ACTIVE OXYGEN PRODUCTION IN MELON CELL-FREE HOMOGENATES ELICITED WITH HYPHAL WALL COMPONENTS OF *FUSARIUM OXYSPORUM* F.SP. *MELONIS* II. INVOLVEMENT OF A LIPOXYGENASE/FATTY ACID SYSTEM

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SUMMARY

The release of active oxygen (AO) species as a consequence of lipoxygenase (LOX) activity was observed in callus cell-free homogenates of two melon cvs, 'Retato degli Ortolani' and 'Jerac', respectively susceptible and resistant to Fusarium oxysporum f.sp. melonis race 0, treated with hyphal wall components (HWCs) of race 0 and race 1,2. LOX activity and AO generation were found in 'Jerac' homogenates combined with HWCs of race 0 or with arachidonic and linoleic fatty acids, but not in compatible combinations between 'Jerac' with race 1,2 or between 'Retato' and each of the races. LOX activity and H_2O_2 production were promoted on soybean LOX by the elicitor of race 0 only. It is suggested that the relative content of LOX in the plant and unsaturated fatty acids in the fungus could play a role in some melon-F. oxysporum f.sp. melonis incompatible combinations.

RIASSUNTO

GENERAZIONE DI SPECIE ATTIVE DELL'OSSIGENO IN OMOGENATI DI MELONE ELICITATI CON COMPONENTI DI PARETE DI *FUSARIUM OXYSPORUM* F.SP. *MELONIS*. II. COINVOLGIMENTO DEL SISTEMA LOX/ACIDI GRASSI. In omogenati acellulari di callo di due cultivar di melone, "Retato degli Ortolani" e "Jerac", rispettivamente suscettibile e resistente a Fusarium oxysporum f.sp. melonis razza 0, elicitati con estratti di parete (HWCs) di F. oxysporum f.sp. melonis, razza 0 e razza 1,2, è stato osservato il rilascio di specie attive dell'ossigeno (AO). Attività lipossigenasica (LOX) e generazione di AO sono state riscontrate nell'omogenato di "Jerac" trattato con HWCs della razza 0 o con gli acidi grassi arachidonico e linoleico, ma non nelle combinazioni compatibili tra la cv. "Jerac" e la razza 1,2 e tra la cv. "Retato" ed entrambe le razze. Attività LOX e produzione di H_2O_2 sono state promosse in LOX di soia solo da HWCs della razza 0. Si suggerisce che il contenuto in LOX della pianta e in acidi grassi del fungo giochino un ruolo in alcune combinazioni incompatibili Fusarium-melone.

Key words: oxidative burst, plant-pathogen interaction, homogenates, Fusarium oxysporum f.sp. melonis.

Early production of active oxygen (AO) species is a response commonly associated with disease resistance in plants (Levine et al., 1994; Medhy, 1994). In a melon–Fusarium system (De Donato et al., 1997), elicitation of H_2O_2 production was observed in combinations of hyphal wall components (HWCs) of race 0 of the pathogen Fusarium oxysporum f.sp. melonis (Leach et Currence) Sn. et Hans. with cells or callus cell-free homogenates of the resistant melon 'Jerac' possessing the resistance genes Fom1 and Fom2. There was no elicitation in compatible combinations between cv. 'Jerac' with the race 1,2 or the cv. 'Retato degli Ortolani', lacking resistance genes, with both the races. The involvement of the transmembrane oxidase system commonly considered to be responsible for the oxidative burst in animals and plants (Mehdy, 1994; Baker and Orlandi, 1995) was excluded because the oxidative activity was recovered at maximum level in the soluble fraction and in protein extracts of the callus homogenate, and did not require an exogenous electron donor.

As an alternative mechanism we suggested that the oxidative burst observed in the incompatible melon-Fusarium interaction depends on a lipoxygenase (LOX)-fatty acid reaction. LOX is also localized in the cytosol and is present in plants in very variable amounts. Moreover the procedure adopted for preparing the elicitor implies the presence of free fatty acids. Such a mechanism has been substantiated in this work by measuring the expression of LOX-like activity in the homogenate-elicitor interactions, the presence of substrates of soybean LOX in the fungal cell wall elicitors, and the capacity of the melon callus homogenates to promote both LOX-like activity and production of H_2O_2 from unsaturated fatty acids.

