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USING INFRARED SPECTROSCOPY AND RAMAN SPECTROSCOPY TO EVALUATE THE CONFORMATION OF BIOMOLECULES IN MAIZE (*Zea mays* L.) LINES

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Abstract

Currently, there are few non-invasive methods that allow you to control the content and conformation of molecules in plant cells and tissues not only in the laboratory but also in the field. Infrared microscopy and Raman spectroscopy (IR and Raman spectroscopy) are actively used to analyze the role of molecules of certain substances in crop breeding. Using methods of vibronic spectroscopy, we investigated changes in the content and conformation of chloroplast molecules of various maize lines. Using infrared (IR) spectroscopy (3500-3000 cm^{-1}) it was found that in the chloroplasts, the proportion of vibrations of OH-groups and intramolecular and intermolecular H bonds is maximum for the maize line ZPPL 186, and vibrations of NH-groups of amides (proteins) is minimal for ZPPL 225. It has been proven that ZPPL 186 chloroplasts are characterized by the maximum proportion of stretching vibrations from alkane molecules, carboxylic acids (region 2920-2860 cm^{-1}) and deformation vibrations of aromatic structures (band at 1000 cm^{-1}), and for the line M1-3-3-sdms, the fraction of stretching vibrations of O=C=O bonds (band at 2300 cm^{-1}) is characteristic. Using Raman spectroscopy (ranges of 1250-500 cm^{-1} and 1535-1400 cm^{-1}), it was found that differences in the chloroplasts of different maize lines are associated with changes in the conformation of chloroplast carotenoid molecules (rather than cellulose molecules). It was found that in two samples (except ZPPL 225), carotenoid molecules are in the 15-trans form with different conformation of the polyene chain. We note that the conformation of carotenoids of the ZPPL 186 line is characterized by a minimum amount of rotation outside the plane of the polyene chain and has more pronounced vibrations of the lateral CH_3-group. It was assumed that carotenoids of leaf chloroplasts of various maize lines lack interactions with aromatic amino acids of proteins. According to the authors, the combined use of IR and Raman spectroscopy of the leaf chloroplast fraction can be recommended for monitoring the content and conformation of biomolecules in maize breeding.

Keywords: *Zea mays* L., inbred line, leaf, chloroplast, Raman spectra, infrared spectra, conformation changes, carbohydrates, carotenoids, proteins, maize breeding

The growth of the world's population increases the requirements for yields

and profitability of agricultural products, which can be achieved through fundamental research that allows the development of effective analytical methods for use in plant breeding [1-3]. Currently, there are few non-invasive methods that allow you to control the content and change in the conformation of molecules of bioactive organic compounds in plant cells and tissues not only in laboratory but also in the field. Infrared microscopy and Raman spectroscopy (IR and Raman spectroscopy) are actively used to analyze the role of molecules of certain substances in crop breeding [3-6]. In most cases, measurements using these methods can be carried out in the field directly on plant tissues, as well as on fractions isolated from the plant material. Both methods of vibronic spectroscopy make it possible not only to record spectra, the bands of which characterize the content and conformational changes of certain molecules [7-9], but also to distinguish plant genotypes based on these data [1, 2]. The ability to quickly monitor the amount and physicochemical state of bioactive organic compounds and cellular compartments (for example, carotenoids and chloroplasts) in plant leaf homogenates and to identify the correlation of these parameters with economically significant traits would make it possible to use the parameters recorded by spectroscopy as markers for assessing the agronomic efficiency of the studied forms in plant breeding. In addition, these methods can be used in the manufacturing industry to quickly check the quality of incoming raw materials, as well as for control in continuous production [10-12]. Based on the analysis of IR spectra in plants, additional characteristics were obtained not only of the structure of various molecules, but also modifications of their conformation (by changing the proportion of characteristic vibrations of chemical bonds in molecules) [13-15].

Unlike IR spectroscopy, Raman spectroscopy makes it possible to monitor changes in the structure of molecules in cells and tissues without drying the preparations [3, 12, 16-18]. Using this method, the features and differences in the distribution of protein and pigment molecules in plant cells in different hybrids and lines of maize are described. It is worth noting that this approach makes it possible to reveal the presence of a positive correlation between the change in chlorophyll fluorescence in chloroplasts and the content and structure of carotenoid molecules in whole leaves of maize lines and hybrids [3, 18].

