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Short Communication

Increase in peroxidase activity in tea callus in response to darkness, 2,4-dichlorophenoxyacetic acid and carbohydrates

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Abstract The peroxidase (POD) reaction for the industrial synthesis of etoposide proceeds effectively when suspension-cultured plant cells are used as catalysts. Tea (*Camellia sinensis*) suspension cultures express higher POD activity and produce a higher yield of reaction products than those of other plant cells. We intend to investigate the conditions necessary to efficiently increase the POD activity in a suspension-cultured tea callus. Here we examined the influence of photoenvironmental conditions, phytohormones, and carbohydrates on POD activity in the callus grown on solidified medium. POD activity in the callus was increased 1) in the dark; 2) on medium containing 2,4-dichlorophenoxyacetic acid (2,4-D) at 10 mg l^{-1}; or 3) on medium containing 0.3 M sucrose and 0.1 M mannitol. The highest POD activity, achieved under the third set of conditions, was about 3.6-fold higher than that achieved on control medium (with 0.09 M sucrose).

Key words: Carbohydrate, dark, peroxidase, suspension culture, tea (*Camellia sinensis*).

Peroxidase (POD) plays an important role as the rate-limiting factor in the industrial synthesis of the anticancer drug etoposide, which was originally derived from a plant source (*Podophyllum peltatum*) (Dawson 1998). When etoposide is produced in the conventional process with horseradish peroxidase (HRP), hydrogen peroxide (H_2O_2) is required but triggers other reactions in the intermediates, which decrease the yield of etoposide (Kutney et al. 1996).

When suspension-cultured plant cells are used as catalysts, the reaction proceeds favorably without H_2O_2 (Takemoto et al. 2002). This technique is superior to the HRP-based technique in "enzymatic stability" (Kutney et al. 1996), and is amenable to recycling by immobilization of the cells with calcium alginate (Takemoto et al. 1995). The mass production of etoposide could thus be achieved at a lower cost with cultured cells. Recently, tea (*Camellia sinensis*) suspension cultures were shown to express higher POD activity and produce a higher yield of reaction products than do other cultured plant cells, such as *Nicotiana tabacum*, *Daucus carota*, *Catharanthus roseus*, and *P. peltatum* (Takemoto et al. 2002). Therefore, tea suspension cultures could be useful in industrial applications.

The cultivation of tea under high levels of light or with phytohormones, such as ethylene, indole acetic acid, or 2,4-dichlorophenoxyacetic acid (2,4-D), efficiently increases POD activity in the leaves (Saijo and Takeo 1974). POD activity has also been reported to increase under the osmotic stress induced by some carbohydrates, such as mannitol or sucrose, particularly in the cell-suspension cultures of some plants (Wagih and Coutts 1982; Tsutsumi and Sakai 1993; Nose et al. 1995). Our ultimate goal is to maximize POD activity in a tea suspension cell culture. As a first step, we examined the effects of 2,4-D, carbohydrates, and photoenvironmental conditions on POD activity in callus grown on solidified gellan-gum medium to clarify the conditions that would possibly increase POD activity in suspension cell cultures.

Callus were derived from the cotyledons of the seeds of *Camellia sinensis* cv. "Sayamakaori" on Murashige & Skoog (MS) medium (pH 5.8) (Murashige and Skoog 1962) containing 0.2 mg l^{-1} indole butyric acid, 2 mg l^{-1} benzyl adenine, 0.09 M sucrose, and 0.3% gellan gum in 9 cm Petri dishes at 27°C with a 16 h photoperiod (8.5–11.3 W m^{-2}, white fluorescent light) for about 30 days. The proliferated callus, characterized as milky-white colored and compact, was subcultured about every 60 days on MS medium (pH 5.8) containing 0.09 M sucrose and 0.3% gellan gum at 27°C with a 16 h

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; H_2O_2, hydrogen peroxide; HRP, horseradish peroxidase; MS, Murashige & Skoog; POD, peroxidase.
This article can be found at http://www.jspcmb.jp/

photoperiod for about 16 weeks. The subcultured callus, divided into fragments of about 5 mm in diameter, was placed on the same medium used to subculture it, and was incubated at 27°C in the dark or with a 16 h photoperiod for 60 days. The proliferated callus was homogenized with 0.05 M sodium phosphate buffer (pH 6.0). After centrifugation, the supernatant containing the crude enzymes was collected. The POD activity was then measured spectrophotometrically at 480 nm using *o*-aminophenol as the substrate (Kawaoka et al. 1994).

