Étude de l'effet de faibles concentrations de clomazone sur les processus photosynthétiques chez le tabac (*Nicotiana tabacum* L.)

Étude de l'effet des faibles concentrations de clomazone sur les processus photosynthétiques chez le tabac (*Nicotiana tabacum* L.)

Ce chapitre est divisé à deux parties:

La première partie est présentée sous la forme d'un article publié dans *Journal of Stress Physiology and Biochemistry* (Darwish et al., 2013).

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Publication 1

Study of photosynthesis process in the presence of low concentrations of clomazone herbicide in tobacco (*Nicotiana tabacum* L.)

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L'objectif de ce travail a été de rechercher si les mesures des paramètres de fluorescence chlorophyllienne permettent de mettre en évidence l'existence d'un stress photooxydatif en réponse au clomazone chez deux variétés de tabac *Xanthi* et *Virginie*.

Expérimentation

Les plantules de chaque variété au stade trois feuilles ont été traitées par de faibles concentrations de clomazone (10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, 0 µM) pendant 14 jours. Le clomazone est ajouté à la solution nutritive qui est renouvelée tous les deux jours. L'expérimentation a été effectuée dans une chambre de culture, avec un cycle jour/nuit de 16 h/8 h, une température de 22 °C pendant le jour et 17 °C la nuit, une humidité relative de 60 % et une intensité lumineuse de 70 µmol photons m^{-2} s^{-1}. Les mesures effectuées ont été les dosages des pigments photosynthétiques (chlorophyll (*a*, *b*) et caroténoïdes) et les paramètres photosynthétiques calculés de test *OJIP* à partir des mesures de la cinétique rapide de la fluorescence chlorophyllienne.

Principaux résultats

Le clomazone réduit la teneur en chlorophylle totale (*a*+*b*) et en caroténoïdes, ainsi que la teneur en LHC (le ratio chlorophylle *a*/*b*). L'efficacité photochimique maximale (F_v/F_m) du photosystème II (PSII) diminue dans les feuilles jeunes par rapport aux feuilles adultes de manière plus importante chez *Virginie* que chez *Xanthi*. De très faibles concentrations (10^{-4} µM) affectent les paramètres du test *OJIP*. Leur sensibilité se révèle très efficace pour discriminer les variétés. Pour un grand nombre de paramètres, la variété *Virgini*e est plus affectée par le clomazone que la variété *Xanthi*.

Conclusion

Les résultats montrent que le clomazone induit un stress photooxydatif et que la variété *Virginie* est plus sensible au clomazone que la variété *Xanthi* bien que sa teneur en caroténoïdes soit plus élevée. Hors, l'effet toxique du clomazone est décrit comme étant le résultat de l'inhibition de l'activité de l'enzyme DXS, première enzyme de la voie des isoprénoïdes, voie qui conduit à la biosynthèse des caroténoïdes. La teneur en pigments chlorophylliens n'est donc pas le seul paramètre lié à la toxicité du clomazone.

Les mesures de la fluorescence de la chlorophylle *a* et du test *OJIP* montrent que plusieurs paramètres (PI_{abs}, $1–V_J$, ABS/RC, DI0/RC, TR0/RC, ET0/RC et ET0/ABS) sont affectés par le clomazone sans relation directe avec la synthèse des pigments.

Le test *OJIP* peut être utilisé avec efficacité pour étudier les effets du clomazone sur la chaîne de transport des électrons. La compréhension des mécanismes d'action du clomazone sur la chaîne de transport des électrons nécessite des études plus approfondies.

Study of photosynthesis process in the presence of low concentrations of clomazone herbicide in tobacco (*Nicotiana tabacum* L.)

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The effect of chemical residues of clomazone on photosynthetic processes has been studied by using several low concentrations of the herbicide (0, 1, 0.1, 0.01, 0.001, 0.0001 and 0.00001) µM and seedlings of two varieties of tobacco (*Nicotiana tabacum* L. *cv.Virginie vk51*; *Nicotiana tabacum* L. *cv. Xanthi*). The content of photosynthetic pigments, the parameters of the chlorophyll *a* fluorescence and the JIP-test were performed on an adult leaf (AL) and a young leaf (YL), which gave a complementary design to know the action's mode of clomazone on the plant physiological processes. Clomazone reduced the total chlorophyll (*a+b*), carotenoids pigments (reduction in size antenna pigments judged by an increase in the chlorophyll *a/b* ratio) in young leaves more than adult leaves. The maximal photochemical efficiency (F_v/F_m) of photosystem II (PSII) decreased significantly in young leaves compared to adult leaves and in *Virginie* than *Xanthi*. Among the parameters calculated of the JIP-test most affected by the treatment, PI_{abs}, $1–V_J$, ABS/RC, DI0/RC, TR0/RC, ET0/RC, ET0/ABS, which indicated a comparable effects of clomazone(1 µM, 0.1 µM, 0.01 µM) between the two types of leaves and the varieties used. More, the results showed that the concentration (1 µM) was the most effective among the other low concentrations used and the *Virginie* variety is more sensitive than the *Xanthi* variety. We conclude that clomazone has probably two combined functions (physiological, toxic) judged by the different behavior of both types of leaves in the presence of the herbicide.

Keywords*: Chlorophyll fluorescence; JIP-test; Photosynthesis; Photosystem II; Tobacco*

1. Introduction

The plant uses light energy to synthesize organic compounds. The light reactions of photosynthesis in higher plants are conducted through the cooperation of two photosystems, PSI and PSII, which are physically distinct in the stroma and grana of the thylakoid membrane, respectively (Whitmarsh and Govindjee 1999; Dekker and Boekema 2005). Today, because of the current development in agricultural production, pesticides and herbicides that are named plant protection products have become a major environmental danger which menaces the plants on earth's life. Herbicides are chemicals commonly used to control weeds in agriculture and most of them are continually discharged into aquatic environments through surface runoff (Kloeppel et al., 1997). Many studies have been performed to determine the action's mode of herbicides in the production of singlet oxygen ($^1O_2$) and the three intermediate redox states (superoxide, peroxide and hydroxyl radical) which are more reactive (Wong 2000; Frankart et al., 2003). Other studies have been conducted in particular to elucidate the action's mechanism of herbicides on plant physiology (growth rate, pigment content and chlorophyll fluorescence) and vegetable production (Waldhoff et al., 2002; Kaňa et al., 2004; Walters and young 2010; Souza et al., 2012). In the case of plants exposed to herbicides, and under a continuous light, electron transport will be blocked and Q_A will stay in reduced state, promoting the formation of reactive oxygen species, such as ($^1O_2$), *via* chlorophyll triplet ($^3Ch^*$) (Rutherford and Krieger-Liszkay 2000; Fufezan et al., 2002). During recent years, an intensive use of herbicides has raised increasing concern mainly because of their massive pollution of the environment. Glyphosate, Clomazone and Paraquat are the most widely used broad-spectrum herbicides (Rosso et al., 2010).

Clomazone [2-(2-chlorobenzyl)-4,4-dimethyl-1,2-oxazolidin-3-one] is a pre-emergence herbicide used against broad-leafed and grassy weeds (Chang and Konz 1984; Warfield et al., 1985). It is widely used to control weeds in canopies of soybean, cotton, sugar cane, maize, rice, tobacco (Chang et al., 1997). Clomazone is used in 78% of the planted area. Before flooding the fields for cultivation of the rice, clomazone alone or in combination with other herbicides is applied for control of weeds (Saldain and Deambrosi 2009). Clomazone is absorbed by roots and emerging shoots, and transported with the transpiration stream in the xylem (Senseman 2007). It is generally accepted that clomazone prevents the accumulation of chloroplast pigments and plastidic isoprene evolution (Duke and Kenyon 1986; Zeidler et al., 2000). All results showed that the impact of clomazone on carotenoids and chlorophyll

biosynthesis leads to bleaching of leaves. Recently it was shown that the toxicity of clomazone is not induced by clomazone itself, but rather by its break down product, 5-ketoclomazone that blocks 1-deoxy-Dxylulose-5-phosphate (DXP) synthase, the first enzyme of the plastidic isopentenyl diphosphate(IPP) synthesis pathway (Mueller et al., 2000; Ferhatoglu et al., 2001). More, (Souza et al., 2012) indicated that combined clomazone with ametryn herbicide had caused an oxidative stress in the *Emilia coccinea* plants. In reality, most the experiments which have used high concentrations of clomazone indicate the toxic effect of clomazone on plastid enzymes (DXP, MEP, IPP) and therefore on the biosynthesis of photosynthetic pigments (chlorophyll (*a*, *b*) and carotenoids). Limited reports have been published on the effects of low concentrations of clomazone on photosynthesis using chlorophyll *a* fluorescence parameters and particularly in cultivar tobacco. The aim of this work was to study the low concentrations' effects (chemical residues which may exist in the environment) on the physiological process by using several low concentrations of the clomazone. And also if the herbicide leads to an oxidative stress by special regard to changes in photosynthetic pigments, parameters of the chlorophyll *a* fluorescence and by using the JIP-test as a method to evaluate the photosynthesis in adult leaves and young leaves of tobacco's seedling growing in the medium containing clomazone.

2. Materials and methods

2.1. Plant material and chemical treatment

Seeds of two varieties of tobacco (*Nicotiana tabacum* L. *cv. Virgenie vk51*; *Nicotiana tabacum* L. *cv. Xanthi*) were cultivated in soil for two weeks. Germination was carried out in sterile conditions. Tobacco seedlings were obtained in three leaves stage after 5 weeks of the germination. Then, seedlings were placed for 14 days in a nutrient solution of Auckland (1 M KNO_3 (101 g/L) 1 M $Ca(NO_3)_2.H_2O$ (236 g/L) 1 M $MgSO_4.7H_2O$ (246.5 g/L) 1 M KH_2PO_4 (136.08 g/L), 0.01 M FeEDDHA (4.352 g/L)) with low concentrations (0, 1, 0.1, 0.01, 0.001, 0.0001, 0.00001) µM of the clomazone herbicide (Sigma-Aldrich, USA) in tubes (15 mL). The culture solution was refreshed two times a week. The plants were grown in a growth chamber with temperature of 22/17 °C, humidity 60%, 16 h light/8 h dark cycle and the flux of photons (PAR) 70 µmol m^{-2} s^{-1}, to minimize the effect of light and its role in the phenomenon of bleaching, (Duke and Kenyon 1986) shown that the use of the light intensity 150 µmol photons PAR m^{-2} s^{-1} caused the photobleaching in seedlings of cowpea primary.

2.2. Determination of chlorophyll and carotenoids content

Total chlorophyll (*a*+*b*) and the total carotenoids were determined by extraction using pure acetone (100%) as solvent. Samples were incubated 15 min on ice. Then the samples were centrifuged 5 min at 15000 *g* and 4 °C. Quantification of chlorophyll and carotenoids was performed immediately after extraction. Absorbance readings were made at 662 and 645 nm for chlorophyll pigments and 470 nm for carotenoids. The content was calculated using the formula of Lichtenthaler (1987).

