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DETECTION OF PEROXIDASE AND ESTERASE ISOZYMES IN TYPES OF TISSUE OF INTACT TOBACCO PLANTS AND DERIVED PLANT REGENERATES[1]

L. MARŠÁLEK, E. PROVAZNIKOVÁ[2]

Department of Botany and Plant Breeding, University of Agriculture, Brno, Czechoslovakia

Summary. Peroxidase and esterase isozymes of intact plants, tissue cultures and tobacco regenerates (*Nicotiana tabacum* L.) were compared. Samples of leaf tissue, pith tissue and tissue culture of intact plants and plant regenerates were taken for polyacrylgel (PAG) electrophoresis. Two series were ovaluated. There were no marked differences between isozyme patterns of similar tissue types of intact plants and regenerates except esterase isozymes in leaf tissue. The obtained results confirmed a specific function of the studied enzymes depending on the type of tissue. Anodic peroxidase isozymes are obviously coded by the same allelic series in both leaf and pith tissues, whereas those of nonspecific esterases are fully effective in leaf tissue and considerably repressed in pith tissue. In callus tissues, both enzymes were characterized by two to three bands of isozymes with different relative mobility. In callus tissues, isozyme patterns of both enzymes markedly differed from those occurring in leaf and pith tissue and were probably controlled by two allelic series.

Recently, new and progressive methods have been introduced into plant breeding. The aim of all these methods is to shorten the process of breeding from the original material to a new variety and, thus, to obtain a marked economic effect (Phillips Collins, 1979, Dambroth, Mixi, 1980, Kasperbauer et al., 1980). Besides, these new methods permit to obtain such forms which could not be selected by conventional methods of breeding. As an example, the application of somatic hybridization or fusion of protoplasts of different genera, species and other taxa of both plants and animals can be mentioned here (Wetter, 1977, Eichhorn, 1979, Cocking, 1979, Szabados et al., 1981, Gatenby et al., 1980, Chaleff, 1981). It is not necessary to emphasize that in all these methods it is important to transform organ, tissue or cell of an intact plant into a complete regenerate (Novak et al., 1980, Sato et al., 1979, Hussey, 1980, Angiboust, 1980). However, this transformation is still complicated by many unsolved problems.

Using peroxidase and esterase isozymes, we have tried in this study to describe genetic effects of these enzymes within the cycle: intact plants — tissue culture — plant regenerate in tobacco, and to study manifestations of these isozymes for the type of tissue.

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[2] First author: Docent Dr. ing. Second author: ing. Present address: Zemědělská 3c, Brno, Czechoslovakia.

MATERIAL AND METHODS

The cultivar Virginia Gold A, a yellow barley with the genotype $Ar_1Ar_1Ar_2Ar_2$ $yb_1yb_1yb_2yb_2$, was used for detection of anodic peroxidase and non-specific esterase isozymes. Seed material was obtained from the Research Institute of Tobacco Industry, Bab near Nitra, Czechoslovakia. Plants were raised in a growth chamber. For the purpose of PAG electrophoresis samples of leaf tissue and pith tissue of the central part of the stem were taken during the elongation phase and primocultures were established at the same time. Samples of callus cultures were obtained after 6 and 12 weeks of culture. Plants were grown on Murashige-Skoog medium supplemented with kinetin (2 mg 1000 ml^{-1}); IAA (2 mg 1000 ml^{-1}) and 2.4D (0.2 mg 1000 ml^{-1}). After six weeks of culture, some calluses were transferred to a medium without 2.4D to regenerate the entire plants. These plant regenerates were raised under the same conditions as intact plants. Tissues from plant regenerates were sampled in the way this was done for intact plants. Thus two series of experiments were analyzed.

