in 30 min The whole is then passed to the spectrophotometer to read the absorbance at 595 nm. In each aliquot, the protein concentration, in mg / g MF, is determined by reference to the calibration curve established using various concentrations of a BSA solution ranging from 0 to 250 µg / ml.

II.1.3.4. Antioxidant extraction
The antioxidant enzymes (Peroxidase and catalase) extraction process is realized by taking 0.5 g of a fresh plant (leaves) together with 8 ml of cooled phosphate buffer (pH 7.0, containing 1% (w/v) polyvinylpyrrolidone) and 0.2 g quartz sand , mixing the whole and keeping in test tubes. The homogenate was centrifuged at 15000 xg for 20 min. The supernatant is going to be kept at 4°C and used in assays of enzyme activity.

II.1.3.5. The Peroxidase activity
The specific activity of peroxidase is measured relative to the protocol identified by [11], in their method with some modifications. For guaiacol

peroxidase acivity assay; the reaction mixture (3.0 ml) contained 0.1 M phosphate buffer (pH 6.80), guaiacol (30 mM), H_2O_2 (30 mM) and 0.3 ml enzyme extract. Changes in the absorbance of reaction solution at 470 nm were determined every 20s.

II.1.3.6. The Catalase activity

The activity of catalase is measured using the method of [11]. The catalase reaction solution (3 ml) contained 50 Mm phosphate buffer (pH 7.0), 15 Mm H_2O_2 and 0.1 ml enzyme extract. The reaction was initiated by adding enzyme extract. Changes in the absorbance of the reaction solution at 240 nm has been red every 20s. One unit catalase activity was defined as an absorbance change of 0.01 unit/ min.

The results are subjected to that one of ANOVA and Newman-Keuls multiple range tests way. The averages were performed as compare means to determine differences between existed parameters in three different elevations of temperature.

III. Results and discussion

III.1. The accumulation of osmolytes

As can be seen in figure 1, there is a clearly observed correspondence between the accumulation of osmolytes (proline, soluble sugar) and temperature increases. The results obtained indicate that a change in proline and soluble sugar content occurred in the different organs; the highest concentrations were recorded at the root level at 44°C with a rate of increase ranging from 976.92% to 987.96% from one side. From the other side, a significant accumulation of protein was recorded at 42°C, it was as follows: 56.64% in the leaves, 21.80% in the stems and finally 17.2% in the roots comparing to the control; however, this accumulation declined at 44 ° C, but remained above the control.

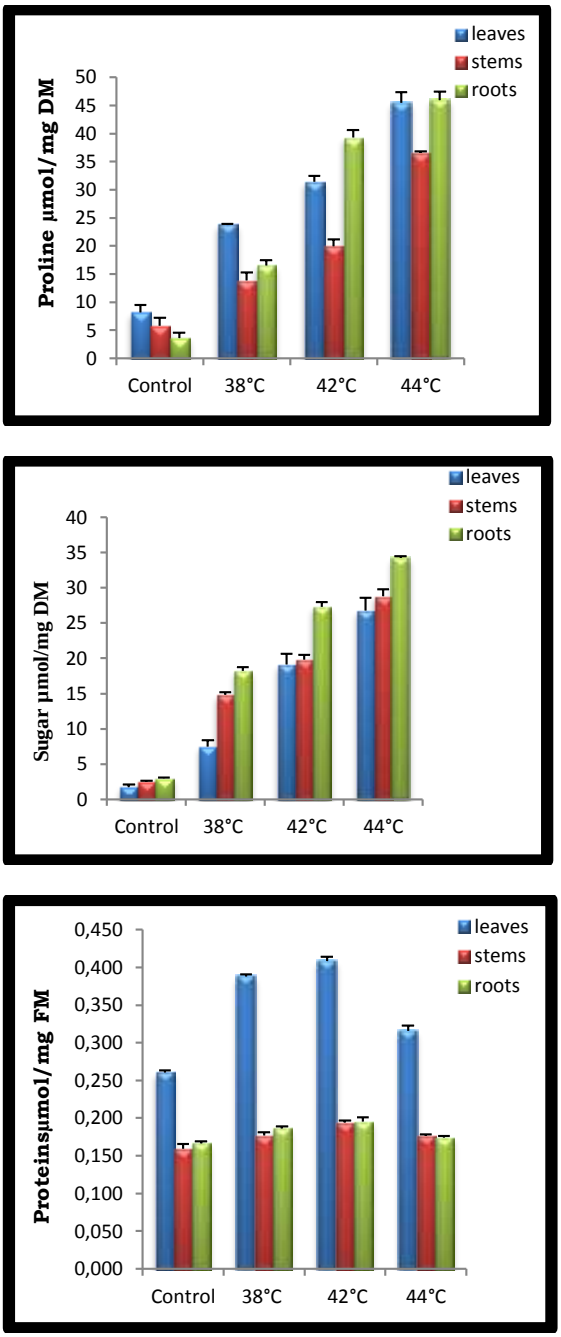

Figure 1. *Variation in proline, soluble sugar and protein contents in the different organs of Melia azedarach under heat treatment at high temperatures*

Statistical analysis of the results obtained from proline soluble sugar and protein by the Newman and Keuls test illustrated in
Table 1 Show that proline and soluble sugar reveal four homogeneous groups; the first group (A) corresponds to the temperature 44°C with the highest average, the second group (B) corresponds to the temperature 42°C, the third group (C) corresponds to the temperature 38°C, and the last group (D) includes the witness that comprises the lowest content. Indeed, for the protein content, the same test reveals the existence of 2 homogeneous groups, the first group (A) corresponds to temperatures 42°C and 38°C. The second group (B) corresponds to the temperature 44°C and the witness with the lowest average.