2.3. Chlorophyll fluorescence and the JIP-test

The behavior of photosystem II (PSII) can be evaluated using a rapid kinetics of the chlorophyll *a* fluorescence emitted by the leaves of plants adapted to darkness. The Chlorophyll *a* fluorescence was used to evaluate the state of seedlings in two different varieties of tobacco. The chlorophyll *a* fluorescence was determined in the dark, seedlings are adapted for 20 minutes in the dark, an adult (AL) and a young leaf (YL) was taken from each seedling for the measurement. The chlorophyll *a* fluorescence (expressed in relative units) was measured using a portable Handy (PEA, Hansatech, UK). A strong pulse of light (3,000 µmol m^{-2} s^{-1}) is used to determine the chlorophyll *a* fluorescence. The fluorescence parameters were calculated automatically, $\Phi p0 = F_v/F_m = (F_m – F_0)/F_m$, which represents the maximal quantum yield photochemical of PSII (Kitajama and Butler 1975), and the quantum yield of open centers of PSII (Maxwell and Johnson 2000). The JIP-test was performed on all measures of fluorescence transient and is based on a simple model, how the flux of photons absorbed by photosynthetic pigments antenna (ABS) is dissipated as heat (DI) and fluorescence or transported as the trapped flux (TR) of reaction centers to be converted into energy redox by reducing the plastoquinone (Q_A) to (Q_A^-). Then (Q_A^-) is reoxidized to (Q_A), therefore the creation of an electron transport (ET) that leads to CO_2 fixation (Heerden et al., 2004). These fluxes are expressed in specific energy flux (per reaction center) or as proportions of other flux (ratios or yields). The values of fluorescence are corresponding to time intervals, the steps O-J-I-P were recorded and used as data origins in the JIP-test (Strasser et al., 2000). Including: the fluorescence intensity maximum (F_m), the fluorescence intensity at 50 µs (F_0), 300 µs (F_{300}), and 2 ms (F_J). The calculation of energy flux and ratios of specific flux was fully explained by Strasser and coworkers (Strasser and Strasser 1995; Tsimilli-Michael et al., 1995; Strasser et al., 2000; Hermans et al., 2003).

Flux of specific energy expressed by the reaction center (RC)

Slope at the origin of the of normalized fluorescence rise Slope at the origin of the of normalized fluorescence rise: **$M0 = (F_{300}–F_0) / (F_m–F_0) / 0, 25$ ms**

Observed rate of Q_A reduction: **$TR0/RC = (M0/V_J) = (ABS/RC) (F_v/F_m)$**

Rate of electron transport beyond Q_A^-: **$ET0/RC = (TR0/RC) (1–V_J)$**
$= (TR0/RC) (ET0/TR0)$

Rate of photon absorption: **$ABS/RC = (TR0/RC) / [(F_m–F_0)/F_m]$**

Rate of heat dissipation: **$DI0/RC = (ABS/RC) – (TR0/RC)$**

Relative variable fluorescence at 2 ms: **$V_J = (F_{2ms}–F_0) / (F_m–F_0)$**

Efficiencies (or flux ratios)

Maximum efficiency with which an absorbed photon results in Q_A reduction:
$TR0/ABS = (TR0/RC) / (ABS/RC) = (F_m–F_0)/F_m$

Efficiency with which an absorbed photon results in electron transport beyond Q_A^-:

$ET0/ABS = (ET0/RC) / (ABS/RC)$

Density of reaction centers per the chlorophyll Functional reaction centers per cross-sectional leaf area: **$RC/ABS = (RC/TR0) (TR0/ABS)$**

Efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A^-: **$ET0/TR0 = (ET0/RC) (TR0/RC) = 1–V_J$**

Performance index

Compound function of light energy absorption, efficiency of Q_A reduced and conversion energy to electron transport:

$PI_{abs} = [RC/ABS] [(TR0/ABS)/(F_0/F_m)] [(ET0/TR0)/V_J]$

The subscript "**0**" indicates the quantification of PSII behavior at the onset of fluorescence induction.

ABS is proportional to the concentration of photosynthetic pigments of chlorophyll antenna Chl_{ant}.

2.4. Statistical analysis

Differences between treatment means were compared using the Wilcoxon test with R statistical software at the 95% probability. Data are presented for values ±SE. The Significance levels are represented by letters (a, b, c, d) to determine significant differences between treatments.

3. Results and discussion

For a long time, carotenoids have been given a central role in photoprotection of the photosynthetic apparatus by limiting the destructive reactions ($^1O_2$) or by quenching the triplet state of Chl (Chl*), which is sensitive to the formation of reactive oxygen species (Cogdell et al., 1992; Telfer et al., 1994; Bartley and Scolnik 1995). Levels of chlorophyll (Chl *a*, Chl *b*) and total carotenoids were significantly reduced in leaves of the both tobacco's varieties at the highest concentration of clomazone (1 µM) used (Fig. 1C, D, G and H). Agrees with previous results showing that the photosynthetic pigments (Chls, carotenoids) decreased with increasing the concentration of clomazone (Lutzow et al., 1990; Weimer et al., 1992; Kaňa et al., 2004). But we find that the decrease in chlorophyll and carotenoids is more significant in young leaves of *Virginie* variety (Chls 55.77% (*Xanthi*), 77.91% (*Virginie*), carotenoids 51.09% (*Xanthi*), 73.61% (*Virginie*)) than adult leaves of *Xanthi* variety (Chls 5.78% (*Xanthi*), 38.88% (*Virginie*), carotenoids 3.08% (*Xanthi*), 33.82% (*Virginie*)). Clomazone caused a significant increase in ratio (chl *a/b*) in young leaves at the concentration (1 µM) in *Xanthi* variety and (1, 0.1 µM) in *Virginie* variety (Fig. 1B and F). The increase in ratio (chl *a/b*) indicates that the amount of pigments antenna (LHCII) reduced in the PSII of the thylakoid membrane (Lichtenthaler et al., 1982; Anderson et al., 1998; Kaňa et al., 2004). In addition, with (1 µM) of clomazone, there is a significant reduction in the ratio (chl (*a*+*b*)/car) in both type of leaves in *Virginie*, compared with *Xanthi* which shows a reduction not significant in the young leaves (Fig. 1A and E). This reduction expresses that carotenoids are more affected than Chls by the herbicide.

Chlorophyll fluorescence has been widely used in research of photosynthesis, such as studying the effect of several herbicides on the photosynthetic performance (Frankart et al., 2003; Kaňa et al., 2004; Vaillant-Gaveau et al., 2007). A significant decrease in (F_v/F_m) was observed at the (1 µM) treatment in adult leaves of *Virginie* variety and it was not significant in *Xanthi* variety, (Fig. 2C and F). Related to young leaves, F_v/F_m decreased significantly at the concentration (1 µM) (F_v/F_m= 0.279 (*Virginie*); F_v/F_m= 0.454 (*Xanthi*). This result don't

corresponds with result of Kaňa et al. (2004) showing that clomazone cause a small reduction not significant in F_v/F_m, and with results of Duke and Kenyon (1986), who observed no direct effect of clomazone on electron transport in PSII. In addition, the performance index (PI_{abs}) had provided information on the seedlings tobacco's state treated with clomazone. We notice that clomazone (1 µM, 0.1 µM) affected both types of leaves, and the young leaves were the most affected. We found that there is a large reduction compared to the control for this parameter ((1 µM) of clomazone PI_{abs}= 0.054 for (*Virginie*) and PI_{abs}= 0.163 for (*Xanthi*)) (Fig. 2B and E). This parameter is related to one hand with the density of reaction centers per chlorophyll, and the other hand with the maximum quantum yield (TR0/ABS) and the efficiency with which a trapped electron can go beyond Q_A^- in chain electron transport (1–V_J= ET0/TR0) (Strasser et al., 2000).

The J step considered to be associated with an accumulation of $Q_A^-Q_B^-$ from as demonstrated by experimental results and theoretical simulations. Therefore it is necessary to follow the JIP-test to determine whether the effect of the herbicide on the photosynthetic apparatus is limited on the toxicity of clomazone, which inhibits the synthesis of (Chls, carotenoids), or there is another effect on the process of photosynthesis (the status of electron transport) accordingly. The JIP-test has been used to access the sensitivity of plants to different environmental conditions (Tsimilli-Michael et al., 1995; Krüger et al., 1997; Ripley et al., 2004; Appenorth et al., 2001; Strauss et al., 2006; Bussotti et al., 2007). Our study confirmed that electron transport at the acceptor side of PSII was a clomazone-induced inhibitory site when tobacco's seedlings exposed to clomazone. A decrease value in the efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A^- (1–V_J= ET0/TR0) in exposure to (1 µM) clomazone in both young and adult leaves. But, the decrease was more pronounced in the young leaves and the *Virginie* variety (Fig. 2A and D). A significant increase in photons absorbed by the reaction center (ABS/RC) was observed in adult leaves of *Virginie* at the concentration (1 µM), compared to *Xanthi* and other concentrations of the herbicide. In the young leaves, the effect of clomazone (1 µM) touched the two varieties with a greater increase of (ABS/RC) in *Virginie* variety (Fig. 3E and J), agrees with Hermans et al. (2003), who noted that the total flux of photon absorbance of PSII by the number reaction center (ABS/RC) was decreased in plants collected, which have a poor performance, and with Xia and Tian (2009), who reported that (ABS/RC) increased in *Chlorella pyrenoidosa* exposed to excess Cu. The parameter (TR0/RC) is proportional to the excitation flux trapped by the reaction center (RC) (Strasser et al., 2000). A significant increase (TR0/RC) was only observed in adult leaves of the variety *Virginie* with clomazone

treatment (1 µM) (Fig. 3D). In the young leaves, (1 µM) of clomazone increased the energy trapped by the reaction center in both varieties, but the increase was greater in *Virginie* than *Xanthi* (Fig. 3I). This result is contradictory with Hermans et al. (2003), indicating that the excitation flux trapped by reaction centers of the PSII reduced with a poor performance of the plants collected. Our result corresponds to results obtained by Kaňa et al. (2004), who found that the (TR0/RC) parameter increased (30%) for barley plants cultivated in the presence of 500 µM clomazone compared to the control, that can be due to the increase in yield of light in the leaves, this may caused the acceleration of supply and excitation to the reaction center. In our case, this increase (TR0/RC) is due to the increase in photons absorbance (ABS) flux compared with number of reaction centers (RC). Energy dissipated into heat by the reaction center was only significantly increased in adult leaves treated with (1 µM) of clomazone in *Virginie* (Fig. 3C). In young leaves, (1 µM) clomazone leads to increase levels of energy dissipation (DI0) by the reaction center (RC), and the more increase was in *Virginie* (DI0= 30.27) compared to *Xanthi* (DI0= 3.59) (Fig. 3H). We find nevertheless an increase in the energy trapped by the reaction center. There is an important amount of energy that was dissipated as heat and this corresponds to Hermans et al. (2003) who reported the increase of energy dissipated into heat for plants of poor performance compared with control, agrees with Khamis et al. (1990) and Verhoeven et al. (1997), who indicated that the increase of dissipation into heat of the excited energy is probably due to the intervention of the xanthophyll cycle under conditions of treatment (a deficiency of nitrogen and light intensive). In addition, it is possible that the dissipation of energy into heat by the reaction center has increased with the reduction of carotenoids (LHCs), and therefore it has reduced the ability of pigments to protect the xanthophyll cycle (Kaňa et al., 2004). The electrons transported (ET0) by the reaction center (RC) were significantly reduced for adult leaves in *Virginie* at (1 µM) of clomazone and no difference was found in *Xanthi* (Fig. 3B). In young leaves, clomazone (1 µM) decreased the electrons transported beyond Q_A^- in both varieties, but the high decrease was in *Virginie* (50.79% compared to the control) compared to *Xanthi* (7.21% compared to the control) (Fig. 3G).

