PEROXIDASE ACTIVITY IN *EUSTOMA GRANDIFLORUM* PLANTS TRANSFORMED BY *AGROBACTERIUM RHIZOGENES*

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The effects of inoculation with wild type of *Agrobacterium rhizogenes* strains: A4, 8196 and LBA 9402, on the protein content and on peroxidase activity and isoenzymes in the normal and transformed plants of *Eustoma grandiflorum* Griseb. (roots, hairy root and leaves) were evaluated. Three cultivars of *in vitro* micropropagated plants of *E. grandiflorum* cultivars: 'Heidi Pink', 'Heidi Blue' and 'Echo White' were used for the experiments. Among the cultivars the highest level of the peroxidase production was archived in the *Heidi Blue* hairy roots induced following infection with both *A. rhizogenes* strains 8196 and LBA9402. By contrast, peroxidase activity was increased in *Echo White* hairy roots induced by *A. rhizogenes* strain A4. The highest protein content was found in *Echo White* hairy root induced by *A. rhizogenes* strain 8196. These results demonstrate that the total protein content and peroxidase activity were significantly affected by genotype and by *A. rhizogenes* strain used in the infection process.

Key words: *Eustoma grandiflorum* Griseb., *Agrobacterium rhizogenes*, hairy root, peroxidase activity.

INTRODUCTION

The genus *Gentiana* includes several herbaceous perennials with important value for their ornamental and medicinal properties. *Eustoma grandiflorum* Griseb. (Gentianaceae), commonly known as lisianthus or prairie gentian, is an ornamental plant cultivated for cut flower and pot plant production. Methods for *in vitro* propagation and genetic transformation of lisianthus using wild-type *Agrobacterium rhizogenes* strains were described by several authors (Handa, 1992; Ruffoni *et al.,* 1993; Semeria and Allavena, 1993; Popa *et al.,* 2006). The hairy root disease caused by a soil bacterium, *Agrobacterium rhizogenes*, affected a wide range of dicotyledonous plant species. This disease is characterized by the formation of a large number of roots at the site of the bacterial infection. The growth of these roots is practically not-limited and is independent of the presence of the bacteria. In other words, hairy roots may be grown under axenic culture

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conditions (Tepfer and Tempé, 1981). During plant infection a segment of DNA, the T-DNA, that originates from the large root- inducing (Ri) plasmid, is transferred from the bacteria to the plant cell, and further integrated into the nuclear plant genome (Chilton *et al.*, 1982). In *A. rhizogenes*, various Ri plasmids are found that show some differences in terms of genetic organization. Expression of T-DNA genes is responsible for the disease symptoms.

Plant hairy roots have become of interest due to their active proliferation on phytohormone-free medium and their capacity to produce valuable plant secondary metabolites accumulated at a comparable level of the original plant root (Flores *et al.*, 1987). In contrast to cell suspension cultures, hairy root cultures are characterized by high biosynthetic capacity and genetics as well as biochemical stability.

In this paper we investigated changes in peroxidase activity in normal and transformed plants (roots, hairy root and leaves) obtained following infection with different wild-type *A. rhizogenes* strains. Another approach in our studies was the comparative analysis of total protein extract from normal and transgenic plants.

MATERIALS AND MEDHODS

Plant material and culture conditions. *In vitro* micropropagated plants of *Eustoma grandiflorum* cultivars 'Heidi Pink', 'Heidi Blue' and 'Echo White' were used for the experiments. The plants were regenerated on MS (Murashige and Skoog, 1962) basal medium containing 3% sucrose, 3.0 mg l^{-1} BAP (6- benzyl aminopurine) and multiplied on the same medium supplemented with 1.0 mg l^{-1} BAP and 5.0 mg/l GA_3 (gibberellic acid). All media components were mixed and adjusted to an appropriate pH (5.7) before autoclaving at 121°C for 20 min. Cultures were incubated at 23±2°C in a growth room under a 16 hrs light photoperiod. Subculturing was carried out every 3 weeks.

Bacterial culture and plant infection. Two agropine strains of *A. rhizogenes*: LBA 9402, A4 and a mannopine *A. rhizogenes* strain 8196 were used for the infection. The *A. rhizogenes* strains, which were kindly provided by David Tepfer Ph.D. (I.N.R.A., Centre de Versailles, France), were grown for 48 h at 28°C on M16 medium with 10g glucose, 10 g yeast extract, 1g $(NH_4)_2SO_4$ and 0.25 g KH_2PO_4.

Microcuttings of 2–3 cm in length were prepared from 8 old weeks *in vitro* shoot cultures. The infections were carried out by immersing in 2 ml bacterial suspension the fresh wounded plant material. Infected plants were co-cultivated for 48 h, in darkness at 25±2°C, on MS liquid medium without hormones and antibiotics. After co-cultivation the explants were transferred to the same medium containing 0.6 % agar, 3 % sucrose and 300 mg l^{-1} cefotaxime. The culture was kept at 25° C under constant illumination.

