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Estimation of Cellular Damages Caused by Paraquat and Lead Using a Cell Culture System

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Abstract

A cell culture system of poplar *(Populus alba × P. glandulosa)* was established to test four different methods for evaluation of cellular stresses. Two different kinds of stresses were given to the cultures by adding either $Pb(NO_3)_2$ or paraquat and the cellular responses were monitored during a week period. While fresh weight reduction was observable in two days after the treatment of $Pb(NO_3)_2$, such changes were apparent only in later stage in paraquat treated cultures. Cells in paraquat treated cultures in the first 3 days showed no alteration in fresh weight as compared to untreated cultures, but had their MTT reducing activities completely inhibited. Neither Evans blue staining nor ion conductivity of the medium was consistent with fresh weight changes of the cultures. Overall, cell clumps formed during suspension culture appeared to interfere with staining and washing reactions and thus cause the assays unreliable. Among the four methods examined, fresh weight changes and MTT reducing activity appeared to be the most reliable and consistent.

Introduction

Plant's response to stress is a complex process involving many different biochemical and physiological reactions. Currently, one of the important stresses to plants is heavy metal poisoning due to soil acidification. Phytotoxicity of heavy metals has been extensively studied in field throughout the world (see review by Balsberg, 1989). However, the assessment of symptoms is very difficult due to a number of factors including temperature, moisture, light, and pH (Wersuhn et al., 1994). In many cases, greenhouse pot study or hydroponic culture is required to minimize the variability in developing response curves to stress materials (Toda et al., 1999). Although these systems are effective and able to offer a fairly uniform response, they have some limitations related to cost and reproducibility. Furthermore, they may not be ideal for some studies that monitor small cellular changes. In many cases, roots play an important role as a barrier against transport of heavy metals to other parts of plant. Thus it is very difficult to assess the biochemical and physiological effects of stress agents on cellular metabolism using intact plants.

Cell cultures might be a quick, reliable and reproducible alternative to whole plant systems to assess cellular damage following stress treatments (Minocha et al., 1996). The inhibition of post-treatment growth has been used as a marker for the dose effect. However, some other biochemical as well as physiological symptoms that might be accompanied by the cellular stress could also be used. Koyama et al. (1995) demonstrated that tissues having different stresses exhibited different stainability with fluorescin diacetate and propidin iodide. Putrescine has also been reported as an early indicator of stress caused by heavy metals (Zhou et al., 1995). Ion coductivity of culture medium was correlated with cell growth in the medium (Kwon et al., 1999). Ikekawa et al. (1998) demonstrated that inhibition of post-treatment growth was strongly related to both permeability of Evans blue by plasma membrane and

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MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) reducing activity of mitochondrial enzymes. However, a number of obstacles have to be overcome to standardize cell culture systems, as much variation exists among cell lines in genetic as well as physiological states (Noh and Minocha, 1990). The mode of action of chemical and physical stress agents has to be understood before setting up experiments.

Lead and paraquat have different physiological toxicity to plants (Aery and Jagetiya, 1997; Upham and Hatzios, 1987). In this report, we have evaluated four different methods to estimate cellular damages caused by these compounds using a cell culture system.

Materials and Methods

Establishment of cell culture

Calli were induced by culturing stem and leaf segments of *Populus alba* × *P. glandulosa* on MS agar medium (Murashige and Skoog, 1962) containing 0.01 mg/L BA, 0.1 mg/L NAA, and 1.0 mg/L 2,4-D. About 5 grams of cells were collected and passed through an 1 mm sieve by pushing with a spatule followed by pouring liquid MS medium through the sieve into a 500 mL sterile beaker. The cells were then concentrated by sedimentation and divided into five 125 mL flasks containing 50 mL of fresh MS medium. The flasks were maintained on a gyratory shaker at 180 rpm. Throughout the experiment, the suspensions were kept in a culture room under 16 hour photoperiod provided by cool white fluorescent lights at 30 moL $m^{-2}s^{-1}$. The temperature was maintained at 25± 2℃.

