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Response of *Kalanchoe daigremontiana* to wounding and infection with *Agrobacterium tumefaciens*

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Abbreviations:
CAT – catalase
DNMRT – Duncan's New Multiple Range Test
G-POD – guaiacol peroxidase
HR – hypersensitive response
PAGE – polyacrylamide gel electrophoresis
PPO – polyphenol oxidase activity
P-POD – pyrogallol peroxidase
ROS – reactive oxygen species

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Abstract

Background and Purpose: *Transformation of plant tissue with* Agrobacterium tumefaciens *includes wounding of plant and subsequent infection by bacteria. Polyphenol oxidase activity and oxidative stress parameters – the content of H_2O_2, as well as activity and isoenzymes of antioxidative enzymes catalase, pyrogallol and guaiacol peroxidase were investigated as markers of plant response to wounding and infection.*

Materials and Methods: *Five tissue types – healthy tissue, wounded tissue, tissue in immediate proximity of the wound, tumour tissue and tissue next to the tumour were collected on 7^{th}, 14^{th} and 21^{st} day after wounding or infection. Activities of antioxidative enzymes were evaluated spectrophotometrically. Isoenzymes were separated electrophoretically in native conditions. H_2O_2 content was estimated by measuring the titanium hydro-peroxide complex.*

Results and Conclusion: *All measured parameters were remarkably different in tumour in comparison to healthy tissue. Activities of peroxidases were significantly increased, accompanied with induction of new isoenzymes. After two weeks of the experiment catalase activity was also significantly increased in tumour tissue as well as in tissue next to the wound. H_2O_2 content was lower in tumour after three weeks. Aside from tumour tissue, polyphenol oxidase activity was significantly increased in tissue next to the tumour. Remarkably increased peroxidase and polyphenol oxidase activity in tumour tissue, additional isoenzymes of peroxidases as well as decreased H_2O_2 content, indicated changes in tissue organisation and induction of defence mechanisms against pathogen, in which these two groups of enzymes are involved. Wounded tissue, as well as tissue next to the wound or infection, did not show significant variation from healthy tissue.*

INTRODUCTION

As sessile organisms, plants are continuously exposed to variable biotic and abiotic environmental conditions. Biotic factors include interactions with other organisms, for example infection by pathogens (viruses, bacteria, fungi, nematodes), mechanical wounding by herbivores and effects of symbiosis or parasitism. Examples of abiotic factors are drought, salinity, extreme temperatures, light intensity or air and soil pollution. Plants possess a broad range of strategies to cope with stress conditions, which include morphological, biochemical and genetic properties. For example, resistance to pathogen attack depends on mechanical barriers such as cuticle, synthesis of secondary metabolites

and other inducible responses, which can disable successful pathogen-plant interaction. Signalling molecules that induce synthesis of antimicrobial compounds and strengthening of cell walls also take part in this response (*1, 2*).

Interactions between pathogens and plants represent a mode of communication between organisms from different kingdoms (*3*). *Agrobacterium tumefaciens* is a soil bacterium that infects mostly dicotyledonous plant species and causes the development of crown-gall tumours. The tumours are usually not fatal for the host, but reduce growth (*4*), can be a potential site for secondary pathogen infections (*3*) and increase the plants' sensitivity to abiotic stress. Virulent strains of *A. tumefaciens* contain the tumour-inducing (Ti) plasmid. During infection, a small fragment of Ti-plasmid, the T-DNA, is transferred to the plant cell nucleus and integrated into the chromosome. T-DNA genes encode enzymes involved in the synthesis of plant growth hormones – auxins and cytokinins that causes tumour formation. The ability of T-DNA to integrate stably into the plant genome has led to its widespread use as a vector for introduction of foreign genes into plant cells (*2*). For that purpose, the bacterial genes that encode proteins responsible for tumour formation are removed and substituted by genes of interest.