It is obvious that the formation of a methodology for studying the functional state of a plant using spectral methods will reveal new molecular mechanisms that can be used in the breeding of maize.

In this work, it has been proved that the combined use of IR and Raman spectroscopy of a plant leaf can be recommended as a minimally invasive method for monitoring the content and possible differences in the conformation of biological molecules when testing lines and hybrids of maize.

The aim of this work was to comprehensively analyze changes in the content and conformation of protein, carbohydrate and pigment molecules in leaf chloroplasts in maize inbred lines by IR and Raman spectroscopy.

Materials and methods. Maize (*Zea mays* L.) lines ZPPL 186, ZPPL 225, and M1-3-3-sdms (originated by Maize Research Institute, Zemun Polje, Belgrade, Serbia) were used in the study. The lines possess high grain quality, yields and are adapted to cultivation technologies [1, 3, 12]. Thirty seeds of each line (weighing 313±9, 382±17 and 196±5 mg, respectively), after treatment with hydrogen peroxide for 30 min and washing with water, were incubated in a Petri dish at 22 °C with constant non-contact wetting until the appearance of roots with a length of at least 5 mm. The germinated seeds were placed in the ground and grown at 16-hour day length until the three-leaf stage (laboratory test). The quality of the seeds was assessed by germination rate (the ability of seeds to germinate and give normally developed seedlings under certain conditions within the accepted time frame; GOST 13056.6-75) and

germination energy (the ability of seeds to germinate in a shorter period of time, from 1 to 15 days; GOST 13056.6-75).

To isolate chloroplasts, cooled (0-4 °C) leaves (5 g portions, samples were kept in a polyethylene bag or wet filter paper) were separated from the veins, chopped with scissors and homogenized at 0-4 °C three times (10 s each) in a buffer (0.04 M sucrose, 20 mM Tris-HCl, pH 7.8, 35 mM NaCl, 1 mM EDTA) chilled to 4 °C. The homogenate was filtered through four layers of nylon and centrifuged (1500 rpm, 5 min, 0-4 °C). The supernatant was poured into precooled tubes and centrifuged again (5000 rpm, 44 °C for 10 min). The supernatant was discarded; the resulting pellet was resuspended in 5 ml of isolation medium. Glycerin (30% of the volume obtained) was added and frozen at –73 °C. Before recording the Raman and IR spectra, the suspension was thawed and diluted with a buffer (15 mM NaCl, 400 mM sucrose, 50 mM Mes-NaOH, pH 6.5) at a ratio of 10 μl of suspension per 5 ml of buffer [19, 20].

Cellulose was isolated from maize leaves according to the description [21] using 3% sodium hypochlorite solution, 5% hydrogen peroxide solution and a mixture of 3% sodium hypochlorite solution and methanol.

Raman scattering of carotenoids in leaf chloroplasts was recorded using a DFS 24 Raman spectrometer (JSC LOMO, Russia) with a laser (Ciel, Eurolaser GmbH, Germany) (wavelength 473 nm), registration system MORS 1/3648 (LLC MORS, Troitsk, Russia) based on a linear CCD matrix TCD1304DG (Toshiba, Japan) with an LPO2-473RS-50 filter (Semrock, USA). The laser power on the sample was 3 mW, the signal registration time was 10 s [23].

Fourier-transform infrared spectroscopy (FTIR spectrometry) in the range of 400-4000 cm^{-1} was carried out using an IR-Prestig 21 IR spectrometer (Shimadzu Corp., Japan) with a measurement step of 4 cm^{-1}. Before the experiment, the leaf homogenate suspension (see above) was thawed and diluted with buffer (15 mM NaCl, 400 mM sucrose, and 50 mM Mes-NaOH, pH 6.5; 10 μl suspension per 5 ml buffer), dried and rolled into a tablet with bromide potassium (KBr) (mixing ratio 1:50) [12, 23].

