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The Use of 2, 3, 5-Triphenyltetrazolium Chloride in Assessing Leaf Protoplast Metabolic Activity

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ABSTRACT

The method of evaluating leaf protoplast metabolic activity by use of triphenyl tetrazolium chloride (TTC) has been established. The procedure for the TTC test is as follows: leaf protoplasts were isolated from *Brassica napus* cv. "Bronowski" and purified by floatation on 0.7M sucrose. The leaf protoplasts were suspended in 0.6M sorbitol-0.05M phosphate buffer (pH 7.4) containing 0.05% TTC and incubated in dark at 30°C for 16 hours. Reduced water insoluble formazan was extracted from the protoplasts with 80% ethanol. Record the absorbance at 520nm. The purified protoplasts reduced TTC, whereas celluar or subcelluar debris gave negative TTC test. In order to estimate the relationship between TTC reduction rate and plating efficiency, leaf protoplasts of *Petunia hybrida* were treated with N-methyl-N'-nitro-nitrosoguanidine(MNNG). TTC reduction rate decreased with increasing concentration of MNNG, but the plating efficiency decreased conspicuously as MNNG concentration reached to 25 μg/ml. Consequently, it could correlate with the plating efficiency. The TTC reduction test described here offers a simple test for prediction of leaf protoplast metabolic activity.

Introduction

Plant protoplast viability may be defined as the capacity of an isolated protoplast to continue to grow and to form callus or plantlets. The process of protoplast isolation must render their viability extremely changed from their initial stage of the cells. However, it is clear that a proportion of the isolated protoplasts will not survive in the following culture. This proportion depends upon the types of material employed as protoplast sources. Therefore, protoplasts need to be checked for viability at an early stage prior to protoplast culture. In order to determine a portion of damaged protoplasts that appeared during isolation procedures or in the following culture, a number of tests have been applied to check protoplast viability. Dye exclusion methods can indicate the viability of protoplasts by virtue of plasmalemma intactness. The test that relies on the ability of the plasmalemma to block the entry of dye molecules is normally performed with Evans blue(Glimelius et al.[3], Kanai and Edwards[4]). The dead or dying protoplasts stain blue, whilst those with an intact plasmalemma remain unstained. Percentage viality can be assessed by counting. If vacuole is disrupted, protoplast will die. The intactness of the vacuole can be assayed by use of neutral red, a vital dye(Basham and Bateman[1]). The dye transverses the intact plasmalemma and is concentrated in vacuoles. Dead or damaged cells will be stained completely. Fluorescein diactetate(FDA) staining method depends on plasmalemma integrity and an associated esterase activity(Larkin[7]). The esterase cleaves the acetate from the FDA as it passes into the protoplast, the fluorescein fluoresces on irradiation with ultraviolet. In contrast to the dye exclusion methods, only viable protoplasts will fluoresce in this method. This paper reports the use of triphenyltetrazolium chloride(TTC) as a protoplast viability test. It has been formerly used for evaluation of seed germinability(Porter et al,[8]), cold injury of plant tissue (Purcell and Young[9]) or that of suspension cultured cells(Kuriyama et al[6]). In contrast to FDA staining that depends on plasmalemma integrity and its associated esterase activity, TTC reduction rate is due to some dehydrogenase activities and may contribute to estimate metabolic activities of protoplasts more directly. However, TTC reduction tests described

previously have not directly applied to leaf protoplasts because of endogeneous pigment interference. The present study provides a method for evaluating leaf protoplast metabolic activity by the use of TTC.

Materials and Methods

Plant materials

Seeds of *Brassica napus* cv. "Bronowski", a maintainer of Shiga's cytoplasmic male sterile line were obtained from National Institute of Agrobiological Resources. Seeds of *Penunia hybrida* cv. "Recovery White" were commercially obtained.

