Effet d'un prétraitement par une alternance de cycles courts lumière/obscurité sur la tolérance du tabac (*Nicotiana tabacum* L.) vis-à-vis du clomazone

Effet d'un prétraitement par une alternance de cycles courts de lumière/obscurité sur la tolérance du tabac (*Nicotiana tabacum* L.) vis-à-vis du clomazone

Ce chapitre est divisé à deux parties:

La première partie est présentée sous la forme d'un article publié dans *Journal of Photochemistry and Photobiology B: Biology* (Darwish et al., 2014).

La deuxième partie est présentée sous la forme d'un article soumis à *Journal of Photochemistry and Photobiology B: Biology*.

Publication 3

Pretreatment with Alternation of Light/Dark Periods Improves the Tolerance of Tobacco (*Nicotiana tabacum* L.) to Clomazone Herbicide

M. Darwish, F. Lopez-Lauri, M. El Maataoui, L. Urban, H. Sallanon, Pretreatment with Alternation of Light/Dark Periods Improves the Tolerance of Tobacco (*Nicotiana tabacum*) to Clomazone Herbicide, *Journal of Photochemistry and Photobiology B: Biology* 134 (2014) 49-56

Dans le chapitre précédent, il a été montré que le clomazone induit un stress photooxydatif chez la variété la plus sensible *Virginie*.

Des donnés bibliographiques ont décrit des effets stimulant la croissance et les mécanismes antioxydants chez des algues lors d'application de lumière intermittente (Sharkey et al., 1986; Nedbal et al., 1996; Merchuk et al., 1998).

Dans ce travail nous avons appliqué à la variété *Virginie* un prétraitement de 3 jours pendant lesquels la durée de l'alternance lumière/obscurité était de 16 min/ 8 min, une semaine avant le traitement avec l'herbicide.

Comme dans les chapitres précédents, ont été suivi les teneurs en pigments, l'efficacité du PSII *via* les paramètres de la fluorescence chlorophyllienne et le test *OJIP*, les teneurs en H_2O_2 et les activités antioxydantes des enzymes du cycle Asada-Halliwell.

Expérimentation

La variété de tabac *Nicotiana tabacum* L. *Virginie vk 51* a été utilisée. Une partie des plantules au stade trois feuilles a été exposée à une alternance lumière/obscurité de 16 min/8 min sous une intensité lumineuse de 50 µmol photons m^{-2} s^{-1} pendant 3 jours. L'autre partie des plantules est cultivée sous une alternance lumière/obscurité de 16 h/ 8h. Après une semaine de croissance à 22 °C le jour et 17 °C la nuit, avec une humidité relative de 60 %, des cycles jour/nuit de 16 h/8 h et une intensité lumineuse de 50 µmol photons m^{-2} s^{-1}, les plantules ont été traitées avec 1 µM de clomazone pendant 14 jours.

Principaux résultats

Le prétraitement par l'alternance de cycles courts de lumière/obscurité a amélioré la tolérance des plantules de tabac face au stress photooxydatif induit par le clomazone. Ce prétraitement a

augmenté la surface foliaire de la plante, l'efficience photochimique maximale du PSII (F_v/F_m), l'efficacité réelle du PSII (Φ_{PSII}), l'indice de performance (PI_{abs}), le flux d'électrons au-delà de Q_A ($1–V_J$), et a également diminué la dissipation d'énergie sous forme de chaleur (DI0/RC).

De plus, ce prétraitement a conduit à une diminution de l'accumulation d'H_2O_2 et une augmentation de l'activité des enzymes de détoxification (APX, MDHAR, SOD, GR et DHAR).

Conclusion

Le prétraitement par l'alternance de cycles courts de lumière et d'obscurité améliore la tolérance des plantules de *Nicotiana tabacum* L. *Virginie vk 51* au clomazone. Cette amélioration semble provenir de la stimulation de l'activité des enzymes antioxydante dans le chloroplaste qui protège l'appareil photosynthétique des effets directs des ERO et élimine des électrons en excès dans la chaîne de transport.

Journal of Photochemistry and Photobiology B: Biology 134 (2014) 49–56

Contents lists available at ScienceDirect

Journal of Photochemistry and Photobiology B: Biology

journal homepage: www.elsevier.com/locate/jphotobiol

Pretreatment with alternation of light/dark periods improves the tolerance of tobacco (*Nicotiana tabacum*) to clomazone herbicide

Majd Darwish *, Félicie Lopez-Lauri, Mohamed El Maataoui, Laurent Urban, Huguette Sallanon

EA 4279, Laboratoire de Physiologie des Fruits et Légumes, Université d'Avignon et des pays de Vaucluse, 301, rue Baruch de Spinoza, BP 21239, 84916 Avignon cedex 9, France

ABSTRACT

This work analyses the effects of alternation of light/dark periods pretreatment (AL) in tobacco plantlets (*Nicotiana tabacum* L. *cv.Virginie vk51*) growing in solution with low concentration of the clomazone herbicide. The experimentation has been carried out by exposing the plantlets to successive and regulated periods of light (16min light/8min dark cycles, PAR 50 μmol m^{-2} s^{-1}) for three days. The photosynthesis efficiency was determined by mean of the chlorophyll fluorescence and JIP-test. The AL pretreatment improved the clomazone tolerance; this has been observed by the increase in the leaf area of the plant, the maximal photochemical quantum efficiency of PSII (F_v/F_m), the actual PSII efficiency (ΦPSII), the performance index (PI_{abs}), the electron flux beyond Quinone A ($1–V_J$), and also by the diminution of the energy dissipating into heat (DI0/RC). Furthermore, AL pretreatment led to low accumulation of hydrogen peroxide (H_2O_2) which proves that the scavenging enzymatic system have been activated before clomazone treatment. In the plantlets pretreated with AL, with regard to the ascorbate content, some of antioxidant enzyme whose function is associated with it have continued to scavenge reactive oxygen species (ROS) induced by clomazone, such as ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR). So, the observed photooxidative damages induced by clomazone herbicide were noticeably reduced.

Keywords: Light**;** Chlorophyll fluorescence**;** JIP-test**;** Photosynthesis**;** Photosystem II**;** *Nicotiana tabacum***;** Tolerance

Abbreviations: ABS absorption flux.- APX ascorbate peroxidase.- AsA ascorbate.- Chls chlorophyll (a+b).- DI0 dissipation flux.- DHA dehydroascorbate.- DHAR dehydroascorbate reductase.- ET0 electron Transport flux.- F_0, F_v, F_m minimal, variable, maximal chlorophyll fluorescence.- F_v/F_m maximal photochemical efficiency of PSII.- GR glutathione reductase.- H_2O_2 hydrogen peroxide.- LHCs light harvesting complex of photosystem I (II).- MDHAR monodehydroascorbate reductase.- $^1O_2$ singlet oxygen.- PAR photosynthetic active radiance.- PI_{abs} performance index.- PSI photosystem I.- PSII photosystem II.- ΦPSII actual PSII efficiency.- Q_A Quinone A, primary quinone electron acceptor of PSII.- *q*P photochemical quenching.- RC reaction center.- ROS reactive oxygen species.- SOD superoxide dismutase.- TR0 energy flux trapping.

1. Introduction

The photosynthetic reactions in higher plants are piloted through the cooperation of the two photosystems, PSI and PSII [1,2]. Light energy is collected by the antenna of PSII and PSI (LHCs) and is channeled to the reaction centers (RC). In most of the cases, where plants are exposed to herbicides, the electron transport will be blocked and the Q_A will stay in a reduced state, thus promoting the formation of reactive oxygen species, such as singlet oxygen ($^1O_2$), *via* the chlorophyll triplet $^3Ch^*$ [3,4]. $^1O_2$ is an active form of the reactive oxygen species (ROS); it can provoke to not only the destruction of plastidial structure, but also the formation of the other ROS, such as superoxide (O_2^{-}), peroxide hydrogen (H_2O_2) and hydroxyl radical (OH^{-}). However, $^1O_2$ seem to be the major reactive oxygen species involved in photooxidative damage to plant [5]. The herbicide clomazone belongs to the class isoxazolidinone [2-(2-chlorobenzyl)-4,4-dimethyl-1,2-oxazolidin-3-one]. It is a pre-emergence herbicide, widely used in agriculture for the control of annual weeds in soybean, cotton, sugar cane, maize, rice and tobacco [6]. Clomazone treatment can lead to decrease in the antenna pigments of the photosynthetic apparatus; consequently, it leads to photooxidative stress which can inhibit the PSII [7].

It is important to know that the variation of intensity and duration of light may produce modulation of the response of plants to biotic and abiotic stress, this in turn can be explained by the accumulation of ROS [8-10]. These ROS are thought to be one of the primary causes of the irreversible loss of photosynthetic activity, which is observed in the plant leaves exposed to light in combination with the environmental stresses [11-13]. However, plants have developed numerous mechanisms to protect the photosynthetic apparatus against photooxidative damages. This involves modifications in the amount of the soluble enzymes, the components of the electrons-transport, and the pigment-protein complexes which are effective mechanisms to match the available amount of light with the very capacity of the plant itself to absorb it for carbohydrate synthesis [14]. Moreover, antioxidant systems are considered as another mechanism by which the photooxidative damage induced by the herbicides is considerably reduced. Indeed, plants have two antioxidant defense systems: enzymatic and non-enzymatic scavenging systems. Enzymatic antioxidants system includes superoxide dismutase (SOD) and catalase (CAT), and the enzymes belonging to Ascorbate-glutathione cycle such as ascorbate peroxidase (APX), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR), whereas non-enzymatic antioxidant system includes some compounds such as ascorbic acid (AsA), glutathione (GSH), α-tocopherol, and

carotenoids [9,15,16]. SOD activity can transmute O_2^- into H_2O_2 [17]. The H_2O_2 generated by the SOD activity is scavenged by APX; this enzyme is also involved in the oxidation of ascorbate to monodehydroascorbate (MDHA), which itself can be converted back to ascorbate *via* the MDHAR. In addition, MDHA can be converted rapidly to dehydroascorbate (DHA) that is converted back to ascorbate by DHAR [9]. Then, the ascorbate-glutathione cycle needs the regeneration of the AsA that relies on GR (a key enzyme in GSH regeneration cycle) [9]. CAT is also a main enzyme used to eliminate the H_2O_2 in the peroxisome [18]. Ascorbate (AsA) is a major antioxidant in plants scavenging the reactive oxygen species [19].

The alternation of light/dark periods has been suggested beneficial to the photosynthetic efficiency by reducing photoinhibitory damage [20-22]. In a recent study, Gergoff Grozeff et al [23] have demonstrated that short light pulses can improve the antioxidant capacity in post-harvested leaves of spinach. More, Kreslavski et al [24] showed that red light preillumination increased the antioxidant enzymes, by consequent the resistance of the Arabidopsis photosynthetic apparatus to the oxidative stress caused by UV irradiation. In our work presented here, we will suppose that the pretreatment with the alternation of light/dark periods can moderate the photosynthetic efficiency and protect the plants from the clomazone stress.

In the present study, tobacco plantlets were pretreated with repetition of light/dark cycles for a limited and regulated periods, and then they were grown in the same light intensity before applying the herbicide. The objective was to investigate whether or not the pretreatment with the alternation of light/dark periods is involved in the protection of tobacco plantlets against the observed photooxidative stress provoked by clomazone; special attention has been paid to modifications in the growth parameters, photosynthetic pigments, H_2O_2, ascorbate content, antioxidant activities, parameters of chlorophyll fluorescence JIP-test as a quick and effective method to evaluate the photosynthetic activity.

2. Materials and Methods

2.1. Plant material, growth conditions and chemical treatments

Tobacco (*Nicotiana tabacum* L. *cv.Virginie vk51*) seeds were germinated in a plastic container with sterilized potting soil for two weeks at 22/17 °C, 16h/8h of light/dark cycle and flux of photons (PAR) 50 µmol m^{-2} s^{-1}. Germination was carried out in sterile conditions and in compliance with the growth chamber conditions, according to Darwish et al. [7]. After 5 weeks, plantlets (three leaves stage) were exposed or not to an alternation of light/dark periods (AL) (16min/8min of light/dark cycle, PAR 50 µmol m^{-2} s^{-1}) in a

growth chamber for three days; the other part remained without an AL to be used as a control. Plantlets were transferred into a hydroponic system containing the nutrient solution of Auckland (1 M KNO_3; 1 M $Ca(NO_3)_2.H_2O$; 1 M $MgSO_4.7H_2O$; 1 M KH_2PO_4; 0.01 M FeEDDHA) in the same environmental conditions of germination phase. Then, clomazone (CL) (Sigma-Aldrich, USA) was added to treated plantlets at the concentrations of 1 µM for 14 days, (Fig. A). The solutions of such a treatments were refreshed twice a week. After 14 days, growth and fluorescence parameters are determined in leaves of the four treatments ((1) Con (Control, without alternation of light/dark periods or clomazone); (2) CL (1 µM clomazone); (3) AL (alternation of light/dark periods); (4) AL+CL (alternation of light/dark periods+ 1 µM clomazone)). Then, leaves are harvested and stored at -80°C for analysis.

2.2. Determination of growth parameters

Fourteen days after the beginning of CL treatment, plant growth was determined by several parameters: leaf area, plant height, length and the fresh weight of the leaves and roots. The leaf area was measured by using image J software.

2.3. Determination of photosynthetic pigments

Total chlorophyll and carotenoids were determined according to Lichtenthaler [25] using pure acetone as extraction solvent. Absorbance at 662, 645 and 470 nm was measured immediately after extraction. Chlorophyll and carotenoids contents were calculated according to Lichtenthaler [25].

2.4. Determination of H_2O_2 content

The hydrogen peroxide levels were determined as described by Murshed et al [26] with some modifications: 250 mg of sample was homogenized in 1 ml 0.1% trichloracetic acid (TCA). The homogenate was centrifuged at 12,000 × *g* for 15 min at 4 °C. Aliquots of 100 µl from each tube were placed in 96-well plates and 50 µl of 10 mM potassium phosphate buffer (pH 7.0) and 100 µl of 1 M KI were added in each well. Control sample were made with water instead of KI to remove the color background of extract at 390 nm. Each plate also contained commercial H_2O_2 to generate a standard curve. Plate was briefly vortexed and the absorbance

readings were taken at 390 nm in a micro-plate reader. The concentration of H_2O_2 was given on a standard curve.

2.5. Determination of the ascorbate content

The assay of the total and reduced AsA contents were carried out on a material stored at -80 °C according to Murshed et al [27] with some modifications. Briefly, 100 mg of tobacco leaf powder was homogenized with 1 ml of ice cold 6% trichloracetic acid (TCA). Samples were centrifuged at 15,000 × *g* for 10 min at 4 °C and the 10 µl of supernatant used in each assay. The total AsA (addition of 20 µM of dithiothreitol (DTT)) and reduced AsA (without (DTT)) were measured on each sample. 10 µl of each sample or standard were distributed into two wells (for three repetitions) of a 96-well microplate and mixed with 10 µl of 20 mM DTT (total AsA assay) or 0.2 M phosphate buffer (pH 7.4) (reduced AsA assay). The plate was incubated at 42 °C for 15 min. Then, 10 µl of *N*-ethyl maleimide (NEM) (total AsA assay) or 0.2 M phosphate buffer (pH 7.4) (reduced AsA assay) were added and mixed, followed by 150 µl of colour reagent (containing H_3PO_4 ; $FeCl_3$ and dipyridil). After incubation at 42 °C for 40 min, the plate was briefly vortexed and the absorbance readings were taken at 525 nm in a micro-plate reader (Power Wave, HT microplate spectrophotometer, BioTek, France). The AsA concentration of the samples was calculated from a standard curve.

2.6. Anti-oxidative enzymes activities:

Extraction of enzymes:

Protein extraction was performed according to Murshed et al [28]. Frozen leaf powder (150 mg) was homogenized in 1 ml of 50 mM MES/KOH buffer (pH 6.0), containing: 40 mM KCl, 2 mM $CaCl_2$, and 1 mM AsA. Solutions were centrifuged at 15,000 × *g* for 15 min at 4 °C, and the supernatants were analyzed immediately for measuring enzyme activities.

Enzymes assays:

All enzymes assays are performed in a final volume of 0.2 ml volume kinetic reactions at 25 °C, using a micro-plate reader (Power Wave, HT microplate spectrophotometer, BioTek, France). APX, DHAR, MDHAR and GR activities were measured by the method of Murshed et al [28]. APX activity was determined spectrophotometrically following the decrease in absorbance at 290 nm (ε 290 = 2.8 $mM^{-1}cm^{-1}$) due to ascorbate oxidation. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 0.25 mM AsA, 5 µl extract and 5

mM H_2O_2. The reaction was started by adding H_2O_2 (200 mM). DHAR was assayed in a reaction mixture consisting of 50 mM HEPES buffer (pH 7.0), 0.1 mM EDTA, 2.5 mM GSH, 0.2 mM DHA and 5 µl extract; activity was determined by measuring the increase in the reaction rate at A265 and was calculated from the 14.0 mM^{-1} extraction coefficient. MDHAR was determined in a reaction mixture consisting of 100 mM HEPES buffer (pH 7.6), 2.5 mM AsA, 0.25 mM NADH, 5 µl extract and 0.4 units ascorbate oxidase. Activity was determined by measuring the decrease in reaction rate at A340 and calculated from the 6.22 mM^{-1} extraction coefficient. GR activity was assayed in a reaction mixture consisting of 50 mM HEPES buffer (pH 8.0), 0.5 mM EDTA, 0.25 mM NADPH, 5 µl extract and 0.5 mM GSSG. Activity was determined by measuring the decrease in the reaction rate at 340 nm and was calculated from the coefficient 6.22 $mM^{-1}cm^{-1}$. CAT activity was measured according to Chance and Maehly [29] in a reaction mixture containing 50 mM KH_2PO_4 buffer (pH 7.0), 15 mM H_2O_2 and 5 µl extract to initiate the reaction. Activity is described in terms of the change in absorbance at 240 nm as the H_2O_2 was lowered. SOD activity was determined using the modified method of Dhindsa et al [30]. SOD activity was assayed in a 0.1 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75 µM nitro blue tetrazolium (NBT), 0.1 mM EDTA, 10 µM enzyme extract, and 2 µM riboflavin. Absorbance at 560 nm was measured immediately, after 5 min illumination, with a light intensity of 1 µmol. m^{-1} s^{-1} at 25 °C. One unit of SOD was defined as the amount of enzyme required to inhibit the NBT photoreduction by 50%.

