Note

ANTIOXIDANT ISOENZYME RESPONSES TO NICKEL-INDUCED STRESS IN TOBACCO CELL SUSPENSION CULTURE

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ABSTRACT: Exposure to nickel (Ni) at high concentrations can lead to production of reactive oxygen species (ROS) resulting in oxidative damage at the cellular level. We investigated the antioxidative responses of *Nicotiana tabacum* cv BY-2 cell suspension to Ni stress (0.075 and 0.75 mM $NiCl_2$) over a 72 h period with special attention to potential alterations in isoenzymes of superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR). Two main SOD isoenzymes were observed, a Mn-SOD (band I) and a Fe-SOD (band II), as well as one CAT isoenzyme and four GR isoenzymes. Activity staining analysis revealed that CAT activity plays a major role in the early response to Ni-induced oxidative stress, particularly when the Ni concentration used was low, whilst a specific GR isoenzyme appears to respond to the Ni-induced oxidative stress when a much higher Ni concentration was used to induce the stress for the same period of treatment. These results illustrate the importance and advantages of determining individual isoenzyme activities.
Key words: antioxidant enzymes, catalase, nickel, superoxide dismutase

RESPOSTA DE ISOENZIMAS ANTIOXIDANTES AO ESTRESSE INDUZIDO POR NÍQUEL EM CULTURA DE CÉLULAS EM SUSPENSÃO DE FUMO

RESUMO: A exposição ao níquel (Ni), em altas concentrações, pode levar à produção de espécies reativas de oxigênio (EAOs), resultando em danos oxidativos em nível celular. Foram investigadas as respostas antioxidativas de células em suspensão do cultivar BY-2 de *Nicotiana tabacum* submetidas ao estresse por Ni (0.075 e 0.75 mM de $NiCl_2$) por 72 h, com atenção especial às alterações potencias em isoenzimas de superóxido dismutase (SOD), catalase (CAT) e glutatione redutase (GR). Duas principais isoenzimas de SOD foram observadas, uma Mn-SOD (banda I) e outra Fe-SOD (banda II), bem como uma isoenzima CAT e quatro isoenzimas de GR. As análises revelaram que a atividade de CAT tem papel principal no momento inicial de resposta ao estresse oxidativo induzido por Ni, particularmente, quando sua concentração foi mais baixa, enquanto uma isoenzima específica de GR parece responder a este estresse na concentração mais alta de Ni, no mesmo período de tratamento. Os resultados ilustram a importância e as vantagens de se determinar a atividade de isoenzimas individuais.
Palavras-chave: enzimas antioxidantes, catalase, níquel, superóxido dismutase

INTRODUCTION

It has been widely reported, particularly in more recent years, the problems related to contamination of the environment with heavy metals. Such contamination is mainly due to anthropogenic activities (Gratão et al., 2005). As a consequence, several aspects of heavy metal research has gained importance and special attention, resulting in a several fold increase in papers published in the literature describing the effect of heavy metal-induced oxidative stress in plants, a trend that is being followed by the study of other important elements (Vitória et al., 2001; Garcia et al., 2006; Gomes-Júnior et al., 2006a, 2006b; Lea & Azevedo, 2006; Gomes-Júnior et al., 2007; Lea & Azevedo, 2007), the effect on ultrastructure alterations (Vitória et al., 2006) and other aspects involving techniques of phytoremediation and heavy metal-soil-plant

interactions (Fontes & Alleoni, 2006; Gonzaga et al., 2006; Mendes et al., 2006; Nascimento, 2006; Sonmez et al., 2006; Pereira et al., 2007).

Plant responses to heavy metal exposure varies depending on plant species, tissue, stage of development, metal concentration and type of metal, triggering a series of defence mechanisms which involve enzymatic and non-enzymatic components (Gratão et al., 2005). In addition to the use of plant seedlings or adult plants, another interesting approach has been the use of *in vitro* cell cultures (Gomes-Júnior et al., 2007), since some of these factors can be controlled better.

