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USE OF INTERNAL REFLECTION SPECTROSCOPY FOR MAIZE (*Zea mays* L.) GRAIN DIAGNOSIS

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Abstract

Infrared (IR) spectroscopy and Raman spectroscopy (RS) are modern methods on the basis of which biotechnological approaches are being successfully developed that allow genetic and functional analysis of individual plant organs and tissues at the molecular level. In the present work, using surface internal reflection spectroscopy (SIR), which is a modification of IR spectroscopy, differences in the content and conformation of biomolecules in grain homogenates and its components (endosperm, pericarp, germ) of ZP 735 maize hybrids were first recorded and revealed. Our goal was to develop a methodology for surface SIR for the identification of organic molecules, their content and conformation in corn seed and its tissues. The grains of the corn hybrid (*Zea mays* L.) ZP 735 (originator Maize Research Institute, Zemun Polje, Belgrade, Serbia) served as the object of the study. Thirty grains were selected by random sampling. To obtain SIR spectra, samples of grains and their parts (endosperm, pericarp, and embryo) were crushed, homogenized, and placed in a special cell of the device. A spectrometer (FT-IR spectrophotometer, Thermo Scientific, USA) with diamond (diamond ATR crystal, Thermo Scientific, USA) was used to record the SIR spectra, and a software package (Thermo Scientific™) was used to analyze the spectra. The SIR spectra were compared in the data library of the Advanced ATR correction Algorithm program. The SIR spectroscopy method is based on the reflection of a light beam at the interface between two phases: the phase of a crystal with a high refractive index, which is part of the SIR device, and the phase of the sample under study with a lower refractive index. During the measurement, the light beam penetrates to a small depth into the phase of the sample, where it is partially absorbed. During subsequent irradiation of the sample, this phenomenon is repeated, and as a result, the SIR spectrum is recorded. It has been proven that in the range from 400 cm^{-1} to 4000 cm^{-1} ATR spectra of whole grain, endosperm, pericarp and embryo of the ZP 735 hybrid are similar to the previously obtained IR spectra of grain, which indicates the

possibility of using a new method for molecular breeding technologies. The location of the bands of the SIR spectrum characterizes various forms of vibrations of the valence bonds of the functional groups of the organic molecules of the seed, which makes it possible to identify not only the presence of certain molecules, but also their conformation. It has been proved that by analyzing the amplitude of the SIR spectrum bands (maximum intensity amplitude and high intensity amplitude), it is possible to control changes in the content of a number of organic compounds in seed tissues: proteins, lipids, sugars, esters, amides, ketones, aldehydes, carboxylic acids, simple ethers, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkenes, alkanes and alkynes. The obtained results are important for testing the presence of the necessary organic compounds in the grain or changes in their conformation during the selection process. The important advantages of SIR spectroscopy compared to IR spectroscopy include, on the one hand, the preservation of the nativeness of the object (the possibility of conducting research without fixing or staining the object) and simple sample preparation, on the other hand, an effective assessment of the content and conformation of molecules with high sensitivity (resolution about 1.0 cm^{-1}). The implementation of the developed methodology for the formation of molecular breeding technology will increase the profitability of cultivation and the efficiency of breeding not only corn, but also other agricultural plants.

Keywords: *Zea mays* L., hybrid, grain, endosperm, pericarp, embryo, surface internal reflection spectroscopy

At present, considerable attention is paid to the development of methodological techniques that allow one to control the content and conformation of various molecules in plant cells and tissues. Infrared (IR) spectroscopy and Raman spectroscopy (RS) are modern methods on the basis of which biotechnological approaches are successfully formed that allow genetic and functional analysis of plant organs and tissues at the molecular level, which is important for crop breeding [1-4]. Rapid molecular monitoring makes it possible to effectively evaluate the results of diagnostics and selection not only in the laboratory, but also in the field. In addition, these methods can serve as the basis for new technologies for prompt and qualified quality control of incoming raw materials for the manufacturing industry [5-7].

