INSTITUTE OF PLANT PATHOLOGY
UNIVERSITY OF NAPLES
F. CERVONE - G. CRISTINZIO

INSTITUTE OF PLANT PATHOLOGY
UNIVERSITY OF TURIN
A. GARIBALDI

Maceration and death of potato tissue caused by polygalacturonase from *Rhizoctonia fragariae*

Estratto dagli « Annali » della Facoltà di Scienze Agrarie
dell'Università di Napoli in Portici
SERIE IV, VOL. XII, I SEMESTRE 1978

1978
Della Torre - Portici

Institute of Plant Pathology
University of Naples
F. CERVONE - G. CRISTINZIO

Institute of Plant Pathology
University of Turin
A. GARIBALDI

Maceration and death of potato tissue caused by polygalacturonase from *Rhizoctonia fragariae* (*)

Introduction

Maceration of plant tissue is common in many diseases of higher plants caused by fungi and bacteria. It has been shown that tissue maceration is closely related to death of protoplasts (9) and that both maceration and death are caused by pectic enzymes (4, 5, 11).

Various methods have been developed for measuring tissue maceration and cell death caused by pectic enzymes (1, 8). For measuring cell death the most widely used method is based on the inability of killed protoplasts to retain the vital due neutral red, which accumulates in the vacuols of living cells (7). Thus living protoplasts retain the stain, whereas dead cells rapidly release it in a plasmolysing solution. Results recording involves visual observation of the stained tissue and subjective assessment of an index, the Neutral Red Index (NRI), which reflect the number of protoplasts that have not retained the stain (2).

We have tried to overcome these difficulties by the use of a quantitative method to assess the dye lost by the cells. This paper describes the extraction of neutral red from plant tissue and its measurement by spectrophotometer.

Materials and methods

Culture filtrates and solution of homogeneous endopolygalacturonases (PG 1 + PG 2) were prepared from *Rhizoctonia fragariae* Husain et McKeen as previously described (3). Enzyme activity was expressed in relative viscometric units (RVU) as previously reported (3). Disks (8 mm diameter) were cut from 4 mm thick slices of medullary tissue of potato tubers, and washed in 50 mM Na-acetate buffer, pH 5, to remove potato starch and cytoplasm debris before adding to the reaction mixture. A disk was placed in 1 ml of culture filtrate (1 RVU) or of a solution of homogeneous enzyme (1 RVU) in 50 mM Na-acetate buffer at pH 5 and 23 °C. The ambient solution was then removed and the disk was washed with 50 mM Na-acetate buffer, pH 5. Maceration was assessed from the ease with which the disk could be pulled apart

(*) This work was supported by a grant from C. N. R., research group « Patologia Piante Ortensi ».

with spatulas on a 0 to 5 linear scale. A rating of « 0 » indicates that cohesion of the tissue is similar to that of freshly cut tissue and a rating of « 5 » indicates complete loss of cohesion. This gives a maceration index (MI) 0 to 5.

To assess killing of protoplast, a disk was placed in 1 ml of a solution containing 0,01 % neutral red in 50 mM phosphate buffer, pH 7, for 20 min., remove and rinsed with several changes of 1 M KNO_3. Cell death was then determined in two ways. The first estimated visually the amount of neutral red retained by cells with a 0 to 5 linear scale to give the Neutral Red Index (NRI) (8). In the second method, neutral red was extracted for 1 hour from a disk with 3 ml of methanol at 23 °C. The methanol was collected, centrifuged and its content of neutral red was estimated measuring adsorbance at 465 nm in a Beckman DB-GT spectrophotometer with pure methanol as blank. Controls were disk no-treated with enzyme and disk killed by boiling for 5 min.

Results and discussion

Table 1 shows the results obtained when potato disks were incubated for different times in culture filtrate or in a solution of purified endopolygalacturonases (PG 1 - 2) from *Rhizoctonia fragariae*. Both culture filtrate and

Table 1 - Maceration (M. I.) and cell death in potato tissue incubated in culture filtrate or a solution of purified polygalacturonases from *R. fragariae* (1 RVU). Cell death was assessed by the NRI and by extracting neutral red retained by cells after treatment with the enzyme. The control were non-treated (L = living tissue) and boiled (D = dead tissue) disk. The percentage of neutral red extracted was calculated by the formula $\frac{A - A^D}{A^L - A^D} \times 100$, where A^L is the adsorbance at 465 nm of neutral red extracted from the « L » disk, A^D is the adsorbance of neutral red extracted from disk « D », A is the adsorbance of neutral red extracted from the disk treated with enzyme. Each value was the average of 6 repetitions. Further details are given in the text.