2.7. Determination of the chlorophyll fluorescence transients

The behavior of the photosystem II (PSII) can be evaluated using the rapid kinetics of the fluorescence of chlorophyll *a* emitted by the leaves of plants adapted to darkness and light with a light saturation. Before any measurement takes place, leaves were dark-adapted for 20 min. The polyphasic chlorophyll *a* fluorescence (expressed in relative units) was measured using a portable Handy-PEA (Hansatech, Kings Lynn, UK). The fluorescence of leaves was determined after an illumination with a light intensity of 3,000 µmol photons m^{-1} s^{-1}. The fluorescence parameters were calculated automatically $F_v/F_m = (F_m – F_0)/F_m$, which represents the maximal quantum photochemical yield of PSII [31] and the quantum yield of the open centers of PSII [32]. The JIP-test is based on the energy flux theory in bio membranes developed by Strasser et al [33]. This model describes how the flux of photons absorbed by the photosynthetic pigments antenna ABS is dissipated as heat (DI) and fluores-cence or

transported as the trapped flux (TR) of the reaction centers to be converted into energy redox by reducing plastoquinone Q_A to Q_A^-. Then Q_A^- is reoxidized to Q_A, and therefore the creation of an electron transport (ET) ensues which leads to CO_2 fixation [34]. These fluxes are described in terms of a specific energy flux (per RC) or as proportions of other fluxes (ratios or yields). The calculation of the energy flux and ratios of a specific flux was fully explained by Strasser and his coworkers [35-37]. The actual PSII efficiency ΦPSII= $(F_m'–F_s)/F_m'$ and photochemical quenching coefficient *q*P= $(F_m'–F_s)/(F_m'–F_0')$ [37] were measured using a pulse-modulated fluorimeter system (FMS1, Hansatech, Kings Lynn, UK); the steady-state fluorescence level F_s and the maximum chlorophyll fluorescence level F_m' were measured using an actinic light 500 µmol photons m^{-2} s^{-1} and a saturating light pulses of 6,000 µmol photons m^{-2} s^{-1} with 1 s duration, respectively according to Pompelli et al [38]. The actinic light was removed and the minimal fluorescence in the light-adapted state (F_0') was determined.

2.8. Statistical Analysis

Statistical analysis was performed with R statistical software using an ANNOVA with Tukey test. Results were displayed by means of 8 replicates ± SE. Results were considered significant at the $P<0.05$.

Fig. A. The experimental protocol showing the preparation of tobacco plantlets and chemical treatment by herbicide clomazone

3. Results

3.1. Plant growth

The negative effect of clomazone on the fresh weight of the roots and plants was shown by a significant decrease about 50% and 38% as compared to the control, respectively. In contrast, the fresh weight of the roots and plants was significantly increased in plantlets of the AL+CL treatment. Moreover, the AL pretreatment enhanced the growth of plantlets in AL+CL treatment to the extent that its fresh weight has increased about (32%) as compared to the control (Table 1).

Table. 1. Measure of fresh weight, length and plant leaf area of tobacco plantlets grown under alternation of light/dark periods and clomazone treatments. **Con** without alternating of light/dark periods, or clomazone**; CL** treatment with 1 µM clomazone**; AL** alternation of light/dark periods**; AL+CL** pretreatment of alternation of light/dark periods+ 1 µM clomazone treatment. Different letters denote significant differences for each parameter between means within each traitment (P<0.05, ANNOVA-Tukey test). Data are expressed as means ±SE, n= 8.

	Fresh weight (g)		Length (cm)		
Treatment	**root**	**plant**	**root**	**plant**	**plant leaf area (cm^2)**
Con	0,40 ± 0,04 b	1,4 ± 0,17 b	10,6 ± 0,7 a	1,9 ± 0,2 a	79 ± 10 a
CL	0,20 ± 0,04 c	0,8 ± 0,19 c	8,6 ± 0,9 b	1,4 ± 0,1 b	50 ± 9 b
AL	0,57 ± 0,09 a	1,6 ± 0,21 b	9,5 ± 0,4 ab	1,9 ± 0,2 a	81 ± 9 a
AL+CL	0,44 ± 0,07 b	2 ± 0,20 a	10,6 ± 0,8 a	1,9 ± 0,2 a	94 ± 6 a

The roots and the plantlets length in the CL treatment has already decreased as compared to the control (19% ; 28%), respectively. However, the AL pretreatment was able to regulate the plant growth with no negative effects of clomazone to be found on roots or plantlets length (Table 1). Similarly, the leaf area of plantlets has considerably increased in the AL+CL treatment as compared to CL treatment (47%) (Table 1).

3.2. Photosynthetic pigments (chlorophyll and carotenoids)

Clomazone reduced the total chlorophyll content in the plantlets about (22%) as compared to the control. The plantlets of AL+CL treatment showed an increase in the total chlorophyll which was about (40%) as compared to CL treatment alone (Table 2).

The level of carotenoids was also reduced in the CL treatment. However, the herbicide had no effect on the carotenoids content in the case of plantlets pretreated with AL (Table 2).

Moreover, a significant increase in Chl *a/b* ratio was found in the CL treatment as compared to the control. However, this increase was removed in AL+CL, which had no significant difference as compared to the control (Table 2).

Table. 2. Chlorophyll (**Chl *a***, **Chl *b***, **Chl *a+b***) and carotenoides (**Car**) contents per fresh weight in the control (**Con**), clomazone (**CL**), alternation of light/dark periods (**AL**) and alternation of light/dark periods+ clomazone (**AL+CL**) leaves. Different letters denote significant differences for each parameter between means within each traitment (P<0.05, ANNOVA-Tukey test). Data are expressed as means ±SE, n= 8.

Treatment	Chl *a* (µg. g^{-1} FW)	Chl *b* (µg. g^{-1} FW)	Chl *a+b* (µg. g^{-1} FW)	Chl *a/b*	Car (µg. g^{-1} FW)
Con	586 ± 28 a	375 ± 42 b	962 ± 52 b	1,71 ± 0,18 b	145 ± 7 a
CL	509 ± 13 b	242 ± 20 c	750 ± 32 c	2,18 ± 0,12 a	120 ± 6 b
AL	602 ± 33 a	535 ± 39 a	1136 ± 51 a	1,30 ± 0,13 c	152 ± 10 a
AL+CL	581 ± 27 a	476 ± 62 ab	1157 ± 51 ab	1,43 ± 0,24 bc	142 ± 11 a

3.3. H_2O_2 and antioxidant systems

H_2O_2 content (Fig. 1A) increased in the tobacco plantlets treated with clomazone as compared to the control, AL pretreatment was also able to protect the tobacco plantlets from the large accumulation of H_2O_2. Similarly, AL+CL treatment has shown a high decrease in the H_2O_2 content (61%) as compared to the CL treatment.

The total ascorbate (Fig. 1B) decreased in the CL treatment as compared to the control: about 19%, while in the AL and AL+CL treatments, the total ascorbate increased about (16%; 26%) as compared to the control, respectively. This has been observed by the higher content of oxidized ascorbate (DHA) with the AL pretreatment, and the reduced (AsA) and oxidized (DHA) ascorbate with the AL+CL treatment.

In the CL treated leaves, the activities of the DHAR (Fig. 2B), MDHAR (Fig. 2C) and GR (Fig. 2D) were significantly decreased (P<0.05) in comparison with the control, while the activities of APX (Fig. 2A) and CAT (Fig. 2E) seem to have had no difference. The SOD (Fig. 2F) activity was significantly higher than the one found in the control.

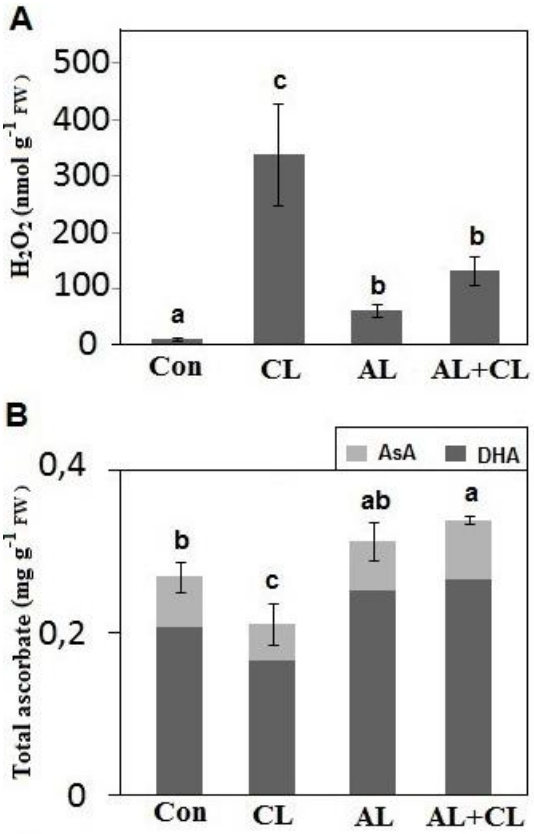


Fig. 1. Effects of clomazone and alternation of light/dark periods on $\mathbf{H_2O_2}$ content and total ascorbate in tobacco (*Nicotiana tabacum* L. *cv.Virgenie vk51)*. **Con** without alternating of light/dark periods, or clomazone**; CL** treatment with 1 µM clomazone**; AL** alternation of light/dark periods**; AL+CL** pretreatment of alternation of light/dark periods+ 1 µM clomazone treatment. All data represent means, SE for n= 8 and different letters to determine the significant difference between means within each treatment (P<0.05, ANNOVA-Tukey test).

In the AL+CL treatment, the APX activity has shown a significant increase as compared to the Con and CL treatments. Moreover, the activities of DHAR, MDHAR and GR were enhanced in this treatment as compared to the CL treatment.

AL treatment seems to have improved the APX, MDHAR and CAT activities as compared to the control, while the activities of DHAR, GR and SOD prove to have had no significant differences between the AL and Con treatments.

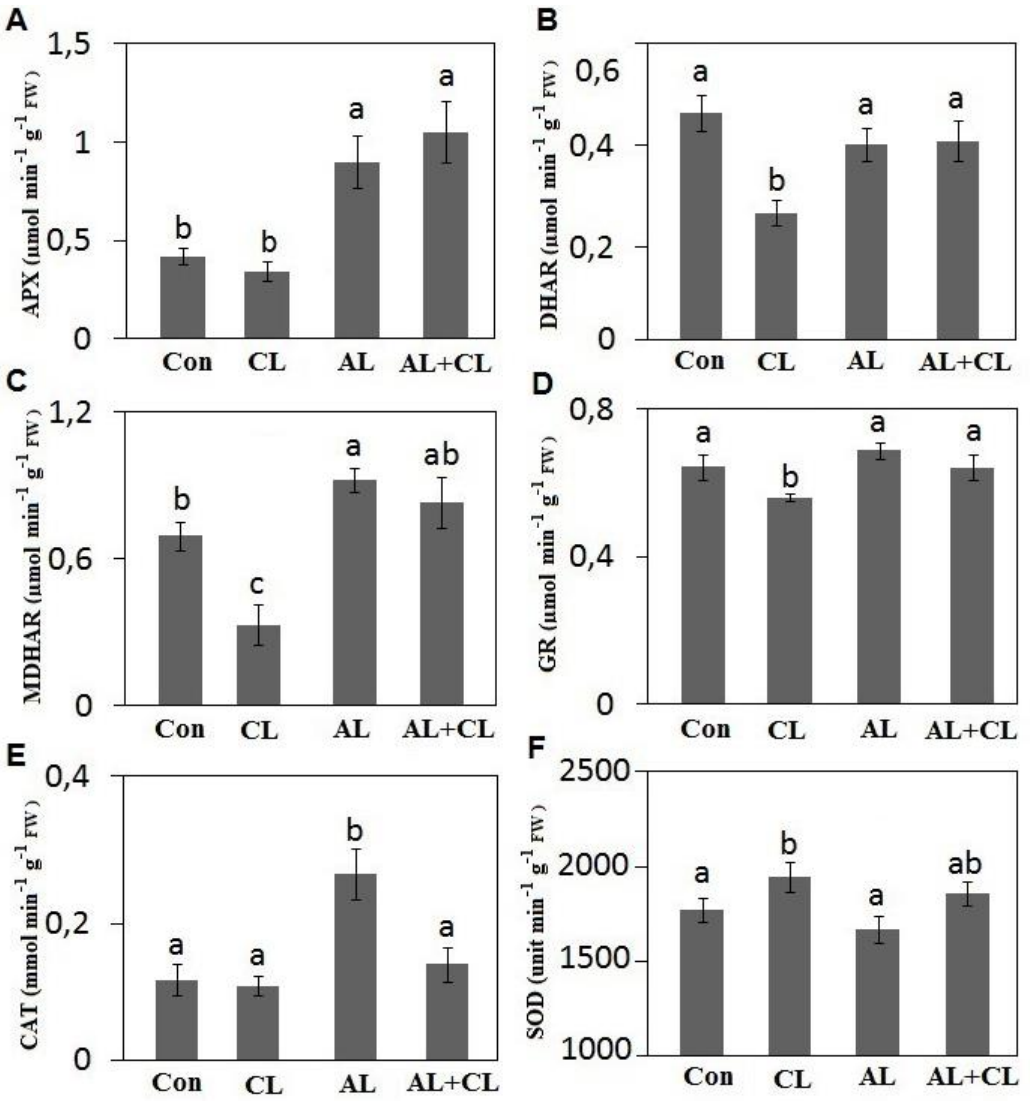


Fig. 2. Effects of clomazone and alternation of light/dark periods on **APX** (A), **DHAR** (B), **MDHAR** (C), **GR** (D), **CAT** (E) and **SOD** (F) activities in tobacco (*Nicotiana tabacum* L. *cv.Virgenie vk51)*. **Con** without alternating of light/dark periods, or clomazone**; CL** treatment with 1 µM clomazone**; AL** alternation of light/dark periods**; AL+CL** pretreatment of alternation of light/dark periods+ 1 µM clomazone treatment. All data represent means, SE for n= 8 and different letters to determine the significant difference between means within each treatment (P<0.05, ANNOVA-Tukey test).

3.4. Chlorophyll fluorescence transients

A considerable decrease of the maximal quantum of the photochemical yield of PSII (F_v/F_m) was found in plantlets treated with CL as compared to the Con treatment. In contrast, the plantlets of the AL+CL treatment have shown a significant increase (P<0.05) in F_v/F_m as compared to the plantlets treated with clomazone alone (Table 3).

Table.3. Effect of clomazone and alternation of light/dark periods treatments on Chl fluorescence intensity (F_0, minimal fluorescence; F_m, maximal fluorescence; F_v/F_m, maximal efficiency photochemical of PSII) and on the performance index (PI_{abs}) in the J step of flurescence rise. **Con** without alternating of light/dark periods, or clomazone**; CL** treatment with 1 µM clomazone**; AL** alternation of light/dark periods**; AL+CL** pretreatment of alternation of light/dark periods+ 1 µM clomazone treatment. Different letters denote significant differences for each parameter between means within each traitment (P<0.05, ANNOVA-Tukey test). Data are expressed as means ±SE, n= 8.

Treatment	F_0	F_m	F_v/F_m	PI_{abs}	$1–V_J$
Con	347 ± 5 b	1912 ± 40 a	0,818 ± 0,001 ab	2,07 ± 0,13 a	0,37 ± 0,007 a
CL	385 ± 10 a	1621 ± 49 c	0,75 ± 0,01 c	0,89 ± 0,15 c	0,29 ± 0,01 c
AL	327 ± 5 bc	1929 ± 37 a	0,830 ± 0,002 a	2,10 ± 0,15 a	0,36 ± 0,004 a
AL+CL	352 ± 10 b	1770 ± 62 b	0,79 ± 0,01 b	1,48 ± 0,20 b	0,33 ± 0,01 b

In comparison with the control, we have noticed that the CL treatment has considerably decreased the PIabs value, which was about (PI_{abs}= 0,9). In contrast, PIabs value has increased in the plantlets of AL+CL treatment (PI_{abs}= 1,481) as compared with CL treatment (Table 3).

In fact, the JIP-test has been used to assess the sensitivity of plants towards different environmental conditions [7,37,39,40,41]. It is considered that the J step is associated with an accumulation of $Q_A^-Q_B$ which has been demonstrated by the experimental results and the theoretical simulations. The results have shown that the electron transport at the PSII acceptor side was a clomazone-induced inhibitory site when the tobacco plantlets were exposed to clomazone [7]. A low value in the efficiency $1–V_J$ was found in the case of an exposure to clomazone. However, the AL pretreatment proves to have reduced the negative effect of clomazone, and the value of $1–V_J$ parameter in the AL+CL treatment was more important as compared to CL treatment (Table 3).

In the plantlets of the CL treatment, the total flux of the photons absorbed by the reaction center ABS/RC and the excitation flux trapped by the reaction center TR0/RC have significantly increased (P<0.05) as compared to the other treatments (Fig. 3A and B).

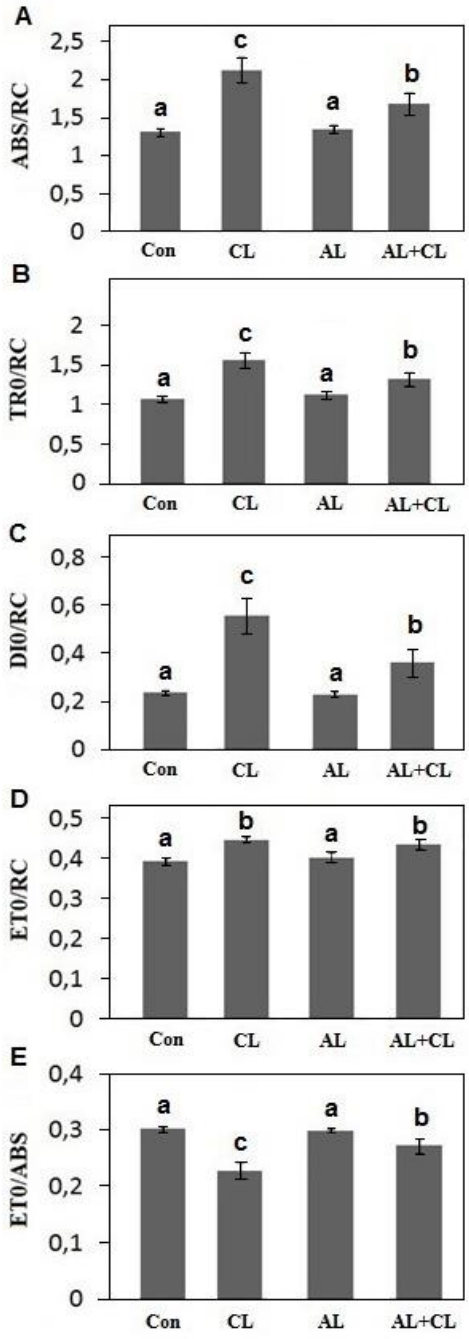


Fig. 3. Effects of clomazone and alternation of light/dark periods on **ABS/RC**, **TR0/RC**, **DI0/RC**, **ET0/RC** and **ET0/ABS** in leaves of tobacco plantlets (*Nicotiana tabacum* L. *cv.Virgenie vk51)*. **Con** without alternation of light/dark periods, or clomazone**; CL** treatment with 1 µM clomazone**; AL** alternation of light/dark periods**; AL+CL** pretreatment of alternation of light/dark periods+ 1 µM clomazone treatment. All data represent means, SE for n= 8 and different letters to determine the significant difference between means within each treatment (P<0.05, ANNOVA-Tukey test).