Most of the published reports have presented data on total enzyme activity and isoenzyme activity profiles using non-denaturing PAGE. Our group has concentrated on the study of antioxidant enzyme responses to several distinct heavy metals in a series of plant species. We now report the effect of Ni on the activity of three important antioxidant enzymes in tobacco cell suspension culture. Such a study allows the better understanding of specific isoenzyme responses which is not possible if only total enzyme activity is determined.

MATERIAL AND METHODS

Tobacco BY-2 (*Nicotiana tabacum* L. cv. Bright Yellow 2) cells were cultured as described by Vitorello & Haug (1996). Cultures were grown in the dark on a refrigerated rotary shaker (MA830/A, MARCONI, Brazil) at 160 rpm at 27ºC and growth was evaluated by packed cell volume following centrifugation at 500 rpm for 5 min. BY-2 cells were maintained in liquid medium for seven days and 3 mL of cell inoculum were subcultured into new medium (50 mL). Two-day-old cell cultures (beginning of log phase of growth) were submitted to preliminary trials on the effect of several $NiCl_2$ concentrations (0, 0.05, 0.075, 0.1, 0.15, 0.2, 0.5, 0.75, 1 and 2 mM) for up to 72 h (beginning of stationary phase). Two concentrations were chosen for the main experiments, 0.075 mM $NiCl_2$, which exhibited growth and cell viability levels similar to the control, and 0.75 mM $NiCl_2$, which exhibited about half the growth and cell viability at 72 h of treatment when compared to the control (data not shown). Two-day-old cell cultures were submitted to the treatments and cells were harvested at different periods following exposure (12, 36 and 72 h), were suction-dried and weighed for cell mass determination and storage at -80°C for further analyses.

The following steps were carried out at 4°C unless stated otherwise. The BY-2 cells were homogenized (2:1 buffer volume: fr. wt) in a mortar with a pestle with 100 mM potassium phosphate buffer (pH 7.5) containing 1 mM ethylene-diaminetetra-acetic acid (EDTA), 3 mM DL-dithiothreitol and 5% (w/v) insoluble PVPP (Azevedo et al., 1998). The homogenate was centrifuged at 15,000 rpm for 30 min and the supernatant was kept stored in separate aliquots at -80°C, prior to non-denaturing PAGE and SOD, CAT and GR analyses.

SOD, CAT and GR non-denaturing PAGE activity determinations, electrophoresis buffers and gels, and SOD isoenzyme classification were carried out as described by Gomes-Júnior et al. (2007). Briefly, electrophoresis was carried out in 8% polyacrylamide non-denaturing gels and a constant current of 20 mA/gel was applied for 8 h (CAT gel) or 4h (SOD and GR gels) and the temperature maintained at 4°C. Equal amounts (60 mg) of protein were loaded on to each gel lane.

Isoenzyme profiles were also subjected to a densitometric analysis for band intensity through the use of Kodak Digital Science - 1D - Image Analysis Software version 3.0.1. in a Power Look 1120 imagine system (Umax Technology, Texas, U.S.A).

Protein concentration for all samples was determined by the method of Bradford (1976) using bovine serum albumin as a standard in a Lambda 40 spectrophotometer (Perkin-Elmer Corporation, USA).

The experimental design was randomized with three replicates for each flask/treatment/time interval. The results of the densitometric analysis were expressed as mean of three independent replicates of CAT, GR and SOD for one of the experiments. The gel images shown are from one of the replicates.

RESULTS AND DISCUSSION

In this study, SOD, CAT and GR isoenzyme bands were observed following staining of native PAGE gels for enzyme activity of tobacco BY-2 cells subjected to Ni-induced oxidative stress (Figure 1). Band intensity changes were also assessed based on a densitometry analysis (Table 1). Tobacco BY-2 cell cultures revealed two SOD, four GR and one CAT isoenzymes (Figure 1). SOD bands were classified, according to the inhibition patterns to hydrogen peroxide and KCN, as Mn-SOD (SOD I - resistant to both inhibitors) and Fe-SOD (SOD II - inactivated by one of the inhibitors) (Figure 2), which have been shown to be located in distinct cell compartments in plants species. The tobacco SOD I is likely to be located in the mitochondria whereas SOD II in the plastid.

