Plant Physiol. (1991) 97, 402–408
0032-0889/91/97/0402/07/$01.00/0

Received for publication January 2, 1991
Accepted May 14, 1991

In Vitro Flower Bud Formation in Tobacco: Interaction of Hormones[1]

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ABSTRACT

External application of auxin and cytokinin is required for the formation of flower buds on thin-layer tissue explants of *Nicotiana tabacum* cv Samsun. Interaction between both plant growth regulators during this regenerative process has been demonstrated with respect to speed of flower bud initiation and the number of flower buds formed. Separation in time of the hormone application during culture revealed that the cytokinin benzyladenine plays a key role in flower bud initiation whereas auxin (indoleacetic acid) stimulates in particular the differentiation of flower buds. The uptake of each hormone was proportional to the concentration supplied in the medium, and the uptake of either hormone appeared independently of the presence of the other. Metabolism studies showed the conversion of indoleacetic acid by the tissue to at least 13 metabolites after 24 h of culture. In addition, indoleacetic acid metabolism was demonstrated not to be influenced by the uptake and metabolism of benzyladenine. Taken together the results indicate that the interaction of auxin and cytokinin with respect to *in vitro* flower bud formation is indirect, *i.e.* does not take place at the level of hormone uptake or metabolism but at some step in the cascade of processes they initiate.

Flowering is a unique developmental event in the life cycle of a higher plant and results from a redetermination of the vegetative shoot meristem. In contrast to the reiterative leaf-forming activity of the vegetative meristem, the floral meristem is characterized by the sequential differentiation of all cells into morphologically and functionally different organs that constitute the flower. Floral determination of a competent terminal or axillary meristem is imposed by signals originating outside the meristem, *i.e.* by environmental factors like temperature and photoperiod as well as by factors transmitted from other plant parts, *e.g.* hormones (3, 17).

McDaniel *et al.* (11) found that in tobacco floral determination is apparently not an all-or-none state. Stem explants from a flowering plant of *Nicotiana tabacum* cv Wisconsin 38 regenerated both floral and vegetative shoots but showed a strong downward gradient with respect to floral determination, *i.e.* decreasing regeneration of floral shoots on explants excised towards the base of the plant. The capacity of tobacco cells to form a flower *in vitro* is an expression of a determined developmental state of tissue that under normal development on the intact plant will not form flowers. The determination can be revealed when meristems are induced *in situ* or in culture.

Similarly, examination of *in vitro* cultures of thin cell layers consisting of epidermis and subepidermal cortex from *N. tabacum* cv Samsun reveals determination by the formation of subepidermal meristems induced by hormones that, depending upon the type of tissue, may develop into either vegetative shoots or floral buds (2, 19–21, 25). Our studies are focused on floral bud formation on thin-layer tobacco pedicel explants that regenerate only flower buds directly from epidermal and/or subepidermal cells without intermediate callus formation (25).

In this *in vitro* system, regeneration of flower buds can be studied without interference by external signals from other plant parts and the environmental factors can be controlled. Two hormones, auxin and cytokinin, are required for the induction of flower bud initiation on tobacco thin-layer explants. The (interactive) effects of these hormones are expressed in three ways: (a) the number of flower buds formed per explant, (b) the speed of bud formation, and (c) the distribution of flower buds over the explant surface (2, 12, 14, 23). Cytokinin determines in particular whether flower buds are formed, whereas auxin mainly determines the position of buds on the explants. In addition, it was shown that both hormones were rapidly metabolized and that the level of flower bud formation is regulated by the concentrations of the free, nonmetabolized hormones (14, 23).

The aim of this study was threefold: (a) to compare the effect of the natural auxins (IAA)[3] and IBA and the synthetic NAA with respect to *in vitro* induction of flower buds; (b) to investigate the interaction between auxin and cytokinin during flower bud induction over a wide range of concentrations; and (c) to determine the temporally regulatory effect on flower bud formation of each hormone during culture.

MATERIALS AND METHODS

Plant Tissue Culture

Nicotiana tabacum L. cv Samsun plants were raised from seed in the greenhouse as described previously (20, 21). Tissue

[1] This research was supported by a grant from the Foundation for Biological Research in the Netherlands (BION-421.121).