The POD activity in callus cultured in the dark was about 1.6-fold higher than that in callus cultured with a 16 h photoperiod (Figure 1). Thus, culture in the dark efficiently increased POD activity of callus conventionally subcultured and grown in the light. Thus, the effect of light on the POD activity of tea callus differed from the effect of light on the POD activity of tea leaves. Light has been reported to increase POD activity in some plants (Sharma and Biswal 1976; Jain et al. 1978; Mishra et al. 1993; Repka and Fischerova 1997/98; Morimura et al. 1999). Conversely, there have been several reports of increased POD activity induced by darkness in plant cells conventionally cultured under illumination (Leu et al. 1975; Druart et al. 1982). In both cases, photoenvironmental changes possibly affected the increase in POD activity. In this experiment, the increase in POD activity in the callus might have been induced by the change in photoenvironmental conditions. However, to verify the effects of photoenvironmental conditions on maintaining POD levels in cultured cells, we need to investigate in detail the influence of these conditions on POD activity during the culture period.

Subcultured callus were grown on MS medium with 2,4-D at concentrations of 0, 1, 5, or 10 mg l^{-1}. Consistent with the photoenvironmental results described above (Figure 1), these callus were cultured at 27°C for 60 days in the dark. POD activity was examined after the proliferation of the callus.

When the callus was cultured on MS medium containing 2,4-D (10 mg l^{-1}), POD activity increased 2.4-fold relative to that observed under control condition when no phytohormones were applied (Figure 2). Generally, the addition of 2,4-D effectively increases and maintains total POD activity in suspension-cultured cells during the culture period (Krsnik-Rasol 1991; Agostini et al. 2000). This experiment demonstrated that 2,4-D increases the POD activity in suspension-cultured tea cells. To identify the optimal conditions, we have yet to examine the effects of 2,4-D concentrations above 10 mg l^{-1} on POD levels.

Subcultured callus were placed on MS medium with a wide range of carbohydrate concentrations. Sucrose and mannitol at concentrations of 0–0.4 M were added to the medium in various combinations. Medium containing 0.09 M sucrose was used as the control. The callus was

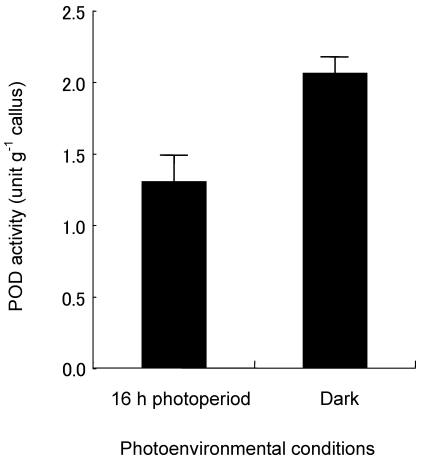

Figure 1. Effect of photo-environmental conditions on POD activity in callus. POD activities were expressed as units per gram fresh weight of proliferated callus, which were obtained from total 20 explants cultured on the MS medium (pH 5.8) with 0.09 M sucrose and 0.3% gellan gum under expressed photoenvironmental conditions at 27°C for 60 days. Each value is the means of triplicate experiments. Error bars are SEs.

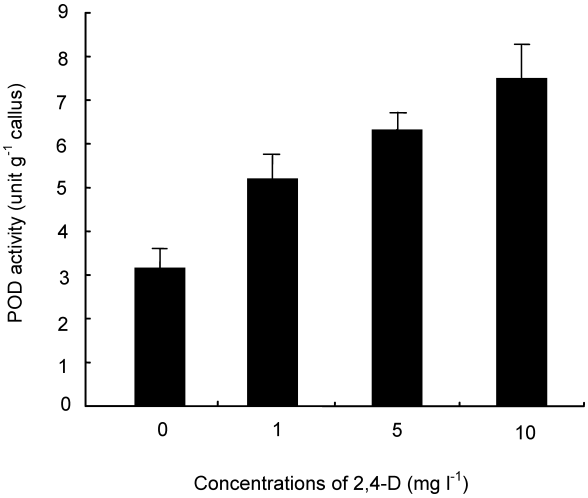

Figure 2. Effect of 2,4-D in the medium on POD activity in callus. POD activities were expressed as units per gram fresh weight of proliferated callus, which were obtained from total 20 explants cultured on the MS medium (pH 5.8) with 2,4-D at expressed conditions, 0.09 M sucrose and 0.3% gellan gum under dark condition at 27°C for 60 days. (0 mg $^{-1}$ 2,4-D: control condition). Each value is the means of triplicate experiments. Error bars are SEs.

cultured for 60 days in the dark, and the POD activity in the callus was measured after proliferation.

