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A New Method for Differential Determination of Basic Natural Forms of Indolyl-3-acetic Acid

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Abstract—A new ELISA method is proposed for differential quantitative determination of free (indolyl-3-acetic acid; IAA) and bound (indolyl-3-acetyl-L-aspartate) forms of natural auxins. There is similarity in results obtained by this and some traditionally used methods. The standard error of determination of the active form of IAA by our method is 1.5-2.0 times less than that using the traditional method. The method of quantitative differential determination of the main natural auxins does not require preliminary sample preparation, and this shortens assay time. The developed method has been used for practical determination of different forms of endogenous IAA in wheat and dandelion ovaries subjected to minimal treatment. This method can be used to investigate changes in the ratio of various hormonal forms of auxins that differ in their physiological activity in reproductive organs of angiosperms at various stages of reproduction.

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Auxins represent one of six groups of phytohormones constituting a biochemical basis for hormonal regulation in plants; together with other systems, they are involved in maintenance of integrity of the plant during its whole life cycle. Auxins are involved into apical domination and attraction [1-3], but these are only a small part of auxin effects known to date. They are also involved in onto- and morphogenesis, growth and motility, and adaptive and reparative processes [1, 2]. Formation of roots, stems, and leaves, conducting bundles, and some other organs and tissues are also auxin-dependent processes. In grains, auxins inhibit tillering [1].

Centers of auxin synthesis are localized in subapical meristems and so auxin transport occurs basipetally mainly via phloem cells. However, mechanisms responsible for auxin transport remain poorly understood.

At the cellular level, auxin may influence characteristics of cell wall, protoplasm, osmosis, and cell respiration. It is suggested that auxins activate functioning of acid hydrolases and H^+ pump; this results in acidification and wall matrix softening required for cell tension. Formation of cell wall *de novo* also depends on auxins [1, 2].

Auxin signaling involves specific cell receptors. The auxin signal influences plasmalemma and polyribosome functioning. It is suggested that auxins influence synthesis of all types RNAs. Expression of groups of early nuclear and late nuclear and plastid aux-genes depends on auxins [2].

Auxin interaction with target cell is often accompanied by rapid transformation of a major proportion of free auxins into bound forms; this explains why standard methods detect small quantities of free auxins, which cannot give clear information about total auxin content in particular place [1].

This brief overview accounts for the interest of phytologists in practical methods of reliable and accurate quantitative determination of auxins *in situ*. It is particularly interesting and important to assay differentially

Abbreviations: ANAA) *p*-aminonaphthyl acetic acid; BSA) bovine serum albumin; IAA) indolyl acetic acid (free form); NAA) α-naphthyl acetic acid; OV) ovalbumin; TPS) 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl and 0.1% Triton X-100.

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microquantities of free (indolyl acetic acid (IAA)) and bound (indolyl-3-acetyl-L-aspartate) forms of auxins. This was the goal of this study.

MATERIALS AND METHODS

The following forms of the phytohormone have been used in this study: IAA, the natural hormone (OlChemim Ltd, Czech Republic); NAA (α-naphthyl acetic acid), the synthetic hormone (Aldrich Chemical Company, USA); IAA methyl ester, the synthetic analog of a bound hormone (the analog of the reserve form) (OlChemim Ltd), and also synthesized *p*-aminonaphthyl acetic acid (ANAA). For sorption on polystyrene plates, all these phytohormones were used as conjugates with ovalbumin (OV) to avoid nonspecific reactions between components of the immunochemical system. The conjugates of the phytohormones with bovine serum albumin (BSA) were used as the immunogens for antibody production.

ANAA was synthesized as follows. Concentrated sulfuric acid (5 ml) was heated to 70°C under intensive stirring and then sequential portions of potassium nitrate (final amount 1.2 g) and NAA (0.8 g) were added under stirring and heating. After addition of these reagents, the reaction mixture was stirred and maintained at this temperature for 30 min. The reaction mixture was then filtered and washed onto this filter with ethyl acetate. After several washings with ethyl acetate, the resultant light-yellow powder of nitro-NAA was dissolved in 5 ml of 0.5 M alkaline solution. The resulting solution was mixed with 2.5-fold excess of sodium hydrosulfite (which was added in several portions under stirring). The reaction was carried out at 60-70°C for 15-20 min. The reaction mixture was cooled to room temperature, pH was adjusted to neutral values with 0.5 M HCl, and the mixture was evaporated using an evaporator. ANAA was subsequently purified by thin layer chromatography using chloroform–methanol–water (40 : 50 : 4 v/v) mixture as the mobile phase [4].