To estimate anodic peroxidase isozymes (E.C.1.11.1.7), the samples were homogenized in cooled mortars with extraction buffer pH 6.8 in a ratio 1 : 1. Homogenates were filtered through a fine cloth and centrifugated at 3500 g and 4°C for 40 minutes. The supernatant was thickened with Sephadex G-25 to half its initial volume. For electrophoresis, a 7.5% acrylamide gel was used (Safonov, Safonov 1971). Samples were stained with a benzidine mixture (0.005 M benzidine dissolved in 96% ethylalcohol and acetate buffer 0.1 M pH 4.8 in a ratio 1 : 1). Gels were stained in this mixture for 30 minutes at 4°C. Thereafter, 1 ml of 1.5% hydrogen peroxide was added. Gels were stained in darkness for 10 - 15 minutes.

To estimate the non-specific esterase isozymes (E.C.3.1.1) electrophoresis on 7.5% acrylamide gel was done according to the method of Davis (1964) modified by Novacky at al. (1966). In other respects the method was the same as that for the identification of anodic peroxidase isozymes. Esterases were detected with 0.1 M phosphate buffer pH 7.4 — naphtylacetate, Fast Blue RR Salt (Sahulka, Benes, 1969). Gels were stained in darkness for approximately 15 minutes. After incubation they were rinsed with 7% acetic acid. Stained bands were drawn on the paper in the form of zymograms and the Rm values were calculated.

RESULTS AND DISCUSSION

1st series (Fig. 1 and 2, Tab. 1)

A. INTACT PLANTS

There were no differences in intact plants in the distribution and numbers of peroxidase isozymes (IEP) occurring in leaf and pith tissue. In both types of tissue, 6 isozymes were detected with Rm values ranging from 0.48 to 0.67. A significant

Table 1. Rm values and isozyme numbers — (lst series)

No. of isozymes	Tissue of								Callus after							
	leaf				pith				6 weeks				12 weeks			
	Isozymes															
	peroxidase		esterase		peroxidase		esterase		peroxidase		esterase		peroxidase		esterase	
	plants															
	intact	regene-rates	intact	regene-rates	intact	regene-rates	intact	regene-rates	intact	regene-rates	intact	regene-rates	intact	regene-rates	intact	regene-rates
1	0.48	0.11	0.25	0.10	0.48	0.37	0.28	0.28	0.20	0.21	0.14	0.16	0.18	0.17	0.18	0.27
2	0.51	0.50	0.30	0.15	0.50	0.50	0.34	0.34	0.24	0.24	0.19	0.21	0.24	0.21	0.25	0.33
3	0.54	0.53	0.37	0.26	0.55	0.54	0.39	0.40	0.27	0.28	0.32	0.26	0.49	0.24	0.31	0.38
4	0.60	0.56	0.38	0.33	0.60	0.57			0.53	0.53	0.58	0.33	0.52	0.49	0.57	0.65
5	0.63	0.61	0.48	0.35	0.63	0.61			0.57	0.55	0.64	0.37	0.55	0.53	0.63	0.70
6	0.67	0.64	0.52	0.38	0.67	0.66					0.69	0.64		0.56	0.66	0.73
7		0.68	0.66	0.40		0.70										
8			0.70	0.51												
9				0.54												
10				0.63												
11				0.69												

difference was found in callus cultures. In this case, two zones of IEP distribution were found, viz. a new one with a low relative mobility (Rm 0.18 - 0.27) and another with a higher rate of migration (Rm 0.49 - 0.57). As compared with leaf and pith tissue, the major part of the isozymes was repressed in this second zone.

Non-specific esterase isozymes (IEE) showed a greater variation in individual tissues. The highest number of IEE' s (i.e.8) was found in leaf tissue and the lowest (i.e.3) in pith tissue. The leaf tissue was found to have three zones of IEE distribution (Rm 0.25 - 0.38; Rm 0.48 - 0.52; Rm 0.66 - 0.70) whereas the pith tissue had only one with Rm 0.28 - 0.39; other zones were repressed. In calluses, two zones were detected.

