Mass spectrometric analysis of the carbon isotope composition in plant leaves via sample gasification using oxidation by yeast

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Abstract. The paper describes a new developed method of sample gasification using yeast oxidation of monosaccharides in plant tissue for \(^{13}\)C/\(^{12}\)C isotope ratio mass spectrometric analysis. Variations in the carbon isotopic composition of the leaf surface have been shown; the \(\delta^{13}\)C value for chlorophytum varies from -37 ‰ to -32 ‰ from the tip of the leaf to its base. It has been shown first that the spectral composition of radiation affects the ratio of carbon isotopes in plant leaves: the predominance of blue component in the spectrum results in accumulation of heavy \(^{13}\)C isotope up to \(\delta^{13}\)C = -25 ‰.

1. Introduction

Carbon isotope fractionation is observed in a number of key metabolic reactions in plants [1-3]. Differences in the properties of isotopes (atomic masses, the electronic term positions, and the nucleus magnetic moment) determine a wide range of isotope effects in plant life-sustaining activity [4]. The following processes can be distinguished leading to the isotope distribution between fractions of a substance: 1) isotope exchange reaction; 2) kinetic isotope effects; 3) diffusion [5]. Kinetic isotope effects can occur both in the process of selective absorption of carbon from atmospheric carbon dioxide, and in biochemical reactions in plant tissues. Note that both inter-component and intramolecular isotopic heterogeneity of biomass is possible [6].

Selective absorption and fractionation of carbon isotopes in the key process of plant life – photosynthesis is of great interest. The isotope distribution between air and photosynthesis products is determined by reactivity of molecules with different isotopic composition [7]. Moreover, that isotope will be accumulated in the reaction products – monosaccharides (simple sugars), which provides then higher reaction rate. Experiments with labeled radioactive carbon \(^{14}\)C and \(^{11}\)C showed that plants extract mainly light isotope of carbon from the ambient air due to the selective assimilation of the initial carbon dioxide [8]. Most plants intensively accumulate \(^{12}\)C, and the relative content of this isotope is 15–25 ‰ higher in them than in the atmosphere. Presumably, the differentiation of isotopes in photosynthesis proceeds in two stages: at the first stage, atmospheric air \(^{12}\)CO\(_2\) is absorbed preferably and dissolute in cytoplasm due to kinetic effect; on the second stage, the fraction enriched in the \(^{12}\)C isotope is extracted from the \(\mathrm{CO}_2\) dissolved in the cytoplasm in synthesis of organic compounds [9].

The most accurate and common method for determining the \(^{13}\)C/\(^{12}\)C isotope ratio is mass spectrometry. For its implementation, it is necessary to convert the target carbon-containing compound into the form of carbon dioxide, and its concentration in the sample must exceed 1% in order to
achieve the necessary measurement accuracy. The most common method for converting a solid into a gaseous state is its burning in a combustion chamber with excess of oxygen, which ensures the total conversion of the entire plant pool into carbon dioxide [10].

Study of the isotopic composition of reaction products produced directly by photosynthesis, namely monosaccharides, is more interesting for photosynthesis research than that for products accumulated throughout the whole plant’s life. The method of the sample gasification by burning transfers the whole carbon pool into the gaseous state and is not applicable to determine the carbon isotopic composition of only simple sugars in the tissues of plants. We propose another method of selective sample gasification by oxidation of a plant tissue with yeast to produce CO2. It is based on two facts: 1) it is known that yeast decomposes preferably monosaccharides with the formation of carbon dioxide at the lag-phase; 2) earlier, a method for studying the carbon isotopes fractionation by heterotrophic microorganisms using yeast was proposed [11].

Thus, the aim of this work was to develop and use the method of sample gasification by oxidation with yeast for mass spectrometric studies of the plant leave isotopic composition under varying of growing conditions.

2. Materials and methods
The analysis of the $^{13}\text{C}/^{12}\text{C}$ ratio was carried out on the specialized isotope static magnetic mass spectrometer «Helicomass» developed by the Ioffe Institute [12] and calibrated using a Thermo Scientific Delta mass spectrometer. The registered molecular ions with the following mass numbers $m/z$: 44 corresponding to the main isotopic modification $^{12}\text{C}^{16}\text{O}^{16}\text{O}$; and 45 which is the sum of the isotopic modifications $^{13}\text{C}^{16}\text{O}^{16}\text{O}$ and $^{12}\text{C}^{17}\text{O}^{16}\text{O}$. The isotopic abundance of $^{17}\text{O}$ was accounted in the calculation and contribute very slightly to the resulting $^{13}\text{C}/^{12}\text{C}$ ratio [13].

