BIOLOGIA PLANTARUM (PRAHA)

33 (2) : 135–144, 1991

Photoautotrophy Established in Multiple-Shoot Cultures of *Ruta graveolens*

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Abstract. During the growth of multiple-shoot cultures of *Ruta graveolens*, oxygen and carbon dioxide exchanges were continuously and simultaneously measured. The shoots subcultured on a medium containing 166 mM glucose showed a marked respiration rate. Even under light, CO_2 concentrations reached 4000 to 6000 $cm^3\ m^{-3}$. Photosynthesis never compensated for respiration. These cultures were photomixotrophic. A change of respiration and photosynthesis occurred between the 30th and the 32nd day of culture, with a high respiration rate. When the shoots were subcultured on a medium containing 41 mM glucose, it was possible to obtain photoautotrophy after two weeks under high irradiance (150 $\mu mol\ m^{-2}\ s^{-1}$), and after three weeks under low irradiance (60 $\mu mol\ m^{-2}\ s^{-1}$), the CO_2 concentrations being 1100 and 600 $cm^3\ m^{-3}$ respectively.

The ability of a large number of medicinal plants to form multiple-shoot cultures from axillary meristems or shoot explants has opened an alternative *in vitro* system of some economical importance for the production of plant constituents (Heble 1985). It seems reasonable to think that the refining of culture conditions would lead, among organized cultures *in vitro*, to the same metabolite patterns as in the mother plants.

Ruta graveolens and its tissue culture have been extensively studied (see Petit-Paly *et al.* 1989, for a review) : it is easy to obtain multiple-shoot cultures giving high yields of coumarins, alkaloids and volatile oils (Corduan and Reinhard 1972, Czygan 1975, Ramawat *et al.* 1985). As shown in several papers, the metabolic pattern of *in vitro* cultures of *R. graveolens* is altered by irradiance (Reinhard *et al.* 1968, Drawert *et al.* 1984, Eilert *et al.* 1983). Thus, photoautotrophy may be a useful tool to modify secondary metabolism (Drawert *et al.* 1984).

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Received January 1, 1990; accepted June 17, 1990

Our aim was to study the influence of culture conditions on growth and metabolism of *R. graveolens* multiple-shoot cultures. In this first paper, we showed evidence that a photoautotrophic growth state is attainable after a photomixotrophic growth state, when the cultures are grown on a low-level glucose medium. Respiraroty and photosynthetic gas exchanges were measured by using a closed circuit apparatus previously described (Leclerc and Abd el Rahman 1988).

MATERIALS AND METHODS

Plant material

Multiple-shoot cultures of *Ruta graveolens* L. (*Rutaceae*) were established from nodal meristems of aseptic seedlings on agar medium (Gamborg *et al.* 1968), supplemented with 166 mM glucose, 4,4 μM benzyladenine and 5.7 μM indole-3 -acetic acid. This medium will be referred to as G-medium. The shoot buds arose from the unorganized calli which had developed at the base of explants. Cultures were grown in 250 ml Erlenmeyer flasks, under continuous white light (Philips TL 40 W – 34; 20 $\mu mol^{-2}\ s^{-1}$) at 24 ° ± 1 °C. They were subcultured every month. For experiments, we used either G-medium or G/4 medium (as G-medium but glucose was reduced to 41 mM). In this last case, multiple-shoots were subcultured three times prior to experiments.

O_2 and CO_2 simultaneous exchanges

The original apparatus described by Leclerc and Abd el Rahman (1988) was slightly modified to be used for several days of continuous measurements (Fig. 1). The cultures were raised for about one month in one litre pyrex glass flasks filled with 250 ml G-or G/4-medium under a continuous gas flow (100 $l\ h^{-1}$). Inlet and outlet of the flasks were protected by sterile filters (Sartobran II, Sartorius). A carbonate buffer bubbling chamber provided CO_2 and transpiration compensation of the shoots. All cultures were maintained at 24 ° ± 2 °C. Light was provided by a 400 W PHYTOCLAUDE high pressure mercury lamp, during 12 ± 1 h, either at low (60 $\mu mol\ m^{-2}\ s^{-1}$ = LI) or high irradiance (150 $\mu mol\ m^{-2}\ s^{-1}$ = HI), alternatively with 12 ±1 h dark periods. A microcomputer (CBM 4032) with printer (COMMODORE 4022 L) and floppy disk (COMMODORE 2031) peripherals, drove multichannel (SCHLUMBERGER 7010) and a microvoltmeter (SCHLUMBERGER 7066) connected to O_2 and CO_2 probes. Thermocouples measuring the temperatures of the probes were connected to the system in order to adjust measurements. As the CO_2 concentrations varied along cultures, a calibration curve was built (Fig. 2). Both CO_2 and O_2 variations showed a ± 0,2 cm^3 accuracy on one hour measurements; on 12 h measurements an accuracy of ± 0.05 $cm^3\ h^{-1}$ was obtained. The whole closed circuit had a volume of 2 to 4 l depending on the number of flasks used.

