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Factors Influencing the Incidence of Habituation for Cytokinin of Tobacco Pith Tissue in Culture

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Abstract. Pith tissue of *Nicotiana tabacum* L. cv. "Havana 425" exhibits a gradient in its tendency to habituate for cytokinin on an auxin-containing medium at 35° C, about 10° C above the standard culture temperature. Explants of pith from below the 8th to 11th internode, counting from the bottom of the plant, rarely habituate for cytokinin; explants from above this threshold habituate rapidly. The explants must also be above a critical size, about 20–30 mg, to habituate. There was a pronounced interaction between size and position effects; the threshold position for cytokinin habituation shifted upward with decreasing explant size.

Key words: Cytokinin habituation – Habituation, cytokinin – *Nicotiana* – Position effects (habituation) – Size effects (habituation) – Tissue culture.

Introduction

The development of higher plants involves stable phenotypic changes, which, in some cases, persist when cells divide (for a recent review see Meins and Binns 1979). Cytokinin habituation provides a model system in which changes of this type can be induced and reversed at will under precisely defined conditions in culture. When explants of tobacco pith are incubated on an auxin-containing medium at 35° C, approx. 10° C above the standard culture temperature, hyperplastic nodules, which no longer require exogenous cytokinin, appear on the tissues (Meins and Lutz 1980). This change from the cytokinin-requiring to the cytokinin-autotrophic state, known as cytokinin habituation, is thought to have an epigenetic basis, since it is directed, potentially reversible, and leaves the heritably altered cell totipotent (for further discussion see Meins and Binns 1978).

Earlier, we used the formation of hyperplastic nodules to measure the rate of habituation and found that this rate varied widely from $5.6 \cdot 10^{-4}$ to $1.2 \cdot 10^{-2}$ per cell generation in different experiments (Meins and Lutz 1980). Some of this variation had a seasonal basis. The incidence of habituation was sevenfold higher for explants isolated in the spring than for explants isolated in the winter. In this study we have identified two additional sources of variation; habituation rate depends on both the position in the plant from which pith explants were isolated and the size of the explant.

Material and Methods

Pith parenchyma tissue from *Nicotiana tabacum* L. cv. "Havana 425" plants was isolated, cultured, and induced to habituate as described previously (Meins and Lutz 1980). In brief, tissues were excised from the internode indicated, counting from the bottom of the plant, and were incubated on a basal medium modified from Linsmaier and Skoog (1965) containing 2.0 mg·l^{-1} α-naphthaleneacetic acid as an auxin. Growth is expressed as fresh weight (W) after 21 d incubation minus initial weight (W_0), divided by initial weight, i.e., $(W - W_0)/W_0$.

Cytokinin habituation, unless indicated otherwise, was induced by incubating explants of pith tissue in the dark at 35° C on basal medium for 7 d. Explants were then incubated for an additional period of about 21 d in the light at 25° C, and scored for the appearance of habituated nodules, which are clearly discernible by transillumination against a background of friable, non-habituated tissue. Incidence of habituation is expressed as the fraction of explants bearing nodules.

The competence of pith tissue to habituate in response to cytokinin was assayed as described previously (Meins et al. 1980). This involved incubating explants of pith tissue at 25° C on basal medium supplemented with 0.3 mg·l^{-1} kinetin (6-furfurylaminopurine) and then comparing the growth of the tissues on basal medium with and without kinetin.

The effect of position in the stem on the capacity of pith tissue to habituate for cytokinin was detected by cutting equal numbers of explants weighing approx. 25 mg from different internodes of the same plant. The explants were induced to habituate by 35° C treatment and then scored for habituated nodules.

To detect effects of explant size on incidence of habituation,

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complete sets of cube-shaped explants, with lengths of 1.8, 2.5, 3.0, 3.2, 3.5, 3.7, 4.0, and 4.5 mm, were cut from different internodes of the same plant and were induced to habituate by 35° C treatment. Explants cut in this way gave a roughly uniform distribution of weights in the range 3–80 mg.

