Auxin-Kinetin Interaction in Tissue Cultures of Nicotiana Species & Tumor-Conditioned Hybrids [1, 2]

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Introduction

Historically, much of the research in plant tumor physiology and growth has utilized cultures derived from bacterial infection and wounding. Observation of these systems has demonstrated that the transformation of a normal cell to a tumor cell in the crown-gall disease involves progressive activation of certain biosynthetic systems. According to Braun and Wood (3) transformed cells become autonomous for auxins and kinins as well as for other compounds. On the other hand, it has been reported that tumor cells of *Picea glauca* have an absolute auxin requirement for growth (6).

In earlier work with Nicotiana species and hybrids on the conversion of tryptophan to auxin it was suggested that high auxin levels might contribute to undifferentiated growth in the tobacco hybrid system (8). Thus, the Nicotiana parental species, which do not form tumors, and their tumor forming hybrids and amphiploids seem to offer excellent experimental material to explore and further clarify the suggested differences in the hormonal requirements of tumor and nontumor forming tissues. If an imbalance in the auxin system is in fact responsible for tumor induction in vivo, it should be possible to demonstrate a differential hormone requirement for the tissue cultures of nontumorous parental species and their tumor forming hybrids.

One purpose of this study was to determine the tissue culture requirements of tumor and nontumor forming tissues of Nicotiana. Another purpose was to test specifically with the tobacco hybrid system Braun's findings (1, 2) regarding fundamental changes accompanying the conversion of normal cells to tumor cells by crown-gall bacteria. The data presented show not only a marked difference in the auxin requirement of tumorous vs. nontumorous tissue but indicate that exogenous kinetin is an important and consistent requirement for growth of tissues of the nontumor forming types. There is also a synergistic effect of auxin and kinetin.

Materials & Methods

Plant Material. The plants used in this study consisted of the parental species *Nicotiana langsdorffii* Weinm. and *N. suaveolens* Lehm. The synthetic amphiploid hybrids used in the study were *N. suaveolens-langsdorffii* and *N. glauca.* (Grah.)-*langsdorffii* (Weinm). The nontumorous mutant of *N. glauca-langsdorffii* was obtained from x-rayed seeds by Izard (7).

Isolation of Tissue. Tissues of species and genetic combinations used in this study were isolated from stem sections and cultured on supplemented White's basic medium (16). The isolation procedure involved first removing the stem of a young rapidly-growing plant and cutting it into 10 cm sections. These were placed in Alconox for 15 minutes and then transferred to 5 % Clorox (a commercial product consisting of 5.25 % by weight of sodium hypochlorite) solution for 15 minutes for surface sterilization. Next, sections were dipped into sterile water and then into 95 % ethanol for flaming. The flaming not only reduced surface contamination but facilitated the subsequent peeling operation. The surface tissue external to the vascular cambium was aseptically peeled from 2.5 cm stem sections which were then split longitudinally. The sections were placed into the culture medium with the split surface exposed to the air. Successive transfers were made every 4 to 6 weeks by placing approximately 50 mg of callus into the new medium. The size of the callus piece transferred was approximately four millimeters on a side.

Tissue Transfer and Growth Measurements. Tissues representing the tumorous and nontumorous genotypes were used for experimental material as soon as enough growth had occurred for starting large experiments. Enough tissue was usually available after the third transfer. Small pieces of tissue, weighing approximately 50 mg, were aseptically transferred to 33 ml of culture medium in 125 ml Erlenmeyer flasks. The flasks were stoppered with cotton plugs and maintained at 23 to 25° for 3 to 8 weeks under continuous cool-white fluorescent light of approximately ten foot candles. Growth was measured by taking the final wet weight of the cultures. The average of individual treatments represents data from 4 to 10 replications. The number of replications varied because some cultures were sacrificed for successive transfer and photography.