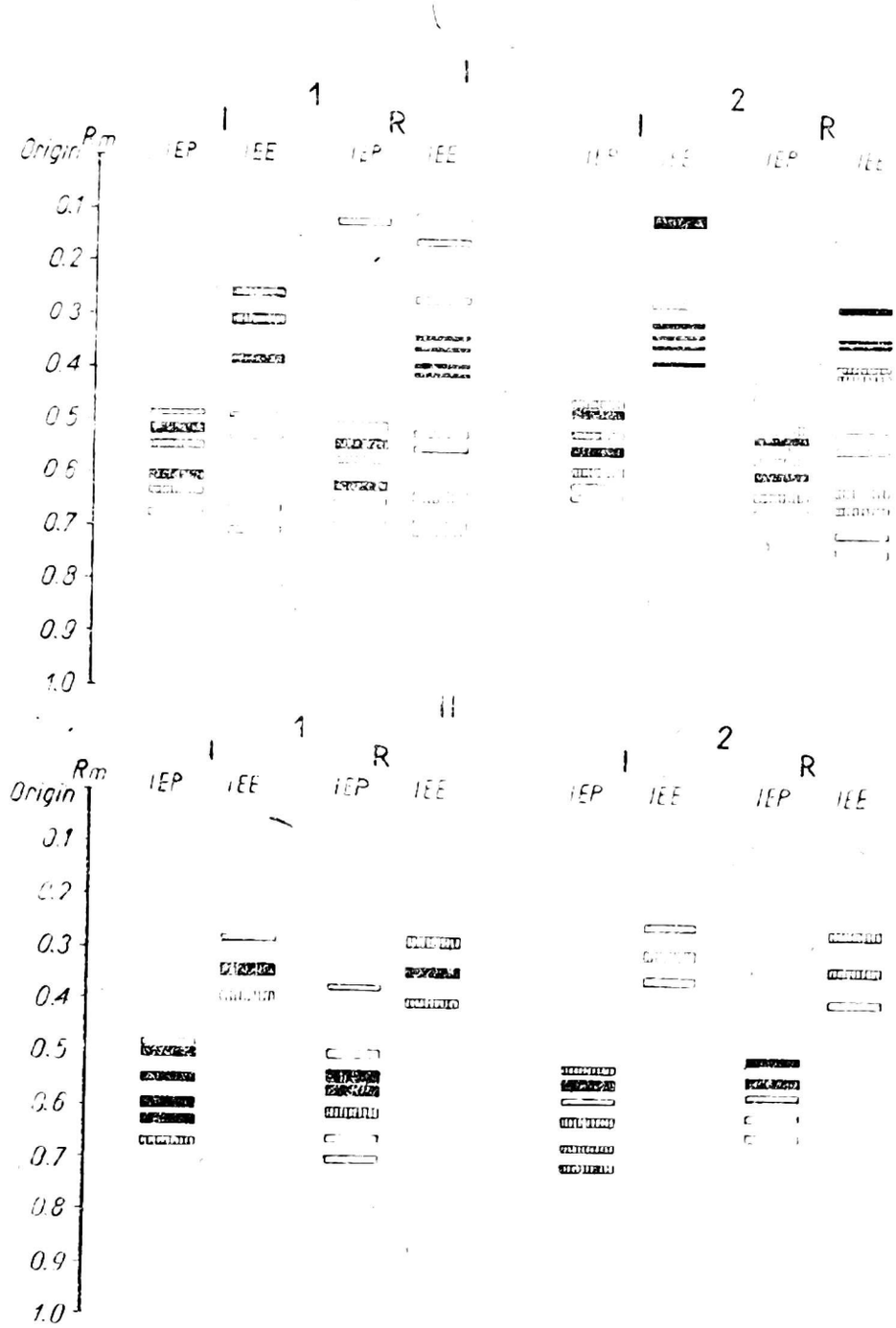

Fig. 1. Zymograms of peroxidase (IEP) and non-specific esterase (IEE) isoenzyme patterns in leaf (I) and pith (II)