Paraquat and lead treatment

The cells grown for a week after subculture to fresh medium were pooled into an 1 liter round-bottomed flask containing fresh medium. To achieve homogeneity of suspensions, the cells were constantly stirred at a low speed with magnetic stirrer during the period of transfer as described in Zhou et al. (1995). Ten ml of the culture containing approximately 300 mg were diluted to the equal volume of fresh media. The resulting twenty ml of cell suspension were grown in 125 ml flask for 3 days and then treated with 3 different concentrations of paraquat (0, 1, or 5 μM) or $Pb(NO_3)_2$ (0, 300, or 600 μM). The flasks were incubated under agitation for 1, 2, or 4 more days before harvest. Each treatment consists of 3 replicates and repeated three times.

Cell harvest and fresh weight determination

Cells were harvested by pouring the suspension into a filtration unit consisting of Whatman #2 filter, a vacuum, and flasks. Vacuum was applied for 10 seconds right after the liquid medium was drained out from the cell mass. The cell mass on the filter paper was carefully collected and weighed on a balance.

FDA assay

Cells were harvested by 10 mL pipett and stained with fluorecein diacetate (FDA, 5 μg/mL) for 3 min as described in Koyama et al. (1995). The stained cells were placed on slide glass and observed with a confocal microscope (Zeiss Co., FRG).

Ion conductivity test

Ion conductivity of culture medium was measured immediately prior to cell harvest. The probe of the ion conductometer was directly soaked into the media to measure the conductivity as suggested by manufacturer (Hach Co., USA).

MTT assay

MTT assay was performed according to Ikekawa et al. (1998) with a slight modification. Harvested cells (100 mg) were transferred to 1.5 mL microfuge tubes and resuspended in 1 mL of MS medium containing 250 mg/mL MTT. The tubes were then incubated at room temperature under gentle shaking (60 rpm) on a rocker for 3 hours. The cells were spun down at 13,000 rpm for 10 min. After the supernatant was discarded, the pellet was washed five times in 1 mL sterile distilled water. Formazan was released by adding 1 mL of acid propanol (0.4M HCl in isopropanol) to the last pellet followed by 10 min centrifugation at 13,000 rpm. The supernatant was transferred to a cuvette for spectrophotometric analysis at 590 nm.

Evans blue assay

The loss of plasma membrane integrity was evaluated by a spectrophotometric assay of Evans blue stain retained by cells as described in Ikekawa et al. (1998) with minor modifications. Harvested cells were transferred to 1.5 mL microfuge tubes and resuspended in 1 mL of 0.05% Evans blue. The tube was then incubated at room temperature by gentle shaking (60 rpm) on a rocker for 15 min. The cells

were then spun down at 13,000 rpm for 10 min. After the supernatant was discarded the cellular Evans blue dye was released by sonication of the cells for 2 min after addition of 1 mL of 1% SDS. The cell extract was centrifuged for 10 min at 13,000 rpm and the supernatant was transferred to a cuvette for spectrophotometric analysis at 600 nm.

Standard curves using quick killed cells

To estimate the relationships between MTT reducing activity or Evans blue staining and the proportion of dead cells in a cell population, different levels (0, 20, 40, 60, 80, or 100%) of dead cells (those killed by boiling) were added to harvested cells. The mixed cells were then assayed for MTT reducing activity as well as Evans blue staining.

Results and Discussion

Fresh weight growth

The fresh weight doubled within 4 days after subculture (Figure not shown). A notable characteristic of the growth of the cells was the formation of cell clumps during the suspension cultures. Tiny clumps in size of 2 to 3 mm in diameter were formed and broke off in the course of suspension culture. The faster increase in fresh weight was observed after day 6. However, when either $Pb(NO_3)_2$ or paraquat was added to the culture, the growth of the cells was markedly inhibited. There was a dose-dependent inhibitory effect of $Pb(NO_3)_2$ on growth (as measured by change in fresh weight per culture) within 4 days (Figure 1). On the other hand, there was no significant effect of paraquat on cell growth upto 2 days (Figure 2). The inhibitory effect of paraquat became visible at the day 4. Incubation with 1 μM paraquat resulted in 21% reduction in fresh weight of the cultures in 4 days. However, about 40% reduction in cell growth was seen at 5 μM (Figure not shown). The tolerance shown by the cultured cells to paraquat in the first 2 days might be due to their limited dependence on photosynthetic activity since sugars were provided in the culture medium. Paraquat exerts its phytotoxicity by accepting photosynthetic electrons from PS1 and transferring them to oxygen species producing toxic oxygen species which cause lipid peroxidation (Upham and Hatzios, 1987). According to Kwon et al. (1999), the inhibitory effects of herbicides on tobacco cell culture were visible only 6 to 12 days after treatment.

