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Effects of Continuous Light/Dark and 2,5-Norbornadiene (1,5-Cyclooctadiene) on Glucose-6-phosphate Dehydrogenase Activity in Spinach Leaves

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Effects of continuous light/dark and the cyclic olefin 2,5-norbornadiene (NBD; 1,5-cyclooctadiene) on the glucose-6-phosphate dehydrogenase activity (G6PD, EC.1.1.1.49) in spinach leaves were investigated. Plants were grown in sand culture under greenhouse (12:12-h light/dark) condition for 30 d. To examine the effect of NBD; 2 µL pure NBD, 5 µL pure NBD and 5 µL NBD/10 mL water were applied to the plants. The results show that G6PD activity was inhibited in the light and was reactivated in the dark both of the opened control and the closed control. In this study, it is shown for the first time that G6PD can have low and high activity form in the continuous light and the dark regimes. The G6PD shows circadian rhythm. In the leaves with NBD, G6PD activity values increased with respect to the control group under both light and dark periods. Since G6PD is affected by the cyclic olefin NBD, an inhibitor of ethylene action, ethylene because plants have not NBD in normal conditions can have a role in regulation process of this enzyme or NBD can be a stress factor for plants.

Key Words: Glucose-6-phosphate dehydrogenase, Spinach, 2,5-Norbornadiene.

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PD, EC.1.1.1.49) is one of the key enzymes of the oxidative pentose-phosphate cycle, known to be encoded by nuclear DNA and synthesised by cytoplasmic ribosomes[1]. Two isoenzymes of the G6PD have been described to occur in green plants, but little is known concerning the mechanism of regulation of the G6PD at the molecular level. One of the isoenzyme is localized in the chloroplast, the other in the cytosol[2,3].

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2,5-Norbornadiene (1,5-cyclooctadine, NBD) an inhibitor of ethylene action, delays or almost completely inhibits the germination of excised *Avena fatua* embryos[4]. Pea germination was strongly inhibited by NBD application and this inhibition was mostly counteracted by ethephon[5]. The increase in the length of pea seedlings has been observed until the concentration of *ca.* 1000 µL/L of NBD. However, the further increase in the NBD concentration cause decrease in the epicotyl's length[6]. The inhibition of ethylene action by NBD was associated with the inhibition of dormancy breaking effect of stratification, ethylene, GA_3 and BA in the resprouter[7]. Continuous NBD treatment prevented in-rolling symptom of petals and maintained fresh flowers for a long time and delayed senescence without wilting[8]. The effect of NBD can be inhibited by the ethylene-induced protein phosphorylation[9]. Treatment of the tissue with NBD resulted in substantial reduction in lipoxygenase and ascorbate peroxidase activities. Guaiacol peroxidase activity was reduced only slightly, if any, by NBD[10].

A number of physiological studies with NBD confirm the ability of this cyclic olefin to block ethylene action in a number of plants[11]. So far, it is known that light inactivates G6PD but effects of continuous light/dark are not known and no study has been reported about the effects of ethylene and NBD on G6PD activity in the literature. For this reason, a series of experiments were performed using the reversible inhibitor of ethylene action, NBD. Therefore, the aim of this paper is to investigate the effects of continuous light/dark and NBD on G6PD activity.

EXPERIMENTAL

Growth conditions: Seeds of spinach (*Spinacia oleracea* L. cv. Gladiator) were grown in sand culture and supplied with the standard Hoagland's solution every 2 d. They were maintained in growth chambers under control conditions (12-h light/22 ºC, 70 % RH and a 12-h dark/18 ºC, 50 % RH) for 30 d with a light intensity of 400 µmol m^{-2} s^{-1}. For NBD treatment, some of the control plants were covered with stretch film with and without NBD (2 µL pure NBD, 5 µL pure NBD and 5 µL pure NBD/10 mL water) (E. Merck, Germany) for 1 d. Spinach leaves growing in these different conditions were harvested (first real leaves collected from plant) at 3 h intervals, frozen in liquid nitrogen and stored at -80 ºC for G6PD activity assays. The enzyme activities were measured in all leaf samples. For each treatment were used plants in three plastic pots (it has a number of plants).

