Additional phosphate stabilises uninterrupted growth of a *Dioscorea deltoidea* cell culture

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**Abstract**

Suspension cells of *Dioscorea deltoidea* Wall (strain D-1) were maintained in a semicontinuous culture (SCC) in shake flasks at a high growth rate. It was shown that continuous propagation growth of this culture is unstable on Murashige's and Skoog's (MS) medium due to P starvation. On a P-enriched MS-medium the SCC was stable even at mean specific growth rates \( > 0.3 \text{ day}^{-1} \). Highest volumetric concentrations of furostanol glycosides were obtained, when a P-enriched SCC was not further subcultivated but fed with sucrose. The investigated culture is able to control phosphate uptake and to prevent toxicity on media with excess P. High concentrations of cellular Pi did not effect the ratio of furostanol to starch. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

**Keywords**: *Dioscorea deltoidea*; Furostanol glycosides; Phosphate limitation; Plant cell culture; Secondary metabolism; Semicontinuous culture

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**1. Introduction**

Uninterrupted plant cell propagation at continuous or discontinuous dilution with fresh medium has found useful applications [1–8]. The semicontinuous culture (SCC) is a repeated batch culture with relatively short culture periods between the dilution steps. By an appropriate choice of the length of these periods and adjusting the dilution rate \( (\delta) \) to a value slightly below the maximum specific growth rate of the culture, a steady state of uninterrupted growth is reached, whereby mean value of the specific growth rate \( (\mu) \) equals \( \delta \). For special biotechnological purposes and also physiological studies [9,10] it can be of great value that the fraction of resting or differentiating cells is small or even negligible in a SCC at sufficient high values of \( \delta \).

The transfer of a batch culture to a continuous dilution regime is not successful in every case. Kandarakov et al. [11] tried to establish chemostat cultures of *Dioscorea deltoidea* Wall. In the fermentor, the cell density increased with a high specific growth rate before the chemostat regime was established. At continuous dilution of the culture by fresh medium, the growth rate could be adapted to the dilution rate for certain periods only, whereafter the growth rate and the percentage of viable cells decreased rapidly. This breakdown phenomenon occurred at dilution rates which were significantly lower than the maximum specific growth rate.

The relatively low phosphate concentration of the applied medium (defined by Murashige and Skoog [12]) was found to be suboptimal for the growth of a *Chenopodium album* L. suspension culture at high cell density [13]. We investigated in this study whether P deficiency is the reason of the reported break-down of the *D. deltoidea* cell cul-
ture in the regime of uninterrupted growth and whether an increased P concentration effects the production of furostanol glycosides by this culture.

2. Materials and methods

2.1. Plant cell culture

The investigated strain D-1 of a *D. deltoidea* suspension had been initiated from a rhizome [14]. The suspension cultures (200 or 150 ml in each culture vessel) were grown in 500 ml flasks on a rotary shaker (radius 2.5 cm) at 200 rpm and 27°C in dim light. The mineral medium described by Murashige and Skoog [12] was applied in combination with 40 g l\(^{-1}\) sucrose, 0.25 mg l\(^{-1}\) 2,4-dichlorophenoxyacetic acid and 0.1 mg l\(^{-1}\) kinetin (N\(^6\)-fururylaminopurine). The phosphate concentration was either 0.17 g KH\(_2\)PO\(_4\) l\(^{-1}\) standard MS-medium) or 0.4 g KH\(_2\)PO\(_4\) l\(^{-1}\) (P-enriched MS-medium). Uninterruptedly growing semicontinuous cultures were established by periodically replacing a certain portion (\(a\)) of the suspension broth by fresh medium. The mean dilution rate (\(\delta = 1/\Delta t \ln f\)) was obtained from the dilution factor (\(f = 1/(1-a)\)) and the culture period (\(\Delta t\)). Cells previously grown on SCC were transferred to the stationary phase by repeated addition of 10 ml of concentrated sucrose solution (300 g l\(^{-1}\)) to the suspension (150 ml). The sucrose solution was added when the refractometric index of the medium was lower than 2%. Stock suspensions were kept on standard MS-medium with a culture period of 14 days and a dilution factor of 6. Samples for analysis were obtained either at the end of the subculture period in the SCC, when defined volumes of the suspension were removed and replaced by fresh medium, or by withdrawal of 10 ml suspension from batch cultures.