Previously, with the IR and Raman spectroscopy, important characteristics were obtained not only of the structure of individual molecules, but also of changes in their conformation (change in the proportion of characteristic vibrations of chemical bonds in molecules) [8-11]. For example, using Raman spectroscopy, we revealed changes in the content and conformation of carotenoid molecules in the chloroplasts of maize hybrids [7]. Using IR spectroscopy (range 3500-3000 cm^{-1}), it was found that in chloroplast molecules (water, carbohydrates, proteins) the proportion of vibrations of OH groups and intra- and intermolecular H-bonds was maximum in the ZPPL 186 line, and the proportion of vibrations The N−H groups of amides (proteins) turned out to be minimal in the ZPPL 225 line. ZPPL 186 chloroplasts were characterized by the maximum proportion of stretching vibrations from molecules of alkanes, carboxylic acids (range 2920-2860 cm^{-1}) and bending vibrations of aromatic structures (1000 cm^{-1}), and for the line M1-3-3-sdms, the fraction of stretching vibrations of O=C=O bonds (2300 cm^{-1}). Using Raman spectroscopy (regions 1250-500 cm^{-1} and 1535-1400 cm^{-1}) it was found that differences in chloroplasts in maize lines are associated with changes in the conformation of carotenoid molecules, and not cellulose. In all samples, except for ZPPL 225, carotenoid molecules were in the 15-trans form with different conformations of the polyene chain. It is important that the conformation of carotenoid molecules of the ZPPL 186 line is characterized by the minimum rotation of the molecular axis outside the plane of the polyene chain and more pronounced vibrations of the $C-CH_3$ side methyl group. It is assumed that carotenoids from the chloroplasts of the leaves of various maize lines do not interact with the aromatic amino acids of proteins [7].

The introduction of these methods for analyzing the conformation of molecules in plant tissues, along with molecular genetic technologies, contributes to

the formation of molecular breeding methods in agriculture [7].

In the present work, using surface internal reflection spectroscopy (ATR-IR), which is a modification of IR spectroscopy, for the first time, differences in the content and conformation of biomolecules in the grain, endosperm, pericarp, and germ of ZP 735 maize hybrids were recorded and revealed.

Our goal was to develop a methodology for surface internal reflection spectroscopy to identify the content and conformation of organic molecules in whole maize seed and its tissues.

Materials and methods. The grains of the corn hybrid (*Zea mays* L.) ZP 735 (originator Maize Research Institute, Zemun Polje, Belgrade, Serbia) served as the object of the study. Endosperm, pericarp, and grain germ were separated according to the described method [12]. Agronomic, morphological and chemical-physiological properties of the ZP 735 corn hybrid, including breeding, seed production and technological characteristics, are described in detail in M. Radosavljević et al. [13].

Thirty grains were randomly selected. To obtain internal IR-reflexion spectra (IRS), samples of whole grains and their parts (endosperm, pericarp, and embryo) were crushed, homogenized, and placed in a special cell of the instrument. The TER spectra were recorded using a spectrometer (Nicolet™ iS20 FTIR Spectrometer, Thermo Scientific, USA) with a diamond (diamond ATR crystal, Thermo Scientific, USA), and a software package (Thermo Scientific, USA) was used to analyze the spectra). The characteristics of the device were as follows: spectral range 3800-375 cm^{-1}, resolution about 1.0 cm^{-1}, signal-to-noise ratio more than 20000:1, ordinate linearity 0.07% T, wavenumber accuracy 0.01 cm^{-1}, maximum speed 40 scans/s. The spectra were recorded in 32-fold repetitions and identified using data libraries (more than 1500 compounds). The .ATR-IR spectra were compared in the data library of the Advanced ATR correction Algorithm program (Thermo Scientific, USA).

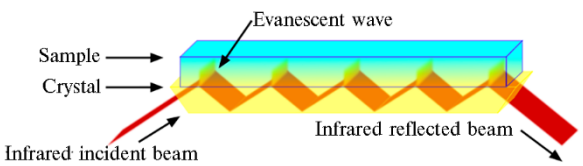

Fig. 1. Optical path of infrared beams in a crystal of IR total internal reflection (ATR-IR) **spectroscopy.**

