

Effect of Calcium and Calmodulin Antagonists on Tracheary Element Differentiation in *Zinnia mesophyll* Cell Suspension Cultures

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ABSTRACT. *Tracheary element (TE) differentiation in Zinnia mesophyll cell suspension was studied with a view understand the process of TE differentiation and to explain the effects of certain factors on the process. The involvement of calcium in TE differentiation was studied indirectly through the use of calcium chelator to deny the access of calcium in the process and, through the use of specific antagonists of calmodulin; a protein with which calcium binds in order to elicit the various responses it is capable of. Isolated leaf mesophylls cells were treated with 1. Calcium chelator EGTA (Ethylene Glyco-bis N, N Tetra Acetic acid) and 2. Two calmodulin inhibitors; chlorpromazine (CPZ) and trifluoperazine (TFP). In the initial study 0.1 and 0.5 mM EGTA, 10, 20 and 40 micro M TFP and 10 micro M CPZ were used. Subsequently, CPZ and TFP at concentrations of 10, 20 and 40 micro M were used. The total number of TEs/ml of cell suspension and the percentage of TEs/total number of live cells were taken at peak TE differentiation (after 96 hrs of culture). It was evident from the results that Ca⁺⁺ plays an important role in TE differentiation. The highest number of TEs were observed without TFP or CPZ while it was significantly lower with EGTA 0.1 mM, CPZ 10 micro M and TFP 10 micro M treatments compared to control. TE formation was completely inhibited by EGTA 0.5 mM, TFP 20 and 40 micro M concentrations. This inhibition may clearly indicate the necessity of Ca⁺⁺ for differentiation process. Cell death was observed at 0.5 mM EGTA and 40 micro M TFP probably due to non specific toxic effects of the high concentration of chemicals.*

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INTRODUCTION

Differentiated cells in plants can redifferentiate *in vitro* to other types of cells, tissues, organs and even whole plants. The first step of such *in vitro* redifferentiation is cytodifferentiation occurring in individual cells. TE differentiation is an excellent example of cytodifferentiation *in vitro* in higher plants (Fukuda and Komamine, 1980). TE formation in leaf mesophyll cell suspension cultures of *Zinnia elegans* where 30-60% of isolated single mesophyll cells differentiate into TEs synchronously within 60 and 80 hrs of culture by the supplement of two plant hormones, 0.5 micro M naphthalene acetic acid (NAA) and 0.5 micro M benzyl adenine (BA), has been used as a model system to analyze physiological, cytological and biochemical aspects of cytodifferentiation extensively.

Calcium mediates various physiological processes elicited by extra cellular signals such as light, hormones and gravity. Many of the effects of Ca^{++} are actually mediated by a Ca^{++} binding regulatory protein, Calmodulin (Poovaiah and Reddy, 1987). Induction of xylogenesis in cultured explants of *Lactuca* is also claimed to require Calmodulin action (Roberts and Baba, 1987). Calcium sequestration is said to accompany the on set of cell wall thickening during TE formation in *Zinnia* cells. Yet, experimental evidence regarding the involvement of calcium and calmodulin in xylogenesis of *Zinnia* mesophyll cells is rather limited. Therefore, the following experiments were conducted asses the effects of calcium by using calmodulin antagonists and calcium chelator on TE formation in the *Zinnia* system.

MATERIALS AND METHODS

Preparation of cell cultures

Zinnia elegans (L) cv Envy seedlings were raised in a climate chamber (MB Teknik) under controlled environmental conditions; 14 hrs light with cool fluorescent light (40 W, Phillips) at a photon flux density of $136 \text{ mM m}^{-2} \text{ s}^{-1}$ of photosynthetically active radiation, a constant temperature of 25 C and 75% RH. Leaf mesophyll cells were aseptically isolated from 14 day old leaves by mechanical means and cultured in Fukuda and Komamine (1980) medium containing 0.5 micro M NAA and 0.5 micro M BA as specified by Church and Galston (1988) at pH 5.6. Cell density was adjusted to 150,000 cells/ml. Cultures were incubated on an orbital shaker at 65 rpm in dark at 27 C.

Chemical treatments

The calcium chelator Ethylene Glycol-bis N,N Tetra Acetic acid (EGTA) and two calmodulin phenothiazine inhibitors: Chlorpromazine (CPZ) and Trifluoperazin (Sigma Chemical Co., USA) were used at the following concentrations. Experiment 1: 0.1 and 0.5 mM EGTA, 10, 20 and 40 micro M TFP and 10 micro M CPZ, Experiment 2: 10, 20 and 40 micro M of TFP and CPZ. The chemicals were filter sterilized and added at the beginning of the incubation.

Cell counts

Cell counts were taken after staining the cell with Evans blue. Cells in the suspension can be divided into 3 groups as live cells (L) which are not stained with Evans blue, tracheary elements (T) which can be easily identified by wall banding patterns and dead cells (D) which are stained with Evans blue. The percentage of live cells represents $((T+L)/(T+L+D)) \times 100$ and the percentage of TEs represents $(T/(T+L)) \times 100$.