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[3] Abbreviations: IAA, indoleacetic acid; BA, benzyladenine; IAAala, IAA conjugate with alanine; IAAasp, IAA conjugate with aspartic acid; IAAgly, IAA conjugate with glycine; IBA, indolebutyric acid; NAA, naphthaleneacetic acid.

strips, consisting of epidermis and a few cell layers of subepidermal cortex, were cut with a razor blade from pedicels at anthesis.

Ten tissue strips, approximately 1 × 8 mm in size, were cultured in Petri dishes (ϕ 9 cm) containing 25 mL of Murashige and Skoog medium, 125 mM glucose, 1% agar, and several combinations of auxin and cytokinin concentrations as indicated in the particular experiments.

IAA is known to be destroyed by light (8), the presence of Murashige and Skoog salts (7), and the combination of both (7). This process was also found to occur in our medium of tobacco tissue cultures (data not shown). To exclude the effect of IAA destruction, the explants were transferred to fresh IAA-containing media every 2 d. This was achieved by culturing the tissue strips on nylon gauze. Control tissues were cultured continuously for 19 d without transfer to fresh medium.

Bud numbers were determined 9, 14, and 19 d after the onset of culture. The results were evaluated by analysis of variance after logarithmic transformation, ln (bud number + 2), because it has been shown that this transformation resulted in a normal distribution of bud numbers (20). LSD is calculated at P = 0.05 and is presented as a bar.

Separation of the Auxin and Cytokinin Effect in Time

To separate the hormone application, it was necessary to apply them during a limited period of time without losing the flower bud regenerative capacity. We first established this period for each hormone by culturing explants on both hormones and replacing the media after different periods of time with media containing only one hormone. Flower buds were counted 14 d after the onset of culture.

Hormone Uptake

[8-^{14}C]BA and [5(n)^{3}H]IAA were purchased from Amersham. Specific activity of [^{14}C]BA was 2.1 MBq/μmol and of [^{3}H]IAA was 1.1 TBq/mmol. Each Petri dish with 10 explants contained 11.1 MBq of [^{14}C]BA and 27.8 MBq of [^{3}H]IAA and was supplemented, if necessary, with unlabeled BA and/or IAA to obtain the desired concentration. Each sample consisted of two explants (in triplicate) and was digested in 0.5 mL of Lumasolve (Lumac) in a scintillation vial at 50°C for 4 to 24 h. Afterwards, 4 mL of Lipoluma was added, and the radioactivity was measured in a Philips PW 4540 liquid scintillation analyzer coupled to a Hewlett-Packard 9815A calculator programmed for automatic quench correction. Results are expressed in picomoles per explant.

Hormone Metabolism

Explants were cultured as described above with media containing 2-[^{14}C]IAA (specific activity, 2.06 MBq/μmol; Amersham) and BA at the concentrations indicated.

Samples of explants were collected at appropriate times after the onset of culture into cold methanol (90% v/v with 0.01% butylated hydroxy toluene) and were kept overnight at −20°C. This extraction was carried out twice. The recovery of the label was generally more than 90%. The combined extracts were dried in vacuo at 35°C with a rotary evaporator and resuspended in 25 to 50 μL of absolute ethanol.

The sample extracts were analyzed by TLC on precoated silicagel 60 glass plates with fluorescent indicator (F_{254}) (Merck), and chloroform:methanol:acetic acid (75:20:5 v/v) were used as solvent. Radioactive spots were localized by autoradiography. Exposure for autoradiography was for 3 to 10 d at −80°C with Kodak XAR-5 x-ray film. The spots were scraped off directly into vials, lumagel (Lumac) was added, and the spots were analyzed for radioactivity. Hydrolysis experiments with scraped-off spots were performed as described by Vijayaraghavan and Pengelly (24). The reference compounds IAAasp, IAAala, and IAAgly were obtained from Research Organics Inc. and IAA and BA were from Sigma.