To eliminate the instrumental error of mass spectrometric measurements and for data comparability, the carbon isotope ratio is expressed in $\delta^{13}\text{C}$ (‰) as deviation of the carbon isotope ratio of the target compound $^{13}\text{C}/^{12}\text{C}$ from the isotope signature of the generally accepted standard Dee Belemnite (PDB) ($^{13}\text{C}/^{12}\text{C})_{\text{standard}} = 1123.72 \cdot 10^3$ [5]:

$$\delta^{13}\text{C} = \left( \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} - 1 \right) \cdot 1000\text{‰}$$

Sowing oats was chosen as an object for research. Plants were grown in climatic chambers under variation in the spectral composition of the light medium. Sampling was carried out on the 13th day of the plant development.

The developed method of sample gasification consisted of placing homogenized plant tissue, water, and dry yeast in a sealed tube. Reagent concentrations were selected experimentally. After 15 minutes of the reaction, the synthesized carbon dioxide is transferred to input system of the «Helicomass» mass spectrometer for carbon isotope analysis.

To determine the course of the reaction and to identify possible isotopic interference, the method was verified by recording the full mass spectrum using a MS 7-100 mass spectrometer (IAI RAS).

3. Results and discussion
3.1 Method verification
We conducted an experiment on picking up the reagent concentrations as the monosaccharide concentration in the tested plant tissues is not exactly known. Homogenized oat plant tissue in the amount of 0.5 mg, 1 mg and 2 mg were added to the 1 mg of yeast and 1 ml of water. $\delta^{13}\text{C}$ measurement was carried out 3 times for each concentration option. There were no significant differences in the obtained $\delta^{13}\text{C}$ values for the various concentrations of leaves in oxidation reaction by yeast (figure 1). In further experiments, the following proportion of reagents was taken as a basis: 1 mg of plant tissue, 1 mg of yeast per 1 ml of water. This means that yeast was taken in excess to accelerate the reaction.
Figure 1. The values of $\delta^{13}C$ depending on the used plant tissue amount during its gasification by yeast oxidation. The dashed line indicates the average value $\delta^{13}C=-33\pm1\%_0$.

It was essential to measure the total amount of residual organic admixtures in the sample gas, as their fragmental ions can lower isotopic measurement accuracy by interfering with target ions. To do this, the dynamics of changes in the composition of gas components during oxidation of the leaf by yeast was taken using the MS 7-100 mass spectrometer. The complete mass spectrum of the gas mixture in the tube at 15 minutes of reaction is shown on figure 2. The intensity of peak with $m/z=44$ (corresponding to CO$_2$) was increased in 50 times relative to atmospheric air during 15 minutes of the reaction. The total amount of organic impurities in gas was less then $10^{-5}$ relative to CO$_2$, so one can believe that the sample is free from possible interferences, and ready for isotopic measurements.

Figure 2. Mass spectrum: a – atmospheric air in the reaction volume, b – gas mixture formed in oxidation of plant leaves by yeast.
Ethanol is the most important organic admixture interfering with target peaks in mass-spectrometric measurement of $^{13}\text{C}/^{12}\text{C}$ ratio [14]. Fragmental ion CH$_3$O$^+$ with $m/z=31$ produces the largest peak in the ethanol mass-spectrum, its intensity being 6 times higher than ethanol fragmental ion C$_2$H$_5$O$^+$ with $m/z=45$ and nearly 20 times higher than molecular ion C$_2$H$_6$O$^+$ with $m/z=46$. The measured spectra show that $m/z=31$ peak intensity I=$4\cdot10^{-7}$ a.u. does not grow in yeast oxidation, evidencing ethanol absence in the sample.

The ratio of plant tissue carbon isotopes was measured each 15 minutes during 3 hours oxidation by yeast, to study the reaction and identify possible isotopic discrimination over time. The results are presented in figure 3. One can see that the sample carbon isotopic composition was $\delta^{13}\text{C} = -30\pm1\%$ and remained unchanged during the oxidation.

