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Effects of the Growth Regulator 4PU-30 on Growth, K^+ Content, and Alkaloid Production in Tobacco Callus Cultures

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Abstract. Growth, K^+ content, and alkaloid production were compared in nonorganogenetic callus cultures of *Nicotiana tabacum* cv. Burley 21 grown at 25°C in the dark on two different media: a basal medium with 1 μM α-naphthaleneacetic acid and 1 μM kinetin, and one with 1 μM α-naphthaleneacetic acid and 1 μM 4PU-30 (N-(2-chloro-4-pyridyl)-N′-phenylurea). These callus tissues behaved differently not only in growth and K^+ content but also in alkaloid production. In comparison to cultures grown with kinetin, those grown with 4PU-30 showed a significantly higher fresh weight and dry weight and K^+ content during the growth period studied. The data clearly indicate a positive correlation between K^+ uptake rate stimulated by 4PU-30 and cell enlargement rate. However, the alkaloid biosynthesis in the callus tissues was activated by the supply of kinetin and diminished by that of 4PU-30. It thus appears that cellular enlargement of meristematic tissue stimulated by 4PU-30 limited alkaloid production.

The presence of a purine ring is not essential for cytokinin activity. A number of substituted phenylureas possess high cytokinin activity of which N-(4-pyridyl)-N′-phenylurea (4PU) derivatives are a good example (Capelle et al. 1983). Of these, N-(2-chloro-4-pyridyl)-N′-phenylurea (4PU-30) shows outstanding activity, promotes vigorous growth of callus tissue, and gives a fresh weight of callus somewhat lower at its optimal concentration of 4×10^{-3} μM (0.001 ppm) than that of the optimal concentration 4×10^{-2} μM (0.01 ppm) of kinetin or N^6-benzyladenine (BA) in the bioassay devised by Takahashi et al. (1978). Therefore, 4PU-30 is one of the most active cytokinins known to date.

We have demonstrated previously in tobacco callus that, in the presence of 1 μM kinetin used to supply the cytokinin requirement of tissue, it was the auxin

concentration in the culture medium that controlled the rate of nicotine synthesis, which follows a course almost parallel to callus growth (Piñol et al. 1984). The present study was undertaken to obtain more information about 4PU-30, where in the presence of 1 μM NAA, the optimal auxin concentration for nicotine production according to Ohta et al. (1978) gave a higher fresh weight of tobacco callus at its optimal concentration of 1 μM in the culture medium than that of the optimal concentration (1 μM) of kinetin (Piñol, unpublished). The specific objectives of this study were to determine and compare the effects of kinetin and 4PU-30, both 1 μM, on growth, K^+ content, and alkaloid production (measured as nicotine) in nonorganogenetic *N. tabacum* cv. Burley 21 callus cultures grown with optimal conditions for nicotine synthesis. Furthermore, the callus tissues were examined cytologically to determine whether microscopic changes had occurred.

Materials and Methods

Midrib segments excised from the basal part of leaves of 3-month-old *Nicotiana tabacum* L. cv. Burley 21 plants were inoculated on Murashige-Skoog (1962) basal medium solidified with 0.8% Bacto agar and supplemented with 11.5 μM α-naphthaleneacetic acid (NAA) and 1 μM kinetin (6-furfurylaminopurine), the concentrations being appropriate to supply, respectively, the auxin and cytokinin requirement of tissue (Kinnersley and Dougall, 1980). After callus induction (~3–4 weeks), six pieces of callus tissue individually weighing 40 ± 0.4 mg were inoculated onto each of two Murashige-Skoog's media solidified with 0.8% Bacto agar. The first medium, optimal for nicotine synthesis (Piñol et al. 1984, 1985), referred to as "kinetin," was supplemented with 1 μM NAA and 1 μM kinetin. The second, referred to as "4PU-30" medium, was supplemented with 1 μM NAA and 1 μM N-(2-chloro-4-pyridyl)-N'-phenylurea (4PU-30). The callus cultures were grown in Petri dishes (100 × 25 mm) containing 20 ml of medium at 25°C in the dark over a culture period of 7 weeks.

Measurement of callus growth was as given previously by Piñol et al. (1984). To obtain the dry weights, fresh tissues were dried at 60°C for 48 h. Water content (weight loss after 48 h at 60°C) was measured in all the callus pieces used for the measurement of callus growth on both media (48 pieces each week) and expressed in g (g dry weight)$^{-1}$.

