EFFECTS OF THE GAS EXCHANGE RATE IN THE CULTURE VESSELS ON THE PHOTOSYNTHESIS AND THE CARBON METABOLISM OF MICROPROPAGATED FRUIT PLANTLETS (APPLE ROOTSTOCK MM 106)

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ABSTRACT

The specific in vitro conditions cause a modified structure and physiological functions of the photosynthetic apparatus which result in poor survival rate of plantlets during acclimatization. The type of vessel closure affects the gaseous composition and hence affects the growth of plantlets in culture. In tightly closed vessels concentrations of metabolically generated gases increase because their rate of diffusive escape is depressed. Different approaches have been proposed to enhance the photosynthetic ability of plants in vitro in striving to favour the success of further acclimatization to conditions ex vitro. The aims of the present research are to find the closure material which is gas permeable and to study the effect of the improved gas exchange on the growth and the photosynthetic ability of the micropropagated apple rootstock MM 106. The polyester wool - one of the tested types of closure, provides good gas exchange rate and preserves the culture from contamination. The data of this investigation show that there is a positive correlation between the improved gas exchange and accumulation of dry matter, light harvesting pigments as well as the ability of plantlet to assimilate $^{14}CO_2$. In plantlets, cultured with polyester wool, the relative part of the labelled low -molecular photosynthetic products (sugars, amino acids, organic acids) decreases, while the part of labelled high-molecular fractions (lipids, pigments and starch) increases. The improvement of gas exchange leads to rise of the content of sorbitol and the ratio sorbitol : saccharose. This might be a sign of a better carbon metabolism of in vitro cultivated apple rootstock MM 106.

Introduction

Isolated plant parts growing aseptically and heterotrophically on an appropriate medium are known as plant tissue cultures (13). The conventional *in vitro* environment is characterized as having the followings: high relative humidity (RH), constant temperature, low photosynthetic photon flux density (PPFD), large diurnal fluctuation in CO_2 concentration, the high concentration of sugar, salt and growth-regulating substances in the medium, the accumulation of toxic substances and the abscence of microorganisms (1). These conditions often cause a modified structure and modified physiological functions of the photosynthetic apparatus which result in poor survival rate of plantlets during acclimatization.

Enhancing photosynthetic ability of plants *in vitro* may improve growth conditions in the culture tubes as well favour the success of further acclimatization to conditions *ex vitro*. In recent research it is generally accepted that changing environmental factors, such as

sucrose level in the medium, light intensity or CO_2 concentration, may affect the development of photosynthetic characteristics *in vitro* (12).

The type of vessel closure affects the gaseous composition and hence affects the growth of plantlets in culture. In tightly closed vessels concentrations of metabolically generated gases increase because their rate of diffusive escape is depressed. For this reason, poorly aerated tissues are often enriched of respiratory CO_2, especially in the dark, and with ethylene (6).

Different approaches have been proposed to correct or to control the gaseous composition. Some of them are rather expensive, other are difficult to apply in large-scale micropropagation.

The aims of the present research are to find the closure material which is gas permeable and to study the effect of the improved gas exchange on the growth and the photosynthetic ability of the micropropagated apple plantlets (apple rootstock MM 106).*

Materials and Methods

Experiment 1. Determination of the gas exchange of the containers with different closures

The radioisotope method developed in the Radioisotope Laboratory in our Institute was used. The schematic diagram is presented in **Fig. 1.** The chamber (1) with volume 5,4 l with installed Geiger - Muller counter (2) and microventilator (3) was designed. The culture vessel (5)/ a jar with volume 0.6 l / with an equipment for emission of $^{14}CO_2$ (4) was placed in the chamber. The jar is closed with a closure device. After hermetic sealing of the chamber, the emission of $^{14}CO_2$ ($Ba^{14}CO_3$ + HCl) starts, the reading with GM counter begins at the same time.

The exponential character of the gaseous

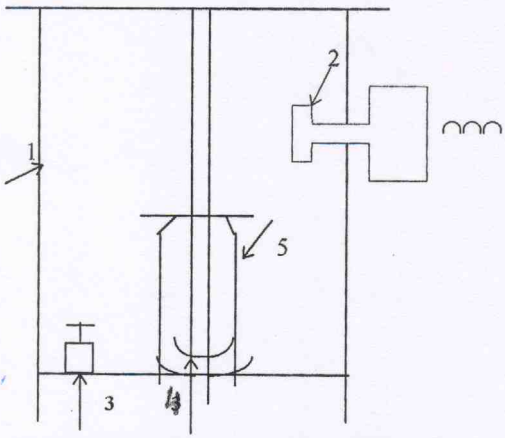

Fig. 1. Schematic diagram of the chamber for determination of gas permeability of the vessels closures.

diffusion from the jar in the chamber allows mathematical interpretation of the data by the following formula:

$C_t = C_p (1 - e^{-kt})$, where C_t = cpm $^{14}CO_2$ in the chamber at a moment,

C_p = cpm $^{14}CO_2$ at the balance between $^{14}CO_2$ in the vessel and the chamber.