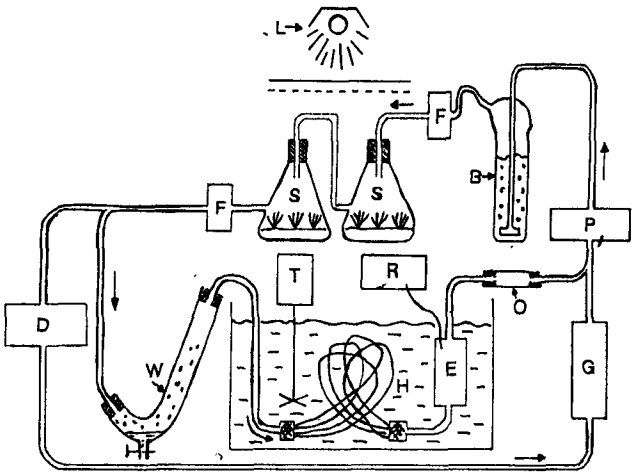

Fig. 1. Diagram of the experimental system used in O_2 and CO_2 simultaneous measurements.
P: air pump – B: bubbler for air humidity and CO_2 control – F: sterile filter – S: experimental flasks – D: air dryer with $CaCl_2$ – G: infra-red gas analyser – W: large and slightly cooled tube, for partial condensation of water vapour – H: thermostated water bath, with 4 coiled stainless steel tubes, 3 m lenght each – E: chamber for O_2 probe – O: chamber for opening the circuit – T: thermostat – R: recorder for O_2 and CO_2 signal, electrode and flasks temperature – L: lighting system with infra-red and neutral filters.

RESULTS

Cultures on G-Medium

The photosynthetic state of cultures grown on their subculture G-medium was first studied. The values of respiration and photosynthesis were monitored from the 8th day up to the 36th day of culture. A large part of the CO_2 used in photosynthesis came from the "glucose respiration", the glucose being absorbed from the medium by the cells. Probably a small part of CO_2 obtained was assimilated, the larger part being (both under dark and light) set free in the flask atmosphere. We found very high CO_2 concentrations: around 2000 to 4000 cm^3m^{-3} at the end of a light period, and up to 6000 cm^3m^{-3} at the end of the dark period, as reported for grapevine shoots (Fournioux and Bessis 1986). The high glucose consumption led to a high respiration rate (Fig. 3 and 4) increasing during the culture growth . The dark respiration was maximal on the 30–32nd day: the rate calculated on the whole tissues (callus and shoots) basis was 2.0 cm^3 (O_2 absorbed) $h^{-1}g^{-1}$ (dry mass). On the shoot basis, we found 3.2 cm^3 (O_2 absorbed) $h^{-1}g^{-1}$ and 4.1 cm^3 (CO_2 evolved) $h^{-1}g^{-1}$. The excess in CO_2 emission compared with the O_2 absorption showed a supernumerary fermentation phenomenon.

The gross photosynthetic rate (P_G) was estimated by the difference in gas rate exchanges in light and darkness. The comparison of the curves obtained (Fig. 3 and

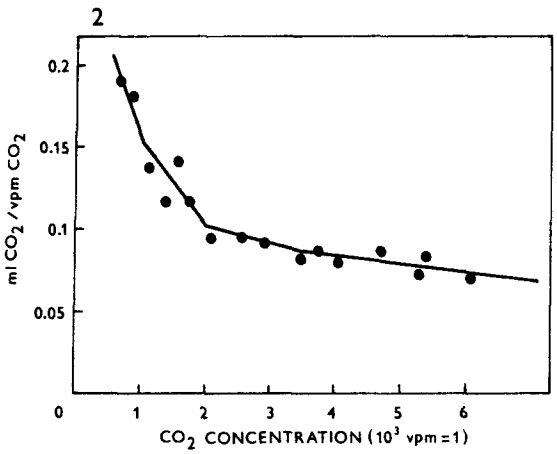

Fig. 2. Calibration curve for calculation of absolute CO_2 variations.
The curve is used during measurement, after observations of cm^3m^{-3} variations. A calibration curve is made for each set of cultures included in the closed circuit.