Transformation efficiency of plant cells by *A. tumefaciens* depends not only on successful colonization and recognition of plant tissue by the bacteria, but also on overcoming plant cell responses to infection. There are some reports on the development of necrosis and low survival rate of infected tissue during the T-DNA transfer into the plant cell which can be the result of hypersensitive response (HR). Besides plant cell-bacterium interaction, infection process involves plant wounding. Both processes cause defense responses which include overproduction of reactive oxygen species (ROS) and increased activity of some enzymes, such as peroxidases (POD) and polyphenol oxidases (PPO) which are involved in plant cell wall strengthening, wound healing and synthesis of antimicrobial agents (*5*, *6*). ROS are highly toxic derivatives of molecular oxygen such as superoxide radical, hydroxyl radical, hydrogen peroxide, singlet oxygen and organic hydroperoxides. Since ROS can damage cell components, for example proteins, nucleic acids, membrane lipids and photosynthetic pigments, the serious consequence of increased ROS production can be disturbance of cellular metabolism, including major metabolic processes – photosynthesis and cellular respiration. However, it has been demonstrated that certain amounts of ROS are produced under normal conditions and that they are included in lignification process, programmed cell death, signal transduction, and some other cellular processes (*7*). Moreover, rapid production of ROS, called oxidative burst, usually observed immediately after pathogen attack, can serve in protection against invading pathogen but could also be a signal for activation of further defense reactions, including the HR of infected cells (*8*). In order to control ROS production and minimize ROS-mediated cellular damage, plants have evolved a highly effective defense system that includes low-molecular antioxidants (i.e. glutathione, ascorbate, tocopherols and carotenoides) and antioxidant enzymes. The most important antioxidant enzymes in plants are superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and various peroxidases (PODs). SOD catalyzes the conversion of superoxide radical into H_2O_2, while CAT degrades H_2O_2 to oxygen and water. PODs remove the H_2O_2 and catalyze the oxidation of various compounds such as glutathione, ascorbate and cytochrome *c*. Moreover, H_2O_2 and POD are involved in physiological and developmental processes, such as cross-linking of cell wall structural proteins, lignification and suberinization, catabolism of indol-3-acetic acid (IAA), biosynthesis of ethylene and healing of wounds. Under *in vitro* conditions, POD can use a wide range of artificial electron donors, e.g. guaiacol or pyrogallol, so they are usually referred as guaiacol or pyrogallol peroxidases (G-POD and P-POD), respectively. H_2O_2 is ROS with important role as diffusible signal molecule (*9*). Polyphenol oxidases (PPOs) are a group of enzymes involved in disease resistance (*6*, *10*). They are widely distributed within the plants and act by hydroxylizing monophenols to o-diphenols and oxidizing these compounds to quinones which are often more toxic to microorganisms than the original phenolic compounds (*10*, *11*).

Transformation of plant cells by *Agrobacterium* is not only interesting as a natural phenomenon, but also represents a very interesting tool for introduction of new genes into plants. In order to optimize the transformation process, researchers developed protocols to control plant defense against *Agrobacterium*-mediated T-DNA transfer (*3*, *4*, *12*). Since the response of plant tissue to both wounding and subsequent infection by *Agrobacterium* can influence the transformation process, the object of our research was to investigate plant defense mechanisms in the leaves of *Kalanchoe daigremontiana* during wounding and subsequent infection with *A. tumefaciens*, as well as during wounding alone. Plant response at wounding and infection sites was evaluated through PPO activity and oxidative stress parameters – the content of H_2O_2 as well as activity and isoenzymes of CAT, P-POD and G-POD.

MATERIALS AND METHODS

Plant material and infection with *Agrobacterium tumefaciens*

Kalanchoe daigremontiana Raym.-Hamet & H.Perrier were grown in greenhouse conditions. Average height of plants taken for the experiment was 20 cm.

Agrobacterium tumefaciens, strain B6S3 was grown on solid nutrient medium (pH 7.0) containing bovine extract (5 g/L), yeast extract (1 g/L), peptones (1 g/L), sucrose (5 g/L) and agar (15 g/L).

On each plant chosen for the experiment, two leaves of approximately the same size and age were wounded

with a sterile needle. The two wounds, 3 cm long were made on both sides of the leaf blades – on the right and left of the main vein. Then, one of the two wounded leaves was inoculated with bacteria. Tumour development and the healing of wounds were monitored during two weeks and the tissue samples were collected on the 7th, 14th and 21st day after inoculation. For the analysis, samples from healthy uninjured leaf tissue, tumour tissue, tissue in immediate proximity of the tumour, wounded tissue, and tissue in immediate proximity of the wound were taken. At least four replicas were prepared for each tissue.

Enzyme assays

Fresh tissue (150 mg) was homogenized in 0.5 mL of cold 50 mM potassium phosphate buffer (pH 7.0), containing 1 mM ethylenediaminetetraacetic acid (EDTA) and 5% (w/v) polyvinylpyrrolidone. The homogenate was centrifuged at 30 000 *g* and 4 °C for 30 min and the supernatant was used for catalase and peroxidase assays.