The energy dissipated into heat (DI0/RC) has significantly increased (P<0.05) in the plantlets treated with clomazone. In comparison with the CL treatment, the AL+CL treatment has shown a significant decrease (P<0.05) in the rate of heat dissipation (DI0/RC= 0,558 ;

DI0/RC= 0,361), respectively (Fig. 3C). The electrons transport beyond Q_A^- per the reaction centre (ET0/RC) had no significant difference (P>0.05) in the CL treatment as compared to the AL+CL treatment (Fig. 3D). In addition, the transferred electrons ET0 beyond Q_A^- per flux of the photons absorbed by the photosynthetic pigments antenna ABS have decreased in the CL treatment, while they have remarkably increased in the AL+CL treatment (Fig.3E).

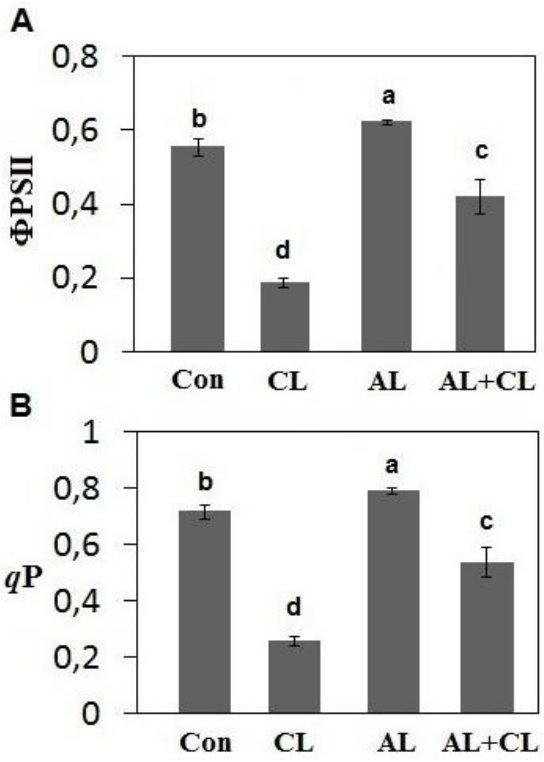


Fig. 4. The actual PSII efficiency (**ΦPSII**) and photochemical quenching coefficient (***q*P**) in the control, alternation of light/dark periods and clomazone treatments of tobacco leaves *(Nicotiana tabacum* L. *cv.Virgenie vk51*). All data represent means, SE for n= 8 and different letters to determine the significant difference between means within each treatment (P<0.05, ANNOVA-Tukey test).

The actual photochemical efficiency of the PSII (ΦPSII) and the photochemical quenching of the varying chlorophyll fluorescence (*q*P) (Fig. 4A and B) have noticeably decreased in the CL as compared to the other treatments. In contrast, ΦPSII and *q*P increased in the AL+CL treatment as compared to the CL treatment.

4. Discussion

The tobacco plantlets were subjected to cycle of an alternation of light/dark periods followed or not by herbicide treatment. To determine the effect of the light/dark alternative periods pretreatment on clomazone treatment in tobacco plantlets, we have used the following

parameters: the plant growth, the pigments content, the fluorescence parameters of the chlorophyll *a* (F_v/F_m, *q*P, ΦPSII), the JIP-test parameters (PI_{abs}, $1–V_J$, ABS/RC, TR0/RC, DI0/RC, ET0/RC, ET0/ABS), the H_2O_2 and AsA contents, and the antioxidant activities.

Our experiment shows that clomazone caused not only a reduction in chlorophyll and carotenoid levels but also a decline in the content of LHCII (Chl *a/b* ratio) in tobacco plantlets (Table 2). A similar decrease was observed in the case of a higher concentration of clomazone in the adult leaves of tobacco and tomato [7,42], and the adult leaves of barley seedlings [43]. Due to the effect of clomazone in the reduction of the antenna pigments (LHCII), an excessive influx of the excitation ABS is transferred into the active reaction centre (RC) of the photosystem II according to Kaňa et al [43]. During this process, the formation of the reactive singlet oxygen $^1O_2$ *via* the ^{3}Chl* was excepted according to Fufezan et al [4], especially with the reduction of the carotenoids which have an important role in protecting the chlorophyll from photooxidation during the plant growth [44]. The singlet oxygen $^1O_2$ can be considered as an important species, which causes damages in the reaction center during the photoinhibition [45]. The inactivation of some RCs led to the increase in trapping rate TR0 and electrons transport ET0 by the active reaction center (RC) (Fig. 3A, B and D). This in turn has caused a more reduced PQ pool as a result of the absence of active acceptor of electrons further than Q_A^- (see the reduction of $1–V_J$) (Table 3), and consequently increased the energy dissipating into heat DI0/RC (Fig. 3C) as a protection mechanism which causes photoinhibition at the acceptor side of PSII (see the reduction in F_v/F_m, PI_{abs}, *q*P, ΦPSII and ET0/ABS) [46]. In turn, the PQ pool in a very reduced state is a favorable condition to the formation of $^1O_2$ by the recombination reactions in PSII [47].

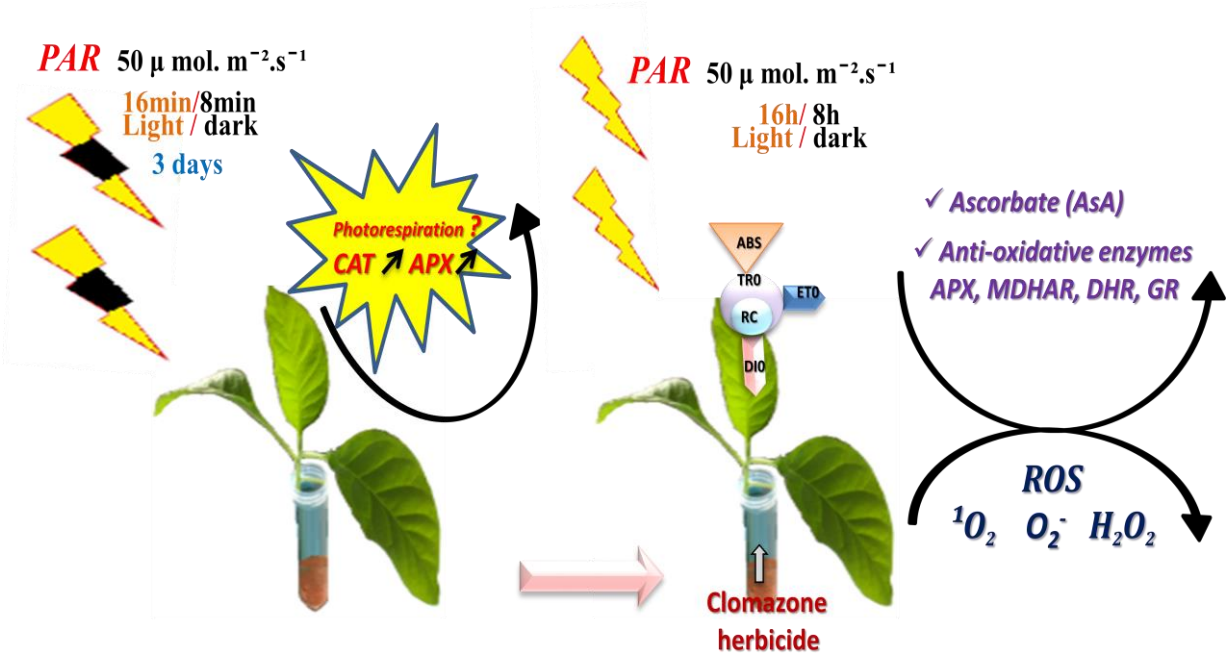
These processes lead to decease the formation of reduced NADPH and consequently to accumulate of O_2^- as result of the reduction of O_2 by ferredoxin (Fd) [48]. SOD activity was noticed to have increased in the clomazone treatment (Fig. 2F); and considering the decrease in the APX and CAT activities (Fig. 2A and E), this can partially explain the high accumulation of H_2O_2 (Fig. 1A) which may be also able to attack the protein D1 and destroy it, and to induce cell death [46]. This was also supported by the low content of total ascorbate which itself is accompanied by a considerable decline in the activities of its recycling enzymes, including the DHAR, MDHAR and GR enzymes (Fig. 2B, C and D).

Considering the ability of clomazone to reduce the PSII activity (see above) and to produce ROS, the parameters of growth have decreased in the tobacco plantlets (Table 1). It has been confirmed that clomazone leads to decrease the growth of both corn and pea seedlings [49,50].

In contrast, the AL pretreatment has enhanced the growth of the plantlets treated with clomazone, which was observed in all the parameters used (Table 1), especially the leaf area of the plant which increased about (47%) in the plantlets of the AL+CL treatment as compared to the CL treatment. At this point, these positives changes in the growth parameters were partially related to the efficiency of CO_2 fixing in the photosynthesis process. More clearly, the capacity of the AL+CL plantlets to benefit from the absorbed photons by antenna pigments (Table 2) can be confirmed by the increase of F_v/F_m, PI_{abs}(Table 3), *q*P and ΦPSII (Fig. 4A and B), and consequently, by the decrease of energy dissipating into heat DI0/RC (Fig. 3C). Moreover, the AL+CL treatment has shown an enhancement in the electron flux beyond Q_A^- and a regulation of the plastoquinone function (see the increase in $1–V_J$) (Table 3), especially in the presence of a high electrons flux beyond Q_A^- (ET0/RC) (Fig. 3D).

We can say that the positive effect of the AL pretreatment on the photosynthesis apparatus is due to the systemic capacity of the plantlets to avoid the stress state. With regard to the antioxidant activities, the ascorbate (Fig. 1B) contributes to reducing the photooxidative stress induced by the high accumulation of H_2O_2. This was observed in the AL+CL plantlets in the light of the increase in the APX activity (Fig. 2A) and the recycling enzymes of the ascorbate such as the DHAR, MDHAR and GR (Fig. 2B, C and D). In Consequence, the plantlets were more able to scavenge H_2O_2, and thus, a low content of H_2O_2 was found in the plantlets of the AL+CL treatment as compared to the CL treatment alone (Fig. 1A). Considering both the H_2O_2 content and the antioxidant system in the AL pretreatment, we can say that H_2O_2 content induced by the AL was might due to its effect in stimulation the photorespiration, according to Igamberdiev et al [51] which reported photorespiratory flux and mitochondrial contribution to energy and redox balance in the light and during light-dark transitions. This was able to motivate the antioxidant enzymes at the level of chloroplast and peroxisome (see APX, MDHAR and CAT activities). Some of the enzymes activated in the AL pretreatment have continued to scavenge H_2O_2 after the treatment with clomazone such as APX and MDHAR, while the CAT activity was of little importance during clomazone treatment. This also confirms that scavenging H_2O_2 mainly relates to the activity of the ascorbate and antioxidant enzymes whose function is associated with the ascorbate content; thus, the plantlets were well protected to reduce the photooxidative damages induced by clomazone, the herbicide.

In summary, AL pretreatment through the stimulation of antioxidant activities not only protects the photosynthetic apparatus from direct effect of ROS, but also relax the photon (electron) excess stress.



The possible effect of the pretreatment with alternation of light/dark periods to reduce the photooxidative stress induced by clomazone.

5. Acknowledgements

This work was supported by the Syrian Ministry of Education and Research, and by laboratory of fruits and vegetables of university of Avignon and Vaucluse countries.

6. References

[1] J. Whitmarsh, Govindjee, in: G.S. Singhal, G. Renger, K.D. Irrgang, S. Sopory, Govindjee (Eds.), The photosynthetic: Concept in Photobiology: Photosynthesis and Photomorphogenesis, Narosa Publishers, Kluwer Academic Publishers, New Delhi, 1999, pp. 11–51.
[2] P.J. Dekker, E.J. Boekema, Supramolecular organization of thylakoid membrane proteins in green plants, Biochim. Biophys. Acta 1706 (2005) 12–39.
[3] W.A. Rutherford, A. Krieger-Liszkay, Oxidative stress induced by herbicides in the photosystem II, Trends Biochem Sci. 26 (2001) 648-653.
[4] C. Fufezan, A.W. Rutherford, A. Krieger-Liszkay, Singlet oxygen production in herbicide-treated photosystem II, FEBS Lett. 532 (2002) 407-410.
[5] C. Triantaphylidès, M. Krischke , F.A. Hoeberichts, B. Ksas, G. Gresser, M. Havaux, F. Van Breusegem, M.J. Mueller, Singlet oxygen is the major reactive oxygen species involved in photo-oxidative damage to plants. Plant Physiol. 148 (2008) 960–968.
[6] H.J. Chang, M.J. Konz, E.A. Aly, R.E. Sticker, K.R. Wilson, N.E. Korg, P.E. Dickson, in: D.R. Baker, J.G. Fenyes, W.K. Moberg, B. Cross (Eds.), 3-Isoxalidones and related compounds a new class of herbicides, Synthesis and Chemistry of Agrochemicals, American chemical society, Washington, 1997, pp. 10–23.
[7] M. Darwish, F. Lopez-Lauri, H. Sallanon, Study of photosynthesis process in the presence of low concentration of clomazone herbicide in tobacco (*Nicotiana tabacum L)*, JSPB 9 (2013) 229-245.
[8] B.C. Osmond, in: N.R. Baker, J.R. Bowyer (Eds.), What is photoinhibition? Some insights from comparisons of shade and sun plants, Photoinhibition of Photosynthesis-from Molecular Mechanisms to the Field, Bioscientific Publishers, Oxford, 1994, pp. 1-24.

[9] K. Asada, The water-water cycle in chloroplasts: scavenging of active oxygens and dissipation of excess photons, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 601–639.
[10] P. Müller, X.P. Li, K.K. Niyogi, Non-photochemical quenching: a response to excess light energy, Plant Physiol. 125 (2001) 1558-1566.
[11] R. Allen, Dissection of oxidative stress tolerance using transgenic plants, Plant Physiol. 107 (1995) 1049-1054.
[12] K.K. Niyogi, Photoprotection revisited: genetic and molecular approaches, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 333-359.
[13] J. Kruk, R. Szymańska, Singlet oxygen and non-photochemical quenching contribute to oxidation of the plastoquinone-pool under high light stress in Arabidopsis, Biochim. Biophys. Acta 1817 (2012) 705-710.
[14] E.H. Murchie, K.K. Niyogi, Manipulation of Photoprotection to Improve Plant Photosynthesis, Plant Physiol. 155 (2011) 86-92.
[15] H.C. Foyer, A. Trebst, G. Noctor, in: B. Demmig-Adams, W.W. Adams (Eds.), Advances in Photosynthesis and Respiration: Photoprotection, Photoinhibition, Gene Regulation and Environment, Springer Science Publishers, Netherlands, 2005, 241–268.
[16] A. B. Logan, D. Kornyeyev, J. Hardison, A.S. Holaday, The role of antioxidant enzymes in photoprotection, Photosynth. Res. 88 (2006) 119–32.
[17] C. Bowler, M. Van Montagu, D. Inzé, Superoxide dismutase and stress tolerance, Annu. Rev. Plant Physiol. Plant Mol. Biol. 43 (1992) 83–116.
[18] S. Shigeoka, T. Ishikawa, M. Tamoi, Y. Miyagawa, T. Takeda, Y. Yabuta, Y. Kazuya, Regulation and function of ascorbate peroxidase isoenzymes, J. Exp. Bot. 53 (2002) 1305 – 1319.
[19] C. Massot, Analyse des variations de la teneur en in vitamine C dans le fruit de la tomate fruit en rôle de l'environnement lumineuse. PhD-thesis: Biology and Agronomy, Aix-marseille Academic, France, 2010, pp. 24-25.
[20] U.J. Grobbelaar, Microalgal biomass production: challenges and realities, Photosynth. Res. 106 (2010) 135-144.
[21] C. Vejrazka, M. Janssen, M. Streefland, R.H. Wijffels, Photosynthetic efficiency of *Chlamydomonas reinhardtii* in flashing light, Biotechnol. Bioeng. 108 (2011) 2905-2913.
[22] J.C. Merchuk, M. Ronen, S. Giris, S.M. Arad, Light/dark cycles in the growth of the red microalga *Porphyridium sp*, Biotechnol. Bioeng. 59 (1998) 705-713.
[23] E.G. Gergoff Grozeff, A.R. Chaves, C.G. Bartoli, Low irradiance pulses improve postharvest quality of spinach leaves *(Spinacia oleraceae* L. cv Bison), Postharvest Biol. Tec. 77 (2013) 35-42.
[24] V.D. Kreslavski, G.N. Shirshikova , V.Y. Lyubimov, A.N. Shmarev, A.M. Boutanaev, A.A. Kosobryukhov, F-J Schmitt , T. Friedrich, S.I. Allakhverdiev, Effect of preillumination with red light on photosynthetic parameters and oxidant-/antioxidant balance in Arabidopsis thaliana in response to UV-A, J. Photochem. Photobiol. B: Biol. 127 (2013) 229–236.
[25] H.K. Lichtenthaler, in: S.P. Colowick, N.O. Kaplan (Eds.), Chlorophylls and carotenoids pigments of photosynthesis biomebranes, Methods in Enzymology, Academic Press, New York, 1987, pp. 350–382.
[26] R. Murshed, F. Lopez-Lauri, H. Sallanon, Effect of water stress on antioxidant systems and oxidative parameters in fruits of tomato (*Solanum Lycopersicon* L, cv Micro-tom), Physiol. Mol. Biol. Plants (2013) (doi: 10.1007/s12298-013-0173-7).
[27] R. Murshed, F. Lopez-Lauri, H. Sallanon, Effect of salt stress on antioxidant systems depends on fruit development stage, Physiol. Mol. Biol. Plants (2013) (doi: 10.1007/s12298-013-0209-z).
[28] R. Murshed, F. Lopez-Lauri, H. Sallanon, Microplte quantification of enzymes of the plant ascorbate-glutathione cycle, Anal. Biochem. 383 (2008) 320-322.
[29] B. Chance, A.C. Maehly, Assay of catalase and peroxidase, Methods Enzymol. 2 (1955) 764-775.
[30] R.S. Dhindsa, P. Plumb-Dhindsa, T.A. Thorpe, Leaf senescent correlated with increased levels of membrane permeability and lipid peroxidation, and decreased levels of superoxide dismutase and catalase, J. Exp. Bot. 32 (1981) 93-101.
[31] M. Kitajama, W.L. Butler, Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone, Biochim. Biophys. Acta 376 (1975) 105-115.
[32] K. Maxwell, G.N. Johnson, Fluorescence of chlorophyll-a, a practical guide, J. Exp. Bot. 51 (2000) 659–668.
[33] R.J. Strasser, A. Srivastava, M. Tsimilli-Michael, in: M. Yunus, U. Pathre, P. Mohanty (Eds.), The fluorescence transient as a tool to characterize the situation photosynthetic samples, Probing photosynthesis: mechanisms, regulation and adaptation, Taylor & Francis, London, 2000, pp. 445–483.
[34] P.D.R. Heerden, R.J. Strasser, G.H.J. Stress, Reduction in dark chilling N2-fixation in soybean by nitrate indicated by the kinetics of the florescence of chlorophyll-a, Physiol. Plant. 121 (2004) 239–249.
[35] B.J. Strasser, R.J. Strasser, in: P. Mathis (Ed.), Measuring fast fluorescence of the fluorescence transient in response to environmental questions: the JIP-test, Kluwer Academic Publishers, Netherlands, 1995, pp. 977-980.