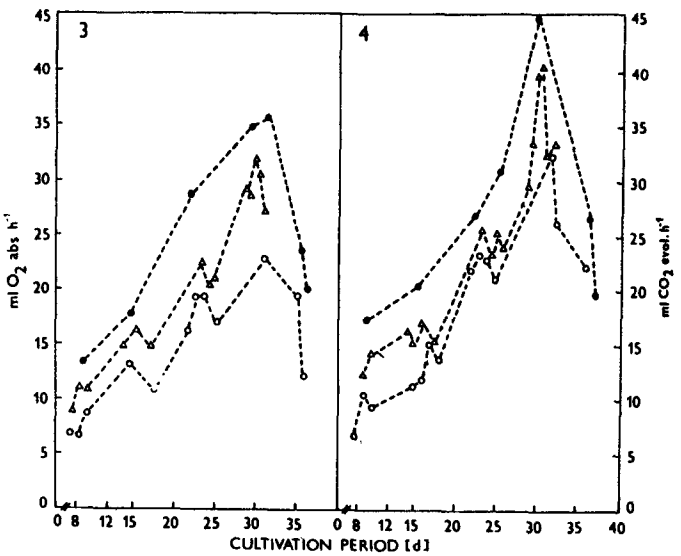

Fig. 3. O_2 absorption in shoot cultures grown on G-medium. Results are given for 4 experimental flasks.
Fig. 4. CO_2 evolution in shoot cultures grown on G-medium. Results are given for 4 experimental flasks.
•: dark – Δ: low irradiance (LI = 60 μmol m^{-2} s^{-1}) – o: high irradiance (HI = 150 μmol m^{-2} s^{-1}).

Fig. 4) showed that the P_G sharply fell at the 30th–32nd day both under LI and under HI, and increased again until the 36th day.

The hourly activity variations were studied. Some examples are shown in Fig. 5. Throughout the dark period, the respiration rate was rather stable when the cultures were young (*e.g.* 9th–10th day, but decreased when the cultures were older (*e.g.*

22nd–23th day). Under light, the net photosynthetic rate (P_n) of the shoots was always negative, but stable before the "crisis" of the 30th–32nd day. The comparison of the figures 5A and 5B shows that during this crisis, the HI P_n fell slightly. We observed also an excess of released CO_2 as compared to absorbed O_2. Finally, as a drop in the respiration rate was observed from the 32nd up to the 36th day (Fig. 3–4), one might suppose that a positive P_n would have been obtained around the 38th–40th days, thus completing photoautotrophy; however a slight necrosis occurred from the 32nd day among the shoots, which hindered the experimentation beyond the 35th or 36th day.

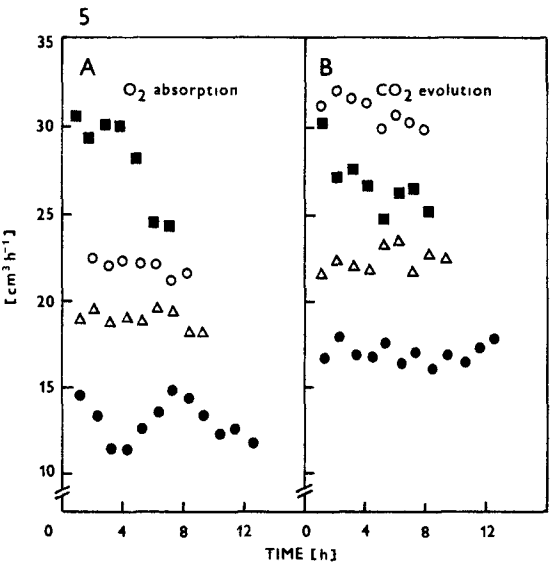

Fig. 5 – Hourly variations in O_2 absorption (A) and CO_2 evolution (B) in shoot cultures grown on G-medium.
– during a dark period between 9th- 10th days (•) or 22nd–23rd days (▪)
– during a low irradiance (LI) period (24th days: Δ) or a high irradiance (HI) period (31st day: o)

Cultures on G/4–medium

In order to accelerate the photomixotrophy-photoautotrophy transitions, we used cultures grown on a medium containing reduced sugar level (41 mM glucose). Gas exchange measurements (Fig. 6) show a positive P_n from the 13th day, under HI, and from the 18th day, under LI. From the 13th day up to the 17th day, the cultures were able to make better use of HI. On the 14th day, the P_G (difference between the values obtained with cultures grown in light or darkness) was: 1 cm^3 (O_2 evolved) h^{-1} $flask^{-1}$ (HI) or 0.45 ml h^{-1} $flask^{-1}$ (LI). Between the 16th and 18th days, we also remarked that P_n was rather stable under HI, indicating a stationary photosynthetic

potential; on the contrary, P_n increased under LI. The dark respiration rate remained almost stable except at the end of the experiment where the increase of CO_2/O_2 ratio perhaps revealed some fermentation process.