For P-POD activity assay, the increase in absorbance at 430 nm due to the oxidation of pyrogallol ($\varepsilon = 2.6$ mM^{-1} cm^{-1}) was monitored during 2 min, essentially as described by Chance and Maehly (*13*). The results were expressed as μmol of purpurogallin (a product of pyrogallol oxidation) per min and gram of fresh weight.

G-POD activity was assayed by measuring the increase in absorbance at 470 nm due to the oxidation of guaiacol ($\varepsilon = 26.6$ mM^{-1} cm^{-1}) during 2 min. The results were expressed as μmol of tetraguaiacol per min and gram of fresh weight (*13*).

For CAT activity, the decrease in absorbance at 240 nm was measured according to Aebi (*14*) and the results were expressed as μmol of decomposed H_2O_2 ($\varepsilon = 36$ mM^{-1} cm^{-1}) per min and gram of fresh weight.

PPO activity was measured as an increase in absorbance at 420 nm due to the oxidation of catechol. Reaction mixture contained 0.1 M potassium-phosphate buffer (pH 7.0) 0.05 mM EDTA and 50 mM catechol. The enzyme activity was expressed as changes in absorbance per min and gram of fresh weight.

Electrophoretical separation of isoenzymes

For isoenzyme separation, polyacrylamide gel electrophoresis (PAGE) was performed without sodium dodecylsulfate on 8% (w/v) polyacrylamide gels (*15*). The protein content in enzyme extracts was determined by a dye-binding technique (*16*) using bovine serum albumin as a protein standard. Approximately equal amounts of proteins, 15 μg per well, were loaded and electrophoresis was performed at 4 °C.

For P-POD detection, the gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0), for 30 min, then incubated in a 50 mM potassium phosphate buffer (pH 7.0) containing 20 mM pyrogallol and 8 mM H_2O_2, until the appearance of brown bands (*13*).

G-POD was stained according to the Chance and Maehly (*13*) procedure. After electrophoresis, the gels were equilibrated with 50 mM potassium phosphate buffer (pH 7.0) for 30 min followed by incubation in the same buffer containing 18 mM guaiacol and 8 mM H_2O_2, until brown bands appeared due to guaiacol oxidation by G-POD.

For CAT detection, the gels were incubated in distilled water for 45 min, and then in H_2O_2 solution (0.003%, v/v), for 10 min. Afterwards the gels were washed in distilled water and stained in 1:1 mixture of 2% (w/v) $FeCl_3$ and 2% (w/v) $K_3Fe(CN)_6$, for 10 min (*17*).

Gels were photographed and processed using image processing software Adobe Photoshop 6.0. The data resulted from three independent experiments.

H_2O_2 determination

Fresh tissue (150 mg) was homogenized in 1 mL of ice-cold acetone and centrifuged at 1000 *g* and 4 °C for 3 min. Supernatant was mixed with 400 μL of titanium sulphate and 500 μL of concentrated ammonium solution and centrifuged at 10 000 *g* for 10 min at 4 °C. The precipitate was dissolved in 1 mL 2 M H_2SO_4. The samples were centrifuged again at 10 000 *g* and 4 °C for 10 min. The absorbance of the supernatant was measured at 415 nm *(18)*. The amount of hydrogen peroxide was calculated from the amount of titanium hydro-peroxide complex using an extinction coefficient of 1.878 mM^{-1} cm^{-1} and expressed on a fresh weight basis.

Statistics

The results of H_2O_2 determination and enzyme activities were calculated as the mean value of at least three replicates ± standard error. Statistical analysis was performed using the STATISTICA 7.1 (StatSoft, Inc., USA) software package. Significant differences between mean values were established by one-way ANOVA followed by Duncan's New Multiple Range Test (DNMRT). Differences were considered significant at $P < 0.05$.

Isoenzyme analyses were repeated at least two times.

RESULTS

Tumour development and wound healing

After inoculation with *A. tumefaciens,* the development of tumour and wound healing were observed during three weeks. The appearance of tumour was noticed after the first week, while its growth increased during the following two weeks. Also, slow healing of wounds was noticed in the course of experiment (Figure 1).

