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Short Communication

Uptake and Metabolism of NAA and BAP in Explants of Tobacco in Relation to *In vitro* Flower Bud Formation

G. W. M. Barendse, A. F. Croes, M. Bosveld, W. M. van der Krieken, and G. J. Wullems

Department of Botany, University of Nijmegen, Toernooiveld 6525 ED Nijmegen, The Netherlands

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Abstract. *In vitro* flower bud initiation and development depend on the presence of two hormones in the culture medium—auxin (NAA) and cytokinin (BAP). The uptake of both NAA and BAP by the explants was shown to be proportional to the concentrations supplied in the medium over a period of 4 days after the onset of culture. However, when supplied at equal concentrations for 24 h, the NAA uptake was up to 10-fold higher than the BAP uptake. Both hormones are rapidly metabolized by the explants. Nevertheless, the concentrations of free hormones inside the explants appeared to be high and in the case of NAA exceeded the concentration in the medium by more than 1 order of magnitude within 24 h. Apparently flower bud initiation in tobacco explants requires relatively high concentrations free NAA and BAP in the tissue maintained by a continuous supply in the medium. There are at present no indications that the products of hormone metabolism are directly involved in bud formation.

Hormonal control of plant development is mainly studied by application of hormones and/or the determination of their endogenous levels (e.g., Letham and Palni 1983). This approach implies that a correlation exists between hormone levels and quantitative aspects of development. Knowledge about the manner in which endogenous levels of hormones are controlled is still very limited. The hormone concentration in a particular tissue is determined by biosynthesis, translocation, and (in)activation through metabolism. Few studies

deal with hormone metabolism in tissue culture, and very little is known about the effect of hormone metabolism on free-hormone concentrations in the tissue. Moreover, the relation between these hormone concentrations and *in vitro* organogenesis has not been established in most systems. This study deals with the uptake and metabolism of naphtaleneacetic acid (NAA) and benzylaminopurine (BAP) and their levels in explants from pedicels of *Nicotiana tabacum* cv. Samsun. These explants consisting of epidermis plus epidermal cortex regenerate flower buds when cultured on an agar medium containing basic nutrients and varying concentrations of hormones. Under appropriate conditions, 20–30 buds can be formed on one explant.

The number of flower buds formed depends strongly on the BAP concentration supplied in the medium, and the NAA concentration determines in particular the position of the buds on the explants resulting in "polar" and/or "random" bud formation (Van den Ende et al. 1984b,c).

The role of these hormones with respect to *in vitro* flower bud development in tobacco was studied by analyzing the uptake of the hormones from the medium and their subsequent metabolism in the explants. NAA is known to be rapidly converted to conjugates in a variety of systems (Barendse et al. 1986, Caboche et al. 1984, Cohen and Bandurski 1982, Goren and Bukovac 1973, Vijayaraghavan and Pengelly 1986, Zenk 1962). Conjugation leads to products with low auxin activity (Vijayaraghavan and Pengelly 1986, Kazemie and Klämbt 1969). BAP is also rapidly metabolized (Letham and Palni 1983), but some of its metabolites may be active.

From the data obtained, the concentrations of free NAA and BAP during early flower bud initiation were calculated.

Materials and Methods

Flowering plants of *Nicotiana tabacum* cv. Samsun were raised from seed in the greenhouse under 16-h days of natural daylight supplemented with 400-W Philips HPLRG lamps and at 21°C as described previously (Van den Ende et al. 1984b,c). Tissue strips ~ 8 × 1 mm were excised from the pedicels of flowers at anthesis.

The explants were cultured in Petri dishes on 1% agar containing revised Murashige-Skoog medium (Murashige and Skoog 1962), 150 mM glucose, and various concentrations of NAA and BAP, pH 5.6. In some experiments, glucose was replaced by mannitol. In the absence of glucose, no buds develop on the explants (Van den Ende et al. 1984a). The numbers of buds were determined 14 days after the onset of culture. In general, each treatment consisted of at least 20 explants.

To determine hormone uptake and metabolism, [^{14}C]NAA (spec. act. 2.1 GBq/mmol) and [^{14}C]BAP (spec. act. 1.85–2.2 Bq/mmol) or [^{3}H]BAP (spec. act. 55 GBq/mmol) purchased or custom-synthesized by Amersham (England) were supplied in the medium. The explants were exposed continuously to the labeled hormones for 1, 2, 3, or 4 days. Hormone uptake was determined by collecting the explants directly into minivials containing 250 µl 2 N NaOH.

