Research Article

Phytochemical Analysis

Received: 1 July 2010; Revised: 31 August 2010; Accepted: 13 September 2010 Published online in Wiley Online Library: 27 February 2011

(wileyonlinelibrary.com) DOI 10.1002/pca.1275

Quantitative Determination of Superoxide in Plant Leaves Using a Modified NBT Staining Method

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ABSTRACT:

Introduction – In plants, the ROS (reactive oxygen species) level is tightly regulated because their accumulation produces irreversible damage leading to cell death. However, ROS accumulation plays a key role in plant signaling under biotic or abiotic stress. Although various methods were reported to evaluate ROS accumulation, they are restricted to model plants or provide only qualitative information.

Objective – Develop a simple method to quantify superoxide radicals produced in plant tissues, based on the selective extraction of the formazan produced after nitroblue tetrazolium (NBT) reduction in histochemical staining.

Methodology – Plant leaves were stained with a standard NBT method and the formazan precipitated in tissues was selectively extracted using chloroform. The organic phase was dried and formazan residue dissolved in dimethylsulfoxide–potassium hydroxide and quantified by spectrophotometry. The method was tested in strawberry plant leaves under different stressing conditions.

Results – Formazan extracted from leaves subjected to stress conditions showed similar absorption spectra to those obtained from standard solutions using pure formazan. Calibration curves showed a linear relationship between absorbance and formazan amounts, within the range 0.5–8 μg. Outcomes suggested that formazan was retained in the solid residue of leaf tissues. This protocol allowed us to quantify superoxide radicals produced under different stress conditions.

Conclusions – Chloroform allowed a selective formazan extraction and removal of potential endogenous, exogenous or procedural artefacts that may interfere with the quantitative determination. This protocol can be used to quantify the superoxide produced in plant tissues using any traditional qualitative NBT histochemical staining method.

Keywords: formazan; superoxide radical; nitroblue tetrazolium; reactive oxygen species; strawberry

Introduction

Hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\cdot -}$) radicals are the main ROS generated in cell with aerobic metabolism and their deleterious effects are neutralised by specific enzymes. Whereas hydrogen peroxide is reduced by catalases or peroxidases, superoxide radicals can be rapidly dismuted to hydrogen peroxide by superoxide dismutases.

In the past, ROS were considered to be toxic by-products which needed to be rapidly disposed by the enzymes mentioned above. However, in recent years, it was demonstrated that plants actively produce ROS as signalling molecules involved in many complex biological processes such as programmed cell death and other stressing conditions such as pathogen attack, drought and desiccation, salt, cold, heat, heavy metals, radiations, air pollutants, mechanical and light stresses (Heath, 2000; Fryer *et al*., 2002; Wohlgemuth *et al*., 2002; Xiong *et al*., 2002; Faize *et al*., 2004; Höglund *et al*., 2005; Vicente *et al*., 2006). It has been demonstrated, that during plant–pathogen interaction, a rapid and transient ROS accumulation (known as oxidative burst) takes place after the recognition of the pathogen and this reaction plays a key role in host plant cells as an early signal of plant defense response. This scenario suggests, therefore, that the ROS levels in plants should be tightly regulated because the accumulation of any of the chemical species can produce irreversible damage in membrane lipids, proteins and nucleic acids, leading to cell death. Consequently, plant cells have developed several mechanisms to regulate ROS level in tissues, such as antioxidant and ROS-scavenging systems (Lamb and Dixon, 1997; Grant and Loake, 2000; Foyer *et al*., 2009).

With the aim to evaluate plant responses to stress, many quantitative methods were reported to measure ROS level (Brennan and Frenkel, 1977; Warm and Laties, 1982; Wolff, 1994; Bozsó *et al*., 2005). Although those protocols are adequate and efficient in model plants, they usually are not suitable for other species due to the presence of interferences and inhibitors that make its application difficult. However, methods based on histochemical staining were successfully used by many authors to demonstrate the oxidative burst in plants, but results were only qualitative and visual. To overcome this problem some authors used digital

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image analysis that required special computer software to quantify these results (Wohlgemuth *et al*., 2002; Bozsó *et al*., 2005; Díaz-Vivancos *et al*., 2008).

Nitro blue tetrazolium staining is widely used as a histochemical method to detect *in situ* production of superoxide radicals in plant tissues (Doke, 1983). The latter showed high sensitivity and specificity and was successfully used in many plant species to detect superoxide accumulation in different tissues such as leaves, root nodules, tuber, pollen, protoplasts and endosperm (Hauser and Morrison, 1964; Doke, 1983; Robertson and Earle, 1986; Wohlgemuth *et al*., 2002; Groten *et al*., 2005; Jeng *et al*., 2005; Salazar *et al*., 2006).

