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Activity of glutathione *S*-transferase toward some herbicides and its regulation by benoxacor in non-embryogenic callus and in vitro regenerated tissues of *Zea mays*

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Abstract

A procedure was developed to obtain non-embryogenic callus and regenerated lines from root segments of *Zea mays* grown in aseptic conditions. The activity of glutathione-*S*-transferases (GSTs), for non-embryogenic callus, was determined toward 1-chloro-2,4-dinitrobenzene (CDNB) and it was compared with that obtained for corn seedlings grown without hormones. For the callus masses, increases of specific activity toward CDNB and the kinetic parameter V_{max} were observed with respect to corn seedlings. The procedure permitted the regenerating of tissues from callus explants, therefore the GST(CDNB) activity and the effect of the safener benoxacor on its expression were investigated for the regenerated tissues grown in agarized substrate and in liquid medium. These explants showed a constitutive GST(CDNB) activity higher than corn seedlings and this activity was increased, for both tissues, in response to the presence of the safener benoxacor in the growing medium. The GST activity for the above tissues was also assayed toward benoxacor and terbuthylazine, metolachlor and fluorodifen herbicides. Measurable GST activity was found toward some of the above chemicals and it was found to be significantly enhanced in response to benoxacor treatment.

Keywords: Terbuthylazine; Metolachlor; Fluorodifen; Glutathione *S*-transferase; Benoxacor; Regulation; *Zea mays*; Callus; Regenerated tissues

1. Introduction

The first report of regeneration from tissue culture of *Zea mays* was by Green and Phillips [1]. Since then, in many works corn plants were regenerated by somatic embryogenesis from immature embryos, which are the best source of callus with regeneration competence [2]; while few studies have been done on regeneration by non-embryogenic lines.

Callus cultures have been recognized to be important as a viable means of growing regenerable cereal tissue cultures, while little attention is given to the feasibility of using these tissues and the regenerated lines as a suitable plant material to investigate and point out the role of particular biochemical factors involved in plant metabolism, such as the activity of specific enzyme systems. Several studies have been performed in the past to show the phytotoxicity of herbicides and their mode of action by using plant tissue cultures [3–6]. To clarify the pathways of herbicide detoxification in plants, it appears to be of relevance to standardise a protocol that gives plant material suitable for carefully elucidating the expression and induction of enzymes able to detoxify exogenous synthetic compounds as herbicides. In particular, a family of detoxifying enzymes of plants, named glutathione-*S*-transferases (EC 2.5.1.18; GST),[1] was recognized as being able to conjugate the tripeptide glutathione (GSH) with a large range of different toxic electrophilic compounds, both endogenous and exogenous, by

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[1] *Abbreviations used:* GST, glutathione *S*-transferases; CDNB, 1-chloro-2,4-dinitrobenzene; GSH, glutathione; 1D SDS–PAGE mono-dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis; 2,4-D, 2,4-dichlorophenoxyacetic acid.

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a nucleophilic substitution reaction to give less or no longer toxic derivatives [7,8]. It was demonstrated that the GSTs are involved in the detoxificative pathway of many herbicides, such as chloroacetanilides, chloro-*s*-triazines, thiocarbamates, triazinone sulfoxides, solfonylureas, aryloxyphenoxypropionates, diphenylethers, thiodiazolidines, and sulfonamides [8–13]. It was also shown that GSTs are constitutively composed by subunits, some of which can selectively detoxify specific herbicides, determining their selectivity [7,14,15].

It is of common use in agronomic practice to employ a group of different synthetic compounds, named herbicide safeners, which protect crop-plants from herbicide damage without reducing their action against the target weed species. The enhancement of the activity of some enzymes involved in herbicide metabolism was recognized to be the predominant mechanism of safener action [16]. Some plant GSTs can be differentially inducible by herbicide safeners, with the consequent increase of herbicide tolerance and selectivity [13,17,18]. It has been ascertained that this enhancement of herbicide resistance is due to the safener ability to induce specific GST isoenzymes having affinity for the herbicides as substrates. Therefore the safeners allow a selective control of botanically related weeds, and this strategy has been applied successfully in crops such as corn, rice, wheat, and sorghum [9,12,19–21]. Among the safener compounds, benoxacor [(±)-2,2-dichloro-1-(3,4-dihydro-3-methyl-2H-1,4-benzoxazin-4-yl)ethanone] is known to be very effective in inducing GST activity in corn plants [8,22–25].

