Acta Bot. Croat. 41, 33—39, 1982.

CODEN: ABCRA2
YU ISSN 0365—0588

UDC 582.982:577.158.52=20

ISOPEROXIDASES — EARLY INDICATORS OF SOMATIC EMBRYOID DIFFERENTIATION IN PUMPKIN TISSUE

MARIJANA KRSNIK-RASOL, SIBILA JELASKA* and DRAŠKO ŠERMAN***

(*Department of Botany, Faculty of Science and
**Department of Biology, Medical Faculty, University of Zagreb)

Received October 15, 1981

Total peroxidase activity was determined in several callus lines of the pumpkin and electrophoretic patterns of their isoenzymes were obtained. The highest enzyme activity was determined in the callus lines DE and NA_{∞}, which grow in the presence of growth regulators and show high capacity for embryoid formation. Peroxidase activity is considerably lower in the habituated tissue (line $Z_{5b/T}$) but the increase in enzyme activity was observed prior to embryoid appearance. Various callus lines differ from one another with the respect to their electrophoretic isoenzyme patterns. Although these electrophoretic patterns are influenced by growth regulators, the patterns still reflect the developmental stage of the culture.

Introduction

The enzyme peroxidase catalizes the oxidation of a variety of substrates during which the reduction of hydrogen peroxide takes place (Betz 1974). It is well known that many enzymes appear in multiple molecular forms, like isoenzymes. Most of these varieties have a genetic basis, which makes isoenzymes ideal natural indicators in biological research (Scandalios and Sorenson 1977). Isoperoxidases represent the enzymatic complex involved in the metabolism of auxins and the synthesis of lignin. Their role in the regulation of growth and differentiation is also known (Siegel and Galston 1967, Wolter and Gordon 1975, Kochba et al. 1977, Thorpe et al. 1978a, b, Rücker and Markotai 1978, van Huystee 1980).

The second author developed a number of pumpkin callus lines which display their rich morphogenetic potentials, above all a high capacity for the genesis of somatic embryoids (Jelaska 1972, 1974, 1977, 1980, Jelenčić 1980). In the present work we have tried to find suitable indicators of metabolic changes which precede the genesis of somatic embryoids, by using the system of peroxidase isoenzymes. We have also tried to detect the differences among the callus lines considering both enzyme activity and the electrophoretic isoenzyme patterns.

Material and Methods

In our experiments the following lines of the pumpkin callus tissue were used: DE, DE_4, NA_3, $Ž_{5b/T}$. In contrast to the original cultures which had been grown on semisolid medium, in these experiments the tissues were grown on agitated liquid MS—medium (Murashige and Skoog 1962), with the addition of plant hormones (lines DE, DE_4, NA_3), or without them as the habituated tissue (line $Ž_{5b/T}$). Under our culture conditions (12 hours light, 3000 Lux, 25° C, shaking frequency of 90 r. p. m.) each passage lasted 8 days.

For enzyme extraction Tris buffer (phosphoric acid 0.26 mol with respect to Tris), pH 6.9 was used. The volume of 2 ml buffer was used for 0.2 g of tissue. The tissue was homogenized by hand in a mortar with the addition of up to 5 % polyvinylpyrolidone. The whole procedure was carried out at temperatures of 273—277 K (0°— 4° C). The homogenate was centrifuged for 20 minutes at 18000 g, and the supernatant was used as crude extract.

The peroxidase activity was measured with the test solution prepared after Siegel and Galstone (1967). For each 3 ml of this solution 0.01 ml of extract was added, which was previously diluted in the case of too high activity. Absorption was measured by a spectrophotometer (Perkin Elmer 550), at 470 nm. Enzyme activity is expressed as possible absorption (A) per minute per 1 g of fresh tissue, or as specific activity in relation to the content of soluble proteins per 1 g of fresh tissue. Soluble proteins were quantitatively determined in extracts by the method of Bradford (1976).