H_2O_2 content

The content of H_2O_2 was determined on the 7th, 14th and 21st day in samples from healthy uninjured leaf tissue, tissue in immediate proximity of the wound, wounded tissue, tissue in immediate proximity of the

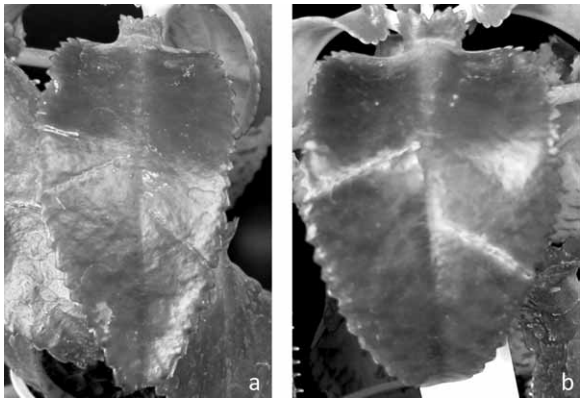

Figure 1. *A leaf of* Kalanchoe daigremontiana *plant: a week after wounding (a) and tumour development a week after inoculation with bacteria (b).*

tumour and tumour tissue. Certain differences among tissues were noticed but they were mostly not statistically significant. An exception was tissue next to the tumour sampled after two weeks and tumour tissue after three weeks, which showed significantly lower content of H_2O_2 in comparison to healthy tissue (Figure 2).

P-POD, G-POD, CAT and PPO activities

Antioxidative enzymes (P-POD, G-POD and CAT) and PPO demonstrated significant differences in activities in wounded and infected tissue.

P-POD activity significantly increased in tumour tissue in comparison with the healthy tissue already after the first week following inoculation with bacteria. After three weeks it was 39 times higher in tumour than in healthy tissue (Figure 3). During the course of the experiment there was no significant change in P-POD activity in the tissue in immediate proximity of the tumour, in wounded tissue as well as in the tissue next to the wound.

G-POD activity showed significant increase in tumour tissue samples during the whole experiment. After three weeks, this activity was 5 times higher than in healthy tissue. In wounded tissue, significant increase was noticed only after three weeks (Figure 4).

Compared to healthy tissue, CAT activity was significantly increased in only two samples – in tumour tissue and tissue next to the wound taken after two weeks. Comparing all three sampling times, CAT activity was the lowest at the end of the experiment (21st day) in all tissues except in healthy tissue (Figure 5).

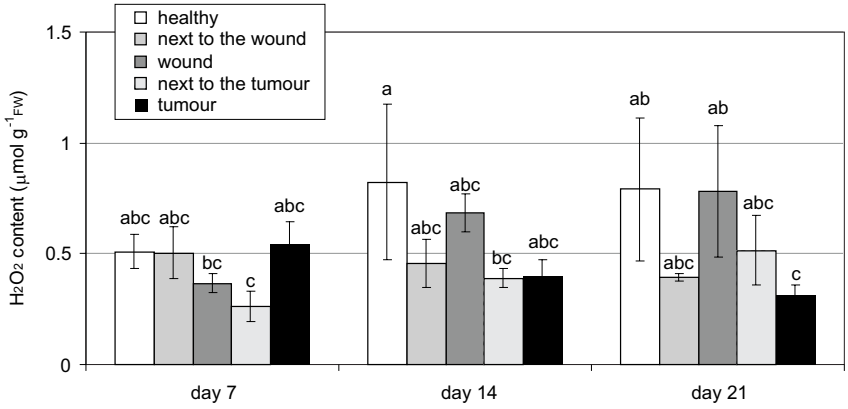

Figure 2. *The content of H_2O_2 in tissue samples taken from infected or wounded plant on the 7th, 14th and 21st day. Results are shown as the mean value from at least four replicates ± standard error. The values were processed by DNMRT, but did not show significant differences ($P \leq 0.05$).*