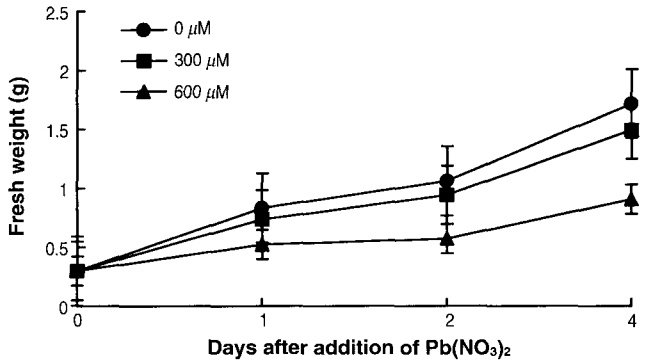

Figure 1. Time course changes in fresh weight of cultured *Populus alba* × *P. glandulosa* cells. $Pb(NO_3)_2$ was added to the cell cultures 3 days after subculturing to new media. Day 0 is the time when $Pb(NO_3)_2$ was added.

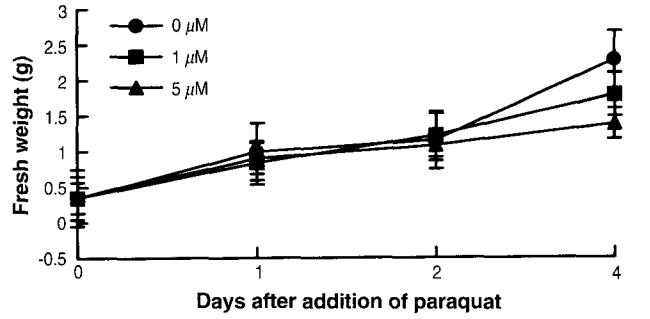

Figure 2. Time course changes in fresh weight of *Populus alba* × *P. glandulosa* cells. Paraquat was added to the cell cultures 3 days after subcultuing to new media. Day 0 is the time when paraquat was added.

Changes in medium pH

The pH value of the media dropped from 5.8 to 4.3 within 1 day of culture. Although it further dropped to 4.1 in 3 days, a slow recovery was seen to the point of around 4.8 after day 5 (Figure 3). However, in the $Pb(NO_3)_2$ treated cultures, no such recovery was observed. In the case of paraquat treated cell cultures, the pH did not change much when compared to $Pb(NO_3)_2$ treated cultures. The decrease in medium pH during the first 3 days of culture has already been reported in many culture systems and been attributed to the uptake of both ammonium ions and amino acids by plant cells (Rayns and Fowler, 1993). This might result in the excretion of H^+ ions which then cause acidification of the medium. The slight recovery of medium pH after day 5 might be the results of excretion of OH^- ions by live cells. However, the cells damaged by lead did not seem to be able to do this. The pH of the medium is known to strongly influence the speciation of ions in solution and thus extremely important when considering the toxicity of heavy meatals including lead, aluminum,

copper and zinc (Clarkson and Sanderson 1989; Kinraide, 1991). The decrease in medium pH could have increased the availability of Pb^{++} and caused more damage to the cells. This was confirmed by Koyama et al. (1995) who examined the effect of aluminum on cell viability by FDA staining. They found that seedlings exposed to low pH (pH 4.5) had decreased viability when compared with those exposed to pH 5.0. In the present study, the viability of the cells examined by FDA staining showed that $Pb(NO_3)_2$ appeared to kill cells on dosage dependent manner (Figure 4). However, it was difficult to quantify the fluorescence using a microscope since many cells grew as aggregated clusters.