[36] M. Tsimilli-Michael, G.H.J. Kruger, R.J. Strasser, in: P. Mathis (Ed.), Suboptimality as driving force for adaptation: a study about the correlation of excitation light intensity and the dynamic fluorescence emission in plants, Kluwer Academic, Netherlands, 1995, 981–984.
[37] B. Genty, J. M. Briantais, N.R. Baker, The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence, Biochim. Biophys. Acta 990 (1989) 87-92.
[38] M.F. Pompelli, S.C. Martins, W.C. Antunes, A.R. Chaves, F.M. DaMatta, Photosynthesis and photoprotection in coffee leaves is affected by nitrogen and light availabilities in winter conditions, J. Plant Physiol. 167 (2010) 1052-1060.
[39] G.H.J. Krüger, M. Tsimilli-Michael, R.J. Strasser, Light stress provokes plastic and elastic modifications in structure and function of photosystem II in camellia leaves, Physiol. Plant. 101 (1997) 65–277.
[40] B.S. Ripley, S.P. Redfern, J. Dames, Quantification.of the photosynthetic performance of phosphorus-deficient Sorghum by means of chlorophyll-a fluorescence kinetics, South African J. Sci. 100 (2004) 615-618.
[41] M.H. Kalaji, R. Carpentier, I.S. Allakhverdiev, K. Bosa, Fluorescence parameters as early indicators of light stress in barley, J. Photochem. Photobiol. B: Biol. 112 (2012) 1-6.
[42] J.E. Scott, L.A. Weston, J. Chappell, K. Hanley, Effect of.clomazone on IPP isomerase and prenyl transferase activities in cell suspension cultures and cotyledons of solanaceous Species, Weed Sci. 42 (1994) 509–516.
[43] R. Kaňa, M. Šponduvá, P. Ilík, D. Lazár, K. Klem, P. Tomek, J. Nauš, O. Prášil, Effect of herbicide clomazone on photosynthetic processes in primary barley (*Hordeum vulgare L*), Pestic. Biochem. Physiol. 78 (2004) 161-170.
[44] B. Biswall, Carotenoids catabolism during leaf senescence and its control by light, J. Photochem. Photobiol. B: Biol. 30 (1995) 3–14.
[45] A. Trebst, B. Depka, H. Holländer-Czytko, A specific role for tocopherol and of chemical singlet oxygen quenchers in the maintenance of photosystem II structure and function in *Chlamydomonas reinhardtii*, FEBS Lett. 516 (2002) 156–160.
[46] F. Poiroux-Gonord, J. Santini, A.L. Fanciullino, F. Lopez-Lauri, J. Giannettini, H. Sallanon, L. Berti, L. Urban, Metabolism in orange fruits is driven by photooxidative stress in the leaves, Physiol. Plant. 149 (2013) 175–187.
[47] N. Adir, H. Zer, S. Shokhat, I. Ohad, Photoinhibition- a historical perspective, Photosynth. Res. 76 (2003) 343-370.
[48] N. Murata, S. Takahashi, Y. Nishiyama, S.I. Allakhverdiev, Photoinhibition of photosystem II under environmental stress, Biochim. Biophys. Acta 1767 (2007) 414-421.
[49] K.W. Vencill, K.K. Hatzios, H.P. Wilson, Growth and physiological responses of normal, dwarf, and albino corn (*Zea mays L*) to clomazone treatments, Pestic. Biochem. Physiol. 35 (1989) 81-88.
[50] G. Sandman, P. Boger, Interference of dimethazone with formation of terpenoid compunds, Z. Naturforsch 41 (1986) 729-732.
[51] A.U. Igamberdiev, E. Romanowska, P. Gardeström, Photorespiratory flux and mitochondrial contribution to energy and redox balance of barley leaf protoplasts in the light and during light-dark transitions, J. Plant Physiol. 158 (2001) 1325- 1332.

Publication 4

Alternation of light/dark periods priming enhances clomazone tolerance *via* improving ascorbate and phenol compounds accumulation, and ROS detoxification in tobacco (*Nicotiana tabacum* L.) plantlets

Majd Darwish*, Félicie Lopez-Lauri, Veronique Vidal, Mohamed El Maataoui, Huguette Sallanon

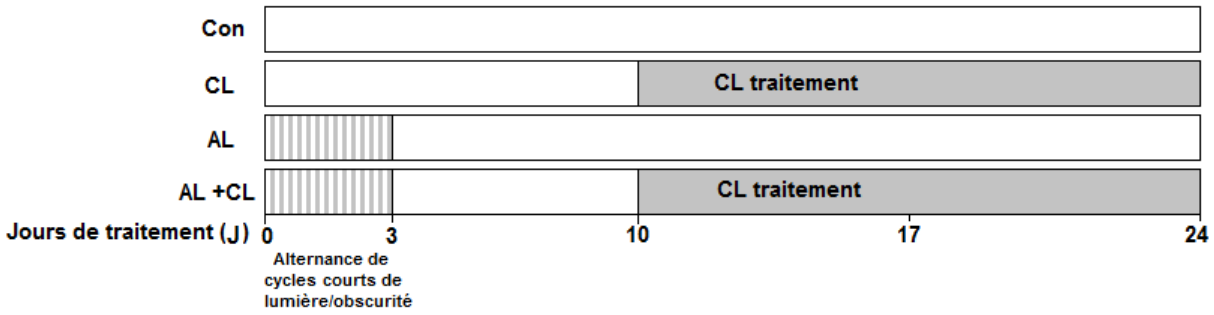
La deuxième partie de ce chapitre est un projet d'article soumis dans *Journal of Photochemistry and Photobiology B: Biology*. Nous avons rapporté précédemment que le prétraitement par l'alternance de cycles courts de lumière/obscurité améliore la tolérance du tabac face au stress photooxydatif induit par le clomazone. Cet effet pourrait être lié à une stimulation des activités des enzymes antioxydantes (APX, DHAR, MDHAR et GR). En particulier, des augmentations plus importantes des activités des enzymes APX et MDHAR et de la teneur en ascorbate ont été observées chez les plantules de tabac prétraitées par l'alternance de cycles courts de lumière/obscurité et ensuite traitées par le clomazone.

L'objectif de ce travail a été d'approfondir la compréhension des mécanismes impliqués dans la tolérance des plantules du tabac prétraités par l'alternance de cycles courts de lumière/obscurité face au stress photooxydatif induit par le clomazone. Pour cela, nous avons étudié le comportement des plantules prétraitées par l'alternance de cycles lumière/obscurité à plusieurs stades (J_0, J_3, J_{10}, J_{17} et J_{24}) en mesurant: (i) les paramètres liés au transport d'électrons photosynthétiques en présence de clomazone au niveau du donneur d'électrons (l'évolution d'oxygène par chloroplastes isolés) et au niveau accepteur d'électrons (la fluorescence chlorophyllienne et test *OJIP*), (ii) les analyses des échanges gazeux et, (iii) les teneurs en H_2O_2, ascorbate et polyphénols. En complément, des études cytohistologiques ont été effectuées à ces différents stades.

Expérimentation

La variété de tabac *Nicotiana tabacum* L. *Virginie vk 51* sensible au clomazone a été utilisée. Une partie des plantules au stade trois feuilles a été exposée à des alternances de cycles courtes de lumière/obscurité (16min jour/8min nuit) avec une densité de flux de photons photosynthétiques (PPFD) de 50 µmol m^{-2} s^{-1} dans une chambre de culture pendant trois

jours. L'autre partie est utilisée comme contrôle. Après une semaine de croissance dans la chambre de culture contrôlée à 22/17 °C, humidité 60 %, 16 h/8 h d'un cycle lumière/obscurité et PPFD de 50 µmol photons m^{-2} s^{-1}, les plantules ont été traitées par du clomazone à 1 µM pendant 14 jours. Le clomazone est ajouté dans la solution nutritive renouvelée tous les deux jours. Le prélèvement des feuilles a été effectué à 0 (J_0), 3 (J_3), 10 (J_{10}), 17 (J_{17}) et 24 (J_{24}) jours de traitement pour les études cytohistologiques, et à 10 (J_{10}) et 24 (J_{24}) jours afin de mesurer l'évolution d'oxygène des chloroplastes isolés.



Représentation schématique du protocole expérimental et des traitements. 0 (J_0), 3 (J_3), 10 (J_{10}), 17 (J_{17}) et 24 (J_{24}) se réfèrent au nombre de jours après le traitement par l'alternance de cycles courts de lumière/obscurité. Con, sans alternance de cycles courts de lumière/obscurité et clomazone; CL, le traitement par 1 µM de clomazone; AL, le traitement par l'alternance de cycles courts de lumière/obscurité; AL + CL, le prétraitement par l'alternance de cycles courts de lumière/obscurité et le traitement par 1 µM de clomazone.

Principaux résultats

Le clomazone induit une diminution de l'efficience photosynthétique du PSII à la lumière, de la photosynthèse nette et des teneurs en ascorbate et polyphénols ainsi qu'une augmentation de l'accumulation d'amidon et d'H_2O_2.

Le prétraitement par l'alternance induit un stress modéré révélé par une diminution de l'efficience photochimique du PSII, et de la photosynthèse nette, ainsi qu'une augmentation de l'accumulation d'amidon et de la dissipation d'énergie sous forme de chaleur. Ce stress peut stimuler les enzymes de détoxification et la biosynthèse d'antioxydants.

Il est proposé que l'effet protecteur de l'alternance vis-à-vis au stress photooxydatif est principalement lié à la stimulation post-illuminatoire de la photorespiration. En effet, en présence d'alternance, un grand nombre de passage lumière/obscurité, conduit à une augmentation de l'activité photorespiratoire (PIB) (Post-illumination burst) qui agit comme

voie alternative de consommation des électrons. Les effets antioxydants des polyphénols dont la synthèse stimulée en présence de l'alternance sont également discutés.

Conclusion

Les mécanismes par lesquels le prétraitement par l'alternance de cycles courts de lumière/obscurité agit sur la tolérance, sont complexes. Indépendamment de la stimulation des activités antioxydantes enzymatique, les teneurs en phénols ainsi que la stimulation de l'activité photorespiratoire semblent impliquée dans la protection de l'appareil photosynthétique.

Alternation of light/dark periods priming enhances clomazone tolerance *by* increasing ascorbate and phenol compounds levels, and ROS detoxification in tobacco (*Nicotiana tabacum* L.) plantlets

Majd Darwish*, Félicie Lopez-Lauri, Veronique Vidal, Mohamed El Maataoui, Huguette Sallanon

Laboratoire Physiologie des Fruits et Légumes (EA 4279), Université d'Avignon et des Pays de Vaucluse, Bât Agrosciences, 301 rue Baruch de Spinoza, BP 21239, F-84916 Avignon cedex 9, France

ABSTRACT

The effect of the alternation of light/dark periods (AL) (16/8 min of light/dark cycles and PPFD of 50 μmol photons m^{-2} s^{-1}, for three days) on activity of PSII and photosynthesis, the content of H_2O_2, ascorbate and phenols compounds, and at the cytological level to clarify the involved mechanisms in clomazone tolerance of tobacco plantlets primed with AL was studied. Clomazone induced decreases in the PSII activity, the net photosynthetic rate (P_n), the ascorbate and total polyphenols contents as well as increases in the H_2O_2 and starch-grains accumulation, and the level of the cells that underwent programmed cell death (PCD). The pretreatment with AL reduced the inhibitory effect of clomazone on the PSII activity and photosynthesis, indicated by the decrease in the H_2O_2, the starch-grains accumulation and the PCD levels, as well as increased the content of the ascorbate and some of phenol compounds such as the chlorogenic acid, the neochlorogenic acid and the rutin. The AL treatment could promoted photorespiration *via* post-illumination burst (PIB) possess effects. This alternative electron pathway photorespiratory could reduce the H_2O_2 generation *via* consumption photochemical energy, such as ATP and NADPH. Furthermore, this process proves to induce a moderate stress at 10 days (D_{10}) of the treatment with AL, which appears to be revealed by decreases in the maximum photochemical quantum efficiency of PSII (F_v/F_m) and the P_n as well as increases in the energy dissipation as heat (DI0/RC) and the starch-grains accumulation. This moderate stress proves to stimulate H_2O_2 detoxification systems *via* increasing the activity of antioxidant enzymes and the biosynthesis of antioxidant components, such as ascorbate acid and some phenolic compounds. So, the PCD levels,

induced by the photooxidative stress provoked by clomazone, at 24 days (D_{24}) of the treatment with AL were noticeably decreased.

Keywords: Light; *Nicotiana tabacum* L.; Photosynthesis; Programmed cell death; PSII effeciency; ROS detoxification; Tolerance

Abbreviations: ABS PSII light absorption flux.- APX ascorbate peroxidase.- AsA ascorbate.- ^{3}Chl* chlorophyll triplet state.- C_i intercellular CO_2 concentration.- DI0 PSII excitation dissipation flux.- DHA dehydroascorbate.- DHAR dehydroascorbate reductase.- ET0 electron transport flux.- ETR photosynthetic electron transport rate.- F_0, F_v, F_m minimal, variable and maximal chlorophyll fluorescence.- F_v/F_m maximum photochemical efficiency of PSII.- GR glutathione reductase.- g_s stomatal conductance.- H_2O_2 hydrogen peroxide.- LHC light-harvesting chlorophyll *a/b* binding protein complex II.- MDHAR monodehydroascorbate reductase.- $^1O_2$ singlet oxygen.- O_2^- superoxide radical.- OH^- hydroxyl radical.- PFDD photosynthetic photon flux density.- PCD programmed cell death.- PI_{abs} performance index on absorption basis.- P_n net photosynthetic rate.- PQ plastoquinone.- PSI, PSII photosystem I(II).- Φ_{PSII} actual PSII efficiency.- Q_A primary quinone electron acceptor of PSII.- RC reaction center.- ROS reactive oxygen species.- RuBP ribulose-1,5-bisphosphate.- R_s dark respiration.- SOD superoxyde dismutase.- T transpiration rate.- TR0 PSII light energy flux trapping.- 1-V_J a measure of the electron flux beyond Q_A.

1. Introduction

In higher plants, the light energy is absorbed by the antenna pigments of the photosynthetic apparatus, and then transferred to the reaction centers (RCs) of the two photosystems, PSI and PSII, to be converted into chemical energy, which can be used in the assimilation of CO_2 and organic matter. When plants are exposed to herbicides inducing photooxidative stress, the light energy absorbed will not be fully used in photosynthesis. In this case, the excess excitation of chlorophyll conducts to the formation of singlet oxygen ($^1O_2$) leading to destruction of the D1 protein and to blockage of the ability of PSII to repair [1]. In addition, $^1O_2$ induces the formation of other ROS such as superoxide ($O_2^{\cdot-}$), peroxide hydrogen (H_2O_2) and hydroxyl radical ($OH^{\cdot}$), which can damage proteins, lipids and DNA [2]. Depending of level of H_2O_2, this later may behave as signaling molecule capable of inducing stress tolerance or leading to damages in cells and programmed cell death (PCD) in a very narrow concentration range [3,4].

The herbicide clomazone [2-(2-chlorobenzyl)-4,4-dimethyl-1,2-oxazolidin-3-one] leads to PSII photoinhibition damages and high H_2O_2 accumulation in leaves of tobacco [5].

The reduction of even small amounts of H_2O_2 may help maintain the ROS concentration, at the level of the whole cell, within a sub-lethal range. In this way, the plant has developed several mechanisms to protect the photosynthetic apparatus to avoid the massive accumulation of ROS and reduce its oxidative damages. This involves antioxidant systems that are considered as a mechanism by which the photooxidative damage induced by the herbicides is considerably reduced. The enzymatic antioxidants system, such as SOD, CAT, APX, DHAR, MDHAR and GR is one of these mechanisms. Moreover, some of phenolic compounds are considered as one of the non-enzymatic antioxidants system. In turn, the accumulation of flavonoids and hydroxycinnamates as phenolic antioxidant compounds and its role to protect the plants against excess light damages has been suggested by several studies [6-10]. The flavonoids have been suggested to perform the inhibition of ROS generation and then scavenge ROS once they are formed [9]. Flavonoids located within or close proximity of centers of ROS generation in stressed plants can constitute a ‘secondary’ antioxidant system that complements the function of antioxidant enzyme activities to scavenge ROS [9,11-13]. The flavonoids in the vacuole indeed have been considered as co-substrates for vacuolar peroxidases that can be in addition of ascorbate peroxidase activity (APX) a potential to reduce H_2O_2 level from the chloroplast [14]. A system constituted by the flavonoids is primarily involved in detoxifying vacuolar H_2O_2 and then flavonoid radicals are

recycled back to their original form by ascorbate acid [15]. Chloroplast envelope-located flavonoids can also, under excess excitation conditions, protect cells plant from photooxidative stress, by limiting the exit of $^1O_2$ involved in the programmed cell death (PCD) [9,16,17]. Quercetin a chloroplast flavonol was suggested to have an antioxidant function at the oxidizing side of the PSII [18]. Rutin (quercetin 3-O-rutinoside) is a flavone derivative, considered as one of the most promising quercetin derivatives. In addition, chlorogenic acid is a major hydroxycinnamic exhibiting antioxidant activity to scavenging ROS [19].