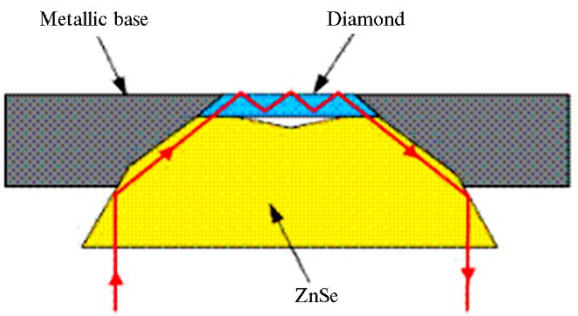

Fig. 2. Scheme of sample holder for IR total internal reflection (ATR-IR) **spectrometer.**

Results. In traditional IR spectroscopy, the spectrum of the emission of light transmitted through the sample is analyzed, in .ATR-IR spectroscopy, infrared radiation reflected from the surface of the sample is recorded. The ATR-IR spectroscopy method is based on the reflection of a light beam at the interface between two phases: the phase of a crystal with a high refractive index, which is part of the ATR-IR device, and the phase (homogeneous surface) of the sample under study with a lower refractive index. During the measurement, the light beam penetrates the sample to a shallow depth, where it is partially absorbed. During subsequent irradiations, this phenomenon is repeated, and as a result, the ATR-IR spectrum is recorded.

When analyzing the composition of a sample using ATR-IR spectroscopy,

the substance or object was placed on the surface of the crystal in the attachment of the ATR-IR spectrometer (Fig. 1). Further, IR radiation was directed through the crystal at a specially selected angle, the intensity of which was then recorded at the exit of the light beam from the crystal. It is important that the material of the crystal used for ATR-IR spectroscopy has a high refractive index (crystals of diamond and zinc selenide) (Fig. 2).