Preparation of callus, callus cell-free homogenate

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and elicitor, and H_2O_2 determination were as reported by De Donato et al. (1997). Most of the experiments were done with supernatant obtained after centrifugation of the crude homogenate at 10,000 **g** for 10 min.

LOX activity was determined spectrophotometrically by measuring the conjugated diene absorption at 234 nm (Tappel, 1962). The reaction mixture contained 1950 µl of 0.05 M MOPS buffer (pH 7.2), 10 µl of 8 mM fatty acid (linoleic or arachidonic acid) or 6 µl of HWC preparation, and 10 to 40 µl of enzyme solution (homogenate or soybean LOX, Sigma L-7395). The reaction, conducted at room temperature, was initiated by the addition of the substrate solution.

Presence of fatty acids was detected by measuring the fluorescence decay of ADIFAB (Molecular Probes, Inc., Eugene, USA). This is a fluorescent indicator of free fatty acids, consisting of a polarity-sensitive fluorescent probe (acrylodan; λ_{ex} ~ 432 nm, λ_{em} 390 nm) conjugated to a protein (FABP; 15,000 daltons) with high binding affinity for free fatty acids. When a fatty acid is added to the fluorophore solution, the fluorescence decay is proportional to the fatty acid concentration (Haugland, 1996).

LOX activity was observed in the melon cell-free homogenate of cv. 'Jerac' challenged by the elicitor of the avirulent race 0 of F. oxysporum f.sp. melonis but not in the compatible interactions 'Jerac'-race 1,2 and 'Retato'-race 0 and race 1,2 (Fig.1). These results coincide exactly with those of H_2O_2 production (De Donato et al., 1997).

LOX-like activity and H_2O_2 generation were promoted on soybean LOX by the elicitor of race 0 only (Fig.2).

Marked dose-dependent decay in fluorescence of the probe ADIFAB occurred with the elicitor from race 0 but not with that from race 1,2, revealing the presence of unsaturated fatty acids in race 0 only (Fig.3).

The AO scavengers Tiron and catalase almost completely inhibited fluorescence decay in race 0-soybean LOX combination without affecting the production of fatty acid hydroperoxides (Table 1), thus confirming that the pyranine fluorescence decay is due to AO produced by the lipoxygenase activity.

LOX-like activity (Fig.4) and H_2O_2 production (Fig.5) were induced by arachidonic and linoleic unsatured fatty acids in the cell-free homogenate of the resistant cv. 'Jerac' but not in the susceptible cv. 'Retato'.

The results confirm the hypothesis that the release of AO by melon cell homogenate challenged by F. oxysporum f.sp. melonis cell wall elicitors is LOX dependent. Moreover they show that LOX activity is markedly higher in the melon cultivar 'Jerac', containing the resistance genes Fom1 and Fom2 and that only the fungal cell wall preparation of the avirulent race 0 of F. oxysporum f.sp. melonis contains substrate for LOX.
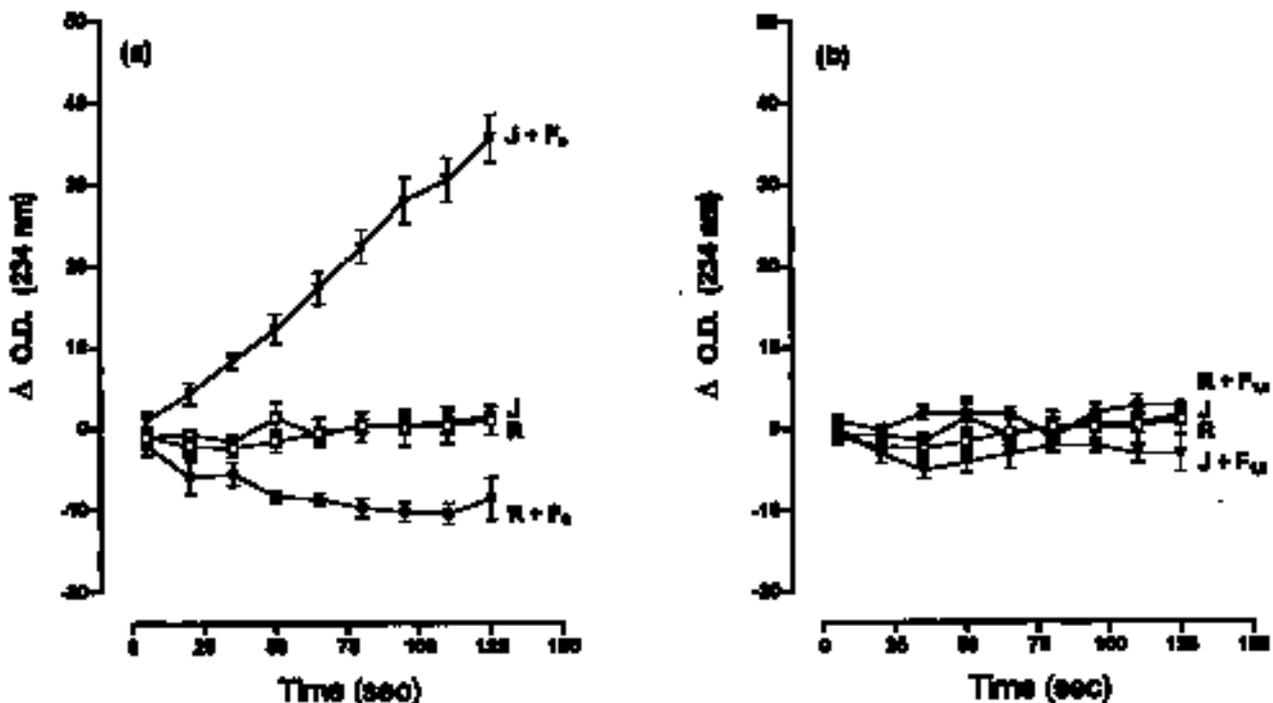