Results

1. Position Effects. If position in the plant does not affect the tendency of pith tissue to habituate, then the incidence of habituation in explants isolated from different internodes should be the same, i.e., the number of explants with habituated nodules should be uniformly distributed. Thus, a plot of the cumulative fraction of habituated explants after 35° C treatment as a function of internode number (1), should give a straight line with a positive slope. This relationship was not obtained. The plots were clearly biphasic in shape (Fig. 1). Below a threshold position, which varied from the 8th to the 11th internode in different experiments, there was little or no habituation. Above this position, the incidence of habituation increased in a roughly linear fashion. These results clearly show that the incidence of habituation depends on position in the plant. Near the top of the plant, explants from different internodes habituate readily and with roughly the same incidence, whereas, near the bottom of the plant, the explants have little or no tendency to habituate.

Pith tissue of tobacco consists of at least two populations of cells: competent cells, which habituate readily when incubated at 35° C on basal medium or at 25° C on basal medium plus 0.3 mg l^{-1} kinetin, and cells unable to habituate under these conditions (Meins et al. 1980). Thus, the observed position effect could result from an abrupt change in the distribution of competent cells along the length of the stem. If this is the case, then the incidence of habituation in response to kinetin treatment should depend upon position as well. The experimental results did not support this hypothesis. All explants, whether isolated from above or below the threshold position, habituated after a single transfer on kinetin medium (Table 1). Moreover, explants isolated from the two positions exhibited the same degree of habituation after kinetin treatment; the relative growth rates of the different tissues, with and without kinetin, were the same (Table 2). It appears that the threshold does not result from an abrupt change in the abundance of competent cells.

2. Effect of Explant Size. If habituation events occur randomly distributed in a tissue, then a large explant will have a greater chance of habituating than a small explant. The exact relationship between the probability of finding a habituated explant, $P(W)$, and explant size, expressed as weight (W), can be expressed by the equation:

$$\ln|1/(1-P(W))| = \lambda W - \lambda W' \text{ for } W \geqq W' \qquad (1)$$

where λ is the number of habituation events per mg tissue and W′ is the weight of the smallest explant

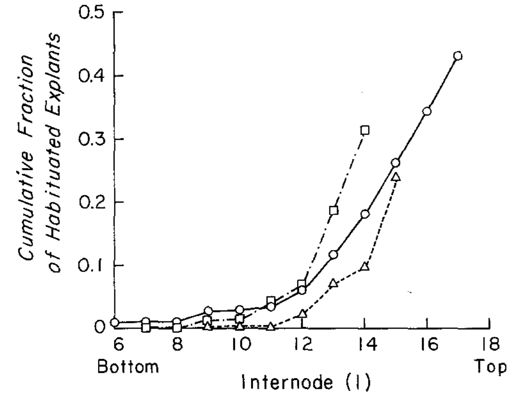

Fig. 1. The cumulative fraction of habituated pith explants as a function of position in Havana 425 tobacco plants. Results of three different experiments with individual plants performed on 19 Jan 78 (○), 30 Mar 79 (□), and 7 Jun 79 (△); and with at least eight explants per internode

Table 1. Competence of pith tissues from the top and bottom of a Havana 425 tobacco plant for kinetin-induced habituation

Source of pith explants (internode No. from below)	Pretreatment[a]	Explants habituated (%)[b]
13	−kinetin	67
	+kinetin	100
9	−kinetin	11
	+kinetin	100

[a] Explants incubated for 21 d, as indicated, on basal medium or on basal medium plus 0.3 mg l^{-1} kinetin

[b] Percent of 9 explants after two successive transfers of 21 d duration each, on basal medium

Table 2. Degree of habituation of pith explants from the top and bottom of a Havana 425 tobacco plant induced by kinetin treatment

Source of pith explants (internode No. from below)	Growth $(W-W_0)/W_0$[a]		A/B
	−kinetin (A)	+kinetin (B)	
13	62.0±11.5[b]	11.1±0.85	5.59
9	61.9± 9.75	12.3±3.20	5.04

[a] Growth of explants pretreated for 21 d on basal medium plus 0.3 mg l^{-1} kinetin after two successive transfers of 21 d duration each, on the medium indicated