When $^{14}CO_2$ is precisely dosed in the chamber, Cp is a constant. This makes it possible to determine *k* after several measurements of $^{14}CO_2$ in the chamber using the formula:

$$-k = \frac{1}{t}\left(1 - \frac{C_t}{C_p}\right)$$

The physical meaning of the gas exchange constant (*k*) gives expression about the part of the volume of the container which is exchanged for a unit of time (1 minute in this case).

The permeability of the following closures have been tested:

-glass cap, tightly closed with a transparent folio (a conventional method of covering) - the control

-gas permeable folio

-polyethylene folio with an opening closed with a plastic foam

-polyethylene folio with an opening with a bacterial filter (Milipore)

*Abbreviations : **DW**-dry weight, **FW** - fresh weight, **BAP**- 6-benzylaminopurine, **GM counter** - Geiger-Muller counter, **IAA** - indoleacetic acid, **Chl** - chlorophyll, **cpm** - count per minute, **LMC** - low-molecular carbohydrates, **HMC** - high-molecular compounds

-polyester wool under the glass cap
-covertan - two layers

Experiment 2. Influence of the improved gas exchange on the photosynthetic ability of micropropagated plantlets

Plant material and growth conditions

Micropropagated shoots of apple rootstock MM 106 subcultured at 4 weeks on the MS nutrient medium (10) supplemented with 1.00 $mg.l^{-1}$ BAP, 0.01 $mg.l^{-1}$ IAA and 30 $g.l^{-1}$ sucrose were used. In jar (volume 600 ml) with 100 ml medium 30 explants were inoculated.

Cultures were kept in a growth chamber at 22+2°C and under a photoperiod of 16 h light (40 $\mu molm^{-2}s^{-1}$ PPFD, fluorescent lamps OSRAM 40 W) and 8 h dark. Three variants have been evaluated.

1. Variant - tightly glass cap, 4 weeks on MS medium, supplemented with BAP and IAA.

2. Variant - polyester wool and glass cap, 4 weeks on MS medium, supplemented with BAP and IAA.

3. Variant - polyester wool and glass cap, 2 weeks on MS medium + 2 weeks on MS medium without growth regulators.

After 4 weeks were observed: dry matter (%), light harvesting pigments, intensity of $^{14}CO_2$ uptake and metabolism of the photofixed $^{14}CO_2$.

The content of light harvesting pigments was determined spectrophotometrically in 80 % acetone extract (9). The intensity of $^{14}CO_2$ uptake and the initial metabolism of the photofixed ^{14}C were determined. Plantlets from the three variants were placed in a common assimilating chamber. The exposition was for 30 minutes at 50 $\mu molm^{-2}s^{-1}$ PPFD and at 22+2°C.

After that a part of plantlets from each variant were fixed for 3 min in 80 % boiling ethanol, the other part was weighed and dried out at 60°C for assessing the total quantity of assimilated $^{14}CO_2$. The fixed in ethanol material was extracted by conventional method. The water-soluble fraction was separated by ion exchange chromatography (Dowex 1 in HCO_3- form and Dowex 50 in H^+ form) of amino acids, organic acids and free low molecular carbohydrates (sugars+sorbitol).

Water non-soluble fraction was separated of: lipids and pigments - chloroform fraction, starch -(extract with 1 % sulphosaliylyc acid) and high - molecular structural compounds - non - soluble residium.

The compounds of the LMC fraction was separated on a paper chromatography in two systems: (n- butanol : acetic acid : H_2O = 4:1:1) and (saturated boric acid : ethylmethyl ketone : acetic acid = 9 :1 :1). The content of ^{14}C in the components was detected radiometrically after autoradiography.

Results and Discussion

Experiment 1.