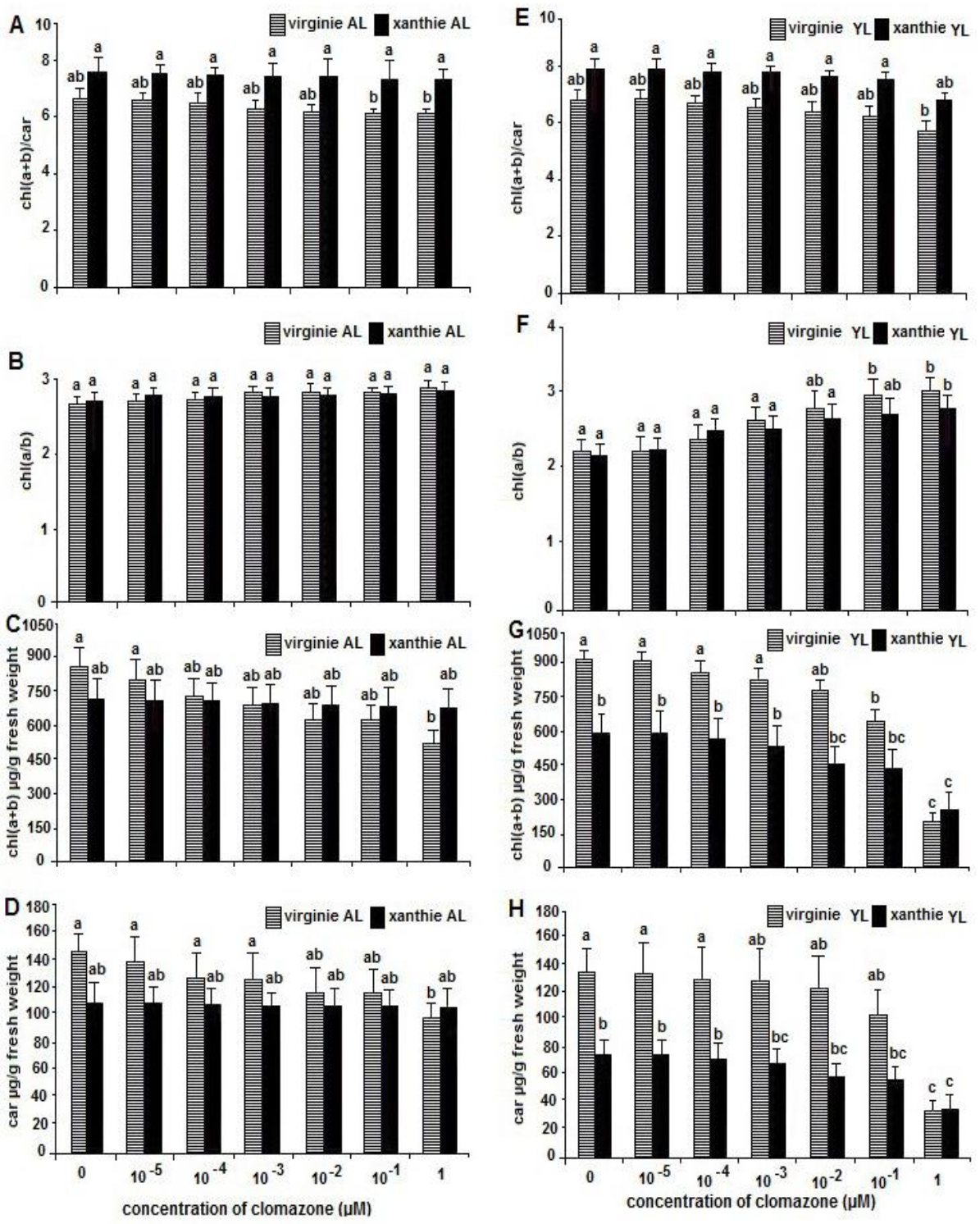

Fig 1. Influence of several low concentrations of clomazone on pigment content in leaves of two varieties of tobacco. All data represent means, SE for n= 8 and different letters (a, b, c, d) to determine significant differences between the treatments.

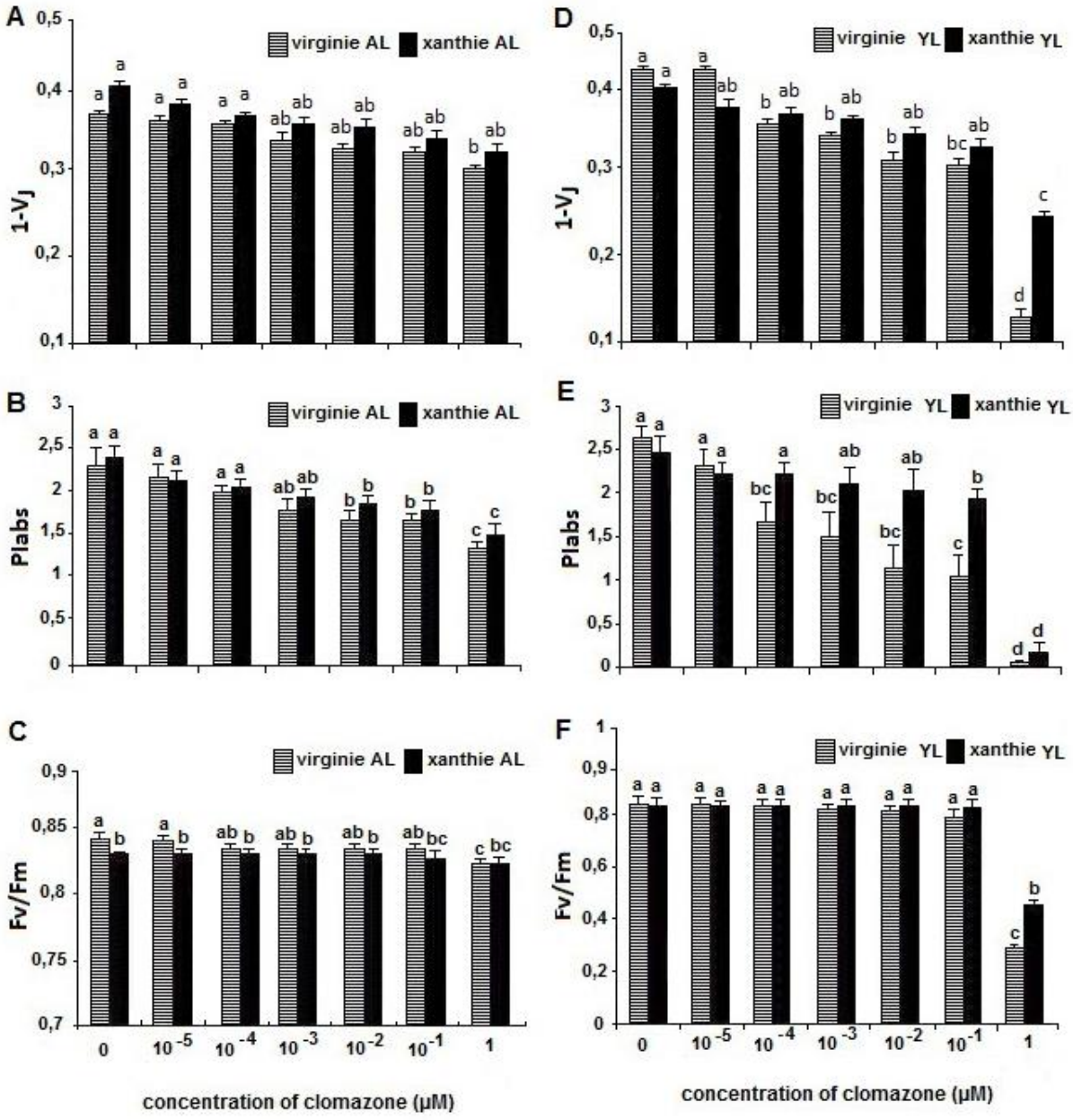

Fig 2. Effect of clomazone treatments in leaves of two varieties of tobacco on Chl fluorescence intensity (F_v/F_m, maximal effeciency photochemical of PSII) and on the performance index (PI_{abs}) in the J step of fluorescence rise.

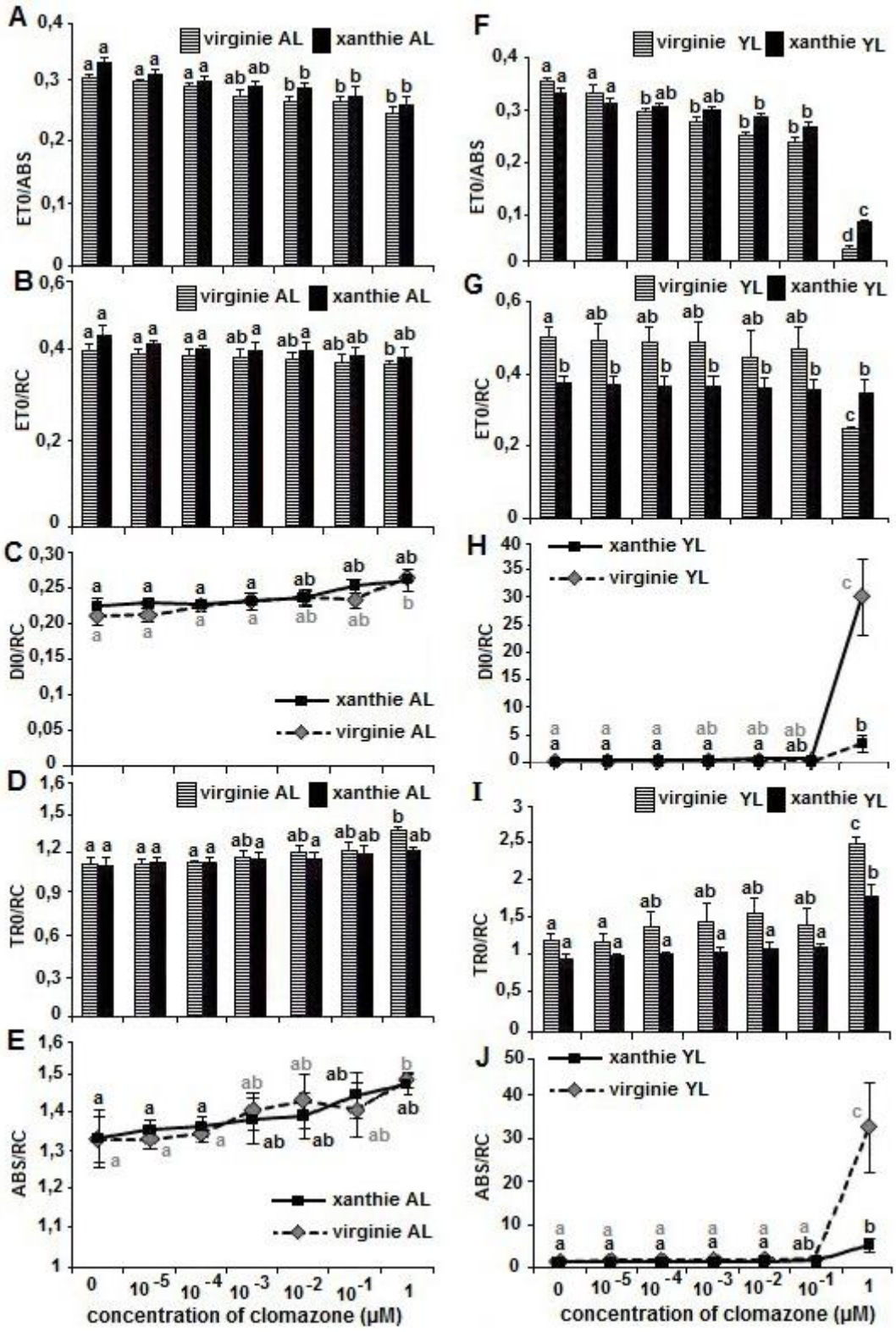

Fig 3. ABS/RC, TR0/RC, DI0/RC, ET0/RC and ET0/RC in the seedlings leaves of two varieties of tobacco, which are treated by several low concentrations of clomazone. All data represent means, SE for n= 8 and different letters (a, b, c, d) to determine significant differences between the treatments.

