Estimation of ethylene production and 1-aminocyclopropane-1-carboxylic acid content in plants by means of gas chromatography

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ABSTRACT

The paper deals with problems associated with preparation and collection of samples when estimating the production of ethylene and content of ACC (1-aminocyclopropane-1-carboxylic acid) in plants by means of gas chromatography. A proper method of sampling can significantly influence not only the reliability of obtained data but also their interpretation. Attention was paid to cultivation of plant material, sampling vessels, conditions of ethylene production, sampling procedure, and storage of gaseous samples. The estimation of ACC as a precursor of ethylene is more laborious but it supplements the information about the endogenous level of ethylene in a given part of the plant organism. The authors describe the sampling procedure, methods of sample preservation, extraction and purification, and also the method of oxidation of ACC to ethylene. In the final part of this study the authors evaluate the time consumption and difficulty of individual methods and describe their advantages and disadvantages as compared with other, alternative methods.

Keywords: plant cultivation; vessels; sampling; preservation of gaseous samples

Ethylene influences processes of plant growth and development. Its physiological effects, chemical and biochemical properties, and biosynthesis were studied by many authors (Macháčková and Ullmann 1987, Bleecker and Kende 2000, Chang and Bleecker 2004, Pierik et al. 2006). Gaseous ethylene is released into the environment from the intercellular space where its concentration is in equilibrium with the amount of ethylene dissolved in the cytoplasm. ACC, which originates from the amino acid L-methionine, is considered to be the immediate precursor of ethylene (Adams and Yang 1979). ACC conjugates to N-malonyl-ACC, the metabolism of which was described by Matilla and Gómez-Jiménez (2001).

At first, ethylene was estimated with bioassays (Neljubov 1901, Crocker et al. 1932); later, these procedures were gradually replaced by instrumental methods. Ethylene was fixed by means of sorbents (Hradilík et al. 1986) and thereafter released to a gas chromatograph column. Recently, methods of estimation of ethylene production by means of a direct sampling from cultivation environment (Thompson 1977, Dundelová et al. 1993, Fišerová et al. 2001) were elaborated enabling to measure its biosynthesis in intact plants. Regarding the fact that in individual plant species ethylene production is different [e.g. Radin and Loomis (1969) observed an inhibiting effect of ethylene (2–3 μl/1) on the formation of lateral roots in radish (*Raphanus sativus* var. *radicola*), Dimasi-Theriou et al. (1993) recorded an increase in number of adventitive roots at the concentration of ethylene 0.01–10 μl/l when regenerating petunia (*Petunia hybrida*) leaves, and/or Burg (1968) mentioned that a majority of physiological reactions took place within the range of 0.01–1.0 μl/l of volume concentration of ethylene] it is possible, when using a suitable experimental layout, to obtain a response in the order of nanolitres (Fišerová et al. 2006, 2007).

In green plants, there are diurnal changes in production of ethylene, and as a rule, the biosynthesis of this gas is decreasing (Yang and Hoffman 1984).

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The biosynthesis of ethylene is influenced also by temperature and its maximum occurs within the range of 25–35°C (El-Abd et al. 1999).

The closed environment of sampling vessels, which is necessary for accumulation of gases, is favourable also for the proliferation of some phytopathogens that can influence ethylene biosynthesis (Lund et al. 1998). Under sterile conditions, such an infection can be prevented and in a non-sterile environment, the infestation can be reduced by application of various disinfectants (e.g. SAVO).

In isolated parts of plants, the estimation of ethylene production is rather difficult due to the release of stress ethylene and for that reason it is more suitable to try to estimate only the content of ACC. An indirect estimation of ACC by means of its chemical oxidation to ethylene, followed by gas chromatography, is a relatively reliable analytical method (Lizada and Yang 1979).

MATERIAL AND METHODS

Preparation of samples for the estimation of ethylene production

When trying to estimate ethylene produced by plants directly from samples its concentration is very low. For that reason it is necessary to find a right cultivation vessel; it must be suitable for gas sampling and it should keep the volume as small as possible and at the same time enable the growth of plants and prevent the escape of gas released in the course of cultivation. Under normal conditions, plants are cultivated in vessels used in laboratories and/or in food industry (Figure 1), which can be sealed either with caps with a plastic septum (for repeated gas sampling) or with a latex membrane (for a single sampling).