In this paper we describe a rapid and simple method based on the formazan solubility in dimethylsulfoxide-potassium hydroxide (Muñoz and Alfaro, 2000) to quantify from NBT stained leaves the superoxide radicals production of plants exposed to different stressing conditions.

Experimental

Plant materials

Strawberry plants (*Fragaria × ananassa)* of the cv. 'Pajaro' used in experiments were kindly provided by the strawberry BGA (Banco de Germoplasma Activo at University of Tucumán, Argentina). Healthy plantlets were obtained from *in vitro* cultures and rooted in pots with sterilised substrate, humus : perlome (2 : 1). Before using in experiments, plants were maintained for 15 weeks in growing cabinets at 28°C, 70% relative humidity with a light cycle of 16 h per day, and watered every other day with 50 mL of distilled water. All senescent leaves and petioles were removed periodically until 10 days before the inoculation experiments, leaving only three to four young healthy leaves.

Biotic stress induction

Biotic stress was induced by infecting strawberry plants with an avirulent fungal pathogen. Isolate F7 of *Colletotrichum fragariae*, characterised in our laboratory (Salazar *et al*., 2006), was used in these infection assays; it was grown on potato dextrose agar (PDA) supplemented with streptomycin (300 µg/mL) and maintained at 28°C under continuous white fluorescent light (200 µmol/m^2 s). Plates were incubated for 20 days or until the colony completely covered the plate. The culture surface was gently scraped with a sterile scalpel, conidia recovered in 3 mL of sterile distilled water and suspensions filtered through sterile gauze to remove mycelial debris. Suspensions were then diluted with sterile distilled water (containing 0.01% Tween 20) to a final concentration of 1.5×10^6 conidia/mL and applied to plants by spraying the leaves up to runoff using a hand pump sprayer. Immediately after inoculation, plants were placed in a dew chamber at 28°C, 100% relative humidity during 4 h in dark. Plants sprayed with sterile distilled water containing 0.01% Tween 20, incubated under same conditions, were used as non-infected controls.

Abiotic stress induction by Paraquat treatment

Application of *N,N'*-dimethyl-4,4'-bipyridinium dichloride or Paraquat (PQ) on strawberry plants was used as abiotic stress. Plants were sprayed with 1 mM PQ in distilled water containing 0.01% Tween 20, and incubated in a greenhouse at 28°C under white fluorescent light during 16 h. Control plants were treated only with distilled water containing 0.01% Tween 20 and incubated under same conditions.

Abiotic stress induction by riboflavin–methionine treatment

The riboflavin–methionine photochemical reaction was used to generate superoxide exogenously (Beauchamp and Fridovich, 1971). Briefly, detached leaflets from strawberry plants were completely immersed in 50 mM potassium phosphate buffer (pH 7.8) containing 27 µM riboflavin and 17 mM methionine and infiltrated by one vacuum shock (0.8 bar) during 2 min. Samples were then incubated for 1 h under fluorescent light (without vacuum) before NBT staining. Detached leaves subjected to same conditions but not stained with NBT were used as controls.

Histochemical detection of superoxide

NBT staining was used to detect *in situ* the production of superoxide radicals and was carried out according to Wohlgemuth *et al*. (2002) with minor modifications. Detached leaflets from strawberry plants subjected to the treatments above described and their respective controls were immersed in 50 mM potassium phosphate buffer (pH 7.8) containing 0.1% NBT and 10 mM sodium azide. Leaflets were vacuum infiltrated as described above during 2 min, incubated for 2 h in the dark (without vacuum) and then immersed in 96% (v/v) ethanol to completely eliminate the chlorophyll. Superoxide production was visualised as a purple formazan deposit within leaflet tissues. Leaflets of plants that received no treatment were also infiltrated with 50 mM potassium phosphate buffer (pH 7.8) containing only 10 mM sodium azide and used as control.

Quantification of formazan

A method described by Muñoz and Alfaro (2000) for neutrophil blood cells was adapted to plant tissues. After NBT staining leaf tissues were dried with a heat gun and ground in a mortar to determine sample dry weight. Fomazan was selectively extracted from 20 mg of dried tissue using 1 mL of 2 M potassium hydroxide : chloroform (1 : 1, v/v). The extraction was performed three times at 4°C and light-protected. Chloroformic extracts were pooled and then completely dried under gaseous nitrogen flush at 4°C and light protected. The solid residue was dissolved in 350 µL of DMSO and 300 µL of 2 M potassium hydroxide at room temperature and immediately analysed with a Beckman DU 7500 spectrophotometer. Spectra were obtained by measuring the absorbance in the range of 400–800 nm and formazan quantification was performed at 630 nm.