[b] Mean ± standard error for 9 replicates

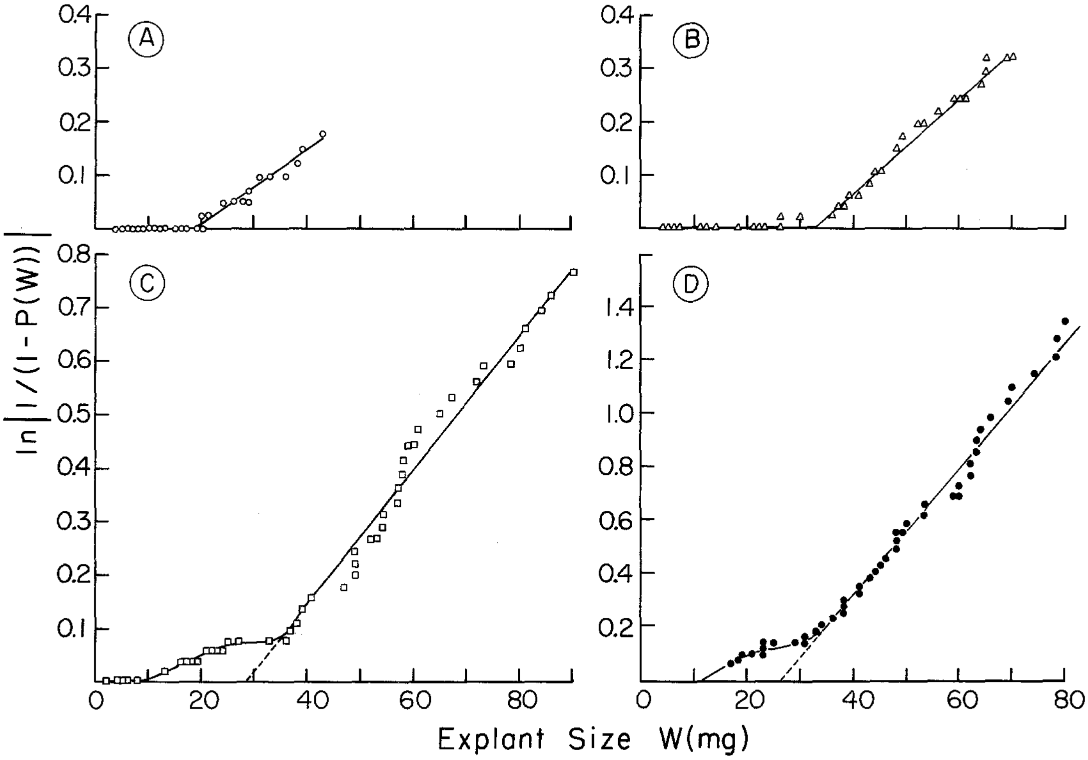

Fig. 2A–D. Relationship between probability of habituating and the size of pith explants from Havana 425 tobacco plants. Results obtained from four experiments with different plants; **A** (○), 24 Aug 78; **B** (△), 12 Jan 78; **C** (□), 22 Mar 79; **D** (●), 17 Jul 79. In each experiment, above a critical explant size, there was a highly significant linear correlation of $\ln|1/(1-P(W))|$ and explant size

in a set of explants uniformly distributed in weight. This equation, which is a statement of the exponential distribution law, is derived in the Appendix. The important point is that a plot of the left side of equation (1) versus W gives a straight line with slope λ provided the variation of $P(W)$ with size only reflects the random distribution of habituation events.

The results obtained in four representative experiments shown in Fig. 2 did not support a random-distribution model. When sets of explants uniformly distributed in weight were incubated at 35° C and the expression on the left side of Eq. (1), calculated from the cumulative fraction of explants with habituated nodules, was plotted as a function of explant size, the plots obtained were biphasic rather than linear in appearance. In experiments A and B, where incidences of habituation were relatively low, explants below 20–30 mg in size did not habituate. In experiments C and D, in which the incidences of habituation were higher, some explants below 20–30 mg in size habituated, but with a low incidence. These results show that explants must be above a threshold size to habituate in response to 35° C treatment. In each case, the plots above this size were linear, which is consistent with the hypothesis that once the threshold is reached, habituation events occur randomly distributed in the tissue with a constant incidence per mg weight.

3. Interactions Between Position and Size Effects. Explants weighing approx. 25 mg, which is below the size threshold, were used in studies of position effects. Nevertheless, tissue obtained from near the top of the plant regularly habituated, indicating that there is an interaction between size and position. Two types of experiments were performed to test this hypothesis. In the first, sets of large and small explants were isolated from internodes 9 and 10, which are below the threshold position, and the explants were incubated at 35° C. Under these conditions, the large, but not the small, explants habituated indicating that the position effect can be overcome by increasing explant size (Table 3).