Priming process that involves a temporally limited experience of an environmental stimulus to prepare the plant to more successfully cope with a future environmental stimulus, has been suggested as a potential to increase the plant tolerance under several abiotic stress, including UV-A [20], oxidative stress of cadmium [21], temperature stress in *Triticum aestivum* [22,23] and salt stress in *Lactuca sativa* [24]. The alternation of light/dark periods is thought to be beneficial in algae by exerting various biological effects such as ROS scavenging [25-27]. By influencing the circadian clock of plant, the transient light/dark cycles could lead to a dynamic control of a host of physiological processes, including the determination of the levels of primary and secondary metabolites, and the anticipation of future environmental constraints [28]. The recent work of Darwish et al. [5], reports that alternation of light/dark periods pretreatment improves tobacco tolerance to clomazone through the stimulation of antioxidant activities enzymes (APX, DHAR, MDHAR and GR) and relaxes the excess photon stress. In this report, more important increases in APX and MDHAR activities and ascorbate content were observed in tobacco plantlets pretreated with alternation and afterwards treated with CL compared to those of the control. Nevertheless, it remains unclear how AL pretreatment induced a greater tobacco tolerance to clomazone herbicide. So, intensive research efforts are still needed to elucidate the involved mechanisms.

The main focus of present work therefore is to better understand the mechanisms involved in the tolerance of tobacco plantlets pretreated with alternation of light/dark periods to clomazone. For this purpose, we measured parameters related to modifications in the H_2O_2, ascorbate and polyphenols contents, chloroplast oxygen-evolving rate, chlorophyll *a* fluorescence and JIP-test, and gas exchanges analysis. Additionally, cytohistological analyses were carried out on the leaves treated by light/dark periods alternation and clomazone.

2. Materials and Methods

2.1. Plant material, growth conditions and chemical treatments

The tobacco seeds of the variety (*Nicotiana tabacum* L. *cv.Virginie vk51*) were germinated in a plastic container with sterilized potting soil for two weeks at 22/17 °C day/night temperature, 16/8 h light/dark cycle and photosynthetic photon flux density (PPFD) of 50 µmol photons m^{-2} s^{-1}. The germination was carried out in sterile conditions and in compliance with the growth chamber conditions, according to Darwish et al. [29]. The experimental design is shown in Fig. 1.

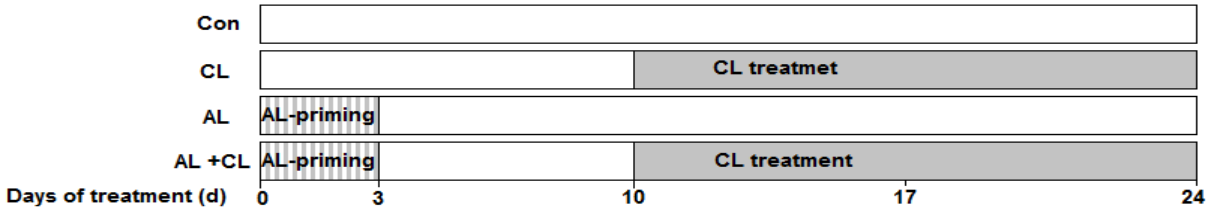


Fig.1. Shematic representation of expirmental design and treatments. 0 (D_0), 3 (D_3), 10 (D_{10}), 17 (D_{17}) and 24 (D_{24}) refer to days after the treatment with the alternation of light/dark periods. Con, without alternation of light/dark periods and without clomazone; CL, treatment with 1 µM clomazone; AL, alternation of light/dark periods; AL+CL, pretreatment of alternation of light/dark periods and treatment with 1 µM clomazone.

In brief, after 5 weeks of germination, the plantlets (three leaf stage) were exposed or not to an alternation of light/dark periods (AL priming) (16/8 min of light/dark cycle, PPFD of 50 µmol photons m^{-2} s^{-1}) in a growth chamber for three days; the other part remained without an AL to be used as a control, as described by Darwish et al. [5]. Then, the plantlets were transferred into a hydroponic system containing Auckland's nutrient solution (1 M KNO_3; 1 M $Ca(NO_3)_2.H_2O$; 1 M $MgSO_4.7H_2O$; 1 M KH_2PO_4 and 0.01 M FeEDDHA) under the same conditions as those used during the germination. After 10 days of the pretreatment with AL, clomazone (CL) (Sigma-Aldrich, USA) was added at the concentrations of 0 or 1 µM for 14 days. The solutions of such a treatments were refreshed twice weekly. At 0 (D_0), 3 (D_3), 10 (D_{10}) and 24 (D_{24}) days of the treatment with AL, chlorophyll a fluorescence parameters and gas exchanges are determined in leaves of the four treatments ((i) Con (Control, without alternation of light/dark periods or clomazone); (ii) CL (1 µM clomazone); (iii) AL (alternation of light/dark periods); (iv) AL+CL (alternation of light/dark periods and treatment with 1 µM clomazone)). Additionally, gas exchanges analysis are also recorded during two cycles of AL treatment, fresh leaves are collected to be used for chloroplast isolation (at D_{10} and D_{24}) and cytohistological

observations (at D_3, D_{10}, D_{17} and D_{24}), and the remaining leaves were stored at -80 °C for biochemical analysis.

2.3. Determination of the H_2O_2 contents

The hydrogen peroxide levels were determined as described by Murshed et al. [30] with some modifications. Briefly, 250 mg of tobacco leaves from plantlets treated or untreated with AL and CL were homogenized in 1 mL of 0.1% trichloracetic acid (TCA). The homogenate was centrifuged at 12,000 × *g* for 15 min at 4 °C. Aliquots of 100 µL from each tube were placed in 96-well plates and 50 µL of 10 mM potassium phosphate buffer (pH 7.0) and 100 µL of 1 M KI were added to each well. Control samples were prepared using water instead of KI to remove the color background of the extract at 390 nm. Each plate also contained commercial H_2O_2 to generate a standard curve. The plate was briefly vortexed and the absorbance readings were collected at 390 nm in a micro-plate reader. The concentrations of H_2O_2 were determined using a standard curve and expressed as nmol g^{-1} dry weight (DW).

2.3. Determination of the ascorbate contents

The assay of the total and reduced AsA contents was performed according to a modified version of the method of Murshed et al. [31] using leaf material that was stored at -80 °C. Briefly, 100 mg of tobacco leaf powder was homogenized with 1 mL of ice-cold 6% trichloroacetic acid (TCA). The samples were centrifuged at 15,000 × *g* for 10 min at 4 °C, and 10 µL of the supernatant was used in each assay. The total AsA (addition of 20 µM dithiothreitol (DTT)) and reduced AsA (without DTT) in each sample were measured. Aliquots of 10 µL of each sample or a standard were distributed into two wells (in triplicate) of a 96-well microplate and mixed with 10 µL of 20 mM DTT (total AsA assay) or 0.2 M phosphate buffer (pH 7.4) (reduced AsA assay). The plate was incubated at 42 °C for 15 min. Then, 10 µL of *N*-ethyl maleimide (NEM) (total AsA assay) or 0.2 M phosphate buffer (pH 7.4) (reduced AsA assay) was added and mixed followed by the addition of 150 µL of color reagent (containing H_3PO_4, $FeCl_3$ and dipyridyl). After incubation at 42 °C for 40 min, the plate was briefly vortexed, and the absorbance was read at 525 nm using a micro-plate reader (Power Wave, HT microplate spectrophotometer, BioTek, France). The AsA concentrations of the samples were calculated using a standard curve and expressed as mg g^{-1} dry weight (DW).

2.4. Extraction and analysis of phenolic compounds

The quantification and identification of phenolic compounds were determined as described by Charles et al. [32] with some modifications. Briefly, 200 mg of lyophilized tobacco leaves were extracted in 2 mL of 80% methanol, containing 0.1% (v:v) hydrochloric acid and methoxyflavone, as internal standard. The extraction tubes were vortexed for 30 s, sonicated for 5 min, incubated in liquid nitrogen 1 min, vortexed and sonicated again for another 30 s and 5 min, respectively. The tubes were centrifuged (14,000 × *g*, 15 min, at 4 °C). The supernatants were transferred to a microfuge tube and the sample was centrifuged once more (14,000 × *g*, 15 min, at 4 °C). Extracts were filtered through a 0.45 µm RC membrane.

The chromatographic separation of phenolic compounds was performed with a Waters (Milford, MA) Alliance chromatography system equipped with a Waters 600 Pump, a Waters 2996 PDA detector and a Waters 717 plus autosampler. The HPLC system was monitored and controlled by Water Empower software version 2. The chromatographic separation was carried out on a Waters SunFire^{TM} C18 column (250 × 4.6 mm I.D; 5 µm particle size) with a C18 guard column (4 × 4.6 mm I.D; 5 µm particle size). Mobile phases consisted of phase A (acetonitrile) and phase B (water) acidified with 0.5% formic acid run at 1 mL min^{-1}. Polyphenolics were separated using a gradient elution system that kept mobile phase A at 5% for 1 min and then changed phase A from 5% to 25% in 30 min, 25% to 100% in 2 min, remained at 100% for 3 min and returned to original conditions in 5 min for the next injection. The injection volume was 10 µL and all analyses were performed three times. Phenolic compounds, namely chlorogenic acid (3-O-caffeoylquinic), neochlorogenic (3-O-caffeoylquinic) and rutin (quercetin-3-rutinoside) were identified by comparison of their retention times and UV spectra with those of authentic standards (Extrasynthèse, Genay, France) and quantified at 280 nm using individual calibration curve. Polyphenolics content was expressed as mg g^{-1} dry weight (DW).

2.5. Chloroplast preparation

The chloroplasts were isolated from the fresh leaves of tobacco plantlets as described by Joly and Carpentier [33] with certain modifications. Briefly, 5 g of dark green leaves was harvested and rinsed with distilled water. The leaves were then ground for 20-30 seconds in 30 mL of buffer A (grinding buffer) containing 660 mM sorbitol, 20 mM KCl, 2 mM $EDTANa_2$ and 100 mM HEPES-KOH (pH 7.9). After grinding, the homogenate was filtered twice through five layers of Miracloth tissue, and the filtrate was quickly centrifuged (1500 ×

g, 2 min, at 4 °C). The supernatant was discarded, and the pellet was resuspended in 1.5 mL of buffer B (resuspension buffer) containing 10 mL of buffer A, 100 µM $MgCl_2$, and 100 µM $MnCl_2$.

The total chlorophyll content of the chloroplast preparation was estimated according to Porra et al. [34] with some modifications. Briefly, 10 µL of the chloroplast preparation was added to 1 mL of acetone 80% (v:v). The mixture was homogenized and vortexed for 2 min. The absorbance of the supernatant was measured at 645 and 663 nm (A_{645} and A_{663}, respectively) using a spectrophotometer (Biochrom Libra S22, Biochrom Ltd., UK). The chlorophyll concentration was calculated using the following equation:

$$\mu g\ Chl\ mL^{-1} = [(12.7\ A_{663} - 2.69\ A_{645}) + (22.9\ A_{645} - 4.68\ A_{663})]$$

2.6. Measurement of oxygen-evolving rate

The oxygen-evolving rate by a suspension of chloroplasts that were broken in hypotonic medium to allow the compounds used as electron acceptors to access into thylakoid membranes was measured. Oxygen evolution measurements were performed polarographically using a Clark-type oxygen electrode (DW1, Oxygraph system, Hansatech Instruments, UK) at 25 °C under saturating light intensity on chloroplast particles equivalent to 80 µg Chl mL^{-1}.

The photosynthetic electron transport of the entire chain was determined as the oxygen evolution using potassium ferricyanide $K_3Fe(CN)_6$ (99% pure, Sigma-Aldrich, USA) as an electron acceptor. The reaction mixture in a final volume of 1 mL contained 2 mM $K_3Fe(CN)_6$ and chloroplast particles equivalent to 80 µg Chl mL^{-1}.

The PSII [H_2O→ benzoquinone (BQ)] rates were measured using BQ (98% pure, Sigma-Aldrich, USA) as an electron acceptor according to Samuel and Bose [35]. The rate of PSII electron transport was measured in terms of oxygen evolution in the presence of BQ (2 mM). The reaction mixture in a final volume of 1 mL contained 100 mM HEPES-KOH (pH 7.9), 20 mM KCl, 2 mM $EDTANa_2$, 0.1 mM $MgCl_2$, 0.1 mM $MnCl_2$, 2 mM BQ and chloroplast particles equivalent to 80 µg Chl mL^{-1}.

All activities were measured in the presence of 1 µM nigericin (98% pure, Sigma-Aldrich, USA) a potent uncoupler of photophosphorylation in isolated chloroplasts. This compound dissipates the proton gradient that occurs between the lumen and the stroma during illumination [36].

All the experiments were independently performed and the presented data were expressed as percent (%) of the inhibition of oxygen evolution as compared with the control.

2.7. Measurement of fast chlorophyll *a* fluorescence transients

The behavior of photosystem II (PSII) can be evaluated based on the fast kinetics of chlorophyll *a* fluorescence emitted by leaves of plantlets that are adapted to darkness. At 0 (D_0), 3 (D_3), 10 (D_{10}) and 24 (D_{24}) days of the treatment with AL, fast chlorophyll *a* fluorescence transients were measured on 8 leaves of tobacco plantlets. Prior to any measurement, the leaves were dark-adapted for 20 min. The polyphasic chlorophyll *a* fluorescence (expressed in relative units) was measured using a portable Handy-PEA (Hansatech, Kings Lynn, UK) under illumination with a light intensity of 3,000 µmol photons m^{-2} s^{-1}. The fluorescence parameter $F_v/F_m = (F_m-F_0)/F_m$ represents the maximum photochemical quantum yield of PSII [37]. In turn, the JIP-test has been found as a potential to be very sensitive to stress caused by changes in different environmental conditions [5,29,38-41]. The JIP-test is based on the energy flux theory of the thylakoid membrane. This model describes how the energy of the flux of photons that are absorbed by the photosynthetic pigments in the PSII antenna (ABS) is dissipated as heat and fluorescence (DI), trapped by PSII reactions centers (RCs) (leading to Q_A reduction), and converted into electron transport flux (ET). These fluxes are described in terms of a specific energy flux (per RC) or as proportions of other fluxes (ratios or yields). The calculation of the energy flux and ratios of a specific flux was fully explained by Strasser and his coworkers [38,40,42-46]. The appendix shows some JIP-test parameters, their biophysical or biochemical meanings and how they are calculated from the original data (F_0, F_{300}, F_J, F_m) derived from fast chlorophyll *a* fluorescence transients. The effective quantum yield of PSII ($\Phi_{PSII} = (F_m'-F_s)/F_m'$) [47,48] was measured using a leaf chamber fluorometer (LI-6400-40 LCF, LI-Cor, Lincoln NE, USA), and PPFD of 500 µmol photons m^{-2} s^{-1}. The rate of light-saturated electron transport through PSII was calculated as $ETR = \Phi_{PSII} \times PPFD \times A \times 0.5$ [49], where A was the proportion of incident PPFD absorbed by the sample and the factor 0.5 accounted for the presence of two photosystems, assuming equal involvement in linear electron flow.

2.8. Determination of gas exchanges

The net photosynthetic rate (P_n), the stomatal conductance (g_s), the intercellular CO_2 concentration (C_i) and the transpiration rate (T) were measured at D_0, D_3, D_{10} and D_{24} using a

portable photosynthetic analyzer (LI-Cor, Lincoln, Nebr. USA) which was equipped with a leaf chamber fluorometer (LI-6400-40 LCF). The sample leaf was placed in the cuvette that was maintained at ambient temperature and humidity, and exposed or not to PPFD of 50 µmol photons m^{-2} s^{-1} and ambient concentration CO_2 of 400 µmol mol^{-1} using a LI-6400-01 CO_2 injector with a high-pressure liquid CO_2 cartridge source. Additionally, gas exchanges analysis (P_n, g_s, C_i, T and the dark respiration rate (R_s)) are recorded by LI-COR for 2 cycles of alternation of light/dark at D_3 in the Con and AL plantlets using PPFD of 50 µmol photons m^{-2} s^{-1} and ambient concentration CO_2 of 400 µmol mol^{-1}.

2.9. Cytohistological analyses

To evaluate the effects of clomazone at the cytological level, leaf fragments were collected from each treatment and prepared for cytohistology as previously described [50]. Briefly, the tissue samples (approximately 5 mm square) were excised from the middle parts of the leaves and immediately fixed at 4 °C in 4% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2). To promote penetration of the fixative, the samples were placed under vacuum for 20 min. After 48 h of fixation at 4 °C, the specimens were rinsed in distilled water and stored in 70% ethanol at 4 °C until required. The samples were then dehydrated in an alcohol series and embedded in methacrylate resin (Kit Technovit 7100, Heraeus-Kulzer GmbH, Wehrheim, Germany) according to the manufacturer's instructions. Transverse sections (3-µm thickness) were serially cut using a retraction microtome (Reichert-Young Supercut 2065, Wien, Austria), collected on microscope slides, allowed to dry and stored in dust-proof containers. The slides were hydrated in distilled water before staining to visualize insoluble carbohydrates (cellulose, pectin and starch) using the periodic acid-Schiff's reagent (PAS) technique and proteins using naphthol blue black (for details and references, see [50]). Observations and imaging were performed using a Leica DM 2000 photomicroscope (Leica Instruments, Nussloch, Germany) equipped for bright field and UV illumination. The images were captured using Leica DFC 300 FX digital camera (Leica) and analyzed with LAS software (Leica). Approximately 5 leaf fragments were examined for each treatment. Particular attention was paid to structural alterations affecting the photosynthetic mesophyll cells.

