BIOLOGIA PLANTARUM 42 (4): 599-607, 1999

Improved histochemical test for *in situ* detection of hydrogen peroxide in cells undergoing oxidative burst or lignification

V. REPKA

Laboratory of Molecular Biology and Virology, CRIVE, Matúškova 25, SK-833 11 Bratislava, Slovakia

Abstract

An improved version of a simple histochemical test for the *in situ* assaying the production of hydrogen peroxide in living plant tissue was demonstrated. The test solution containing 50 mM KI in a 4 % potato starch solution was directly applied to the fresh cut surface of the tissue to be tested. Incorporation of an enhancer potassium permanganate (1 % final concentration) into the test reagent resulted in a ten times greater hydrogen peroxide mediated oxidation of iodide ions to iodine, especially in the case when, *e.g.* suboptimal concentration of H_2O_2 is present or endogenous catalase decomposes the H_2O_2 in tissue as quickly as it is evolved. Subsequently, iodine is complexed by the starch to form a coloured product. H_2O_2 production by wound-induced oxidative burst or lignification can be easily discriminated due to the dual colour response.

Additional key words: *Cucumis*, *Kalanchoe*, lignin, *Nicotiana*, peroxidase, starch/KI reagent, wounding.

Introduction

Reactive oxygen species (ROS) resulting from many metabolic processes have been implicated in numerous developmental and adaptive responses in both animal and plant cells (Dypbukt *et al.* 1994, de Marco and Roubelakis-Angelakis 1996, Lamb and Dixon 1997). Among them, the hydrogen peroxide which performs a plethora of phytochemical roles (Elstner 1987) is of particular interest. In this respect much attention has been given to two H_2O_2 associated events, particularly the lignification and defense-related oxidative burst.

Received 18 January 1999, *accepted* 15 July 1999.

Abbreviations: CAT - catalase, HR - hypersensitive reaction, PRX - peroxidase, ROS - reactive oxygen species.

Acknowledgement: The skilful and dependable assistance of Mrs. Irma Fischerová is gratefully acknowledged.

Fax: (+421) 754 775436, e-mail: repka@crive.axonpro.sk

Lignification starting with a phenylalanine ammonia-lyase catalyzed reaction to produce lignin precursor alcohols, and terminating with oxidative coupling of lignin monomers in a process that requires H_2O_2 and cell-wall-bound peroxidase (Gross *et al.* 1977). In addition of direct antimicrobial activity of hydrogen peroxide (Peng and Kuc 1992), a novel important roles of H_2O_2 from the oxidative burst have recently been postulated. Bradley *et al.* (1992) reported that H_2O_2 produced during the oxidative burst cross-links a set of soluble proteins to the plant cell wall and thus reinforces it against pathogen penetration. The production of H_2O_2 has been noted when plants are challenged either with particular microbial pathogen (Mehdy 1994, Bollwell and Wojtazsek 1998) or isolated microbial elicitor (Apostol *et al.* 1989, Lamb and Dixon 1997). Moreover, likewise animal apoptosis (Raff *et al.* 1993), H_2O_2 from the oxidative burst also participates in programmed cell death, *i.e.* hypersensitive reaction (HR) in plants (Greenberg *et al.*1994) and triggers defense response in adjacent cells around the vicinity of the site of infection (Dietrich *et al.* 1994). More importantly, Levine *et al.* (1994) clearly showed that H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response and induces transcription of genes involved in systemic acquired resistance (Low and Merida 1996)