When the callus was cultured on MS medium containing total sucrose and mannitol concentrations greater than 0.3 M and sucrose concentrations of more than 0.2 M, the total POD activity of the callus increased above that of the control callus grown with 0.09 M sucrose (Figure 3). Therefore, culture in high concentrations of carbohydrates effectively increased POD activity. In this study, the highest POD activity achieved, which was about 3.6-fold greater than that

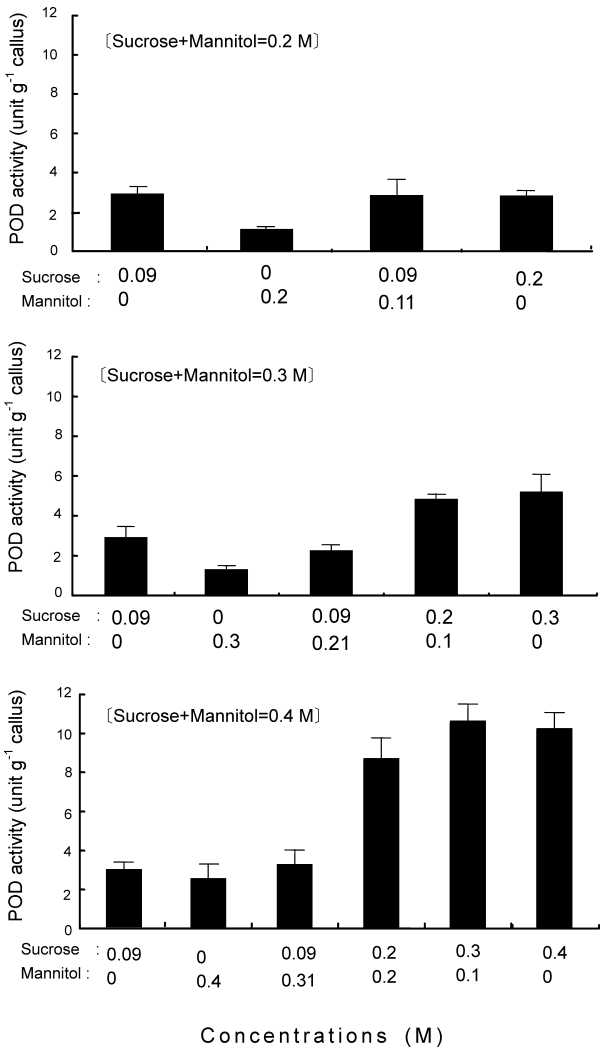

Figure 3. Effect of high concentrations of sucrose and mannitol in the medium on POD activity in callus. POD activities were expressed as units per gram fresh weight of proliferated callus, which were obtained from total 20 explants cultured on the MS medium (pH 5.8) with sucrose and mannitol at expressed concentrations and 0.3% gellan gum under dark condition at 27°C for 60 days. (0.09 M sucrose: control condition). Each value is the means of triplicate experiments. Error bars are SEs.

achieved in the control medium (with 0.09 M sucrose), was on medium containing 0.3 M sucrose and 0.1 M mannitol. In this experiment, we surveyed a wide range of carbohydrate concentrations to determine the effects of carbohydrates on increasing POD activity. Further experiments are required to determine the optimal concentrations.

In some plant cell suspension cultures, carbohydrate-induced osmotic stress increases and maintains POD activity during the culture period (Wagih and Coutts 1982; Tsutsumi and Sakai 1993; Nose et al. 1995; Bueno et al. 1998). In the present study, high levels of POD activity were achieved by culturing the callus for relatively long periods with mannitol and sucrose, with a concentration of sucrose slightly higher than that of mannitol. In general, sucrose is believed to act not only as an osmoticum, but also as a substrate in carbon metabolism and as an energy source in living organisms, whereas mannitol acts only as an osmoticum (Halford et al. 1999; Satoh et al. 2000). Therefore, there might be factors other than osmotic stress contributing to the increase in POD activity induced by carbohydrates.

Generally, as the POD activity increased, the proliferation rate of the callus decreased (data not shown). As the main purpose of this experiment was to produce callus that expressed maximal POD activity, we did not address the issue of callus proliferation. However, for reference, the callus proliferation rate under the conditions producing the highest POD activity (0.3 M sucrose and 0.1 M mannitol) was 391% over 60 days, whereas the rate under control conditions (0.09 M sucrose) was 867% over 60 days.

Here, we identified techniques that increase the POD activity in tea callus. This was achieved by culturing the callus in the dark, on MS medium supplemented with higher concentrations of 2,4-D or carbohydrates (sucrose and mannitol). These results constitute an initial investigation of the optimal conditions for suspension-cultured cells, with the ultimate goal of using tea callus in the industrial synthesis of anticancer drugs.

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