After 8 weeks of *in vitro* culture, hairy roots and leaves from normal and transformed plants were used for peroxidase activity quantification.

Enzyme extraction and activity assay. Plant tissue (~0,5 g) was crushed in a mortar under liquid nitrogen. Soluble proteins were extracted in 50 mM potassium phosphate buffer (pH 7,0. 0,1 mM EDTA, 4 % polyvinylpyrrolidone (PVP) and 0.2 mM ascorbic acid. After centrifugation at 10000 × for 10 min. at 4°C, the supernatant was used for enzyme activity assay.

Estimation of peroxidase (POX) activity. For POX extraction sodium acetate buffer 0.2M (pH 6.5) containing 1% PVP, 15 mM/l β-mercaptoethanol and 0.25% Triton x100 was used. The reaction mixture contained enzyme extract 0.1 ml, 30 mM guaiacol, 1 ml sodium acetate buffer 0.1 M (pH 5.4), and 0.05 H_2O_2 3%.

Absorption was measured at 470 nm for 5 min at intervals of 60s. Enzyme activity was expressed in units/ ml enzyme. One unit of enzymatic activity represents the changes in DO with 0.1 units/min.

Electrophoresis was performed in 9% polyacrylamide gel (PAGE), according to Laemmli (1970) with some modification. Proteins were stained with Coomassie Brilliant Blue in a fixative solution and enzyme activity was revealed with benzidine reagent.

RESULTS

Three *E. grandiflorum* cultivars: 'Heidi Pink', 'Heidi Blue' and 'Echo White' were tested for their peroxidase activity as response to three *A. rhizogenes* strains infections. Changes in peroxidase activity are shown in Table 1.

For all investigated cultivars the values of peroxidase activity were increased in the hairy roots induced by *A. rhizogenes* strain 8196 in comparison with the control.

Among cultivars the highest level of the peroxidase production was recorded in the *Heidi Blue* hairy root induced following infection with both *A. rhizogenes* strains 8196 (9,07 U/ml) and LBA9402(4.47 U/ml) and *Echo White* hairy roots induced by *A. rhizogenes* strain A4 (8.38 U/ml) (Table 1).

Table 1

Peroxidase activity in normal and transformed plant tissues

Genotype	Peroxidase activity (U/ml enzyme)			
	Control	*A. rhizogenes* A4	*A. rhizogenes* LBA9402	*A. rhizogenes* 8196
Heidi Pink	**1.69**	**2.70**	**1.82**	**4.31**
Heidi Blue	**4.14**	**2.28**	**4.47**	**9.07**
Echo White	**0.77**	**8.38**	**2.42**	**3.29**

For extraction of total proteins and peroxidase isoenzymes separation we used roots, hairy roots and leaves from normal and transformed plants of *Echo White* cultivar. Electrophoretic pattern of total proteins showed significant differences between proteins content in transformed roots and leaves in comparison with the controls (lanes P1and P5) (Fig. 1).

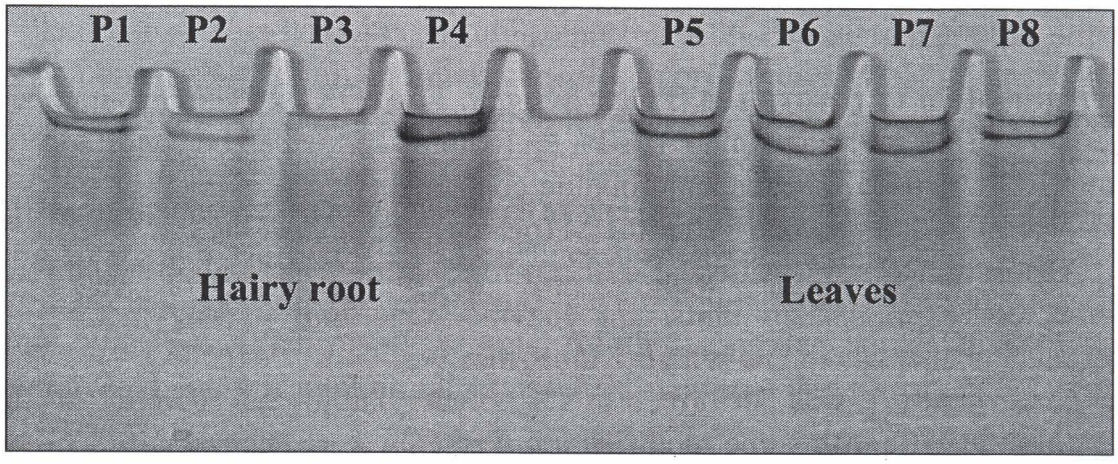

Fig. 1. Electrophoretic pattern of proteins extracted from *Echo White* normal and transformed plant. Lane P1- roots control; lane P2- *hairy roots* sample from transformed plant by *A. rhizogenes* strain A4; lane P3- *hairy roots* induced by *A. rhizogenes* strain LBA 9402; lane P4- *hairy roots* induced by *A. rhizogenes* strain 8196; lane P5-leaves control; lane P6- leaves sample from transformed plant by *A. rhizogenes* strain A4; lane P7- leaves from transformed plant by *A. rhizogenes* strain LBA 9402; lane P8- leaves from transformed plant by *A. rhizogenes* strain 8196.