I — intact plants, R — regenerates plants, 1 — 1st series, 2 — 2nd series

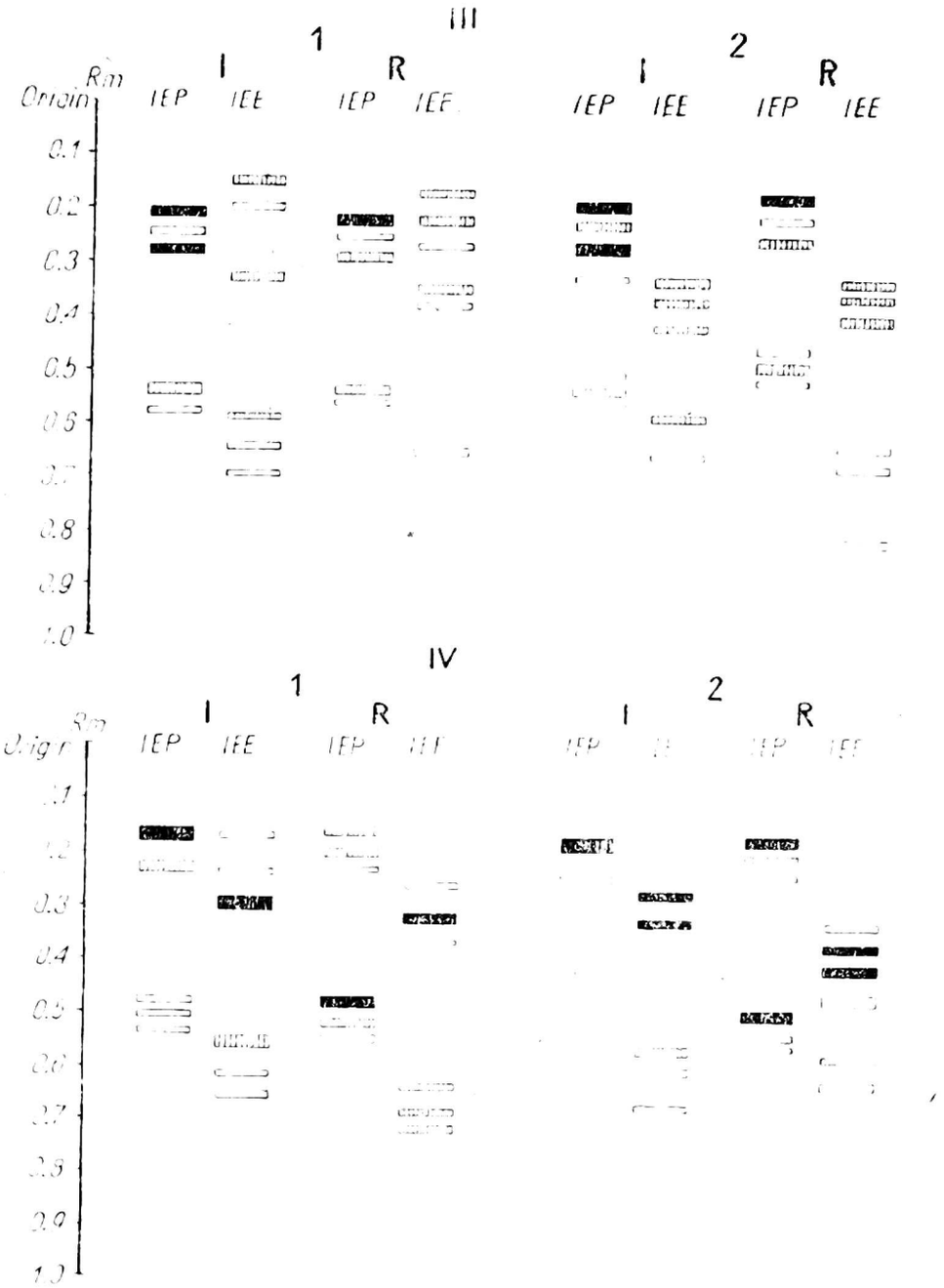

Fig. 2. Zymograms of peroxidase and non-specific esterase isozyme patterns in callus after 6 weeks (III) and 12 weeks (IV)