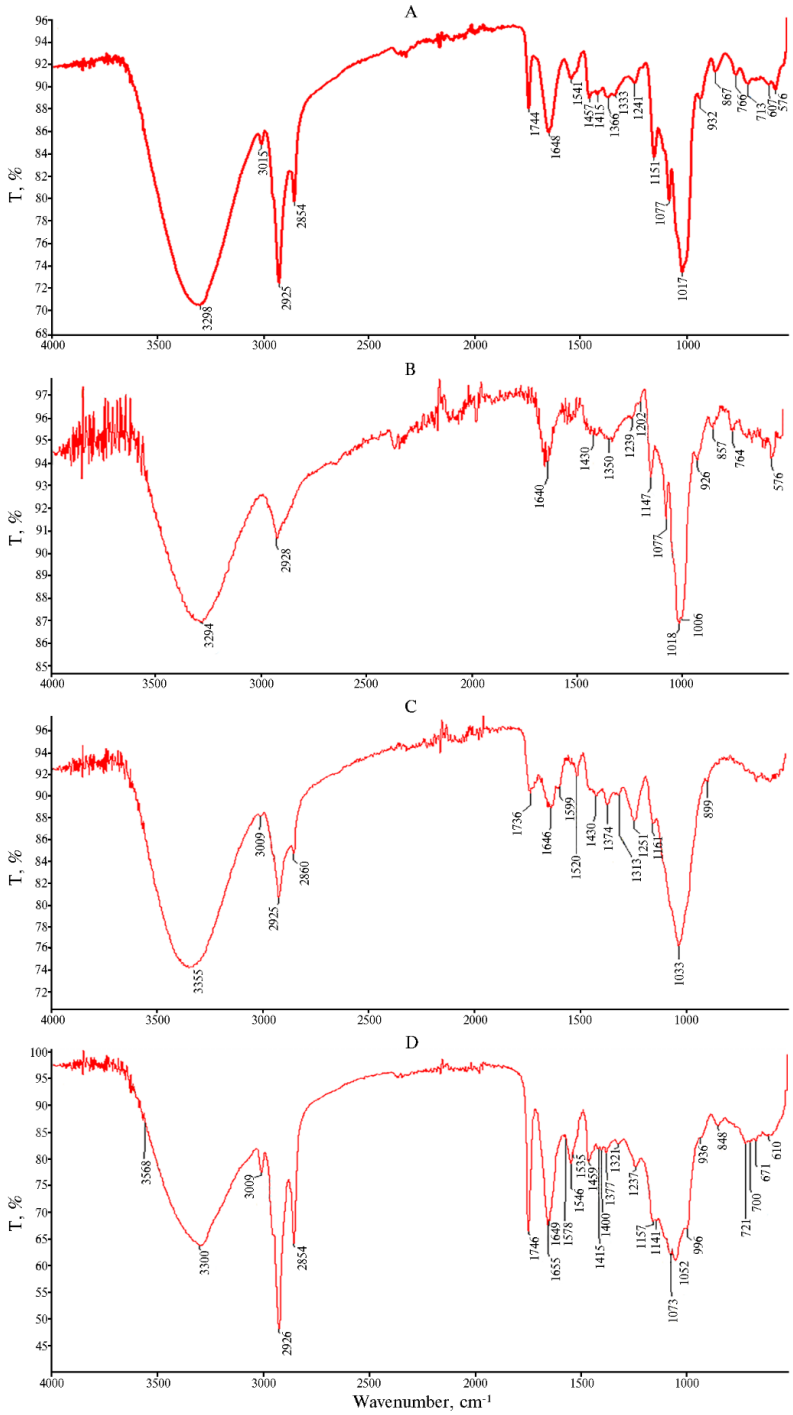

Fig. 3. ATR-IR spectra of whole grain homogenate (A)**, endosperm** (B)**, pericarp** (C)**, germ** (D) **of corn (*Zea mays* L.) hybrid ZP 735. T — transmittance (the ratio of the intensity of light passing through a**

sample to the intensity of light falling on the sample, i.e., the fraction of the incident light that passes through the test sample).

A significant advantage of ATR-IR spectroscopy is that it allows one to study opaque native samples, as well as to do without lengthy sample preparation of the object and to carry out analysis directly in the field. The proposed method makes it possible to assess the state (conformation) and the content of various biomolecules in a whole tissue (for example, a leaf, a root, etc.).

The grain of the hybrid maize ZP 735 is 84.75% endosperm, 5.30% pericarp, 9.95% germ [13]. The air defense spectrum of the ZP 735 hybrid maize grain homogenate (in the range from 400 cm^{-1} to 4000 cm^{-1}) (Fig. 3) was characterized by 20 bands, which differed from each other in intensity amplitude and frequency. All bands of the ATR spectrum both for the homogenate of the whole grain and for the homogenates of the endosperm, pericarp, and embryo were divided into four groups according to the amplitude of the maxima (T, %).

1. Characterization of ATR-IR spectrum bands of the homogenate of whole grain, endosperm, pericarp and embryo of the maize (*Zea mays* L.) hybrid ZP 735

Order of registration of spectral bands	Intensity, %	Wavenumber, cm^{-1}	Spectral bands of maximum and high intensity
		Whole grain	
1	20.8	3298	Proteins, lipids, carboxylic acids, sugars, esters, amides, ketones, aldehydes, nitro compounds, amines, ethers, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkynes, alkenes, alkanes
2	12.5	2925	
3	2.9	2854	
4	7.0	1744	
5	6.1	1648	
6	1.9	1541	
7	3.6	1457	
8	1.8	1413	
9	0.8	1366	
10	0.2	1333	
11	0.4	1241	
12	8.0	1151	
13	6.0	1077	
14	9.2	1017	
15	0.2	932	
16	1.8	867	
17	1.4	766	
18	0.8	713	
19	0.2	607	
20	0.2	576	
		Endosperm	
1	17.9	3294	Carboxylic acids, lipids, proteins, sugars, esters, amides, ketones, aldehydes, nitro compounds, amines, esters, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkynes, alkenes, alkanes
2	4.0	2928	
3	6.0	1640	
4	2.6	1430	
5	0.8	1350	
6	1.1	1239	
7	1.2	1202	
8	6.6	1147	
9	6.0	1077	
10	12.0	1018	
11	11.8	1006	
12	0.4	926	
13	0.4	857	
14	1.8	764	
15	1.6	576	
		Pericarp	
1	18.0	3355	Proteins, lipids, carboxylic acids, sugars, esters, amides, ketones, aldehydes, nitro compounds, amines, ethers, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkynes, alkenes, alkanes
2	0.2	3009	
3	7.0	2925	
4	0.2	2860	
5	5.0	1736	
6	2.4	1646	
7	0.2	1599	
8	1.8	1520	
9	2.0	1430	
10	2.0	1374	
11	2.5	1313	
12	4.0	1251	
13	10.0	1162	
14	11.0	1033	
15	0.2	899	