Protoplast isolation

Leaf protoplasts were prepared from leaves of 8 to 10 week old plants grown in a growth room with 14h photoperiod of 50 μmol $m^{-2}s^{-1}$ of fluorescent tubes and at 25/20°C for light/dark periods. Leaf blades were sterilized with 70% ethanol for 10 sec. then a 1.2 % final solution of hydrochlorite for 5 min. followed by several washes in autoclaved water. The leaves of *B. napus* were cut into narrow strips and incubated overnight in an enzyme solution containing 0.2% Cellulase Y-C, 0.1% Pectolyase Y-23, 5mM MES(pH 5.8) and 0.6M sorbitol. Lower epidermis were peeled off from leaves of *P. hybrida* and incubated in an enzyme solution containing 0.2% Cellulase Y-C, 0.1% Pectolyase Y-23, 5mM MES(pH 5.8) and 0.6M sorbitol. Lower epidermis were peeled off from leaves of *P. hybrida* and incubated in an enzyme solution containing 0.1% Cellulase Y-C, 0.02% Pectolyase Y-23, 5mM MES(pH 5.8) and 0.6M sorbitol. Isolated protoplasts were sieved through 4 layes of gauze and purified by floatation on 0.7M sucrose.

TTC reduction test

Leaf protoplasts in 15ml of screw capped centrifuge tubes were suspended in 3ml of 0.05M K_2HPO_4-KH_2PO_4-0.6M sorbitol buffer(pH 7.4) containing different concentration of TTC(w/v), and incubated overnight at 30°C in dark. The TTC solutions were sterilized with 0.45 μm of membrane filter. All procedures described above were conducted in aseptic condition. TTC redution rate was determined by the following methods.

Protoplasts were collected by centrifugation at 180xg for 10 min. Water insoluble formazan was extracted from the protoplasts with 5ml of 95% (v/v) ethanol in a boiling water bath for 5 min.(Steponkus and Lanphear[12]). The extract was cooled and made up to 10ml volume with 95% ethanol. Absorbance at 520nm was recorded for each sample.

MNNG treatment

Leaf protoplasts of *P. hybrida* were treated with 25, 50 and 100μg/ml of MNNTG for 1 hour at 25°C in light(3 μmol $m^{-2}s^{-1}$). The MNNG solutions were discarded, and washed the protoplasts three times with 10 ml of 0.6M sorbitol. Protoplasts for TTC reduction test were transferred to screw capped centrifuge tubes and cultured in 1ml of MS liquid medium supplemented with 1.0mg/l of 2,4-D, 0.5mg/l of BAP, 2% sucrose, 1% glucose and 0.6M sorbitol(pH 5.8) at 25°C for 5 days in dark.

Protoplast culture

Leaf protoplasts of *P. hybrida* were cultured in 10cm petris dishes at a cell density of 8.0×10^5 per ml in the MS meduim described above that was solidified by 0.6% agar. They were maintained in dark at 25°C. After 2 months, the number of small calli that were appeared on the surface of agar medium were determined.

Results

Effect of TTC concentration and incubation time on TTC reduction rate

To evaluate the effect of TTC concentration, leaf protoplasts of *B. napus* were treated with four concentrations of TTC for 16 hours. Fig. 1 shows that as the concentration of TTC increased to 0.05%, the absorbance at 520nm of reduced water insoluble formazan increased considerably, but the TTC assay value (absorbance at 520nm) showed no further increase over 0.05% concentration of TTC. Fig. 2 shows the effect of incubation time on the TTC assay value. TTC assay value increased exponentially up to 10 hour incubation time, however longer incubation did not increase the value as much. Conseqently, 0.05% TTC and 16 hour incubation time were chosen for the TTC reduction assay.

