Gartenbauwissenschaft, **65** (4). S. 170–173, 2000, ISSN 0016–478X. © Verlag Eugen Ulmer GmbH & Co., Stuttgart

Analysis of Cytokinin Activity in Commercial Aqueous Seaweed Extract

Analyse der Cytokininaktivität in handelsüblichem wäßrigem Algenextrakt

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Summary

TTC viability staining method as a bioassay for cytokinin activity was refined using the soybean (*Glycine max* L.) suspension cultures by detecting cell viability and plotting a standard curve ($R^2 = 0.98$) and using different concentrations of kinetin in soybean medium as control. When kinetin concentrations are between 0.02 and 2 mg/l, it can improve cell viability. Soybean cell cultures are sensitive to the presence of kinetin. When cytex dilutions are between 5x and 5000x, it can improve cell viability as well. Thus it is concluded that cytex contains the biological activity similar to cytokinin. Establishment of a standard curve ($R^2 = 0.91$) using enzyme-linked immunosorbent assay (ELISA) for a natural cytokinin (DHZR), we found that cytex contains about 6.5 pmole/0.1ml DHZR which shows cytokinin like biological activity in soybean cell culture. The results are consistent with TTC method. We conclude that TTC can be one of the bioassays for cytokinin in suspension cell culture.

Zusammenfassung

Die Lebensfähigkeit der Zellen in Suspensionskulturen von Sojabohne (*Glycine max* L.), die durch Färbung mit TTC (2,3,5 – Triphenyltetrazoliumchlorid) ermittelt wurde, diente als Test für die Cytokinin-Aktivität. Eine Standardkurve wurde erstellt ($R^2 = 0.98$). Kinetin diente als Kontrolle, wobei dessen Konzentration zwischen 0.2 und 2 mg/L liegen sollte, da Zellkulturen von Sojabohne auf Kinetin empfindlich reagieren. Die Lebensfähigkeit der Zellen kann ebenso gut sein, wenn Cytex (Meeresalgen-Extrakt) angewendet wird, wenn die Verdünnungen zwischen 5x und 5000x liegen. Daraus wird geschlossen, dass Cytex eine ähnliche biologische Aktivität entwickelt wie Cytokinin. Nach Ermittlung einer Standardkurve ($R^2 = 0.91$) für das natürliche Cytokinin DHZR (Dehydrozeatinriborose) mit Hilfe des ELISA-Tests wurde festgestellt, dass Cytex etwa 6.5 pmol/0.1 ml DHZR enthält, da eine ähnliche biologische Aktivität in den Zellkulturen der Sojabohne gefunden wurde. Diese Ergebnisse stimmen mit der TTC-Methode überein. Es wird daraus geschlossen, dass TTC einer der Biotests für Cytokinin in Zellsuspensionskulturen sein kann.

Introduction

There are many bioassays to detect cytokinin activity, such as tobacco pith callus bioassay (NOGUE *et al.*, 1995), soybean callus bioassay (RODAWAY 1993), carrot root secondary phloem explants bioassay (HONG *et al.* 1995). These plant tissues are sensitive to cytokinin and increase in fresh weight with increasing cytokinin concentration in the medium. Recently, a novel bioassay method was developed to detect the cytokinin activity based on the expression of a chimerical gene in transformed tobacco (BOERJAN *et al.* 1992). Suitable ranges of cytokinin activity resulting from biological systems differ, so do their sensitivities to different kinds of cytokinins. Bioassays are time-consuming methods and the loss and destruction during purification process decrease the activity, while the activity of cytokinin analogues might interfere in the detection of cytokinins. High performance liquid chromatograph (HPLC) (LUKASZEWSKA *et al.* 1994; WAGNER and BECK 1993) and gas chromatography-mass spectrometry (GC/MS) (SOEJIMA *et al.* 1992) are physiochemical methods to detect cytokinins. Different kinds of cytokinin have different retention time, which can be identified by comparing with standard samples using HPLC. Furthermore, the purity can be analysed by using a high-speed photodiode array spectrophotometric detector (LIN and LIN 1990). Similarly, GC/MS can analyse the molecular constructors of unknown substances based on the ion mass/charge. Besides, the cytokinin containment in the cells can be known by labeling radioisotope on cytokinin or its precursors and detecting the radioactivity. Highly sensitive and specific assays have been developed to measure the abundance of cytokinin at high resolution by enzyme-linked immunosorbent assay (ELISA) (NICANDER *et al.* 1993, ULVSKOV *et al.* 1992, STRAND *et al.* 1992). It is based on monoclonal antibodies having high specificity to cytokinin and changing colour with different binding percentages. The understanding of the types, presence and activities of cytokinins in unknown substances is through establishing a suitable standard curve using suitable biological system or physiochemical methods.