RESULTS

Hormone Interaction with Respect to Flower Bud Formation

The results with respect to flower bud formation on tissue explants at 28 different hormone concentration combinations of BA and IAA are shown in Figure 1. Bud numbers were determined 9 (panel A), 14 (panel B), and 19 (panel C) d after

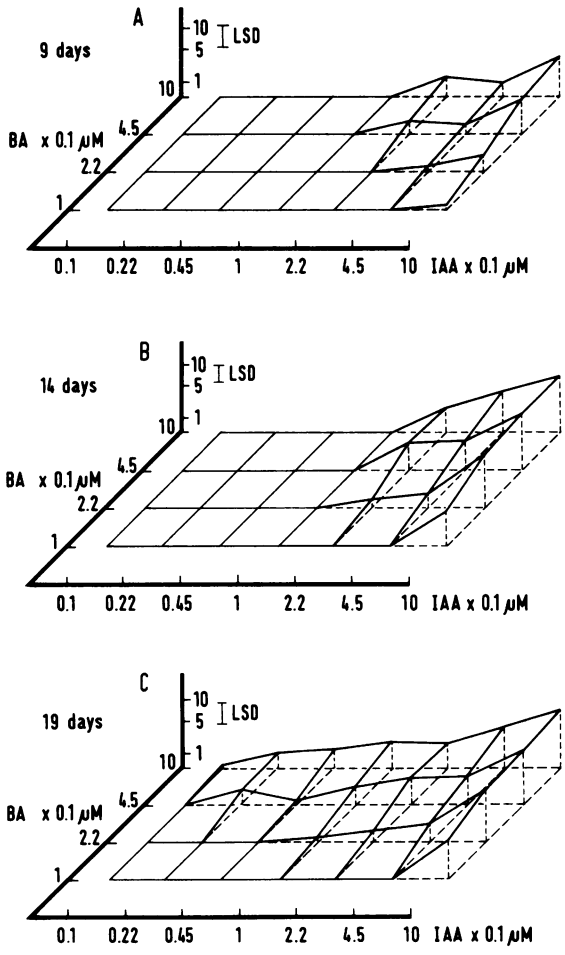

Figure 1. Effect of IAA and BA on *in vitro* flower bud formation at 28 hormone concentration combinations. Bud numbers were determined at (A) 9, (B) 14, and (C) 19 d after the onset of culture. Bar indicates LSD, P = 0.05.

the onset of culture. Early bud formation occurs at high auxin concentrations, *e.g.* 1 μM, even when the cytokinin concentration is low, *e.g.* 0.1 μM (Fig. 1A). Figure 1C illustrates that at high cytokinin concentrations, *e.g.* 1 μM, and low auxin concentrations, *e.g.* 0.01 μM, flower buds are formed much later, *i.e.* at about 19 d as compared with the early bud formation at about 9 d after the onset of culture. In addition, this experiment shows that at the same suboptimal hormone concentration combinations both auxin and cytokinin enhance the number of flower buds formed when the concentration of either hormone is increased whereas optimal flower bud formation is reached at 1 μM auxin and 1 μM BA. Similar experiments were carried out with the natural auxin IBA and the synthetic auxin NAA. The results of these experiments were essentially the same as those with IAA and are therefore not shown.

Culturing explants for 19 d without refreshing the medium resulted, in the case of IAA, in very poor-looking, barely living explants, indicating the instability of IAA under culture conditions. In contrast, experiments with IBA and NAA showed no significant differences in flower bud formation either under continuous culture or when the medium was refreshed every 2 d.

Figure 1 shows flower bud formation at four characteristic auxin/cytokinin concentration combinations. Under the combination 0.01 μM IAA and 0.1 μM BA, the explants stay alive without flower bud formation; the combination 1 μM IAA and 0.1 μM BA gives early bud formation; under the combination 0.01 μM IAA and 1 μM BA, flower bud formation is delayed compared with the combination 1 μM IAA and 1 μM BA, at which optimal flower bud formation takes place. These results show that at suboptimal concentrations auxin and cytokinin interact with regard to flower bud initiation.

Separation of the Auxin and Cytokinin Effect in Time

To separate the effects of the individual hormones in time it was necessary to establish the minimal duration of hormone application for a given concentration of both auxin and cytokinin at which still significant regeneration of flower buds was obtained. It was determined that this period was 4 d for NAA (16) and 3 d for BA (22), both at a concentration of 1 μM.

