A BIOASSAY OPTIMIZATION FOR EVALUATING CELL VIABILITY OF In Vitro RICE PLANTS (Oryza sativa L.) USING 2, 3, 5 TRIPHENILTETRAZOLIUM

Lydia García, Martha Hernández, A. Gutiérrez and Saturnina Mesa

ABSTRACT. Different factors changing cell viability response were evaluated when 2,3,5 triphenilteterazolium chloride (TTC) is used *in vitro* rice plants var. LP-1. The evaluated factors were : pH, TTC concentration, applying time of TTC and incubation time with necrosing agent. The results obtained show the optimum conditions for each of these factors: pH = 7.5, 0.6% TTC, 20 hours of applying TTC and six hours of necrosing incubation agent. Furthermore, it is demonstrated that this bioassay can be used to evaluate the effect of biotic agents on cell viability in rice cultivar.

Key words: rice, *Oryza sativa*, necrosis, viability

RESUMEN. Se evaluaron diferentes parámetros que afectan la respuesta de la viabilidad celular cuando se utiliza 2,3,5 cloruro de trifeniltetrazolium (TTC) en vitroplantas de arroz variedad LP-1. Los parámetros evaluados fueron: pH, concentración de TTC, tiempo de aplicación del TTC y tiempo de incubación con el agente necrosante. Los resultados obtenidos muestran las condiciones óptimas para cada uno de estos factores: pH = 7.5, 0.6 % de TTC, 20 horas de aplicación del TTC y seis horas de incubación del agente necrosante. Además, se demuestra que este bioensayo puede utilizarse para evaluar el efecto de agentes bióticos sobre la viabilidad celular en el cultivo del arroz.

Palabras clave: arroz, *Oryza sativa*, necrosis, viabilidad

INTRODUCTION

Photosynthesis, respiration, vital colorants, among others, have been used to determine cell viability in plant tissues. Among vital colorants, 2,3,5 triphenilte-trazolium has been largely used with good results.

When viable tissues are present, the TTC colourless solution produces a less water soluble red compound named phormazan.

When TTC is applied to living cells, it is reduced by respiration enzymes : deshydrogenases of malic, succinic and isocitric acids (Lorin, 1951). Therefore, the amount of red phormazan produced is positively correlated with cell respiratory efficiency and it is considered a cell viability measuring parameter.

Butchelli *et al.* (1990) evaluated cell viability using radioisotopic leucin, fluorescein diacetate and TTC in rice cell suspensions and found that the three methods can be used to measure this parameter.

Steponkus and Lanpheaar (1967) reported a good test for leaf discs, stem segments and cell suspensions, in which the red phormazan produced is extracted with ethanol. The evaluation of cell viability has been used in different biologic assays. At present, it is used to value the necrosing effect of carbohydrase enzymes attacking plant cell wall (Dean and Timberlaf, 1989) and to determine experimentally the effect of cell wall derived oligosaccharines when necrosis inducing the characteristic necrosis of hypersensitive answer, a defensive mechanism of plants shown at host-pathogen incompatibility interactions (Pierre *et al.*, 1985).

The present paper is proposed to determine the optimum conditions to value cell viability of *in vitro* rice plants with TTC as indicator.

MATERIALS AND METHODS

In vitro rice plants var. LP-1 were obtained after growing in Murashige and Skoog's medium for 30 days (Tascón and García, 1985, and Roca and Mroginishi, 1991).

The optimum bioassay conditions to evaluate cell viability with TTC were determined after testing different pH values (6-9), TTC concentrations (0.5-0.9 %) and TTC application times (5 - 25 hours).

Three mililitres TTC were added to the selected seedlings which were incubated in the dark. Afterwards, the red phormazan was extracted with ethanol 95 %. The absorbability read using a Spekol Karl Zeis (MOD-10) was 530 nm; according to literature, this wave lenght is reported as the least interferring photosynthetic pigment (Steponkus *et al.*, 1967).

Data were processed through a Variance Analysis of Simple Classification and four observations per treatment. Duncan's Multiple Range Test was used to detect significative differences among treatments.

Once the optimum conditions of this method were determined, the most adequate incubation time was recorded for a necrosing agent obtained from a sugar

Dra. Lydia García, Profesor Asistente, Martha Hernández, Profesor Auxiliar y Saturnina Mesa, Técnico Docente del Departamento de Química, Instituto Superior de Ciencias Agropecuarias de la Habana, Gaveta Postal No. 18-19, San José de las Lajas; A. Gutiérrez, Investigador Agregado del Laboratorio de Oligosacarinas. Departamento de Fisiología y Bioquímica Vegetal, Instituto Nacional de Ciencias Agrícolas, Gaveta Postal No. 1, San José de las Lajas, La Habana, Cuba.

cane hemicellulose enzymatic hydrolysate using a commercial enzyme with xylanase activity.