The results were statistically processed using Microsoft Excel 2013 (Microsoft Corp., USA) and Statistica v.10 (StatSoft, Inc., USA). The primary processing of the Raman and IR spectra was carried out using the Origin Pro 2017 package (OriginLab Corp., USA). Statistical hypotheses were tested using a nonparametric Kruskal-Wallis H-test for a set of independent variables at the significance level of $p = 0.05$ ($n = 10$).

Results. The objectives of our study included recording the IR spectra of chloroplasts in three maize lines and analyzing the differences between them for protein and carbohydrate molecules, as well as performing additional Raman spectroscopy to detect changes in carotenoids contained in chloroplasts. In the IR spectra of chloroplasts in the range 4000-400 cm^{-1}, bands were found due to vibrations of bonds in the molecules of a number of organic compounds, including cellulose, proteins, carbohydrates, ethers, phenols (Fig. 1).

It was found that in the IR spectrum of chloroplasts in the region of 3700-3100 cm^{-1} there were bands of vibrations of hydroxyl groups, and in the region of 1500-900 cm^{-1} to vibrations of C–H, C–O and O–H groups of glycosidic bonds and glucopyranose ring of cellulose molecules [13-15]. Thus, the 3340 cm^{-1} band of the IR spectrum characterizes the O–H and CH_2–OH vibrations of cellulose bonds, the 2900 and 1374 cm^{-1} bands are characteristic of the C–H group bending vibrations, and the 1170 and 1059 cm^{-1} bands of C–O–C and C–OH groups, respectively. In the IR spectrum of the leaf chloroplasts, there was a band at 3414 cm^{-1} caused by stretching vibrations of hydroxyl groups and a band at 2904 cm^{-1} which characterizes the C–H vibrations of methylene and methine

groups of molecules. The IR spectrum also revealed a band at 1654 cm^{-1}, due to H−O−H vibrations of crystallized water, a band at 1375 cm^{-1} caused by deformation vibrations of the C−H bond, and a band at 1317 cm^{-1} of bending vibrations of CH_2-groups. The IR band at 1165 cm^{-1} is the stretching vibrations of the C−O −C bond, but it is also referred to as the bending vibrations of C−O or O−H in the C−OH groups. The IR band at 1085 cm^{-1} is the vibrations of the C−O−C bond in the glucopyranose ring. The band at 1058 cm^{-1} is the stretching vibration of the C−O bond in the C_3H−OH group, and the bands at 796 and 777 cm^{-1} correspond to vibrations of the glucopyranose ring associated with vibrations of CH- and CH_2-groups. The bands identified in the IR spectra in the range of 1500-1650 cm^{-1} correspond to proteins.

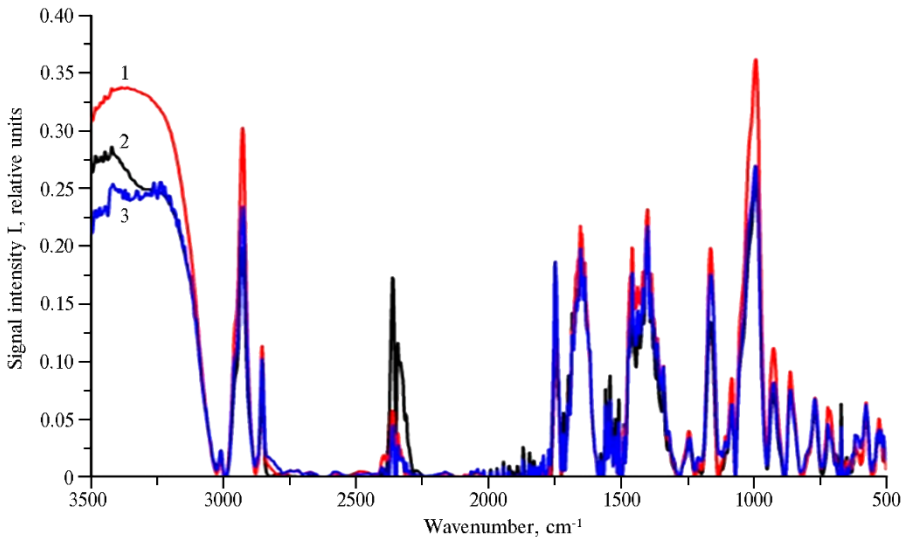

Fig. 1. **IR spectrum** (Fourier transform infrared spectroscopy) **of maize (*Zea mays* L.) leaf chloroplasts at the three-leaf stage, the lines ZPPL 186** (1)**, M1-3-3-sdms** (2), **and ZPPL 225** (3) (originator is Maize Research Institute, Zemun Polje, Belgrade, Serbia) (laboratory test). Fig. 1 shows typical normalized IR spectra.