Material and Methods

Soybean suspension cultures were obtained using the procedure as described by HORN *et al.* (1983). The cultures were subcultured once every 2 weeks and maintai-

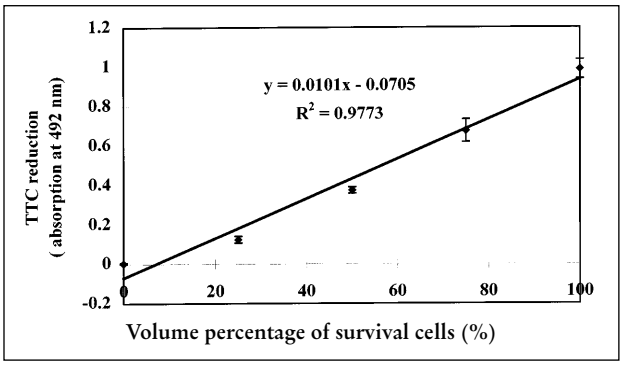


Fig. 1. Standard curve for TTC staining method. Soybean suspension culture cells were mixed with different percentage of dead cells pretreated with liquid nitrogen before treating with TTC. (R^2 = 0.98). Bars indicate SE (n ≥ 3). *Standardkurve für die TTC-Färbung. Vor der TTC-Behandlung waren Zellkulturen der Sojabohne mit verschiedenen Anteilen toter Zellen gemischt worden, die mit flüssigem Stickstoff behandelt worden waren. Striche zeigen Standardabweichung (n ≥ 3).*

ned in soybean medium (1.65 g/l NH_4NO_3, 1.9 g/l KNO_3, 370 mg/l $MgSO_4 \cdot 7H_2O$, 22.3 mg/l $MnSO_4 \cdot 4H_2O$, 0.025 mg/l $CoCl_2 \cdot 6H_2O$, 37.2 mg/l Na_2-EDTA $\cdot 2H_2O$, 27.8 mg/l $FeSO_4 \cdot 7H_2O$, 0.1 mg/l thiamin-HCl, 2 mg/l α-naphthalene acetic acid (NAA), 0.2 mg/l kinetin, 10 g/l sucrose, pH 5.7). Every time, 5 ml of culture cells were transferred into 125 ml flask containing 35 ml of fresh medium. The cultures were agitated at 120 rpm on a gyratory shaker under light at 24 h photoperiod. The incubation temperature was 25°C.

FRENCH and PARKIN'S (1993) 2,3,5-triphenyl-tetrazolium chloride (TTC) method with partial modification was used for viability staining of cells. Two ml of the suspension cells was taken on day 7 of culture and centrifuged at 2400 rpm for 10 minutes. The supernatant was discarded and the cells were added with 1 ml 0.4% (w/v) TTC in phosphate buffer, pH 7. The reaction mixture was incubated at RT in dark for 12 h. The cells were spinned down and the extra TTC reagent was discarded. The cells were homogenized after the addition of 50% alcohol (1 ml) using polytron grinder (PT3000KINEMATICA AG) at 28 500 rpm. TPF is extracted through hexane partition and centrifuged to obtain the top phase suspension by eliminating the chlorophyll using 8 N hydrochloric acid. The absorbance was measured at 492 nm using spectrophotometer (U2001, HITASHI). The degree of absorbance indicates the viability index.

Phytodetek-Dehydrozeatin-riborose (DHZR, a kind of natural cytokinin) kit (Catalogue # P-04D-096. Idetek, Inc. San Bruno, Campus Association) was used for ELISA test. DL-dihydrozeatin riboside was dissolved in N, N-Dimethylformamide (DMF). Four concentrations (at 0.05, 0.2, 0.5 and 2.0 pmole/0.1 ml) of DL-dihydrozeation riboside were prepared in 25 mM tris buffered saline; pH 7.5, along with a 100 pmole/0.1 ml (NSB) and B_0 for the standard curve. 100 µl of standard or sample was added to each well of strips. 100 µl of diluted tracer was added using a multichannel pipette. The contents were mixed and cells were covered and incubated in refrigerator at 4°C for three hours. The wells were rinsed twice by adding 200 µl of the wash solution. 200 µl of substrate solution was added. Wells were covered and incubated at 37°C for 60 minutes. 1 drop of stopping reagent was added and absorbance was read using vertical light path photometer for microtitration plates fitted with 405 nm filter.