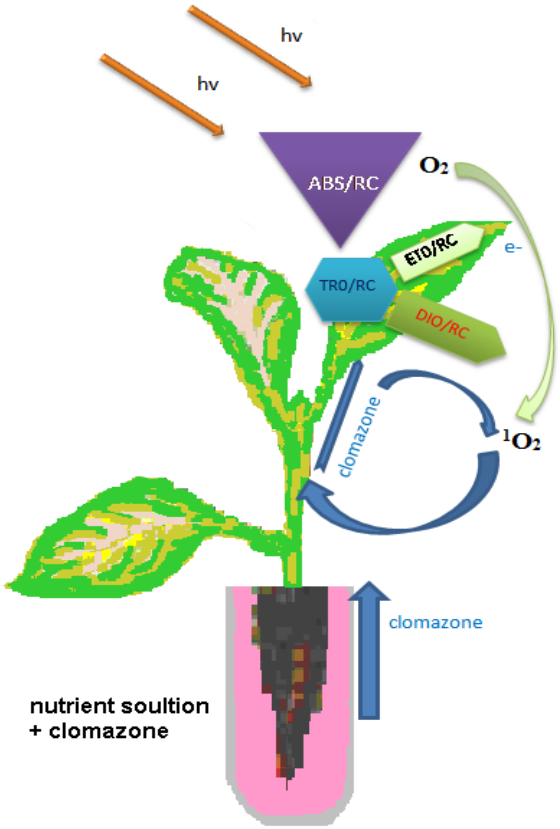

Fig 4. Energy pipeline models of the performance of tobacco seedlings treated with clomazone herbicide. The leaf models show the phenomenological fluctuations which are expressed as total absorption flux of PSII antenna chlorophyll (ABS), energy flux trapping (TR0), energy dissipated into heat (DI0) and electron transport flux (ET0) divided by the number of active reaction centers (RCs) (in the sense of Q_A reduction).

Even though, there is a good capture of energy excited by the reaction center (TR0/RC was increased), but still the number of electrons transported by the reaction center has been reduced, according to Hermans et al. (2003) who showed that the parameter (ET0/RC) was decreased in the leaves of plants that have a low performance (PI_{abs}).

In addition, the large excited energy dissipation heat can explain the reduction in the transfer of electrons from the reaction center (ET0/RC). The electrons transported (ET0) beyond the Q_A^- by photons flux (ABS absorbed by photosynthetic pigments antenna Chls) were reduced more significantly in young leaves than adults leaves. This decrease was found at clomazone treatments (1, 0.1, and 0.01) µM in both *Virginie* and *Xanthi* varieties (Fig. 3A). In the young leaves, *Virginie* was affected more than *Xanthi* with the concentration (1 µM) of clomazone, and there is a significant difference between the two varieties with this treatment (Fig. 3F). As (ET0/ABS) is related to the density of chlorophyll per reaction center (RC/ABS) and the electrons transported by the reaction center (ET0/RC). It is normal that the number of

electrons transported (ET0) by the flux of photons absorbed (ABS) decrease, with a decrease found in number of electrons transported (ET0), the increase in the dissipation into heat (DI0/RC) (Appenorth et al., 2001), and the decrease in total chlorophyll. Hermans et al. (2003) found a good relationship between the relative performance of the electron transferred (ET0/ABS) and the amount of chlorophyll in the leaves of plants selected.

4. Discussion

The effect of clomazone herbicide on the process of photosynthesis and photosynthetic pigments content has already been studied in barley seedlings by Kaňa et al. (2004), who measured several fluorescence's parameters of chlorophyll *a* (F_0, F_m, F_v/F_m, V_J, 1–*q*P, *q*n, Rfd, TR0/RC). In this work, we used the content of pigments (carotenoids, chlorophyll (*a*+*b*), chl *a*/*b*, chl (*a*+*b*)), the parameters of the fluorescence of chlorophyll *a* (F_0, F_m, F_v/F_m) and the parameters of the JIP-test (PI_{abs}, $1–V_J$, ABS/RC, TR0/RC, DI0/RC, ET0/RC, ET0/ABS) as highly relevant to well determine the effect of clomazone on photosynthesis in two varieties of tobacco. For this, all measurements were performed on an adult and a young leaves. Chlorophyll and carotenoids are strictly correlated with each other, because carotenoids have an important role in protecting the chlorophyll from photooxidation during the growth (Biswall 1995).

Our work confirms that clomazone reduces the content in pigments of the leaves (total chlorophyll, carotenoids) with the highest concentration used (1 µM) in young leaves for both varieties and in adult leaves for *Virginie* variety. A similar decrease was obtained with the high concentration of clomazone in adult leaves of tomato and tobacco (Scott et al., 1994) and seedlings' adult leaves of barley (Kaňa et al., 2004). But in our case, the difference between the two types of leaves means that clomazone probably affects photosynthesis. Especially we observed in *Xanthi* variety that clomazone had not any effect significant to decrease the pigments content in adult leaves, and its effect was significant in young leaves. It gives us an indication that clomazone probably retards the kinetics of photosynthesis, thus the biosynthesis of pigments in young leaves. The behavior of *Virginie* variety which more sensitive support this idea, there is an effect on adult leaves and the effect was multiplied on young leaves. It means that the efficiency of photosynthesis is reduced more with a first influence on photosynthesis. The second influence comes from the reduction in the biosynthesis of photosynthetic pigments, and this probably related to the inhibition of enzymes, which synthesize the chlorophyll and carotenoids. After reduced the levels of

pigments in the leaves, the efficiency of photosynthesis decreases through the reduction of photons absorbed. The correlation between changes in the ability to absorb of photons and parameters of photosynthesis was also observed for leaves growing with different irradiation conditions (Kurasová et al., 2002). Although the parameters of chlorophyll *a* fluorescence, as F_0, F_m, F_v/F_m, none gives often satisfactory results. The F_v/F_m is often not correlated with the chlorophyll content of leaves (Maxwell and Johnson 2000; Lazár 2006). This parameter is an indicator of stress (Maxwell and Johnson 2000). A decrease in F_v/F_m could be due to an increase in protection by increasing non-radiative energy dissipation or damages of photoinhibition of the reaction center of PSII (Maxwell and Johnson 2000). In our work, the F_v/F_m is a good indicator that the seedlings are under stress. Especially, to follow this parameter in an adult and a young leaves, where we find that *Virginie* was more affected for both type of leaves. But the decline in F_v/F_m was higher in young leaves. This also supports our idea, that the effect of clomazone is not limited to its toxic effect, but also it has another function, which affects the PSII reaction center.

In addition, to study the effect of clomazone on the transfer of electrons from the acceptor side of PSII, the JIP-test was used to explain the progressive flux of energy through the reaction center's level of PSII. This test was used for a wide range of physiological processes, such as evaluation of the quality of urban trees in a comparative study of physiological processes by following the flux of energy by the JIP-test (Hermans et al., 2003), the quantification of photosynthetic performance of phosphorus deficiency with the kinetics of fluorescence of chlorophyll *a* in sorghum (Ripley et al., 2004), and measurements of chlorophyll florescence to evaluate the time and storage temperature of the leaves of vegetables Valeriana (Ferrante and Magiore 2007). Among several indices that can be calculated with JIP-test, PI_{abs}, $1–V_J$, ABS/RC, TR0/RC, DI0/RC, ET0/RC, ET0/ABS, they are the best parameters that showed differences between tobacco's seedlings for the two varieties (*Virginie* and *Xanthi*) and both types of leaves (Fig. 2,3).

The PI_{abs} encompasses fluorescence transient changes associated with changes in antenna conformation and energy fluctuations, therefore PI_{abs} helps to estimate plant vitality with high resolution (Hermans et al., 2003). The PI_{abs} indicated a difference response of PSII in the adult and the young leaves treated with clomazone (1, 0.1, and 0.01) µM. The *Virginie* variety was the most affected according to this parameter, and the decline in the PI_{abs} was higher in young leaves than adult leaves, but for the another variety *Xanthi*, this parameter was reduced only in young leaves. This gives an indicator that the seedlings of tobacco were in stressed state.

In addition, it is normal to find the PI_{abs} decreases as a result of the reduction in the maximum efficiency of PSII (F_v/F_m= TR0/ABS) and efficiency with which a trapped exciton can move an electron into the electron transport chain further than Q_A^- ($1–V_J$). The flux of absorbance energy (ABS) and energy dissipation (DI0) by the reaction center increased at the concentration (1 µM) in both types of leaves in *Virginie* variety and in young leaves in *Xanthi* variety. These results suggest that Chl antenna size of PSII might decreased by clomazone treatments, agrees with Kaňa et al. (2004), who confirmed that clomazone reduced the formation of pigment-protein complexes in the thylakoid membrane, and Din et al. (2012) reported that reduction of antenna size led to a lower chlorophyll content and the chlorophyll loss had little effect on the antenna size. Although, there is an increase in (TR0/RC), and reduction in (ET0/RC) and (ET0/TR0) in clomazone treatment (1 µM), that may be a result of the inactivation of reaction centers (RCs), conversion of excitation energy absorbed into heat (DI0), and partially to the transfer of excitation energy with the PSII antenna. Din et al. (2012) indicated that the transfer excitation energy with the antenna does not impose a rate limitation on the trapping of energy (TR0).

5. Conclusion

Chlorophyll *a* fluorescence parameters, followed by the JIP-test can be very useful in detecting the effects on the plant physiological processes caused by low concentration of clomazone (1 µM, 0.1 µM, 0.01 µM), and its gave us an alternative view on the action mechanism of clomazone on photosynthesis in seedlings of tobacco treated. Our work showed that there is a difference between the two varieties treated with clomazone, and *Virginie* variety was more sensitive than *Xanthi* variety. We find that the difference between the two types of leaves in response to clomazone treatments is probably due to effect of two functions of clomazone: the first function on the kinetics of photosynthesis and in particular the reaction center of PSII. It is probable that the clomazone blocks electron transfer beyond Q_A^- (see Fig. 2A and D), hence the formation of chlorophyll triple state (^{3}Chl*) is expected, leading to formation of singlet oxygen ($^1O_2$). The accumulation of ($^1O_2$) increasingly contributes to oxidative stress that touches photosynthesis and the biosynthesis of photosynthetic pigments, the adult leaves response in presence of clomazone supports this idea. In addition, through the time, accumulation of ($^1O_2$) and the other (ROS) may help trigger the second toxic function of the herbicide. Ferhatoglu and Barrett (2006) observed that clomazone was metabolized and oxidized in plants (clomazone→ clomazone-OH→ keto-clomazone), then ketoclomazone

inhibits DXP synthase, the first enzyme in the chain of the isoprenoid biosynthesis of chloroplast, which can also confirms our idea Fig.4.

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Publication 2

Tolerance to Clomazone Herbicide is linked to the state of LHC, PQ-Pool and ROS Detoxification in Tobacco (*Nicotiana tabacum* L.)

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Nous avons observé précédemment que bien que plus riche en caroténoïdes, la variété *Virginie* est plus sensible au clomazone que la variété *Xanthi*. Le clomazone est connu pour inhiber la première voie de synthèse des isoprénoïdes et donc des caroténoïdes. Par ailleurs, le rôle important des caroténoïdes dans les mécanismes de photoprotection a été largement décrit. Il semble donc que le stress photooxydatif du clomazone n'est pas directement et seulement lié à son action sur la synthèse des caroténoïdes.

L'objectif des travaux présentés dans ce chapitre a donc été d'approfondir les effets du clomazone et d'identifier les mécanismes liés à la tolérance de la variété *Xanthi*.

Le fonctionnement de la chaîne de transport des électrons a été étudié:

- au niveau du PSII et de l'émission des électrons jusqu'aux plastoquinones, par les mesures de la fluorescence et le test *OJIP*.
- au niveau de la chaîne de transport du PSII au PSI par des mesures d'évolution d'O_2 par des chloroplastes isolés.