2.10. Statistical Analyses

Statistical analyses were performed with the R statistical software using an ANOVA with Tukey's test. The results are displayed as means ± SE and were considered significant at $P<0.05$.

3. Results

3.1. H_2O_2 and ascorbate contents

The tobacco plantlets that were treated with clomazone exhibited a higher H_2O_2 level (Fig. 2A) than control. We also note that AL+CL-treated plantlets showed significant decrease ($P<0.05$) in the H_2O_2 content by approximately 55% compared to that of the CL treatment alone.

Total ascorbate (Fig. 2B) significantly decreased ($P<0.05$) in the CL treatment by approximately 15% compared to that of the control, while in the AL and AL+CL treated plants, total ascorbate significantly increased by approximately 51% and 50% compared to those of the control. In addition, the AL+CL treatment showed a high increase in the total ascorbate by approximately 78% compared to that of the CL treatment alone.

3.2. Phenolic compounds

The total polyphenols (Fig. 2C) decreased in the CL treatment by approximately 17% compared to that of the control, while in the AL+CL treatment, the total polyphenols increased by approximately 37% compared to that of the CL treatment alone. However, the AL and AL+CL treatments had no difference significant ($P>0.05$) compared to the control.

Specifically, the chlorogenic acid content (Fig. 2D) decreased in the CL treatment by approximately 29% compared to that of the control, while the AL+CL-treated plantlets showed significant increase ($P<0.05$) in the chlorogenic acid content by approximately 23%, 40% and 75% compared to those of the control, AL and CL treatments, respectively.

The neochlorogenic acid content (Fig. 2E) increased in the AL+CL treatment by approximately 30%, 40% and 45% compared to those of the control, AL and CL treatments, respectively.

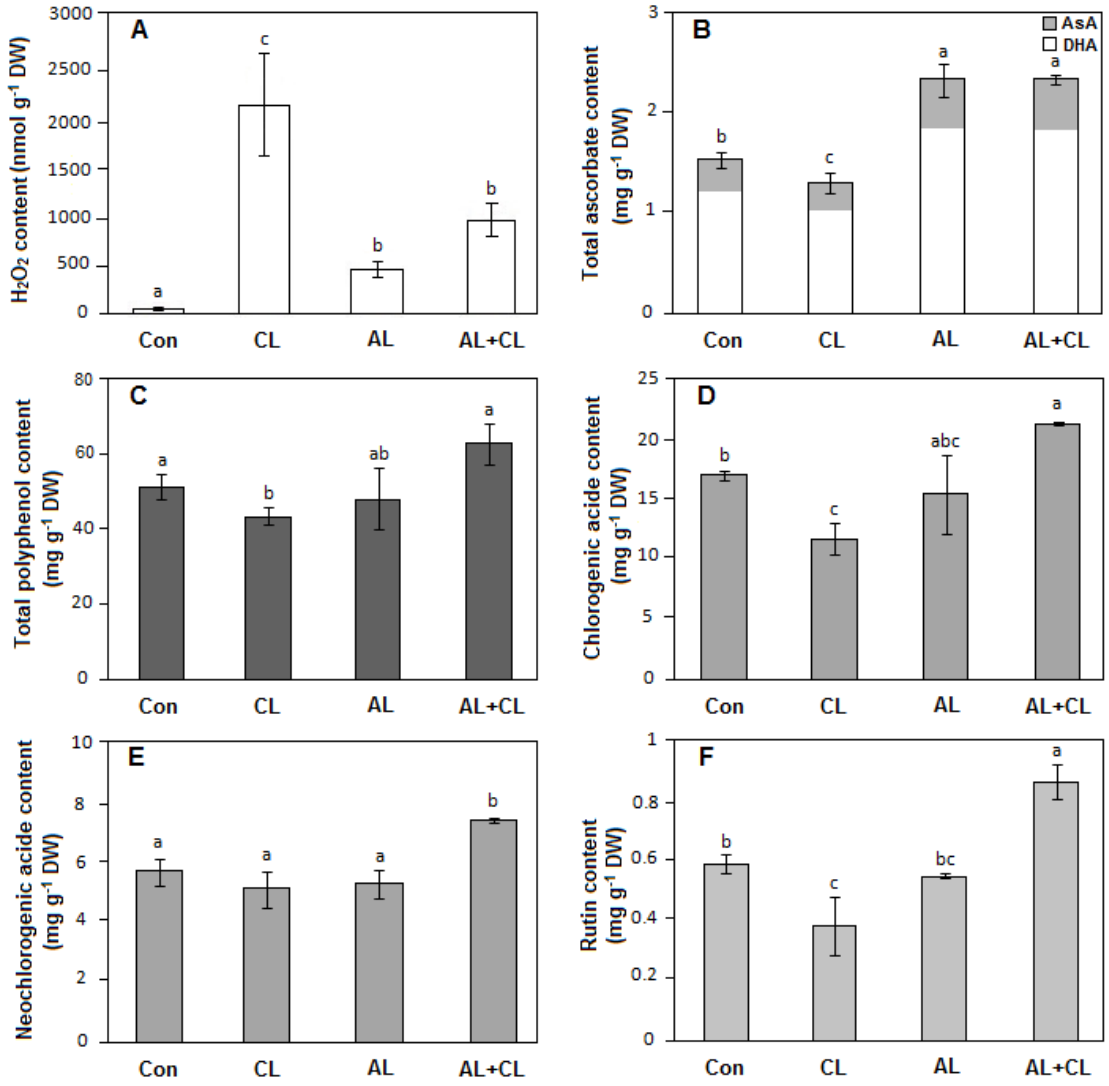


Fig.2. Effect of clomazone and alternation of light/dark periods treatments on H_2O_2, ascorbate and phenolic compounds contents in tobacco plantlets (*Nicotiana tabacum* L. *cv. Virginie vk51*). Con, without alternation of light/dark periods and without clomazone; CL, treatment with 1 µM clomazone; AL, alternation of light/dark periods; AL+CL, pretreatment of alternation of light/dark periods and treatment with 1 µM clomazone. Measurements were performed at 24 (D_{24}) days after the treatment with AL. Reported data are obtained from eight replicates, n= 8. Different letters indicate significant difference within each treatment according to an ANOVA-Tukey test at the 95% confidence level.

Similarly, the rutin content (Fig. 2F) showed significant decrease (P<0.05) in the CL treatment by approximately 36% compared to that of the control, while it significantly increased (P<0.05) in the AL+CL treatment by approximately 45%, 56% and 126% compared to those of the control, AL and CL treatments, respectively.

3.3. Chloroplast oxygen evolution

At D_{10}, the AL treatment decreased chloroplast oxygen-evolving rate by approximately 6% in the presence of ferricyanide compared to that of the control. Similarly in presence of benzoquinone, the AL treatment reduced chloroplast oxygen evolution [$H_2O \rightarrow$ BQ] by approximately 5% compared to that of the control. While at D_{24}, the AL pretreatment had no significant effects on chloroplast oxygen-evolving rate using either ferricyanide or benzoquinone as electron acceptors compared to the control. In comparison with the control, the CL treatment significantly decreased (P<0.05) chloroplast oxygen-evolving rate in presence of benzoquinone or ferricyanide at D_{24} (Table 1).

Table 1. Inhibition of chloroplast oxygen-evolving rate (nmol mg^{-1} min^{-1}) measured in thylakoids (80 µg Chl mL^{-1}) as percent (%) compared to the control. CL, treatment with 1 µM clomazone; AL, alternation of light/dark periods; AL+CL, pretreatment of alternation of light/dark periods and treatment with 1 µM clomazone. D_{10}, after 10 days of the treatment with AL, chloroplasts were isolated from the Con and AL plantlets and then treated with clomazone; D_{24}, after 24 days of the treatment with AL or 14 days of the treatment with CL, chloroplasts were isolated from the Con, CL, AL and AL+CL plantlets. $K_3Fe(CN)_6$, potassium ferricyanide as an electron acceptor; BQ, benzoquinone as an electron acceptor in the PSII. Reported data are obtained from four replicates, n= 4. Different letters indicate significant difference within each treatment according to an ANOVA-Tukey test at the 95% confidence level.

Treatment	D_{10}		D_{24}	
	$K_3Fe(CN)_6$	BQ	$K_3Fe(CN)_6$	BQ
CL			12% c	16.5% c
AL	6% b	5% b	0.5% a	0.5% a
AL+CL			6% b	6.5% b

At D_{24}, chloroplast oxygen-evolving rate showed significant increases (P<0.05) by approximately 6% (using ferricyanide) and 6.5% (using benzoquinon) in the AL+CL-treated plantlets compared to those of the CL-treated plantlets, respectively (Table 1).

3.4. Chlorophyll *a* fluorescence transients

Compared to the control, the F_v/F_m of the AL-treated plantlets significantly decreased (P<0.05) at D_{10}, after the F_v/F_m value enhanced and no significant deference (P>0.05) was found at D_{24}. The results showed a significant decrease (P<0.05) in the F_v/F_m of the CL-treated plantlets at D_{24}, while the F_v/F_m value of the AL+CL-treated plantlets increased by approximately 5% compared to that of the CL-treated plantlets (Table 2).

The performance index on absorption basis (PI_{abs}) of the AL treatment significantly decreased (P<0.05) at D_{10} and then it showed an enhancement compared to the control. The CL

treatment considerably lowered (P<0.05) the PI_{abs} at D_{24} by approximately 34% and 66% compared to those of the control, respectively. In contrast, the AL pretreatment increased the PI_{abs} value in the AL+CL-treated plantlets by approximately 73% compared to that of the CL-treated plantlets at D_{24} (Table 2).

Table 2. Effect of clomazone and alternation of light/dark periods treatments on chlorophyll *a* fluorescence intensity (F_0, minimum fluorescence; F_m, maximum fluorescence; F_v/F_m, maximum photochemical efficiency of PSII) and on the performance index (PI_{abs}) evaluated from the fast chlorophyll fluorescence curves (i.e., OJIP transients). Con, without alternation of light/dark periods and without clomazone; CL, treatment with 1 µM clomazone; AL, alternation of light/dark periods; AL+CL, pretreatment of alternation of light/dark periods and treatment with 1 µM clomazone. Measurements were performed at 0 (D_0), 3 (D_3), 10 (D_{10}) and 24 (D_{24}) days after the treatment with AL. Reported data are obtained from eight replicates, n= 8. Different letters indicate significant difference within each treatment according to an ANOVA-Tukey test at the 95% confidence level.

Treatment	Time	F_0	F_m	F_v/F_m	PI_{abs}	$1\text{-}V_J$
Con	D_0	458 ± 11 b	2251 ± 51 a	0.79 ± 0.008 a	1.9 ± 0.09 b	0.38 ± 0.002 a
	D_3	466 ± 20 b	2319 ± 40 a	0.80 ± 0.011 a	1.8 ± 0.18 b	0.37 ± 0.003 ab
	D_{10}	379 ± 10 a	2078 ± 58 b	0.81 ± 0.009 a	1.9 ± 0.07 b	0.36 ± 0.005 b
	D_{24}	368 ± 13 a	2043 ± 15 b	0.81 ± 0.006 a	2.2 ± 0.19 ab	0.38 ± 0.003 b
CL	D_{24}	565 ± 26 c	1865 ± 50 c	0.69 ± 0.015 d	0.75 ± 0.11 d	0.34 ± 0.002 c
AL	D_3	360 ± 18 a	2076 ± 50 b	0.82 ± 0.01 a	1.7 ± 0.21 b	0.34 ± 0.004 c
	D_{10}	427 ± 9 b	1891 ± 64 c	0.77 ± 0.007 b	1.6 ± 0.18 bc	0.37 ± 0.004 ab
	D_{24}	369 ± 10 a	2091 ± 54 b	0.82 ± 0.004 a	2.4 ± 0.13 a	0.36 ± 0.009 b
AL+CL	D_{24}	539 ± 22 bc	2033 ± 114 bc	0.74 ± 0.01 c	1.3 ± 0.01 c	0.39 ± 0.003 a

The $1\text{-}V_J$ parameter of the AL-treated plantlets decreased at D_3 and it increased in the following stages. The CL treatment significantly decreased (P<0.05) the $1\text{-}V_J$ parameter at D_{24} compared to the control. However, the AL pretreatment proves to have reduced the negative effect of clomazone, and the increases in the $1\text{-}V_J$ values in the AL+CL-treated plantlets were significant (P<0.05) at D_{24} compared to those of the CL-treated plantlets (Table 2).

In the CL-treated plantlets, the flux of photons absorbed by PSII antenna per reaction center (ABS/RC) and the excitonic flux trapped per reaction center (leading to Q_A reduction) (TR0/RC) increased compared to those of the control. Similarly, the part of the absorbed energy that was dissipated as heat and fluorescence (DI0/RC) significantly increased (P<0.05) in the CL-treated plantlets. In contrast, the AL+CL-treated plantlets showed significant decreases (P<0.05) in the ABS/RC, TR0/RC and DI0/RC parameters. The electrons transporting beyond Q_A^- per the active reaction centre (ET0/RC) significantly decreased (P<0.05) in the AL+CL-treated plantlets compared to that of the CL-treated plantlets. In

addition, the quantum yield of the electron transport beyond Q_A^- (ET0/ABS) significantly decreased (P<0.05) in the CL-treated plantlets, while it has remarkably increased in the AL+CL-treated plantlets compared to that of the CL-treated plantlets (Table 3).

Table 3. Effect of clomazone and alternation of light/dark periods treatments on the ABS/RC, TR0/RC, DI0/RC, ET0/RC and ET0/ABS. Con, without alternation of light/dark periods and without clomazone; CL, treatment with 1µM clomazone; AL, alternation of light/dark periods; AL+CL, pretreatment of alternation of light/dark periods and treatment with 1 µM clomazone. Measurements were performed at 0 (D_0), 3 (D_3), 10 (D_{10}) and 24 (D_{24}) days after the treatment with AL. Reported data are obtained from eight replicates, n= 8. Different letters indicate significant difference within each treatment according to an ANOVA-Tukey test at the 95% confidence level.

Treatment	Time	ABS/RC	TR0/RC	DI0/RC	ET0/RC	ET0/ABS
Con	**D_0**	1.2 ± 0.01 a	1 ± 0.01 a	0.25 ± 0.011 b	0.37 ± 0.005 b	0.30 ± 0.001 a
	D_3	1.3 ± 0.05 b	1 ± 0.03 a	0.27 ± 0.023 bc	0.39 ± 0.007 b	0.29 ± 0.005 a
	D_{10}	1.3 ± 0.03 b	1.1 ± 0.04 a	0.24 ± 0.009 ab	0.39 ± 0.008 b	0.30 ± 0.003 a
	D_{24}	1.3 ± 0.07 b	1 ± 0.05 a	0.23 ± 0.021 ab	0.39 ± 0.02 bc	0.31 ± 0.003 a
CL	**D_{24}**	1.8 ± 0.19 d	1.2 ± 0.15 ab	0.54 ± 0.057 e	0.43 ± 0.023 c	0.25 ± 0.011 b
AL	**D_3**	1.5 ± 0.07 c	1.2 ± 0.05 b	0.26 ± 0.025 b	0.42 ± 0.011 c	0.28 ± 0.006 a
	D_{10}	1.3 ± 0.08 b	1 ± 0.05 a	0.30 ± 0.026 c	0.37 ± 0.017 b	0.29 ± 0.005 a
	D_{24}	1.1 ± 0.03 a	1 ± 0.03 a	0.19 ± 0.002 a	0.32 ± 0.017 a	0.30 ± 0.009 a
AL+CL	**D_{24}**	1.4 ± 0.04 bc	1 ± 0.03 a	0.37 ± 0.021 d	0.39 ± 0.009 b	0.29 ± 0.004 a

The actual photochemical efficiency of the PSII (Φ_{PSII}) and the photosynthetic electron transport rate (ETR) (Fig. 3 A and B) noticeably decreased (P<0.05) in the CL treatment at D_{24} compared to the other treatments. These parameters showed significant increases (P<0.05) in the AL-treated plantlets especially at D_3 and D_{24}, while at T_{10} these parameters were decreased compared to the control. In the AL+CL treatment, the Φ_{PSII} and ETR prove to have increased compared to those of the CL treatment alone.

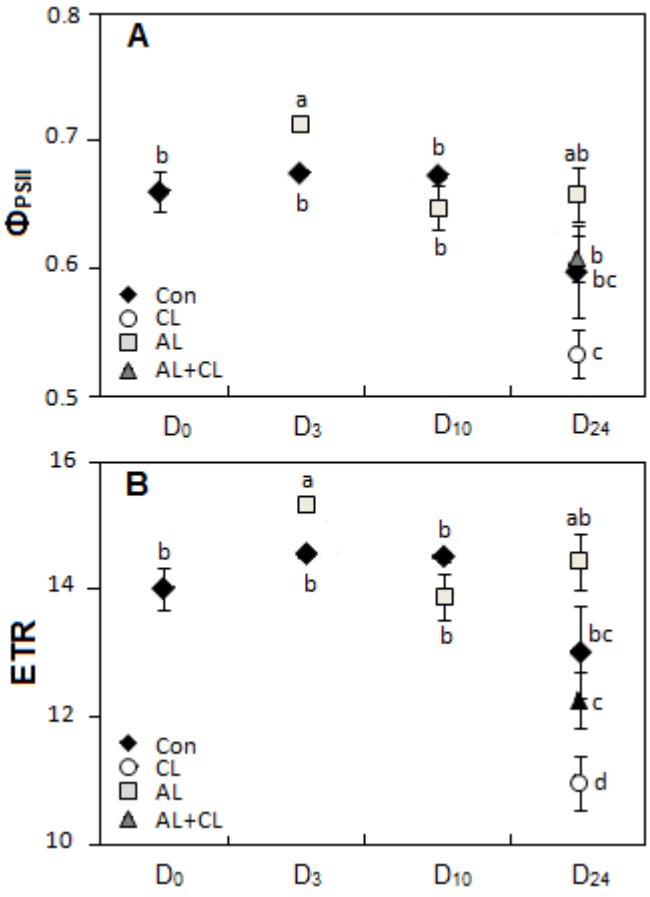


Fig.3. The actual photochemical efficiency of the PSII (Φ_{PSII}) and the photosynthetic electron transport rate (ETR) in the control, alternation of light/dark periods and clomazone treatments of tobacco leaves (*Nicotiana tabacum* L. *cv*. *Virgenie vk51*). Con, without alternation of light/dark periods and without clomazone; CL, treatment with 1 µM clomazone; AL, alternation of light/dark periods; AL+CL, pretreatment of alternation of light/dark periods and treatment with 1 µM clomazone. Measurements were performed at 0 (D_0), 3 (D_3), 10 (D_{10}) and 24 (D_{24}) days after the treatment with AL. Reported data are obtained from eight replicates, n= 8. Different letters indicate significant difference within each treatment according to an ANOVA-Tukey test at the 95% confidence level.