Table 1 shows the CO_2 concentration values in flasks. When the shoot development was such as to allow a positive P_n in HI only (13th–17th days), the CO_2 concentrations under light remained high (around 1000 $cm^3\ m^{-3}$). When the P_n was positive both under LI and HI (18th–23rd days), the CO_2 concentrations were around 500 $cm^3 m^{-3}$. In young cultures (5th–10th days), the CO_2 concentrations were between 650 and 1100 $cm^3\ m^{-3}$ (results not shown).

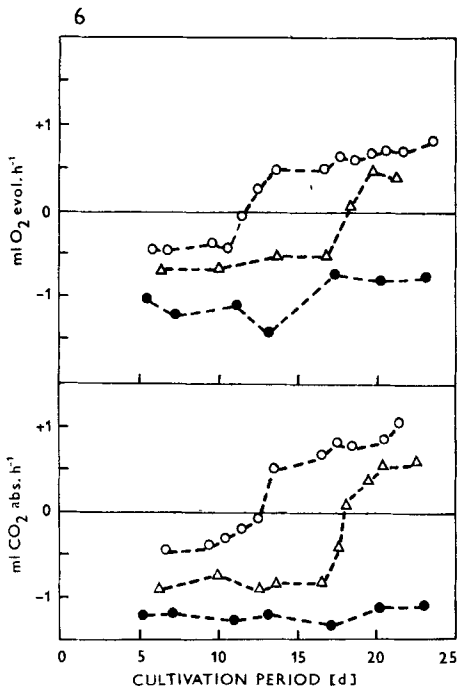

Fig. 6 – CO_2 absorption and O_2 evolution in shoot cultures grown on G/4 – medium. Results are given for 2 experimental flasks. See Fig. 3 for symbols.

On the 18th day, P_n became positive under LI (Fig. 6). However, a study of hourly variations of O_2 and CO_2 exchanges (Fig. 7) shows that between the 4th and the 8th h, the P_n had a tendency to revert to a negative state. Such a "hesitation" remains to be explained. At the end of the experiment, P_n under HI increased slowly from day to day (Fig. 6). We also noticed hourly changes during the light period. Fig. 7 gives an example on the 22nd day: both for O_2 and CO_2 exchanges, lowering of activities were observed after the fourth hour of light, as is there were a "fatigue" somewhere in the enzymatic systems of photosynthesis.

Table 1

Average CO_2 concentrations effectively observed during the photomixotrophic-photoautotrophic transition in cultures grown on G/4-medium.

Time [d]	11	12	13	14	18	19	20	21	22	23
Irradiance [$\mu mol\ m^{-2}s^{-1}$]	60	150	150	150	150	60	150	60	60	60
CO_2 Concentration [cm^3m^{-3}]	810	1050	980	1120	680	650	610	740	570	490

DISCUSSION

Photoautotrophy is generally achieved *in vitro* differently from the way it is here: cultures are grown in sugar-free media, under high CO_2 level (Yamada *et al.* 1978, 1982). In *R. graveolens*, the first photoautotrophic tissue cultures were obtained by Corduan (1970). The fundamental photosynthetic functions were observed by Yamaya *et al.* (1977) in suspension cultures, but the cells were unable to grow without exogenous carbohydrates.

In cell and tissue cultures of several species grown in sugar-supplemented media under light and high CO_2 concentrations, glucose and fructose are absorbed and phosphorylated (Neumann and Bender 1987). The phosphorylated products are then channeled into metabolism via glycolysis. In shoot forming cultures of *Digitalis purpurea* (Hagimori *et al.* 1984), higher photosynthetic characteristics were obtained more easily with photoautotrophic cultures (but under 1 % CO_2) than with corresponding photomixotrophic cultures (the CO_2 for photosynthesis mainly comes from glycolysis). Photoautotrophic cell suspensions of *Chenopodium rubrum* exhibit many photosynthetic enzyme activities (Herzbeck and Hüseman 1985) and recycling of $^{14}CO_2$ originating from decarboxylation processes. Another decarboxylation mechanism was shown in *R. graveolens* tissue cultures (Ignatiev and Polevaya 1986) and a stimulation of respiration by high saccharose level was observed by Smolov *et al.* (1978). More recently, Solárová *et al.* (1989) showed that *in vitro* tobacco plantlets cultivated without saccharose grow better under high than low CO_2 levels.