Incubation time (hours)	Enzyme	M. I.	N. R. I.	% Neutral red extracted (*)
L	—	—	0	100
D	—	—	5	0
1	C. F.	0	0	97
2	C. F.	0,5	1	77
4	C. F.	1	2	55
	PG 1 - 2	0,5	1	77
5	C. F.	1,5	4	19
	PG 1 - 2	1	1	68
7	C. F.	3	5	3
	PG 1 - 2	2	5	3

M. I. — Maceration Index; 0, no maceration; 5, complete maceration.
N. R. I. — Neutral Red Index; 0, no cell death; 5, all cells death.
C. F. — Culture filtrate.
PG 1 - 2 — Solution of PG 1 and PG 2.
(*) Percentage of neutral red extracted from treated tissue compared to that from living tissue (L).

the PG 1-2 solution macerated potato tissue; the culture filtrate was the more active, possibly because of the presence of non-pectolytic macerating factors similar to those detected in culture filtrates of other fungi (6, 10, 12). The culture filtrate and the PG 1-2 solution also rapidly killed cells of potato tissue. Results with different amounts of polygalacturonases are shown in table 2.

TABLE 2 - Maceration and cell death in potato tissue incubated for 5 hours with different amounts of PG 1-2 solution. Each value was the average of 6 determinations. (See table 1 and text for further details)

Enzyme amount (RVU)	M. I.	N. R. I.	% Neutral red extracted
0	0	0	100
1	1	1	68
2	1	1	64
3	2	3	30
4	3	5	2

By extracting the neutral red retained by the cells it was possible to determine the extent of cell death caused by treatment with enzyme. The values obtained showed a good inverse proportionality between the percentage of neutral red extracted and the amounts of enzyme used. The values of neutral red extracted avoided visual assessment of neutral red indices and allowed detection of small differences among various samples.

Ricevuto il 9-5-1978

SUMMARY

A quantitative method for measuring cell death caused by pectic enzyme is reported. According to the method, the vital dye neutral red, which accumulates in the vacuols of living cells, was extracted with methanol from the plant tissue and measured by spectrophotometer. The values of neutral red extracted avoided visual assessment of neutral red indices and allowed detection of small differences among various samples.

RIASSUNTO

Un metodo quantitativo è stato messo a punto per la stima delle cellule vegetali morte in seguito ad attacco di enzimi pectici. Secondo questo metodo il rosso neutro, un colorante che si accumula nei vacuoli delle cellule vegetali vive, viene estratto con metanolo e misurato spettrofotometricamente.

Il metodo presenta notevoli vantaggi rispetto a quelli finora in uso perché evita qualsiasi valutazione soggettiva della morte delle cellule e rileva anche piccole differenze fra i diversi campioni.

REFERENCES

(1) Basham H. G. and Bateman D. F. 1975 - Killing of plant cells by pectic enzymes; the lack of direct injurious interaction between pectic enzymes or their soluble reaction products and plant cells. Phytopathology *65*, 141-153.

(2) Basham H. G. and Bateman D. F. 1975 - Relationship of cell death in plant tissue treated with a homogeneous endopectate lyase to cell wall degradation. Physiol. Pl. Path. *5*, 249-262.

(3) Cervone F., Scala A., Foresti M., Cacace M. G. and Noviello C. 1976 - Endopolygalacturonase from *Rhizoctonia fragariae*. Purification and characterization of two isoenzymes. Bioch. et Bioph. Acta *482*, 379-385.

(4) Garibaldi A. and Bateman D. F. 1971 - Pectic enzymes produced by *Erwinia chrysanthemi* and their effects on plant tissue. Physiol. Pl. Path. *1*, 25-40.

(5) Hall J. A. and Wood R. K. S. 1974 - Permeability changes in tissue and other effects on cell separating solution from soft rots caused by *Corticium praticola* and *Erwinia atroseptica*. Ann. appl. Biol. *38*, 129-140.

(6) Kern H. and Naef-Roth S. 1971 - Phytolysin, ein durch pflanzenpathogene Pilze gebildeter mazerierender Faktor. Phytopath. Z. *71*, 231-246.

(7) Mahlberg P. 1972 - Localization of neutral red in lysosome structures in hair cell of *Tradescantia virginiana*. Can. J. Bot. *50*, 857-859.

(8) Stephens G. J. and Wood R. K. S. 1975 - Killing of protoplast by soft rot bacteria. Physiol. Pl. Pathol. *5*, 165-181.

(9) Tribe H. T. 1955 - Studies in the physiology of parasitism XIX. On the killing of plant cell by enzyme from *Botrytis cinerea* and *Bacterium aroideae*. Ann. Bot. *19*, 351-368.

(10) Tani T. and Namba H. 1969 - Qualitative nature of macerating activities in the culture filtrates of *Botrytis cinerea*. Ann. Phytopath. Soc. Jap. *35*, 1-9.

(11) Tseng T. G. and Maent M. S. 1974 - Toxicity of endopolygalacturonase trans-eliminase, phosphatidase and protease to potato and cucumber tissue. Phytopathology *64*, 229-236.

(12) Yoshikawa M., Masago H. and Minuora S. 1977 - A non-pectolytic protein from *Phytophthora capsici* that macerates plant tissue. Physiol. Pl. Pathol. *11*, 61-70.