2.2. Parameter determinations

Inorganic phosphate (P\(_i\)) in the medium and in the cells (after extraction with 7% trichloroacetic acid) was measured by the molybdenum blue-method. Viability was estimated as the percentage of unstained cells after mixing one volume of cell suspension with one volume of 0.1% phenosafranin or Evan’s blue. Sucrose consumption was calculated from the decrease in refractometric index. Yield coefficients (\(Y\)) of phosphate and sucrose were calculated from data obtained with suspensions growing in SCC at a relatively constant dry wt-level in subsequent culture periods [15]:

\[
Y = \frac{C_t - C_0}{S_0 - S_t} \quad (\delta = \mu),
\]

where \(C_t\) and \(C_0\) are the cell dry wt-concentrations (g l\(^{-1}\)), and \(S_0\) and \(S_t\) the concentrations of the respective substrate (mmol l\(^{-1}\) or g l\(^{-1}\)) in the medium after (index 0) and prior (index t) to dilution. The cell number was counted in a hemacytometer (Fuchs–Rosenthal) after maceration of cell aggregates in 20% chrome acid for 30 min at 60°C and passing twice through a fine needle (0.8 mm in diameter, 40 mm in length). Cell cluster-size distribution was analysed with a laser particle analyzer. The mitotic index and the ploidy spectrum were investigated after fixation of the cells with ethanol–acetic acid, 3:1 (v/v), and staining with aceto-orcein. At least 100 metaphase plates were analyzed on squashed preparations. Furostanol glycosides were determined colorimetrically [16]. The standard of furostanol glycosides was obtained from Professor V. Paseshnichenko, Bach Institute of Biochemistry (Moscow).

The cell wall cut-off, a measure of wall pore size, was analyzed by a method described previously [13,17]. It represents the Stokes’ radius of a dextran fraction, which reaches 50% of the inner space of ethanol-denatured cells (diffusion time 30 min).

3. Results

3.1. Uninterrupted propagation of cells on standard and P-enriched MS-medium

To check whether the previously described growth instabilities of *D. deltoidea* on standard MS-medium were due to the regime of continuous dilution, discontinuous dilution with a relatively long interval (\(\Delta t = 7\) days) was carried out, whereby the mean dilution rates were high enough to prevent a stationary phase. Fairly constant final cell densities were maintained for more than 15 cycles. Finally, however, the specific growth rate and the percentage of viable cells decreased sud-
ddenly and the cultures diluted out (Table 1A). The semicontinuous culture (SCC) was then established on a MS-medium with enhanced concentration of KH$_2$PO$_4$. Here the dilution rate could be raised to more than 0.3 day$^{-1}$. The culture was able to keep the cell dry weight (dry wt.-concentration at a relatively high level (10–15 g$^{-1}$ before dilution). No break-down was observed (Table 1B).

To confirm that P limitation is responsible for the break-down phenomenon, a SCC was established on the P-enriched medium, and repeatedly, parts of this culture were subcultivated on standard medium. Fig. 1 shows that uninterrupted growth was stable on P-enriched and unstable on standard medium.

To compare the cultures growing with different phosphate levels, parallel SCCs ($\delta = 0.23$ day$^{-1}$, $\Delta t = 72$ h) were established on both standard and P-enriched MS-medium (Table 2). The data obtained for the mitotic index and the polyploidy pattern (not shown here) were not suitable for a quantitative comparison, as these values showed strong fluctuations at both P-levels. Obviously, the SCCs were not in an ideal asynchronous state. After a transition period of about 12 days a relatively constant final cell dry wt. concentration was obtained in 15 subsequent cycles at both P levels. $P_i$ was completely absorbed from the P-enriched medium during the second day. Yield coefficients ($Y$) of P were higher (5.36 g dry wt. per mmol P) on the standard MS-medium than on the P-enriched medium (3.88 g dry wt. per mmol P). This means that at the end of one subculture period the cells harvested from the P-enriched medium had a higher total P concentration (sum of free and esterified) in their dry wt. (258 μmol per g dry wt.) than the cells harvested from standard medium (187 μmol per g dry wt.). On the standard MS-medium the P$_i$ content in the cells at the end of the subculture period was 0.6–1.0 μmol per g dry wt. (ca. 0.5% of the total cellular P). During break-down of cell growth on the standard medium, when cell concentration decreased and more phosphate was available per cell (Fig. 1), cellular P$_i$ increased to 5–6 μmol per g dry wt. On the P-enriched medium, cellular P$_i$ was 3.7–5.1 μmol g$^{-1}$ and thus remained small in comparison to total P (ca. 2%).