We have elaborated these experiments for IAA in combination with BA and found that the best regeneration took place at a concentration of 10 μM IAA applied for 5 d (Fig. 2) and 10 μM BA applied for 3 d (data not shown). These results enabled us to examine the effects of the hormones on flower bud formation separated in time. The results in Figure 3 show that application of BA before IAA leads to a higher bud number than in the reverse situation (compare lines 3 and 2), although in both cases bud formation is less in the control with continuous application (6). Continuous application of BA in combination with a 5-d IAA application enhances flower bud formation to the control level (compare lines 5 and 6), whereas in the reverse situation, *i.e.* continuous IAA and 3-d BA, no such enhancement occurs (compare lines 4, 5, and 6). In addition to the effect on flower bud formation, an effect on the fresh weight of the explants was also observed. It appears that simultaneously applied hormones or continuous application of BA leads to explants with a weight comparable with the control explants (compare lines 1, 5, and 6). Simultaneous application of IAA and BA for a limited period results in the same number of flower buds as with application of BA followed by IAA (compare lines 1 and 3). However, in this case, the fresh weight of the explants is comparable to the control (compare lines 1 and 6).

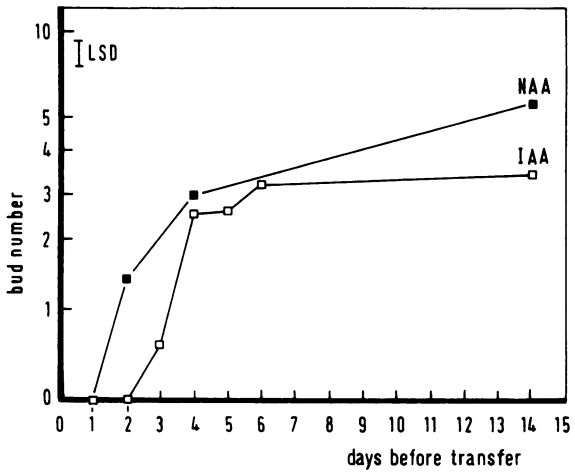

Figure 2. Determination of minimal duration of IAA or NAA application at which optimal regeneration takes place. BA was applied continuously, and both hormones were at a concentration of 1 μM. Bar indicates LSD, P = 0.05.

Not only the bud number and fresh weight were determined at several hormone regimes, but also the time at which the first flower buds appeared on the explants. Figure 4 shows that flower buds appear at the 6th d after the onset of culture when continuous 1 μM BA and 1 μM IAA are applied. The same results are obtained when 3 d of 10 μM BA followed by 5 d of 10 μM IAA are applied. Flower bud formation is postponed in the reverse situation: at d 8 or 9, the first flower buds are detectable.

Hormone Uptake and Metabolism

With respect to the hormone combinations A, B, and C in Table I, which lead, respectively, to no flower bud formation, early flower bud formation, and late flower bud formation as shown in Figure 1, double-label uptake experiments were carried out with [^{14}C]BA and [^{3}H]IAA. The results show a linear or near-linear uptake with time of both IAA and BA (Table I). The uptake of IAA appears independent of the concentration of BA but with a slight tendency for a higher uptake of IAA at the higher BA concentration after 48 h (Table I; compare A and C, column 1). Furthermore, BA uptake becomes more rapid after 4 d of culture at a higher IAA concentration (Table I; compare A and B, column 2). The uptake of IAA is proportional to the concentration supplied in the medium, *e.g.* a 100-fold-higher concentration gives rise to approximately 100-times-higher uptake of this hormone at all times (Table I; compare A and C with B). This is also the case for BA, considering that the actual [^{14}C]BA concentration, because of its low specific activity, was somewhat higher than 0.1 μM, *i.e.* actual concentration of 0.116