B. PLANT REGENERATES

Plant regenerates showed no significant differences between IEP patterns occurring in the leaf and pith tissue. In both cases totally 7 isozymes were found. In callus cultures, again, only 2 zones were found, viz. one with a lower rate of migration (Rm 0.17 - 0.28) and the other with a higher rate (Rm 0.49 - 0.56). IEE showed a greater variation. The highest number of IEE's (11) was found in the leaf tissue, the lowest in the pith tissue. In the leaf tissue totally 4 zones were discovered, viz. with Rm 0.10 - 0.15; Rm 0.26 - 0.40; the other zones were repressed. The callus tissue were found to have 2 - 3 zones of IEE.

2nd series (Fig. 1 and 2, Tab. 2)

A. INTACT PLANTS

Intact plants showed no significant differences in the distribution of IEP's in the leaf and pith tissue. Rm values ranged from 0.45 to 0.71. Callus cultures had two zones of distribution, Rm 0.18 - 0.32 and Rm 0.50 - 0.56. After 12 weeks of culture, the second of IEP's was repressed in the calluses.

IEE showed a greater variation. The highest number of IEE (7) was found in the leaf tissue and the lowest (3) in the pith tissue. The leaf tissue had three zones of IEE, Rm 0.10, Rm 0.26 - 0.37 and Rm 0.82. The callus tissue had two zones, Rm 0.28 - 0.41 and Rm 0.57 - 0.68.

B. PLANT REGENERATES

The highest number of IEP's (7) was found in the leaf tissue and the lowest (5) in the pith tissue. The distribution of IEP was similar in both types and ranged from Rm 0.40 to 0.65. Again, a significant difference was found in tissues where IEP patterns were distributed in two zones. The highest number of IEE was detected in the leaves and the lowest (3) in the pith. The leaves had totally three zones with Rm 0.26 - 0.38; Rm 0.50 and 0.53; and Rm 0.61 - 0.72. The calluses were found to have only two zones.

On the basis of the evaluation of isozyme patterns occurring in intact plants and plant regenerates of both series the following conclusions may be drawn.

In intact plants, IEP patterns in the leaf and pith tissue were very similar. The callus tissue was found to have two zones of IEP distribution, which were very similar after 6 and 12 weeks of culture. In the second zone (Rm 0.49 - 0.57), repressed IEP's were detected in the callus tissue. A similar trend was observed also in tissue of derived plant regenerates. This means that IEP's in the second zone (Rm 0.45 - - 0.71) are determined by only one allelic series in all types of tissue. Callus cultures have one zone with a low relative mobility (Rm 0.16 - 0.28) which is determined by one allelic series; the second zone is similar to that occurring in the leaf and pith tissues but 3 to 4 IEP's are repressed.

With IEE, 3 - 4 zones were detected according to the rate of migration from the start. In intact plants, differences were found between all types of the investigated tissue. The lowest number of IEE (3) was detected in the pith tissue and the highest in the leaf tissue. All tissues showed the same pattern (Rm 0.25 - 0.41), which is probably controlled by one allelic series. In callus cultures 2 - 3 zones were detected. A similar situation occurred also in plant regenerates.

Esterase and peroxidase isozymes are especially suitable for studies on genetic problems, because they are polymorphic in numerous plant species and also because they can be detected by relatively simple methods (Schmidt-Stohn 1979). Isozymes of different enzymes are used as markers especially in those cases where no morphologic markers are available (Hayward, Hacker 1980). Until now

Table 2. Rm values and isozyme numbers — (2nd series)