The BSA–IAA conjugate was synthesized as follows: IAA (10 mg) was dissolved in freshly distilled absolute dimethylformamide and N,N′-dicyclohexylcarbodiimide (10 mg) and N-hydroxysuccinimide (5 mg) were then added in 2 ml of dimethylformamide. The reaction mixture was incubated at room temperature for 60 min under stirring. The sediment formed was separated by centrifugation at 8000 rpm for 5 min using 5415C-Eppendorf microcentrifuge (Eppendorf, Germany). The supernatant containing modified IAA was mixed with 10 mg BSA dissolved in 3 ml of 0.2 M potassium phosphate buffer, pH 7.4. The mixture was incubated under stirring: initially at room temperature for 60 min and then stirred at 4°C overnight. The sediment formed was separated by centrifugation at 8000 rpm for 5 min using the 5415C-Eppendorf microcentrifuge. The supernatant was dialyzed against distilled water for 3 days at 4°C. Synthesis of the OV–IAA conjugate employed the same method as above. However, we varied molar ratio of OV/IAA in the reaction mixture to obtain immobilized conjugates with various protein–hormone ratios.

The BSA–ANAA conjugate was synthesized using the two-step glutaraldehyde method. BSA (10 mg) was dissolved in 2 ml of 0.1 M potassium phosphate buffer, pH 6.8, containing 0.15 M NaCl. The resulting solution was cooled to 4°C and mixed with 10% aqueous solution of freshly distilled glutaraldehyde to its final concentration of 0.2%. The reaction mixture was incubated at 4°C for 60 min and at room temperature for 2 h under constant stirring. Excess glutaraldehyde was removed by dialysis against 0.15 M NaCl, and 10 mg of ANAA was added in 1 ml of dimethylformamide to the dialyzed solution. The pH value was adjusted to 9.0 using 0.5 M Na_2CO_3. The resulting solution was incubated at room temperature for 24 h under constant stirring. After this incubation, the reaction mixture was then dialyzed at 4°C against distilled water and centrifuged at 8000 rpm for 10 min using the 5415C-Eppendorf microcentrifuge. Synthesis of the OV–ANAA conjugate was carried out using the same method as in the case of BSA–ANAA. However, we varied molar ratio of OV/ANAA in the reaction mixture to obtain optimal properties of the immobilized conjugates.

Rabbits were immunized with 1 mg of the BSA–IAA or BSA–ANAA conjugates for 3-4 months with a two-week interval between immunizations. The first immunization included subcutaneous administration of conjugates in complete Freund's adjuvant (1 : 1) into the withers, during the second immunization conjugates were injected subcutaneously into several belly points in incomplete Freund's adjuvant (1 : 1). Subsequent immunization included intramuscular injections of pure conjugates BSA–IAA (or BSA–ANAA) into femoral muscles. Blood was collected from the marginal ear vein 8-10 days after the seventh immunization.

Concentrations of endogenous forms of IAA were determined in plant aqueous extracts by the method of competitive ELISA using labeled antispecies antibodies [5]. The conjugates OV–IAA and OV–ANAA were used as the antigens immobilized onto the solid phase. Antigens were adsorbed onto polystyrene plates with high sorption capacity (Nunc, Maxi Sorp, Denmark). The sorption was carried out in 0.2 ml (at protein concentration 0.5 μg/ml) 0.02 M sodium carbonate buffer, pH 9.5, at 4°C for 12 h. Plate wells were then washed five times with 0.3 ml of TPS (0.01 M potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl and 0.1% Triton X-100) using a PPA-01 automated plate washer (Proplan, Russia). After the washing, we added 0.1 ml of a sample (or 0.1 ml standard solution of IAA or its methyl ester) and then 0.1 ml of a homologous antiserum using dilutions 1 : (30,000-50,000) in TPS. The mixtures were incubated at 4°C overnight for binding of antigens with antibodies; after the incubation,