Further and more precise studies of above mentioned phenomena require a simple and sensitive *in situ* histochemical tests for hydrogen peroxide. Despite diverse and very important roles proposed for H_2O_2 in plants, histochemical applications of the organic analysis spot test for peroxides (Emich and Feigl 1932) have been rare, other than those that include the addition of polyamine oxidase catalyzed reactions (Smith 1970, Kaur Sawhney *et al.* 1981, Scorer *et al.* 1989). A different histochemical assay for H_2O_2 depended on endogenous peroxidase and the addition of an exogenous peroxidase substrate (*e.g.* guaiacol, Goldberg *et al.* 1985), as well as on the conditions activating that enzyme. Alternatively, H_2O_2 production was assessed cytochemically via determination of cerium perhydroxide formation after reaction of $CeCl_3$ with endogenous H_2O_2 (Bestwick *et al.* 1997). Unfortunately, to date only two studies examined fresh, undigested tissue (Angelini *et al.* 1990, Olson and Varner 1993). The principle of the later very simple histochemical test for H_2O_2 is based on the peroxide-mediated oxidation of iodide ions to iodine followed by complexation with the starch. Despite the simplicity of this assay, there are some drawbacks in its application. As suggested by the authors and experienced empirically by us, under specific circumstances, *e.g.* high rate of H_2O_2 turnover, suboptimal concentration of hydrogen peroxide or presence of strong endogenous reductants, we frequently failed in H_2O_2 detection when using this assay. Therefore, to overcome these problems and enhance the sensitivity of the test we have adopted the principle of manganometry as a sensitive analytical tool to stain of H_2O_2 using potassium permanganate. In this report we apply the improved reagent to fresh hand-cut sections of stem tissue in order to examine and verify H_2O_2 production in relation to the process of lignification and in the course of oxidative burst induced by wounding.

Materials and methods

Plants: Tobacco (*Nicotiana tabacum* cv. White Burley) and cucumber (*Cucumis sativus* L. *cv.* Laura) were grown from surface sterilized seeds for two months in sterilized poting compost mix in a glasshouse (temperature 20 - 32 °C). *Kalanchoe daigremontiana* L. plants were collected from the greenhouse at CRIVE campus. Plants were watered to saturation daily and fertilized weekly with *Substral* (*Henkel AS*, Bratislava, Slovakia).

Wounding: In order to study the association of the oxidative burst-induced H_2O_2 with the wounding response several layers of cells were crushed using the unsharpened edge of a razor blade in some fresh hand-cut sections from tobacco stem. The region of crushed tissue extended from the epidermis into the pith parenchyma. Comparative observations were made between crushed and unwounded cells in respect of the rate of H_2O_2 production. The effect of exogenously added catalase and the presence of reductant (100 mM ascorbic acid) were tested on wounded tissue using the protocol described below.

Lignin staining: Lignin was stained histochemically either with 2 % phloroglucinol (*Sigma*, Deisenhofen, Germany) in 30 % HCl (Jensen 1962) or with Maule reagent (1 % $KMnO_4$ followed by destaining in 30 % HCl) according to standard protocol (Srivastava 1966). Staining for lignin was done either on fresh tissue sections or on the same section on which H_2O_2 has been assayed before. In the later case, acidified phloroglucinol was applied on the section within 5 min when the starch-I_2 complex decolorized under acidic conditions spontaneously.

Peroxidase localization: Peroxidases were localized in fresh-cut tissue sections by adding a few drops of *p*-phenylenediamine solution (20 mM in sodium acetate buffer pH 5.2 containing 0.03 % H_2O_2) to the section.

Peroxide localization: The presence of H_2O_2 in living tissue was assayed by two different methods: *1*) method of Olson and Varner was performed exactly as described in published protocol (Olson and Varner 1993), and *2*) improved method. In the later case fresh hand-cut sections were first incubated for 2 min in aqueous solution of potato starch (4 % m/v) and potassium iodide (100 mM). Tissue sections were transferred for 10 s to an enhancer solution containing 1 % $KMnO_4$ and then to 30 % HCl. Localization of H_2O_2 is indicated by the development of dark starch-I_2 complexes on the cut surface. Several control were used to evaluate the reproducibility and sensitivity of this improved H_2O_2 assay. Aliquots (0.01 cm^{-3}) of catalase [from bovine liver, 105 mg protein cm^{-3}, 30 000 U mg^{-1}, 2 × crystalline suspension in water containing 0.1 % thymol (*Sigma*, Deisenhofen, Germany)] were mixed in separate 0.1 cm^{-3} aliquots of starch-KI reagent and applied to different tissue sections. Alternatively, the sections were coated either with starch-KI reagent alone or reagent containing various concentrations of ascorbic acid (10 - 100 mM, *Duchefa Biochemie*, Haarlem, The Netherlands) and observed for indications of H_2O_2 production. Control sections in water were also prepared for comparison.