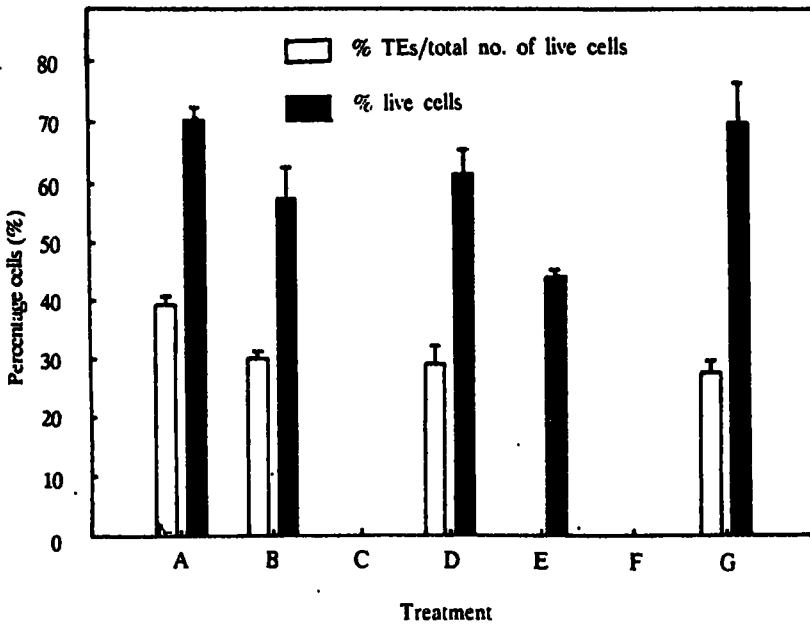
RESULTS AND DISCUSSION

Experiment 1

The number of TEs were significantly higher in the control (5.937 ± 1.4) compared to all the other treatments. The differences of TE numbers recorded with EGTA 0.1 mM (3.352 ± 0.8), CPZ 10 micro M (3.330 ± 0.8) and TFP 10 micro M (3.102 ± 2.0) treatments were not statistically significant, when observed with TFP 20 micro M (0.000) and 40 micro M (0.000) and EGTA 0.5 mM treatments.

Results indicate that the Ca^{++} chelator (EGTA) and calmodulin inhibitors CPZ and TFP drastically affect the TE differentiation process. EGTA at 0.5 mM and TFP at 20 and 40 micro M concentrations completely inhibit xylogenesis. Even at low concentrations, 0.1 mM EGTA, 10 micro M of TFP and CPZ significantly reduce the TE formation compared to the control. There was no significant difference in the mean number of live cells between the control, EGTA 0.1 mM and CPZ 10 micro M treatments probably exhibiting its inhibitory effect on TE formation with minimum toxic effect on live cells. This behaviour was further evident in TFP treatment

where 0 micro M concentration completely inhibited the TE formation compared to 10 micro M concentration without affecting the total number of live cells significantly (Figure 1).



A = Control. B = EGTA 0.1 mM, C = EGTA 0.5 mM.
D = TFP 10 μ M. E = TFP 20 μ M and G = CPZ 10 μ M.

Figure 1. Effect of EGTA, CPZ and TFP on TE differentiation of *Zinnia mesophyll* cells.

Complete cell death was observed with 0.5 mM EGTA and 40 micro M TFP, probably due to non specific toxic effects of these compounds at higher concentrations. Hepler and Wayne (1985) reported that calcium-calmodulin complex activate several enzymes, specially kinases taking part in processes such as mitosis, protoplasmic streaming growth. High concentrations of EGTA and calmodulin antagonists (TFP and CPZ) may have seriously disturbed the metabolic activities of cells further to its inhibitory effect on TE formation ultimately causing cell death.

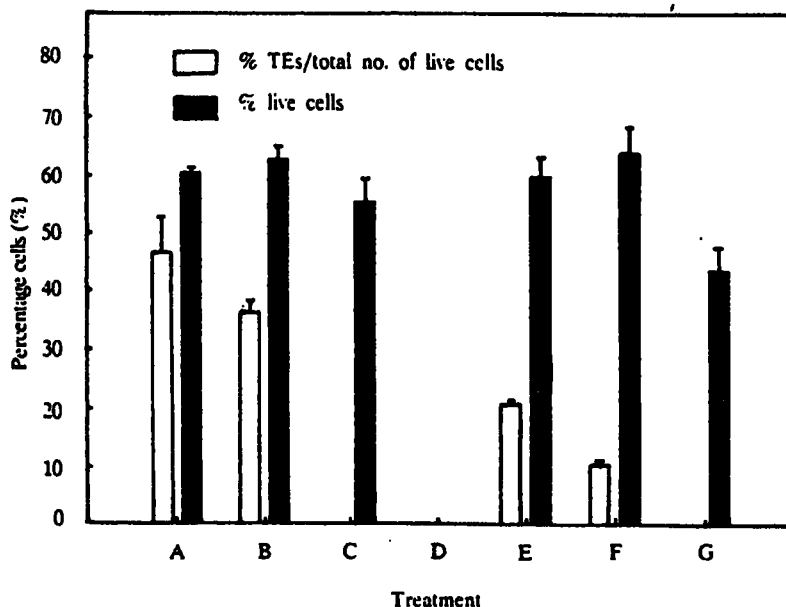
The effect of calmodulin antagonists (TFP and CPZ) was studied further in this experiment. Results of the experiment is presented in Table 1.