Vibrations of free and bound OH-groups, intra- and intermolecular H-bonds, stretching vibrations of N−H bonds in primary and secondary protein amides, vibrations of hydroxyl OH-group (water, carbohydrates, amino acids), and stretching vibrations of NH-group (proteins, amino acids and their derivatives) are maximum for the leaf chloroplasts of ZPPL 186 line and minimum for the ZPPL 225 line (3500-3000 cm^{-1} region). Leaf chloroplasts of the ZPPL 186 line are also characterized by the maximum contribution of stretching vibrations of alkanes, carboxylic acids (range 2920-2860 cm^{-1}) and bending vibrations of bonds in aromatic compounds (band at 1000 cm^{-1}), the line M1-3-3-sdms shows vibrations of O−C−O bonds (band 2300 cm^{-1}). Thus, we have revealed differences in the composition of protein and carbohydrate molecules in chloroplasts of leaves of different maize lines. These differences may be due to the synthesis of new molecules of cellulose, proteins, carbohydrates, ethers, phenols. For example, a higher content of molecules of alkanes, carboxylic acids, and aromatic compounds in chloroplasts of a leaf was characteristic of ZPPL 186.

However, by using the IR spectroscopy, we were unable to reveal differences in the region from 1100 to 1600 cm^{-1} (see Fig. 1). It is known that this region is characteristic of symmetric and antisymmetric stretching vibrations of atoms, as well as in-plane and out-of-plane bending vibrations of molecules [14, 15]. Possible candidates for such differences could be cellulose and carotenoid

molecules from leaf chloroplasts. The content of starch, fiber and carotenoids in the tissues of the leaves of the studied maize lines is high [22]. Therefore, in the next series of experiments, we obtained IR spectra of cellulose, which had bands in the region of 4000-2500 cm^{-1} (stretching vibrations of O−H and C−H bonds) and bands in the region of 1500-500 cm^{-1} (stretching vibrations of C−C, C−O bonds and deformation vibrations of C−H and O−H bonds). The range of vibrations of OH-groups included three bands corresponding to free hydroxyl groups (at carbon atoms C2 and C6) and an OH-group, which are involved in hydrogen bonds (Fig. 2).