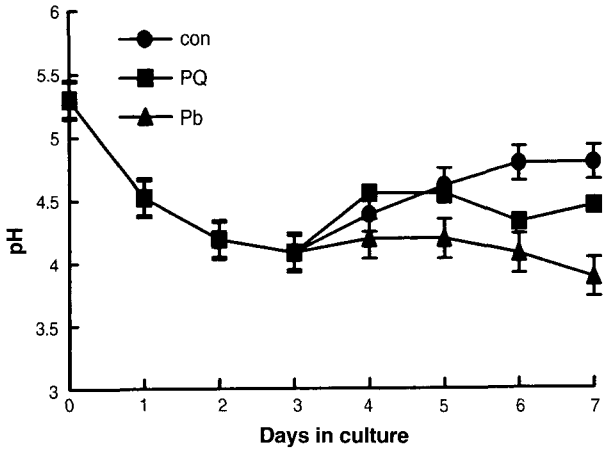

Figure 3. Time course of pH in cell suspensions of *Populus alba* × *P. glandulosa*. The day 3 is the time when either paraquat or $Pb(NO_3)_2$ was added.

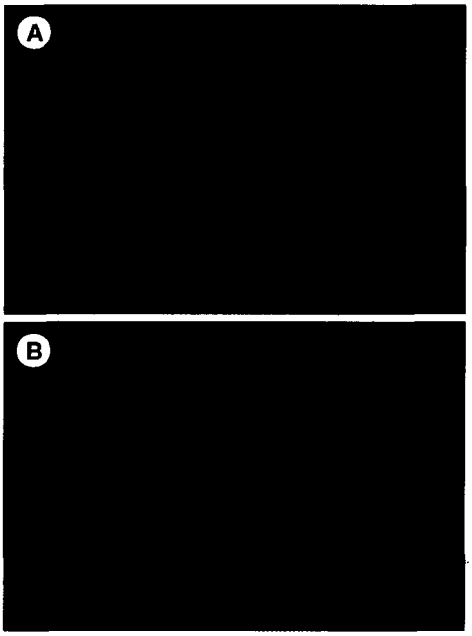

Figure 4. Fluorescence of cultured cells of *Populus alba* × *P. glandulosa* after staning with FDA. A; Control cells, B; $Pb(NO_3)_2$ treated cells.

Ion conductivity of the media

There was much variation and fluctuation in the ion conductivity of the cultures after the addition of $Pb(NO_3)_2$ (Figure not shown). However, the cultures treated with paraquat showed a drastic decrease in ion conductivity at day 5 (Figure 5). The decrease in ion conductivity coincided with the decrease in the fresh weight growth. The apparent insensitivity to paraquat during the first 3 days in culture may be due to slower cell damage by the herbicide in culture. Kwon et al. (1999) reported that ion conductivity was reversely correlated with cell growth inhibition data in tobacco cell cultures treated with herbicides. When cells are damaged, ions leak causing change in the medium conductivity. However, the correlation could not be observed in $Pb(NO_3)_2$ treated cultures. The addition of $Pb(NO_3)_2$ itself might have changed the conductivity of the medium.

Changes in MTT reducing activity

As shown in Figure 6. MTT reducing activities of mitochondria estimated by absorbance at 590nm was reversly proportional to dead cells in the population. The inhibition of fresh weight growth by $Pb(NO_3)_2$ coincided with the decrease in MTT reducing activity (Figure 7). As early as 1 day after the addition of $Pb(NO_3)_2$, a significant effect on MTT reducing activity was observed at 600 μM $Pb(NO_3)_2$.