Les activités antioxydantes des enzymes impliquées dans la protection contre le stress oxydatif au clomazone ont également été mesurées. Enfin, des analyses cytohistologiques ont été effectuées pour visualiser dans les tissus l'impact du clomazone chez les deux variétés.

Expérimentation

Deux variétés de tabac *Nicotiana tabacum* L. ont été utilisées: la variété *Xanthi* plus tolérante au clomazone et la variété *Virginie vk 51* plus sensible. Les plantules de chaque variété au stade trois feuilles ont été traitées par une faible concentration de clomazone (1 µM) pendant 14 jours. Le clomazone est ajouté à la solution nutritive qui est renouvelée tous les deux jours. L'expérimentation a été effectuée dans une chambre de culture avec un cycle jour/nuit est de 16 h/8 h, une température de 22 °C pendant le jour et 17 °C la nuit, une humidité relative de 60 % et une intensité lumineuse de 50 µmol photons m^{-2} s^{-1}. Le prélèvement de feuilles a été

effectué avant et après traitement par le clomazone pour isoler les chloroplastes et mesurer l'évolution d'oxygène, et après le traitement par le clomazone pour les analyses cytohistologiques.

Principaux résultats

Les résultats confirment que le clomazone réduit la teneur en pigments chlorophylliens (Chl + Car) et le ratio Chl *a/b* chez la variété *Virginie* alors qu'il n'a pas d'effet chez la variété *Xanthi*, chez laquelle les teneurs en pigments sont de 1,5 à 2 fois inferieures en présence ou non de clomazone.

Les mesures de fluorescence (test *OJIP*) et d'évolution d'O_2 des chloroplastes isolés ont montré que la variété *Virginie* en présence de clomazone montre des signes de photoinhibition du PSII dus à une accumulation de Q_A^- et du pool de plastoquinones réduites.

L'activité de transport des électrons par le PSI n'est pas affectée par le clomazone.

Chez la variété *Virginie*, le clomazone entraine une forte accumulation de H_2O_2, tandis que le fonctionnement des enzymes antioxydantes APX, DHAR, CAT, et SOD est significativement plus faible que chez la variété *Xanthi*. Des symptômes de mort cellulaire sont visibles lors des observations microscopiques chez la variété *Virginie* traitée par le clomazone.

Conclusion

La tolérance face au stress photooxydatif induit par le clomazone semble être indépendante de la teneur en pigments chlorophylliens et en particulier des caroténoïdes.

La différence de sensibilité entre les deux variétés parait être liée à l'état de LHC, à l'état rédox du pool de plastoquinones et aux activités des enzymes antioxydantes impliquées dans la détoxification de H_2O_2.

Tolerance to Clomazone Herbicide is linked to the state of LHC, PQ-Pool and ROS Detoxification in Tobacco (*Nicotiana tabacum* L.)

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ABSTRACT

In this study, plantlets of two tobacco (*Nicotiana* tabacum L.) varieties that are clomazone-tolerant (cv. *Xanthi*) and clomazone-sensitive (cv. *Virginie vk51*) were subjected to low concentration of clomazone herbicide. The oxygen-evolving rate of isolated chloroplasts, chlorophyll *a* fluorescence transients, JIP-test responses, hydrogen peroxide contents, antioxidant enzyme activities, cytohistological results and photosynthetic pigment contents were recorded. The results indicated that the carotenoid content was 2-fold higher in *Virginie*, which had greater clomazone sensitivity than *Xanthi*. *Virginie* exhibited noticeable decreases in the LHC content (Chl a/b ratio), the maximum photochemical quantum efficiency of PSII (F_v/F_m), the performance index on the absorption basis (PI_{abs}), and the electron flux beyond the first PSII Q_A evaluated as ($1-V_J$) with $V_J= (F_J-F_0)/ (F_m-F_0)$ as well as increases in the rate of photon absorption (ABS/RC) and the energy dissipation as heat (DI0/RC). These results suggest that PSII photoinhibition occurred as a consequence of more accumulated Q_A^- and reduced PQ-pool. The oxygen evolution measurements indicate that PSI electron transport activity was not affected by clomazone. The more significant accumulation of H_2O_2 in *Virginie* compared to *Xanthi* was due to the absence of ROS-scavenging enzymes, and presumably induced programmed cell death (PCD). The symptoms of PCD were observed by cytohistological analysis, which also indicated that the leaf tissues of clomazone-treated *Virginie* exhibited significant starch accumulation compared to *Xanthi*. Taken together, these results indicate that the variable tolerance to clomazone observed between *Virginie* and *Xanthi* is independent of the carotenoid content and could be related to the state of the LHC, the redox state of the PQ-pool, and the activity of detoxification enzymes.

Keywords: Chlorophyll *a* fluorescence transients; Chloroplast oxygen evolution; Clomazone; *Nicotiana tabacum*; Photosystem II; Tolerance

Abbreviations: ABS, PSII light absorption flux; BQ, benzoquinone; CAT, catalase; 3Chl*, chlorophyll triplet state; DCPIP, dichlorophenolindophenol; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; DI0, PSII excitation dissipation flux; ET0, electron transport flux; F_0, F_v, F_m, minimal, variable, maximal chlorophyll fluorescence; F_v/F_m, maximum photochemical efficiency of PSII; H_2O_2, hydrogen peroxide; $^1O_2$, singlet oxygen; $^3O_2$, oxygen triplet state; $O_2^{\cdot-}$, superoxide radical; OEC, oxygen evolving complex; PAR, photosynthetically active radiation; LHCs, light-harvesting chlorophyll *a/b* binding protein complexes; MV, methylviologen; PCD, programmed cell death; PI_{abs}, performance index on absorption basis; PQ, plastoquinone; PSI, photosystem I; PSII, photosystem II; Q_A, primary quinone electron acceptor of PSII; Q_B, secondary quinone electron acceptor of PSII; RC, reaction center; ROS, reactive oxygen species; SOD, superoxide dismutase; TR0, PSII light energy flux trapping; $1–V_J$, a measure of the electron flux beyond Q_A.

1. Introduction

The environmental fate of herbicides is a matter of concern, given that only a small fraction of these herbicides reaches the target plants (Pimentel, 1995). Investigations of the effects induced by such toxic products are of great interest, not only for a better understanding of the interactions and involved mechanisms, but also with respect to the tolerance of non-target plants to chemical residues in the environment.

Photosynthesis is, in fact, a sensitive process that can be easily perturbed by several toxic species of herbicides, pesticides and heavy metals. Herbicides as one of these species can induce noxious effects, ranging from slowing oxygen emission rates and photosynthetic electron transport, to changes in the structure of the photosynthetic apparatus (Walters, 2005). The photosynthetic reactions in higher plants take place through the cooperation of two photosystems, PSI and PSII, in the thylakoid membrane (Whitmarsh and Govindjee, 1999; Dekker and Boekema, 2005). Light energy is collected by the antenna of PSII and PSI (LHCs) and is channeled to the reaction centers (RCs) where a charge separation occurs. Two quinones are located on the acceptor side of PSII RC: the primary acceptor Q_A receives the electron from the reduced pheophytin $Pheo^-$ (the primary PSII electron acceptor), after which the electron is transferred to the secondary acceptor Q_B by Q_A^-; the plastoquinol (PQH_2) is formed *via* double reduction of Q_B. PQH_2 is then exchanged for a PQ molecule in the thylakoid membrane (Petrouleas and Crofts, 2005). On the donor side, the oxygen-evolving complex (OEC) goes through four successive oxidation steps (called S states) in order to spilt 2 H_2O into O_2 and 4 H^+ (Gauthier et al., 2010).

In most cases in which plants are exposed to the herbicides, the charge recombination in PSII can result in the formation of a chlorophyll triplet state (3Chl*), which can react with $^3O_2$ to form $^1O_2$ (Rutherford and Krieger-Liszkay, 2001; Fufezan et al., 2002). $^1O_2$ contributes to photodamages at PSII RCs (Trebst et al., 2002), leading to the formation of other ROS, such O_2^- and H_2O_2 (Ledford and Niyogi, 2005; Vass, 2012). This formation of ROS inhibits photosynthesis as the result of photoinhibitory damage or photosynthetic electron transport. However, the presence of $^1O_2$ and H_2O_2 which inhibit the repair of photodamaged PSII RCs during the photoinhibition (Nishiyama et al., 2006) seems to be responsible for programmed cell death (PCD) initiation and tissue damage (Dat et al., 2003; Gechev and Hille, 2005; Gechev et al., 2006).

The herbicide clomazone [2-(2-chlorobenzyl)-4,4-dimethyl-1,2-oxazolidin-3-one] (labeled CL) is widely used in agriculture to control annual weeds in soybean, cotton, sugar cane,

maize, rice and tobacco crops (Chang et al., 1997). Clomazone treatment can lead to decreased carotenoid biosynthesis, which leads to chlorophyll bleaching (Duke and Kenyon, 1986; Norman et al., 1990; Souza et al., 2012). In a previous study using photosynthetic pigment content, chlorophyll *a* fluorescence parameters and JIP-test results, we compared the effects of low concentrations of clomazone on photosynthesis in *Xanthi* and *Virginie* varieties of tobacco (*Nicotiana tabacum* L.) (Darwish et al., 2013). This study demonstrated that *Xanthi* was more tolerant, despite the typical difference in the total chlorophyll and carotenoid contents between the two varieties (i.e., approximately 2-fold higher in *Virginie* than in *Xanthi*).

Carotenoids are involved in the photoprotection mechanism in plants; scavenging 3Ch* and $^1O_2$ is one of their major roles (Biswall, 1995). This role of carotenoids is important when plants are exposed to herbicides that inhibit photosynthetic electron transport. Thus, it is surprising that the *Virginie* variety, having double the amount of carotenoids, is more sensitive to clomazone compared to *Xanthi*. To better understand this conflicting issue, and to determine the parameters that are involved in the herbicide tolerance of the *Xanthi* and *Virginie* varieties, it is necessary to study the electron transport chain in the presence of clomazone. Therefore, Clark-type electrodes and chlorophyll *a* fluorescence analysis were used as sensitive and effective methods.

Clark-type electrode has been used to measure electron transport chain activity of the photosynthetic apparatus in many previous studies, especially in algae and higher plants (Samuel and Bose, 1987; Koblìzek, 1998; Croisetiere et al., 2001; Kroger et al., 2002). Using this technique, oxygen evolution measurements coupled with fluorescence emission analysis has been used to detect the effect of a low concentration (less than 10^{-7} M) of herbicides on electron transfer activity of spinach chloroplasts, thylakoid membranes and isolated PSII membranes (Ventrella et al., 2010).

Chlorophyll *a* fluorescence can provide detailed information on the function of the photosynthetic apparatus, especially on PSII (Strasser et al., 2004). Fluorescence measurements have been used to investigate electron transport processes on the acceptor and donor sides of PSII, as well to study antenna states of PSII and many properties of the electron transport chain as a whole (see, Van Gorkom, 1986; Papageorgiou and Govindjee, 2004, and reviews therein). The JIP-test has been used to assess the sensitivity of plants to different environmental conditions (Tsimilli-Michael et al., 1995; Krüger, 1997; Ripley et al., 2004; Gauthier et al., 2010; Kalaji et al., 2012; Darwish et al., 2013, 2014). The JIP-test is based on the energy flux theory in the thylakoid membrane that was developed by Strasser

and co-workers (Strasser et al., 2000; Strasser et al., 2004; Stirbet and Govindjee, 2011). In this test, the fluorescence signals at the O, J, I, and P steps are used to calculate different parameters that are related to the energy and electron fluxes in PSII.