The polyacrylamide gel system No. 1 was used for electrophoretic separation towards the anode, and No. 5 for separation towards the cathode (Maurer 1968).

The gels were stained by enzymatic reaction in the test solution already mentioned ($5 \cdot 10^{-6}$ mol guayacol, $5 \cdot 10^{-6}$ mol H_2O_2 in $2 \cdot 10^{-1}$ mol phosphate buffer, pH 5.8).

Results

Our experiments show that the callus lines studied differ somewhat among themselves in their content of soluble proteins (Table 1). These differences, however, vary only within the narrow range of 2 mg per gram of fresh tissue. Repeated measurements of peroxidase activity indicate differences among the lines studied. The highest enzyme activity was found in lines DE and NA_3, which at the same time displayed the most pronounced capacity for embryogenesis, and grew in the presence of plant hormones. The enzyme activity was significantly lower, however, in the embryoids isolated from the callus line DE (Tab. 1).

Table 1. Soluble protein content and peroxidase activity in pumpkin callus lines DE_1 - isolated embryoids, DE_2 - callus, $\bar{Z}_{5b/T^1}$ - nonembryogenic culture, $\check{Z}_{5b/T^2}$ embryogenic culture

Callus line	Growth regulator	Protein content (mg · g^{-1} fr. weight) a	Peroxydase activity (A_{470} min^{-1} · · g^{-1}) b	Specific peroxidase activity b/a
DE_4	2,4—D ($1.35 \cdot 10^{-6}$ mol)	8.0	592.0	74.0
DE_1	2,4—D ($4.5 \cdot 10^{-6}$ mol)	7.2	2016.0	280.0
DE_2	"	8.1	432.0	53.3
NA_3	NAA ($5.37 \cdot 10^{-6}$ mol) adenin sulfat (10^{-6} mol)	6.2	1720.0	277.4
$\bar{Z}_{5b/T^1}$	0	6.8	98.7	14.51
$\check{Z}_{5b/T^2}$	0	7.1	296.0	41.66

The habituated tissue showed the lowest enzyme activity. This tissue had a slightly lowered potential for embryogenesis under conditions of our experiments. We were unable to determine which external factor stimulated embryogenesis, but the increase in peroxidase activity was determined immediately prior to the appearance of embryoids (Table 1).

Electrophoretic patterns of peroxidase isoenzymes show differences among callus lines. The line DE_4 (Fig. 1 a) is characterized by the well developed capacity for embryogenesis, but their embryoids are mostly restricted in the initial stages of development. The electrophoretic pattern of peroxidase isoenzymes shows three bands in the anodic group and two bands in the cathodic group of isoenzymes (Fig. 2 b i c).

The line DE (8 year old culture, Jelaska 1980) is morphologically somewhat different from DE_4 (2 year old culture), although it grows also in the presence of 2,4-D, but at its higher concentration (Fig. 2 a). This is a very loose tissue showing many separated cells dispersed in the growth medium. The appearing embryoids develop from the initial globular stage to a heartlike one, and finally to the torpedo stage (Fig. 3 a). In this line three bands were present in the anodic group of isoenzymes, and only one with slow relative mobility in the cathodic group (Fig. 2 b i c). The isoenzyme pattern of the isolated embryoids is slightly different (Fig. 3 b i c): one of the two anodic groups moves relatively faster, which happens also with the only band in the cathodic group of isoenzymes.

The line NA_3 (Fig. 4 a) shows a very high embryogenic potential, so that the culture consists mainly of embryoids in all the different developmental stages, of malformed plantlets, and of relatively scarce undifferentiated tissue. In spite of the very high enzyme activity detected, only two faint isoenzyme groups are seen on the anodic side (Fig. 4 b), and only one with slow mobility on the cathodic side. This isoenzyme group is probably responsible for most of the enzyme activity (Fig. 4 c).

