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CARBOHYDRATE OXIDATION DURING *NICOTIANA TABACUM* CALLUS GROWTH

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Abstract—Tobacco callus was cultured in light or dark, with or without giberellic acid, and with various carbon sources in the medium, and the growth rate and activities of some enzymes of the Embden–Meyerhof–Parnas and pentose phosphate pathway were determined. No changes in the specific activities of enzymes of either pathway could be correlated with growth but there was a light-dependent stimulation of the pentose phosphate pathway enzymes examined

INTRODUCTION

CULTURES of plant tissues *in vitro* normally require an exogenous source of carbon and energy.[1] For most plants sucrose is the most effective carbon source.[2] In carrot tissue cultures, sucrose was followed by glucose, maltose, raffinose, fructose, galactose, mannose and lactose in decreasing order of utilization.[3] Tobacco, sunflower, periwinkle, paris-daisy and marigold tissues in culture grew relatively well on media containing sucrose, glucose, fructose, and with the exception of sunflower, on maltose as well.[4] In that study, the growth response to other carbohydrates was species specific; with tobacco growing moderately well on media containing mannose, cellobiose, dextrin and pectin, but poorly or not at all on galactose, lactose, raffinose, starch or inulin. Sucrose (final concentration 3%) was found to give the highest yield in fresh and dry wts for tobacco callus grown on a high salt medium.[5] In addition, gibberellic acid markedly increased the fresh wt of that tissue.

In previous studies, it was shown that tobacco callus grown on sucrose produced the largest number of shoots in light or darkness.[6] In light-grown tissue, maltose-grown tissue was the next best carbon source, followed by glucose plus fructose mixture, fructose, glucose and raffinose. Tissue grown on raffinose-medium produced distinctly smaller shoots. In dark-grown cultures, fructose was second in effectiveness to sucrose, followed by glucose, glucose plus fructose, maltose and raffinose as carbon sources. Sorbitol-grown tissue remained alive but produced no shoots in light or darkness, while galactose- and lactose-containing media were toxic. Furthermore, these various carbon sources maintained the correlation between starch accumulation and shoot formation.[7,8]

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[2] BUTENKO, R. G. (1964) *Plant Tissue Culture and Plant Morphogenesis*, p. 25, Science Press, Moscow. Translated from Russian by Israel Program for Scientific Translations, Jerusalem (1968)
[3] GAUTHERET, R. J. (1959) *La Culture des Tissus Vegetaux Techniques et Realisations*, Masson, Paris.
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[5] MURASHIGE, T. and SKOOG, F. (1962) *Physiol Plant* **15**, 473
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Thus, it seemed possible that the differences in growth of cultured tissue, grown under different culture conditions and on various carbon sources, might be related to differences in the ability of the tissue to oxidize the carbohydrates. In plants two major pathways of carbohydrate oxidation are present; the Embden–Meyerhof–Parnas (EMP) glycolytic pathway and the pentose phosphate pathway (PPP).[9] Indeed, we have found higher levels of activity of enzymes of these pathways in shoot-forming tobacco callus than in corresponding non-shoot-forming cultures.[10] In this paper, the activities of key enzymes of each of these pathways were examined during culture of tobacco callus under various culture conditions, and in the presence of various carbon sources. The enzymes examined were glucokinase (ATP:D-glucose 6-phosphotransferase, E.C. 2.7.1.2), aldolase (fructose-1,6-diphosphate-D-glyceraldehyde-3-phosphate-lyase, E.C. 4.1.2.b), glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate:NADP oxidoreductase, E.C. 1.1.1.49) and phosphogluconate dehydrogenase [6-phospho-D-gluconate:NADP oxidoreductase (decarboxylating), E.C. 1.1.1.44]