SOD activity staining did not reveal any specific isoenzyme alteration, but there were changes in activity, particularly at 36 h of exposure to 0.075 mM $NiCl_2$ when both SOD bands exhibited increases in ac-

Table 1 - SOD, CAT and GR densitometry analysis of non-denaturing PAGE presented in Figure 1. The pixel intensity of each band was used to calculate the relative change (%) with respect to the control (zero Ni) of each exposure period.

Isoenzyme	0.075 mM $NiCl_2$			0.75 mM $NiCl_2$		
	12 h	36 h	72 h	12 h	36 h	72 h
SOD I	1.45	92.31	-2.5	1.67	-9.59	1.91
SOD II	5.09	78	3.56	2.56	1.95	-2.8
CAT I	40.36	59.35	*	-39.18	-41.9	*
GR I	*	*		*	*	-3.70
GR II	*	*	*	**	**	*
GR III	2.41	-3.27	-4.36	1.90	1.85	-3.42
GR IV	-4.65	-1.15	-4.93	5.75	-1.12	-6.43

*band not was identified and **band was identified.

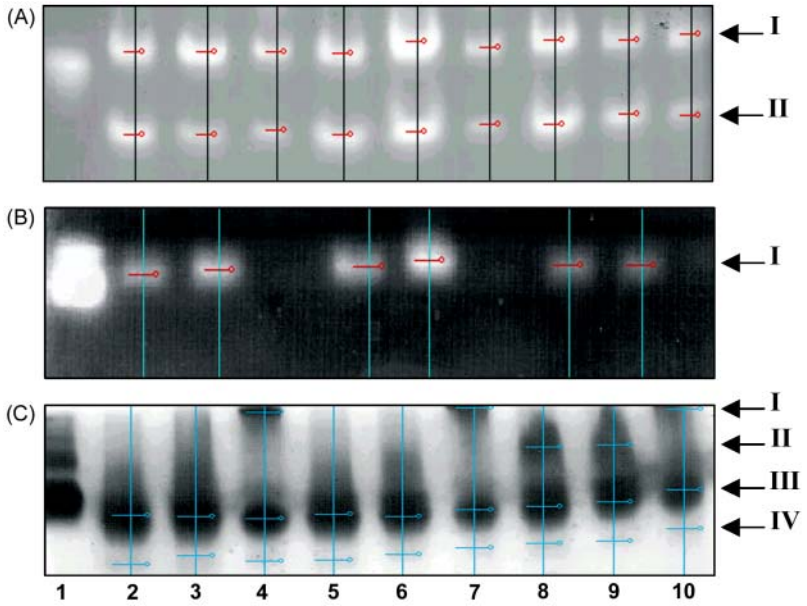

Figure 1 - Activity staining for (A) superoxide dismutase (SOD), (B) catalase (CAT) and (C) glutathione reductase (GR) following non-denaturing PAGE of extracts of tobacco BY-2 cells. Lane 1, bovine SOD, bovine liver CAT and *Saccharomyces cerevisiae* GR standards for (A), (B) and (C), respectively; lane 2, control (zero Ni), 12 h; lane 3, control, 36 h; lane 4, control 72 h; lane 5, 0.075 mM $NiCl_2$, 12 h; lane 6, 0.075 mM $NiCl_2$, 36 h; lane 7, 0.075 mM $NiCl_2$, 72 h; lane 8, 0.75 mM $NiCl_2$, 12 h; lane 9, 0.75 mM $NiCl_2$, 36 h; lane 10, 0.75 mM $NiCl_2$, 72 h.

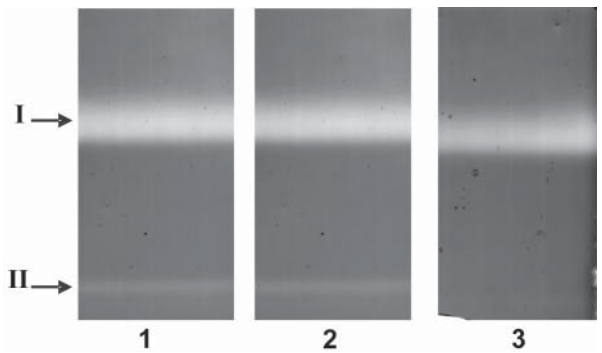

Figure 2 - SOD isoenzyme classification of tobacco BY-2 cell cultures. Lane 1, control SOD activity; lane 2, plus 2 mM potassium cyanide and lane 3, plus 5 mM hydrogen peroxide.