The isotopic composition has been measured for two sugars produce from sugar cane and from sugar beet to test the proposed method. Samples were produced from plants that differ in the type of photosynthesis due to the amount of carbon involved in the metabolism – the first product of the CO$_2$ fixation is three-carbon 3-phosphoglyceric acid for C$_3$ plants and four-carbon oxaloacetic acid for C$_4$ plants. It is results in different carbon isotopic composition typical for C$_4$ and C$_3$ plants. It was measured that $\delta^{13}\text{C} = -33 \pm 2\%$ for the beet sugar extracted from C$_3$ plants and $\delta^{13}\text{C} = -14 \pm 1.5\%$ for cane sugar synthesized from C$_4$ plants. The values are consistent with the literature data [11] for these types of photosynthesis, which allows us to apply the proposed methodology for a wide range of objects.

So, yeast oxidation is indeed an effective method of monosaccharide’s gasification, and the sample is free from admixtures able to misrepresent isotopic measurements.

3.2. Measurements for parts of the leaf
We applied the method for two interesting problems. First, we measured variations in $\delta^{13}\text{C}$ values for various parts of a leaf. An essential difference in the carbon isotopic composition has been found: $\delta^{13}\text{C} = -36.8 \pm 0.2\%$ for the top of the chlorophytum leaf, $\delta^{13}\text{C} = -36.3 \pm 0.2\%$ for the middle, and $\delta^{13}\text{C} = -31.1 \pm 0.9\%$ for the lower part. Probably, the observed effect is due to the rate of photosynthesis, and, accordingly, with the nature of carbon dioxide assimilation – this means that metabolic reactions are more intense in the new part of the plant – the top of the leaf. Earlier, the similar results were got by authors of [15] for parts of the tree Betula pendula: leaves, cambial tissue, bodywood, branches, roots, soil.

This means that variations in the isotopic composition for the same phytotest are most likely due to the difference in $^{13}\text{C}/^{12}\text{C}$ ratio for different parts of the plant, as well as for their development period and growth conditions. Standardization of growth conditions, e.g. using phytotron technologies [16], and selection of specific sampling zones are required.
3.3. Role of the light spectrum
A study has been conducted of the radiation spectral characteristics influence on the carbon isotopic composition in the oats leaves on the early stages of their development. Phyto-test objects were grown in six chambers of a laboratory phytotron with different lighting conditions. Plants were illuminated during growth by various light spectra from specially created lamps containing red, blue, white LEDs and their combinations. The total number of photons, i.e. photosynthetic photon flux density (PPFD) for each variant, was calculated according to the algorithm proposed in [16]. The isotopic composition of oats leaves was measured by the proposed method 3 times. Table 1 shows the $\delta^{13}$C values for carbon compounds in the leaves of the plant; they vary depending on the radiation spectrum. It is observed that heavy isotope $^{13}$C content in plant leaves increases in exposure to light with a predominant blue component, which is inhibiting the development of plants. On the contrary, irradiation with red light results in larger amount of $^{12}$C isotope, probably because molecules with $^{12}$C react faster in photosynthesis due to greater affinity for enzymes.

| Value | LEDs | Red | Blue | Red, Blue | Red, Blue and White | Warm White | Cold White |
|-------|------|-----|------|-----------|---------------------|------------|------------|
| PPFD, мкмоль·м$^{-2}$·с$^{-1}$ | 40 | 44 | 71 | 71 | 190 | 175 |
| $\delta^{13}$C, ‰ | -34.9±0.6 | -25.7±0.3 | -32.4±0.6 | -31.6±0.6 | -33±0.3 | -25±0.3 |

4. Conclusion
The developed method of the sample gasification by yeast oxidation for mass spectrometric analysis allows to measure the carbon isotopic composition of plant tissue in express mode. The sample preparation process is simple and suitable for a wide range of objects.

A mass spectrometric study of the isotopic composition of the oat leaf surface was carried out under varying growing conditions using the proposed methodology for obtaining carbon dioxide samples from monosaccharides contained in leaves by their biochemical oxidation by yeast. The influence of the spectral composition of the light environment on the carbon isotopes ratio in plant tissue has been observed first. An increase in the $^{12}$C concentration in plant tissues is potentially associated with an acceleration of the photosynthesis reaction.

Thus, mass spectrometric measurement of the $^{13}$C/$^{12}$C isotope ratio in plant leaves via their gasification by yeast oxidation is a promising method for measuring metabolic processes in plants, photosynthesis rate and assimilation of carbon dioxide, as well as the influence of external factors on plant development.

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