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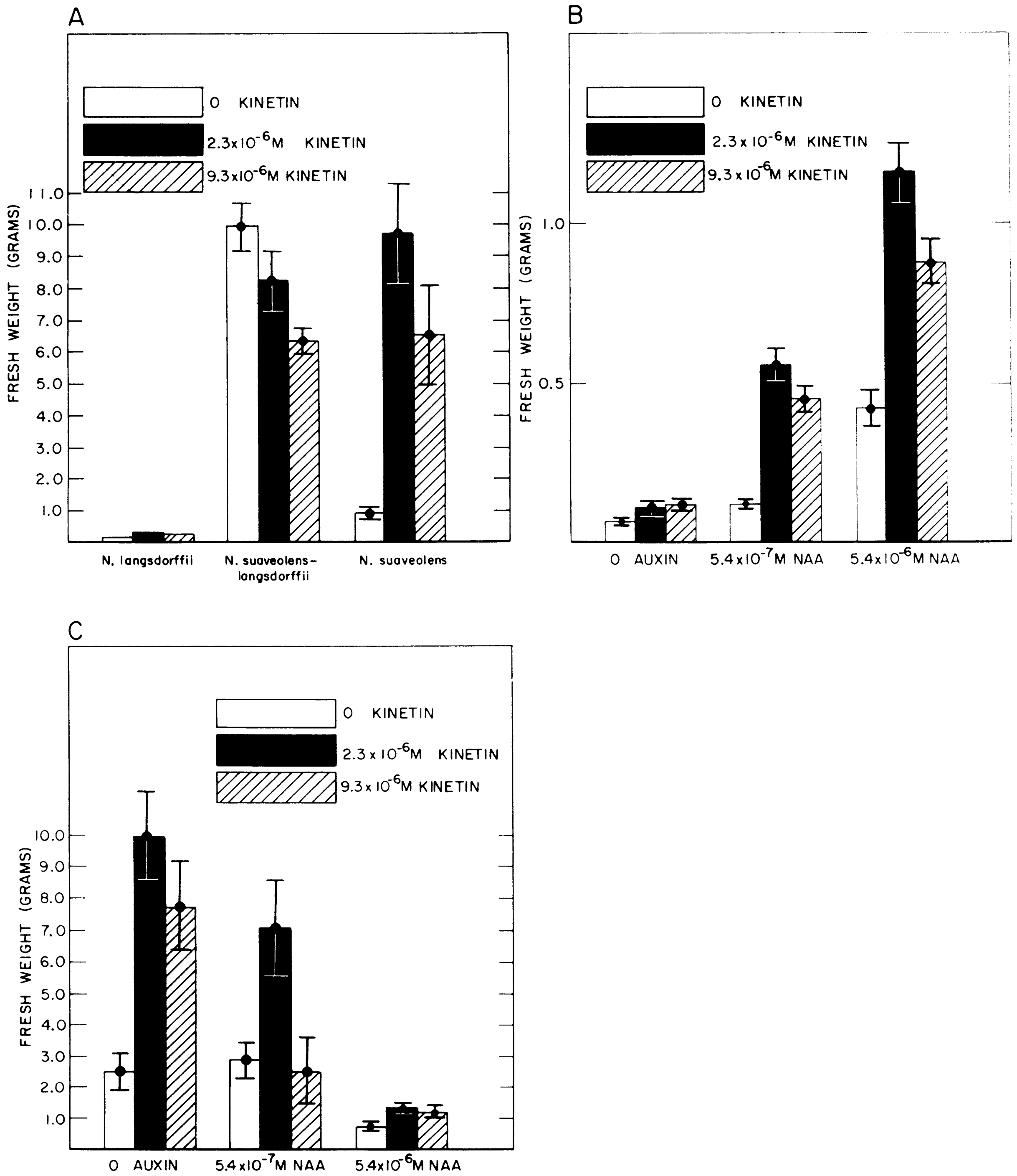

FIGURE 1

There were some losses due to contamination.

Tissue Culture Medium. The tissues were routinely cultured on White's basic medium (16) supplemented with 5.4×10^{-3} M KCl and 5.5×10^{-5} M tetrasodium ethylenediamine-tetraacetic acid (EDTA) chelated with 4.5×10^{-5} M $FeSO_4 \cdot 7H_2O$. Other important changes were made: the level of all the inorganic salts was increased to five times the original White's basic medium except for the chelated iron and the medium was supplemented with 5.0×10^{-4} M cytidylic acid, guanylic acid, hypoxanthine, and *myo*-inositol. The medium was also supplemented with 10^{-3} M L-glutamine. IAA, α-naphthaleneacetic acid (NAA) and 6-furfurylaminopurine (kinetin) were used at varying levels. Sucrose was added to a concentration of 4 %. The medium was prepared with 0.75 % Difco certified agar. All the constituents of the media were mixed and the solutions were adjusted to pH 5.5 with NaOH. After the pH adjustment the solutions were autoclaved.