RESULTS

The growth of tobacco callus for 35 days in light and darkness on the sucrose-medium in the presence or absence of gibberellic acid (GA_3) is shown in Fig. 1. Tissue grown in the dark remained colourless, while that exposed to continuous illumination turned green after 7 days. A typical sigmoidal growth curve was observed for fresh weight increase of dark-grown tissue in the absence of GA_3. The lag phase of growth which lasted for about 2 weeks was followed by the log phase for the next 2 weeks after which growth slowed down. The free sugar content of the tissue remained high at 5–6 weeks in culture, being in excess of 20 mg/g fresh wt. For light-grown tissue in the absence of GA_3 there was a steady increase in fresh weight throughout the culture period. The effect of GA_3 was very pronounced on growth in both light and dark, particularly beyond 2 weeks in culture. The increase in fresh weight in the dark-grown cultures was accompanied by increased friability of the tissue, while the light-grown tissue remained firm. The tissue cultured in the presence of GA_3 remained in the log phase of growth up to the end of the 5 week culture period. Examination of the dry weights of the tissue (Fig. 1) showed that there was a steady accumulation of non-aqueous material, light-grown tissue in the presence of GA_3 having the highest dry weight, followed by dark-grown GA_3-treated tissue, dark-grown tissue and finally light-grown tissue. In the early stages of growth (up to 2 weeks) the rate of dry weight increase was higher for light-grown than for dark-grown cultures.

Examination of the activities of glucokinase and aldolase, showed that there were no differences in the activities of these EMP enzymes in tobacco callus cultured in the light or in the dark in the presence or absence of GA_3 (Fig. 2), when sucrose was used as the energy source. For light-grown tissue there was a very slight increase in glucokinase activity over the dark-grown tissue during the last 2 weeks of culture. In general, the activity of aldolase increased slightly during the first 3 weeks of culture in both light and dark-grown cultures. This increase was greater in the dark-grown than in the light-grown tissues during the early period. With respect to glucose 6-phosphate dehydrogenase the activity of this enzyme was higher in dark-grown tissue during the initial period. However, by 2 weeks the activity of this enzyme in light-grown cultures was significantly higher and the

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differences increased with age over the dark-grown tissues. The level of activity of this enzyme was higher in the GA_3-grown tissue in the light than in the absence of this growth regulator. For dark-grown tissue after the initial rise in activity there was a steady decline in the level of activity of this enzyme. With respect to phosphogluconate dehydrogenase, there were no differences in activity in the different treatments early in culture, but by 14 days the activity of this enzyme in light-grown cultures began to increase and continued to do so for the remainder of the culture period. Here again the activity of the GA_3-grown tissue in the light was higher than the corresponding tissue in the absence of GA_3. The reverse was seen in the dark-grown cultures, which maintained a steady level of enzyme activity. The activity in the presence of GA_3 was less than in its absence

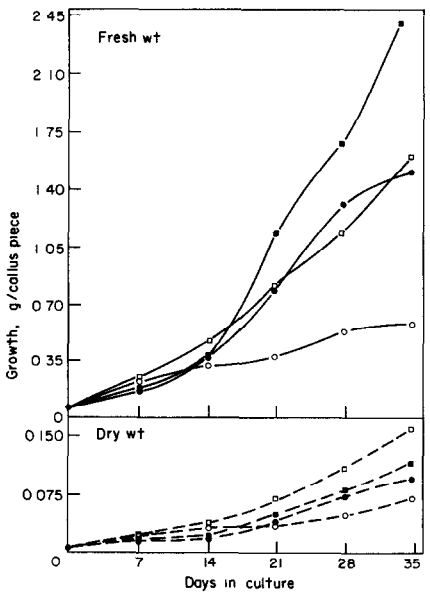

FIG 1 GROWTH OF TOBACCO CALLUS ON SUCROSE MEDIUM IN THE LIGHT (OPEN SYMBOLS) AND DARK (SOLID SYMBOLS) IN THE ABSENCE (CIRCLES) OR PRESENCE (SQUARES) OF GIBBERELLIC ACID (GA_3, 5×10^{-7} M)

