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ORIGINAL PAPER

The use of excised roots from in vitro culture for the determination of superoxide production in plants

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Abstract The production of reactive oxygen species (ROS) in plants is a common event in metabolic and physiological processes as well as in the response to biotic and abiotic stress. In this paper we will report that root tissue from axenically grown tomato cultivars and *Lycopersicon* wild species can be used for the determination of superoxide production. Superoxide generation was evaluated following the treatment of root tissues with two general elicitors of the defence response: laminarin and calcium ionophore A23187. Results demonstrated that elicitor reactivity in terms of superoxide generation of the tomato cultivars and the wild species used was different. This suggested varying levels of competence for non-specific active defence. The proposed technique merges the advantages of in vitro cultures and of whole tissues and also demonstrates that root tissue is a suitable material for evaluating free radical release.

Keywords Biotic stress · *Lycopersicon* · Reactive oxygen species · Tissue culture

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Abbreviations

ANOVA	Analysis of variance
CAT	Catalase
Cyt *c*	Cytochrome *c*
HPRGs	Hydroxyproline-rich glycoproteins
HR	Hypersensitive response
LS	Linsmajer and Skoog medium
NADPH	Nicotinamide adenine dinucleoside phosphate
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
SOD	Superoxide dismutase

Introduction

Reactive oxygen species (ROS) i.e. superoxide radical ($O_2^{\cdot-}$), hydroxyl radical ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2) are formed in plants during normal metabolic reactions such as photosynthesis and respiration. They are involved in important physiological processes such as senescence and fruit ripening. Environmental injury from salinity stress, excess of heavy metals, mechanical shock, UV light, exposure to ozone, water deficiency and pathogen attack also leads to the production of ROS that can damage cellular macromolecules and even result in cell death (Mittler 2002). Sources for ROS in plant cells include the organelles (chloroplasts, mitochondria, microbodies), the detoxifying reactions

catalysed by cytochromes in the cytoplasm and the endoplasmic reticulum, and the transmembrane nicotinamide adenine dinucleoside phosphate (NADPH) oxidases probably activated by an influx of extracellular Ca^{2+} ions (Apel and Hirt 2004).

In order to avoid oxidative damage plants are equipped with a series of enzymatic and non-enzymatic tools for ROS scavenging. These include superoxide dismutases, catalases, peroxidases, ascorbate and glutathione (Mittler 2002; Apel and Hirt 2004).

As ROS production is a crucial event in several aspects of a plant's life, this paper aims at describing the usage of fast growing and readily available in vitro grown root tissue for routine superoxide determination as well as for preliminary screening.

Current methods for superoxide evaluation are based mostly on plant cell cultures and protoplasts (Doke 1983a; Able et al. 1998) or on whole organs such as leaves or tuber discs (Doke 1983b; Doke and Ohashi 1988). While suspension cells or protoplasts cannot be considered representative from the physiological point of view as in vivo plants, the detailed study of physiological responses in whole plant tissues may be difficult because of the unreliable level of effective penetration and diffusion of exogenous reporter molecules and inhibitors. However, the importance of tissue integrity has been demonstrated in several instances. No evidence for the induction of HO_2^-/O_2^- production and hypersensitive response (HR) was in fact observed in tobacco protoplasts challenged with harpin from *Pseudomonas syringae* pv. *syringae* or with oomycete zoospores respectively, suggesting the requirement of close contact between the pathogen and the host cell wall (Hoyos et al. 1996; Able et al. 1998).