Seedling Culture Medium. The seedlings were grown aseptically in 125 ml Erlenmeyer flasks containing White's basic medium (16), including $FeSO_4 \cdot 7H_2O$ as the source of iron, a vitamin fraction, and 2 % sucrose. White's basic medium was supplemented, however, with 10^{-3} M KCl. The media were prepared in 1 % Difco certified agar. The solutions were adjusted to pH 5.5 with NaOH and autoclaved for sterilization. The only variable in the study on seedling growth and seedling callus formation was NAA which was used at 5.4×10^{-7} M and 5.4 and 10^{-6} M.

The surface of the seeds was sterilized by soaking for 7 minutes in 1 % Kromet, a clorox-detergent prepared by the Wyndotte Chemical Corporation. The seeds were then carefully rinsed in sterile water to remove the detergent. The plants were maintained in cotton-stoppered flasks in a constant temperature chamber at 24° with an 18-hour photoperiod at 600 to 650 ft-c. Observations were made 29 days after sowing.

Results

Effect of Auxin and Kinetin on Tissues of N. suaveolens, N. langsdorffii, and N. suaveolens-langsdorffii. In some of our earlier experiments with *N. suaveolens* and *N. suaveolens-langsdorffii* it became evident that *N. suaveolens* tissue responded favorably to 9.3×10^{-6} kinetin in the medium that was also supplemented with 2.9×10^{-6} M IAA whereas *N. suaveolens-langsdorffii* showed no such response. This effect is illustrated (table I) by the greater growth of treated compared to nontreated tissue of *N. suaveolens*. IAA appeared to stimulate growth for both types of tissue. Kinetin with IAA greatly stimulated the growth of the tissues of the nontumorous parental species *N. suaveolens*. In figure 1A further data are represented which show that *N. suaveolens-langsdorffii* tissue grows better without kinetin but *N. suaveolens* is greatly stimulated by the levels of kinetin used. Although the growth of *N. langsdorffii* tissue was slow and only small cultures were produced, the growth pattern was similar to that of the other parental species.

Figures 1B and 1C clearly show: A. pronounced stimulation of growth by 2.3×10^{-6} M kinetin particularly at a relatively high auxin level for *N. langsdorffii* and at a relatively low level for *N. suaveolens*, B. a high auxin requirement for *N. langsdorffii* and questionable auxin requirement for *N. suaveolens*. It should be noted that *N. langsdorffii* tissue could not be cultured from stem sections without addition of kinetin to the medium. The stimulation of growth of *N. suaveolens-langsdorffii* tissue by either kinetin or auxin is questionable as illustrated in table I and figure 1A. This is in agreement with current concepts of the physiology and biochemistry of tumorous vs. nontumorous cell growth (1, 2, 3, 8).

The preceding results suggest that the hormone systems of *N. suaveolens* and of *N. langsdorffii* may differ considerably from each other. Growth of *N. suaveolens* tissue is definitely inhibited by high NAA

FIG. 1A (*upper left*). Effect of three levels of kinetin upon the growth of Nicotiana tissues cultured on modified White's basic medium (5 × unit concentration of inorganic salts) supplemented with L-glutamine, *myo*-inositol, hypoxanthine, cytidylic acid, guanylic acid and 2.9×10^{-6} M IAA. (*Left*): Growth of *N. langsdorffii* tissues cultured for 51 days, mean of 10 replications. (*Center*) Growth of *N. suaveolens-langsdorffii* tissue cultured for 34 days, mean from a minimum of six replications. (*Right*) Growth of *N. suaveolens* tissue cultured for 25 days, mean of four replications. Standard errors are indicated by the line symbols at the top of the histogram.

FIG. 1B (*upper right*). Effect of NAA and kinetin upon the growth of *N. langsdorffii* tissue cultured on modified White's basic medium (5 × unit concentration of inorganic salts) supplemented with L-glutamine, *myo*-inositol, hypoxanthine, cytidylic acid and guanylic acid. Histograms represent the mean growth of at least eight replications of the tissue cultured for 51 days. Standard errors are indicated by the line symbols at the top of the histogram.