The basic precondition of successful cultivation of plants is to maintain a required humidity in vessels. This can be assured in different ways: (i) by cultivation in special stands (Figure 2B) (for pea seedlings); (ii) by submersion of plant bases into water without any support (for stem cuttings); (iii) by cultivation on wet filter paper, cellulose wadding, perlite or other material with the necessary water-holding capacity (for germination of seeds); (iv) by cultivation in liquid or solid growing media, e.g. agar, gerlite etc. (for tissue cultures).

Ethylene is produced in presence of oxygen (Lieberman 1979) and during its biosynthesis the plants release carbon dioxide and hydrogen cyanide (Yang and Hoffman 1984). These compounds, similarly as an increased level of ethylene, can influence the growth and development of plants and it is thus necessary to ventilate the sampling vessels at regular intervals to preserve identical experimental conditions. Depending on the type of plant material and size of the sampling vessel this time interval can range from 1 to 24 h.

Gas samples are collected with a syringe (Braun Melsungen AG) with the volume of 2 ml and a needle (Beeton Dickinson Ltd., Ireland) with the diameter of 0.5 mm. This enables to sample 1.5–2.0 ml of gas from the sampling vessel. Immediately before the analysis the sample volume is adjusted to only 1 ml.

Testing the security of sampling vessels by means of an ethylene standard

To eliminate technical errors it is necessary to test the security of sampling vessels by using ethylene standards. A comparison of results obtained when testing the tightness of sampling vessels within a period of 48 h is presented in Figure 3.

Storage of samples

Because of a high number of replications and variants of gas estimation it is not possible to perform the analysis of samples in the gas chromatograph immediately after sampling. Each analysis lasts approximately 3 min and for that reason the samples can be stored in syringes, which are inserted into the rubber cap (Vitrum Rožnov s.r.o., Figure 2A) of a flask. These samples can be stored in darkness at a constant room temperature for as much as 24 h without any condensation of water vapours and/or changes in volumes of sampled gases. Changes in concentration of ethylene stored in syringes for a period of 48 h are presented in Figure 4.

Preparation of samples for the estimation of ACC, extraction, and purification

In the corresponding stages of development, samples of 0.3–1.0 g of studied plants were taken off, frozen in liquid nitrogen and/or dry or normal ice, and stored at temperatures ranging from –18°C to –70°C. For longer periods of storage it is necessary to use lyophilisation.

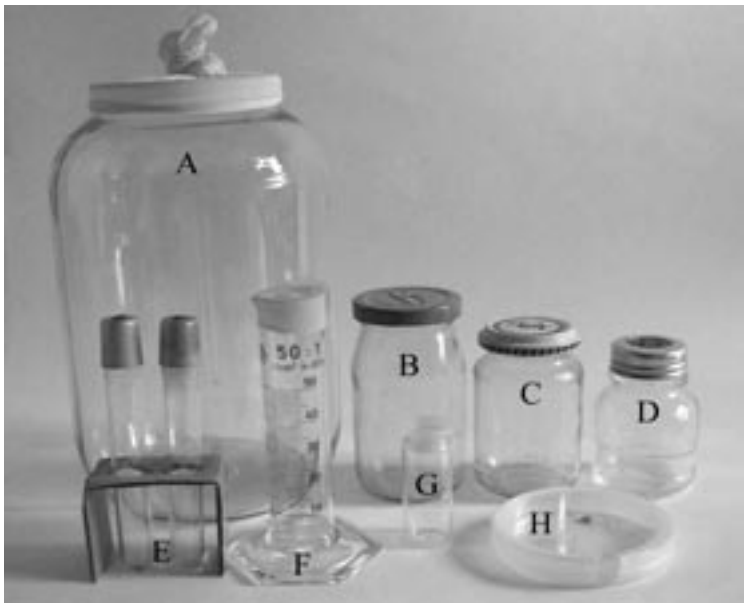

Figure 1. Presentation of sampling vessels: (A) a glass vessel with the volume of 5 000 ml closed with a latex membrane (suitable for potato tubers or pot plants of different size), (B) a glass vessel with the volume of 260 ml closed with a metal cap with septum (suitable for seedlings with hypogeic cotyledons), (C) a glass vessel with the volume of 200 ml closed with a metal cap with septum (suitable for tissue cultures), (D) a glass vessel with the volume of 133 ml closed with a metal cap and rubber padding (suitable for studies on germination of caraway achenes and/or isolated buds), (E) test tubes with aluminium caps with septum and parafilm sealing (suitable for tissue cultures), (F) a glass cylinder with the volume of 50 ml closed with a latex membrane (suitable for stem cuttings), (G) a glass vessel with the volume of 20 ml closed with a plastic cap with septum (suitable for isolated buds), (H) plastic Petri dishes with the diameter of 100 mm with a septum and parafilm sealing (suitable for studies on germination of different crops)