For cytological examination, callus tissues were fixed for 2–4 h with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and postfixed in buffered 1% OsO_4 solution for 2 h. After dehydration through an increasing acetone series, the samples were infiltrated with Araldite and polymerized for 48 h at 40°C. The blocks were cut on a microtome, and the sections (1–1.5 μm) were stained in methylene blue for 1.5 min at 100°C.

For the measurements of K^+ and alkaloid content, 10 dried samples from each culture medium were analyzed each week over the growth period studied. The K^+ content of callus tissues was determined by flame photometry (flame photometer Zeiss model PF5) of acid-digested tissue samples; dried samples were weighed with a precision of ±0.1 mg and digested by heating with a

mixture of concentrated HNO_3 and H_2SO_4 at a ratio of 8:3 (v/v). Alkaloid content, measured as nicotine, of the callus tissues was determined using the colorimetric method described by Schmid and Serrano (1948). K^+ and alkaloid content were expressed in mg (g dry weight)$^{-1}$.

To compare the effects of both the kinetin and 4PU-30 media on growth, K^+ content, and alkaloid production in the callus cultures, the respective data were subjected to analysis of variance.

NAA, kinetin, and nicotine were purchased from Sigma Chemical Co. (St. Louis, MI, USA). 4PU-30 was supplied by Ferrer Internacional, S.A. (Barcelona, Spain). Other reagents used were of the highest quality available.

Results and Discussion

The nonorganogenetic callus tissues grown on the nutrient agar medium containing 1 μM NAA and 1 μM kinetin (purine cytokinin; "kinetin medium") developed into brownish-yellow compact calli. Those grown on the nutrient agar medium containing 1 μM NAA and 1 μM 4PU-30 (phenylurea cytokinin; "4PU-30 medium") developed much more, as yellowish, friable calli.

The curves for tissue growth obtained over the 7-week subculturing period are presented in Figure 1. 4PU-30 in the culture medium significantly stimulated the growth of callus pieces measured both as an increase in fresh weight ($p \leq 0.001$) and dry weight ($p \leq 0.001$ until week 6, and $p \leq 0.01$ in week 7) over the 7 weeks considered. In the kinetin and 4PU-30 media, the fresh weight of the callus tissues increased until week 7, when the experimental period ended and the fresh weights were respectively at that moment 20-fold and 48-fold higher than the inoculum's fresh weight. The dry weight of the callus tissues in the kinetin medium increased steadily until week 6 and then practically stopped. In the 4PU-30 medium it increased from week 1 to week 6; in the following week the calli lost dry weight, presumably owing to the faster exhaustion of nutrients in this culture medium. Maximum dry weight of calli grown on the kinetin medium was 20-fold higher, and that of calli grown on the 4PU-30 medium 24-fold higher, than the inoculum's dry weight.

As to the water content, there were large differences between the calli grown on both media (Fig. 1). The water content of callus tissues grown on the 4PU-30 medium showed little change during the first 3 weeks and afterward increased significantly ($p \leq 0.001$) until week 7, when the experimental period ended. In contrast, the callus tissues grown on the kinetin medium showed a decrease in the water content during the first 3–4 weeks and reached a plateau at about week 4.

The histological examination of callus pieces at week 7 (Fig. 2) showed that the cells of callus tissues grown on the kinetin medium did not lengthen as in callus tissues grown on 4PU-30 medium, since mainly meristematic cells are conspicuous in methylene blue–stained sections of calli cultured on the former medium and practically only large mature cells are conspicuous in those of the calli cultured on the latter medium. The previous data and the results of the histological examination suggest that the 4PU-30–promoted increase in fresh weight of callus pieces after 3 weeks was due primarily to cell enlargement,