Fig.1. Effect of fungal elicitors on LOX-like activity, measured as hydroperoxide formation in cell-free homogenates of melon (100 µl ml^{-1}). Fig. 1a: F_0 = HWCs (3 µl ml^{-1}) from F. oxysporum f.sp. melonis, race 0, virulent on cv. 'Retato' (R) and avirulent on cv. 'Jerac' (J). Fig. 1b: $F_{1,2}$= HWCs (3 µl ml^{-1}) from F. oxysporum f.sp. melonis, race 1,2, virulent on both cvs 'Retato' (R) and 'Jerac' (J). Vertical bars represent SE (n = 3).
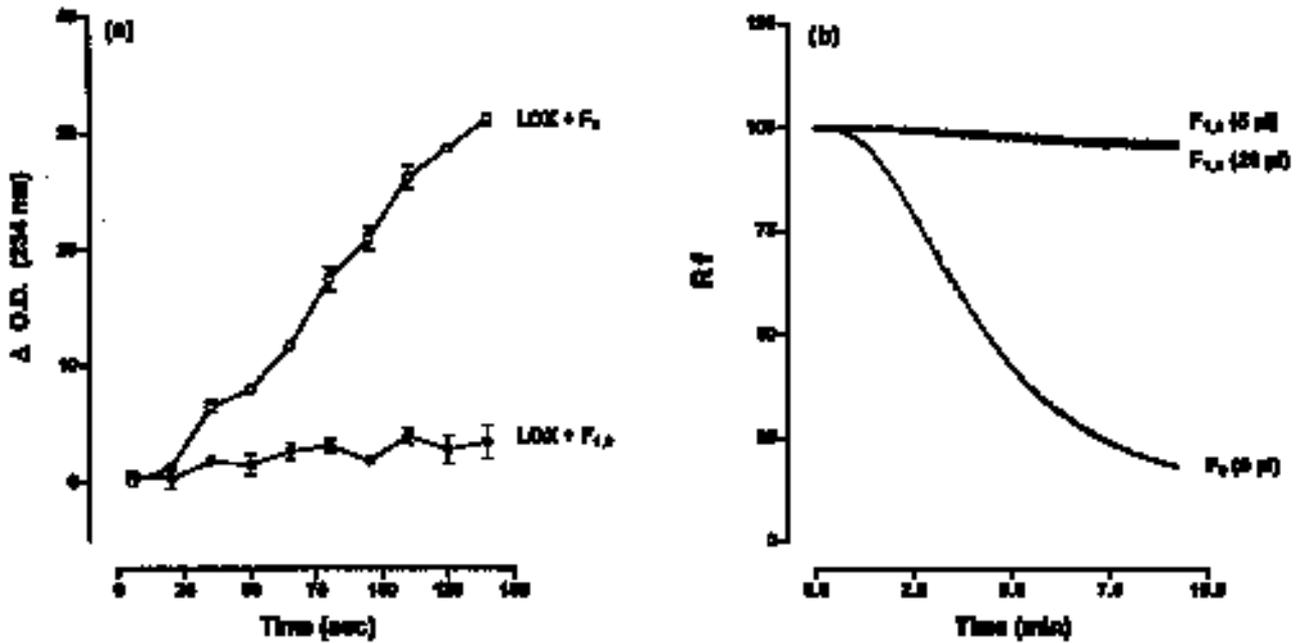
Fig.2. Effect of fungal elicitors on the formation of hydroperoxide (a) and quenching of pyranine fluorescence following production of H_2O_2 (b) in soybean LOX (500 U ml^{-1}). F_0 = HWCs from F. oxysporum f.sp. melonis, race 0, virulent on cv. 'Retato' (R) and avirulent on cv. 'Jerac' (J). $F_{1,2}$ = HWCs from F. oxysporum f.sp. melonis, race 1,2, virulent on both cvs 'Retato' (R) and 'Jerac' (J). Vertical bars represent SE (n = 3).
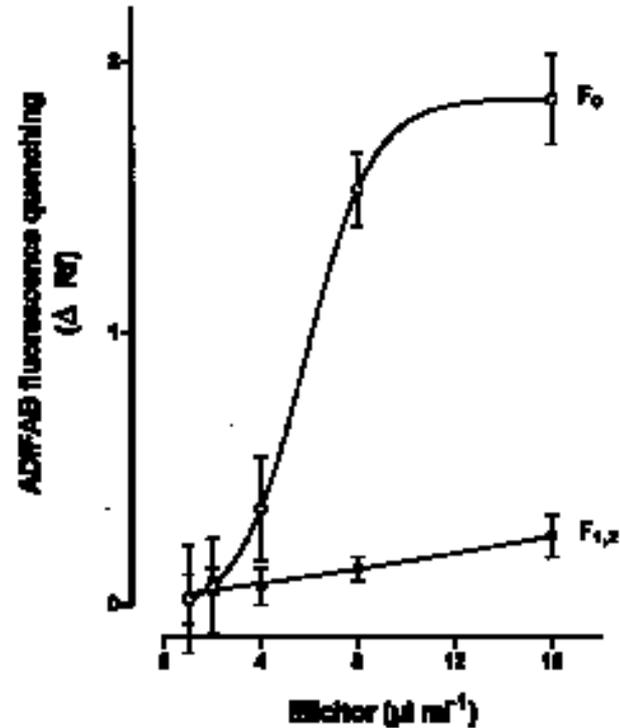