In *Nicotiana tabacum* cells cultured in a medium containing 30 $g\ l^{-1}$ saccharose under a rather high irradiance, photoautotrophic growth was obtained (the saccharose being probably totally consumed), and a low CO_2 concentration in the flasks was observed after a photomixotrophic first phase of growth giving a high CO_2 level in the flasks (Nato *et al.* 1982). In our work, shoot forming cultures of *R. graveolens*, after a photomixotrophic state, became photoautotrophic at the end of the growth,

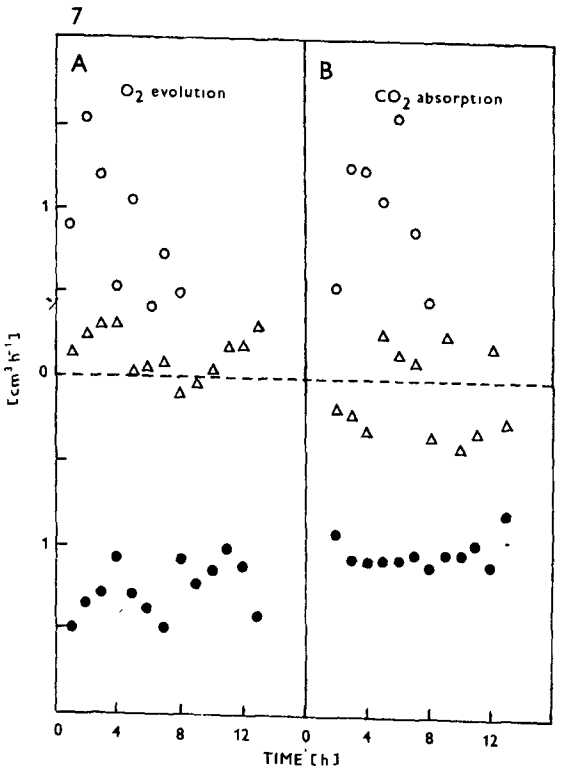

Fig. 7 – Hourly variations in O_2 evolution (A) and CO_2 absorption (B) in shoot cultures grown on G/4 – medium.
– during a dark period between 7th–8th days (•)
– during a low irradiance (LI) period (18th day: Δ) or a hight irradiance (HI) period (22nd day: o)

when the glucose was certainly consumed, and under CO_2 concentrations similar to those occuring in natural conditions.

As suggested by Nato *et al.* (1982), the dependence of *in vitro* cell cultures upon the carbohydrates may be compared with the transition from an heterotrophic (or a photomixotrophic) to an autotrophic condition during the maturation of young leaf cells. Moreover, the seedlings of higher plants consume their carbohydrate reserves before their photoautotrophic life. As previously shown with maize seedlings (Leclerc and Abd el Rahman 1988), going from heterotrophy to autotrophy is not only a simple replacement of one mode of metabolism by another; photosynthesis only develops freely in harmony with the other activities of the plant, after a crisis includes the disappearance of the heterotrophic metabolism. As with the maize seedlings, we observed that the *R. graveolens* shoots present some signs of crisis when the glucose has probably been consumed: imbalance between O_2 and CO_2 exchanges in shoot cultures grown on G-medium, "hesitation" for the positive P_n passage in the shoot cultures grown on G4-medium.

R. graveolens cultures grown on a medium containing 166 mM glucose presented well formed shoots and leaves, but photoautotrophy was not attainable because the shoots had grown too old when the glucose was completely consumed. On the contrary, with cultures grown on G/4-medium, photoautotrophy was achieved but the shoots were small with abnormal leaves. Apparently, good photosynthesis and good morphogenesis were not correlated. It would probably be necessary to change some hormonal parameters in order to get rapidly both normal photosynthesis and normal morphology. As shown by Heble (1985) in some species, the obtaining of interesting secondary metabolites is sometimes dependent upon structural organization, but the relationship between secondary metabolism and photoautotrophy is not simple: in preliminary experiments, we found that the alkaloid content in shoots grown on G/4-medium was lower than in shoots grown on G-medium. Probably, a clue for a good alkaloid production in multiple-shoot cultures lies both in their energetic status (photosynthesis and respiration activities) and in their hormonal balance.

Using the apparatus for simultaneous CO_2 and O_2 exchange measurements is simple. In a multiple assay system for hormonal and light conditions, it could be adapted to test repeatedly the photosynthetic development of many samples, without contaminating or sacrificing them. Multipoint measurements are possible with the analog scanner and its associated microvoltmeter. Air oxygen probes for photosynthesis can be purchased at low cost.

Acknowledgement

The authors wish to thank Dr. Damphousse (Tours University) for revising the English manuscript.

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