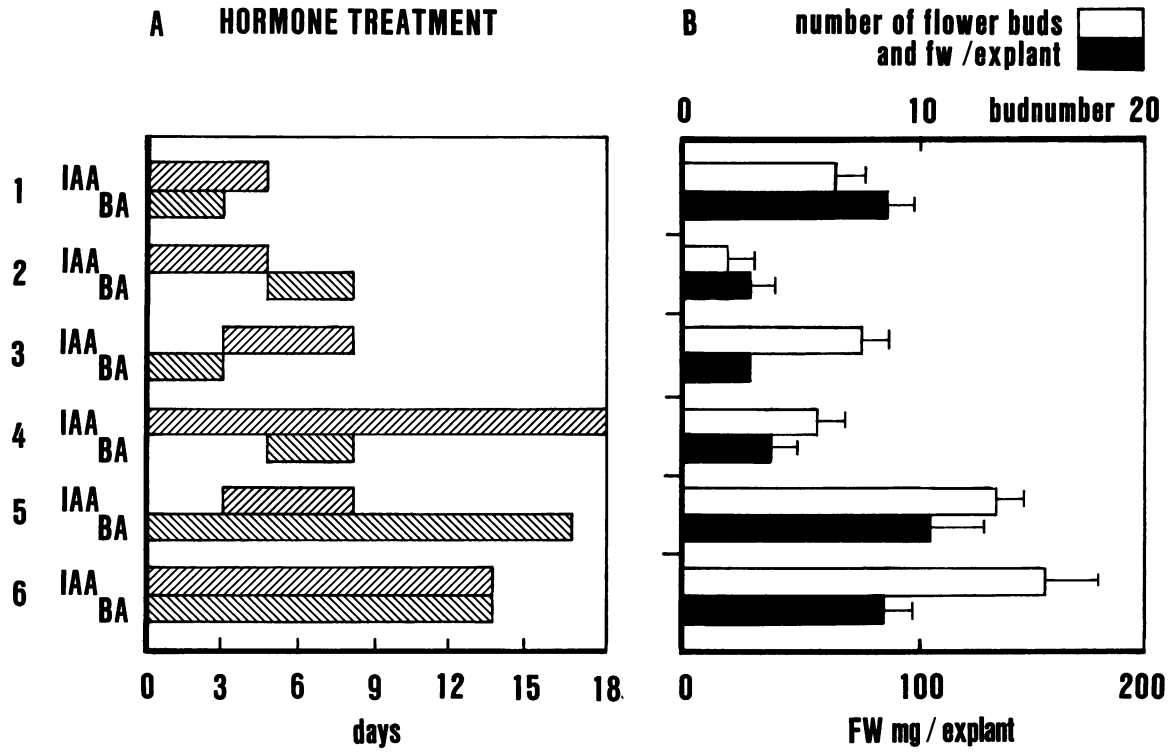

Figure 3. A. Hormone treatment: 10 μM IAA and 10 μM BA were applied in the medium for different periods of time. Continuous hormone application (longer than 10 d) is at a concentration of 1 μM. B. Number of flower buds formed and the fresh weight per explant (milligrams) at given hormone treatment. Bud numbers and fresh weight were determined 14 d after the application of the second hormone. Bar indicates SE of 30 explants.

μM. Table I shows that the difference between uptake of IAA and BA (compare B, column 1, and C, column 2) is not as great as previously reported for NAA and BA (2). The rate of IAA uptake is on average about twice the rate of BA uptake compared with about four- to sixfold for NAA.

Furthermore, we have investigated whether an interaction exists between both hormones at the level of metabolism. Figure 5 shows that IAA is rapidly metabolized once it has been taken up by the explants. After 2 h, most of the absorbed IAA is still present as free IAA. However, other compounds, metabolites of IAA, have already been formed. With time, metabolism proceeds and the amount of free IAA rapidly decreases to a constant level of 1 to 3% of the total radioactivity. After 24 h, at least 13 metabolites are formed. Most of these metabolites are present at a very low level (1 to 5% of total radioactivity), but a few metabolites make up 10 to 40% of the total 2-[^{14}C]IAA input.