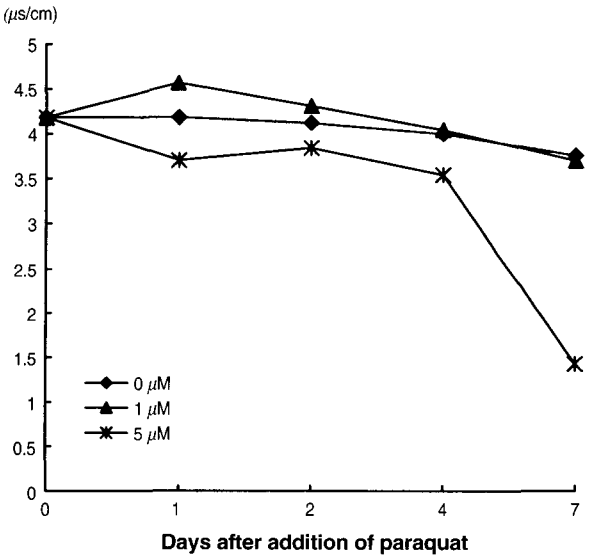

Figure 5. Time course changes in ion conductivity of suspensions of *Populus alba* × *P. glandulosa* cells after addition of paraquat.

Incubation with 300 μM $Pb(NO_3)_2$, however, had no effect on both growth of the cell cultures and MTT reducing activity. In paraquat treated cultures, however, the reduction in MTT activity was seen 2 days after the addition. MTT reducing activity declined faster than fresh weight reduction. An interesting observation is that the cells treated with 1 M paraquat appeared to recover MTT activity in the later culture period (Figure not shown). The reason is not clear. The cells might have adjusted the low concentration of paraquat or the cells that might have survived proliferated since the paraquat in the medium is being depleted. In any event, most of the cells treated with paraquat were not killed during the culture period. Plants are known to contain some enzymes that could detoxify paraquat. These include ferritin and glutathionein. They might have been able to handle a limited amount of paraquat in the medium. However, when the concentration was raised to 5 μM, no such a recovery was seen.

Ikekawa et al. (1998) demonstrated that inhibition of post-treatment growth of tobacco cell cultures was strongly related to Evans blue uptake, but not to MTT reduction. However, both fresh weight growth and MTT reducing activity appeared to be good early indicators of cell damage caused by $Pb(NO_3)_2$ in poplar cell cultures.

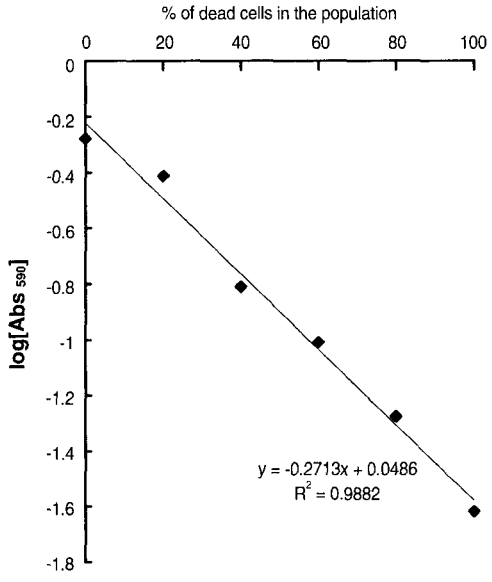

Figure 6. Relationship between MTT reducing activity and the percentage of dead cells in the cell cultures. MTT reducing activity estimated by log[Abs $_{590}$] was negatively correlated to the percentage of dead cells.

Evans blue assay

As in the case of MTT reducing activities of quick killed cells, Evans blue staining was proportional to the percentage of dead cells in the population (Figure 9). Evans blue uptake was used to test cell viability by Archambault et al. (1996) to confirm that cells remained viable through the experimental period. However, in the case of both $Pb(NO_3)_2$ and paraquat treated cells, much variation was observed between as well as within treatments. The reduction of fresh weight growth by the addition of $Pb(NO_3)_2$ did not correspond to the change in Evans blue staining. In the case of paraquat treated cells, little variation was observed among the treatments. However, the staining activity decreased with days after the addition of paraquat. This might be due to aggregation of cells in culture. Poplar cells tend to aggregate during suspension culture and form nodule like structure. This property might have made Evans blue very difficult to pass through cell wall in the clumps. Thus, the dye was