Rate of H_2O_2 accumulation: The rates of accumulation of H_2O_2 in living tissue were evaluated by visual comparison of colour intensity with known concentration of hydrogen peroxide spotted on filter paper. Aliquots (2 - 5 mm^3) of H_2O_2, ranging in concentration from 5 - 100 mM were spotted using *Slot-Blot Apparatus* (*Hoefer Scientific Instruments*, San Francisco, CA, USA) on to *Whatman* No. 3 filter paper. A thin film of starch-KI reagent was spread over these. Intensity of colour was compared with that in fresh tissue sections.

Photodocumentation and anatomy comparison: Tissue section screening and sequential analysis of H_2O_2 production were documented using *Leica Stereomicroscope MZ12* (*Leica AG*, Heerbrugg, Switzerland) equipped with the Trinocular video/phototube and colour CCD camera. Scanings were analysed using *Adobe Photoshop* software (*Adobe Systems Inc.*, San Jose, USA). Hardcopy prints were produced on *Mitsubishi* videoprinter device (*Mitsubishi Corp.*, Tokyo, Japan). For the observation of tissue anatomy, the fresh hand-cut sections were stained with 0.025 % toluidine blue and photographed in bright field as described above.

Results

Oxidative burst-induced accumulation of H_2O_2 during wounding response: In our initial experiments we examined the effects of wounding on the accumulation of H_2O_2 in fresh hand-cut sections. In the presence of the enhancer (1 % $KMnO_4$)

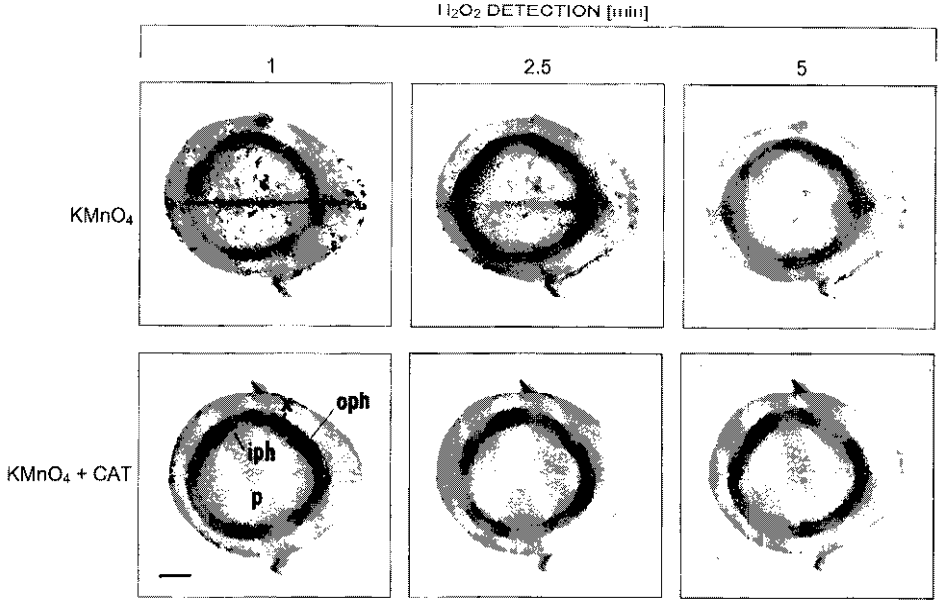

Fig. 1. Cross-sections of tobacco stems wounded with the blunt end of a razor blade, and stained for indicated periods either with 1 % $KMnO_4$ alone or with added catalase (x - xylem, oph - outer phloem, iph - inner phloem, p - pith parenchyma; *bar* - 300 μm).