The aims of this work were to standardise a procedure to obtain non-embryogenic callus and regenerated lines having suitable characteristics for studying the expression of GST activity as a "model" factor of herbicide detoxification, and to evidence benoxacor's potential to enhance herbicidal metabolism in the in vitro regenerated lines.

2. Materials and methods

2.1. Plant material and culture initiation

Two lots of thirty mature seeds of corn (*Zea mays* L.) (var. Belgrano) were surface sterilized, respectively, in 0.1 and 1.0% sodium hypochlorite solution containing Tween 20 for 10 min. After three rinses in sterile distilled water, each seed was placed into a glass tube containing 10 mL of a germination medium (GM), which consisted of the macronutrients, micronutrients and vitamins of MS [26] supplemented with 2.0 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 15.0 g L^{-1} sucrose. The medium was solidified by 8.0 g L^{-1} of agar–agar and the pH was adjusted to 5.5 with NaOH before autoclaving at 115 °C for 20 min. The cultures were transferred into the growth chamber at 22 ± 1 °C and with a 16-h light photoperiod, under cool white fluorescent light (40 μE m^{-2} s^{-1}) for 2 weeks.

2.2. Callus culture initiation

Segments 8–10 mm long of leaves and roots were excised from seedlings obtained in aseptic conditions, and six explants were placed in each of 20 Petri dishes (10 for roots and 10 for leaves) containing 25 mL of a modified GM medium supplemented with 6.0 mg L^{-1} 2,4-D to induce initial callus formation. All cultures were transferred into the growth chamber for 2 weeks under darkness and 2 weeks in light conditions.

2.3. Organogenesis

Parts of the large initial callus masses developed were employed, taking 1.0 g samples and placing them into flasks containing 100 mL of MS liquid medium supplemented with 0.5 mg L^{-1} naphthaleneacetic acid (NAA), 0.05 mg L^{-1} kinetin and 30.0 g L^{-1} sucrose (MDS); the pH was adjusted to 5.7. Six flasks of the above sterile cultures were transferred into the growth chamber and kept on a rotary shaker (100 rpm) under darkness conditions. After 14 days, half cultures were treated with 100 μM of benoxacor and placed in the darkness; 2 days later, samples of the masses developed with and without benoxacor were collected and submitted to enzymatic assays.

To obtain regenerated explants, 10 initial callus fragments of 4–5 mm diameter were placed into each Petri dish containing 25 mL of a regeneration agarized medium, composed of inorganic and organic elements of MS, with the addition of 2.0 mg L^{-1} kinetin and 30.0 g L^{-1} sucrose called MDR; the pH of the medium was adjusted to 5.8. The cultures were transferred into the growth chamber under darkness. After 2 weeks the dishes were placed in light. This procedure was repeated for two subsequent subcultures refreshing the culture medium, and then one part of the masses developed was collected and another part was transferred onto MDR free of growth regulators; the masses were divided into two tests performed in the absence or presence of 100 μM benoxacor, and placed in the growth chamber. Samples of the cultures were collected after 4 weeks and submitted to the procedures for GST extraction and assay.

2.4. GST extraction and purification

GST extraction was carried out according to the procedure of Cummins et al. [14]. Samples of callus and regenerated tissues (10.0 g) were powdered in liquid nitrogen using a mortar and pestle. The powders were suspended in extraction buffer (1/5, w/v), composed of 100 mM Tris–HCl (pH 7.5), containing 2 mM EDTA, 1 mM dithiothreitol, and polyvinylpolypyrrolidone (1.5%, w/v). After filtration through two layers of muslin, the homogenate was centrifuged at 15,000 rpm for 20 min and the supernatant was adjusted to 80% saturation with respect to $(NH_4)_2SO_4$ to precipitate the proteins (4 °C for 3 h). The resulting suspension was centrifuged at 15,000 rpm for 10 min and the