No. of isozymes	Tissue of								Callus after							
	leaf				pith				6 weeks				12 weeks			
	isozymes															
	peroxidase		esterase		peroxidase		esterase		peroxidase		esterase		peroxidase		esterase	
	plants															
	intact	regene-rates	intact	regene-rates	intact	regene-rates	intact	regene-rates	intact	regene-rates	intact	regene-rates	intact	regene-rates	intact	regene-rates
1	0.45	0.40	0.10	0.26	0.52	0.50	0.25	0.26	0.18	0.16	0.32	0.32	0.18	0.17	0.28	0.33
2	0.47	0.49	0.26	0.32	0.55	0.54	0.30	0.33	0.22	0.20	0.36	0.35	0.25	0.20	0.33	0.37
3	0.51	0.51	0.30	0.33	0.58	0.57	0.35	0.39	0.26	0.24	0.41	0.39		0.24	0.57	0.41
4	0.54	0.55	0.32	0.37	0.62	0.61			0.32	0.45	0.58	0.63		0.50	0.61	0.47
5	0.58	0.58	0.84	0.38	0.67	0.65			0.50	0.48	0.65	0.67		0.54	0.68	0.58
6	0.61	0.62	0.37	0.50	0.71				0.53	0.51		0.81		0.56		0.63
7	0.63	0.65	0.82	0.53					0.56							
8				0.61												
9				0.64												
10				0.69												
11				0.72												

only few papers have dealt with the comparison isozyme patterns in callus cultures and/or plant regenerates on the one hand and in intact plants, on the other. Some authors (Lee 1973, Mader et al., 1976, Maršálek 1979) found different patterns of isoperoxidases in tobacco callus tissues and in intact plants. Chaleff (1981) observed that patterns of isoperoxidases in the leaves of the somatic hybrid *Nicotiana glauca* × *N. langsdorfii* were identical with those in leaves of sexual hybrids and that they represented a combination of isozyme patterns of both parental species. In our experiments a similar relationship between isozyme patterns of peroxidase was found in intact plants and plant regenerates. A greater polymorphism was observed with esterase isozymes both in intact plants and plant regenerates. These results are consistent with data published by other authors (Clegg, Allard 1973, Hvid, Nielsen 1977, Schmidt, Wandelt 1979, Quiros 1980).

REFERENCES

1. Angiboust A. (1980). Arboricult. fruit. 27: 41 - 46.
2. Clegg M. T., Allard R. W. (1973). Journal of Heredity. 1: 3 - 6.
3. Dambroth M., Mix G. (1980). Agrar-übersicht. 11: 22 - 24.
4. Davis B. J. (1964). Ann. NY. Acad. Sci. 121: 404 - 427.
5. Eichhorn M. (1979). Biol. Rdsch. 5: 321 - 322.
6. Gatenby A. A., Zapata F. J., Cocking E. C. (1980). Z. Pfl.-Zücht. 84: 1 - 8.
7. Hayward M. D., Hacker J. B. (1980). Euphytica. 29: 347 - 356.
8. Hussey G. (1980). Grower. 93: 17 - 23.
9. Hvid S., Nielsen G. (1977). Hereditas. 87: 155 - 162.
10. Chaleff R. S. (1981). Genetics of higher plants. Applications of coll culture. Camb. Univ. Press.
11. Kasperbauer M. J., Buckner R. C., Springer W. D. (1980). Crop Sci. 20: 103 - 107.
12. Lee T. T. (1973). Physiol. Pl. 29: 198 - 203.
13. Mader M., Meyer Y., Bopp M. (1976). Planta, 129: 33 - 38.
14. Maršálek L. (1979). Acta univ. agric. Brno. 27: 29 - 39.
15. Novacky K., Macko V., Hassler J. (1966). Biológia (Bratislava). 21: 64 - 68.
16. Novák F. J., Zadina J., Horáckova V., Masková I. (1980). Potato Res. 23: 155 - 166.
17. Phillips G. C., Collins G. B. (1979). Crop Sci. 19: 59 - 64.
18. Quiros C. F. (1980). Crop Sci. 20: 262 - 264.
19. Safanov V. I., Safanova M. P. (1971). In Russ. Sb. Biokhimicheskie Metody v Fiziologii Rastenii. Moskva.
20. Sahulka J., Benes K. (1969). Biol. Plant. 11: 23 - 33.
21. Sato M. S., Imanishi S., Hiura I. (1979). Jap. J. Breed. 29: 33 - 38.
22. Schmidt J., Wandelt W. (1979). Wissensch. Zeitschrift der Pädag. Hochsch. Güstrow, 1: 75 - 90.
23. Schmidt-Stohn G. (1979). Z. Pfl. Zücht. 83: 155 - 162.
24. Szabados L., Hadlaczky G., Dudits D. (1981). Planta. 151: 141 - 145.
25. Wetter L. R. (1977). Molec. gen. Genet. 150: 231 - 235.