Results and Discussion

The major function of cytokinins is to promote cell division. Soybean cells in suspension culture grow rapidly after subculture. The log period is reached on day 7, which means cells have the best activity and rapidly progressing cell division. That is why the cells were taken on day 7 after subculture. A standard curve was plotted using a refined TTC method. TTC staining has become a widely used index for evaluating biological viability in many aspects such as the microbial activity, the metabolism of seeds, the viability of flower buds, the degree of plant damage in temperature stress and the viability of suspension culture cells. The basis of TTC staining test is the reduction of colourless TTC to red formazan by active enzyme in the electronic transport chain of the cell mitochondria to transfer hydrogen and electron from organic acid. When the cell dies, due to halt of its respiration, TTC can not be reduced and the reagent remains colourless. Depending on the degree of TTC reduction, the absorbance varies in the red wavelength and it can be used as an index in deciding biological ability. Figure 1 shows the absorbance and the percentage of the survival cells which are positively related (R^2 = 0.98). TTC method is correct and sensitive in evaluating cell viability values.

In plant cell culture, cytokinin is normally required for callus formation. Different types and concentrations of cytokinin are closely associated with cell culture and effect growth and development of cells. If there is no cytokinin activity in medium, cells will grow slowly. Soybean cells were grown aseptically in a liquid medium with NAA (2 mg/l) and proper nutrients with different concentrations of kinetin for 7 days. Figure 2 shows lower cell viability when there is no exogenous cytokinin in the medium, indicating exogenous kinetin still has an effect on stimulation of cell viability of this

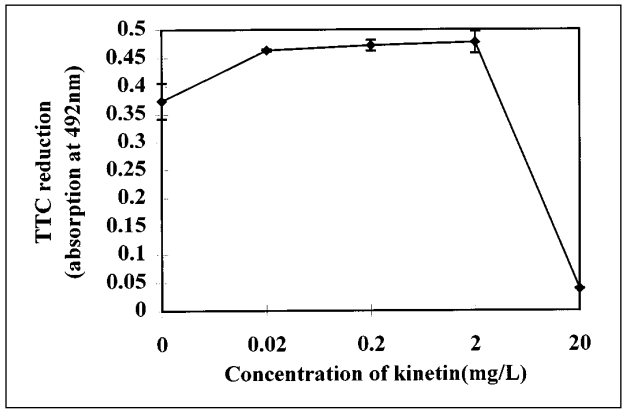


Fig. 2. Effect of different kinetin concentrations on cell viability of soybean suspension cultures. Bars indicate SE (n ≥ 3). *Wirkung verschiedener Kinetin-Konzentrationen auf die Lebensfähigkeit der Zellen von Suspentionskulturen der Sojabohne.*

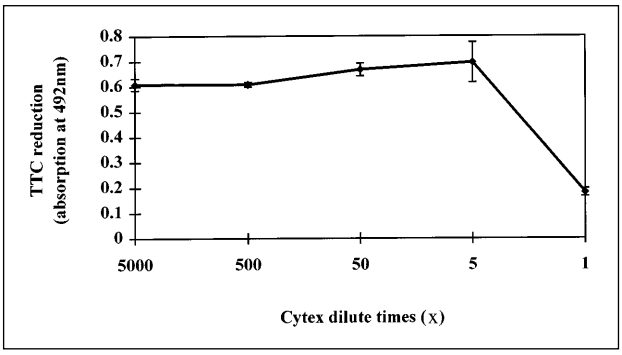


Fig. 3. Effect of different cytex dilutions in the absence of kinetin on cell viability of soybean suspension cultures. Bars indicate SE (n ≥ 3).
Wirkung verschiedener Cytex-Verdünnungen ohne Kinetin auf die Lebensfähigkeit der Zellen in Suspensionskulturen der Sojabohne.

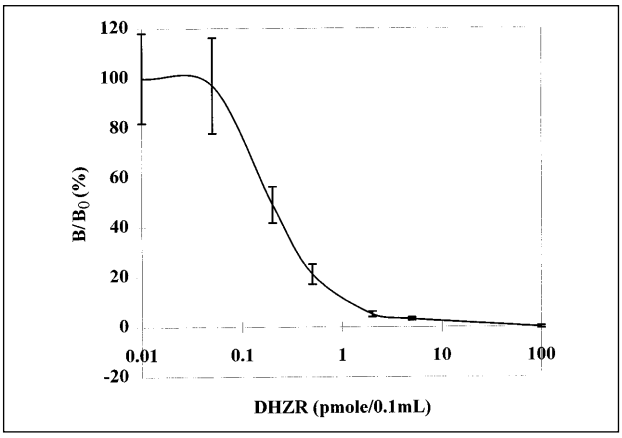


Fig. 4a. Standard curve for DL-Dihydrozeatin Riboside ELISA (B/B_0 (%) vs. Concentration)
Standardkurve für DL-Dihydrozeatin-Ribosid ELISA (B/B_0 (%) vs. Konzentrationen).