In the second type of experiment, we compared the incidence of habituation as a function of position using explants uniformly distributed in weight above and below the size threshold. Sets of explants from experiments A and C shown in Fig. 2 were used to obtain the data. In experiment A, where there was a low incidence of habituation, the threshold for large pieces was the 11th internode, whereas the threshold for small pieces was displaced upward to the 14th internode (Fig. 3). Similarly, in experiment C, where there was a higher incidence of habituation, the threshold for large pieces, less than the 10th internode, was shifted upward to the 12th internode for small pieces. These results clearly show that the position of the threshold depends upon explant size.

Table 3. The effect of explant size on the incidence of cytokinin habituation of pith tissue obtained from the 9th and 10th internodes of a Havana 425 tobacco plant

Explant size (mg)[a]	Habituated explants/ total No. explants[b]	Explants habituated (%)
3.0±0.0	0/13	0
5.3±1.2	0/14	0
49.3±1.5	3/14	21.4
70.7±4.3	12/13	92.3

[a] Mean ± standard error, 3 replicates

[b] After 7 d incubation at 35° C followed by 21 d incubation on basal medium

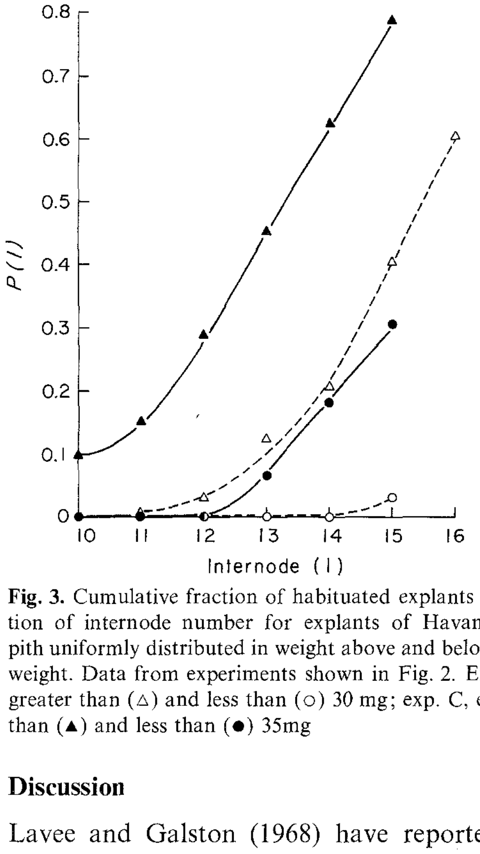

Fig. 3. Cumulative fraction of habituated explants P(I) as a function of internode number for explants of Havana 425 tobacco pith uniformly distributed in weight above and below the threshold weight. Data from experiments shown in Fig. 2. Exp. A, explants greater than (△) and less than (○) 30 mg; exp. C, explants greater than (▲) and less than (●) 35mg

Discussion

Lavee and Galston (1968) have reported that pith explants from different regions of Wisconsin 38 tobacco plants differ in both tissue morphology and growth rate when cultured on a complete medium containing auxin and cytokinin. The present studies confirm that pith is not homogeneous with regard to growth potential in culture; the tendency of pith tissue to habituate depends upon its position in the plant. There is a threshold, near the bottom of the plant, below which habituation rarely occurs. Above this threshold, plots of the cumulative fraction of habituated explants versus internode number are roughly linear as expected for an uniform distribution of habituation events. Thus, it appears that near the top of the plant the incidence of habituation is nearly independent of position. Our results also show that there is a pronounced interaction of position and explant size in determining the incidence of habituation; the threshold position shifts upward with decreasing explant size.

Similar position effects have been reported for auxin habituation (Cheng 1972). Pith explants obtained from the lower internodes of tumor-prone *Nicotiana glauca* × *langsdorfii* plants show an absolute requirement for auxin when grown on an otherwise suitable culture medium. In contrast, pith explants obtained from internodes near the top of the plant can grow without added auxin. As is the case for cytokinin habituation, this position effect can be overcome by treating explants from the lower internodes with the growth factor. After one transfer on indoleacetic-acid-containing medium, these explants subsequently grow on media without auxin.