The aim of this work was: (i) to clarify the mechanisms involved in the tolerance of *Xanthi* and *Virginie* tobacco varieties to clomazone; and (ii) to determine whether these mechanisms are linked to the inhibition of carotenoid biosynthesis. The effects of low concentration of clomazone on the function of the photosynthetic apparatus were analyzed using oxygen evolution measurements, chlorophyll *a* fluorescence and JIP-test parameters, antioxidant activities and cytohistological analyses.

2. Materials and methods

2.1. Plant materials, growth conditions and chemical treatments

The tobacco seeds of two varieties (*Nicotiana tabacum* L. *cv. Xanthi* and *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 photon flux (PAR) of 50 µmol photons m^{-2} s^{-1}. The germination was took place under sterile conditions, and in the growth chamber configured according to Darwish et al. (2013, 2014). After 5 weeks of germination, the plantlets were transferred to 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. This nutrient solution was refreshed twice weekly. The plantlets were used for two experiments:

- Experiment A: The effect of clomazone (CL) (Sigma-Aldrich, USA) on oxygen evolution was studied in chloroplasts that were isolated from control plantlets of both varieties. Clomazone at 0-1 µM was directly added to the isolated chloroplasts during the measurement.
- Experiment B: CL was added to the nutrient solution at concentrations of 0-1 µM for 14 days. The leaves were then collected for various analyses. Fresh leaves were used for chloroplast isolation and cytohistological analysis, and the remaining leaves were stored at -80 °C for biochemical analysis.

2.2. Determination of photosynthetic pigments

The total chlorophyll and carotenoid contents were determined according to Lichtenthaler (1987) using pure acetone as the extraction solvent. The absorbance at 662, 645 and 470 nm was measured immediately after extraction.

2.3. Chloroplast preparation

The chloroplasts were isolated from the fresh leaves of tobacco plantlets as described by Joly and Carpentier (2011) 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. (1989) 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:

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

2.4. Measurement of oxygen evolution

The evolution of oxygen by a suspension of chloroplasts that were broken in hypotonic medium to allow the compounds used as electron acceptors to have access into thylakoid membranes was measured. The 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 (1987). 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}.

The PSI [plastoquinone pool→ PSI acceptor side] rates were determined in terms of oxygen uptake in the presence of methylviologen (MV) (98% pure, Sigma-Aldrich, USA) as an exogenous electron acceptor substituting for ferredoxin in PSI, according to Samuel and Bose (1987) with some modifications. Here, dichlorophenolindophenol (DCPIP) was used as an artificial electron donor to PSI (Michelet and Krieger-Liszkay., 2012). The reaction mixture in a final volume of 1 mL contained 5 mM ascorbate, 100 µM DCPIP, 0.1 mM MV 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) as an uncoupler. This compound dissipates the proton gradient that occurs between the lumen and the stroma during illumination (Farineau et al., 2011).

2.5. Measurement of fast chlorophyll *a* fluorescence transients

The behavior of photosystem II (PSII) can be evaluated based on the fast chlorophyll *a* fluorescence emitted by leaves of plantlets that are adapted to darkness. After fourteen days of treatment with clomazone, fast chlorophyll *a* fluorescence transients were measured on 8 leaves of tobacco plantlets (experiment B). 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 (Kitajama and Butler, 1975). The JIP-test is based on the energy flux theory of the thylakoid membrane (Strasser et al., 2004; Stirbet and Govindjee, 2011). This model describes how the energy 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). 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.

2.6. Determination of the H_2O_2 contents

The hydrogen peroxide levels were determined as described by Murshed et al. (2013) with some modifications. Briefly, 250 mg of tobacco leaves from clomazone treated or untreated plantlets 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.

2.7. 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. (2014) 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.

2.8. Antioxidative enzyme activities

Extraction of enzyme:

Proteins were extracted according to Murshed et al. (2008). 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. The solutions were centrifuged at 15,000 × *g* for 15 min at 4 °C, and the supernatants were analyzed immediately to determine the enzyme activities.

Enzyme assays:

All enzyme assays were performed in 0.2-mL kinetic reactions at 25 °C using a micro-plate reader (Power Wave, HT microplate spectrophotometer, BioTek, France). The APX, DHAR and MDHAR activities were measured using the method of Murshed et al. (2008). The APX activity was spectrophotometrically determined by measuring the decrease in absorbance at 290 nm (ε=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 of 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 of extract; the activity was determined by measuring the increase in the reaction rate at A_{265} (ε=14.5 $mM^{-1}cm^{-1}$). The MDHAR activity 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 of extract and 0.4 units of ascorbate oxidase. The activity was determined by measuring the decrease in the reaction rate at A_{340} (ε=2.8 $mM^{-1}cm^{-1}$). The CAT activity was measured according to Chance and Maehly (1955) in a reaction mixture containing 50 mM KH_2PO_4 buffer (pH 7.0), 15 mM H_2O_2 and 5 µL of extract to initiate the reaction. The activity was described in terms of the change in absorbance at 240 nm (ε=39.4 $mM^{-1}cm^{-1}$) as the H_2O_2 level decreased. The SOD activity was determined using the modified method of Dhindsa et al. (1981). The 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. The absorbance at 560 nm was measured immediately after 5 min of illumination with a light intensity of 100 µmol m^{-1} s^{-1} at 25 °C. One unit of SOD was defined as the amount of enzyme that was required to inhibit the NBT photoreduction by 50%.

2.9. Cytohistological analyses

To assess the effects of clomazone at the cytological level, leaf fragments were collected from each treatment and prepared for cytohistology as previously described (El Maataoui and Pichot, 1999). 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 good 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% alcohol 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 El Maataoui and Pichot, 1999). 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. Photosynthetic pigments (chlorophyll and carotenoids)

In the *Virginie* variety, the clomazone treatment decreased the total chlorophyll and carotenoid contents by approximately 22% and 17%, respectively, compared to the control. However, in the *Xanthi* variety, clomazone had no effect on the total chlorophyll and carotenoid contents compared to those of the control (Table 1).

The clomazone treatment significantly decreased ($P<0.05$) LHC contents (Chl *a/b* ratio, for details and references, see Björkman, 1973; Anderson, 1986; Yang et al., 2001) in the thylakoid membrane of *Virginie* leaves by approximately 21% compared to those of the control. However, in the *Xanthi* variety, LHC contents (Chl *a/b* ratio) were not significantly different ($P>0.05$) between the clomazone and control treatments (Table 1).

The control in the two varieties showed a difference concerning the photosynthetic pigment contents, as the chlorophyll and carotenoids levels were approximately 2-fold higher in *Virginie* compared to *Xanthi*.

Table 1. Chlorophyll (Chl *a*, Chl *b*, Chl *a*+*b*) and carotenoid (Car) contents per fresh weight in the control (Con) and clomazone (CL) treatments of tobacco leaves (*Nicotiana tabacum* L. *cv. Xanthi* and *Nicotiana tabacum* L. *cv.Virgenie vk51*). Different letters denote significant differences for each parameter between means within each traitment (P<0.05, ANOVA-Tukey test). Data are expressed as means ± SE, n=8.

Variety	Treatment	Chl *a* (µg g^{-1} FW)	Chl *b* (µg g^{-1} FW)	Chl *a*+*b* (µg g^{-1} FW)	Car (µg g^{-1} FW)	Chl *a*/*b*
Xanthi	**Con**	363 ± 14 c	269 ± 23 b	632 ± 25 c	53 ± 8 c	1.34 ± 0.04 c
	CL	382 ± 27 c	265 ± 11 bc	647 ± 26 bc	47 ± 7 c	1.44 ± 0.04 c
Virginie	**Con**	586 ± 28 a	375 ± 42 a	961 ± 52 a	145 ± 7 a	1.71 ± 0.18 bc
	CL	509 ± 13 b	242 ± 20 c	751 ± 32 b	120 ± 6 b	2.18 ± 0.12 a

3.2. Chloroplast oxygen evolution

The effect of clomazone on oxygen evolution was measured in chloroplasts that were isolated from the two tobacco varieties in presence of ferricyanide as an electron acceptor and nigericin as an uncoupler.

Several preliminary experiments demonstrated that 5 min of incubation was sufficient to determine the effect of clomazone on the electron flow (not shown). In experiment A, the chloroplasts were incubated with 1 µM clomazone for 5 min before monitoring the oxygen evolution. Clomazone reduced the oxygen evolution by approximately 3% and 4% in the *Xanthi* and *Virginie* varieties, respectively, compared to the control (Table 2). In experiment B, the oxygen evolution of isolated chloroplasts that were treated with clomazone decreased in the two varieties compared to the control, and this decrease was greater in *Virginie* (12%) than in *Xanthi* (9%).

Table 2. Inhibition of oxygen evolution (nmol mg^{-1} min^{-1}) measured in thylakoids (80 µg Chl mL^{-1}) as percent (%) in two tobacco varieties (*Nicotiana tabacum* L. *cv. Xanthi* and *Nicotiana tabacum* L. *cv. Virginie vk51*). Experiment A, chloroplasts isolated from control plantlets and then treated with clomazone; Experiment B, chloroplasts isolated form plantlets treated with clomazone. $K_3Fe(CN)_6$, potassium ferricyanide as an electron acceptor; BQ, benzoquinone as an electron acceptor in PSII; DCPIP, dichlorophenolindophenol as an artificial electron donor to PSI; MV, methylviologen as an exogenous electron acceptor which substitutes ferredoxin in PSI. Con, without clomazone; CL, treatment with 1 µM clomazone. Reported data are obtained from four replicates, n=4. Different letters denote significant difference within each treatment (P<0.05, ANOVA-Tukey test).

Variety	Treatment	Experiment A			Experiment B		
		$K_3Fe(CN)_6$	BQ	DCPP → MV	$K_3Fe(CN)_6$	BQ	DCPP → MV
	Con	0% a	0% a	0% a	0% a	0% a	0% a
	CL	3% b	3.5% b	0.5% a	9% b	12.5% b	1.7% a
Xanthi							
Virginie	**CL**	4% b	4.5% b	0.2% a	12% c	16.5% c	2% a

The oxygen evolution in PSII was measured using BQ as an electron acceptor. In experiment A, clomazone reduced the oxygen evolution by approximately 3.5% and 4.5% in *Xanthi* and *Virginie*, respectively, compared to the control (Table 2). In experiment B, more significant decreases of approximately 12.5% and 16.5% were observed for the *Xanthi* and *Virginie* varieties, respectively (Table 2).

The rate of PSI electron transport was analyzed using DCPIP and MV. In both experiments (A and B), clomazone had no significant effect on the electron flow that was mediated by DCPIP and MV in both varieties (Table 2).

3.3. Chlorophyll *a* fluorescence transients

While clomazone induced a significant decrease (approximately 9%) in the F_v/F_m in the leaves of *Virginie* plantlets, it had no significant (P<0.05) effect on the F_v/F_m in the *Xanthi* plantlets (only 2%) compared to that of the control (Table 3).

The performance index on absorption basis (PI_{abs}) decreased in plantlets that were treated with clomazone. Compared to the control, PI_{abs} decreased by approximately 21% and 57% in *Xanthi* and *Virginie* plantlets that were treated with CL, respectively (Table 3). The decrease in 1-V_J parameter was more pronounced in *Virginie* than in *Xanthi* plantlets following exposure to clomazone (22% and 6%, respectively) (Table 3).

Table 3. Effect of clomazone 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) in tobacco plantlets (*Nicotiana tabacum* L. *cv. Xanthi* and *Nicotiana tabacum* L. *cv.Virgenie vk51*). Con, control; CL, treatment with 1 µM clomazone. Different letters denote significant differences for each parameter between means within each traitment (P<0.05, ANOVA-Tukey test). Data are expressed as means ± SE, n=8.