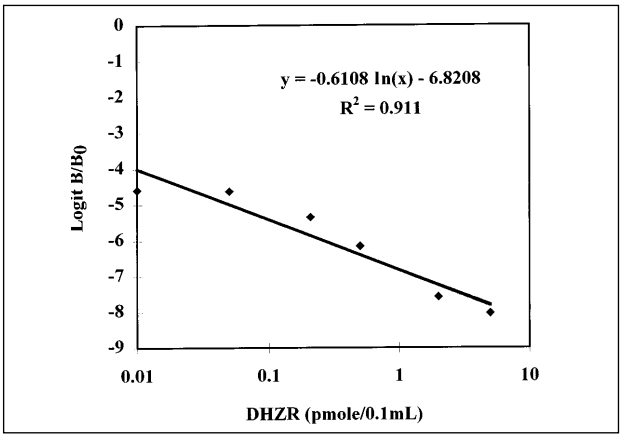


Fig. 4b. Linearized logit/log plot standard curve for DL-Dihydrozeatin Riboside ELISA of the same data. Bars indicate SE (n ≥ 3). B and B<->0 represent binding of tracer in the presence and the absence of DHZR standard, respectively. (R^2 = 0.91).
Linearisierte Logit/Log Standardkurve für DL-Dihydrozeatin-Ribosid-ELISA für die gleichen Daten. B und B_0 geben binding of tracer für Vorhandensein oder Fehlen des DHZR-Standards an (R^2 = 0.91).

biological system grown *in vitro* even though cells might already have endogenous cytokinin activity. Soybean cell cultures are sensitive to the presence of kinetin and reach their maximum viability under concentration range 0.02 to 2 mg/l. In the presence of optimal auxin concentrations, kinetin may promote cell division in the assay system. The amount of growth from new cells serves as a sensitive and specific bioassay for cytokinin level. When the kinetin concentration exceeds 2 mg/L, it can be toxic and lethal to cells. The cell viability is near zero at 20 mg/l kinetin concentration.

Aqueous seaweed extract has been widely used as fertiliser for agricultural and horticultural production, but the plant hormone activity in the extract has to be analysed and demonstrated. In particular, cytokinin is a very useful hormone which helps in modulating genes or biochemical mechanisms, in turn causing physiological responses such as plant development, fruit growth, seed germination etc. It is claimed that the commercial seaweed extract (cytex) contains plant hormone activity such as auxin and cytokinin beneficial for plant growth and development. However, the results obtained have been varying. We have, therefore, taken cytex as a kind of cytokinin fertiliser in absence of kinetin in the soybean cell culture medium. Figure 3 shows that it can enhance the cell viability when the dilutions are be-tween 5x and 5000x when compared to control. Furthermore, compared to the cells cultured with kinetin, cytex contributes to the cell viability better than kinetin. It is assumed that cytex is involved in influencing the cell culture similar to kinetin. It contains substances with biological activity of cytokinin and promotes cell division in the assay system. Thus this response provides a useful bioassay for cytokinins in commercial products. Also, when cytex is not diluted, it has toxic effect on cells.

Further evidence is shown by quantitative determination of cytokinin in cytex by enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody specific to DHZR. The yellow colour produced in the sample is inversely proportional to the DHZR concentration. It is very sensitive in the range of 0.02–5.0 pmole/0.1 ml. Figure 4 shows a standard curve (R^2 = 0.91). At the same time, it is demonstrated that cytex contains

Table 1. ELISA data for DL-Dihydrozeatin Riboside in cytex. "(%) Binding)" represents binding of tracer in different dilute times of cytex and is related to the DHZR concentration by means of the standard curve in Figure 4b
ELISA-Test für DL-Dihydrozeatinribosid in Cytex. „(%) Binding" gibt die binding of tracer verschiedener Verdünnungen von Cytex wieder und wird auf die DHZR-Konzentration der Standardkurve in Abb. 4b bezogen.

Dilute times	Mean	Binding %	Logit B/B_0	X	DHZR (Pmole/0,1ml)
1	0.047	5.39	–7.526	3.20	3.2
5	0.058	9.09	–7.002	1.30	6.5
50	0.263	74.92	–4.845	0.04	2.0
500	0.264	76.26	–4.840	0.04	20.0
5000	0.265	76.09	–4.836	0.04	200.0
50000	0.277	82.83	–4.785	0.04	2000.0

approximately 6.5 pmole/0.1 ml DHZR that leads to relative physiological effect in soybean cell culture (Table 1). The quantity is related to the DHZR concentration by means of the standard curve in Figure 4. The approach is consistent with TTC bioassay, which can be suggested as a new bioassay for cytokinin in suspension cell cultures.

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Eingegangen: 3.5.99/19.10.99

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