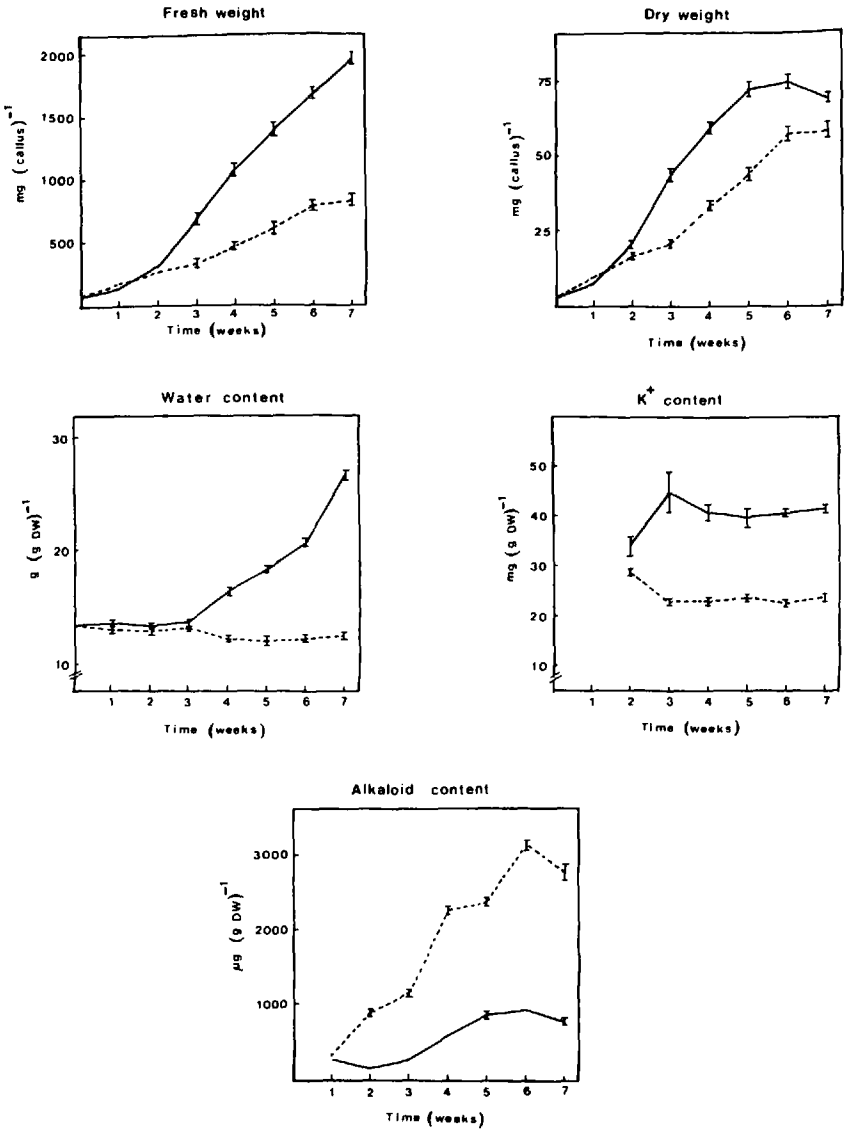

Fig. 1. Response of tobacco callus cultures (Burley 21) to different cytokinins. Tissue pieces were grown in dark for 7 weeks at 25°C on two basal media containing either 1 μM NAA and 1 μM kinetin (kinetin) or 1 μM NAA and 1 μM 4PU-30 (4PU-30). The initial fresh weight of the calli was 0.04 ± 0.004 g (callus)$^{-1}$. Values are means of 10–24 analyses. Bars represent ± SE. Kinetin medium (●---●); 4PU-30 medium (●—●).

whereas the kinetin-promoted increase in the fresh weight of callus pieces was due mainly to cell division during the overall culture period studied.

The time course of K^+ content in the callus tissues grown on each of the two different culture media used are presented in Fig. 1. The calli grown on the