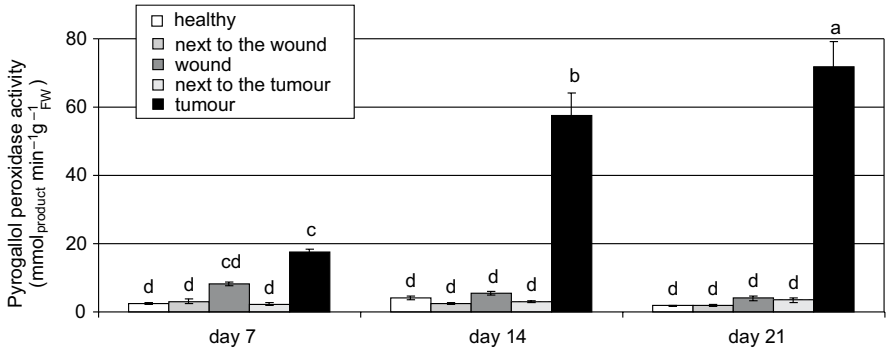

Figure 3. *The activity of pyrogallol peroxidase (P-POD) in the tissue samples taken from inoculated or wounded plant on the 7th, 14th and 21st day. Results are shown as the mean value of at least four replicates ± standard error and processed by DNMRT. The values marked with different letters are significantly different ($P \leq 0.05$).*

In comparison to healthy tissue, PPO activity was significantly increased in tumour tissue in all three sampling periods. After the 1st and 3rd week, the tissue taken immediately close to tumour also showed significantly increased activity. In wounded tissue and the tissue next to the wound, PPO activity was not changed (Figure 6).

P-POD, G-POD and CAT isoenzymes

Three weeks after infection and wounding, five P--POD-isoperoxidases were separated by native PAGE. They were marked as P1-P5. Two bands (P1 and P2) were present in all the tissues, although they were very slightly visible in healthy tissue and the tissue next to the wound. Bands P4 and P5 were revealed only in tumour tissue. Slightly visible band P3 appeared in all tissues except in tumour tissue (Figure 7A).

Six G-POD-isoperoxidases were separated electrophoretically and marked as G1-G6. Isoform G2 was present in all tissues, but it was very slightly visible in healthy tissue and wounded tissue. G1, G4, G5 and G6 isoforms were characteristic for tumour tissue only, while isoform G3 was visible in all tissues except in tumour tissue (Figure 7B).

Electrophoretic analysis revealed two CAT isoenzymes (CAT1 and CAT2) of very low mobility. They were common in all tissues but staining intensity was stronger in tumour and wounded tissue, as well as in the tissue next to the tumour (Figure 7C).

DISSCUSSION

We investigated the response of leaf tissue of *Kalanchoe daigremontiana* on wounding and infection with *Agrobacterium tumefaciens* strain B6S3 by evaluating oxidative stress parameters (the quantity of H_2O_2 in the tissue, activity of antioxidant enzymes P-POD, G-POD and CAT, as well as the number of isoenzymes) and the activity of PPO, an enzyme also involved in the interaction of plant and pathogen. Leaf tissue at the site of the wound, and tumour as well as the surrounding tissues were taken for analyses.

By monitoring the sites of wounding and infection, we observed that tumours increased during the period of three weeks while wounds showed the tendency of healing. The tumour growth indicated the transformation of plant cells, with the T-DNA of *Agrobacterium tumefaciens* carrying the genes for enzymes involved in the

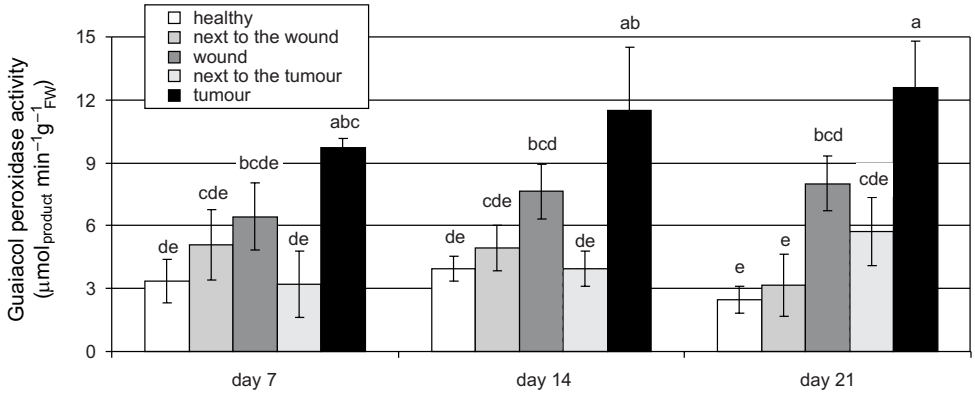

Figure 4. *The activity of guaiacol peroxidase (G-POD) in tissue samples taken from inoculated or wounded plant on the 7th, 14th and 21st day. Results are shown as the mean value of at least four replicates ± standard error, and processed by DNMRT. The values marked with different letters are significantly different (P ≤ 0.05).*