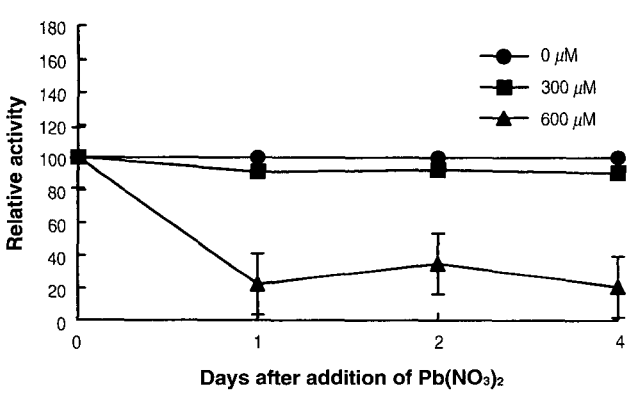

Figure 7. Time course changes in MTT activity of cultured *Populus alba* × *P. glandulosa* cells. $Pb(NO_3)_2$ was added to the cell cultures 3 days after subculturing to new media. The values were calculated by A_{590nm} and converted to the relative value of control.

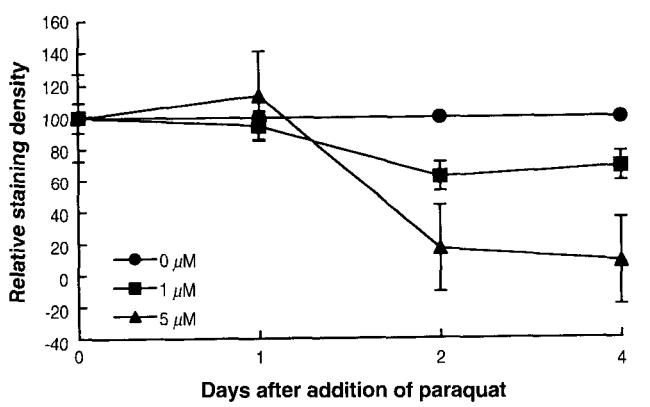

Figure 8. Time course changes in MTT activity of cultured *Populus alba* × *P. glandulosa* cells. Paraquat was added to the cell cultures 3 days after subculturing to new media. Day 0 is time when paraquat was added. The values were calculated by A_{590nm} and converted to the relative values of the control.

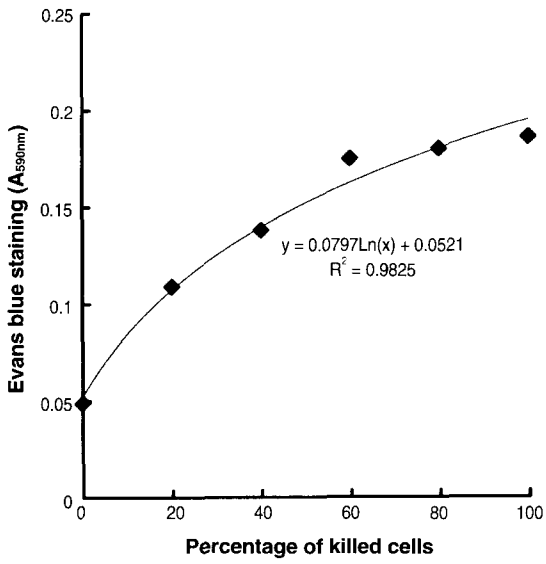

Figure 9. Evans blue staining activity verse the percentage of dead cells in culture. The cells were killed by boiling and mixed with live cells with 6 different levels. (0, 20, 40, 60, 80 or 100%)

either not able to enter the cells or not washed away well due to the aggregated cell clumps. In any event, Evans blue does not seem to be a good choice of estimating cell damage in poplar cells. First of all, the effects varied with cell lines. Second, dose effect was not apparent within two days after treatment. This result is contradictory to the previous reports demonstrating that inhibition of post-treatment growth of tobacco cell cultures was strongly related to Evans blue uptake, but not to MTT reduction (Ikekawa et al., 1998). Different cell types might have resulted in such difference. More work may be needed to apply Evans blue staining to the estimation of cellular damages with poplar cells.

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