Fig.3. Effect of fungal elicitors on the fluorescence quenching of ADI-FAB. F_0 = HWCs from F. oxysporum f.sp. melonis, race 0. $F_{1,2}$ = HWCs from F. oxysporum f.sp. melonis, race 1,2. Successive aliquots of fungal cell-wall extract were added to a 0.2 μM ADIFAB solution in Tris buffer (50 mM Tris, 1 mM EGTA, pH 8.0); the relative fluorescence (Rf) values were recorded within 10 seconds. The data reported are the means of four independent experiments, $\pm$ the confidence interval (P=0.05).

Table 1. Effect of catalase and Tiron on the fluorescence quenching of pyranine and the activity of soybean LOX (hydroperoxide formation measured at 234 nm) in the presence of race 0 elicitor.

Inhibitor	Inhibition (%)	
	Pyranine decay	O.D. increase (234 nm)
Catalase (200 U ml^{-1})	> 90	~ 0
Tiron (2 μM)	> 90	~ 0

This suggests that the relative content of LOX in the plant and unsaturated fatty acids in the fungus could play a role in some melon- F. oxysporum f.sp. melonis incompatible combinations, but no generalization is possible for the moment. Whereas the differences of eliciting activity between the virulent and the avirulent genotype have been constantly reproduced by different isolates and subcultures of the fungus, differences in LOX activity between other melon genotypes, susceptible or carrying the resistance genes Fom1 and Fom2 have been, in preliminary assays, not always consistent.
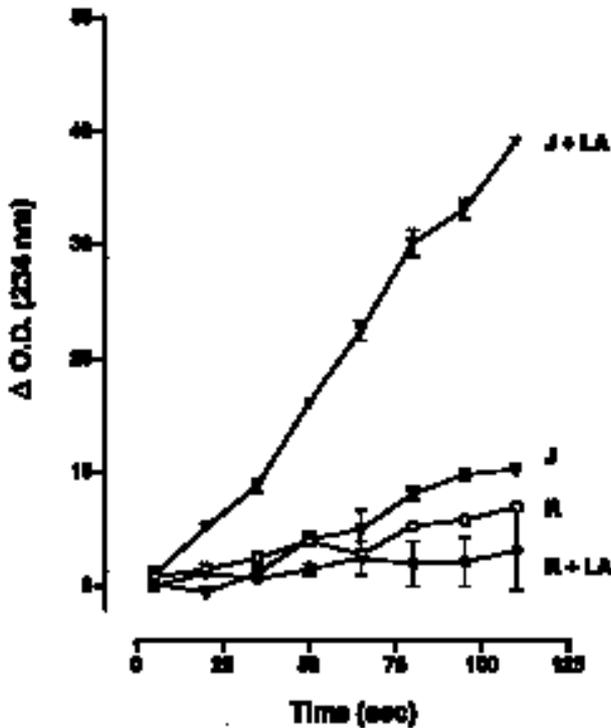