On a fresh (fr. wt.) and dry wt.-basis, the contents of furostanol glycosides were similar in both variants. However, due to the higher dry wt.-concentration, the volumetric productivity of furostanol glycoside synthesis was markedly improved in the P-enriched suspension. The ratio of fr. wt. to dry wt. was independent of the P-level, whereas the cell cluster size was slightly reduced at higher P (Table 2). The cell wall cut off [13] of the uninterruptedly growing cells (3.3 nm) was relatively high in comparison to the value obtained in

<table>
<thead>
<tr>
<th>Medium</th>
<th>Experiment number</th>
<th>Dilution rate, culture period</th>
<th>Cycles investigated</th>
<th>Culture stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>0.26 day$^{-1}$, 7 days</td>
<td>18</td>
<td>After the 16th cycle diluting out and viability loss</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.23 day$^{-1}$, 7 days</td>
<td>22</td>
<td>After the 19th cycle diluting out and viability loss</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
<td>0.2 day$^{-1}$, 7 days</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subsequently 0.2 day$^{-1}$, 3 and 4 days (alternatingly)</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subsequently 0.23 day$^{-1}$, 3 and 4 days</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subsequently 0.27 day$^{-1}$, 3 and 4 days</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subsequently 0.34 day$^{-1}$, 3 and 4 days</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Subsequently 0.31 day$^{-1}$, 3 and 4 days</td>
<td>18</td>
<td>No diluting out until the end of the experiment (324 days)</td>
</tr>
</tbody>
</table>
Fig. 1. Semicontinuous cultures on P-enriched and standard MS-medium. Cells of a batch culture \((Δt = 14 \text{ days}, f = 5)\) growing on the P-enriched medium were adapted to a semicontinuous regime \((Δt = 3 \text{ or } 4 \text{ days}, \text{ alternating, mean } δ = 0.2 \text{ day}^{-1})\) on standard MS-medium (▲) and P-enriched medium (□). At day 78, \(δ\) was fixed to 0.23 day\(^{-1}\) \((Δt = 3 \text{ days})\). At the times indicated by the arrows, a part of the suspension grown on the P-enriched medium was further subcultivated on the standard MS-medium. Cell dry wt.-concentration refers to the end of the subculture period. Each variant is represented by values of two suspensions cultured in parallel. Mean furostanol content (% of fr. wt.) was 0.82 \(±\) 0.08 on standard medium and 0.83 \(±\) 0.10 on P-enriched medium.

Table 2
Parameters obtained for uninterrupted growth on standard and P-enriched medium\(^a\)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Standard medium</th>
<th>P-enriched medium</th>
<th>(n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell dry wt. concentration (\text{g l}^{-1})</td>
<td>6.70 (±) 0.90</td>
<td>11.4 (±) 1.10</td>
<td>14(^b)</td>
</tr>
<tr>
<td>Volumetric biomass production (\text{g dry wt. l}^{-1} \text{day}^{-1})</td>
<td>1.11 (±) 0.62</td>
<td>1.93 (±) 0.41</td>
<td>14(^c)</td>
</tr>
<tr>
<td>Ratio fr. wt. to dry wt.</td>
<td>8.02 (±) 1.07</td>
<td>8.39 (±) 0.63</td>
<td>14(^b)</td>
</tr>
<tr>
<td>Sucrose consumption (\text{g l}^{-1} \text{day}^{-1})</td>
<td>2.20 (±) 0.80</td>
<td>4.60 (±) 0.70</td>
<td>14(^c)</td>
</tr>
<tr>
<td>Yield coefficient for sucrose (\text{g dry wt. g}^{-1})</td>
<td>0.51 (±) 0.20</td>
<td>0.41 (±) 0.07</td>
<td>(^d)</td>
</tr>
<tr>
<td>Yield coefficient for phosphate (\text{g dry wt. mmol}^{-1})</td>
<td>5.36 (±) 1.02</td>
<td>3.88 (±) 0.53</td>
<td>(^d)</td>
</tr>
<tr>
<td>Furostanol glycosides (% dry wt.)</td>
<td>4.17 (±) 0.96</td>
<td>4.87 (±) 0.78</td>
<td>14(^c)</td>
</tr>
<tr>
<td>Furostanol glycosides (mg l(^{-1}))</td>
<td>261.30 (±) 62.60</td>
<td>519.80 (±) 76.70</td>
<td>14(^a)</td>
</tr>
<tr>
<td>Volumetric production of furostanol glycosides (mg l(^{-1} \text{day}^{-1}))</td>
<td>38.50 (±) 18.40</td>
<td>81.10 (±) 21.80</td>
<td>14(^c)</td>
</tr>
<tr>
<td>Cell cluster size (\mu\text{m})</td>
<td>138.25 (±) 4.99</td>
<td>125.30 (±) 5.49</td>
<td>14(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Sampling was started at day 12 of the semicontinuous culture with a 72 h cycle. The figures represent the mean values \(±\) S.D. of \(n\) subsequent determinations, each was obtained at the end of a cycle in the steady state phase.