As mentioned earlier, one of the most studied inducers of ROS production is pathogen attack, which was therefore chosen for our experiments. One of the earliest events in the plant response to pathogen challenge is the so-called oxidative burst represented by a rapid increase of ROS such as superoxide anion and hydrogen peroxide. ROS have multiple roles in the defence response i.e. direct antimicrobial activity, crosslinking of the cell wall Hydroxyproline Rich Glycoproteins (HRGPs) and induction of plant genes involved in cellular protection (Grant and Loake 2000). Moreover, ROS are necessary for the initiation of the HR and are involved in the establishment of Systemic Acquired Resistance (Alvarez et al. 1998). In order to cope with the toxic effect of free radicals, scavenging enzymes such as superoxide dismutases, catalases, peroxidases and antioxidant molecules are differentially induced during incompatible and compatible host-pathogen interactions (García-Limones et al. 2002). Within this context, Storti et al. (1992) have also shown that in callus tissue from tomato cultivars in the presence of *Fusarium oxysporum* all the classes of peroxidases, soluble, ionically bound and covalently bound, are induced to higher levels in the resistant cultivars than in the moderately tolerant or susceptible ones.

The method for superoxide determination from roots has been set up using the tomato (*Lycopersicon esculentum* Mill.) cultivar Tondino then validated on twelve tomato cultivars and on four wild species of the genus *Lycopersicon* treated or not with general elicitors of the defence response.

Materials and methods

Chemicals

Cytochrome *c* (Cyt *c* from horse heart), superoxide dismutase (SOD EC 1.15.1.1, from bovine erythrocytes), catalase (CAT EC 1.11.1.6, from bovine liver), calcium ionophore A23187 (calcimycin, $C_{29}H_{37}N_3O_6$), laminarin (β-1,3 glucan, from *Laminaria digitata*) were purchased from Sigma and dissolved in 10 mM potassium phosphate buffer (pH 7.8). A23187 was dissolved in dimethyl sulfoxide.

Plant cultures

Twelve cultivars of tomato (Table 1) and four *Lycopersicon* species (*L. minutum, L. pennellii, L. peruvianum, L. pimpinellifolium*, obtained from the Centre for Genetic Resources, Wageningen, The Netherlands) were used. Wild species are known sources of resistance to viral, bacterial and fungal pathogens of tomato as well as to nematodes (Williamson 1999; Laterrot 2000).

Tomato seedlings were obtained as described in a previous paper (Buiatti et al. 1987). Four week-old sterile roots were transferred to LS liquid medium (Linsmajer and Skoog 1965) and incubated in the

Table 1 Pathogen resistance/tolerance of the *L. esculentum* cultivars used in this study

Cultivar	Pathogen resistance/tolerance	Source
Calypso	*Verticillium, Fusarium* race 1–2, *Stemphylium*	Technisem (F)
Cayambe	*Fusarium*	CNR-IMOF [a], Napoli (I)
Golden Cherry	TMV, *Fusarium*	Plant Research International, Wageningen (NL)
Goldita	TMV, *Cladosporium fulvum* race 5	de Ruiter Seeds (NL)
Motelle	*Fusarium* race 1–2, *Verticillium*, *Stemphylium*, nematodes	CNR-IMOF [a], Napoli (I)
Petomech	*Verticillium, Fusarium*	Petoseed (I)
Red River PS	*Verticillium, Fusarium* race 1–2,	Petoseed (I)
Rio Grande	*Verticillium, Fusarium* race 1–2	Petoseed (I)
Roma VF	*Verticillium, Fusarium* race 1	Technisem (F)
Royal Chico	*Verticillium, Fusarium*, *Alternaria*, nematodes	Petoseed (I)
San Marzano	–	Four Blumen srl (I)
Tondino	–	Petoseed (I)

[a] Consiglio Nazionale delle Ricerche–Istituto per il Miglioramento Genetico delle Piante da Orto e da Fiore

dark for one week on a rotatory shaker at 100 rpm prior to superoxide determination.

Elicitor treatment

About 200 mg of roots (fw) were used for each experiment. Roots were washed in 10 mM potassium phosphate buffer (pH 7.8) and blotted on sterile filter paper to eliminate excess liquid. They were then cut into 0.5 cm segments with a sterile surgical blade. For controls root segments were transferred to 2 ml of 10 mM potassium phosphate buffer (pH 7.8) and incubated in the same conditions used during growth. Elicitor treatments were performed as follows: A23187 was used at a final concentration of 20 µM by adding 10 µl ml^{-1} from a 2 mM stock solution to the phosphate buffer before immersing the roots. Laminarin concentration was 0.4 mg ml^{-1}. After 1 h the supernatants from both control and elicited samples were collected for spectrophotometric analysis.