Continued Table 1

		Embryo	
1	5.0	3568	Proteins, lipids, carboxylic acids, sugars, esters, amides, ketones, aldehydes, nitro compounds, amines, ethers, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkynes, alkenes, alkanes
2	10.0	3300	
3	2.0	3009	
4	14.0	2926	
5	7.6	2854	
6	13.4	2746	
7	9.1	2655	
8	0.2	1648	
9	2.0	1578	
10	1.6	1546	
11	3.2	1538	
12	0.2	1459	
13	0.2	1415	
14	0.2	1400	
15	0.2	1377	
16	0.2	1321	
17	2.2	1237	
18	4.5	1157	
19	1.6	1141	
20	2.0	1073	
21	2.1	1052	
22	7.4	996	
23	2.0	936	
24	0.4	848	
25	0.2	721	
26	0.2	700	
27	0.2	671	
28	0.2	610	

Note. For all homogenates, the following vibrations of atoms in the molecule were revealed: symmetric and antisymmetric stretching (stretching); deformation planar scissor (scissoring); deformation planar pendulum (rocking); deformation out-of-plane fan (wagging); torsional out-of-plane deformation (twisting); trembling, valence bonds and molecular structures (trembling). The interpretation of the bands of the spectra obtained by us was carried out according to the published data [14-17].

In the whole grain, the maximum intensity amplitude (20% > T > 6%) was noted at 3298, 2925, 1744, and 1151 cm^{-1}, high (6% > T > 3%) at 1648, 1457 и 1077 $см^{-1}$, low (3.0% > T > 1.0%) at f2854, 1541, 1413, 867 and 766 cm^{-1}, and very low (1.0% > T > 0.2%) at 1366, 1333, 1241, 932, 607 and 576 cm^{-1} (Tables 1, 2).

The recorded ATR-IR spectra of the endosperm homogenate, pericarp, and embryo differed from the ATR-IR spectra of the grain homogenate both in intensity and in the frequency of specific bands. It was established that the amplitude of the intensity of the bands of the endosperm air defense spectrum was maximum at 3294, 1147, 1018 and 1006 cm^{-1}, high at 2928, 1640, 1147, 1077, 1018 and 1006 cm^{-1}, low at of 1430, 1239, 1202, 764 and 576 cm^{-1} and very low at of 1350, 926 and 857 cm^{-1} (see Fig. 3, B, Tables 1, 2). The intensity of the bands in the ATR-IR spectrum of the pericarp homogenate was maximum at of 3355, 2925, 1162, and 1033 cm^{-1}, high at of 1736 and 1251 cm^{-1}, low at of 1646, 1520, 1430, 1374, and 1313 cm^{-1}, and very low at 3009, 2860, 1599, and 899 cm^{-1} (see Fig. 3, C, Tables 1, 2). The intensity of the bands of the air defense spectrum of the embryos of the hybrid maize ZP 735 was maximum at 3300, 2926, 2854, 2746, 2655, and 996 cm^{-1}, high at 3568, 1538, 1157, and 996 cm^{-1}, low at 1578, 1538, 1073, 1052, and 936 cm^{-1}, and minimum at 1648, 1459, 1415, 1400, 1377, 1321, 848, 721, 700, 671 и 610 cm^{-1} (see Fig. 3, D, Tables 1, 2).

It is important that earlier in our works, vibrations of the valence bonds of the functional groups of organic molecules were also revealed in the classical IR spectra of whole grains [18, 19].

Thus, we have established that with the help of ATR-IR spectroscopy based not on absorption, but on the reflection of a light beam at the interface between two phases (crystal and biological object), it is possible to record spectra that allow not only to analyze the conformation of molecules of various substances, but and to determine their presence and concentration in seed tissues. For the first

time, the structure (a set of bands) of the ATR-IR spectrum of the grain homogenate of the hybrid maize ZP 735 and its constituent tissues was described in terms of the amplitude of the characteristic bands. These data are proposed to be used to study the content and conformation of various molecules of substances in the tissues of seeds according to the ATR-IR spectra. Such results are important for determining the presence of the necessary organic compounds in the grain or assessing changes in their conformation during the selection process.