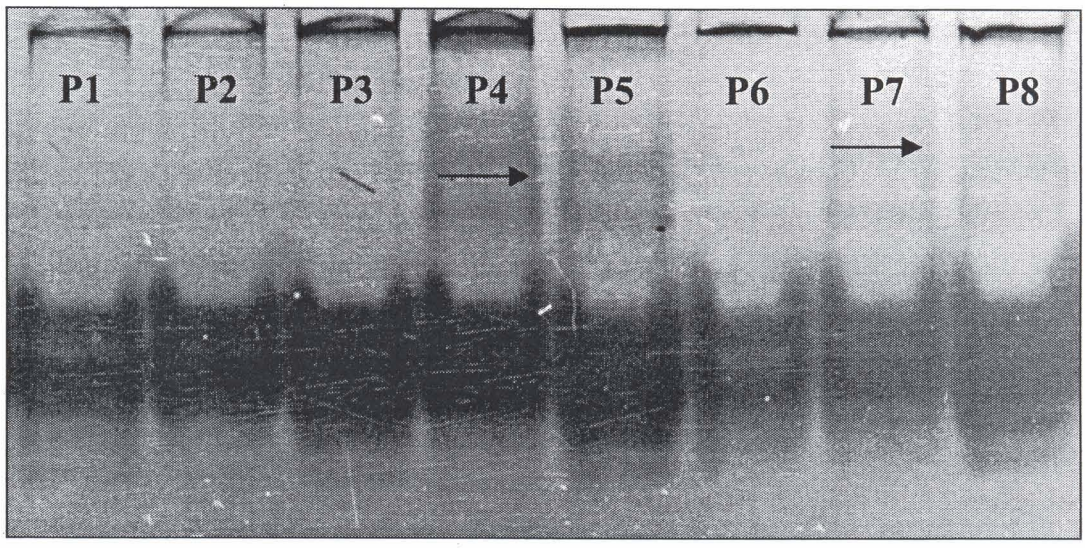

Fig. 2. Electrophoretic pattern of peroxidase isolated from *Echo White* roots and leaves of normal and transformed plants. Lane P1- Roots control; lane P2- *hairy roots* induced by *A. rhizogenes* A4; lane P3- *hairy roots* induced by *A. rhizogenes* LBA 9402; lane P4- *hairy roots* induced by *A. rhizogenes* 8196; lane P5- leaves sample extracted from transformed plant by *A. rhizogenes* 8196; lane P6- leaves control; lane P7- leaves sample extracted from transformed plant by *A. rhizogenes* LBA 9402; lane P8- leaves sample extracted from transformed plant by *A. rhizogenes* A4.

We observed that in hairy root induced by both *A. rhizogenes* strains A4 and LBA 9402, (lanes P2-P3) proteins content decreased in comparison with the leaves samples (lanes P6-P7) extracted from the same plant. The highest protein content in the investigated samples was found in a hairy root extract obtained through *A. rhizogenes* strain 8196 infection (lane P4). Examination of peroxidase zymograms relieved the presence or absence of some bands in the analyzed samples, especially in the rapid electrophoretic mobility area. Thus, in P4, P5, P7 and P8 samples extracted from plants transformed by *A. rhizogenes* strains 8196 (lanes P4 and P5), LBA 9402 (lane P7) and A4 (lane P8) there are present several bands (3 and 2 respectively) (Fig. 2).

We also observed some differences in cathodic peroxidase profiles. In three of the samples analyzed (P3, P5 and P8) a band with different electrophoretic mobility was detected.

DISCUSSION

The results presented in this paper show that the protein content and peroxidase activity were significantly affected by genotype and by *A. rhizogenes* strain used in the infection process.

Between cultivars the highest level of the peroxidase production was recorded in the *Heidi Blue* hairy root induced following infection with both *A. rhizogenes* strains 8196 and LBA9402. By contrast, peroxidase activity was increased in *Echo White* hairy roots induced by *A. rhizogenes* strain A4. The highest protein content in the investigated samples of *Echo White* was found in hairy root induced following infection with *A. rhizogenes* strain 8196. Electrophoretic pattern of peroxidase isolated from roots and leaves of *Echo White* transformed plants by *A. rhizogenes* strains relieved the presence of several bands (3 and 2, respectively).

These results demonstrate that constitutive peroxidase activity levels are associated with the expression of more anionic isoforms, and increased protein expression may be an important component of the plants reaction to *A. rhizogenes* strains infection.

CONCLUSIONS

These results could offer additional information about transformed nature of some plant tissues. The higher level of peroxidase activities could be a consequence of transformation events, and could contribute to an increased

resistance of regenerated plants to pathogens. Enhanced peroxidase activity in transformed plant cells might be a useful biochemical marker for transformation.

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