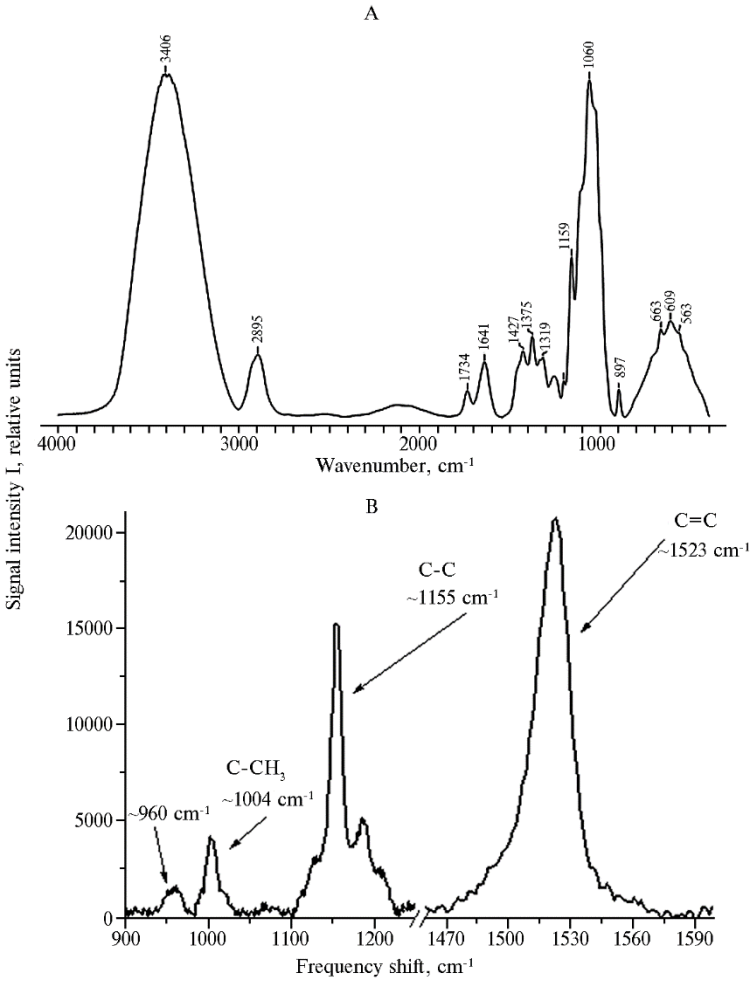

Fig. 2. IR spectra (Fourier transform infrared spectroscopy, A) **of cellulose from leaf tissue and Raman spectra (RS) of carotenoids in chloroplasts of leaves** (B) **in maize (*Zea mays* L.) line ZPPL 186** (originator is Maize Research Institute, Zemun Polje, Belgrade, Serbia) **at the three-leaf stage** (laboratory test)**.** Fig. 2 shows typical normalized IR and RS spectra.

Intramolecular hydrogen bonds between hydroxyl groups at the C2 and C6 positions (as well as at C3) are formed even in the presence of small number of OH-groups. In the IR spectrum in the range of 1500-900 cm^{-1}, we recorded the total intensity of vibrations of three hydroxyl groups of each glucopyranose unit of a cellulose molecule (C−H, C−O. and O−H vibrations). The bands at 1375 and 1319 cm^{-1} characterized the presence and manifestation of bending vibrationsof C−H- and CH_2-groups, respectively, in the cellulose molecule [8, 14]. However, using the IR spectroscopy, we were unable to detect differences

in the investigated region of wavenumbers for cellulose molecules.

In the next series of experiments, we used Raman spectroscopy to refine the results of IR spectroscopy in the 800-1800 cm^{-1} region. It is important that it is the maize leaf chloroplasts' Raman spectra in this region that are characterized by high-amplitude bands of carotenoids and low-amplitude or combined bands of cellulose molecules (the band at 1095 cm^{-1} corresponds to C–O–C vibrations, at 1477 cm^{-1} to H–C–H groups of atoms of the cellulose molecule). Thus, using Raman spectroscopy in the 800-1800 cm^{-1} range, one can additionally investigate the conformation of carotenoid molecules in chloroplasts (see Fig. 2, Table) [24-27]. In the Raman spectra, the 1523 cm^{-1} band originates from vibrations of the C=C bonds in the carotenoid molecule, the 1155 cm^{-1} band from the C–C bonds. In the case of the trans-conformation of double bonds in the carotenoid molecule, the band at 1155 cm^{-1} changes and acquires two pronounced arms, at 1190-1193 and 1210 cm^{-1}. The band at 1004 cm^{-1} corresponds to vibrations of the side methyl group C–CH_3. The 960 cm^{-1} band arises from out-of-plane C–H vibrations in the C–C bond. An increase in the intensity of this band is observed when the planar configuration of the molecule is disturbed: the greater the amount of pigment bound to the protein, the less pronounced out-of-plane twists between the C11 and C12 and, as a consequence, the intensity of the indicated band in the Raman spectrum.

The intensity (I) and band intensity ratios in Raman spectra of carotenoids in chloroplasts of leaves in the studied maize (*Zea mays* L.) lines at the three-leaf stage (n = 10)