In vitro formazan production

Pure formazan obtained from photochemical reaction according to Beauchamp and Fridovich (1971) was used to obtain calibration curves. Reaction mixture containing 27 µM riboflavin, 17 mM methionine and 1 mg/mL of NBT, in 50 mM potassium phosphate buffer (pH 7.8) was prepared. After incubation for 1 h at room temperature, the purple precipitate was washed five times with distilled water, five times with 96% (v/v) ethanol and finally dried to determine weight of formazan yield. Standard solutions were prepared by dissolving the pure formazan with DMSO and 2 M potassium hydroxide (1.6:1, v/v) and diluting up to the desirable concentration immediately prior to use. With the aim to estimate the formazan retained in leaves tissues (cellulose matrix) a calibration curve was obtained with 0.5 mL of freshly prepared standard solutions that were mixed with 20 mg dried leaf tissue obtained from NBT controls. Both solution series were further extracted with chloroform, dried, dissolved and diluted to the original concentration in DMSO and 2 M potassium hydroxide (1.6:1, v/v) and measured at 630 nm.

Results and Discussion

Extraction experiments were carried out to ensure that the formazan precipitated within leaf tissues was selectively extracted and that no interference was pulled out from leaf extracts. The analysis consisted of comparing the visible absorption spectra of pure formazan and the formazan obtained from leaf extracts of plants treated with different oxidative stress inducers. Spectra of pure formazan (Fig. 1, line A) and the formazan extracted from plant leaves exposed to a biotic stress caused by the isolate F7 of *C.*

fragariae (Fig. 1, line B) presented highly similar profiles. Also, similar spectra were obtained from plant leaves exposed to abiotic stresses induced by superoxide exogenously (Fig. 1, line C) or endogenously (Fig. 1, line D) generated, through treatments with riboflavin-methionine or PQ, respectively. These results indicate that the formazan extraction was highly selective and independently of previous plant treatment. Extracts obtained from leaves exposed to no treatment (Fig. 1, line F) or infiltrated only with sodium azide buffer (Fig. 1, line E) showed very low absorbance, indicating that the extraction procedure did not incorporate significant content of interfering compound in the chloroform extracts. Plant leaves exposed to the same stressing conditions and extraction procedures (above described) but not stained with NBT, also showed similar profiles to line E and F (data not shown).

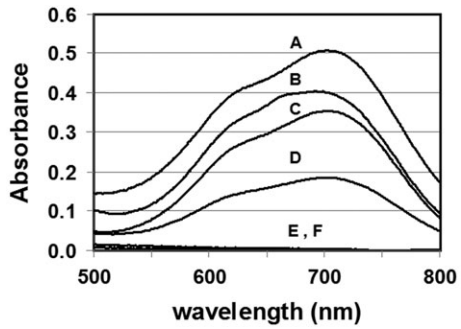

Figure 1. Comparisons of formazan absorption spectra obtained from a pure formazan solution (A), NBT stained leaves infected with the avirulent isolate F7 (B), NBT stained leaves treated with riboflavin-methionine (C), NBT stained leaves treated with PQ (D), leaves only infiltrated with sodium azide-buffer (E), and leaves exposed to no treatment and not stained (F).

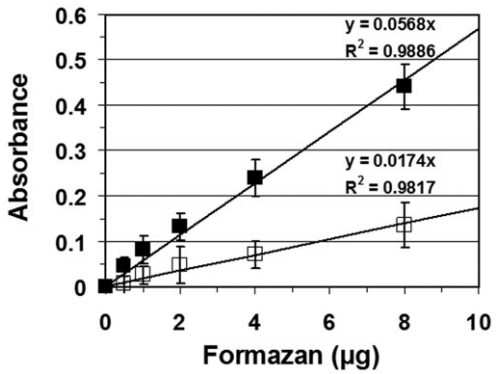

Figure 2. Formazan calibration curves. Different amounts of pure formazan were chloroform-extracted and the absorbance measured at 630 nm. Calibration curves were obtained in absence (solid symbols) or in the presence of leaf tissues (empty symbols).

Before any attempt to quantify the formazan accumulated in stressed plant leaves the formazan retained by the cellulosic plant debris after the extraction was evaluated. Accordingly, calibration curves obtained with solution series prepared with pure formazan and formazan plus leaf cellulosic residues (see Material and Methods) were compared. Formazan in both solution series were chloroform extracted and measured at 630 nm. Figure 2 shows from one side that the absorbance values at 630 nm exhibited a linear correlation with formazan amounts within the range 0.5–8 µg (in both cases), and on the other side revealed that, although the extraction procedure was successful, the cellulosic residue retained 70% of the formazan formed. The second curve allowed us to correct the estimation of superoxide production for formazan losses during extraction procedure.