The callus line $\bar{Z}_{5b/T}$ (grown on auxin free medium) forms very compact tissue aggregates, and there are no loose cells dispersed in the

nutrient medium (Fig. 5 a). Some cultures within this line display a reduced potential for embryogenesis which is accompanied by some morphological changes (the compactness of the tissue is reduced, Fig. 6 a). So we could within the same line differentiate embryogenic from the nonembryogenic cultures. These two types of culture differ in their electrophoretic patterns of peroxidase isoenzymes. The anodic group of isoenzymes displays four bands in the nonembryogenic callus (Fig. 5 b), while in the embryogenic callus this isoenzyme group displays five bands (Fig 6 b). Differences between the cathodic groups of isoenzymes are even more pronounced, so that in the embryogenic cultures there are four bands, and in the nonembryogenic one there are only two strong bands and a faint one (Figs. 5 c and 6c).

Discussion

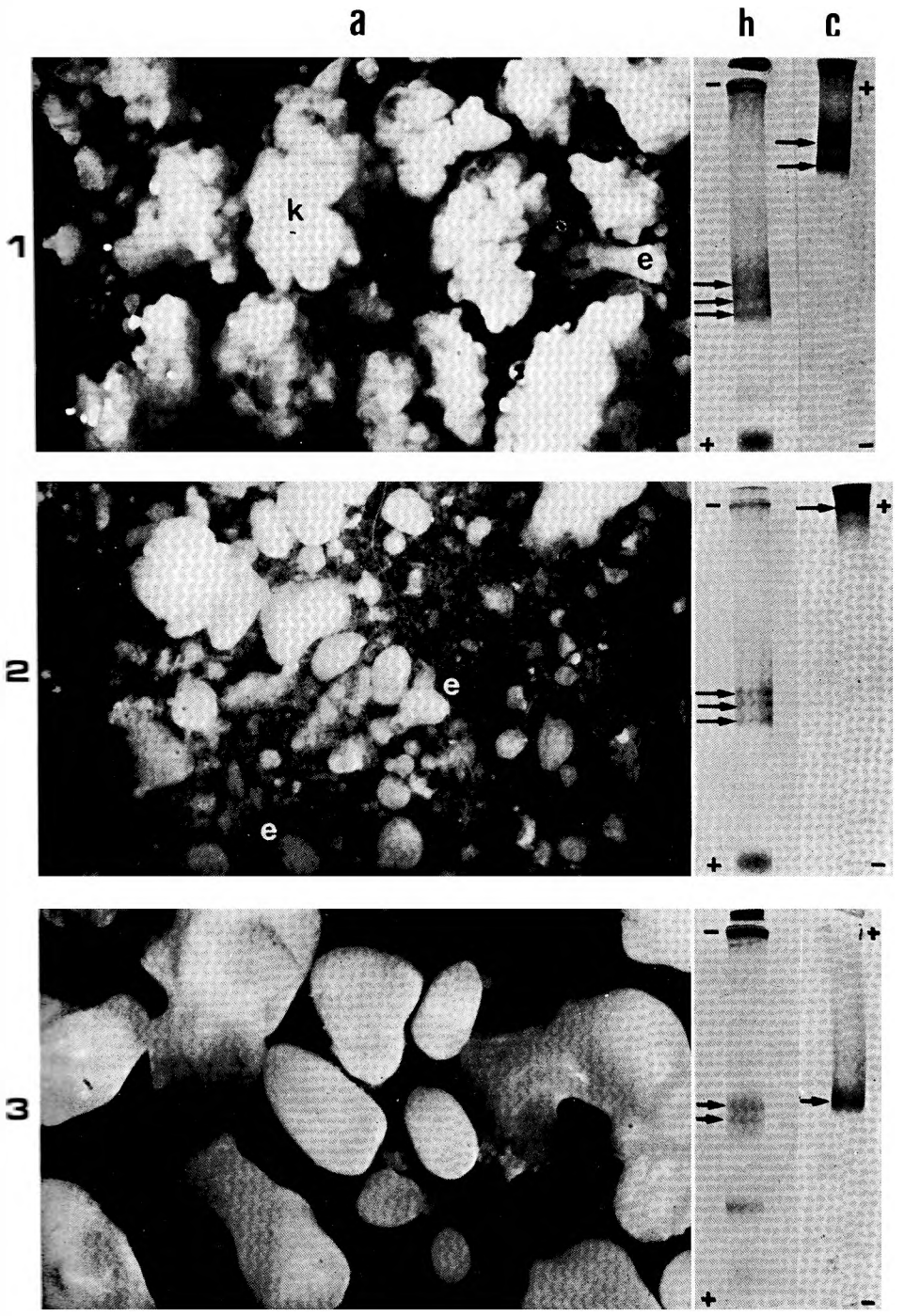
Considering a fact that significantly lower enzyme activity of peroxidase was detected in embryoids isolated from the DE line (specific activity 53.3) as compared to the callus tissue of the same line (specific activity 280.0) we can conclude that high enzyme activity is characteristic of the very undifferentiated callus, in which embryoids are to be developed only in the next developmental step. Low peroxidase activity was also found in embryos isolated from seeds of wheat (Gaspar et al. 1977) and in pine embryos (*Pinus nigra*, our preliminary results). It is known that 2,4-D influences the activity and the pattern of peroxidase isoenzymes as well as that of the IAA oxidases. That influence is dependent on 2,4-D concentration (de Fooz et al. 1966, Lee 1972 a, b, Paul and Öhrman 1980). The differences between the DE_1 and DE callus lines may be explained in a similar way.