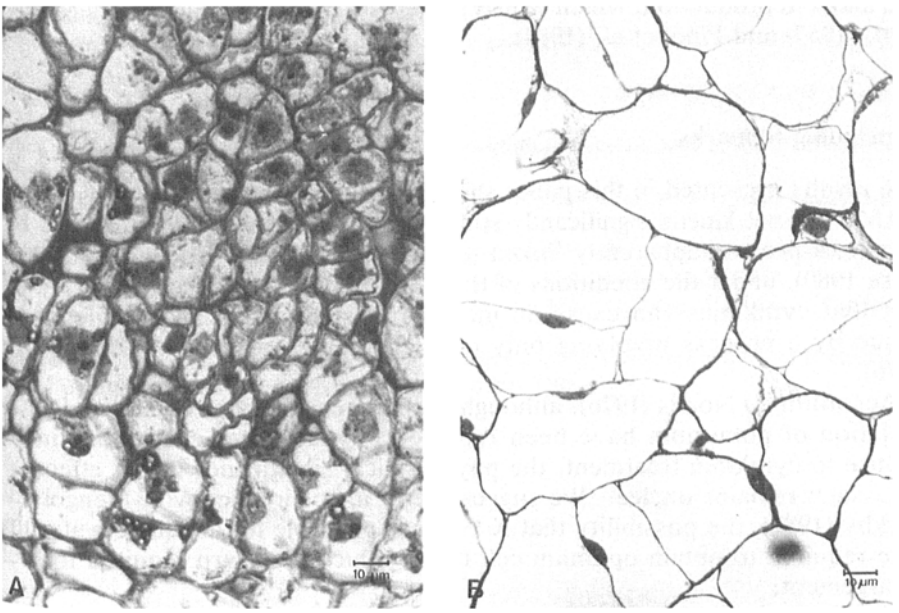

Fig. 2. These light micrographs illustrate that 4PU-30 but not kinetin drastically stimulated the cell enlargement in the callus tissue of *N. tabacum* cv. Burley 21: (A, mainly meristematic cells are conspicuous) 1 μM kinetin, and (B, practically only mature cells are conspicuous) 1 μM 4PU-30. The experimental conditions are given in Fig. 1.

kinetin medium showed significantly lower concentrations of K^+ than those grown on the 4PU-30 medium after week 2 ($p \leq 0.01$ week 3 and $p \leq 0.001$ weeks 4–7). In the medium with kinetin, the average K^+ content ranged from 23.00 to 29.59 mg (g dry weight)$^{-1}$, and in the medium with 4PU-30 it averaged between 34.37 and 45.14 mg (g dry weight)$^{-1}$. The results described show that in the presence of a constant concentration of auxin (1 μM NAA) in the culture medium, the calli grown with 4PU-30 (1 μM) took up more K^+ and water (Fig. 1) (significantly so after week 3–4) than those grown with the same concentration of kinetin, which have to be considered K^+-deficient according to the data cited by Evans and Sorger (1966).

On the other hand, as shown in Figure 1, although alkaloid production followed a similar time course in the callus cultures grown on both media, there was a significant quantitative variation between the accumulation of alkaloid in the calli grown on the two different culture media from week 1 to week 7. The results clearly demonstrated that in the presence of a constant amount of auxin (1 μM NAA), the alkaloid content from the first week onward was significantly higher ($p \leq 0.001$) in the calli grown with a concentration 1 μM of kinetin in the culture medium than those grown with the same concentration of 4PU-30 in the medium. The average alkaloid content of the culture kinetin measured as nicotine was found to be 1852 μg (g dry weight)$^{-1}$, more than threefold higher than in the culture 4PU-30 (577 μg (g dry weight)$^{-1}$). It therefore appears that cellular enlargement of meristematic tissue stimulated by 4PU-30 (Fig. 2) lim-

ited alkaloid production, which would agree with the data published by Mothes et al. (1957) and Piñol et al. (1984).

Concluding Remarks

The results presented in this paper show clearly that in tobacco callus tissue, 4PU-30 but not kinetin significantly stimulated the cell enlargement and the K^+ uptake, a process apparently linked to such enlargement (Cocucci and Dalla Rosa 1980), under the conditions of this experiment. This is supported by the fact that cytokinins can cause an increase in size of leaf and cotyledonary tissue by a process involving only cell enlargement (Letham 1971, Norris 1976).

According to Norris (1976), although both increased water uptake and accumulation of potassium have been reported for cucumber cotyledons in response to cytokinin treatment, the physiological significance of the effects of potassium remains unclear. We suggest here, in accordance with Mengel and Kirkby (1982), the possibility that K^+ is indispensable for adequate water uptake required to obtain optimum cell turgor which is in turn required for cell enlargement.

Finally, it should be pointed out that although the replacement of kinetin in the culture medium by the same concentration of 4PU-30 caused the alkaloid production to be much lower in tobacco callus tissues, this does not presuppose that the result will be the same when the product is sprayed on the aerial organs of the intact plant. We base our opinion on the fact that in tobacco plants nicotine synthesis is confined to the root and from there is transported to the aerial part, where it accumulates in the leaves during the vegetative state of the plant.

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