Different hydrolysate concentrations were sprayed on *in vitro* plants, which were incubated with it for six and 18 hours. Experimental results were processed by means Variance Analysis Bifactorial Rules, a randomized complete block design and four observations per each treatment. Duncan's Multiple Range Test was used to determine significative differences among treatments.

RESULTS AND DISCUSION

Table I shows the effect of pH on red phormazan production observing markedly significative differences among treatments. It was found that the optimum pH range for these experimental conditions were found to be between 7 and 8, neutral or slightly acid, corresponding to maximum absorbability values. Towill and Mazur (1975) obtained similar results with cell suspensions.

The effect of TTC concentration on red phormazan production is reported in Figure 1, obtaining highly significant differences among treatments. As shown in the figure, at the beginning when TTC concentration increases, absorbability increases too, but at higher concentrations than 0.6 %, it decreases abruptly; therefore, the optimum TTC concentration under these conditions corresponds to 0.6 %.

As regard to cell suspensions Towill and Mazur reported the optimum TTC concentration, indicating that each cultivar response to TTC concentration should be investigated, since this toxicity has been reported (Parker, 1953 and Palmer, 1968).

The effect of TTC application times on red phormazan production is demonstrated in Figure 2, obtaining highly significant differences among treatments. As it is observed, when the application time is longer, absorbability increases until 20 hours; thus, this is the best time for these conditions. Maha *et al.* (1986) worked with soybean cell suspensions at 24 hours of application. Towill and Mazur reported an optimum period between 18 and 20 hours.

The effect of necrosing agent incubation times on red phormazan production at different hemicellulose enzymatic hydrolysate concentrations is shown in Figure 3 and no significant interaction differences were found between incubation times and concentration nor incubation times, that were 6 and 18 hours. Similar results were obtained by Maha *et al.* (1980) in soybean cell suspensions. However, markedly significant differences were found among the means when measuring cell viability at different necrosing agent concentrations independently of the incubation time, indicating this is an adequate method for measuring cell viability when varying the concentration of active species that might affect cell viability.

Table I. The effect of pH on red phormazan production

pH	Absorbability
6	0.69 b
7	0.72 a
8	0.70 ab
9	0.65 c

SE = 0.01 ** p < 0.01
VC = 2.30 %
Means with common letters do not differ significantly

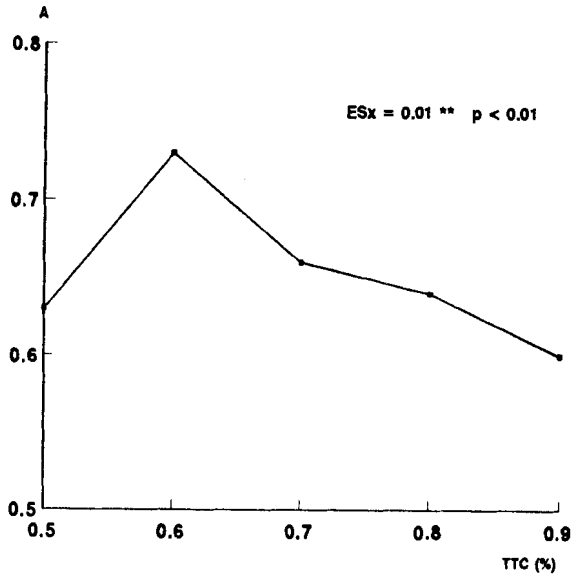

Figure 1. The efect of TTC (%) on red phormazan production (A)

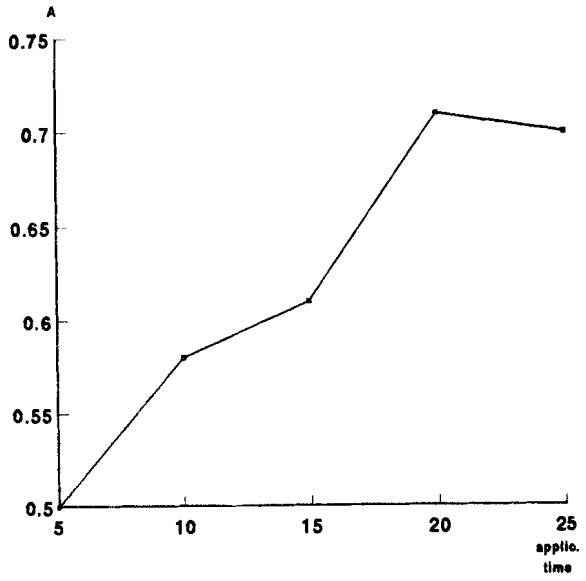

Figure 2. The effect of TTC application times on red phormazan production (A)