Variety	Treatment	F_0	F_m	F_v/F_m	PI_{abs}	$1–V_J$
Xanthi	**Con**	332 ± 14 a	1736 ± 65 b	0.81 ± 0.002 a	1.41 ± 0.11 b	0.33 ± 0.007 b
	CL	361 ± 19 ab	1753 ± 38 bc	0.79 ± 0.009 a	1.11 ± 0.12 bc	0.31 ± 0.011 bc
Virginie	**Con**	347 ± 5 ab	1912 ± 40 a	0.82 ± 0.001 a	2.07 ± 0.14 a	0.37 ± 0.007 a
	CL	385 ± 9 b	1621 ± 46 c	0.75 ± 0.013 b	0.89 ± 0.15 c	0.29 ± 0.014 c

In the CL treatment, 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) significantly increased (P<0.05) in the *Virginie* plantlets compared to those of the control (Table 4). Similarly, the part of the absorbed energy that was dissipated as heat and fluorescence (DI0/RC) significantly increased (P<0.05) in the *Virginie* plantlets that were treated with clomazone. In the *Xanthi* plantlets, clomazone had no significant effect (P>0.05) on the ABS/RC, TR0/RC or DI0/RC parameters compared to those of the control. In the *Virginie* plantlets treated with CL, although the electron transport beyond Q_A^- per reaction center (ET0/RC) increased, the quantum yield of the electron transport beyond Q_A^- (ET0/ABS) decreased compared to the control (Table 4).

Table 4. Effect of clomazone on ABS/RC, TR0/RC, DI0/RC, ET0/RC and ET0/ABS in tobacco plantlets (*Nicotiana tabacum* L. *cv. Xanthi* and *Nicotiana tabacum* L. *cv.Virgenie vk51*). Con, control; CL, treatment with 1 µM clomazone. Different letters denote significant differences for each parameter between means within each traitment (P<0.05, ANOVA-Tukey test). Data are expressed as means ± SE, n=8.

Variety	Treatment	ABS/RC	TR0/RC	DI0/RC	ET0/RC	ET0/ABS
Xanthi	**Con**	1.51 ± 0.06 b	1.22 ± 0.05 b	0.29 ± 0.01 a	0.39 ± 0.008 ab	0.26 ± 0.006 b
	CL	1.71 ± 0.12 b	1.36 ± 0.09 bc	0.35 ± 0.03 a	0.41 ± 0.013 b	0.24 ± 0.008 bc
Virginie	**Con**	1.31 ± 0.06 a	1.06 ± 0.05 a	0.28 ± 0.01 a	0.39 ± 0.009 a	0.30 ± 0.005 a
	CL	2.12 ± 0.16 c	1.57 ± 0.09 c	0.55 ± 0.07 b	0.45 ± 0.008 c	0.22 ± 0.014 c

3.4. H_2O_2 and ascorbate contents

The H_2O_2 contents (Fig. 1A) increased in the tobacco plantlets that were treated with clomazone in the two varieties compared to that of the control. After CL treatment, the plantlets of the *Virginie* variety exhibited an accumulation of H_2O_2 approximately five-fold higher than that of the *Xanthi* variety.

In the *Virginie* and *Xanthi* varieties (Fig. 1B), the total ascorbate content decreased in the CL treatment compared to that of the control.

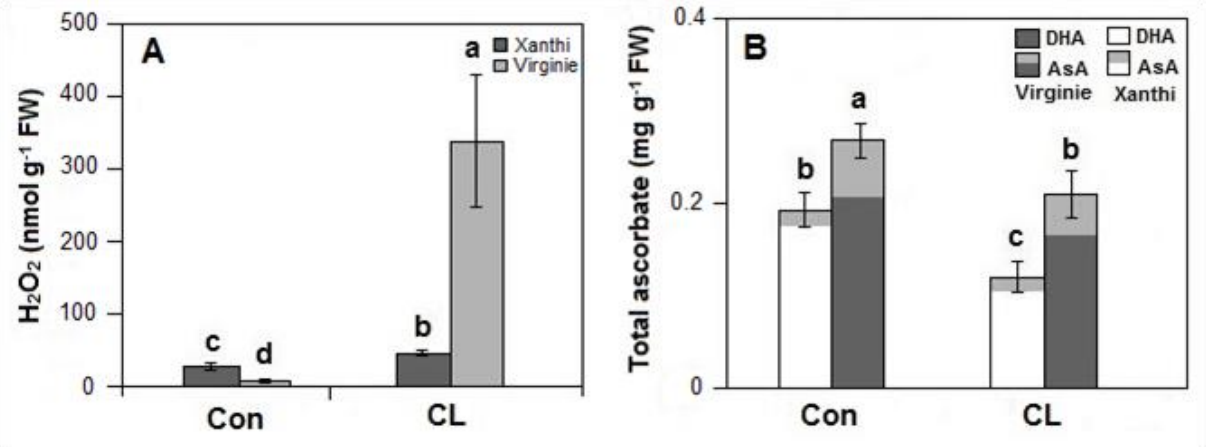

Fig. 1. Effects of clomazone on H_2O_2 content (A) and total ascorbate (B) in two varieties of tobacco (*Nicotiana tabacum* L. *cv. Xanthi* and *Nicotiana tabacum* L. *cv. Virginie vk51*). Con, without clomazone; CL, treatment with 1µM clomazone. All data represent means, SE for n=8 and different letters to determine the significant difference between means within each treatment (P<0.05, ANOVA-Tukey test).

3.5. Antioxidant enzyme activities

There was a difference in both tobacco varieties concerning the response of the antioxidant enzymes to the CL treatment.

In leaves of the *Virginie* variety, the activities of APX (Fig. 2A), MDHAR (Fig. 2B) and DHAR (Fig. 2C) significantly decreased (P<0.05) compared to those of the control, whereas the CAT (Fig. 2D) activity did not differ (P>0.05) between the CL and Con treatments. The SOD activity (Fig. 2E) was significantly (P<0.05) higher than that of the control.

In contrast, the APX (Fig. 2A) and MDHAR (Fig. 2B) activities increased in leaves of the *Xanthi* variety, whereas the CAT (Fig. 2D) activity decreased compared to that of the control. However, the DHAR (Fig. 2C) and SOD (Fig. 2E) activities were not significantly different (P>0.05) between the CL treatment and the control.

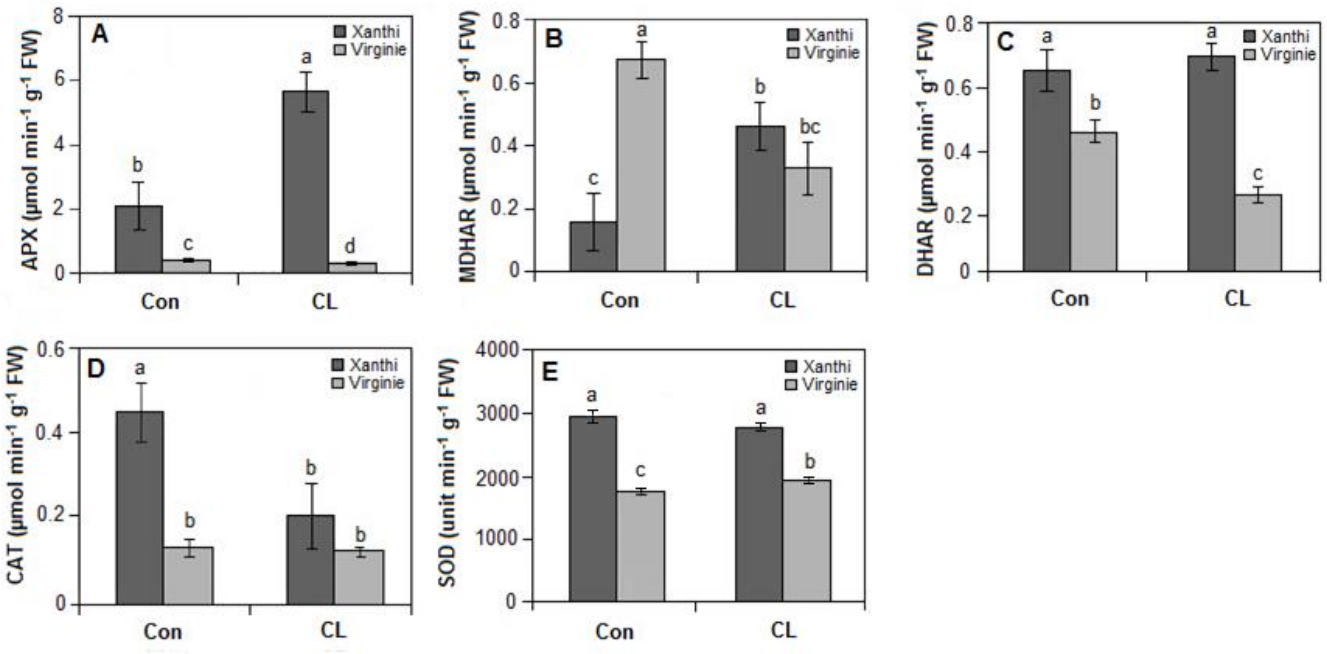

Fig. 2. Effects of clomazone on APX (A), MDHAR (B), DHAR (C), CAT (D) and SOD (E) activities in tobacco plantlets (*Nicotiana tabacum* L. *cv. Xanthi* and *Nicotiana tabacum* L. *cv. Virginie vk51*). Con, without clomazone; CL, treatment with 1µM clomazone. All data represent means, SE for n=8 and different letters to determine the significant difference between means within each treatment (P<0.05, ANOVA-Tukey test).

3.6. Cytohistological analyses

A microscopic study of sections from the control plantlets showed that in both varieties, the leaf structure was similar and typical of dicotyledonous plants. The photosynthetic mesophyll consisted of thin-walled parenchymatous cells that were filled with numerous starch-containing chloroplasts (Fig. 3A and C). These cells were cytoplasm-rich due to the presence of highly invaginated vacuoles, and there were no obvious cytomorphological differences between the palisade and spongy parenchyma. Stomata were present in the both adaxial and abaxial epidermises.

Sections of the leaves of clomazone-treated plantlets displayed mesophyll cells with a large, well-structured vacuole occupying the majority of cell volume in the two varieties (Fig. 3B and D). In presence of clomazone, the chloroplasts of both tobacco varieties appeared to have deposited more starch than did the controls, as evidenced by the prominence of starch grains, particularly in *Virginie* (compare Fig. 3A to Fig. 3B and Fig. 3C to Fig. 3D). A thorough study of the sections from clomazone-treated plantlets of *Virginie* revealed the presence of numerous mesophyll cells exhibiting cytological alterations that are characteristic of programmed cell death (PCD). These alterations consisted of cell wall lysis (Fig. 3D), vacuole

regression, nuclear and cytoplasm condensation and cell shrinkage. The mesophyll in clomazone-treated *Xanthi* leaves appeared to be composed of cells that maintained their morphological integrity (Fig. 3B).