FIG. 1C (*lower left*). Effect of NAA and kinetin upon the growth of *N. suaveolens* tissue cultured on modified White's basic medium (5 × unit concentration of inorganic salts) supplemented with L-glutamine, *myo*-inositol, hypoxanthine, cytidylic acid and guanylic acid. Histograms represent the mean growth of four replications of the tissue cultured for 43 days. Standard errors are indicated by the line symbols at the top of the histogram.

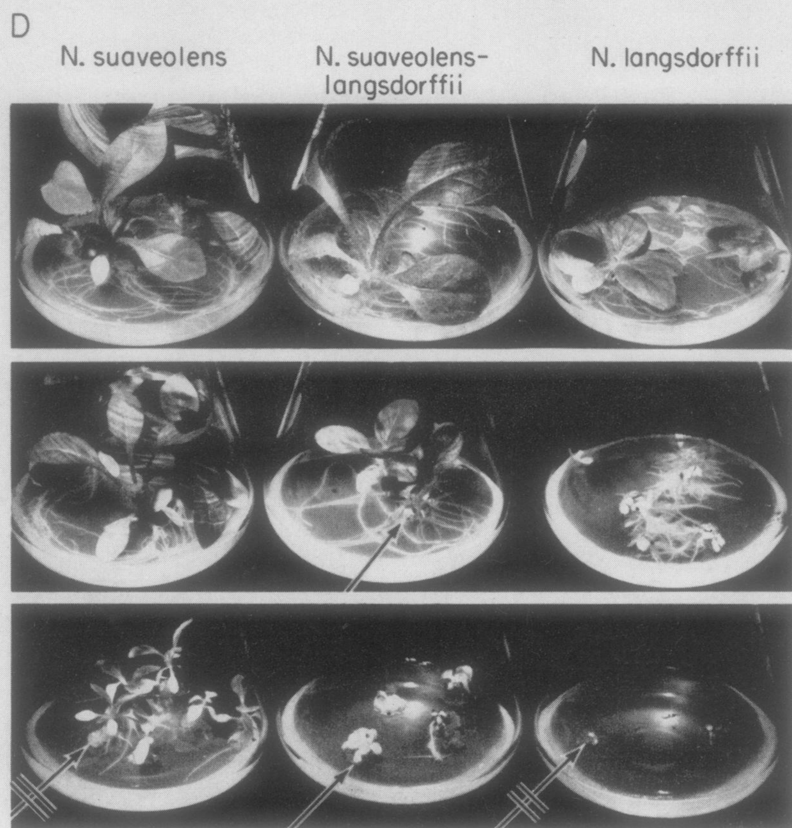

FIGURE 2

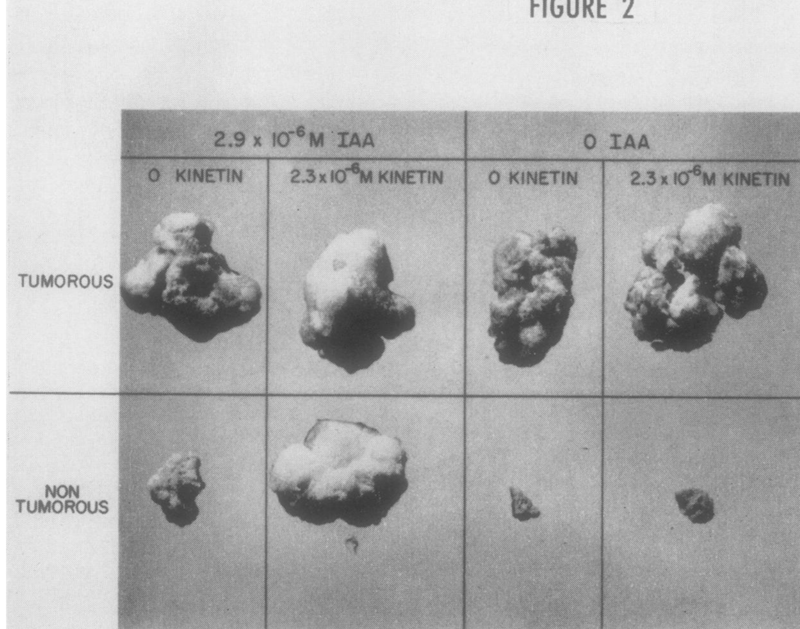

FIGURE 3

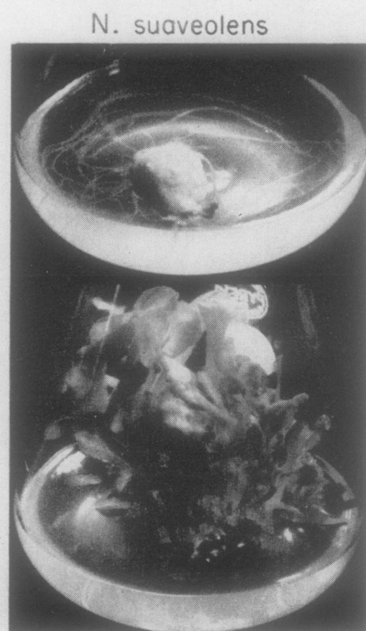

FIGURE 4

Table I

Effect of Indole-3-acetic Acid & Kinetin upon Growth of Nicotiana Tissues Cultured on Supplemented White's Basic Medium

Treatment	No. of replications	Days of culture	Avg wet wt/callus (g) ± SE	Ratio IAA &/or kinetin treated / untreated
N. suaveolens-langsdorffii				
Untreated	7	24	4.3 ± 0.36	...
IAA*	8	24	5.6 ± 0.28	1.31
Kinetin**	7	24	4.9 ± 0.30	1.14
IAA* + kinetin**	8	24	5.6 ± 0.25	1.30
N. suaveolens				
Untreated	6	43	2.5 ± 0.61	...
IAA*	5	43	4.2 ± 0.33	1.70
IAA* + kinetin	6	43	11.6 ± 0.67	4.67

* Final concentration: 2.9×10^{-6} M
** Final concentration: 9.3×10^{-6} M

levels whereas *N. langsdorffii* tissue requires high NAA levels for growth. On the other hand, it can be shown as illustrated in figure 2 that seedlings of *N. langsdorffii* are the most sensitive of the three genotypes utilized in this study to exogenous auxins, whereas seedlings of *N. suaveolens* are the least sensitive in terms of growth inhibition of leaves, stems, and roots. It is of interest to note that *N. suaveolens-langsdorffii* is intermediate between the two parental types in growth response. Undifferentiated growth of a compact nature occurred at a low and high auxin level on the hybrid (tip of arrows). On the other hand, undifferentiated growth of a translucent type appeared only at the higher auxin level on seedlings of the parental types (tip of arrows with crossbars). This compact type of growth observed on the hybrid is similar in appearance to tissue cultures from the parental types obtained on treatment with kinetin. One plausible explanation is that the hybrid is capable of synthesizing kinetin-like substances.