a highly localized oxidative burst occurred in the zone of damaged cells from the epidermis into the pith parenchyma (Fig. 1). The signal persisted on the section at least for 2.5 min and disappeared almost completely within 5 min. With an excess of exogenous catalase present the dramatic oxidation of the starch-KI reagent on wounded tissue did not occur. Interestingly, staining in the lumen of epidermal and pith parenchyma cells also appeared randomly on tissue sections, perhaps due to localized damage of cell during sectioning. Likewise mentioned above, this signal disappeared spontaneously with time and was, even though in excess, catalase sensitive. Comparing the rates of H_2O_2 accumulation in damaged tissue in the presence of $KMnO_4$ enhancer, the response was roughly ten times greater than the oxidative burst seen on wounded sections in starch-KI reagent without $KMnO_4$ (data not shown).

Beside the wound-induced oxidative burst, the starch-KI reagent containing an enhancer showed a sharply localized darkening in phloem and xylem elements undergoing lignification (Fig. 1). Importantly, in the presence of $KMnO_4$, discrimination between oxidative burst-induced and lignification-associated H_2O_2 accumulation is very simple due to the dual colour response. While zones of crushed cells with dramatic oxidative burst and H_2O_2 accumulation stain purple-brown, the zones accumulating H_2O_2 associated with lignification stain dark blue-black. In this

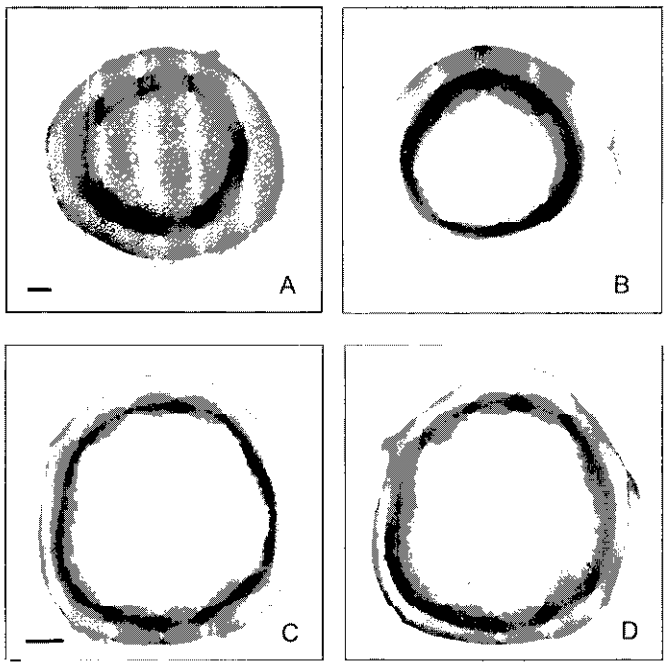

Fig. 2. Cross-sections of tobacco stem: *A* - control section stained with 0.025 % toluidine blue to show anatomy, *B* - section stained with 2 % phloroglucinol to show lignin, *C* - section stained with *p*-phenylenediamine to localize endogenous peroxidase, and *D* - section stained with $KMnO_4$ enhancer reagent showing H_2O_2 production in xylem and phloem. The figures are oriented so that in every case the side of the stem cross-section which subtends the leaf is up (*bars* - 300 μm in all sections).

context it is important to note that H_2O_2-associated signal in lignified components disappeared more slowly than that observed in oxidative burst and was complete with 10 -15 min.

H_2O_2 detection and lignification: More detailed study of lignification-asssociated H_2O_2 accumulation using improved reagent was made with living tissue. Toluidine blue and phloroglucinol defined lignified sectors of phloem and xylem in tobacco stem sections (Fig. 2*A*,*B*). Staining of tissue sections for peroxidase and H_2O_2 with *p*-phenylenediamine and enhancer reagent, respectively, was strongly correlative with those for lignin. On sections stained with *p*-phenylenediamine (Fig. 2*C*) the most intense signal was localized in primary xylem and in a lower extent in outer and inner phloem. In contrast, the signal for H_2O_2 accumulation reached almost the same intensity in both primary xylem and phloem fibres (Fig. 2*D*). Demonstrated variability in staining either for PRX or H_2O_2 appears to be due to the rapid diffusion of the later.