2. Vibrations of the valence bonds of organic molecules in the homogenate of whole grain, endosperm, pericarp and embryo of the corn (*Zea mays* L.) hybrid ZP 735 revealed by ATR-IR spectroscopy

Wavenumber, cm^{-1}				Forms of vibrations of valence bonds of functional groups of organic molecules
whole grain,	endosperm	pericarp	embryo	
				Alcohols ((O—H)
				Amines (N—H), (C—H)
				Alkynes (C≡N), (C≡C)
				Ketones (=C=O)
				Alkenes (C=C)
				Esters (O—CH_2—)
				Lipids (C=O)
				Carbonyl groups (C=O) (esters)
				Amides (N—CH_2—), (—CO—N=)
				Amino groups (—NH—)
				Primary amines (—$CONH_2$)
				Carboxyl groups (—CO_2H)
				Imides (—CO—N—CO—)
				Acid chlorides (—COCl)
				Nitrite (—C≡N)
				Amides (N—CH_2—)
				Carbonyl groups (C=O) (amides)
				Aliphatic carbon-hydrogen bonds
				Aldehydes (—CHO)
3298	3294	3355	3568	
2925	2928	3009	3300	
2854	1640	2925	3009	
1744	1430	2860	2926	
1648	1350	1736	2854	
1541	1239	1646	1746	
1457	1202	1599	1655	
1415	1147	1520	1648	
1366	1077	1430	1578	
1333	1018	1374	1546	
1241	1006	1313	1535	
1151	926	1251	1459	
1077	857	1161	1415	
1017	764	1033	1400	
932	576	899	1377	
867			1321	
766			1237	
713			1157	
607			1141	
576			1073	
			1052	
			996	
			936	
			848	
			721	
			700	
			671	
			610	

N o t e. The interpretation of the bands of the obtained spectra was carried out according to the published data [14, 16, 17].

The data presented by us indicate that with the ATR-IR spectroscopy it is possible to differentiate the state and content of molecules of substances in tissues not only in the laboratory, but also in field tests. The analysis of the ATR-IR spectra by two parameters — the maximum and high amplitude of the bands, makes it possible to found out changes in the content and conformation of various molecules in the tissues of the seeds, while the bands with low and very low

intensity amplitudes, which we noted in the ATR-IR spectra of grain of ZP 735, probably allow only detection of the presence of molecules present in seed tissues in low concentrations (trace amounts).

It is obvious that the new method for studying seeds proposed by us requires a simpler sample preparation compared to IR spectroscopy and, at the same time, allows us to study changes in the conformation and content of individual biomolecules in the whole tissue with high sensitivity. Previously, studies of the semen homogenate were carried out using IR spectroscopy [18, 19]. We proved that the bands of the IR spectra of seeds of corn hybrids are similar to the set of bands of the IR spectra of pure lines of corn: the vibrations of the valence C−H bonds of alkenes and saturated hydrocarbons correspond to the set of bands at 2852 cm^{-1}, 2926 cm^{-1} and 995 cm^{-1}, valence O−H bonds to a set of bands 1161 cm^{-1} and 1082 cm^{-1} of secondary and tertiary alcohols, and vibrations of valence C=O bonds of proteins amide I and amide II to a set of bands 1651 cm^{-1} and 1541 cm^{-1} [20, 21]. In this work, it was found that the bands of the ATR-IR spectra of ZP 735 corn seed homogenates are similar to the set of IR bands: the vibrations of the valence C−H bonds of alkenes and saturated hydrocarbons corresponded to the set of bands at 2854 cm^{-1}, 2925 cm^{-1}, and 932 cm^{-1}, vibrations of valence O−H bonds to a set of bands at 1151 cm^{-1} and 1077 cm^{-1} of secondary and tertiary alcohols, and vibrations of valence C=O bonds of amide I and amide II of proteins — to a set of bands at 1648 cm^{-1} and 1541 cm^{-1}. Using another method, vibronic spectroscopy (Raman spectroscopy), additional signals were detected in the Raman spectra of corn seeds, namely, bands characteristic of the carotenoid molecule (960, 1006, 1156, and 1520 cm^{-1}) corresponding to C−C stretching vibrations. bonds and delocalization of π-electrons in a molecule. It was proved that the structure of carotenoid molecules in hybrids is not the same. The minimum length of the polyene chain of seed carotenoids was found in ZP 335. In other hybrids, this parameter is almost identical: in seeds of pure lines, the I_{1520}/I_{1156} ratio varied from 1.5 (ZP 186, ZP 225) to 1.9 (M1-3-3-sdms). Using Raman spectroscopy, it was found that the carotenoids of whole seeds ZP 341 have the lowest I_{960}/I_{1006} ratio among hybrids, and it is similar to that for seeds of lines ZPPL 186 and ZPPL 225. The ratio I_{1156}/I_{1190} in the Raman spectrum of carotenoids was similar in of all the hybrids studied, and the maximum value of I_{1120}/I_{1190} was revealed in the seeds of ZP 434. The latter probably indicates a high proportion of carotenoids associated with chlorophylls in the seed [18]. Note that all these studies did not provide data on the composition and conformation of metabolites in different tissues of the seed, which is important for breeding and genetics.