Two metabolites cochromatographed with standard IAA conjugates, *i.e.* IAAasp and IAAgly as shown in Figure 5. Hydrolysis of metabolite 1 revealed that it represented an IAA conjugate, IAAasp, as confirmed by HPLC experiments (12). However, hydrolysis of metabolite 4 reveals no free IAA indicating that this cannot be IAAgly. Metabolites 2 and 3, which are the major metabolites, show total hydrolysis into free IAA, indicating that they are conjugates of IAA. In addition, we have examined the metabolism of IAA (1 μM) in the presence of 1 μM BA as well as in the absence of BA.

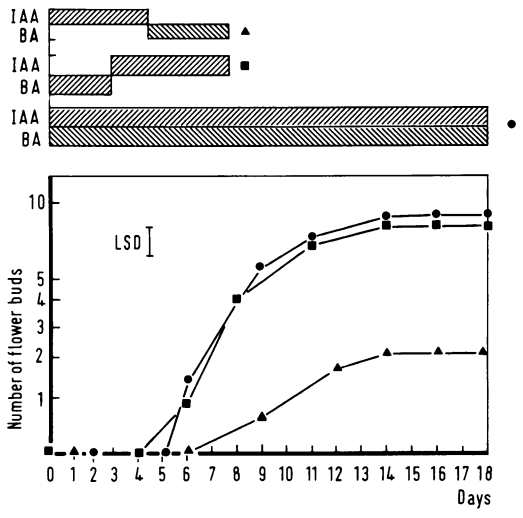

Figure 4. Determination of the time at which the first flower buds are formed at different hormone regimes. ▲, Consecutive application of IAA at 10 μM for 5 d after the onset of culture, followed by BA at 10 μM for 3 d, and then transfer to medium without either hormone. ■, Consecutive application of BA at 10 μM for 3 d after the onset of culture, followed by NAA at 10 μM for 5 d, and then transfer to medium without either hormone. ●, IAA and BA applied continuously from the onset of culture at a concentration of 1 μM. Bar indicates LSD, P = 0.05.

Table I. *Uptake of [^{3}H]IAA and [^{14}C]BA in three different concentration combinations*[a]

Time	A		B		C	
	0.01 μM IAA	0.1 μM BA	1 μM IAA	0.1 μM BA	0.01 μM IAA	1 μM BA
h	*pmol/explant ± SE*					
3	0.1 ± 0.0	0.8 ± 0.1	6.9 ± 0.2	0.9 ± 0.1	0.1 ± 0.0	4.7 ± 0.2
24	0.4 ± 0.0	2.6 ± 0.4	55.5 ± 8.7	1.9 ± 0.3	0.6 ± 0.0	11.9 ± 0.0
48	0.9 ± 0.1	5.0 ± 0.2	115.5 ± 9.0	4.6 ± 0.7	1.0 ± 0.1	22.7 ± 2.3
72	1.5 ± 0.1	10.9 ± 1.0	195.5 ± 9.8	11.1 ± 0.4	2.0 ± 0.1	77.4 ± 2.7
96	2.6 ± 0.1	20.3 ± 1.2	268.5 ± 18.3	19.8 ± 0.7	2.2 ± 0.1	105.9 ± 1.5
120	2.6 ± 0.2	23.7 ± 2.3	395.5 ± 27.9	39.5 ± 3.8	3.0 ± 0.2	150.4 ± 12.7
144	3.2 ± 0.3	40.3 ± 3.1	494.5 ± 25.3	66.8 ± 5.8	4.0 ± 0.3	245.3 ± 21.3

[a] The hormone combinations are A, no flower bud formation; B, early flower bud formation; C, late flower bud formation.

Figure 6 shows that metabolism of IAA is not affected by the absence or presence of BA.

DISCUSSION

By studying the induction of flower buds on thin cell layers of tobacco, we showed that the interaction between auxin and cytokinin concerns both the speed of flower bud initiation and the numbers of buds formed.