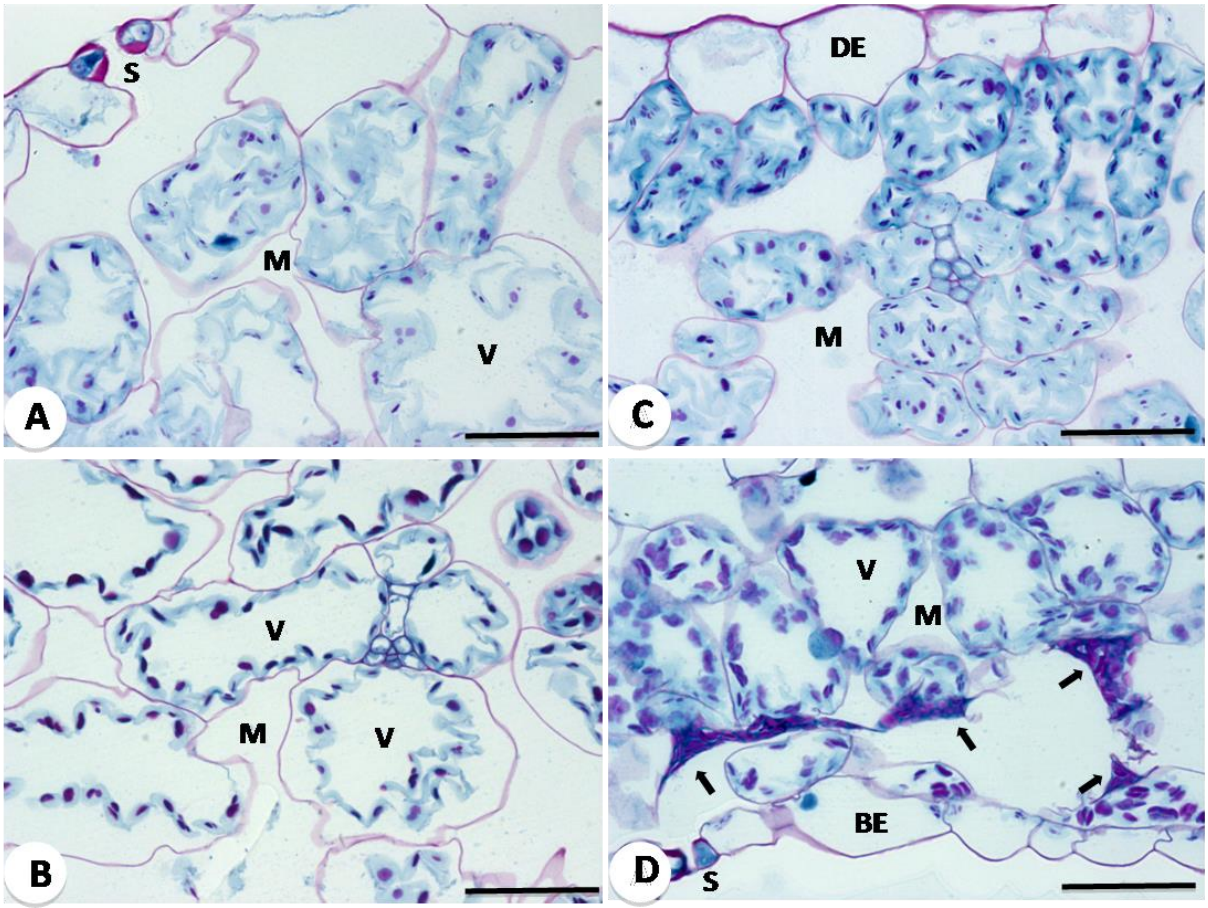

Fig. 3. Cytohistological effects of clomazone on *Xanthi* and *Virginie* varieties of tobacco. A and B, sections from control and clomazone-treated *Xanthi*, respectively. C and D, sections from control and clomazone-treated *Virginie*, respectively. The mesophyll of controls displays structurally similar parenchyma cells in both varieties (Figs A and C). Note the presence of sinuous vacuoles (V) and starchy chloroplasts (pink granules). In general, clomazone increased starch accumulation in chloroplasts of both varieties (Figs B and D) with a remarkable importance in *Virginie*. In addition, the mesophyll of this variety showed cytological alterations resembling those described for programmed cell death (Fig. D, arrows). All scale bars: 50 µm. BE, abaxial epidermis; DE, adaxial epidermis; M, mesophyll; S, stomata; V, vacuole.

4. Discussion

In our previous work, we demonstrated that clomazone differently affected the *Virginie* and *Xanthi* varieties of tobacco based on chlorophyll *a* fluorescence and JIP-test responses (Darwish et al., 2013). In this study, we also used measurements of the O_2 evolution of

isolated chloroplasts, antioxidant systems assessments and cytohistological analyses to elucidate the involved mechanisms. The results confirm that clomazone reduced the pigment contents (total chlorophyll and carotenoid contents) and the LHC content (Chl *a/b* ratio) of *Virginie* leaves compared to those of the control. This negative effect of clomazone in *Virginie* is more pronounced compared to *Xanthi*, where clomazone had no significant effect on the pigment and LHC contents (Table 1).

The PSI [plastoquinone pool→ PSI acceptor side] rates of isolated chloroplasts from both of the varieties demonstrated that clomazone had no effect on the electron transport trough the cytochrome b_6f complex and PSI. When chloroplasts were isolated from the control plantlets (experiment A), the observed reduction in the oxygen evolution in presence of BQ and clomazone suggested that clomazone had a direct effect on PSII function by decreasing of electron transport (Table 2). However, in the experiment B, in which chloroplasts were isolated from clomazone-treated plantlets, the more significant reduction in the oxygen evolution resulted from the effect of clomazone during plantlet growth, which could be due to its effect on the alteration of the stoichiometry of the photosynthetic electron transport chain (Table 2). This alteration is presumably linked to the decrease in the level of LHC content (Chl *a/b* ratio) as induced by clomazone treatment (Kaňa et al., 2004; Darwish et al., 2013). This decrease in LHC content could induce a decrease in F_m values (Dinç et al., 2012), and could thereby explain a significant reduction of the parameter F_v/F_m. This interpretation is supported by the observation of Kaňa et al. (2004) that CL-treated leaves have properties similar to leaves in the early stages of greening. This result agrees with the findings of Darwish et al. (2013), who reported that clomazone more significantly affects the function of the PSII reaction center (F_v/F_m parameter) in *Virginie* than in *Xanthi*. However, the decrease in F_v/F_m could be due as well to PSII photoinhibition (Krause et al., 1990; Osmond et al., 1993; Maxwell and Johnson, 2000; Poiroux-Gonord et al., 2013; Darwish et al., 2014).

The JIP-test was used to study the effect of clomazone on the electron transport from the acceptor side of PSII in two tobacco varieties. The performance index parameter (PI_{abs}) was noticeably decreased in *Virginie* plantlets compared to those in the control and *Xanthi* plantlets (Table 3), indicating that *Virginie* plantlets are more affected by clomazone than are *Xanthi* plantlets. This parameter is related to the number of active PSII reaction centers per total PSII antenna chlorophyll (RC/ABS), the maximum quantum yield (TR0/ABS= F_v/F_m) and the electron flux beyond Q_A ($1\text{-}V_J$=ET0/TR0) (Strasser et al., 2000).

Due the effect of clomazone on the redox state of PQ-pool (see the reduction of $1\text{-}V_J$, Table 3), an excessive influx of excitations is transferred into the active RC of PSII despite the

observed reduction of LHC contents (see the Chl *a/b* ratio, Table 1). This influx of excitations is indicated by an increase in the photon absorption (ABS), photon capture (TR0) and electron transport (ET0) by the active (Q_A^- reducing) reaction center (RC) (Table 4). This induces a more reduced PQ-pool (Tóth et al., 2007) and modifies the functioning of the Q_B and Q_A acceptors (Kyle et al., 1984; Vass et al., 1992). These alterations causes a reduction of electron transport *via* increasing the energy dissipation as heat (see the increase of DI0/RC, Table 4) as a protection mechanism which contributes to photoinhibition on the acceptor side of PSII (see the reduction in F_v/F_m, PI_{abs} and ET0/ABS, Tables 3, 4) (Niyogi, 1999; Raven, 2011; Kalaji et al., 2012). This photoinhibition indicates that the carotenoid content is not a main factor in protecting tobacco plantlets from PSII photoinhibition induced by clomazone (see the carotenoid contents in *Virginie* and *Xanthi*, Table 1); the state of LHC antenna (reflected in changes of Chl *a/b* ratio, ABS/RC and ET0/ABS) is more important in this case.

During the PSII photoinhibition process, the reduction in the pool of plastoquinone contributes to the recombination of the charge separated state $P680^+Pheo^-$, consequently the formation of the reactive singlet oxygen $^1O_2$ *via* 3Chl* is expected according to Fufezan et al. (2002). In turn, the PQ-pool in a very reduced state favors the formation of $^1O_2$ in relation with recombination reactions in PSII RC (Adir et al., 2003). The formed $^1O_2$ can damage the active RC of PSII (see the reduction in F_v/F_m and ET0/ABS in *Virginie*, Tables 3, 4) (Krieger-Liszkay, 2004), which could lead: (i) to the formation of H_2O_2 *via* the modification of the water-oxidizing complex (Fig. 1A) (Vass, 2012); and (ii) to the production of O_2^- at the level of PSI (Ledford and Niyogi, 2005). This O_2^- can be dismutated into H_2O_2 (Fig. 1A) by SOD activity (see SOD activity, Fig. 2E) (Bowler et al., 1992). H_2O_2 (Fig. 1A) at high concentrations becomes cytotoxic, inducing programmed death cell (Foyer et al., 1997; Gechev et al., 2005). To evaluate the H_2O_2 status and its scavenging, we analyzed the activities of the antioxidant enzymes APX, MDHAR, DHAR, CAT and SOD. There was a difference in the response of antioxidant enzymes activities to clomazone between the varieties. In the *Virginie* variety, the SOD activity increased (Fig. 2E), and considering the decrease in the APX and CAT activities (Fig. 2A and D), this increase can partially explain the high accumulation of H_2O_2 (Fig. 1A) in the clomazone treatment. Moreover, this accumulation was also supported by the low activities of MDHAR and DHAR enzymes (Fig. 2B, C and D) and the low content of AsA (Fig. 1B).

Concerning the *Xanthi* variety under CL treatment, despite the decreased CAT activity (Fig. 3D and E), the increased APX and MDHAR activities (Fig. 3A and B) caused low H_2O_2

accumulation (Fig. 1A) and thus, less stress. This antioxidant defense can partially explain the *Xanthi* tolerance to clomazone.

Microscopic analyses allowed us to investigate the effect of clomazone in the two tobacco varieties. The accumulation of starch grains and vacuolization in the sections from the clomazone-treated plantlets could be due to specific cellular response mechanisms to clomazone stress (Fig. 3B and D). Indeed, plant cells have developed mechanisms to exclude and isolate herbicides, and increased vacuolization is one of these mechanisms (Dixon et al., 1998; Jamers and De Coen, 2010; Romero et al., 2011). The data of Ackerson and Hebert (1981) showed large starch granules in stress-adapted plants. Moreover, greater starch accumulation in plants cells was associated with the greater resistance of their photosynthetic apparatus to oxidative stress (Muneer et al., 2014). Compared to those in *Xanthi*, the symptoms of programmed cell death (PCD) that were observed in sections from the clomazone-treated *Virginie* plantlets (Fig. 3D) can be related to the toxic effects of the high accumulation of H_2O_2 (Fig. 1A), which was accompanied by the inactivation of antioxidant enzymes that scavenge H_2O_2.

In summary, we conclude that clomazone has a direct effect on the function of PSII and thus on the photosynthetic electron transport. This effect of clomazone appears to be independent of its influence on the carotenoid contents and could be related to the photooxidative stress and the efficiency of the detoxification mechanisms during plantlet growth. Moreover, this work showed a variable tolerance in both tobacco varieties as a typical response to clomazone; *Virginie* variety was more sensitive than *Xanthi* variety. Furthermore, the sole protective mechanism of heat dissipation after excess photon absorption in *Virginie* could not prevent the accumulation of H_2O_2 in the absence of ROS-scavenging enzymes (APX, CAT, and SOD). In contrast, the efficient activity of antioxidant enzymes (see APX and MDHAR, Fig. 2A and B) in *Xanthi* led to a greater tolerance to clomazone herbicide.

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