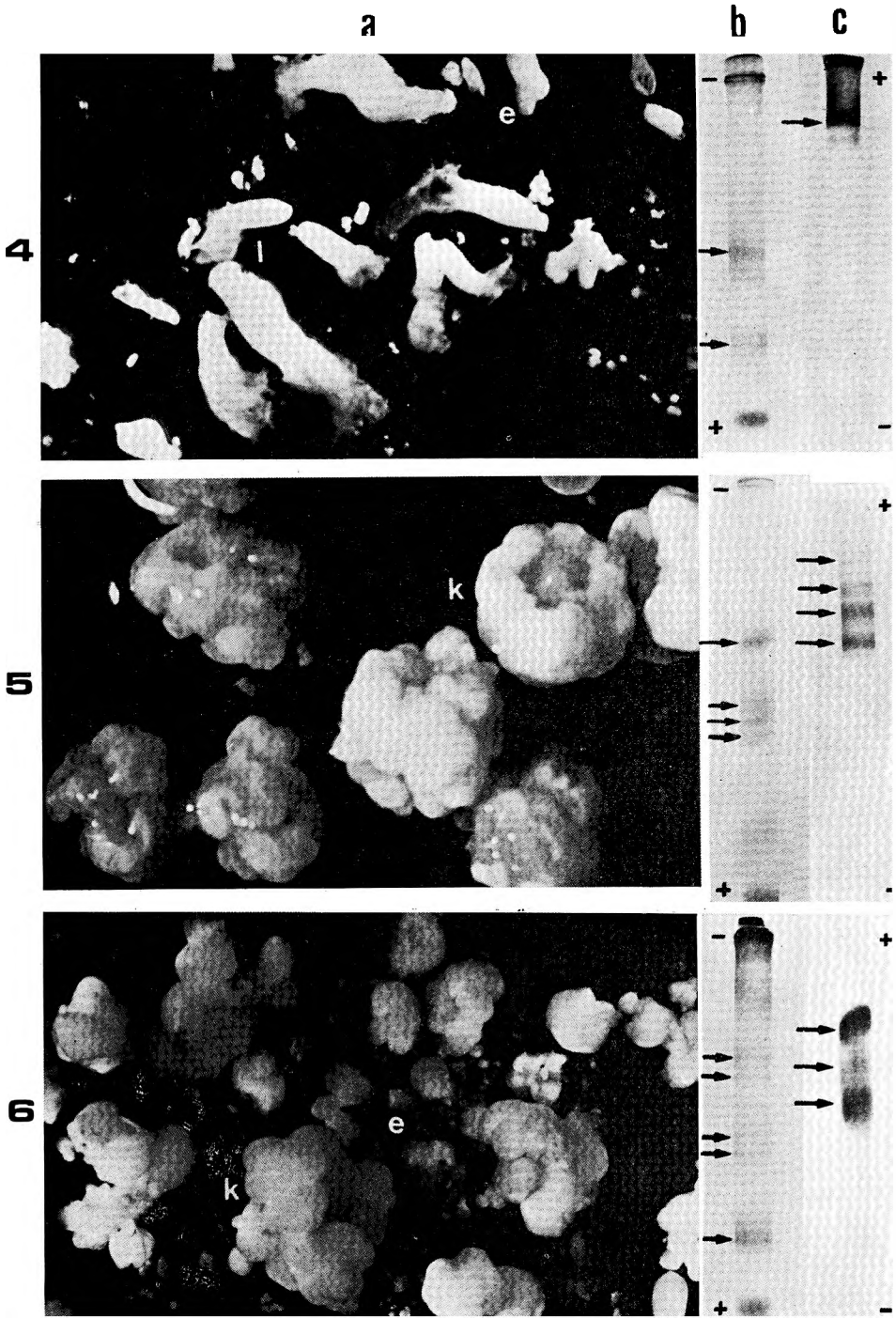
In the habituated tissue the rise in enzyme activity preceded the appearance of embryoids in the culture. This finding is in full agreement with Kochba's results for *Citrus sinensis* (Kochba et al. 1977).