Treatment with NBD: The experiment has five groups and each group contains three plastic pots: **Group I:** at the end of the growing period (30 d), the control plants in the plastic pots (called opened control); **Group II:** at the end of the growing period (30 d), the plants in the plastic pots covered with stretch film (called closed control). **Group III:** at the end of the

growing period (30 d), the plants in the plastic pots covered with stretch film and containing pure 2 µL NBD in sterilized eppendorf tube. **Group IV:** at the end of the growing period (30 d), the plants in the plastic pots covered with stretch film and containing pure 5 µL NBD in sterilized eppendorf tube and **Group V:** at the end of the growing period (30 d), the plants in the plastic pots covered with stretch film and containing pure 5 µL NBD/10 mL water in the petri dishes (called as aqueous NBD solution).

Determination of G6PD activity: The harvested leaves were stored at -80 ºC and 0.5 g of fresh weight of leaf were homogenized with 2 mL of chilled 0.2 M KH_2PO_4/2 mM EDTA containing 5 mM dithiothreitol (DTT) at pH 6.5 buffer for 2 min and filtered. The filtrate was then centrifuged at 27000 g for 20 min. The supernatant was used for the determination of enzyme activities[2,12]. G6PD was assayed routinely in a 0.1 M *tris*-Maleat buffer pH 8.8 containing 2 mM NADP, 0.6 mM G6P and 10 mM $MgCl_2$ at 25 ºC[2,12]. Change in absorbance at 334 nm was recorded. Incubations and assays of enzyme activity (EU/mL. homogenate) were performed at 25 ºC. The G6PD activity determination was done according to the method Beutler[13]. One enzyme unit was defined as the enzyme amount reducing 1 mmol $NADP^+$ per 1 min.

RESULTS AND DISCUSSION

In order to estimate the activation state of G6PD in illuminated or darkened leaves, spinach seedlings were exposed either to full sunlight or to darkness and then extracted as described under experimental section. Since the leaf extract contains both the chloroplastic and the cytoplasmic isoenzyme, their proportions had to be determined by first measuring the combined activities. The results presented in Fig. 1 show that G6PD activity was inhibited in the light and was reactivated in the dark both of the opened control and the closed control. G6PD activity in closed control was higher than the opened control in both light and dark conditions. In addition to this result, it is also shown for the first time that G6PD can have low and high activity forms in the light and dark conditions (Fig. 1). The G6PD shows circadian rhythm. Some researchers also observed a similar result when intact pea plants have been irradiated that is the both plastid and cytosolic forms of G6PD are inactivated[14]. G6PD, one of the key enzymes of the oxidative pentose-phosphate cycle and it can occur both of cytosol and chloroplast. In chloroplasts, pentose-phosphate cycle operates during darkness while light inactivates G6PD, thereby preventing degradation of glucose-6-phosphate and allowing the Calvin cycle to operate faster. One mechanism of deactivation by light is formation of inhibitory NADPH from $NADP^+$ by the thylakoid electron-transport system and another is the ferredoxin-thioredoxin system[15].

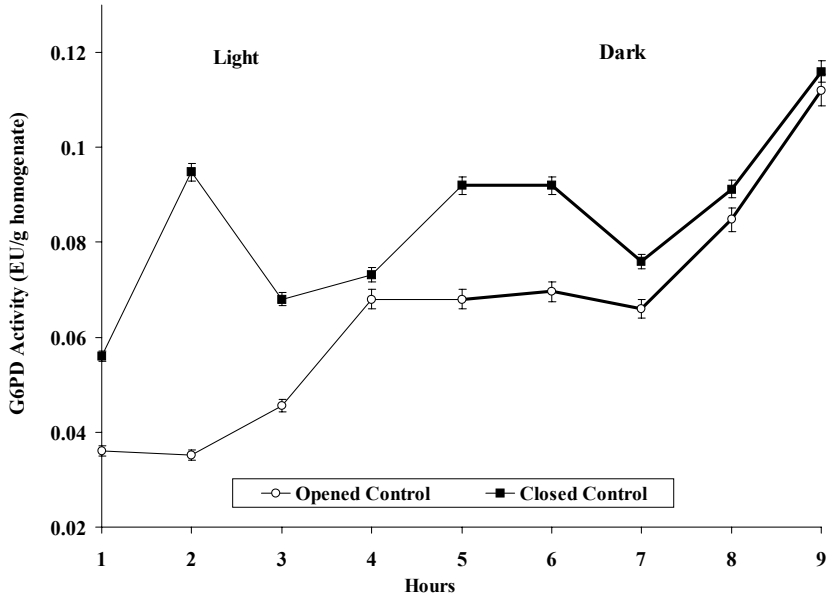

Fig. 1. Effects of light and dark on G6PD activity in spinach leaves
Each value represents the mean ± SD (n = 3)