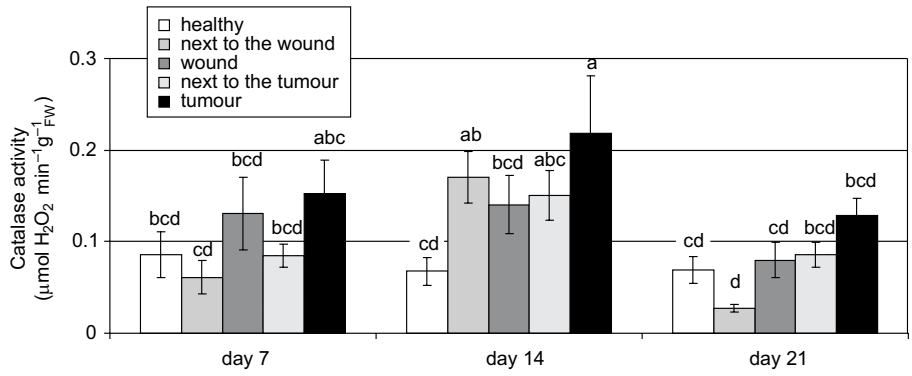

Figure 5. *The activity of catalase (CAT) in tissue samples taken from inoculated or wounded plant on the 7th, 14th and 21st day. Results are shown as the mean value of at least three replicates ± standard error, and processed by DNMRT. The values marked with different letters are significantly different (P ≤ 0.05).*

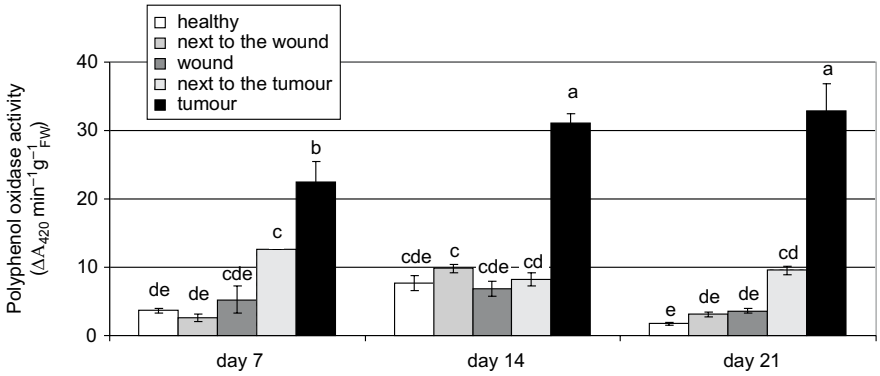

Figure 6. *The activity of polyphenol oxidase (PPO) in tissue samples taken from inoculated or wounded plant on the 7th, 14th and 21st day. Results are shown as the mean value of at least four replicates ± standard error, and processed by DNMRT. The values marked with different letters are significantly different (P≤ 0.05).*

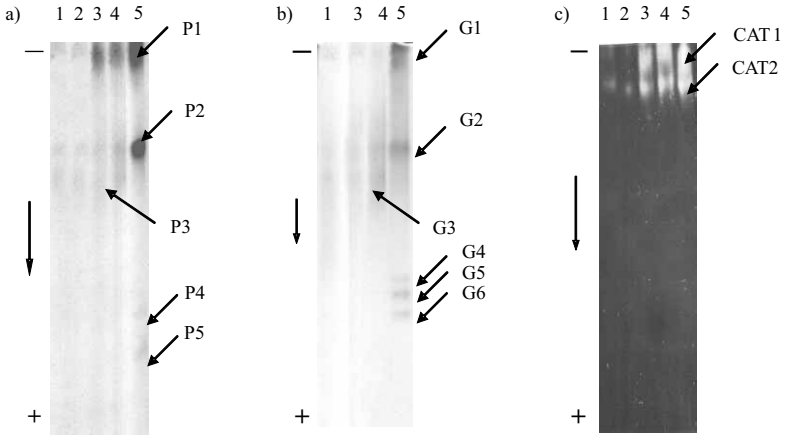

Figure 7. *Isoenzyme patterns of* Kalanchoe daigremontiana *leaf samples taken from inoculated or wounded plant on the 21st day of the experiment. P-POD isoenzymes (A); G-POD isoenzymes (B), CAT isoenzymes (C). 1 – healthy tissue, 2 – tissue next to the wound, 3 – wounded tissue, 4 – tissue next to the tumour, 5 – tumour tissue*

biosynthesis of auxine and cytokinine, which leads to tumour formation (*19*).