$O_2^{\cdot-}$ anion detection

The $O_2^{\cdot-}$ generating activity of root pieces was assayed by spectrophotometrically measuring the reduction of exogenously supplied Cyt *c* at 550 nm (McCord and Fridovich 1969). Cyt *c* was added immediately before the spectrophotometric assay at the final concentration of 2 mM. The amount of $O_2^{\cdot-}$ produced was determined using a molar extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} for Cyt *c* at 550 nm (Massey 1959). To confirm the role of $O_2^{\cdot-}$ in Cyt *c* reduction, a control assay was performed adding to each reaction 300 units ml^{-1} of SOD and 3000 units ml^{-1} CAT, 2 min before addition of the elicitors. Each data point was the average of at least two independent experiments each with two replicates ± standard error.

Statistical analysis

Data were analysed by Student's *t*-test or one-way ANOVA subjected to arcsin transformation for analysis followed by Tukey test.

Results and discussion

Superoxide measurements on roots

In order to assess whether roots could be a suitable material for superoxide determination 200 mg from the cultivar Tondino were used following the protocol of Doke (1983a). As roots were routinely maintained in culture medium at pH 5.8, it was necessary to wash the tissue in potassium phosphate buffer at pH 7.8 in order to avoid (1) possible interactions between components of the culture medium and the radical detection system and (2) the spontaneous dismutation of HO_2^-/O_2^- to H_2O_2, a process whose rate is dependent on pH (Bielski et al. 1985).

For the evaluation of superoxide production a 20 mM Cyt *c* stock solution was added to root supernatant at a final concentration of 2 mM. The reduction of Cyt *c* was then measured spectrophotometrically every min for 10 min.

Results (Fig. 1a) showed that roots indeed produced a detectable level of superoxide that slightly increased up to 3–4 min and then slowly decreased.

The observed time course of superoxide generation was confirmed by repeating the same experiment on two randomly chosen tomato cultivars: Royal Chico and Red River (Table 1), and two *Lycopersicon* wild species: *L. peruvianum* and *L. pimpinellifolium* (Fig. 1a). Each data point is the average of three independent measurements.

Effect of elicitor treatment on roots from tomato cultivars and *Lycopersicon* species

Superoxide production was evaluated in root tissues from tomato cultivars (Table 1) and wild species of the genus *Lycopersicon*, following treatment with elicitors of the defence response.

Firstly, roots were treated with a natural elicitor namely heat-released cell wall components from *F. oxysporum* f. sp. *lycopersici* race 1 (125 μg ml^{-1}