The results presented in Fig. 2 show that the application with NBD caused activation in G6PD in both light and dark when the results were compared with the closed control values. At zero samples of opened control, closed control, 2 µL NBD, 5 µL NBD and aqueous NBD, levels of G6PD activity were 0.020, 0.014, 0.023, 0.018 and 0.037 EU/g homogenate, respectively. Also, the highest activity in the G6PD observed in the aqueous NBD (5 µL/10 mL water) because this concentration is lower than others. To see direct effect of NBD on G6PD activity, it needs *in vitro* studies because this study conducted only *in vivo* conditions. It can be concluded that the increase in the activity of this enzyme was the consequence of enhanced content or activity of the enzyme. Some researchers found that light and dithiothreitol inactivate the chloroplastic G6PD and dark activation of light-inactivated G6PD was inhibited by catalase in a broken pea chloroplast system[16]. Partially purified G6PD from pea leaf chloroplast can be inactivated *in vitro* by dithiothreitol and reactivated by H_2O_2[16]. Increasing Mg^{2+} concentrations can also activate G6PD from pea chloroplasts[17].

The generation of NADPH and pentose phosphate *via* an oxidative pentose-phosphate pathway is required in both cytoplasm and chloroplast components. Only in the chloroplast, however, is it necessary to suppress this pathway in the light. Light-inactivation of the chloroplast isoenzyme prevents the loss of ATP in a futile cycle of CO_2 fixation in the Calvin cycle

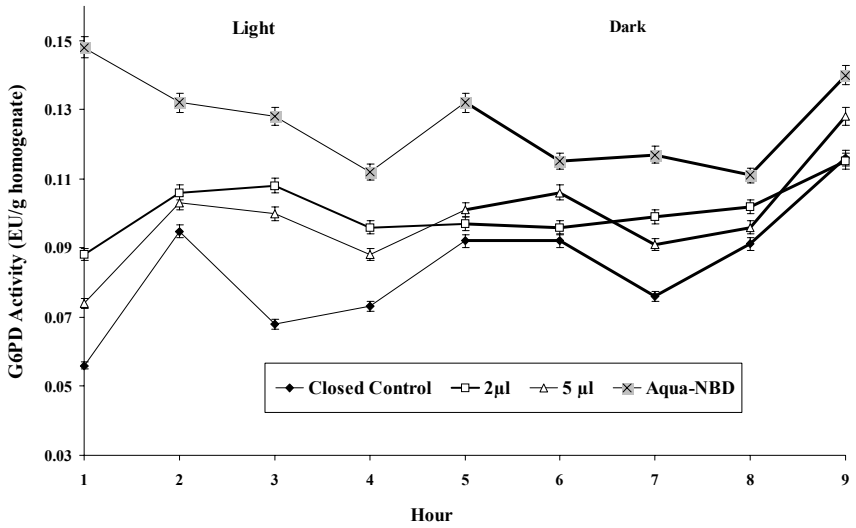

Fig. 2. Effects of NBD concentrations (pure 2 µL, 5 µL and 5 µL/10 mL water) on G6PD activity in spinach leaves. Each value represents the mean ± SD (n = 3)

and concomitant release of CO_2 in the oxidative pentose phosphate pathway. In the light, photosynthetic electron flow provides the chloroplast compartment with sufficient reducing equivalents and the intermediate pools of the Calvin cycle can serve as source for pentose phosphates. In the dark, however, the chloroplastic oxidative pentose phosphate pathway becomes active because of reactivation of the chloroplast G6PD that is inactive in the light[18,19].

Overall, the obtained results from present study will be helpful in further studies related to G6PD regulation because the role of many factors in controlling G6PD transcription, G6PD steady-state protein levels and G6PD activation state are not yet sufficiently understood and requires further investigation.

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