Plant material, homogenized in 70% ethanol (5 ml per 1 g of fresh matter) was stored at –20°C for 12 h (Petruzzelli et al. 2000) and, after dissolving, centrifuged at 10 000 g for 10 min. The supernatant was poured off. Thereafter, the sediment was re-homogenized in 70% ethanol and centrifuged again at 10 000 g for 10 min. Pooled supernatants were evaporated at 40°C to obtain the water phase (with the volume of approximately 1 ml) and sonicated in an ultrasound bath. The content of evaporation flask was transferred with a pipette into an Eppendorf test tube, frozen (to obtain chlorophyll) and centrifuged again. The efficiency of this extraction is usually higher than 80%.

Figure 2. (A) Tubber cap with dug syringes containing gas samples, (B) the stand used for cultivation of plants

The conversion of ACC to ethylene was performed using the method described by Lizada and Yang (1979). In this step, ACC was oxidized in presence of $HgCl_2$ to ethylene using sodium hypochlorite according to the equation:

$$ACC \xrightarrow[NaOCl_2,\ NaOH]{HgCl_2} Cl^- + H_2O + CH_2 = CH_2 + N{\equiv}C\text{-}CO_2^-$$

The efficiency of this oxidation is usually higher than 80%.

The purified samples were placed into a 10 ml test tube with a sealing that enabled gas sampling; thereafter, 0.5 ml of 10 μmol $HgCl_2$ per 1 ml of

sample and 250 µl of a cooled mixture of 5% NaOCl: saturated NaOH (2:1) were added. Samples had to be stored in ice. The content of test tube was mixed for a short time in vortex and stored in ice for 10 min. Thereafter the sample was mixed and 1 ml of gas was sampled for the analysis in the gas chromatograph using a syringe.

GC Analysis of samples

Gas samples were analysed using the method of gas chromatography with a flame-ionising detector (FID), (manufacturer FISSONS INSTRUMENT, Italy; 50 m capillary column Al_2O_3 "S" 15 µm, ID 0.53 mm), spray, column and detector temperatures 230°C, 40°C and 200°C, respectively (Fišerová et al. 2001). The sensitivity of this method can be increased if the normal air is substituted with pure oxygen (Thompson 1977).

RESULTS AND DISCUSSION

If this methodology is properly observed it is possible to assess low concentrations of ethylene. As shown in Figure 3, the glass vessel with the volume of 5000 ml sealed with a latex membrane can be recommended. This is suitable for potato tubers (Fišerová et al. 2004) and/or various plant species cultivated in pots. Further, it is possible to recommend a 260 ml glass vessel with a metal cap and septum (suitable for seedlings with hypogeic cotyledons; Fišerová et al. 2006) and a 133 ml glass vessel with a metal cap and rubber sealing (studies on caraway achenes and cereal kernels; Fišerová et al. 1996) for isolated buds of potato tubers.

As for the method of sampling, the estimation of ethylene by means of a direct sampling from the sampling vessel is quick and time saving. However, two major disadvantages of this method are: (1) a more difficult interpretation of the results obtained from isolated parts of plants (due to the release of stress ethylene) and (2) the impossibility of a long-term storage of gas samples. On the other hand, the main advantages of ACC estimation are: (1) the elimination of effects of stress ethylene and (2) a possibility of a long-term storage of samples. As compared with other methods (namely HPLC), the advantage of an indirect estimation of ACC consists in the fact that the methods of extraction and purification are relatively inexpensive, especially as far as the instrumentation and materials are concerned, and that this precursor can be estimated after its oxidation to ethylene by means of gas chromatography, i.e. using the same apparatus as in the case of direct estimation of ethylene.