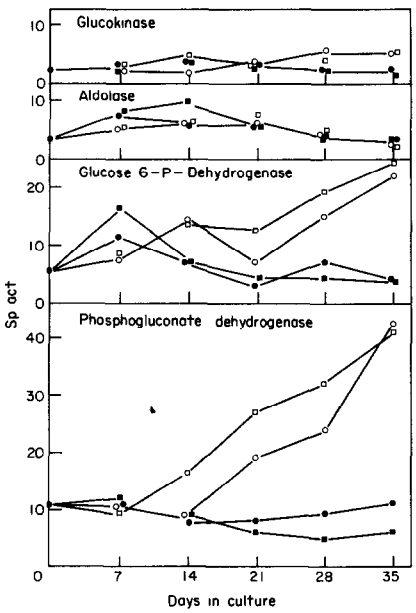

FIG 2 SPECIFIC ACTIVITIES OF GLUCOKINASE, ALDOLASE, GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND PHOSPHOGLUCONATE DEHYDROGENASE IN TOBACCO CALLUS CULTURED ON SUCROSE MEDIUM IN THE LIGHT (OPEN SYMBOLS) AND DARK (CLOSED SYMBOLS) IN THE PRESENCE (SQUARES) OR ABSENCE (CIRCLES) OF GA_3 (5×10^{-7} M)
Specific activity defined in Experimental section

The growth of tobacco callus in the dark on media containing different carbon sources is shown in Fig. 3. Sigmoidal growth curves were obtained for fresh weight growth of tissue grown on sucrose, maltose and raffinose. Tissue grown on glucose was still in the log phase of growth at the end of the culture period. The kinetics of growth of the tissue on sucrose, glucose and maltose were almost the same during the first 3 weeks of growth. Beyond 3 weeks the growth of the tissue on sucrose was superior, leading to the largest increase in weight, followed by glucose, and then maltose. Tissue grown on raffinose grew at a slower rate from the beginning and was significantly slower in growth by 2 weeks, so that the final fresh weight was half that of the tissue grown on maltose and much less than half

that grown on sucrose and glucose. Changes in the dry weight of tissues confirmed the fresh weight findings. During the first 3 weeks there were no differences in dry weight in tissue grown on sucrose, glucose or maltose. Beyond that period the accumulation of matter proceeded at a faster rate in sucrose-grown tissue; while glucose- and maltose-grown tissue increased in dry weight at the same rate. Here again the increase in dry weight was significantly slower in raffinose-grown tissue.

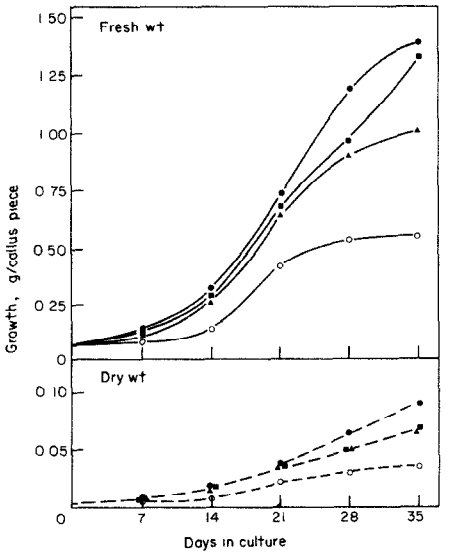

FIG 3 GROWTH OF TOBACCO CALLUS IN THE DARK WHEN CULTURED ON MEDIA CONTAINING 3% (W/V) OF SUCROSE (—●—), GLUCOSE (—■—), MALTOSE (—▲—), AND RAFFINOSE (—○—)