The activity of P-POD and G-POD in tumour tissue increased during three weeks and was significantly higher than in other samples (Figures 3, 4). A possible reason of increased POD activity in tumour tissue is exactly their role in growth and cell wall formation, the processes essential for tumour development. Moreover, POD-mediated cross-linking reactions in the cell wall (*20*) can lead to reinforcement of cell walls, thus helping to prevent further spreading of the pathogen (*21*). Electrophoretic analysis (Figures 7A and 7B) of P-POD and G-POD isoenzymes showed much resemblance. Still, we noticed higher activity of P-POD than that of G-POD, which matches with the results obtained by spectrophotometric measurement of their activity. It is also interesting that certain isoenzymes (P4 and P5, as well as G4, G5 and G6) appear only in tumour, so we can conclude that the induction of these PODs is specific for tumour tissue. In addition, isoenzyme P3, as well as G3, are visible in all samples except in tumour tissue, so there is a possibility that the synthesis of this isoenzyme is inhibited in tumour tissue. Similar results were obtained in a study of POD activity in the tissue of the cactus *Mammillaria gracilis* infected with bacteria *Agrobacterium tumefaciens*. G-POD activity in tumour tissue was 14-times higher than in untransformed tissue, which matches our results. Electrophoretic analysis of isoperoxidases revealed a higher number of isoenzymes in tumour as opposed to untransformed tissue of the plant (*22*), which is also similar to our results gained for the species *K. daigremontiana*. Jang et al (*23*) studied POD activity and expression of POD genes in sweet potato after infection with bacterial pathogen *Pectobacterium chrysanthemi*. Increased POD activity and induction of several POD genes was revealed upon bacterial infection. Moreover, increased POD was also established after fungal infection. An example is infection of sunflower with downy mildew (*24*).

We noticed that after three weeks of the experiment the wounded tissue also showed increase in the activity of

G-POD. Similar to our results, Kruzmane et al. (*25*) reported increased levels of POD at the wounding site, which revealed their involvement in repair of damaged tissue. However, the POD activity in their experiment decreased with time, as the wound healed.

Due to the role of H_2O_2 in defense response during pathogen attack and its importance in signalling pathways that enable activation of other defense mechanisms, we expected a higher quantity of H_2O_2 in infected tissue. Our results, however, did not confirm the accumulation of H_2O_2 in tumour tissue, which can be explained by increased H_2O_2 scavenging by POD.

Taking into consideration that H_2O_2 is also a CAT substrate, we traced its activity as well. It was significantly higher only in tumour tissue two weeks after inoculation (Figure 5). These results can be explained by increased degradation rate of H_2O_2 by POD, so there was no increased induction of CAT activity. Similarly, Jia *et al.* (*26*) did not notice increased levels of CAT in tumour tissue of the plant *Kalanchoe laciniata* infected by *Agrobacterium tumefaciens*. However, García-Limones *et al.* (*27*) noticed the increased activity of CAT in the plant *Cicer arietinum* infected with pathogenic fungi, and they related the increased production of reactive oxygen species with pathogen elimination or an attempt to stop its spread by cell wall reinforcement. Ślesak *et al.* (*28*) confirmed the accumulation of H_2O_2 in wounded tissue of facultative CAM plant *Mesembryanthemum crystallinum*, accompanied by decreased CAT activity.

PPO activity showed correlation with G-POD and P-POD. Activities of all three enzymes were remarkably increased in tumour tissue. It could be explained by already mentioned function of POD in the processes that are essential for tumour growth. On the other hand, the induction of PPO has been observed under conditions of ôstress and pathogen attack (*29*). Moreover, it has been proven that PPO genes are also induced by wounding (*29*), but in our experiment the PPO activity in wounded tissue and in immediate proximity of the wound was at the control level.

Although we expected a plant response to wounding and infection at the proximate distance of infection and wounding, the indicators of stress we used did not show any significant differences in respect to healthy tissue.

CONCLUSION

Remarkably increased POD and PPO activity in tumour tissue and additional isoenzymes of P-POD and G-POD indicated tumour proliferation processes and defense mechanisms against pathogen attack, in which these two groups of enzymes are involved. The wounded tissue, as well as the tissue in vicinity of the wound or infection, did not show significant variation from healthy tissue.

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