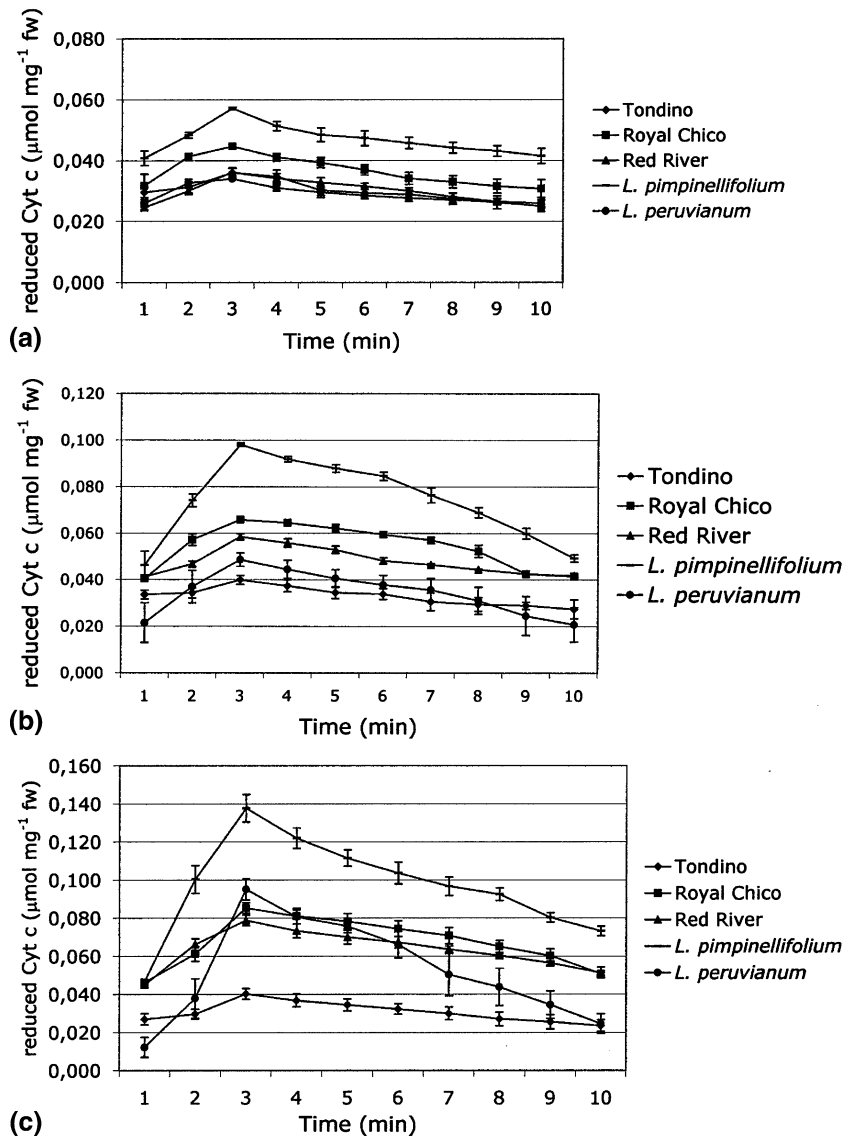

Fig. 1 Superoxide production in tomato roots **(a)** and following treatment with 0.4 mg ml^{-1} of laminarin **(b)** or 20 μM of the ionophore A23187 **(c)**. About 200 mg of roots from axenic culture were used for measuring superoxide release by evaluating the reduction of exogenously supplied Cyt *c*. The concentration of Cyt *c* used was 2 mM. Each data point is the average of three independent measurements

glucose equivalents) extracted as previously described (Buiatti et al. 1985). Superoxide measurements thus obtained were found not to be reliable due to the intrinsic characteristics of the elicitor solution (colour, turbidity and high pH) that severely hampered both the colorimetric reaction and the enzymatic activity of SOD.

It was therefore decided to use two general elicitors, the β-1,3 glucan laminarin and the calcium ionophore A23187. The involvement of poly β-1,3-1,6 glucans in plant-pathogen interactions has been demonstrated in several instances (Shibuya and Minami 2001). Laminarin in particular has been shown to induce a wide array of defence reactions and to stimulate resistance in tobacco (Klarzynski et al. 2000). A23187 has been shown to function as an elicitor activating the phytoalexin biosynthetic pathway in oats where it could also induce DNA fragmentation, one prominent characteristic of the apoptosis linked to the HR (Ishihara et al. 1996; Tada et al. 2001).

In order to check that the kinetics of Cyt *c* reduction was not changed by the addition of the elicitors the same tomato cultivars and *Lycopersicon* wild species reported in the previous section were treated for 1 h with 0.4 mg ml^{-1} laminarin or 20 μM A23187 and superoxide was measured every min for 10 min. Figures 1b and 1c show that the treatment with either laminarin or A23187 did not modify the time course of superoxide production that increased up to 3–4 min.

The same treatments were then applied to the roots of all the tomato cultivars and the wild species. Superoxide was measured by taking only one measure/sample after 3 min of incubation with Cyt *c*.