WYKRYWANIE IZOENZYMÓW PEROKSYDAZY I ESTERAZY W RÓŻNYCH TKANKACH ROŚLIN TYTONIU I POCHODZĄCYCH Z NICH ROŚLIN ZREGENEROWANYCH

Streszczenie

Porównywano izoenzymy peroksydazy i esterazy z wyjściowych roślin, kultur tkankowych i wyprowadzonych z nich zregenerowanych roślin tytoniu (*Nicotiana tabacum* L.). Pobierano próby tkanek liścia i rdzenia roślin wyjściowych i zregenerowanych oraz kultur tkankowych do elektroforezy w żelu poliakryloamidowym (PAG). Nie stwierdzono wyraźnych różnic między wzorami izoenzymatycznymi podobnych typów tkanek z roślin wyjściowych i zregenerowanych, z wyjątkiem izoenzymów esterazy z tkanki liścia. Otrzymane rezultaty potwierdziły specyficzną funkcję badanych enzymów, zależną od typu tkanki. Anodowe izoenzymy peroksydazy kodowane są przez tę samą serię genów allelicznych w tkankach liścia i rdzenia. Geny niespecyficznych esteraz są aktywne w tkance liścia, a ich aktywność w tkance rdzenia jest znacznie obniżona. W tkankach kalusowych, obydwa enzymy charakteryzowały 2 do 3 prążki izoenzymatyczne o różnych ruchliwościach elektroforetycznych. Wzory izoenzymatyczne obu enzymów obserwowanych w tkankach kalusowych różniły się znacznie od występujących w tkankach liścia i rdzenia i były prawdopodobnie kontrolowane przez dwie serie genów allelicznych.

ОБНАРУЖЕНИЕ ИЗОЭНЗИМОВ ПЕРОКСИДАЗЫ И ЭСТЕРАЗЫ В РАЗЛИЧНЫХ ТКАНКАХ РАСТЕНИЙ ТАБАКА И ПРОИСХОДЯЩИХ ИЗ НИХ РЕГЕНЕРИРОВАННЫХ РАСТЕНИЙ

Резюме

В настоящей работе сравниваются изоэнзимы пероксидазы и эстеразы исходных растений, тканевых культур и выведенных из них регенерированных растений табака (*Nicotiana tabacum* L.). Пробы тканок листьев и сердцевины исходных и регенерированных растений, а также тканевых культур брались для электрофореза в полиакрилоамидовом геле (PAG). Не обнаружено отчётливых разниц между спектрами изоэнзимов подобных типов тканок исходных и регенерированных растений, за исключением изоэнзимов эстеразы из листовой тканки. Полученные результаты подтвердили специфическую функцию исследуемых энзимов в зависимости от типа ткани. Анодные изоэнзимы пероксидазы кодировались той же самой серией аллельных генов в тканках листьев и сердцевины. Гены неспецифических эстераз были активны в листовой ткани, а их активность в ткани сердцевины была значительно ниже. В каллусных тканях обеих энзимов наблюдалось от 2 до 3-х изоэнзиматических полос с разной электрофоретической подвижностью. Изоэнзиматические спектры обеих энзимов, наблюдаемые в каллусных тканях, значительно отличались от спектров лиственных тканей и тканей сердцевины и правдоподобно контролировались двумя сериями аллельных генов.