Two types of hypotheses could, in principle, account for position effects in habituation. The first is that there is a gradient along the length of the stem in some intrinsic property of the pith cells. Although pith cells increase in ploidy from 2n to 8n toward the bottom of the tobacco plant (Murashige and Nakano 1966), it is unlikely that this accounts for our results. Chromosome number does not affect the tendency to habituate or degree of cytokinin habituation of pith cells in culture (Binns and Meins 1980).

Another property of pith cells that changes with position is cell size, which increases by about 10-fold toward the bottom of Wisconsin 38 tobacco plants (Lavee and Galston 1968). If the probability of habituating were constant on a per-cell basis, then explants from near the bottom of the plant would contain fewer cells, and, hence, exhibit a lower incidence of habituation than explants of the same size from the top of the plant. Moreover, the position threshold would be shifted downward in the plant with increasing explant size, which is observed experimentally.

The second type of hypothesis is that the threshold effect results from gradients in the concentration of growth regulating substances along the stem. Very low concentrations of the cytokinin, kinetin, about 10^{-9}M, induce the cytokinin habituation of cultured pith explants (Meins and Lutz 1980). This effect exhibits a sharp concentration threshold; thus, a continuous gradient in the concentration of endogenous cytokinin, or a related cell division factor, which is high near the top of the plant and low near the bottom, could generate a discontinuous change in habituation rate with position.

Gradients in cytokinin concentration also provide a plausible explanation for the effect of explant size on habituation rate. We found that there was a critical size below which habituation events were rarely detected. This result is not predicted from a simple model in which variation in the incidence of habituation with explant size arises from a random distribution of habituation events in the tissue. Cell division factors can diffuse out of tissues into the culture medium (Braun and Näf 1954; Tandeau de Marsac and Jouanneau 1972). The rate of diffusion will depend on explant size. For cube-shaped explants, the diffusion cross section increases by the square of the length whereas volume increases by the cube of the length and, hence, small explants should be exhusted of endogenous cytokinin more rapidly large explants. In principle, this would result in a size threshold which depends on cytokinin concentration and time required for induction of habituation, initial cytokinin content of the tissue, and rate of diffusion of cytokinin out of the tissue. Moreover, there should be a pronounced interaction between the effects of size and position on habituation. If there is a gradient in cytokinin concentration, which is high near the top of the plant, then with increasing distance from the top of the plant the minimum explant size required for habituation should increase. This was observed experimentally.

Although our results are consistent with a gradient hypothesis, they do not rule out the possiblity that the observed thresholds in size and position reflect variation in the properties of pith cells along the length of the stem. A more rigorous test would be to measure the cytokinin content of the tissue obtained from different positions in the plant and to compare these values with the concentration of exogenous cytokinin required to induce explants to habituate in culture. According to the gradient hypothesis, the total concentration of endogenous plus exogenous cytokinin required to induce habituation should be constant and independent of position

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Appendix

Relating Incidence of Habituated Explants and Explant Weight

A mathematical expression relating incidence of habituated explants with explant weight was derived following the general methods outlined by Parzen (1960; pp. 148–174). If habituation events occur randomly distributed in a tissue, then the probability of finding an habituated explant is given by the Poisson distribution law as:

$$P = 1 - e^{-m} \qquad (1)$$

where m is the average number of events per explant. Thus, for a constant incidence of habituation events (λ) per weight (W) of tissue, $m = \lambda W$ and Eq. (1) becomes:

$$P(W) = 1 - e^{-\lambda W} \text{ for } W \geqq 0 \qquad (2)$$

which is a statement of the exponential distribution law over a range of weights from 0 to $+\infty$. Since our measurements are made with sets of explants uniformly distributed in weight above some minimum value, W', the probability distribution function $P(W)$ must be normalized over the range W' to $+\infty$. This is accomplished by differentiating Eq. (2) to give the probability density function:

$$p(W) = \lambda e^{-\lambda W}. \qquad (3)$$

Eq. (3) is then integrated between the limits W' and W, and divided by the integral of $p(W)$ over the entire range:

$$P(W) = \frac{\int_{W'}^{W} p(W)\, dW}{\int_{W'}^{+\infty} p(W)\, dW} \qquad (4)$$

to give:

$$P(W) = 1 - \exp\,(\lambda W' - \lambda W) \qquad (5)$$

which is the probability of finding an habituated explant as a function of weight for explants with weights $W \geqq W'$. Finally, rearranging Eq. (5) gives:

$$\ln|1/(1 - P(W))| = \lambda W - \lambda W'. \qquad (6)$$

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