unbound components of the analytical system were removed. Plate wells were washed five times with 0.3 ml TPS and then 0.15 ml of the solution containing the second antibody conjugated with horseradish peroxidase (Ab_2–HRP; 1 nM, evaluated by the marker enzyme; Sigma, USA) was added in TPS. After the incubation at 37°C for 60 min, unbound reagents were removed by repeated quintuple washing with 0.3 ml TPS and 0.15 ml of freshly prepared substrate mixture (4 mg of *o*-phenylenediamine and 4 μl of 30% hydrogen peroxide in 10 ml of 0.1 M sodium citrate buffer, pH 5.0) was added to each well. Plates were incubated for 60 min, the reaction was stopped by addition of 50 μl of 4 M H_2SO_4 into plate wells, and absorbance of reaction product was read at A_{490} using a vertical spectrophotometer for 96-well plates (Uniplan AIFR-01, Russia).

The amphimictic species *Triticum aestivum* L. (wheat) and the apomictic species *Taraxacum officinale* Web. (dandelion) were used as the research objects with egg-cells starting division after fertilization (*T. aestivum*) and without it (*T. officinale*). Wheat ovaries were studied at the following developmental stages: the mature embryo sac with eight nuclei (stage 1), and also ovaries 12 and 24 h after pollination; the latter time intervals corresponded to the stages of zygote interphase (stage 2) and the beginning of its division (stage 3). The dandelion ovaries were studied at the stage of the mature embryo sac (stage 1), in the interphase of the parthenogenetically developing ovule (stage 2) and during its first division (stage 3). The ovaries isolated from flowers [6] (50 mg) were homogenized in 0.5 ml of 0.01 M potassium phosphate buffer, pH 7.4, containing 0.1 M NaCl, 0.01 M sucrose, and 0.1% Triton X-100. The homogenate was centrifuged at 10,000 rpm for 5 min and clear supernatant was analyzed by means of two competitive ELISA system using TPS for its dilution. Concentrations of various forms of IAA in the extracts were calculated using calibrations curves (Figs. 1 and 2). Deviation of optical density from its maximal value represented 60-70% (depending on the system used). This allowed accurate assays of concentrations of IAA and its methyl ester.

RESULTS AND DISCUSSION

The resulting antisera specifically interacted with immobilized homologous OV–hormone. In control wells containing immobilized OV, the absorbance of reaction product (detected the immunochemical complex formed between the immobilized antigen and the antibody onto the polystyrene surface) by means of the Ab_2–HRP corresponded to 2-5% of the A_{490} signal was registered during interaction of the antiserum with the homologous immobilized OV–hormone. In the interaction of antisera against BSA–IAA with the heterologous OV–ANAA immobilized on the polystyrene surface, the antiserum titer sharply decreased (by more than 500 times). Similar behavior was observed in the system of the antiserum to BSA–ANAA and immobilized OV–IAA. This suggests importance of spatial orientation of a hormone molecule versus a protein globule of the conjugated partner for interaction with antibodies. So, in subsequent studies of the systems employed for detection of IAA we considered further only analytical systems of the competitive ELISA with the homologous immunoreagents. The first system included the antiserum to the BSA–IAA conjugate and the immobilized OV–IAA. The second system was based on the antiserum to BSA–ANAA and OV–ANAA as the immobilized conjugate.

The first analytical system was based on the immunoreagents obtained by covalent immobilization of IAA to amino groups of the protein carriers using carboxyl group of the phytohormone. In the second case, we used NAA, the synthetic analog exhibiting auxin properties and higher chemical stability than the natural hormone.

Introduction of amino group at *p*-position of the NAA molecule (i.e. exposed in the opposite orientation versus its carboxyl group) influenced not only chemical nature of the immunogen (substitution of IAA for NAA) but also spatial orientation of the immobilized hormone molecule versus the protein globule in the conjugates with OV and BSA. Comparison of two selected ELISA systems was useful for elucidation of putative influence of hormone carboxyl group esterification on specificity of the immunochemical system used for determination of free IAA and its esterified form (the reserve form of this hormone).