Optimal flower bud formation occurs at relatively high hormone concentrations, *i.e.* at 1 μM auxin (IAA, IBA, and NAA) and 1 μM cytokinin (BA), which is in agreement with previous results obtained with tobacco explants as far as NAA and BA are concerned (20, 21). At low concentrations of these hormones, *i.e.* 0.1 μM, explants stay alive but are incapable of flower bud formation. When either the auxin or cytokinin concentration is increased (Fig. 1), flower buds are formed at particular hormone concentration combinations. Increased auxin concentrations appear to compensate for low cytokinin concentrations and give rise to early bud formation. At high cytokinin and low auxin concentrations, flower bud formation is delayed. This is probably because it takes time to accumulate auxin. However, an increase of either auxin or cytokinin concentration leads to increased bud numbers. Our investigation suggests that the interaction of auxin and cytokinin is complex and shows that both hormones affect flower bud initiation as well as the number of buds formed. From previous investigations (20, 21), it was indicated that cytokinin mainly determined the number of buds formed whereas auxin influenced bud development and callus formation. The

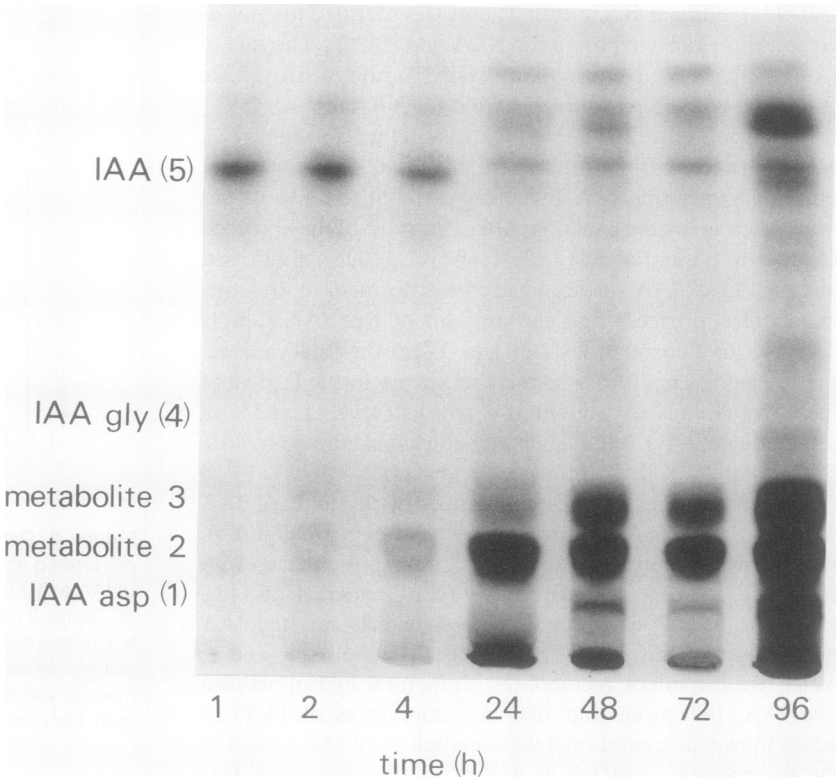

Figure 5. Metabolism of 2-[^{14}C]IAA. At given times, IAA was extracted from three explants analyzed by TLC and visualized by autoradiography. Concentration of both IAA and BA were 1 μM. Metabolites 1 to 5 are explained in the text.

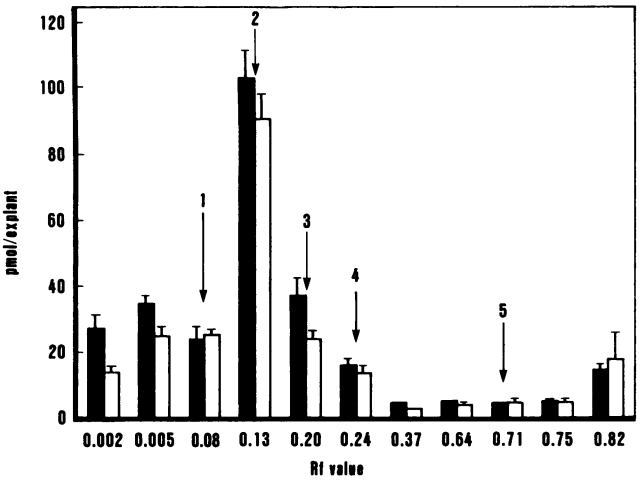

Figure 6. Metabolism of 2-[^{14}C]IAA in the presence of 1 μM BA (■) and in the absence of BA (□). IAA was supplied at a concentration of 1 μM, and metabolism was examined in triplet by TLC with 30 explants after 24-h incubation. Bar indicates SE. Numbers 1 to 5 correspond with metabolites shown in Figure 5.

minimal duration of hormone application that still resulted in flower bud formation was 5 d for IAA (10 μM) and 3 d for BA (10 μM). This is in good agreement with the conclusion of Smulders (15), who showed for NAA that the number of buds formed on the explant depends on the hormone dose rather than on the hormone concentration.