In accordance with the range of responses evaluation the accumulation of H_2O_2 in living tissue using quantitative spot test, an extreme sensitivity of enhancer containing reagent could be helpful to evaluate the rate of H_2O_2 accumulation directly on tissue sections. This statement was demonstrated using three representative plant species (Fig. 3). In *Kalanchoe*, representing the lowest H_2O_2 accumulation rate observed, the presence of H_2O_2 can be estimated directly on the cellular level. On the other hand, among the plant tested in this study, the cucumber stem produced enormous amount of H_2O_2 concentrated tightly around the lignified vascular bundles.

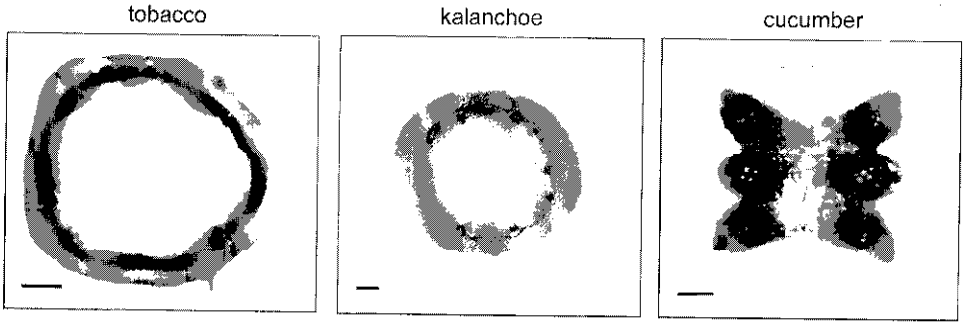

Fig. 3. Cross-sections of tobacco, kalanchoe, and cucumber stems showing a species specific patterns of H_2O_2 production associated with lignification. Sections were stained with improved, an enhancer containing, reagent and photographed within 2 min after the reagent was added to the section (*bars* - 500 μm in all sections).

Discussion

The histochemical test for H_2O_2 using starch-KI reagent developed by Olson and Varner (1993) can serve as a simple method for localizing zones ongoing

lignification or wound-induced oxidative burst. Indeed, we frequently failed in reproducible detection of H_2O_2 in living tissue, especially if strong endogenous reductants like ascorbic acid or if high endogenous catalase activity were present. Therefore, to encounter these difficulties, we have substantially modified and optimized the basic principle by this way that the specific enhancer present in the test reagent more effectively accelerates the iodide oxidation. Incorporation of $KMnO_4$ (1 % final concentration), which is a key component of the sensitive manganometric test for H_2O_2, into the starch-KI reagent under acidic conditions accelerated the speed of response roughly ten times when compared with that without $KMnO_4$. Interestingly, other potential accelerators exist, even if operating via different principle. The malate (10 mM) in 50 mM glutamate buffer (pH 5) was tested in this context (Olson and Varner 1993). They demonstrated that malate accelerated the oxidative burst associated with wounding and this response was roughly three times greater than the oxidative burst in reagent without malate. Since malate had no effect on H_2O_2 production in zones of lignification of fresh unwounded tissue this finding is especially interesting in light of the theory outlined by Gross *et al.* (1977) that suggest that malate dehydrogenase contributes to production of H_2O_2 via providing NADH for the reduction of O_2.