The first analytical system based on OV–IAA and BSA–IAA has already been studied and described in literature [7]. This analytical system is characterized by a unique specificity to the bound form of IAA only. In our experiments, we used methyl ester of IAA (its synthetic analog) as the bound form of this hormone. Cross-reactivity for IAA calculated by its methyl ester did not exceed 0.1%, in the cases of tryptophan and indolyl-3-acetonitrile cross-reactivity was 1%, with indolyl-3-acetaldehyde and indole this parameter was 0.1%. Sensitivity of determination of methyl ester of IAA was 0.5 nM and relative error was 10-13% in the whole range of hormone concentrations studied (Fig. 1).

Our results are consistent with literature data [7, 8]. Consequently, the first analytical system can be employed for quantitative determination of bound form of IAA only. In this case, the antibodies obtained using BSA–IAA as the immunogen do not interact with IAA in which carboxyl group exists in the non-esterified state. So, determination of free IAA using known ELISA kits based on the first immunochemical system usually requires two possible variants of sample preparation: obligate preliminary fractionation followed by subsequent methylation of the sample with diazomethane or methylation of initial

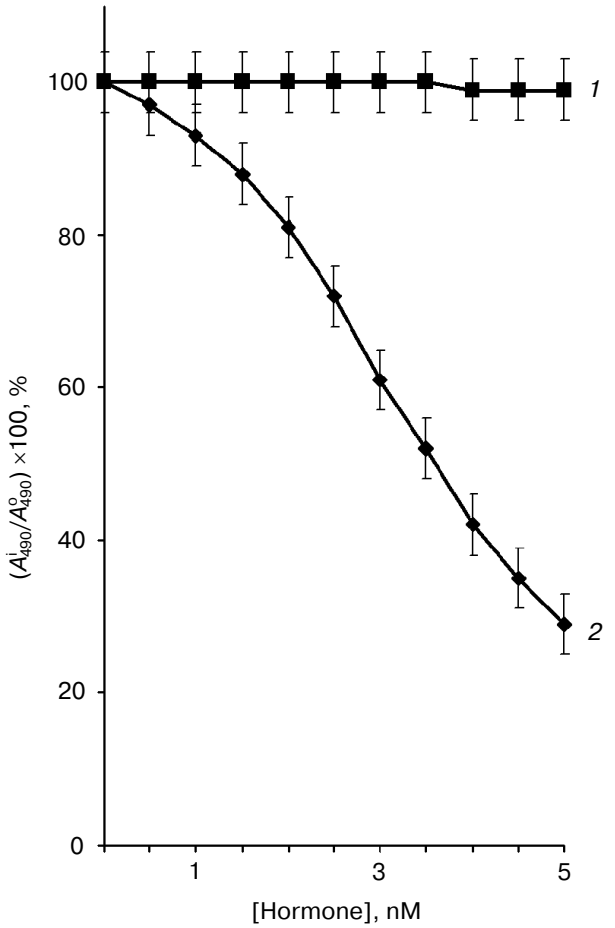

Fig. 1. Dependence of relative binding of antibody against the BSA–IAA conjugate to OV–IAA on concentration of IAA (*1*) and its methyl ester (*2*) in the first ELISA system. Abscissa shows hormone concentration in solution, ordinate shows relative binding of the antibody with the OV–IAA conjugate immobilized onto plate wells, which was measured as the ratio A^i_{490}/A^o_{490}, where A^i_{490} and A^o_{490} are absorbance values of the reaction products obtained in the presence and in the absence of the phytohormone in the immunochemical system.