Cytokinin seems to play a decisive role in flower bud initiation, probably by initiating the cell divisions required for meristem development as indicated by the fact that BA has to be applied before or at least simultaneously with IAA. Application of BA before IAA results in regeneration of flower buds at the same level as the control situation where the hormones were supplied continuously during culture. Application of IAA before BA resulted in a significantly lower number of buds. Furthermore, we showed that flower buds are formed earlier when BA is applied before or simultaneously with IAA than in the situation where IAA is applied first. A high internal concentration of free IAA leads to the differentiation of flower buds. Once the meristem has been formed in the subepidermal tissue, auxin becomes more important for cell enlargement and the differentiation processes leading to flower bud formation. Our results clearly show that the auxin and cytokinin effect can be separated in time and indicate that the interaction between these hormones with respect to flower bud formation is indirect.

Davies and Rubery (6) showed that in stem segments of *Pisum sativum* L. high BA concentration reduces the uptake of IAA because of changes in the internal pH. This is in contrast with our results (Table I), which show an independent uptake of IAA and BA when supplied together in different concentration combinations. In fact, 6 d after the onset of culture, IAA uptake was somewhat higher at a higher BA concentration. The somewhat higher uptake of BA at a higher IAA concentration after 4 d (Table I; compare A and B, column 2) is because the explants become somewhat larger in response to the higher auxin concentration compared with the lower auxin concentration. Barendse *et al.* (2) have shown that the uptake of NAA was considerably higher compared with BA uptake when supplied at equal concentrations. The rate of IAA uptake appears to be lower compared with NAA. When supplied at the same concentrations, the uptake of NAA is about six times the uptake of IAA in our results compared with those of Smulders *et al.* (16). In different tissues, NAA has been reported to possess a low affinity to the uptake carrier of IAA (13, 18) and, thus, the IAA carrier is probably not the mechanism by which NAA is taken up more rapidly than IAA. Smulders *et al.* (16) indicate that the mechanism by which NAA is taken up is not carrier mediated like IAA but has to do with the better solubility of the apolar NAA in the membrane.

Metabolism of IAA in thin-layer tissue strips of *N. tabacum* cv Samsun is quickly initiated and leads to at least 13 detectable metabolites. Most metabolites are present at a very low level. The most abundant metabolites are compounds not yet identified, but the hydrolysis results suggest that they are conjugates. Furthermore, we show that IAA metabolism is not affected by the presence of BA and vice versa (22). Conjugation of IAA with amino acids is regarded as an important mechanism for detoxification, defense against decarboxylation, and a source of free IAA, which is considered to be the biologically active compound (1, 4, 5, 10). Apparently, this mechanism also operates in our tobacco cultures to maintain a stabilized level of free auxin in the tissue (see also ref. 15).

The rate of flower bud formation is ultimately dependent on the endogenous concentrations of each hormone in the tissue. This endogenous hormone concentration reflects, but does not necessarily equal, the concentration supplied in the medium as indicated by the differences in rate of uptake and extent of metabolism of each hormone (16, 23).

This study reveals that neither the physiological response of flower bud formation nor the results with respect to hormone uptake and metabolism indicate the existence of a particular hormone ratio in relation to a particular effect. This is in contrast to the prevailing conceptions (9). Together with our findings that the hormone effects can be separated in time, this indicates that in our system each hormone exerts its effect independently and that their interaction with respect to flower bud formation occurs beyond the level of hormone uptake and metabolism in the cascade of sequential processes that they initiate.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the assistance and collaboration of Mr. G. van der Weerden for raising the necessary tobacco plants in the greenhouse and Dr. A. F. Croes for critically reading the manuscript.

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