Although the physiological role of peroxidases is not completely understood, because of the great number of substrates upon which they act, there is a number of papers which point out the importance of peroxidase isoenzymes as indicators of metabolic changes in tissues undergoing differentiation, during stress, or under the conditions of exogenously applied hormones (Thorpe and Gaspar 1978, Sheoran and Garg 1979, Thomas and Delincee 1979, Cairns et al. 1980). On the basis of our experiments we are aware that callus lines of pumpkin can produce the pattern of peroxidase isoenzymes,

Fig. 1.—6. Pumpkin callus tissue and electrophoretic isoperoxidase pattern (b — separation towards anode, c — separation towards cathode).

1.a — Callus line DE_1. Undifferentiated callus clumps and embryoids.
2.a — Line DE with embryoids in globular and heart stages.
3.a — From line DE isolated embryoids in different developmental stages.
4.a — Line NA_3. Embryoids with green leaf-like cotyledones.
5.a — Line $Ž_{3b/T}$. Typical compact tissue clumps.
6.a — The same line as 5.a with embryoids in early developmental stage. (k = callus, e = embryoids)


a
b
c
1
k
e
2
e
e
3
−
+
+
−


a
b
c
4
e
l
5
k
6
e
k

which — in spite of not being so clear and so rich as the pattern found in the tissue of tobacco plant (Mäder and Bopp 1976, Mäder 1976, Nessel and Mäder 1977, Mäder et al. 1980) — can be used as indicators of the changes taking place in the tissue undergoing differentiation. We have noticed that exogenously applied growth regulators influence the peroxidase isoenzyme pattern. This phenomenon is not surprising because the response of a tissue to added plant hormones could be effected through synthesis of RNA and proteins, including enzymes.

The differences in isoenzyme patterns observed between the embryogenic and nonembryogenic cultures, within the same line of habituated tissue ($\check{Z}_{5h/T}$) are in favour of the fact that peroxidases play a role in the metabolism of endogenous hormones (Raa 1971, Elkinawy and Raa 1973).

Conclusion

On the basis of our experiments the following conclusions may be drawn:

1. High enzyme activity in cultures (DE and NA_3) is correlated with the incidence of embryogenesis.

2. The increase in enzyme activity measured at an as yet undifferentiated stage of culture, with no visible phenotypic changes whatsoever, predicts the appearance of embryoids in the habituated tissue.