Results obtained demonstrated that both elicitors were effective in inducing a significant increase of superoxide production in most of the cultivars and the wild species (Table 2 and Table 3), except for the cultivars Tondino, that was unaffected by both laminarin and A23187, and San Marzano, that responded only to the ionophore albeit less significantly than the other cultivars and the *Lycopersicon* species ($P \leq 0.05$).

Tukey test was performed to compare individual samples showing significant variations in the basal level as well as in the relative increase of superoxide production with a 0.05 probability threshold (data not shown). Control values obtained for the cultivar Petomech were in fact significantly higher with respect to most cultivars and wild species examined, being lower for the cultivar Roma and *L. pimpinellifolium*. Differences in % induction were also significant in most comparisons. In particular the cultivars Tondino and San Marzano had the lowest levels of induction by both elicitors, while values higher than most samples were found for the cultivars Roma and Goldita and the wild species *L pimpinellifolium, L. pennellii* and *L. minutum* in the case of A23187 and for Roma and *L. pimpinellifolium* in the case of laminarin.

Control values of different experiments were subjected to Student's *t*-test and were found to be not significantly different, thus demonstrating the reproducibility of the assay (data not shown).

To check that the observed reduction of Cyt *c* was due to superoxide generated by the roots measurements were taken for all samples after the addition to the reaction mix of the free radical scavenging enzymes SOD and CAT. As expected a significant inhibition of the reduction of Cyt *c* was observed with an average of 62.4% ± 0.8. A minor part of the observed Cyt *c* reduction was therefore superoxide-independent and could involve a second pathway that does not require the presence of oxygen as electron carrier (McCord and Fridovch 1969).

In the present paper we report for the first time that roots from axenically grown plants are a good test system for ROS production as they can be easily grown and allow avoiding time-consuming and tissue-stressing cleaning or sterilising procedures.

Our results also show that the tomato cultivars and the wild species used react differently to general elicitors in terms of superoxide generation, a behaviour that could be indicative of a different 'propensity' for active defence towards non-self through the recognition of pathogen-associated molecular patterns. This 'non-host' resistance, that confers immunity at the species level, is triggered by microbe-associated general elicitors of diverse chemical nature whose recognition induces a wide array of defence reactions (Nürnberger et al. 2004). On the contrary, resistance at the level of individual cultivars is genetically determined and is described by the gene-for-gene hypothesis. In these specific plant-pathogen interactions resistance to a given pathogen is conferred by the presence of a dominant resistance gene in the plant that corresponds to a dominant avirulence gene in the pathogen (Flor 1947).

Table 2 Student's *t*-test analysis for the content of reduced Cyt *c* (μmol mg^{-1} fresh weight) in roots treated with the ionophore A23187 or not (control)

Cultivars/Species	Control (±SE)	A23187 (±SE)	*t* value	% induction (±SE)
San Marzano	0.03626 (±0.00096)	0.04194 (±0.00130)	3.524*	13.5 (±0.4)
Tondino	0.02915 (±0.00336)	0.03626 (±0.00445)	1.274^{ns}	19.4 (±0.6)
Calypso	0.03744 (±0.00144)	0.08152 (±0.00173)	19.552***	54.1 (±0.8)
Cayambe	0.02678 (±0.00196)	0.05474 (±0.00519)	5.044***	50.8 (±1.1)
Golden Cherry	0.03318 (±0.00150)	0.09479 (±0.00232)	22.301***	65.0 (±0.7)
Goldita	0.03531 (±0.00096)	0.11848 (±0.00246)	31.478***	70.2 (±0.2)
Motelle	0.03981 (±0.00109)	0.09147 (±0.00374)	13.250***	56.4 (±0.6)
Petomech	0.04502 (±0.00287)	0.09242 (±0.00404)	9.563***	51.4 (±1.0)
Red River	0.03341 (±0.00342)	0.07773 (±0.00441)	7.942***	57.3 (±2.0)
Rio Grande	0.03981 (±0.00229)	0.10616 (±0.00460)	12.897***	62.6 (±0.5)
Roma	0.02322 (±0.00226)	0.09668 (±0.00392)	16.839***	76.1 (±1.4)
Royal Chico	0.03839 (±0.00133)	0.06967 (±0.00297)	9.614***	44.8 (±0.4)
L. minutum	0.02891 (±0.00113)	0.10521 (±0.00255)	20.630***	72.3 (±2.0)
L. pennellii	0.03033 (±0.00286)	0.10000 (±0.00206)	19.760***	69.8 (±2.2)
L. peruvianum	0.02796 (±0.00140)	0.08815 (±0.00295)	18.448***	68.3 (±0.5)
L. pimpinellifolium	0.02441 (±0.00233)	0.08763 (±0.00340)	11.843***	72.3 (±1.1)