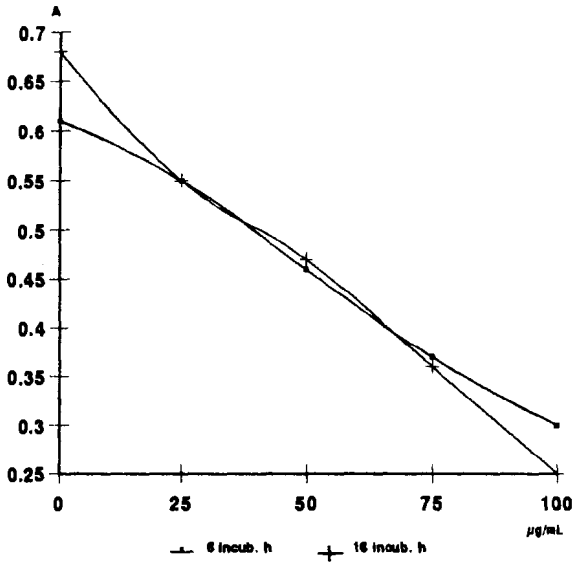

Figure 3. The effect of necrosing agent incubation time on red phormazan production (A)

CONCLUSIONS AND RECOMENDATIONS

Results proved the optimum conditions to evaluate cell viability of *in vitro* rice plants are: pH = 7.5, 0.6 % TTC, 20 hours of TTC application and six hours necrosing agent incubation.

Through this paper a non-reported bioassay for *in vitro* rice plants was standardized and a similar response reported by literature was obtained. Besides, the use of *in vitro* plants makes the work easy, since this bioassay is simpler and cheaper.

It can applied to evaluate the necrossing effect of any biotic agent.

BIBLIOGRAPHY

Buchelli, P. /et al./. Host- Pathogen Interaction. XXXVI Partial Purification and Characterization of Host-Labile Molecules Secreted by the Rice Biast Pathogen that Solubilize Plant Cell Wall Fragments that Kill Plant Cells. Physiological and Molecular Plant Pathology (Dordrech)36:159-173, 1990.

Dean, R. and W. Imberlaf. Production of Cell Wall Degrading Enzymes by Aspergillus nidulans. A Model System for Fungal Pathogenesis of Plants. Plant Cell (New York)1(3): 265-273, 1989.

Lorin, R. Survey of Factors Responsible for Reduction of 2,3,5 Tripheniltetrazolium Chloride in Plant Meristems. Science (Washington)110 (2946): 692-693, 1951.

Maha, A., M. Hamdan and R. Dixon. Differential Biochemical Effects of Elicitor Preparations from Colletotrichum linde Muthianum. Physiological and Molecular Plant Pathology (Dordrech)28: 329-344, 1986.

Palmer, J. M. and M. Kalina. Some Effects of Tetrazolium Salt on the Metabolism of Mithocondria Isolated from Jerusalem Artichoke Tubes. Planta (New York)78: 358-365, 1968.

Parker, J. Some Applications and Limitations of Tetrazolium Chloride. Science (Washington)110 (3055): 77-79, 1953.

Pierre, J. /et al./. Isolation and Characterization of an Elicitor of Necrosis Isolated from Intercellular Fluids of Compatible Interactions of Cladosporium fulvum (Syn. Fulvia fulvis) and Tomato. Plant Physiology (Alexandria)77: 642-647, 1986.

Roca, W. Cultivo de tejidos en la agricultura. /W. Roca, L. Mnoginski.- Cali : CIAT, 1991.

Smith, F. E. Tetrazolium Salt. Science (Washington)110 (2948): 751-754,1951

Steponkus, P. and F. Lamphear. Refinement of the Triphenitetrazolium Chloride Method of Determining Cold Injury. Plant Physiology (Alexandria)(42): 1423-1426, 1967.

Tascón, E. Arroz: Investigación y producción. /E. Tascón, E. García.- En: Cultivo de tejidos en la agricultura.- Cali : CIAT, 1985.

Towill, L. and P. Mazur. Studies on the Reduction of 2,3,5, Triphenil-tetrazolium Chloride As a Viability Assay for Plant Tissue Cultures. Canadian Journal of Botany (Ottawa)53 (11): 1097-1102, 1975.

Recibido: 5 de abril de 1995
Aceptado: 21 de abril de 1995