3.5. Gas exchanges

Effect of treatment with alternation and clomazone on gas exchanges

At D_{24}, the net CO_2 assimilation (P_n) (Fig. 4A) significantly decreased (P<0.05) in the CL-treated plantlets by approximately 70% compared to those of the control, respectively. In contrast, the AL pretreatment increased the net photosynthesis (P_n) in the CL-treated plantlets by approximately 104% at D_{24} compared to those of the CL treatment alone. However, the AL-treated plantlets showed a significant decrease (P<0.05) in the P_n at D_{10} compared to the control.

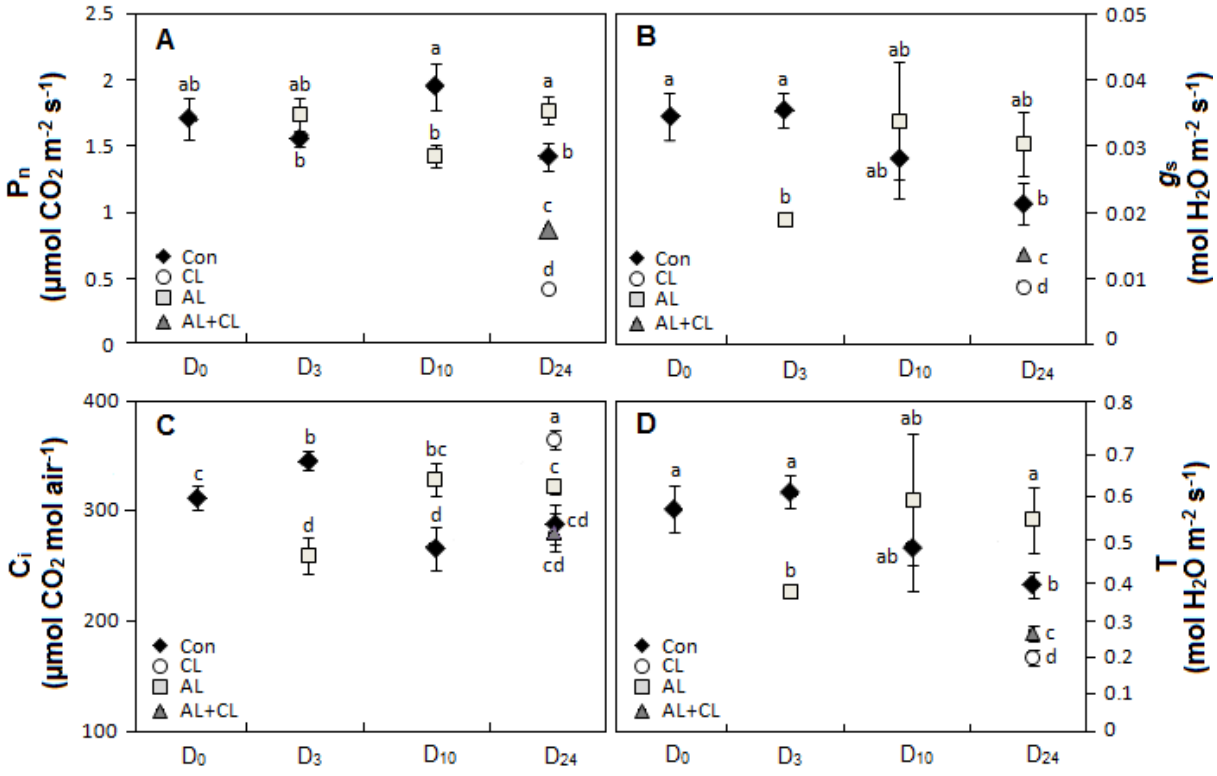


Fig.4. The net CO_2 assimilation (P_n), the stomatal conductance (g_s), the intercellular CO_2 concentration (C_i) and the transpiration rate (T) in the control, alternation of light/dark periods and clomazone treatments of tobacco plantlets (*Nicotiana tabacum* L. *cv. Virgenie vk51*). Con, without alternation of light/dark periods, or clomazone; CL, treatment with 1 µM clomazone; AL, alternation of light/dark periods; AL+CL, pretreatment of alternation of light/dark periods and treatment with 1 µM clomazone. Mesaurments were performed at 0 (D_0), 3 (D_3), 10 (D_{10}) and 24 (D_{24}) days after the treatment with AL. Reported data are obtained from eight replicates, n= 8. Different letters indicate significant difference within each treatment according to an ANOVA-Tukey test at the 95% confidence level.

In the AL-treated plantlets, the stomatal conductance (g_s) and the transpiration rate (T) (Fig. 4B and D) showed significant decreases (P<0.05) at D_3, while they were maximal at D_{24}. In the CL-treated plantlets, the g_s and T showed significant declines (P<0.05) at D_{24}. At D_{24}, AL pretreatment effects to increase the g_s and T in the AL+CL-treated plantlets were more significant to the extent that the g_s and T have increased by approximately 52% compared to those of the CL-treated plantlets.

At D_{24}, the CL treatment increased the intercellular CO_2 concentration (C_i) (Fig. 4C) by approximately 27% compared to that of the control. In contrast, the C_i of the AL+CL-treated plantlets had no significant difference (P>0.05) compared to that of the control. In the AL-treated plantlets, the C_i showed a significant decrease (P<0.05) at D_3, while at D_{10} and D_{24} it significantly increased (P<0.05) compared to that of the control.

Effect of short light/dark cycles on gas exchanges of plantlets treated or not with alternation

During the two cycles of AL (Fig. 5A), the levels of P_n increased in the control plantlets at the second cycle of AL, these increases is more pronounced when compared to those of the control plantlets and the AL-treated plantlets at the first and the second cycles of AL, respectively. The levels of P_n had no changes in the AL-treated plantlets between the first and the second cycles of AL (Fig. 5A).

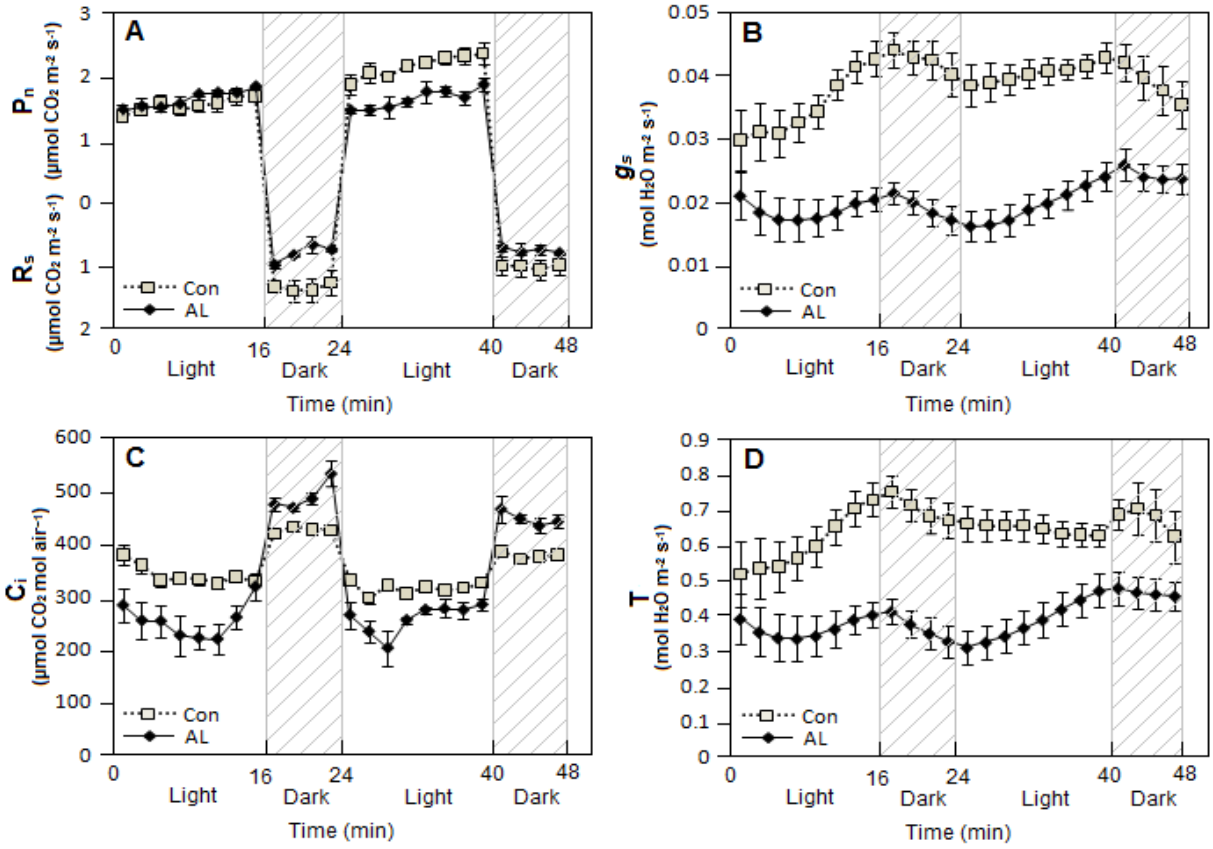


Fig.5. The net CO_2 assimilation (P_n), the stomatal conductance (g_s), the intercellular CO_2 concentration (C_i), the transpiration rate (T) and the dark respiration rate (R_s) recorded for 2 cycles of alternation of light/dark periods (AL) in tobacco plantlets (*Nicotiana tabacum* L. *cv. Virgenie vk51*). Con, without alternation of light/dark periods; AL, alternation of light/dark periods. Mesaurments were performed at 3 (D_3) days after the treatment with AL. Data are expressed as mean ± SE.

The stomatal conductance (g_s) and the transpiration rate (T) (Fig. 5B and D) showed significant decreases (P<0.05) in the AL plantlets compared to those of the control at the two cycles of AL.

The levels of C_i (Fig. 5C) showed significant decreases (P<0.05) in the light periods and significant increases (P<0.05) in the dark periods compared to those of the control.

The levels of the dark respiration rate (R_s) (Fig. 5A) in the AL-treated plantlets decreased compared to those of the control.

3.6. Cytohistological analyses

A microscopic study of sections from the AL-treated plantlets showed that the photosynthetic mesophyll cells not only were in well morphological integrity, but also filled with numerous starch-containing chloroplasts. The chloroplasts appeared to have deposited more starch than did the controls, as evidenced by the prominence of starch grains. These starch grains seems to be visible at D_3 and clearly visible at the beginning of D_{10} when compared to those of the control (Fig. 6A and B). At D_{24}, the vacuole cells revealed small components, which are may be secondary metabolites (Fig. 6D).

The sections from CL-treated plantlets revealed the presence numerous mesophyll cells exhibiting cytological alterations that are characteristic of programmed cell death (PCD). These alterations consisted of cell wall lysis, vacuole regression, nuclear and cytoplasm condensation and cell shrinkage. These alterations were noticeably at D_{24} when compared to the control and the other treatments (Fig. 6D).

The mesophyll of AL+CL-treated leaves in contrast appeared to be composed of cells that underwent to less changes and maintained their morphological integrity, when compared to the control. Compared to the sections from CL-treated plantlets, the mesophyll cells of AL+CL-treated leaves showed well-visible differences: the starch grains presence was less pronounced and the symptoms of PCD apparently were not observed in the sections (Fig. 6C and D).

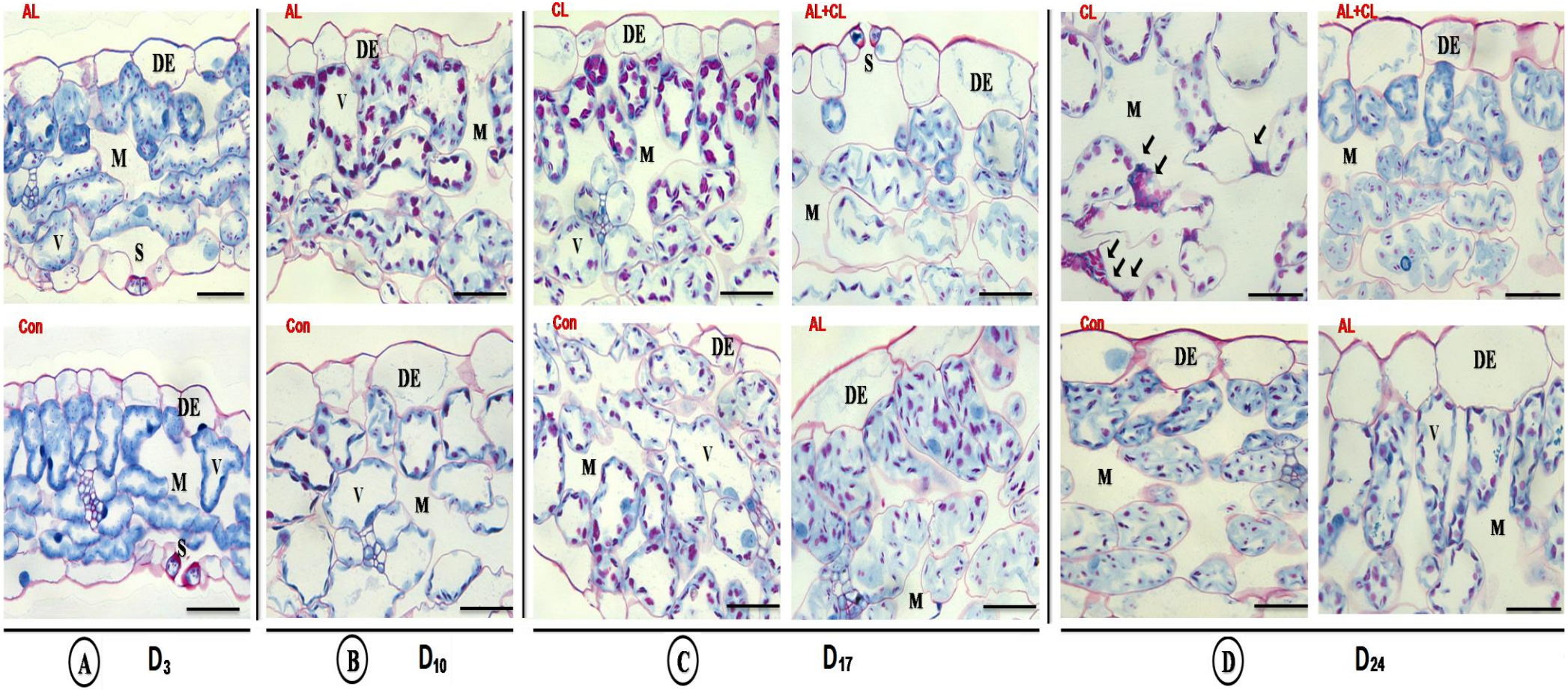


Fig.6. **Fig.6**. Cytohistological effects of clomazone (CL) and alternation of light/dark periods (AL) treatments on tobacco (*Nicotiana tabacum* L. *cv. Virginie vk51*). A, sections from control and AL-treated plantlets at 3 (D_3) days after the treatment with AL; B, sections from control and AL-treated plantlets at 10 (D_{10}) days after the treatment with AL; C, sections from control and the plantlets of CL, AL and AL+CL treatments at 17 (D_{17}) days after the treatment with AL; D, sections from control and the plantlets of CL, AL and AL+CL treatments at 24 (D_{24}) days after the treatment with AL. Note the presence of sinuous vacuoles (V) and starchy chloroplasts (pink granules). In general, clomazone increased grains-starch accumulation in chloroplasts. In addition, the mesophyll cells showed cytological alterations resembling those described for programmed cell death (PCD) at D_{24} (Fig. D, arrows). These alterations in contrast appear to not be found in the AL+CL plantlets. All scale bars: 50 µm. DE, adaxial epidermis; M, mesophyll; S, stomata; V, vacuole.

4. Discussion

Alternation of light/dark periods (AL) to improve clomazone tolerance has already been studied in tobacco plantlets by Darwish et al. [5], who reported that this pretreatment could be responsible for the protection against the oxidative stress induced by clomazone *by* stimulation of APX, DHAR, MDHAR and GR activities. The objective of the present work was to investigate whether or not other mechanisms could be involved in the tolerance of tobacco plantlets pretreated with AL to clomazone.

The findings confirm that clomazone induced a photooxidative stress as evidenced by Darwish et al. [5] (see the reduction in the F_v/F_m and PI_{abs} (Table 2), the Φ_{PSII} (Fig. 3A), and the ET0/ABS (Table 3)). This stress induced by clomazone results from alterations in the structure of the antenna LHC and its function, the function of reaction centers (RCs) and the redox state of PQ-pool (see ABS/RC, TR0/RC, DI0/RC, Table 2, and 1-V_J, Table 1). The impaired electron transport capacity (see the decrease in chloroplast oxygen-evolving rate, Table 1, and ETR (Fig. 3B)) consequently outcomes in the formation of H_2O_2 (Fig. 2A) at the level of PSII [51] and PSI (Mehler reaction). Higher H_2O_2 content in the leaves of the CL-treated plantlets could be toxic for cells, especially in absence of antioxidant system to scavenge the ROS, leading to programmed cell death (PCD). However, the symptoms of the PCD have well been observed in the sections from CL-treated plantlets at D_{24} (Fig. 6D).

Considering the ability of clomazone to reduce electron transport that limits the re-oxidation of NADPH and by consequent RuBP regeneration capacity, the P_n has decreased in the CL-treated plantlets (see Fig. 4A). Furthermore, a limitation in the g_s (Fig. 4B) with no impact on the P_n as well decreases in the Φ_{PSII} and ETR (Fig. 3A and B) mean that all final electron acceptors of the photosynthesis and photorespiration processes are affected by clomazone.

In this work, the accumulation of starch grains that showed the level of stress state displaying in the CL-treated plantlets (see Fig. 6C and D), maybe results in fewer consumption of triose phosphate that could be as a consequence of the limited growth. Although the production of phosphate trioses is thought to be limited (see the decrease of P_n), the starch accumulates. This could be as a cellular response mechanism to clomazone stress [52,53]. However, this mechanism as a specific cellular response to herbicides stress has been reported by the findings of Kaushik and Inderjit in mung bean [54].