Values are the mean of two independent experiments, each with two replicates ± standard error (SE)

^{ns} not significant, * $P \leq 0.05$, *** $P \leq 0.01$

Table 3 Student's *t*-test analysis for the content of reduced Cyt *c* (μmol mg^{-1} fresh weight) in roots treated with laminarin or not (control)

Cultivars/Species	Control (±SE)	Laminarin (±SE)	*t* value	% induction (±SE)
San Marzano	0.03438 (±0.00103)	0.04076 (±0.00070)	2.956^{ns}	15.7 (±1.1)
Tondino	0.02983 (±0.00118)	0.03690 (±0.00148)	2.557^{ns}	19.1 (±1.3)
Calypso	0.03618 (±0.00124)	0.05496 (±0.00205)	7.838 ***	34.1 (±0.2)
Cayambe	0.02819 (±0.00113)	0.04073 (±0.00204)	5.363 ***	30.8 (±0.7)
Golden Cherry	0.03043 (±0.00234)	0.04852 (±0.00285)	4.896 ***	37.5 (±1.2)
Goldita	0.03334 (±0.00151)	0.05816 (±0.00305)	7.302 ***	42.6 (±0.4)
Motelle	0.03629 (±0.00265)	0.05671 (±0.00262)	5.48 ***	36.2 (±1.7)
Petomech	0.04445 (±0.00214)	0.06354 (±0.00243)	5.895 ***	30.1 (±0.7)
Red River	0.03417 (±0.00171)	0.05603 (±0.00360)	5.477 ***	38.8 (±0.9)
Rio Grande	0.03673 (±0.00219)	0.05675 (±0.00352)	4.83 ***	35.2 (±0.2)
Roma	0.02581 (±0.00214)	0.04584 (±0.00231)	6.37 ***	44.0 (±1.8)
Royal Chico	0.03739 (±0.00240)	0.05164 (±0.00295)	3.752 **	27.7 (±0.5)
L. minutum	0.03006 (±0.00192)	0.05154 (±0.00294)	6.12 ***	41.8 (±0.4)
L. pennellii	0.02950 (±0.00117)	0.05015 (±0.00240)	7.723 ***	41.1 (±0.5)
L. peruvianum	0.03013 (±0.00142)	0.04939 (±0.00186)	8.224 ***	39.1 (±0.6)
L. pimpinellifolium	0.02602 (±0.00198)	0.04476 (±0.00213)	6.461 ***	42.1 (±1.7)

Values are the mean of two independent experiments, each with two replicates ± standard error (SE)

^{ns} not significant, * $P \leq 0.05$, ** $P \leq 0.02$, *** $P \leq 0.01$

The ability to respond to elicitors of the defence response with a higher or lower production of superoxide is not always correlated to a higher or lower level of pathogen resistance, as systems have

been reported where ROS generation is uncoupled from hypersensitive cell death and resistance (Torres et al. 2002). Torres et al. (2005) have also demonstrated that the production of reactive oxygen intermediates (ROI) in *A. thaliana* negatively regulated cell death and mutants with reduced ROI generation did not display a corresponding reduction in cell death or increased pathogen growth.

Further work is underway in our laboratory in order to assess the role of the different 'competence' for active defence indicated by a higher or lower production of superoxide upon elicitor treatment in the context of host-pathogen interaction.

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