Next, we compare some of our results on the band amplitudes for different maize seed tissues using IR and ATR-IR spectroscopy. Using IR spectroscopy, it was found that for the seeds of the ZP 735 hybrid, the amplitude of the 1017 cm^{-1} band is maximum in the endosperm homogenate and minimum in the embryo homogenate. Probably, in this region of the IR spectrum (1017-1054 cm^{-1}), the band maxima are due to aromatic CH-planar bending vibrations [20, 21]. In this work, it was also found that the amplitude of the 1018-1052 cm^{-1} band of the ATR-IR spectrum is maximum in the endosperm and minimum in the embryo homogenate. The amplitude of the band at 1648 cm^{-1} in the IR spectrum was maximum in the endosperm and minimum in the embryo homogenate. Probably, these bands are due to C=O stretching vibrations in amides, N−H and C−N bending vibrations in secondary amides of proteins, peptides, and free amino acids, and also characterize vibrations of the OH group of crystalline cellulose water [20-

22]. In the present study, the amplitude of the band at 1640 cm^{-1} of the ATR-IR spectrum was also maximal in the endosperm and minimal in the embryo homogenate.

As is known, their functional activity is associated with the conformational state of photosynthetic pigments [23, 24], which is important, in particular, for selection. Of the spectroscopy methods, IR spectroscopy is most often used to identify and study organic compounds in biological objects [25]. In addition, it should be taken into account that IR exposure has become one of the most common methods for processing plant materials [26, 27], which requires an in-depth study of its physicochemical effects.

It should be noted that in the spectroscopy of surface internal reflection, it is not the absorption of IR radiation of light that is recorded, but its reflection by the sample. Therefore, one of the advantages of ATR-IR spectroscopy is that, unlike IR spectroscopy in which light must pass through the sample, for surface internal reflection spectroscopy, the thickness of the sample does not matter. In addition, in IR spectroscopy, additional preparation is usually needed to obtain a useful spectrum: homogenized grain samples are rolled into a tablet with potassium bromide (KBr, in a ratio of components 1:100), while this is not required in ATR-IR spectroscopy. The proposed approach and the original method will provide a screening study program not only for corn, but also for other plants during selection, diseases, and also when analyzing the impact of extreme environmental factors on plants.

Thus, in the wavenumber range from 400 to 4000 cm^{-1}, all known bands obtained by infrared (IR) spectroscopy were revealed in the spectra of surface internal reflection (SIR) of the whole grain, endosperm, pericarp, and embryo of the ZP 735 hybrid corn. By detecting the bands of the ATR-IR spectrum with maximum and high intensity amplitude, it is possible to control changes in the content of a number of organic compounds (proteins, lipids, sugars, esters, amides, ketones, aldehydes, carboxylic acids, ethers, phenols, alcohols, aromatic hydrocarbons, acyclic compounds, alkenes, alkanes and alkynes) in the tissues of the seed. Important advantages of the ATR-IR spectroscopy method compared to classical IR spectroscopy include, on the one hand, simple sample preparation, which does not affect the physicochemical properties of the sample, and, on the other hand, an effective assessment of the content and conformation of molecules with high sensitivity (resolution of the order of 1.0 cm^{-1}. The implementation of the developed methodology for the formation of molecular breeding technology will increase the profitability of cultivation and the efficiency of breeding not only corn, but also other agricultural plants.

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