\(^b\) Values obtained at the end of a subcultivation cycle.

\(^c\) Values obtained from the difference between the start and final values in a subcultivation cycle.

\(^d\) Calculated on the base of experimental mean values.

the stationary phase (2.5 nm) but did not significantly depend on the P-level.

3.2. Growth, cellular \(P_o\), starch and furostanol in undiluted cultures

Volume fractions of SCC growing on P-enriched medium were not further diluted with fresh medium but instead a concentrated sucrose solution was added, when the refractometric index of the medium indicated sugar depletion. In the high-density cultures thus obtained (final cell dry wt.-concentration about 40 g l\(^{-1}\)), viability was maintained for long periods and the concentration of furostanol glycosides in the dry wt. remained at the level obtained in the SCC. Due to the high dry
wt.-concentration reached, high volumetric furostanol concentrations (1.4 g per l of suspension) were obtained (Fig. 2).

Cells of the suspension growing on the P-enriched medium were transferred to an undiluted batch regime at different P concentrations (2–20 mM). The cellular Pi levels in the obtained high-density cultures were very low at total P concentrations of 2 and 4 mM (Table 3). As no detectable Pi remained in the medium and the dry wt.-concentrations at harvest time were similar in these variants, the content of esterified P in the final dry wt. increased strongly (by nearly 100%) when the total P was raised from 2 to 4 mM. Increased cellular Pi levels at higher total P concentrations (8 and 16 mM) indicate saturation of the cells’ P-assimilation capacity. There was no further increase in cellular Pi, when the total P concentration in the suspension was raised from 16 to 20 mM.

A considerable part of the dry wt. in sucrose-supplemented high density cultures is starch. High Pi in the medium did not reduce the accumulation of starch markedly (Table 3). There was no significant relation between the starch and furostanol contents (data not shown).

4. Discussion

In the batch regime for the maintenance of cell suspension cultures, all detectable Pi of the medium is absorbed rapidly by the cells in the

| Table 3 |
| High density cultures on media with different P concentration |
|----------|-----------------|-----------------|-----------------|-----------------|-----------------|
|          | Experiment number |
|          | Total P concentration in the suspension (mmol l⁻¹) |
|          | 2 | 4 | 8 | 16 | 20 |
| Cellular Pi (μmol per g dry wt.) a | 1 | 3.4 | 18.6 | 150.8 | 270.0 | 285.0 |
| Fr. wt. (g l⁻¹) | 2 | 2.6 | 21.4 | 152.8 | 276.2 | 274.0 |
| Dry wt. (g l⁻¹) a | 1 | 177.6 | 175.7 | 176.4 | 177.0 | 172.0 |
| Starch content (mg glucose per g fr. wt.) | 2 | 206.4 | 209.3 | 202.0 | 220.7 | 284.4 |
| 3 | 22.5 | 24.2 | 23.8 | 22.7 | 23.0 |
| 4 | 33.2 | 35.8 | 30.0 | 29.3 | 28.2 |
| 3 | 37.7 | 37.0 | 32.8 | 30.8 | 29.9 |
| 4 | 51.4 | 40.2 | 40.2 | 40.6 | 32.8 |