Effects of Kinetin upon Differentiation. A pronounced shift in the type and degree of differentiation was noted when *N. suaveolens* tissue previously grown on 5.4×10^{-6} M NAA and 2.3×10^{-6} M kinetin was transferred to media containing: A. no auxin and no kinetin, and B. no auxin but an intermediate level of kinetin (fig 4). The tissues that received kinetin showed pronounced leaf development whereas those tissues similarly treated but without kinetin formed long roots. Evidently kinetin enhances differentiation and growth of leaves. This response was observed numerous times in this study and corroborates the work of others (9, 12) on the effects of kinetin upon differentiation.

Effect of Auxin and Kinetin upon Tumorous and Nontumorous Types of N. glauca-langsdorffii. From the data represented in figure 5 it is clear that the nontumorous mutant type of *N. glauca-langsdorffii* requires auxin and kinetin for rapid growth. Almost no growth occurs without auxin and excellent growth was achieved with high levels of auxin and kinetin. While there appears to be some stimulation of growth in the tumorous type, the overall response to auxin and kinetin is small compared to the growth increase observed with the nontumorous *N. glauca-langsdorffii*. The maximum ratio of weights of treated (auxin &/or kinetin) to untreated cultures is eleven for tissue from the nontumorous type and two for the tumorous type. This is in complete agreement with the results obtained with the nontumorous parental species and the tumorous amphiploid hybrids of *N. suaveolens* and *N. langsdorffii*. Figure 3 also

FIG. 2. Effect of NAA at concentrations of 5.4×10^{-7} M (center row) and 5.4×10^{-6} M (lower row) upon growth of seedlings of *N. suaveolens* (left column), *N. suaveolens-langsdorffii* (center column), and *N. langsdorffii* (right column). Control plants are shown in the top row. Seedlings were grown for 29 days on KCl supplemented White's basic medium containing 2 % sucrose and 1 % agar. Arrows without crossbars show callus formation at high and low auxin levels in the hybrid. Arrows with crossbars show callus formation only at high auxin levels on the parental species.