Absorption spectra of water insoluble formazan

Fig. 3 shows that the chemically reduced formazan has a λ_{max} at about 490nm(Fig. 3B), whereas there is interference with endogenous plant pigments at this

wavelength(Fig. 3A). Steponkus and Lanphear[12] used 530nm, a wavelength still in the region of high absorption by the reduced formazan, but minimum absorption by tissue pigments, for assaying amount of formazan. A result of the TTC reduction test for a fraction of cellular or subcellular debris is presented in Fig. 4. The spectrum shows that there is no water insoluble formazan, since the spectrum is the same as that of extract obtained from protoplasts that were incubated in the TTC-omitted assay medium(Fig. 3C).

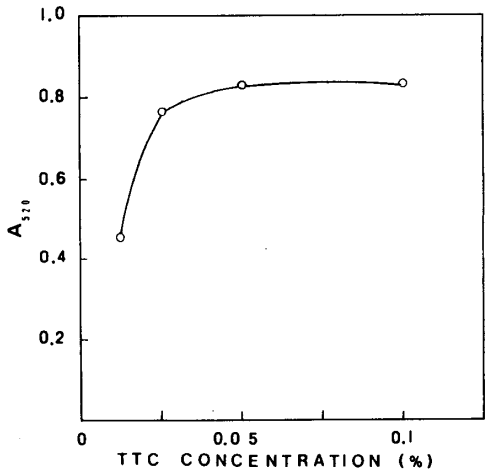

Fig. 1 Effect of TTC concentration on amount of water insoluble formazan extracted with 80% (v/v) ethanol from *B. napus* leaf protoplasts incubated for 16 hours at 30°C.

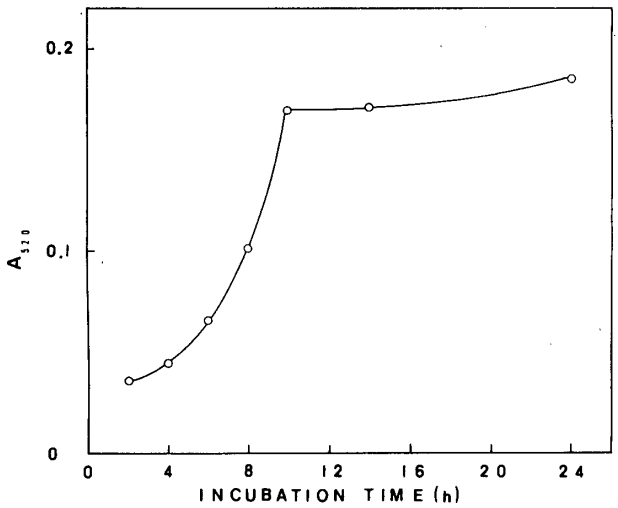

Fig. 2 Effect of TTC incubation time on amount of water insoluble formazan extracted with 80% (v/v) ethanol from *B. napus* leaf protoplasts incubated at 30°C.

Estimation of plating efficiency by TTC reduction test

Petunia leaf protoplasts were treated with MNNG, an alkylating agent which inhibits cell divisions, to determine the relationship between plating efficiency and TTC redution rate. In contrast to *B. napus* leaf protoplasts, *P. hybrida* leaf protoplasts were easy to