Table 1. *Homogeneous groups estimated of proline, soluble sugars, and protein averages according to the Newman and Keuls test at 5% in Melia azedarach under heat treatment at high temperatures*

Temperatures	Proline (µmol/mgDM)	Sugar (µmol/mg DM)	Protein (µmol/mg DM)
Control	05,87 (**D)**	02,43 (**D)**	0,195(**B)**
38°C	18,08 (**C)**	13,49 (**C)**	0,250(**A)**
42°C	32,96 (**B)**	22,73 (**B)**	0,259(**A)**
44°C	43,94 (**A)**	30,38(**A)**	0,221(**B)**

Proline and soluble sugar known to accumulate in plants under different environmental stress conditions [38,48]. In our research, these two metabolites show a significant increase in the different organs in *Melia azedarach* seedlings under heat stress conditions. The strong accumulations are obtained at roots in relation to leaves and stems. These results corroborate those of [54] and [58] which show that both starch and soluble sugar are more prevalent in roots than in stems of the vine and confirm the predominant role of the roots as a carbohydrate storage organ. In fact, [23] study approve that heat stress induces the accumulation of proline and soluble sugar as well as an increase in the percentage of plasma membrane damage in two varieties of durum wheat.
Some researchers interested in such a field of study and investigations [5] claim that the accumulation of osmoprotectants is one of the adaptive strategies stimulated by the plant in response to the environmental constraints.
Some others assume that the stress tolerance level of plants could be related to the quantities of proline accumulated [13], such as the durum wheat variety (Korifla) which is classified tolerant on the basis of proline, soluble sugars, and the resumption of leaf elongation [23].
Several studies made by researchers report protein accumulation due to heat stress [22,34, 26, 47]. Our results show a strong accumulation in *Melia azedarach* leaves at 42°C. This increase in protein levels is due to the acceleration in the synthesis of specific proteins in association with heat stress such as heat shock proteins (HSPs) [24;8; 14]. Recently, [53] isolated heat shock protein genes, *CsHSP17.2* (GenBank accession number: KU244518) from the tea plant and demonstrated highly induced by heat stress. These proteins and ROS-scavenging enzymes are two crucial functional proteins that are activated by heat stress and genes of heat stress-responsive transcription factors (TFs) [27,31]. Thus, the change in protein expression is one of the results of metabolic modification during abiotic stress in plants [25, 51].
Indeed, the thylakoid membrane is a relatively liquid system. It is very vulnerable to high temperatures, because they induce changes in lipid-protein interactions causing protein denaturation [32].Chaperones and heat shock proteins (HSPs) playcrucial roles in protecting plants from stress by restituting normal protein conformation and thus cellular homeostasis [54, 52], so intracellular proteases play an important role in the degradation of damaged or unnecessary proteins, they maintain the protein and membrane structures of the plant cell [20].

III.2. Change in peroxidase and catalase content and their specific activities

The change of peroxidase and catalase content in *Melia azedarach* leaves was illustrated in figure 2; the highest concentration values of these two enzymes were recorded at 38°C with rates of increase ranging from 109.12% to 93.32% respectively. These concentrations decrease as the temperature increases, the lowest values were reported at 44°C with a reduction rate of 42.85% for peroxidase and 26.66% for catalase.
However, the specific activity of peroxidase was significantly elevated in *Melia azedarach* leaves under differential heat shock treatment (38°C and 42°C). The highest values were recorded at 38°C with 50.92%. After that, the rate of the activity decreased at 44°C with a reduction of 49.69%.
In fact, the values obtained from the specific activity of catalase also show a significant increase at 38°C with 167.85%, and then, this latter was decreased comparing to the control at the following

temperatures 42°C and 44°C with a reduction rate of 41.17%, 35.43% respectively.