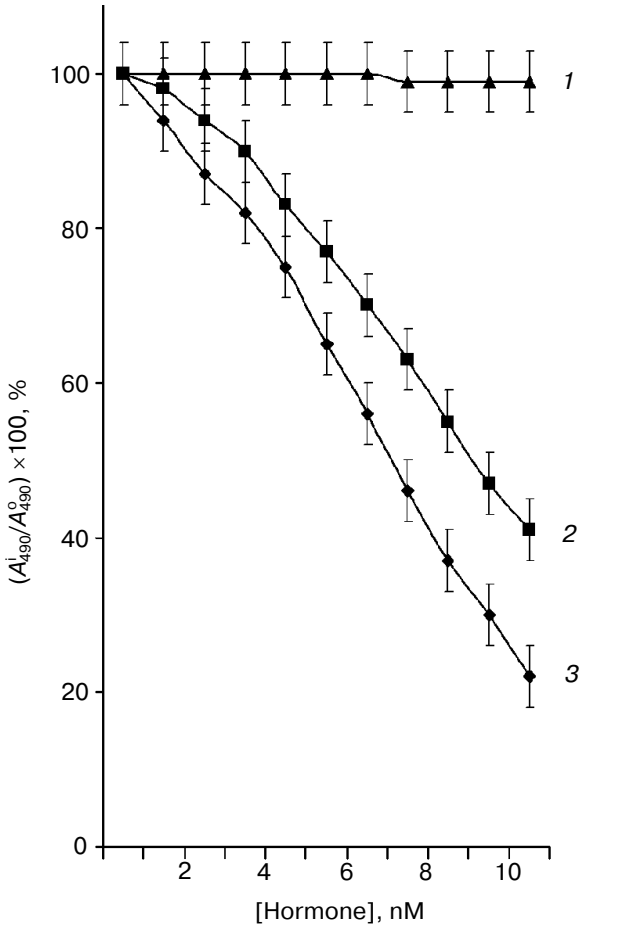

Fig. 2. Dependence of relative binding of antibody against the BSA–ANAA conjugate to OV–ANAA on concentration of methyl ester of IAA (*1*), IAA (*2*), and NAA (*3*) in the second ELISA system. Abscissa shows hormone concentration in solution, ordinate shows relative binding of the antibody with the OV–IAA conjugate immobilized onto plate wells, which was measured as the ratio A^i_{490}/A^o_{490}, where A^i_{490} and A^o_{490} are absorbance values of the reaction products obtained in the presence and in the absence of the phytohormone in the immunochemical system.

sample and determination of the bound (methylated) form of this hormone before and after the methylation reaction.

Since methylation involves not only carboxyl group but also aromatic/heterocyclic moieties of IAA and its metabolites, this reaction is accompanied by formation of a wide spectrum of products. The methylation products have not been studies yet; it is possible that they exhibit various affinity to the antibodies used. Consequently, determined concentrations of IAA cannot represent a simple sum of concentrations of the methylation products in a given sample. This means that measurable IAA concentration cannot reflect true hormone content because it represents some effective parameter, which may significantly differ from real IAA concentration in the sample. IAA content in plant objects is usually very low (<100 nM) and any additional stages of sample preparation may also contribute to deviation of results during the analytical procedure.

The are some other factors influencing the accuracy of hormone determination; these include nonenzymatic oxidation by air oxygen and enzymatic oxidation by air oxygen in the presence of endogenous peroxidases [9], possible loss of IAA due to its adsorption on a glass surface (as methylation occurs under severe chemical conditions accompanied by release of gaseous nitrogen), foaming and spreading of a sample over surface of the reaction chamber [10]. The ratio of various methylation products of IAA and its metabolites also depends on conditions of the methylation reaction. Since this reactions is carried

out under crude conditions (which cannot be well standardized), the set of methylation products may vary. This means that results of analysis of even one sample will depend on methylation conditions; this may influence reproducibility of determination of even effective the parameter of IAA concentration. Thus, previously described methods used for auxin determination [7, 8] are not reliable analytical methods of quantitative differential determination of various forms of IAA. Time consuming methods of sample preparation cannot be recommended for serial assays.

Differential determination of various forms of IAA requires alternative solution of this problem: combined use of the additional ELISA system characterized by higher specificity to free IAA. The second analytical ELISA system, which has been tested in this study, is based on the immunoreagents BSA–ANAA and OV–ANAA, where NAA carboxyl group does not undergo esterification and is located opposite the protein globule in both immunogen and immobilized conjugate. Therefore, the second immunochemical system has significantly higher specificity to both NAA and free form of

Test for detection of IAA and its methyl ester at various ratios of these hormones in standard solution (number of analytical repeats, $N = 10$)