From the viewpoint of time consumption, the preparation of samples for ACC estimation is more complicated because it is necessary to perform extraction, purification and oxidation so that it is possible to prepare 16–25 samples within a period of 8 h. The cost of preparation of samples for ACC estimation is higher, especially due to instrumentation and chemicals. When estimating ethylene by means of a direct sampling, the consumption of time is dependent on the number of samples and the time of closing of cultivation vessels. The number of samples is limited by the

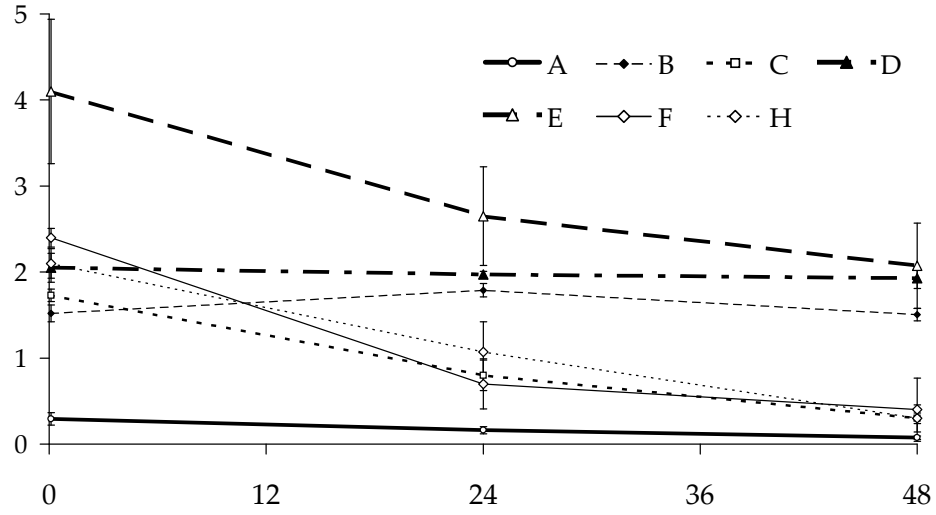

Figure 3. Course of ethylene concentration in sampling vessels within a period of 48 h (*y*-axis = ethylene concentration (nl/l), *x*-axis = time)

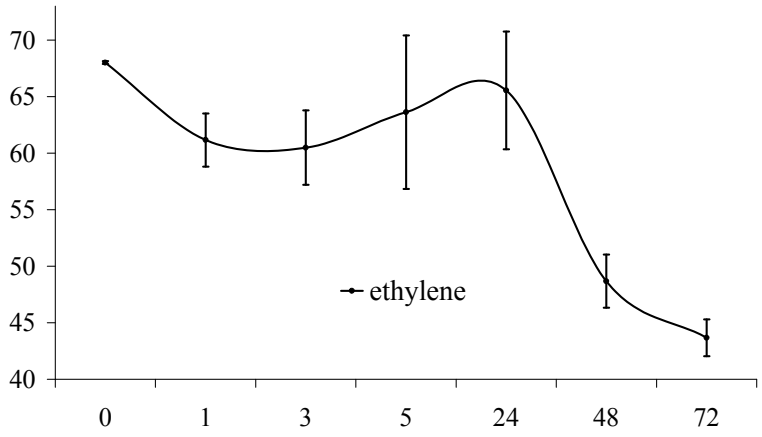

Figure 4. Changes in ethylene concentration when preserving gas samples in syringes (*y*-axis = ethylene concentration (nl/l), *x*-axis = time)

performance of the gas chromatograph (the time consumption is approximately 3 min per sample) and the storage time of samples (24 h). Expenses associated with the preparation of samples for a direct estimation of ethylene are dependent on the price of sampling vessels.

A lower sensitivity of gas chromatography can be improved by concentration of ethylene from the gas mixture using a suitable sorbent (Colson 1963) followed by a thermal desorption in the column of gas chromatograph. This method is time consuming and requires further instrumentation (Hradilík et al. 1986).

Besides the indirect estimation of ACC transformed to ethylene using gas chromatography there are also some direct methods enabling to quantify ACC without its oxidation to ethylene such as GC-MS analysis (Saridge et al. 1983), HPLC-UV (Grady and Bassham 1982), and HPLC-MS-MS (Chauvaux et al. 1997). As a rule, all require a different method of sample purification, different instruments for final quantification (HPLC, GC-MS) and labelled ACC as a standard.

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