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

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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 (16 min light/8 min 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.

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

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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. Material 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, 16 h/8 h 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) (16 min/8 min 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 $Ca(NO_3)_2{\cdot}H_2O$; 1 M $MgSO_4{\cdot}7H_2O$; 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. 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 microplate 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

2.6.1. 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.

2.6.2. 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