Frequency shift, cm^{-1} (M±SD)	Mean I value (M)	Standard deviation (±SD)	Characteristic bands	
			positions	intensity ratios
Line M1-3-3-sdms				
1523.6±0.1	132.5	9.6	962/1006	0.45
1155.6±0.9	148.9	10.9	1006/1157	0.34
1191.3±0.4	46.7	3.7	1157/1190	3.18
1004.9±0.9	51.9	4.2	1006/1525	0.39
960.5±1.3	23.5	1.9	1526/1157	0.88
Line ZPPL 186				
1523.0±0.2	213.6	26.9	962/1007	0.38
1155.8±0.7	195.7	23.5	1007/1158	0.28
1190.4±0.4	55.4	7.3	1158/1190	3.53
1004.0±0.6	55.3	4.5	1007/1526	0.25
960.1±0.5	21.5	2.2	1526/1158	1.09
Line ZPPL 225				
1523.8±0.3	118.2	13.6	960/1005	0.48
1155.2±0.6	132.1	21.2	1005/1157	0.36
1189.7±0.9	43.4	6.7	1157/1189	3.04
1004.6±0.4	47.7	5.6	1005/1526	0.40
960.7±0.3	23.0	2.4	1526/1157	0.89

To normalize the contribution of each type of bonds to the Raman spectra of carotenoids, the ratio of the peak values is used, choosing, as a rule, a constant amplitude Raman band, the changes in which are minimal (intramolecular marker) [23]. Our results indicate that carotenoids in chloroplasts of different maize lines can be in different conformation. Thus, the conformation due to the length of the polyene chain of carotenoid molecules (the value is proportional to I_{1523}/I_{1155}) in leaf chloroplasts of maize lines can vary from 0.88 (M1-3-3-sdms) to 1.09 (ZPPL 186), and in two maize lines (except for ZPPL 225) carotenoid molecules are in the 15-trans conformation. With this conformation, the 1155 cm^{-1} band of the Raman spectrum has one pronounced shoulder at 1190 cm^{-1}. Note that the carotenoids in the leaf chloroplasts of the ZPPL 186 line had the minimum I_{960}/I_{1006} ratio which indicates an insignificant change in conformation caused by rotation of the carotenoid molecule outside the plane of the polyene chain, or the absence of such a change. In the ZPPL 186 line, the carotenoid molecule was characterized by more pronounced vibrations of the side CH_3-group.

It is known that the carotenoid bound to proteins of the light-harvesting complex of photosystem II has a characteristic intense band at 960 cm^{-1} in the Raman spectrum, which practically does not differ in amplitude from the bands at 1156 and 1004 cm^{-1} [24-27]. Note that in our experiments the amplitude of the band at 960 cm^{-1} of the Raman spectrum of carotenoids was significantly less than the amplitude of the bands at 1156 and 1004 cm^{-1} and did not differ in the Raman spectrum of chloroplasts in different lines, which probably indicates the absence of protein-lipid interactions.

In our opinion, the combined use of IR and Raman spectroscopy of the leaf chloroplast fraction can be recommended as a minimally invasive method to control the content and possible differences in the conformation of biological molecules when testing maize lines and hybrids [28-30].

So, using the methods of vibronic spectroscopy, we studied the changes in the content and conformation of chloroplast molecules in different lines of maize. Infrared (IR) spectroscopy (range 3500-3000 cm^{-1}) showed that in the molecules contained in the chloroplasts of the leaf (water, carbohydrates, proteins), the proportion of vibrations of OH-groups and intra- and intermolecular H-bonds is maximal in the maize line ZPPL 186, and the vibrations of NH-groups of amides (proteins) are minimal in the ZPPL 225 line. It was proved that the ZPPL 186 line is characterized by the maximum fraction of vibrations from alkanes, carboxylic acids (region 2920-2860 cm^{-1}) and deformation vibrations of aromatic structures (band at 1000 cm^{-1}) of chloroplasts, and for the M1-3-3-sdms line, the fraction of O=C=O bond vibration (band 2300 cm^{-1}) are typical. Using Raman spectroscopy (ranges 1250-500 cm^{-1} and 1535-1400 cm^{-1}), it was found that the differences in the studied maize lines are associated with changes in the conformation of carotenoid molecules in chloroplasts, but not cellulose molecules. It was found that in two samples (except for ZPPL 225), carotenoid molecules are in the 15-trans form with different conformation of the polyene chain. Note that the conformation of carotenoids of the ZPPL 186 line is characterized by the minimum rotations outside the plane of the polyene chain, while more pronounced vibrations of the side CH_3-group appear. It was suggested that in the studied lines, the carotenoids of leaf chloroplasts do not interact with the aromatic amino acids of proteins.

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