The obtained experiment data is presented in **Table 1.** The access of air from the environment to the jar is practically fully restricted in variant 1 - conventional closure. The jar closure with gas permeable folio, polyethylene folio with plastic foam and polyethylene folio with bacterial filter also leads to low gas exchange between the jar and the environment. Covertan allows significant gas exchange, but was not appropriate because we observed drying of the plantlets and initial necrosis on the leaves. Furthermore, after 4-5 days the nutritional medium was contaminated. The best from the evaluated types of closure was polyester wool (2,712 volumes/hour). Moreover, this type of wool can be

TABLE 1

Gas exchange rate of the jars with different type of closure

Type of Closure	-k	Gas exchange volume/hour
1. Control - glass cap with folio	0,000213	0,013
2. Gas permeable folio	0,009525	0,572
3. Polyethylene folio with a bacterial filter	0,01863	1,112
4. Polyethylene folio with plastic foam	0,0324	1,944
5. Polyester wool	0,0452	2,712
6. Covertan	0,1236	7,416

TABLE 2

Dry matter content (% of fresh weight), content of light-harvesting pigments (mg g^{-1}fw) and intensity of $^{14}CO_2$ uptake (μmol g^{-1}fw s^{-1})

variant	(DW/FW).100	Pigments			chla/b	Chl.(A+B)	intensity of $^{14}CO_2$ uptake
	%	Chl.A	Chl. B	car.		car.	
1	15,20	2,00	0,760	0,419	2,63	6,58	$1,04x10^{-2}$
2	16,00	2,44	0,948	0,472	2,57	7,20	$1,50x\ 10^{-2}$
3	21,50	3,44	1,320	0,675	2,61	7,00	$1,56x10^{-2}$

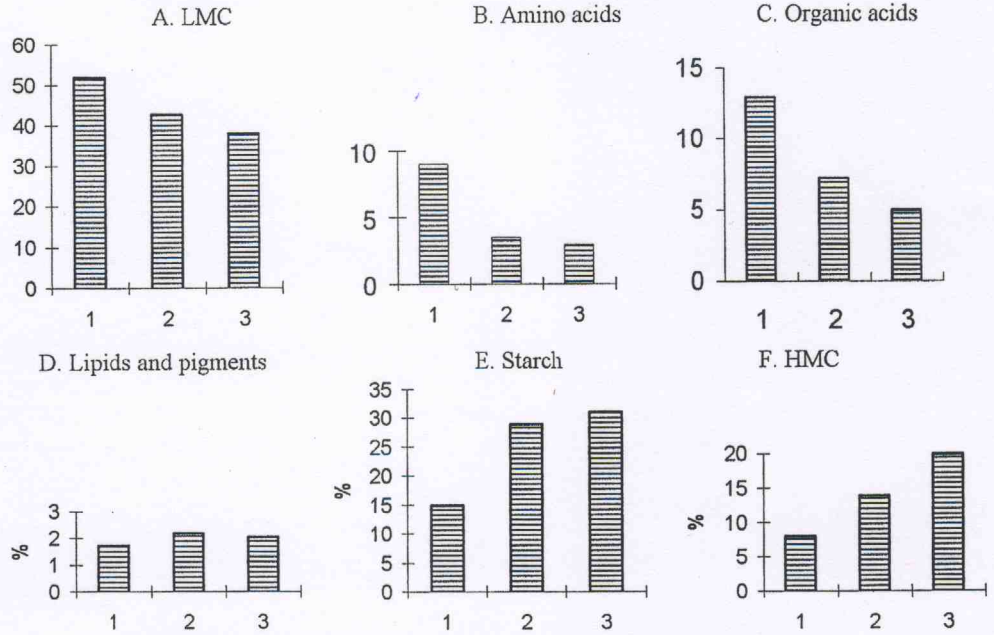

Fig. 2. The distribution of ^{14}C labelled fractions after $^{14}CO_2$ assimilation.

autoclaved - an important circumatance in its practical application.

Experiment 2.

The plantlets from variant 1 were typical *in vitro* plans with elongated nodes and narrow leaves. The shoots from variant 2 were shorter with wide-open leaves and coefficient of multiplication close to that of variant 1. The microplants from variant 3 were tall and had wider leaf blade and more intensive green colour than the plantlets from the control variant. An accumulation of dry matter was proved from variant 1 to variant 3 (**Table 2**). With the improvement of the gas exchange was established an increase in the content of light harvesting pigments (Table 2) but Chl a/b ratio and Chl/Carotenoids ratio were similar in the three variants. The plantlets cultured with polyester wool showed more intensive $^{14}CO_2$ uptake (var. 2 and 3) compared to those, cultivated with conventional type of closure (var.1).