Alternatively, since H_2O_2 detection using $KMnO_4$ enhancer was performed in 30 % HCl, it is possible that highly acidic conditions may equally contribute to accelerated response. Two independent observations strongly support this suggestion. Firstly, we have demonstrated (data not shown) that lowering the concentration of HCl slow-down the speed and sensitivity of response in relation to signal intensity. Secondly, Olson and Varner (1993) have reported that at pH 4 or lower, with or without malate, H_2O_2 appears more rapidly throughout the tissue section. Unfortunately, at the present it is not known whether highly acidic conditions actually increase H_2O_2 production or somehow impede normal cell processes that continuously eliminate H_2O_2. In this connection we note that *in vitro* ascorbate is a reversible inhibitor of catalase (Davison *et al.* 1986).

An additional and very important benefit of the improved method that overbalance the former one is the double colour response in the detection of H_2O_2. At the present the double colour reaction in the presence of $KMnO_4$ is difficult to interpret and more experiments will be needed to understand this phenomenon. Several preliminary observations indicate that this response probably could result from two different scenarios. On the one hand, different colours may reflect the actual cellular redox potential. On the other hand, colour discrimination could also reflects a local pH shifts in cells undergoing either wound-induced oxidative burst or lignification. Although a combination of both scenarios is also a possibility, taking into account the principle of manganometry we anticipate that the first discussed scenario may be the case.

Temporal disappearance of H_2O_2 positive signal in lignified compartment greatly differed from that typical for signal accompanied with wound-induced oxidative burst. Observed delaying effect, as well as the variability in staining appears to be due by the enhancer ($KMnO_4$) alone. Indeed, 1 % $KMnO_4$ is a key element in the Maule stain which is known to react with free syringil units in lignin. Thus, $KMnO_4$

present in the starch-KI reagent probably mimics the Maule stain for lignin residues. This suggestion is strongly supported by the finding (Fig. 1), where 5 min after addition of enhancer reagent the wound-induced signal almost completely disappeared excluding the portions of lignified region (xylem and phloem zone). Time-dependent disappearance of the signal in this region corresponded to that typical for lignification.

A further point of interest of improved method presented in this work is the possibility of the use the same tissue section for subsequent histochemical analysis, *e.g.* H_2O_2 assay followed by lignin staining. Spontaneous disappearance of the H_2O_2 localization signal over a relatively short period of time eliminates the problem associated with the former method. Unlike to our improved test which enables the staining of lignin using acidified phloroglucinol almost immediately after the H_2O_2 stain was done, in the former method of Olson and Varner the starch-I_2, KI must be removed and/or reduced to colourless starch-KI with 100 mM ascorbic acid before applying the acidified phloroglucinol. Thus, eliminating this step from the procedure could save the time especially if so many tissue sections must be processed simultaneously.

Taken together, we hope that our method improves the application of this simple histochemical test for H_2O_2 especially in the cases that meet the major drawbacks of the previous one. Additionally, use of the assay do not provide only evidence of the role of H_2O_2 in wound response and lignification but also could illuminate other areas of plant biology in which H_2O_2 is believed to play a potential role.

References

Angelini, R., Manos, F., Federico, R.: Spatial and functional correlation between diamineoxidase and peroxidase activities and their dependence upon de-etiolation and wounding in chick-pea stems. - Planta **182**: 89-96, 1990.

Apostol, I., Heinstein, P.F., Low, P.S.: Rapid stimulation of an oxidative burst during elicitation of cultured plant cells. - Plant Physiol. **90**: 109-116, 1989.

Bestwick, C.S., Brown, I.R., Bennett, M.H., Mansfield, J.W.: Localization of hydrogen peroxide accumulation during the hypersensitive reaction of lettuce cells to *Pseudomonas syringae* pv. *phaseolicola*. - Plant Cell **9**: 209-221, 1997.

Bolwell, G.P., Wojtazsek, P.: Mechanisms for the generation of reactive oxygen species in plant defence: a broad perspective. - Physiol. mol. Plant Pathol. **50**: 378-388, 1998.

Bradley, D.J., Kjellbom, P., Lamb, C.J.: Elicitor- and wound-induced oxidative cross-linking of a proline-rich plant cell-wall protein: a novel rapid defense response. - Cell **70**: 21-30, 1992.