FIG. 3. Representation of tissue cultures showing the effect of IAA and kinetin upon tumor and nontumor forming types of *N. glauca-langsdorffii*.

FIG. 4. Effect of 2.3×10^{-6} M kinetin (lower portion) upon the growth and differentiation of *N. suaveolens* tissue cultured on modified White's basic medium (5 × unit concentration of inorganic salts) supplemented with L-glutamine, *myo*-inositol, hypoxanthine, cytidylic acid and guanylic acid. Upper portion shows growth without kinetin. Photographed 43 days after transfer.

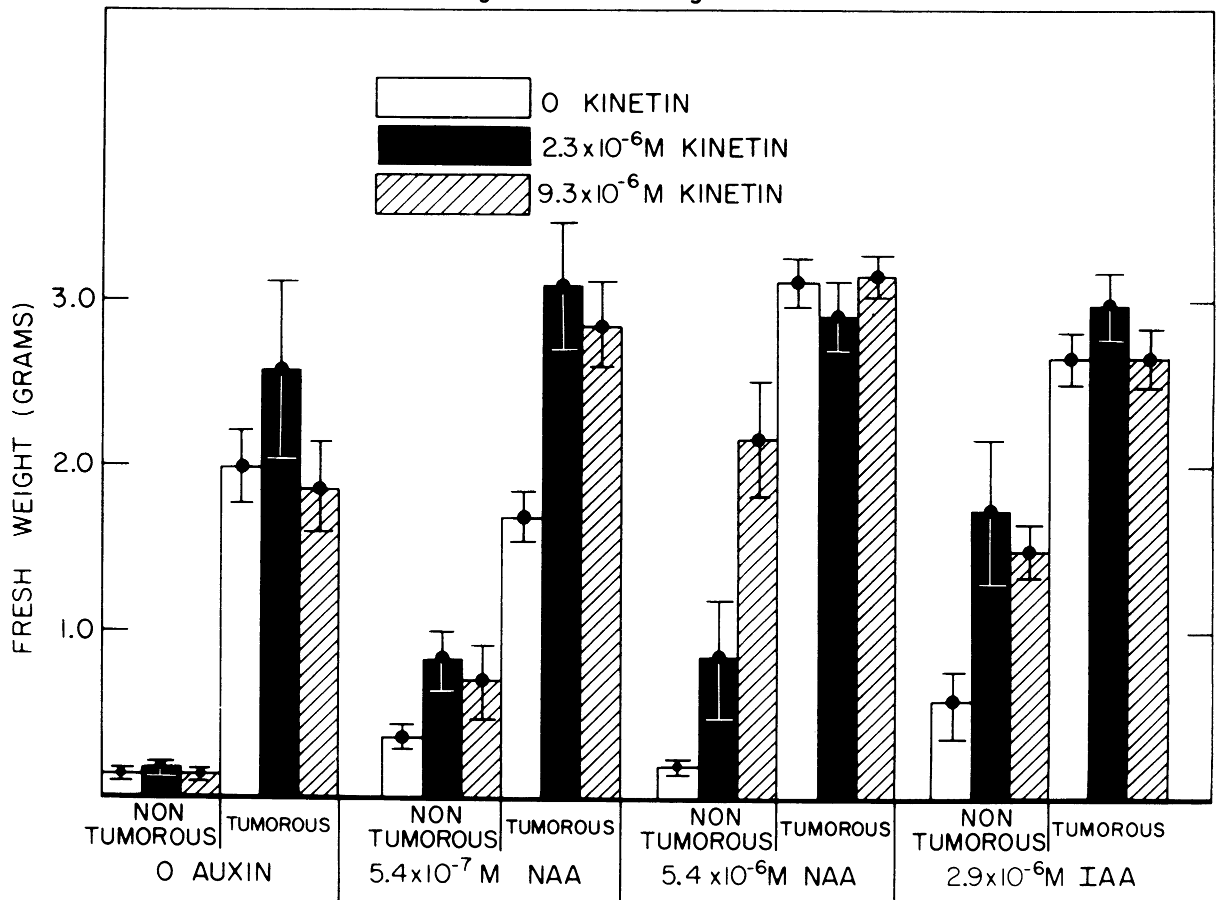

FIGURE 5

FIG. 5. Effect of NAA and kinetin upon tissues from tumor and nontumor forming genotypes of *N. glauca langsdorffii* cultured on a modified White's basic medium (5 × unit concentration of inorganic salts) supplemented with L-glutamine, *myo*-inositol, hypoxanthine, cytidylic acid, and guanylic acid. Histograms represent growth of nontumorous tissue for 41 days (mean of six replications) and tumorous tissue for 44 days (mean of 10 replications). Standard errors are indicated by the line symbols at the top of the histogram.

illustrates the relative magnitude of response of the two genetically different tissues of *N. glauca-langsdorffii* to auxin and kinetin treatments.

Discussion

The consistent growth increase obtained on treatment with kinetin in tissues representing nontumor forming genotypes of Nicotiana is striking. Since the effect of kinetin upon cell division is well-known (4, 5, 10, 11, 12, 15) and the elaboration of kinin-type compounds has been considered an important result of the crown-gall disease (2, 3), it is particularly significant to observe that widely divergent parental species respond similarly and favorably to kinetin whereas their tumor forming hybrids give essentially no response. Apparently tumorous tissues from the hybrid synthesize sufficient auxins and kinetin-like compounds endogenously for rapid growth. The results from these experiments indicate a close relationship between the basic physiology and biochemistry of genetic tumors and the crown-gall tumors. It is of fundamental interest that auxins and kinetin-like compounds appear to be important components of both tumor systems. In addition to the similarities in the crown-gall and genetic tumor systems, certain observations on the response of the parental species to hormone treatment are of fundamental interest. Both the tissue cultures and seedlings of *N. suaveolens* respond differently than *N. langsdorffii* to similar levels of NAA. This indicates that the two species have different hormone sensi-