tivity. Apart from these increases, SOD activity was very similar to control levels in both concentrations tested (Figure 1A). SOD is widely distributed among O_2-consuming organisms and is responsible for the dismutation of $O_2^{\bullet-}$ into H_2O_2, and therefore influencing the concentration of $O_2^{\bullet-}$ and H_2O_2. SOD isoenzymes are compartmentalized in higher plants and three isoenzymes have been detected in plants, which are classified according to their metal cofactor; Mn, Fe and Cu/Zn (Gratão et al., 2005). Mn-SODs are located in the mitochondria and peroxisomes, Fe-SOD has been shown to be associated with the chloroplasts (Gratão et al., 2005), while the Cu/Zn-SODs are located in the cytosol, chloroplasts and peroxisomes (Gratão et al., 2005). SOD activity responses to Ni

stress have been shown to vary considerably depending on plant species, tissue and duration of exposure. We identified and classified two SOD isoenzymes in BY-2 cells, Mn-SOD and Fe-SOD, but up to nine SOD isoforms have been reported in cell suspension cultures of *C. arabica*, two bands corresponding to Mn-SOD and three bands to Fe-SOD isoenzymes (Gomes-Júnior et al., 2006a, 2006b, 2007).

CAT activity staining (Figure 1B) revealed only one CAT isoenzyme in BY-2 cell culture, which was also shown to vary in response to $NiCl_2$, exhibiting an increase in activity in the 0.075 mM $NiCl_2$ treatment at 6 h and 36 h of treatment, whereas in the 0.75 $NiCl_2$ treatment CAT activity was reduced when compared to their respective controls. Interestingly, no CAT activity was detected at 72 h in both control and Ni treatments (Figure 1B). More than one CAT isoenzyme is normally observed in plant species (Azevedo et al., 1998), however, in the present study only one major CAT isoenzyme could be detected following non-denaturing PAGE activity staining. CAT is directly regulated by H_2O_2 levels and the increase in CAT activity particularly during the first 36 h of Ni treatment indicates that CAT is possibly acting in the dismutation of the excess H_2O_2 produced as a result of the Ni-induced oxidative stress. Nevertheless the participation of other peroxidases to dismutate the H_2O_2 cannot be ruled out, particularly in the highest $NiCl_2$ concentration tested, since CAT activity was even slightly reduced when compared to the controls.

The possibility of other enzymes being involved in the response to Ni stress under certain concentrations or periods of treatment was further confirmed by the results observed for GR activity (Figure 1C). Overall, GR activity did not vary much since the isoenzymes III and IV, which accounted for the majority of GR activity, exhibited similar levels of activities among Ni concentrations and controls during the duration of the experiment, however, GR I only appeared after 72 h treatment, but not as a response to Ni-induced stress, since this isoenzyme was also observed in the control at 72 h (Figure 1C). On the other hand, the appearance of GR II isoenzyme at 12 h and 36 h of 0.75 mM $NiCl_2$ treatments is clearly due to the Ni-induced oxidative stress, confirming the importance of non-denaturing PAGE analysis in the identification of such specific changes. In plants, cytosolic and plastidic GR isoenzymes have been identified (Xiang & Oliver, 1998), however in BY-2 cells cytosolic GR isoenzymes are likely to be predominant since the cells were grown in the dark. Although these cells contain numerous plastids, antioxidant activity is expected to be low, since no photochemical reactions are occurring.

In conclusion, differential responses by SOD, CAT and mainly GR isoenzymes may be correlated to specific cell physiological phenomena due to the specific organelle localization of the isoenzymes. It appears that CAT has a major role in dismutating H_2O_2 produced in low Ni concentrations, whereas GR and even other peroxidases take over the process and have a more important role when high concentrations of Ni are used, leading to a more severe and faster establishment of oxidative stress.

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