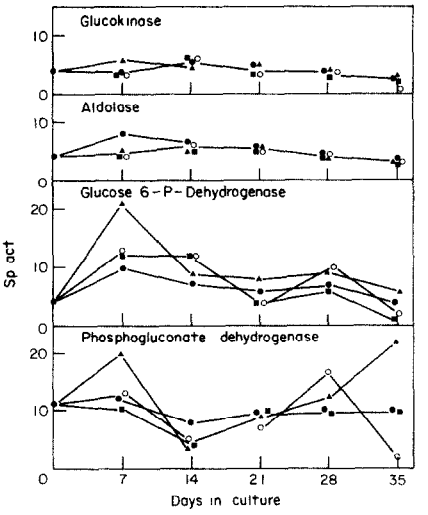

FIG 4 SPECIFIC ACITIVITIES OF GLUCOKINASE, ALDOLASE, GLUCOSE-6-PHOSPHATE DEHYDROGENASE, AND PHOSPHOGLUCONATE DEHYDROGENASE IN TOBACCO CALLUS CULTURED ON MEDIA CONTAINING 3% (W/V) OF SUCROSE (—●—), GLUCOSE (—■—), MALTOSE (—▲—), AND RAFFINOSE (—○—)
Specific activity defined in Experimental section

Figure 4 shows that there were no differences in activity of either glucokinase or aldolase in tobacco callus grown on the four carbon sources examined. By the end of the culture period the activity of glucokinase on raffinose-grown tissue was very low There was a slightly greater increase in aldolase activity in sucrose-grown tissue in the log phase of growth, but no such differences were observed during the remainder of the culture period. With respect to the activity of the PPP enzymes, no differences in the activities of either glucose-6-phosphate dehydrogenase or phosphogluconate dehydrogenase was observed in the tissue grown on the different carbon sources. Tissue grown on maltose showed a significant early stimulation in activity of both enzymes during the lag phase of growth, but during the log phase of growth its PPP activities were similar to those obtained when the tissue was grown on the other carbon sources. Similarly, at 5 weeks in culture the maltose-grown tissue had a significantly higher level of activity for phosphogluconate dehydrogenase. The activities of these PPP enzymes in the raffinose-grown tissue fluctuated quite markedly during culture, with the activity of phosphogluconate dehydrogenase being very low at 5 weeks in culture compared to the other treatments

DISCUSSION

In these studies, we have been able to show that differences in both growth rate and final increase in fresh or dry weight of cultured callus varied with culture conditions Tobacco callus growth in the absence of gibberellic acid, which is not required for growth,[5] was greater for dark-grown cultures than the corresponding light-grown tissues This light-inhibition of callus growth is in keeping with observations made on the intact plant,[11,12] and was also observed in grapefruit callus [13] The presence of GA_3 in the medium increased the growth rate and the final weight of the tissue grown on sucrose in both light and darkness. It has been shown that the effect of GA_3 on callus growth in dimly lit cultures (*ca* 270 lx) was mainly on fresh weight.[5] While this has been confirmed for dark-grown cultures, the effects of GA_3 on increasing dry weight in cultures exposed to higher light intensity (*ca* 2700 lx) was much more pronounced. Also of interest was the finding that cultures in the presence of GA_3 were still in the log phase of fresh weight growth after 5 weeks, when the dark-grown cultures, particularly, had almost entirely covered the agar surface of the flasks. We were also able to confirm the earlier findings with respect to the growth of tobacco callus on different carbon sources in the dark.[4] In terms of fresh weight increase, sucrose was best, followed by glucose, maltose and raffinose, this last carbohydrate being only about half as effective as maltose. In terms of dry weight increase, sucrose was again superior followed by glucose and maltose and lastly, raffinose Of interest was the finding that the differences in growth of the tissue on sucrose, glucose and maltose were mainly related to differences in growth rate during the last two weeks of culture Clearly, in terms of the rate of fresh and dry weight increase and consequently the final weight of the tissue, raffinose was a rather poor carbon source.

Despite the differences in growth obtained above, no corresponding differences in the specific activities of the enzymes examined were obtained. In terms of the EMP glycolytic pathway, glucokinase and aldolase were equally active under all conditions The slightly higher specific activity of glucokinase in light-grown over dark-grown tissue (Fig 2), while possibly real and significant, did not correlate with the growth rates observed With the enzymes of the pentose phosphate pathway there was a pronounced stimulation of the specific activities of these enzymes in light-grown tissue after 14 days. This light dependent stimulation in activity was independent of GA_3, and did not correlate with the fresh or dry weight growth. The significance of this finding is not clear Light-stimulation of enzyme activity in plants is not unknown, e.g. light stimulates the level of activity of ribulose-1,5-diphosphate carboxylase in barley, nitrate reductase in corn, and phenylalanine ammonia lyase in gherkin.[14] We have also found a pronounced stimulation of these enzymes in tobacco callus at the time of meristemoid and shoot-primordium formation in dark-grown cultures.[10] Similarly, while differences in the specific activities of the PPP enzymes in tissue grown in the dark with different carbon sources were found, none of the differences observed could be correlated with the differences in growth of the tissue.