In contrast, AL treatment enhanced the systemic capacity of the plantlets to avoid the photooxidative stress induced by clomazone. The AL treatment induced a moderate stress that is well observed at D_{10} by decreases in the F_v/F_m (Table 1) and the P_n (Fig. 4A) as well as

increases in the DI0/RC (Table 2) and the starch-grains accumulation (Fig. 6B). At this point, the decrease of the P_n at D_{10} accompanied by less production of trios phosphate could explain this starch-grains accumulation. In this regard, Graf et al. [55] concluded that the transient light/dark cycles by its effect on circadian clock regulation improves plant fitness and performance *via* adjusting carbon supplies that could cause starch accumulation. The starch-grains accumulation in plants cells was associated with a greater tolerance of their photosynthetic apparatus to oxidative stress [53].

The AL pretreatment proved to protect the plantlets against the photooxidative stress provoked by clomazone. The AL could enhance the electron flux beyond Q_A^- either by improving the PSII efficiency and regulation of the PQ-pool function (see the increase in the F_v/F_m, PI_{abs}, $1\text{-}V_J$ (Table 2), the ET0/RC and ET0/ABS (Table 3) and the Φ_{PSII} and ETR (Fig. 2B)) or by stimulating alternative electron pathways. Here, the post-illumination burst (PIB) process proves to be important [56]. At light/night transitions, the photorespiration (PR) is maintained while the continuous of photosynthesis (P). So the PR/P ratio under a large number of light-dark transitions during 24 h is higher. The photorespiration by the consumption photochemical energy, such as ATP and NADPH that limit formation of H_2O_2 is considered as one of protection mechanisms against photooxidative damage to PSII [57-59]. Although the decreases observed in the g_s, the C_i and the T during the two cycles of AL, the levels of P_n in the AL-treated plantlets at the first cycle were still as that of the control (Fig. 5B, C and D). This could be explained by the initiation of the photorespiration process. More, the decrease of R_s in the AL plantlets during the two cycles of AL, as well the increases of P_n in the control plantlets at the second cycle of AL can support our idea (Fig. 5A).

By stimulating the ROS detoxification, the AL pretreatment leads to decrease PSII damages (Fig. 2A). In this context, the findings of Darwish et al. [5] have reported that APX and MDHAR activities were continued to scavenging H_2O_2 in the AL+CL-treated plantlets. It is also well know that the activity of these enzymes is linked to the ascorbate recycling and synthesis as showed in AL-treated plantlets (see the ascorbate content, Fig. 2B). This indicated that the scavenging enzymatic system associated to the ascorbate synthesis and its recycling has been activated before clomazone treatment. In other hand, the AL pretreatment stimulated the synthesis of some phenolic compounds when treatment with clomazone (see the AL+CL treatment, Fig. 2C, D, E and F), such as the chlorogenic acid, neochlorogenic acid and the rutin, which proved to have a secondary role in the ROS detoxification. Considering the high generation of H_2O_2 induced by clomazone (see the H_2O_2 content, Fig. 2A), this has required involvement of secondary ROS-scavenging compounds to remove H_2O_2, especially

H_2O_2 diffused out of the chloroplast (or the peroxisomes). This could explain the motivated synthesis of such phenolic compounds [60,61].

In conclusion, the effects of AL through the increase of antioxidant activities associated to the ascorbate, and that coupled to the synthesis of phenolic compounds allow not only to detoxify the ROS and therefore to reduce the photooxidative stress provoked by clomazone, but also to prevent the PCD (see Fig. 6D). The alternation of light/dark pretreatment might be considered as an acclimation mechanism to nearest of priming approaches for agricultural applications.

5. Acknowledgements

This work was supported by the Syrian Ministry of Education and Research, and by laboratory of fruits and vegetables of university of Avignon and Vaucluse countries.

6. References

[1] A. Krieger-Liszkay, Singlet oxygen production in photosynthesis, J. Exp. Bot. 411 (2005) 337–346.
[2] R. Mittler, Oxidative stress, antioxidants and stress tolerance, Trends Plant Sci. 7 (2002) 405–410.
[3] T.S. Gechev, F. Van Breusegem, J.M. Stone, I. Denev, C. Laloi, Reactive oxygen species as signals that modulate plant stress responses and programmed cell death, BioEssays 28 (2006) 1091–1101.
[4] J. Cheeseman, Hydrogen peroxide and plant stress: a challenging relationship, Plant Stress 1 (2007) 4–15.
[5] M. Darwish, F. Lopez-Lauri, M. El Maataoui, L. Urban, H. Sallanon, Pretreatment with alternation of light/dark periods improves the tolerance of tobacco (*Nicotiana tabacum*) to clomazone herbicide, J. Photochem. Photobiol. 134 (2014) 49–56.
[6] C.A. Rice-Evans, N.J. Miller, G. Paganga, Antioxidant properties of phenolic compounds, Trends in Plant Sci. 4 (1997) 152–159.
[7] M. Tattini, C. Galardi, P. Pinelli, R. Massai, D. Remorini, G. Agati, Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress, New Phytol. 163 (2004) 547–561.
[8] M. Galleano, S.V. Verstraeten, P.I. Oteiza, C.G. Fraga, Antioxidant actions of flavonoids: Thermodynamic and kinetic analysis, Arch. Biochem. Biophys. 501 (2010) 23–30.
[9] G. Agati, E. Azzarello, S. Pollastri, M. Tattini, Flavonoids as antioxidants in plants: Location significance, Plant Sci. 196 (2012) 67–76.
[10] C. Brunetti, M. Di Ferdinando, A. Fini, S. Pollastri, M. Tattini, Flavonoids as Antioxidants and Developmental Regulators: Relative Significance in Plants and Humans, Int. J. Mol. Sci. 14 (2013) 3540–3555.
[11] D.C. Close, C. McArthur, Rethinking the role of many plant phenolics – protection from photodamage, not herbivores?, Oikos 99 (2002) 166–172.
[12] S. Pollastri, M. Tattini, Flavonols: old compound for old roles, Ann. Bot. 108 (2011) 1225–1233.
[13] A. Fini, L. Guidi, F. Ferrini, C. Brunetti, M. Di Ferdinando, S. Biricolti, S. Pollastri, L. Calamai, M. Tattini, Drought stress has contrasting effects on antioxidant enzymes activity and phenylpropanoid biosynthesis in *Fraxinus ornus* leaves: an excess light stress affair?, J. Plant Physiol. 169 (2012) 929–939.
[14] G. Agati, C. Brunetti, M. Di Ferdinando, F. Ferrini, S. Pollastri, M. Tattini, Functional roles of flavonoids in photoprotection: New evidence, lessons from the past, Plant Physiol. Biochem. 72 (2013) 35–45.
[15] Y. Sakihama, J. Mano, S. Sano, K. Asada, Y. Yamasaki, Reduction of phenoxyl radicals mediated by monodehydroascorbate reductase, Biochem. Biophys. Res. Commun. 279 (2000) 949–954.
[16] D. Wagner, D. Przybyla, R. Op den Camp, C. Kim, F. Landgraf, K.P. Lee, M. Wursch, C. Laloi, M. Nater, E. Hideg, K. Apel, The genetic basis of singlet oxygen-induced stress responses of *Arabidopsis thaliana*, Science 306 (2004) 1183–1185.

[17] C. Triantaphylides, M. Krischke, F.A. Hoeberichts, B. Ksas, G. Gresser, M. Havaux, F. van Breusegem, M.J. Mueller, Singlet oxygen is the major reactive oxygen species involved in photooxidative damage to plants, Plant Physiol. 148 (2008) 960–968.
[18] U. Takahama, Hydrogen peroxide-dependent oxidation of flavonols by intact spinach chloroplasts, Plant Physiol. 74 (1984) 852–855.
[19] Y. Sato, S. Itagaki, T. Kurokawa, J. Ogura, M. Kobayashi, T. Hirano, M. Sugawara, K. Iseki, In vitro and in vivo antioxidant properties of chlorogenic acid and caffeic acid, Int. J. Pharm. 403 (2011) 136–138.
[20] V.D. Kreslavski, G.N. Shirshikova, V.Y. Lyubimov, A.N. Shmarev, A.M. Boutanaev, A.A. Kosobryukhov, F.J. Schmitt, T. Friedrich, S.I. Allakhverdiev, Effect of preillumination with red light on photosynthetic parameters and oxidant-/antioxidant balance in Arabidopsis thaliana in response to UV-A, J. Photochem. Photobiol. 127 (2013) 229–236.
[21] A. Sebastian, M.N.V. Prasad, Red and blue lights induced oxidative stress tolerance promote cadmium rhizocomplexation in *Oryza sativa*, J. Photochem. Photobiol. 137 (2014) 135–143.
[22] X. Wang, J. Cai, F. Liu, T. Dai, W. Cao, B. Wollenweber, D. Jiang, Multiple heat priming enhances thermo-tolerance to a later high temperature stress via improving subcellular antioxidant activities in wheat seedlings, Plant Physiol. Biochem. 74 (2014) 185–192.
[23] X. Li, J. Cai, F. Liu, T. Dai, W. Cao, D. Jiang, Cold priming drives the sub-cellular antioxidant systems to protect photosynthetic electron transport against subsequent low temperature stress in winter wheat, Plant Physiol. Biochem. 82 (2014) 34–43.
[24] C. Ouhibi, H. Attia, F. Rebah, N. Msilini, M. Chebbi, J. Aarrouf, L. Urban, M. Lachaal, Salt stress mitigation by seed priming with UV-C in lettuce plants: Growth, antioxidant activity and phenolic compounds, Plant Physiol. Biochem. 83 (2014) 126–133.
[25] J.C. Merchuk, M. Ronen, S. Giris, S.M. Arad, Light/dark cycles in the growth of the red microalga *Porphyridium sp*, Biotechnol. Bioeng. 59 (1998) 705–713.
[26] U.J. Grobbelaar, Microalgal biomass production: challenges and realities, Photosynth. Res. 106 (2010) 135–144.
[27] C. Vejrazka, M. Janssen, M. Streefland, R.H. Wijffels, Photosynthetic efficiency of *Chlamydomonas reinhardtii* in flashing light, Biotechnol. Bioeng. 108 (2011) 2905–2913.
[28] A. Sanchez, J. Shin, S.J. Davis, Abiotic stress and the plant circadian clock, Plant Signal. Behav. 6 (2011) 223–231.
[29] M. Darwish, F. Lopez-Lauri, H. Sallanon, Study of photosynthesis process in the presence of low concentration of clomazone herbicide in tobacco (*Nicotiana tabacum L.*), J. Stress Physiol. Biochem. 9 (2013) 229–245.
[30] R. Murshed, F. Lopez-Lauri, H. Sallanon, Effect of water stress on antioxidant systems and oxidative parameters in fruits of tomato (*Solanum Lycopersicon* L, cv Micro-tom), Physiol. Mol. Biol. Plants 19 (2013) 363–378.
[31] R. Murshed, F. Lopez-Lauri, H. Sallanon, Effect of salt stress on antioxidant systems depends on fruit development stage, Physiol. Mol. Biol. Plants 20 (2014) 15–29.
[32] F. Charles, V. Vidal, F. Olive, H. Filgueiras, H. Sallanon, Pulsed light treatment as new method to maintain physical and nutritional quality of fresh-cut mangoes, Innovat. Food Sci. Emerg. Tech. 18 (2013) 190–195.
[33] D. Joly, R. Carpentier, Rapid isolation of intact chloroplasts from spinach leaves, in: R. Carpentier (Ed.), Photosynthesis Research Protocols, Springer, New York, 2011, 321–325.
[34] R.J. Porra, W.A. Thompson, P.E. Kriedemann, Determination of accurate extinction coefficients and simultaneous-equations for assaying chlorophyll-a and chlorophyll-b extracted with 4 different solvents–Verification of the concentration of chlorophyll standards by atomic-absorption spectroscopy, Biochim. Biophys. Acta 975 (1989) 384–394.
[35] K. Samuel, S. Bose, Immediate effects of pyridazinone herbicides on photosynthetic electron transport in algal systems, J. Biosci. 12 (1987) 211–218.
[36] J. Farineau, J.F.M. Gaudry, in: J.FM. Gaudry, J. Farineau (Eds.), Transferts d'électrons dans les thylacoides. Processus physiques, moléculaires et physiologiques, Versailles, Paris, 2011, 141–145.
[37] M. Kitajama, W.L. Butler, Quenching of chlorophyll fluorescence and primary photochemistry in chloroplasts by dibromothymoquinone, Biochim. Biophys. Acta 376 (1975) 105–115.
[38] M. Tsimilli-Michael, G.H.J. Kruger, R.J. Strasser, Suboptimality as driving force for adaptation: A study about the correlation of excitation light intensity and the dynamic fluorescence emission in plants, in: P. Mathis (Ed.), Photosynthesis: from Light to Biosphere, Kluwer Academic, The Netherlands, 1995, pp 981–984.
[39] C. Hermans, M. Smeyers, R.M. Rodriguez, M. Eyletters, R.J. Strasser, J.P. Delhaye, Quality assessment of urban Trees: A comparative study of physiological processes by following the website of the fluorescence by the test-OJIP, Plant Physiol. 160 (2003) 81–90.

[40] A.J. Strauss, G.H.J. Kruger, R.J. Strasser, P.D.R. Van Heerden, Ranking of dark chilling tolerance in soybean genotypes probed by the chlorophyll a fluorescence transient O-J-I-P, Environ. Exp. Bot. 56 (2006) 147–157.
[41] M.H. Kalaji, R. Carpentier, I.S. Allakhverdiev, K. Bosa, Fluorescence parameters as early indicators of light stress in barley, J. Photochem. Photobiol. 112 (2012) 1–6.
[42] B.J. Strasser, R.J. Strasser, Measuring fast fluorescence of the fluorescence transient in response to environmental questions: The JIP-test, in: P. Mathis (Ed.), Photosynthesis: from Light to Biosphere, Kluwer Academic Publishers, The Netherlands, 1995, pp 977–980.
[43] R.J. Strasser, A. Srivastava, M. Tsimilli-Michael, The fluorescence transient as a tool to characterize the situation photosynthetic samples, in: M. Yunus, U. Pathre, P. Mohanty (Eds.), Probing photosynthesis: mechanisms, regulation and adaptation, Taylor & Francis, London, UK, 2000, pp 445–483.
[44] R.J. Strasser, M. Tsimilli-Michael, A. Srivastava, Analysis of the chlorophyll fluorescence transient, in: G.C. Papageorgiou, Govindjee (Eds.), Chlorophyll Fluorescence: A Signature of Photosynthesis, Advances in Photosynthesis and Respiration, Springer, Dordrecht, 2004, pp 321–362.
[45] A. Stirbet, Govindjee, On the relation between the Kautsky effect (chlorophyll *a* fluorescence induction) and Photosystem II: Basics and applications of the OJIP fluorescence transient, J. Photochem. Photobiol. 104 (2011) 236–257.
[46] M. Xiang, S. Chen, L. Wang, Z. Dong, J. Huang, Y. Zhang, R.J. Strasser, Effect of vulculic acid produced by *Nimbya alternantherae* on the photosynthetic apparatus of *Alternanthera philoxeroides*, Plant Physiol. Biochem. 65 (2013) 81–88.
[47] B. Genty, J.M. Briantais, N.R. Baker, The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence, Biochim. Biophys. Acta 990 (1989) 87–92.
[48] B. Genty, J. Harbinson, N.R. Baker, Relative quantum efficiencies of the two photosystems of leaves in photorespiratory and non-photorespiratory conditions, Plant Physiol. Biochem. 28 (1990) 1–10.
[49] J.P. Krall, G.E. Edwards, Relationship between photosystem II activity and CO_2 fixation in leaves, Physiol. Plantarum 86 (1992) 180–187.
[50] M. El Maataoui, C. Pichot, Nuclear and cell fusion cause polyploidy in the megagametophyte of common cypress, *Cupressus sempervirens L*, Planta 208 (1999) 345–351.
[51] I. Vass, Molecular mechanisms of photodamage in the photosystem II complex, Biochim. Biophys. Acta 1817 (2012) 209–17.
[52] D.M. Romero, M.C. Ríos de Molina, A.B. Juárez, Oxidative stress induced by a commercial glyphosate formulation in a tolerant strain of *Chlorella kessleri*, Ecotoxicol. Environ. Safe. 74 (2011) 741–747.
[53] S. Muneer, T. HwanKim, B. ChulChoi, B. SeonLee, J. HyunLee, Effect of CO, NOx and SO_2 on ROS production, photosynthesis and ascorbate–glutathione pathway to induce Fragaria × annasa as a hyperaccumulator, Redox Biol. 2 (2014) 91–98.
[54] S. Kaushik, Inderjit, Phytotoxicity of selected herbicides to mung bean (*Phaseolus aureus* Roxb.), Environ. Exp. Bot. 55 (2006) 41–48.
[55] A. Graf, A. Schlereth, M. Stitt, A.M. Smith, Circadian control of carbohydrate availability for growth in Arabidopsis plants at night, Proc. Natl. Acad. Sci. USA 107 (2010) 9458–63.
[56] A.U. Igamberdiev, E. Romanowska, P. Gardeström, Photorespiratory flux and mitochondrial contribution to energy and redox balance of barley leaf protoplasts in the light and during light-dark transitions, J. Plant Physiol. 158 (2001) 1325–1332.
[57] B. Osmond, M. Badger, K. Maxwell, O. Björkman, R. Leegood, Too many photos: photorespiration, photoinhibition and photooxidation, Trends Plant Sci. 2 (1997) 119–121
[58] S. Takahashi, H. Bauwe, M. Badger, Impairment of the photorespiratory pathway accelerates photoinhibition of photosystem II by suppression of repair process and not acceleration of damage process in Arabidopsis thaliana, Plant Physiol. 144 (2007) 487–494.
[59] S. Takahashi, M. Badger, Photoprotection in plants: a new light on photosystem II damage, Trends Plant Sci. 16 (2011) (doi:10.1016/j.tplants.2010.10.001).
[60] P.M. Mullineaux, S. Karpinski, Signal transduction in response to excess light: Getting out of the chloroplast, Curr. Opin. Plant Biol. 5 (2002) 43–48.
[61] M.M. Mubarakshina, B.N. Ivanov, I.A. Naydov, W. Hillier, M.R. Badger, A. Krieger-Liszkay, Production and diffusion of chloroplastic H_2O_2 and its implication to signaling, J. Exp. Bot. 61 (2010) 3577–3587.