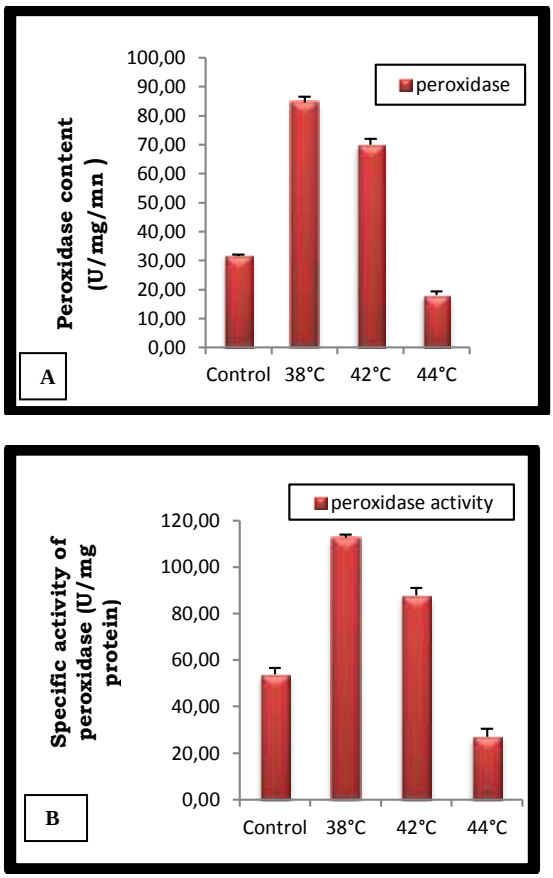

Figure 2. *Catalases and peroxidase content and theirs specific activities in Melia azedarach leaves subjected to high temperatures.*

Statistical analysis of the results obtained from the catalase and peroxidase contents and their specific activities by the Newman and Keuls test have shown four homogeneous groups: the first group (A) corresponds to the temperature (38°C) with the highest average, and the last group (D) includes the lowest content with temperature of 44°C.

Table 2. *Homogeneous groups of catalases and peroxidase content (U/mg /min) and theirs specific activities (U/mg protein) according to the Newman and Keuls test at 5% in Melia azedarach subjected to high temperatures.*

Modality	**Catalase content (U/mg/mn)**	**Specific activity of catalase (U/mg protein)**	**Peroxidase content (U/mg/mn)**	**Specific activity of peroxidase (U/mg protein)**
Control	25,00 **(B)**	42,64**(B)**	31,58 **(C)**	53,86 **(C)**
38°C	48,33 **(A)**	64,35**(A)**	84,59 **(A)**	112,63**(A)**
42°C	20,00 **(C)**	25,08**(D)**	69,82 **(B)**	87,70 **(B)**
44°C	18,33 **(D)**	27,53**(C)**	18,05 **(D)**	27,09 **(D)**

ROS (reactive oxygen species) are produced in a controlled manner under optimal conditions; they have important functions such as cell wall biosynthesis and Redox signaling [18], but they are generated under different stresses, including heat stress. High concentrations of ROS induce oxidative stress and damage proteins, carbohydrates, fats, and DNA [30, 19, 29]. Exposure to heat stress, the antioxidant system composed of low molecular weight antioxidants, such as superoxide dismutases (SOD), catalase (CAT), and peroxidase (POD) could be activated to protect the plant from high-temperature damage and eliminated ROS [2, 30,4,12]. The combined action of the system (SOD / CAT / POD) converts the potentially dangerous superoxide radical (O_2-) and hydrogen peroxide (H_2O_2) into water and oxygen, thus preventing cellular damage [28, 18, 41].
However, a similar increase in the peroxidase activity was also observed in other studies on cold stress [15], and drought [55, 43]. Under environmental stress, the plants feel like to produce the antioxidant enzymes as a defense mechanism [7], this increase is supposed to be an adaptive trait that can help overcome damage to tissue metabolism by reducing toxic levels of H_2O_2 produced during cellular metabolism and protecting against oxidative stress [46].

In this study, catalase activity was significantly elevated in *Melia azedarach* leaves under heat shock treatment at 38°C. Similar results were also appeared in some other studies made on peach trees under salicylic acid stress [35], and ornamental *Amsonia orientalis* under ultraviolet-C irradiation [1]. Indeed, catalase is an oxidoreductase located in peroxisomes, it is considered an important enzyme to counter hydrogen peroxide (H_2O_2) under stress conditions, and it reacts directly with hydrogen peroxide to form water and oxygen [55, 44, 45].
In addition, a catalase molecule can convert ≈ 6 million molecules from H_2O_2 to H_2O and O_2 per minute [19].

However, a decrease in catalase activity in the leaves of *Melia azedarach* was observed under 42°C and 44°C treatments for 3 hours. A similar decline in catalase activity has also been reported in subjects suffering from abiotic stresses, such as heat stress in *Pinus pinea* [49], drought stress in *Zea mays* and *Helianthus annuus* [56,33]. The decrease in catalase activity could indicate its inactivation by accumulated hydrogen peroxide, induced by thermal stress [10] and could be explained in part by the inactivation of the photo enzyme. Under irradiation, catalase inactivation occurs permanently and promoted by light absorption by the heme group bound to an enzyme [17].

IV. Conclusion

The evaluation of biochemical markers (proline, soluble sugar, protein) and antioxidant-enzyme (catalase and peroxidase activities) measured in leaves of *Melia azedarach* seedlings showed significant accumulations of osmolytes and increase of antioxidant activity values under heat stress. All the results show that *Melia azedarach* deduce a tolerance or adaptation subjected to heat stress. A classification grid for urban plantations can, thus be considered and could eventually serve as a decision-making aid to better guide about the choice of species with compensation potential to avoid urban heat stress.

V. Acknowledgments

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