Table 1. Mean number of TEs (x 15625)/ml of cell suspension.

Treatment	Mean TEs/ml
Control	7.185 ± 1.7 ^a
TFP, 10 micro M	6.102 ± 1.7 ^a
CPZ, 10 micro M	2.375 ± 1.1 ^b
CPZ, 20 micro M	1.185 ± 0.3 ^{bc}
TEP, 20 micro M	0.082 ± 0.2 ^c
CPZ, 40 micro M	0.08 ^c
TEP, 40 micro M	0.00 ^c

The results of the second experiment mostly conformed to those of the previous experiment. The highest mean number of TEs was observed in the control. TFP 10 micro M treatment caused 15% inhibition in mean TE number though the difference was not significant compared to the control (Table 1). However, the TFP 20 micro M concentration caused 98.85% inhibition in mean TE number. CPZ, at 10 and 20 micro M concentrations resulted 67% and 83.5% inhibition in the mean TE number, respectively. Further increase of CPZ and TFP up to 40 micro M level almost completely inhibited TE formation by 98.8 & 100%, respectively.

Figure 2 illustrates the changes in percentage of TEs/total number of live cells under different treatments which follows the same trend as the mean number TEs/ml. TFP 10 micro M concentration resulted in 22% inhibition, while 20 and 40 micro concentrations caused complete inhibition of TE formation. At a similar concentration (10 micro M) CPZ, resulted in a much higher inhibition compared to TFP treatment, contrary to the findings of the previous experiment. CPZ 20 micro M concentration inhibited the TE formation by 70% while 40 micro M CPZ caused complete inhibition. These results confirm the findings of the previous experiment, even though the extent of the inhibition was different.



A = Control, B = TFP 10 μ M, C = TFP 20 μ M,
 D = TFP 40 μ M, E = CPZ 10 μ M, F = CPZ 20 μ M and
 G = CPZ 40 μ M.

Figure 2. Effect of CPZ and TFP on TE differentiation of *Zinnia mesophyll* cells.

It may be evident from the results that calmodulin antagonists (CPZ and TFP) and the Ca^{++} chelator (EGTA) inhibit TE differentiation. It is deduced from these results that the inhibition of TE formation by calmodulin antagonists and EGTA is an indication of the necessity of Ca^{++} for TE differentiation in *Zinnia mesophyll* cells. Roberts and Haigler (1990) reported that deprivation of Ca^{++} in the medium, by the use of inorganic and organic Ca^{++} channel blockers and the use of calmodulin antagonists inhibit TE formation. It was further reported that CPZ and TFP inhibited TE differentiation with mean LC_{50} values of 22 and 8 micro M, respectively. Thus, these results are in agreement with other findings. The inhibitory effects observed were probably partly calcium specific, because a significant inhibition occurred without significant cell death at low concentrations. However, at higher doses the number of live cells was reduced, probably due to non-specific effects which could be attributed to the changes in membrane properties causing leakage and cell death.

CONCLUSIONS

Calcium chelator; EGTA and calmodulin inhibitors; trifluoperazin and chlorpromazine inhibit TE differentiation in *Zinnia mesophyll* cell suspension cultures. This inhibition (specially at low concentrations) probably indicates that Ca^{++} plays an important role in TE differentiation.

REFERENCES

- Church, D.L. and Galston, A.W. (1988). Kinetics of determination in the differentiation of isolated mesophyll cells of *Zinnia elegans* to tracheary elements. *Plant Physiol.* 88: 92-96.
- Fukuda, H. and Komamine, A. (1980). Establishment of an experimental system for the study of tracheary element differentiation from single cells isolated from the mesophyll of *Zinnia elegans*. *Plant Physiol.* 65: 57-60.
- Hepler, P.K. and Wayne, R.O. (1985). Calcium and plant development. *Ann. Rev. Plant Physiol.* 36: 397-439.
- Poovaiah, B.W. and Reddy, A.S.N. (1987). Calcium messenger system in plants. *CRC Critical Reviews in Plant Sciences* 6: 47-103.
- Roberts, L.W. and Baba, S. (1987). Exogenous methionine as a nutrient supplement for the induction of xylogenesis in Lettuce pith explants. *Ann. Bot.* 42: 375-379.
- Roberts, A.W. and Haigler (1990). Tracheary element differentiation in suspension cultured cells of *Zinnia* require uptake of extracellular Ca^{++} . Experiments with calcium channel blockers and calmodulin inhibitors. *Planta* 180: 502-509.