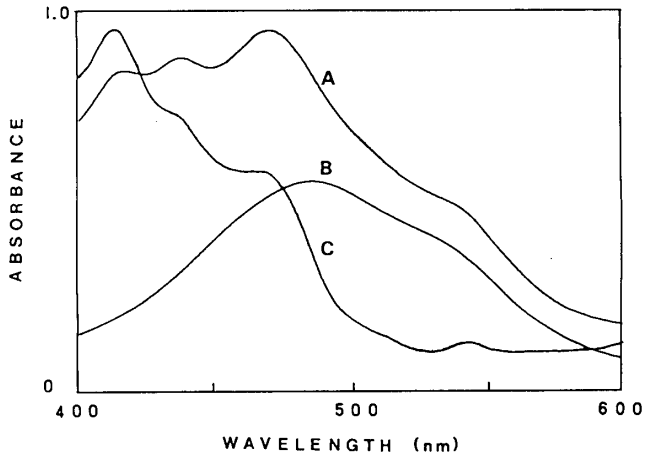

Fig. 3 Absorption spectra of A) 95%(v/v) ethanol extract of *B. napus* leaf protoplasts incubated in the 0.05% TTC assay medium, B) 95%(v/v) ethanol extract of sodium hydrosulfite reduced 0.05% TTC assay medium containing 0.05M K-Pi buffer(pH 7.4) and 0.6M sorbitol, C) 95%(v/v) ethanol extract of *B. napus* leaf protoplasts that were incubated in a TTC omitted assay medium.

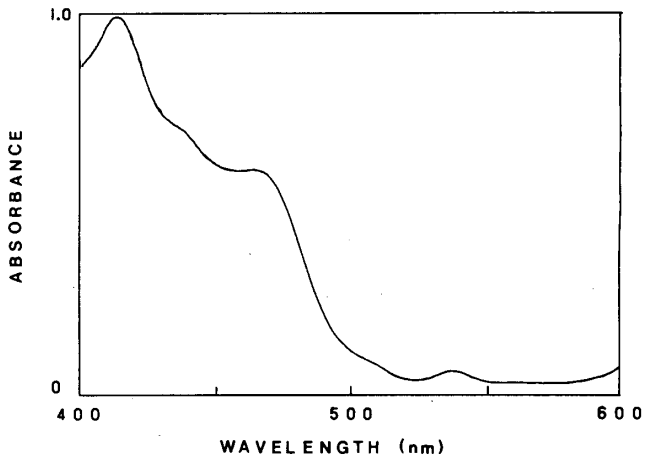

Fig. 4 Absorption spectra of 95%(v/v) ethanol extract of a fraction of cellular or subcellular debris incubated in the 0.05% TTC assay medium.

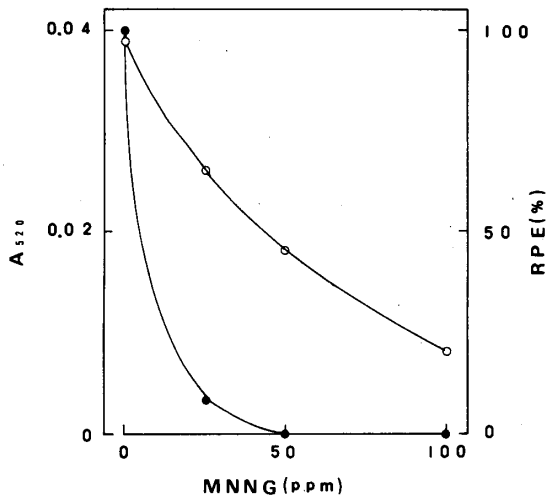

Fig. 5 Relationship between MNNG concentration and plating efficiency or TTC reduction rate. Open circular represents absorbance at 520nm. Closed circular represents relative plating efficiency (RPE). Data are average of two measurements.

initiate cell division and form calli. Therefore, *P. hybrida* leaf protoplasts were chosen fotr this experiment. TTC reduction rate was determined 5 days after MNNG treatment. Plating Efficiency of control was 0.8%. Fig. 5 shows that TTC reduction rate decreased gradually as MNNG concentration increased, while plating efficiency decreased remarkably, as MNNG concentration increased to 25 μg/ml. As a result, TTC reduction rate is able to provide a relative estimation of protoplast plating efficienc at an early stage of culture.