Also of interest in this study, was the observation that no changes in specific activities of key enzymes of either the EMP glycolytic pathway or the PPP could be correlated with a particular phase of growth of the tissue. This finding is different from that observed during growth of sycamore cells in batch suspension culture.[15]

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Here, during the early stages both pathways made appreciable contributions to carbohydrate oxidation, but following the initiation of cell division, carbohydrate oxidation was predominantly by the EMP pathway in the sycamore cells. This difference in behavior may be related to the conditions, i.e. static culture as opposed to the suspension culture used for sycamore. The more or less steady levels of activity of these pathways during callus proliferation also differed from what was observed in this tissue grown under shoot-forming conditions.[10]

The role of the EMP glycolytic pathway in the production of pyruvate, which is utilized in the tricarboxylic acid cycle with concomitant oxidative phosphorylation to produce ATP, is the main pathway for the oxidation of carbohydrate for production of energy. The PPP is also a route of carbohydrate oxidation, but is an important source for producing reducing power (NADPH) for biosynthesis, pentose for nucleic acid synthesis and erythrose-4-phosphate for synthesis of aromatic compounds, via the shikimic acid pathway.[9] The failure in this study to correlate differences in growth of the tissue under different treatments with the specific activities of enzymes involved in carbohydrate oxidation, indicates that the differences in growth observed may be related to factors other than the oxidation of carbohydrate.

EXPERIMENTAL

Plant material and culture conditions. Tobacco (*Nicotiana tabacum* L., cv. Wisconsin 38) callus was isolated from stem pith segments, and maintained on MS medium[5] supplemented with White's organics,[16] inositol (100 mg/l.), indole-3-acetic acid (10^{-5} M), kinetin (2.5×10^{-6} M), Difco casamino acids (1 g/l.) and sucrose (3%). For experimental purposes, the casamino acids and White's organics were omitted, and thiamine·HCl[17] was added. Carbon sources were incorporated into the medium before autoclaving in a final concentration of 3%. Filter-sterilized gibberellic acid (3×10^{-7} M) was added to the medium after it was autoclaved, but before it solidified. Varying numbers of callus pieces (*ca* 50 mg each) from 5- to 6-week-old stock callus were planted in 125 ml conical flasks, which contained 30 ml of medium solidified by Difco Bacto agar (0·9%). Cultures were maintained in darkness or in continuous light (*ca* 2700 lx) at a temperature of 26 ± 1°.

Tissue growth. Ten pieces of tissue per treatment, per treatment day, were weighed and then dried for 48 hr in a drying oven at 80°. Average values per callus piece were computed.

Extraction of enzymes. 2·2–2·4 g of pooled tissue per treatment were ground in a cold mortar with *ca* 0·1 g polyvinylpyrrolidone (PVP) and 10 ml of phosphate buffer (0·1 M, pH 7) then further treated in a Potter–Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 3×10^4 g. The supernatant was dialyzed for 2 hr at 5° against 4 l. of 50 mM Tris·HCl buffer (pH 7·6).

Determination of enzyme activities. Glucokinase, aldolase, glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase were assayed[18–21] with slight modifications in respect to pH for obtaining maximum activities. In each case the substrate was present in a final concentration of 5 μM, and all assays were in a total vol. of 1 ml. The enzymes were measured spectrophotometrically. The specific activity of the enzymes is defined as 1 nmol of NADPH formed or NADH oxidized/min/mg protein. The protein content of the extracts was determined by the Folin–phenol method.[22]

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