Finally, the production of superoxide radicals in plants exposed to different biotic and abiotic stress conditions was evaluated. Leaves of strawberry plants were treated as mentioned above and results are presented in Figure 3. In all cases the method under study showed a significant increase of the level of superoxide radical as compared with control untreated plants.

In an early report, by using the NBT staining technique, Salazar *et al*. (2006) showed that the cv 'Pajaro' of strawberry challenged with avirulent F7 isolated of *C. fragariae*, induced an oxidative burst at 4 h post-infection (hpi) in host leaf tissues. Our results confirmed that observation and let us estimate that the amount of superoxide accumulated at 4 hpi expressed as formazan produced corresponds to 0.53 µg/mg dry weight over the control value.

It is well known that the herbicide Paraquat can uncouple the photosynthetic electron transport chain generating superoxide radicals in plant cells (Fruest and Norman, 1991). In Fig. 3 we can also see that strawberry plants treated with PQ exhibited a high

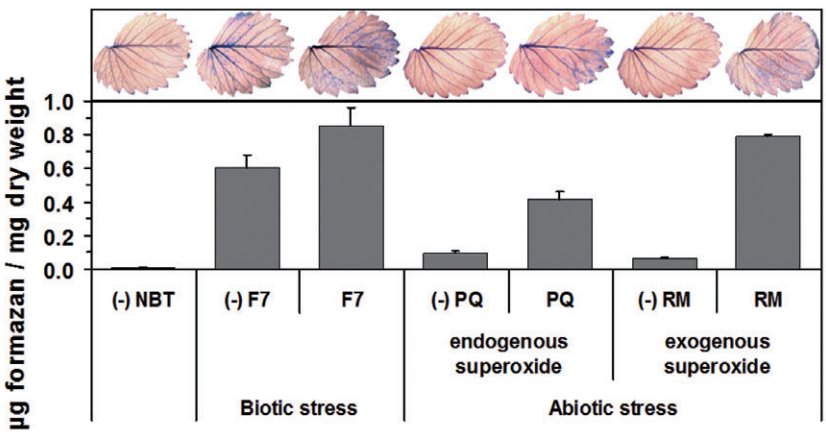

Figure 3. Formazan produced in NBT stained leaves after a biotic or abiotic stress. (-) NBT, corresponds to azide-buffer infiltrated leaves; (-) F7, non infected control; F7, leaves infected with avirulent pathogen F7; (-) PQ, control without paraquat; PQ, leaves treated with paraquat; (-) RM, riboflavin-methionine infiltrated leaves but not stained with NBT; RM, leaves infiltrated with riboflavin-methionine and stained with NBT. Results were obtained from three independent replicates ($n = 3$). Error bars correspond to standard derivation.

accumulation of superoxide radicals 16 h post-treatment (hpt) corresponding to 0.32 µg formazan/mg dry weight over the control value. The latter confirmed the expected effect of PQ on plant leaves and the superoxide accumulation can be easily estimated using the proposed methodology. Likewise, when strawberry leaves were treated with exogenous superoxide, higher levels of formazan was detected (0.72 µg/mg dry weight over the control value), demonstrating the specificity of this method to detect superoxide radicals.

According to electrochemical studies about formazan reduction carried out by Umemoto (1989), a yield factor of 4 electrons/mol of reduced diformazan was assumed. Hence, the amount of superoxide accumulated was 4.1 nmol/mg dry weight in F7 infected plant leaves, 2.5 nmol/mg dry weight in PQ treated leaves and 5.7 nmol/mg dry weight in leaves treated with exogenous superoxide, over their respective control values. We should nevertheless mention that, although the NBT reduction could be almost quantitative within the tissue, there are a number of factors that may influence the formazan production yield such as NBT permeability of tissues, superoxide scavengers or other reducing compounds. Likewise, special attention should be paid to experimental conditions (e.g. temperature, light protection, vacuum infiltration) to reduce spurious redox and photochemical reactions and tissue damage as they may cause false positives.

The protocol described here provide a simple and reliable quantitative procedure that may be useful to perform a time course analysis of superoxide radical accumulation in plant tissues exposed to different stressing conditions.

Acknowledgements

This work was partially supported by the Consejo de Investigaciones de la Universidad Nacional de Tucumán (CIUNT, 26/D423), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 6441) and Agencia Nacional de Promoción Científica y Tecnológica (PICT 08-2105). C.F.G.B. is a fellow and J.C.D.R. is a member of CONICET.

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