Discussion

TTC test has been used fot examining seed viability and cold injury. Steponkus and Lanphear[12] developed a refined TTC test for determining cold injury by using leaf discs and stem segments of ivy. In the present work, TTC test was modified for application to evaluate leaf protoplast viability. Kun and Abood[5] found that tetrazolium is an indicator of succinic dehydrogenase activity of tissue homogenate. However, Roberts[10] described by his inhibition study that no reductase system is responsible for the characteristic reduction in plant tissues, but that a general redox potential level, which was maintained by the operation of several physiologically active system, brings about the reduction of tetrazolium. The TTC test was a two step reaction: substrate + NAD(P) + TTC (colorless) $\xrightarrow{A}$ Product + NAD(P)H + TTC (colorless) $\xrightarrow{B}$ Product + NAD(P) + Formazan (colored), where A = a dehydrogenase, and B = a tetrazole reductase (Gahan[2]). In this experiment, as the substrate and co-factor were omitted from the reaction medium, formazan was produced presumably through the presence of endogenous substrate and co-factor. Difference of an amount of reduced formazan may be due to limitation of substrate and cofactor and hence represent metabolic activity at the time of the test. In fact, cellular or subcellular debris of which the plasmalemma was damaged gave negative TTC test (fig. 4). Plating efficiency and an amount of reduced formazan were relatively correlated. When 25 μg/ml of MNNG containing MS medium was treated, relative plating efficiency of *P. hybrida* leaf protoplast decreased markedly to 8%, wherease absorbance of reduced formazan that was extracted from 5 day-cultured protoplasts decreased slightly from 0.039 to 0.029. The difference between them may be due to their metabolic activity at the time when TTC reduction rate was determined. Because MNNG treated protoplasts survive for more than 5 days, 5 day-cultured protoplasts have some dehydrogenase activities and hence they can reduce TTC. MNNG is an alkylating agent and affects DNA but not dehydrogenase related metabolism. Therefore, differences of TTC reduction rate appears more than 5 days after MNNG treatment. As a result, the TTC reduction test gives some information about metabolic activities involved in dehydrogenases. Robertson and Earle[11] reported a procedure for the use of nitro-blue tetrazolium and fluorescein labelled tetrazolium as stains to detect photosynthetic activity in leaf protoplasts. FDA staining test can only demonstrate plasmalemma integrity and its associate esterase activity. Plasmalemma dependent tests are less important for viability assay, since plasmalemma intact leaf protopasts can be isolated from many plants by purification of sucrose floatation method (Watanabe and Yamaguchi,[13]). All of plasmalemma

intact protoplasts are stained with FDA irrelevant to their metabolic activity. In addition, FDA staining can not quantitatively determine the activities of masses of protoplasts without a spectrofluorimeter, whereas TTC reduction test based on spectrometric method is easy for quantitative analysis. Disadvantage of TTC reduction test is time-consuming. However, it could be overcome by the advantage of TTC test which gives a better information to predict activity of protoplasts.

Except for a limited number of plant materials, regeneration of plantlet from a leaf protoplast is still difficult for many plant species. Some of the difficulties may be due to isolation conditions and its following culture conditions. The method described here may contribute to the search for these conditions.

Acknowledgements

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TTC還元力による葉肉プロトプラスト代謝活性の評価

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摘　要

葉肉プロトプラストのトリフェニルテトラゾリウムクロライド (TTC) 還元力を測定することによって，プロトプラストの代謝活性を評価する方法を確立した．葉肉プロトプラストを0.05%リン酸緩衝液 (pH 7.4) に懸濁し，16時間30°C，暗黒下で培養した．不溶性のフォルマ

ザンは80%のエタノールでプロトプラストから抽出し，520 nmの吸光度を測定した．ショ糖浮遊法で精製したナタネ葉肉プロトプラストはTTCを還元したが，細胞の破片からなる画分はTTC還元力を示さなかった．ペチュニアの葉肉プロトプラストをN-メチル-N-ニトロ-N-ニトロソグアニジン（MNNG）で処理し，TTC還元力とコロニー形成率の関係を調べた．その結果，TTC還元力はMNNG濃度の増加とともに減少したが，コロニー形成率はMNNG濃度が25 μg/mlに達すると急激に減少した．コロニー形成率はプロトプラストの活性を反映しているので，TTC還元力からプロトプラストの代謝活性を推定できるものと思われる．