Initial concentration of IAA, nM	Initial concentration of methyl ester of IAA, nM	Detected concentration of IAA, nM	Detection of IAA, %	Detected concentration of methyl ester of IAA, nM	Detection of methyl ester of IAA, %
2.0	1.0	2.1	105	1.1	107
4.0	1.0	4.1	103	1.1	108
6.0	1.0	6.0	100	1.1	112
8.0	1.0	7.9	99	1.1	113
2.0	2.0	2.1	107	2.0	102
4.0	2.0	4.2	105	2.1	105
6.0	2.0	6.0	100	2.2	110
8.0	2.0	7.9	99	2.3	113
2.0	3.0	2.2	108	3.0	100
4.0	3.0	4.1	103	2.9	98
6.0	3.0	5.9	98	2.9	105
8.0	3.0	8.2	102	3.2	108
2.0	4.0	2.2	108	4.2	105
4.0	4.0	4.1	109	4.2	105
6.0	4.0	6.1	101	4.4	110
8.0	4.0	8.3	104	4.5	112
2.0	0	2.1	104	0	
4.0	0	4.1	101	0	
6.0	0	5.9	98	0	
8.0	0	7.8	98	0	
0	1.0	0		1.1	108
0	2.0	0		2.1	105
0	3.0	0		3.0	101
0	4.0	0		4.1	103

Note: Antigen detection (%) = (detected hormone concentration/true hormone concentration) × 100%.

IAA. Bound methylated form of IAA exhibited very low interaction with such antibody (against BSA–ANAA) (Fig. 2). Cross-reactivity of methyl ester of IAA and other metabolites (tryptophan, indolyl-3-acetaldehyde, indolyl-3-acetonitrile, indole) calculated by IAA did not exceed 1%. So this method can be used for quantitative determination of free form of IAA (up to 1.0 nM) without any significant influence of this assay by the reserve form of this hormone. In the whole concentration range studied, the relative error did not exceed 13% (Fig. 2).

Thus, combined use of two ELISA systems for IAA determinations omits stages of methylation and fractionation of a sample under study; this combination is effective for quantitative determination of both free IAA (the second ELISA system, Fig. 2) and its reserve form (the first system, Fig. 1). This is especially important for analysis of microquantities of plant material (ovary, ovule, embryo, etc.). The test for two ELISA systems for detection of IAA and its methyl ester using standard samples of IAA (2.0-8.0 nM) and its methyl ester (1.0-4.0 nM) differing by ratio of free and the methylated forms has shown significance of changes (table). In all cases, detection of IAA and its methyl ester was 98-109 and 98-113%, respectively. This suggests high significance of quantitative determination of the major natural forms of auxins using our approach.

This method for differential quantitative analysis of auxins *in situ* has been employed for determination of microquantities of various forms of IAA in wheat (amphimict) and dandelion (apomict). This was one of our studies on the problem of hormonal regulation of the reproduction process in angiospermous plants and search of putative hormonal differences in amphimicts (with fertilization) and apomicts (without fertilization) [11-13].

Besides analysis of wheat and dandelion ovaries by means of the new method, we also measured levels of free and bound IAA using traditional method employing methylation of the studied sample followed by hormone determination before and after methylation. Using our method, concentrations of free (4.8 ± 0.9 nM) and bound (11.7 ± 2.1 nM) forms of IAA in extracts of wheat ovules (1 : 100) were determined. Study of free and bound forms of IAA in dandelion ovules gave the values 7.8 ± 1.5 and 14.9 ± 3.0 nM, respectively. Use of traditional method for analysis of the same parameters gave similar values for both wheat (4.0 ± 1.9 and 11.5 ± 2.0 nM) and dandelion (6.9 ± 3.3 and 15.1 ± 3.1 nM) ovules. It should be noted that determination of free form of IAA by traditional method was characterized by insignificant decrease in IAA concentration in samples (compared with our method) and marked (1.5-2.0-fold) increase in standard error (compared with our method). This may be a possible source of wrong interpretation of experimental results. It should be noted that free form of IAA plays a key role in regulation of corresponding physiological processes.

Thus, we have demonstrated here the possibility of determination of various forms of endogenous IAA in microquantities of wheat and dandelion ovules; our method requires minimal sample preparation (homogenization in TPS containing 0.01 M sucrose) without the stage of purification and chemical modification of these hormones. In contrast to the traditional method, the new method described here is applicable for microanalyses of plant material. The data obtained can be used for comparative study of time course of ratio of various natural hormonal forms of auxins, differing by physiological activity in reproductive organs of angiospermous plants at different period of reproduction. Results of the present study are very interesting for modern plant physiology as a tool for studies of mechanism(s) of hormonal regulation of the reproductive processes in the angiosperms and specificity of this regulation in amphimicts and apomicts.

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