tivities or may even have different hormone systems. Further experimentation on the hormone systems of the two parental species is needed in order to clarify the nature of the postulated differences.

In some respects auxin and kinetin appear to function in the control of several different aspects of differentiation. The effect of intermediate levels of kinetin as seen in figure 4 is to stimulate leaf and stem differentiation whereas auxins carried over from previous transfer tend to promote callus growth or growth of roots under these cultural conditions. Root formation in cultures of both parental species under relatively high auxin treatments was frequently observed; however, root formation in all our cultures of tumorous tissues has been exceedingly rare. This suggests a derangement in the normal hormone function in tumorous tissues which favors growth of a less specific type than the root-type of growth observed for normal tissue. This does not necessarily mean that tumorous tissues are incapable of root differentiation at controlled and specific auxin, kinetin, and other growth factor levels. It is recognized that lower concentrations of kinetin than those used in this study could promote root initiation and growth (12) and the observations described here could be explained on the basis of hormone balance (2).

Our results corroborate in general the extensive work of Braun (1, 2, 3) and Wood and Braun (17) on the activation of hormone-synthesizing systems in the plant tumor cell. Perhaps the plant tumor question cannot be completely resolved until the specific mechanisms of action of auxin and kinetin are more completely understood. Additional work is required to understand the mechanisms which lead to loss of control over the hormone-synthesizing systems in genetically conditioned (13, 14) tumor cells of Nicotiana hybrids.

Summary

Tissue cultures of *Nicotiana langsdorffii* Weinm. require both auxin and kinetin for active growth. Growth of *N. suaveolens* Lehm. tissue is greatly enhanced by kinetin but shows some inhibition at higher auxin levels. The tumor forming hybrid *N. suaveolens-langsdorffii* gives little growth response to either auxin or kinetin. Tissues from the tumor forming hybrid amphiploid *N. glauca* (Grah.)-*langsdorffii* (Weinm.) and its nontumor forming mutant show a hormonal growth response which is essentially the same, respectively, as the tumor forming amphiploid hybrid *N. suaveolens-langsdorffii* and its nontumor forming parental species. The results suggest that growth-factor systems activated in the genetic tumor system are very similar to those activated in crown-gall cells.

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