a Mean values of two parallel culture flasks; exp. 1: growth period 10 days, prevention of sucrose exhaustion by adding sucrose at day 5; and exp. 2: growth period 22 days, prevention of sucrose exhaustion by adding sucrose at days 4, 11 and 15.
initial phase of the culture period [18–23]. P limitation of growth can, therefore, be expected in later phases only, when the cell concentration has reached a certain level and the vacuolar $P_i$ has been utilized. In a batch culture the $P_i$ levels of the cells are transiently increased after each subcultivation and $P$ deficiency is restricted to the last cell divisions. Such periodic $P$ limitation is tolerated by the investigated $D. deltoidea$ cells, although these cells do not tolerate an uninterrupted growth limitation by insufficient $P$. $P$ limitation of growth in the latest phase of the batch culture cycle can be even an advantage, since it improves the quality of the inoculum by prolonging the growth period and preventing rapid sucrose starvation [13]. A further reason to keep the $P_i$ level of a basic mineral medium low is the possible toxicity of $P_i$ to the inoculum which can result from excessive $P_i$ uptake at low initial cell concentrations [18,19,24,25].

For the choice of the optimal $P$ concentration in a plant cell culture medium not only the initial but also the final cell densities have to be considered. The results of this and further studies [24,26–29] suggest that a high density culture is usually $P$-limited at the relatively low $P_i$ concentration of the MS-medium and further basic mineral media recommended for plant suspension cultures [30,31]. The instability of uninterrupted growth at $P$ limitation (Table 1, Fig. 1) found cannot be generalized, since $P$ limitation has been used frequently to control cell density in chemostat and semicontinuous cultures [4,6–8,32–34]. In a chemostat, the culture studied here [11] and a further $D. deltoidea$ culture strain (unpublished) were not able to grow uninterrupted with a high specific growth rate on the standard MS-medium. We have shown here that the observed break-down is due to chronic $P$ deficiency. Although we did not find reports on comparable findings in the literature, the observed break-down effect is not necessarily an exceptional case. Nondesired break-down of plant cell cultures after a period of rapid growth is not rare but usually remains unexplained.

Chemostat and semicontinuous cultures resemble organism populations maintained at constant rates of resource renewal, propagation, and removal of individuals. In the considered case, $\mu$ is controlled by competition for $P_i$ [20]. The theoretical treatment of this case shows that the cell density reaches a stable value (proportional to the $P$ concentration) if the physiological affects of $P$ deficiency are reversible. Instabilities (oscillations, chaotic behaviour, break-down) are forecasted when a long lasting reduction of $\mu$ develops in consequence of the physiological deficiency effects [35,36]. Since the cellular $P_i$ content increased strongly during the break-down period, the cells did not lose their capability to absorb $P_i$ but could not utilize the accumulated $P_i$. In the current state we do not know the primary reason of this phenomenon.

At adequate $P$ nutrition, the vacuolar $P_i$ pool may be the largest $P$ fraction in plant tissues, whereas low $P_i$ indicates $P$ deficiency [18,19,37–39]. At SCC on the standard MS-medium the concentration of $P_i$ in the cell volume was less than 1 mM. Since the $P_i$ concentration in the cytoplasm has to be in the range of some mM [37], vacuolar $P_i$ was very low or absent. The $P$ content necessary for the stabilization of uninterrupted growth of the investigated cell culture is more than 180 $\mu$mol per g dry wt. Compared with the $P$ content compatible with adequate growth of a plant shoot (about 60 $\mu$mol per g dry wt. [37,40]), this is a high requirement.

It is known that excess $P_i$ can effect carbohydrate partitioning and often results in a decreased starch content [18,37,41]. Growth, furostanol production and starch accumulation were not inhibited by up to 20 mM $P_i$. The observed insensitivity of growth and carbon allocation to high external $P_i$ (Table 3) is probably due to the cells’ ability to regulate $P_i$ in the cytoplasm by its transport to the vacuole and to control its uptake from the medium at high cellular $P_i$.

Although furostanol glycoside production by $D. deltoidea$ cell cultures is coupled with growth [42], the furostanol content per g dry wt. remained stable and the volumetric product concentration increased markedly when the culture was transferred to the stationary phase with additional $P$ and sucrose. It may be concluded from our study that adequate $P$ nutrition is important both for propagation stability and volumetric productivity, especially when a continuous production regime at high cell density is desired.

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