Fig.4. Elicitation of hydroperoxide formation in cell-free homogenates of melon, cv. 'Jerac' (J) and cv. 'Retato' (R) (100 µl ml^{-1}), treated with linoleic acid (40 µM; LA). Analogous data were obtained with arachidonic acid. Vertical bars represent SE (n = 3).
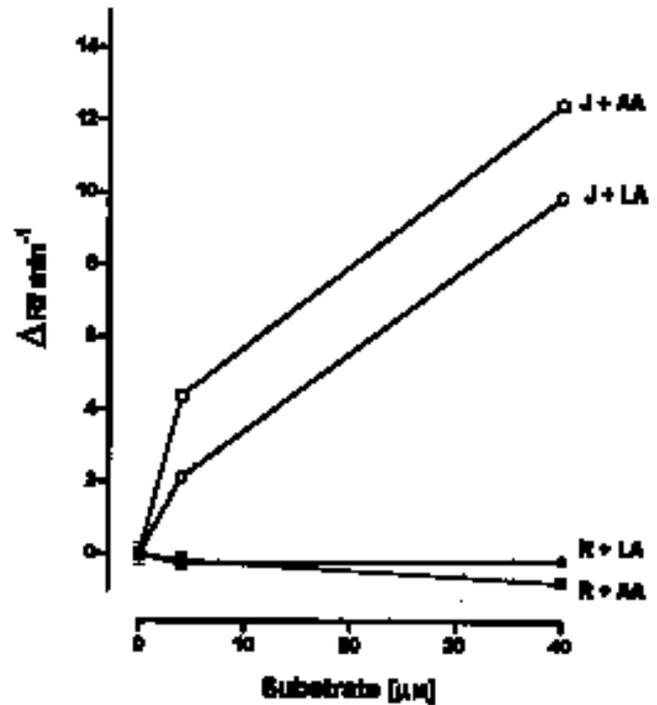
Fig.5. Influence of linoleic acid (LA) and arachidonic acid (AA) concentration on the fluorescence quenching of pyranine elicited in cell-free homogenates of melon. R = cv. 'Retato', susceptible both to race 0 and race 1,2 of F. oxysporum f.sp. melonis; J = cv. 'Jerac', resistant to race 0 and susceptible to race 1,2 of F. oxysporum f.sp. melonis.

REFERENCES

Baker C.J., Orlandi E.W., 1995. Active oxygen in plant pathogenesis. Annual Review of Phytopathology **33**: 299-321.

De Donato M., Mozzetti C., Chiavazza P., Rubino R., Matta A., 1997. Active oxygen production in cell-free melon homogenates elicited with hyphal wall components of Fusarium oxysporum f. sp. melonis. I. A response correlated with race-specific resistance. Journal of Plant Pathology **78**: 35-43.

Haugland R.P., 1996. Handbook of fluorescent probes and research chemicals. 6th Ed. Molecular Probes Inc., Eugene, USA.

Levine A., Tenhaken R., Dixon R., Lamb C., 1994. H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. Cell **79**: 583-593.

Mehdy M.C., 1994. Active oxygen species in plant defense against pathogens. Plant Physiology **105**: 467-472.

Tappel A.L., 1962. Lipoxidase. In: Colowick S.P., Kaplan N.O. (eds.). Methods in enzymology, Vol. 5, pp. 539-542. Academic Press, N.Y., USA.