Davison, A.J., Kettle, A.J., Fatur, D.J.: Mechanism of the inhibition of catalase by ascorbate. - J. biol. Chem. **261**: 1193-1200, 1986.

De Marco, A., Roubelakis-Angelakis, K.A.: The complexity of enzymatic control of hydrogen peroxide concentration may affect the regeneration potential of plant protoplast. - Plant Physiol. **110**: 137-145, 1996.

Dietrich, R.A., Delaney, T.P., Uknes, S.J., Ward, R., Ryals, J.A., Dangl, J.L.: *Arabidopsis* mutants simulating disease resistance response. - Cell **77**: 565-577, 1994.

Dypbukt, J.M., Ankarcona, M., Burkitt, M., Sjoholm, A., Strom, K., Orrenius, S., Nicotera, P.: Different pro-oxidant levels stimulate growth, trigger apoptosis or produce necrosis of insulin secreting RINm5F cells. - J. biol. Chem. **269**: 30553-30560, 1994.

Elstner, E.F.: Metabolism of activated oxygen species. - In: Stumpf, P.K., Corm, E.E. (ed.): The Biochemistry of Plants. Vol. 11. Pp. 253-317. Academic Press, San Diego 1987.

Emich, F., Feigl, F.: Microchemical Laboratory Manual. - Wiley and Sons, Chichester - New York 1932.

Goldberg, R., Thoan, L., Catesson, A.M.: Localization and properties of cell wall enzyme activities related to the final stages of lignin biosynthesis. - J. exp. Bot. **36**: 503-510, 1985.

Greenberg, J.T., Guo, A., Klessig, D.F., Ausubel, F.M.: Programmed cell death in plants: a pathogen-triggerred response activated coordinately with multiple defense functions. - Cell 77: 551-563, 1994.

Gross, G.G., Janse, C., Elstner, E.F.: Involvement of malate, monophenols, and the superoxide radical in H_2O_2 formation by isolated cell walls from horseradish (*Armoratia lapathifolia* Gilib.). - Planta **136**: 271-276, 1977.

Jensen, W.A.: Botanical Histochemistry. - W.H. Freeman and Co., San Francisco 1962.

Kaur-Sawhney, R., Flores, M.E., Galston, A.W.: Polyamine oxidase in oat leaves - a cell wall localized enzyme. - Plant Physiol. **68**: 194-498, 1981.

Lamb, C.J., Dixon, R.A.: The oxidative burst in plant disease resistance. - Annu. Rev. Plant Physiol. Plant mol. Biol. **48**: 251-275, 1997.

Levine, A., Tenhaken, R., Dixon, R., Lamb, C.: H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. - Cell **79**: 583-593, 1994.

Low, P.S., Merida, J.R.: The oxidative burst in plant defense: function and signal transduction. - Physiol. Plant. **96**: 533-542, 1996.

Mehdy, M.C.: Active oxygen species in plant defense against pathogens. - Plant Physiol. **105**: 467-472, 1994.

Olson, P.D., Varner, J.E.: Hydrogen peroxide and lignification. - Plant J. **4**: 887-892, 1993.

Peng, M., Kuc, J.: Peroxidase-generated hydrogen peroxide as a source of antifungal activity *in vitro* and on tobacco leaf discs. - Phytopathology **82**: 696-699, 1992.

Raff, M.C., Barres, B.A., Burne, J.F., Coles, H.S., Ishizaki, Y., Jacobson, M.D.: Programmed cell death and the control of cell survival: lessons from the nervous system. - Science **262**: 695-700, 1993.

Scorer, K.N., Caamal, R.M., Oropeza, C., Loyola-Varagas, V.M.: Amine oxidase activity in *Canavalia ensiformis* during early growth: a histochemical study. - J. Plant Physiol. **135**: 346-350, 1989.

Smith, T.A.: Polyamine oxidase in higher plants. - Biochem. biophys. Res. Commun. **41**: 1452-1456, 1970.

Srivastava, L.M.: Histochemical studies on lignin. - Tappi **49**: 173-183, 1966.