3. There are differences among the callus lines with respect to their electrophoretic isoenzyme patterns. Growth regulators influence the isoenzyme pattern, but this pattern is also reflection of the specific developmental stage of the culture.

This research was sponsored by the Science Research Council of SR Croatia (SIZ-IV).

References

Betz, A., 1974: Enzyme. Gewinnung-Analyse-Regulation. Verlag Chemie GmbH, Weinheim.

Bradford, M. M., 1976: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. *72*, 248—254.

Cairns, E., R. B. van Huystee, W. L. Cairns, 1980: Peanut and horse radish peroxidase isoenzymes. Intraspecies and interspecies immunological relatedness. Physiol. Plant *49*, 78—82.

Elkinawy, M., J. Raa, 1973: Levels of indol-3yl-acetic acid (IAA), IAA-oxidase and peroxidase in developing cucumber seedlings. Physiol. Plant. *29*, 250—255.

Fooz de, G., T. Gaspar, Bouillenne-Walrand, 1966: Acid 2,4-dichlorophenoxy-acetique et catabolisme auxinique chez *Hordéum et Triticum*, Weed Res. *6*, 359—367.

Gaspar, Th., R. Wyndaele, M. Bouchet, E. Ceulemans, 1977: Peroxidase and α-amylase activities in relation to germination of dormant and nondormant wheat. Physiol. Plant. *40,* 11—14.

Huystee, van, R. B., W. L. Cairns, 1980: Appraisal of studies on induction of peroxidase and associated porphyrin metabolism. Botan. Rev. *46,* 429—446.

Jelaska, S., 1972: Embryoid formation by fragments of cotyledons and hypocotyls in *Cucurbita pepo.* Planta (Berl.) *103,* 278—280.

Jelaska, S., 1974: Embryogenesis and organogenesis in pumpkin explants. Physiol. Plant. *31,* 257—261.

Jelaska, S., 1977: The morphology of abnormal embryoids and plantlets obtained from embryogenic callus of pumpkin (*Cucurbita pepo* L.) Acta Bot. Croat. *36,* 63—74.

Jelaska, S., 1980: Growth and embryoid formation in *Cucurbita pepo* callus culture. In: Application de la culture in vitro a l'amméliration des plantes potagéres. Réunion EUCARPIA, Section »Legumes« 172—178. Versailles 16—18 April 1980. INRA.

Jelenčić, B., 1980: Embriogeni potencijal kalusnih kultura bundeve. (Embryogenic potential in pumpkin callus culture). M. Sc. Thesis, Univ. Zagreb.

Kochba, J., S. Lavee, P. Spiegel-Roy, 1977: Differences in peroxidase activity and isoenzymes in embryogenic and non-embryogenic "Shamouti" orange ovular callus lines. Plant and Cell Physiol. *18,* 463—467.

Lee, T. T., 1972a: Changes in indoleacetic acid oxidase isoenzymes in tobacco tissues after treatment with 2,4-dichlorophenoxyacetic acid. Plant. Physiol. *49,* 957—960.

Lee, T. T., 1972b: Interaction of cytokinin, auxin and gibberellin on peroxidase isoenzymes in tobacco tissues cultured in vitro. Can. J. Bot. *50,* 2471—2477.

Mäder, M., 1976: Die Lokalisation der Peroxidase-Isoenzymgruppe G_1 in der Zellwand von Tabak-Geweben. Planta (Berl.) *131,* 11—15.

Mäder, M., M. Bopp, 1976: Neue Vorstellungen zum Problem der Isoperoxidasen anhand der Trennung durch Disk-Elektrophorese und isoelektrische Fokussierung. Planta (Berl.) *128,* 247—253.

Mäder, M., J. Ungemach, P. Schloss, 1980: The role of peroxidase isoenzyme groups of Nicotiana tabacum in hydrogen peroxide formation. Planta (Berl.) *147,* 467—470.

Maurer, R. H., 1968: Disk-Elektrophorese: Theorie und Praxis der diskontinuierlichen Polyacrylamid-Electrophorese. Berlin de Gruyter.