In **Fig. 2** the distribution of ^{14}C labelled fractions after $^{14}CO_2$ assimilation is summarised. The plantlets from variant 1 showed a higher percent of labelled low-molecular photosynthetic products (sugars, aminoacids, organic acids) compared to the other two

variants. (Fig. 2. A-C). The plantlets cultivated with polyester wool have a higher percents of ^{14}C in the high-molecular photosynthetic products (lipids and pigments, starch and the common fraction of the high - molecular compounds - Fig. 2 D-F).

The results from the chromatographic separation of the low molecular carbohydrates showed that the ^{14}C-saccharose of variant 1 was 78.12 % and it decreased in variant 2 and 3 (correspondingly 64 % and 58.65 %). At the same time the percent of the labelled sorbitol almost doubles in variant 2 (30 %) and variant 3 (36.94 %) compared to those of variant 1 (16.46 %).

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TABLE 3

Chromatographic separation of low molecular carbohydrates (%)

Fraction/ Variants	1	2	3
glucose + fructose	5,42 %	5,42 %	3,05 %
saccharose	78,12 %	64,14 %	58,65 %
sorbitol	16,46 %	30,00 %	36,94 %
oligosaccharides		0,41 %	1,36 %
sorbitol : saccharose	0,21 %	0,47 %	0,63 %

The data of chromatographic separation of low molecular carbohydrates (**Table 3**) showed a decrease in the percent of monoses (glucose + fructose) and disaccharides (saccharose) from variant 1 to variant 3 resulted in accumulation of oligosaccharides in variants 2 and 3.

A wide range of factors is known to influence quality and acclimatization of micropropagated plants. Manipulation of the relative humidity of the culture vessel has been shown to improve microplant establishment (11). If this is achieved by allowing moisture to escape from the container, however, the process must be amenable to control such that the culture do not suffer salt stress or other effects due to water loss (5). This approach, and that of using bottom cooling will have the desired effect of increasing transpiration in the microplants in culture thereby facilitating calcium uptake and stomatal function (4).

An alternative strategy to encourage the development of transpiration in microplants is based on the use of membranes with selective permeability. Several reports have indicated positive effects of using a gas-permeable film as the closure on the photosynthesis and the growth rate of plantlets *in vitro* (1). However some of these approaches are expensive and some of them are difficult to apply. We tried to find a closure material which is inexpensive and applicable in mass micropropagation. From the tested closure materials the polyester wool meets to the greatest extent these requirements.

Kubota and Kozai (8) have established a significant growth stimulation of Solanum tuberozum by forced ventilation. The results from Gerbera jamesonii and Ficus lyrata are similar (7). Apart from the possibility of the plantlets to fix CO_2 during the whole photoperiod, the authors emphasize that in this way the accumulation of ethylene and toxic CO_2 concentrations at the end of the dark period is also avoided. The data of this investigation show that there is a positive correlation between the improved gas exchange and the accumulation of dry matter, light - harvesting pigments as well as the plantlet ability to assimilate $^{14}CO_2$ (Table 3). In variants 2 and 3 the relative part of the labelled low - molecular photosynthetic products decreases while the part of the labelled high - molecular fractions increases. Probably this shows that there is a more efficient carbon metabolism in plantlets of variant 2 and especially of variant 3.

The amount of sorbitol in variants 2 and 3 considerably increases (Table 3). The ratio sorbitol : saccharose increases almost twice in variant 2 and three times in variant 3

compared to variant 1. It is known that the sorbitol is basic transport assimilate in woody fruit species of Rosaeceae, so this ratio (sorbitol : saccharose) could be significant for the physiological status of the leaves. Though a lot of studies are still necessary in this field it could be assumed that the increase of this ratio in plantlets *in vitro* might be a sign of a better carbon metabolism.

The obtained results show clearly that when optimizing the environmental factors, the photosynthesis might have a substantial contribution to the carbon balance of the plants *in vitro*. Researchers do not share a common opinion on the issue of the necessity of optimization of photosynthesis at all stages of micropropagation. Besides, the plantlets from the different species react specifically to the optimization of the photosynthetic conditions Walker and al (1988), for example, established that the forced ventilation does not improve the growth of the chrysanthemum plantlets during shoot multiplication. Cuello and al. (2) added that the best medium for chrysanthemum microplants is that of 3 % sucrose and condition which do not control CO_2. This shows that there are no universal conditions for cultivation at all growth stages of micropropagating process and for all plant species. However, there are unquestionable proofs for the necessity of a certain precultivation of plants before the acclimatization. The study on possibilities of enhancing the efficiency of photosynthesis at some stages of the micropropagation might help the successful acclimatization of the plants to the *ex vitro* conditions.

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