Murashige, T., F. Skoog, 1962: A revised medium for rapid growth bioassays with tobacco tissue cultures. Physiol. Plant. *15,* 437—457.

Nessel, A., M. Mäder, 1977: Über die physiologische Bedeutung der Peroxidase-Isoenzym-Gruppen des Tabaks anhand einiger biochemischer Eigenschaften. Z. Pflanzenphysiol. *82,* 235—246.

Paul, K. G., B. Öhrman, 1980: Inertness of horseradish peroxidase to 2,4-dichlorophenoxyacetic acid. Physiol. Plant. *49,* 185—187.

Raa, J., 1971: Degradation of indole-3yl-acetic acid in homogenates and segments of cabbage roots. Plysiol. Plant. *24,* 498—505.

Rücker, W., J. Markotai, 1978: Growth and isoelectric patterns of peroxidase in tissue cultures of tobacco under the influence of cytokinin, phenylcarboxylic acids and aromatic amino acids. Phyton *19,* 1—2.

Scandalios, J. G., J. C. Sorenson, 1977: Isozymes in plant tissue culture (719—730) In: *Reinert, J., Y. P. S. Bajaj* (Eds.), 1977: Plant cell, tissue, and organ culture. Springer Verlag, Berlin-Heidelberg-New York.

Sheoran, J. S., O. P. Garg, 1979: Quantitative and qualitative changes in peroxidase during germination of mung bean under salt stress. Physiol. Plant. *46,* 147—150.

Siegel, B. Z., A. W. Galston, 1967: The isoperoxidases of *Pisum sativum.* Plant. Physiol. *42,* 221—226.

Thomas, P., H. Delincee, 1979: Effect of gamma irradiation on peroxidase isoenzymes during suberization of wounded potato tubers. Ibid. *18,* 917—921.

Thorpe, T. A., Th. Gaspar, 1978a: Changes in isoperoxidases during shoot formation in tobacco callus. In Vitro *14,* 522—526.

Thorpe, T. A., M. Tran Than Van, T. Gaspar, 1978b: Isoperoxidases in epidermal leyers of tobacco and changes during organ formation in vitro. Physiol. Plant. *44,* 388—394.

Wolter, K. E., J. C. Gordon, 1975: Peroxidases as indicators of growth and differentiation in aspen callus cultures. Physiol. Plant. *33,* 219—223.

SAŽETAK

IZOPEROKSIDAZE — RANI POKAZATELJI DIFERENCIJACIJE SOMATSKIH EMBRIOIDA U TKIVU BUNDEVE

Marijana Krsnik-Rasol, Sibila Jelaska, Draško Šerman

(Botanički zavod Prirodoslovno-matematičkog fakulteta Sveučilišta u Zagrebu i Zavod za biologiju Medicinskokg fakulteta Sveučilišta u Zagrebu)

Kod nekoliko kalusnih linija bundeve određena je aktivnost peroksidaze i dobivena elektroforetska slika njenih izoenzima. Najviša aktivnost enzima utvrđena je kod kalusnnih linija DE i NA_3, koje rastu uz dodatak regulatora rastenja i pokazuju visoku sposobnost zametanja embrioida. Kod prilagođenog tkiva (linija $Ž_{5b/T}$) znatno je niža aktivnost peroksidaze, međutim pojavi embrioida prethodi porast aktivnosti enzima. Kalusne linije razlikuju se međusobno u elektroforetskoj slici izoenzima. Na tu sliku utječu regulatori rastenja, međutim ona je također odraz određenog razvojnog stanja u kulturi.

Marijana Krsnik-Rasol, mr biol.,
dr. Sibila Jelaska
Botanički zavod (I) PMF
Roosevelтov trg 6/III P. p. 933
YU-4001 Zagreb (Jugoslavija)

Prof. dr. *Draško Šerman*
Zavod za biologiju
Medicinski fakultet
Šalata 3
YU-41000 Zagreb (Jugoslavija)