The vials were kept overnight at 60°C, and subsequently 250 μl H_2O and 4.5 ml Aqualyte (Baker Chemicals) were added before liquid scintillation counting.

To analyze the metabolism of the hormones taken up by the explants, the tissues were collected directly into cold methanol and kept overnight at −20°C prior to homogenization. The homogenates were filtered, and the filtrates were evaporated to dryness under vacuum. The residue was taken up in 100 μl methanol and stored at −20°C until analysis. The samples were analyzed by reversed-phase high-performance liquid chromatography (HPLC) using a 5- or 10-μm C_{18} Radial-PAK column (Waters-Millipore). NAA, BAP, and their metabolites were separated by isocratic elution with appropriate mixtures of methanol/0.02M acetic acid (e.g., 80:20, v/v, for the 5-μm column; 95:5, v/v, for the 10-μm column), adjusted to pH 3, as mobile phase, at a flow rate of 1.5 ml/min and detection at 270 nm. The eluted fractions were collected and assayed for radioactivity by liquid scintillation counting. Each experiment was carried out at least twice with similar results.

Results

The initiation of flower buds on pedicel explants of tobacco occurs during the first 3 or 4 days of culture at appropriate hormone concentrations, and the first visible flower buds appear 7 or 8 days after the onset of culture (Van den Ende et al. 1984a,b). The growth of the explants (Fig. 1) is slow during the bud initiation period but accelerates thereafter.

When mannitol is substituted for glucose, the explants show little growth and do not form flower buds. Therefore, this system was used for comparison.

The effects of BAP concentrations varying from 0.1 to 10 μM at two concentrations of NAA are shown in Fig. 2. There is a rapid increase in number of flower buds between 0.22 and 1 μM. Higher concentrations of BAP do not lead to a further increase in bud number. The increase in bud number appears to be independent of the NAA concentration.

On the basis of this result, BAP uptake and conversion were investigated at an effective 1-μM and an ineffective 0.1-μM concentration. The same concentrations were used in the experiments on NAA metabolism. At 1 μM of this hormone, the flower buds formed are evenly distributed over the explant surface, whereas at 0.1 μM, bud emergence is restricted to the basal edge of the tissue—i.e., polar bud formation (Van den Ende et al. 1984c).

The uptake of both BAP and NAA, supplied at two concentrations, appears to be proportional to the concentration supplied in the medium (Fig. 3). The uptake of NAA, however, occurs at a much higher rate than the uptake of BAP—e.g., more than 10-fold in the first 24 h when the two hormones are supplied at equal concentrations.

Preliminary experiments had shown that both hormones are rapidly metabolized when taken up by the explants (Barendse et al. 1986). The major products are apparently conjugates, because the free hormones can be set free by appropriate hydrolysis (unpublished results). This metabolism reduces the levels of free, nonmetabolized NAA and BAP inside the explants. To estimate the

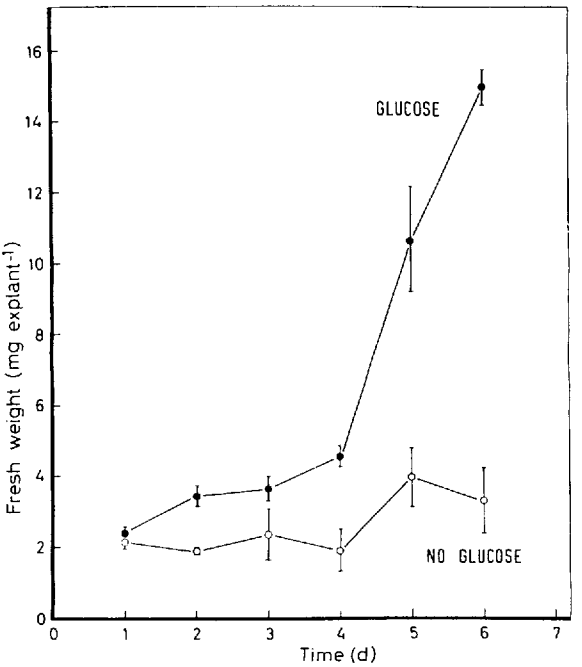

Fig. 1. Growth of tobacco explants on a Murashige-Skoog medium containing 1 μM of NAA and BAP in the presence and absence of glucose.

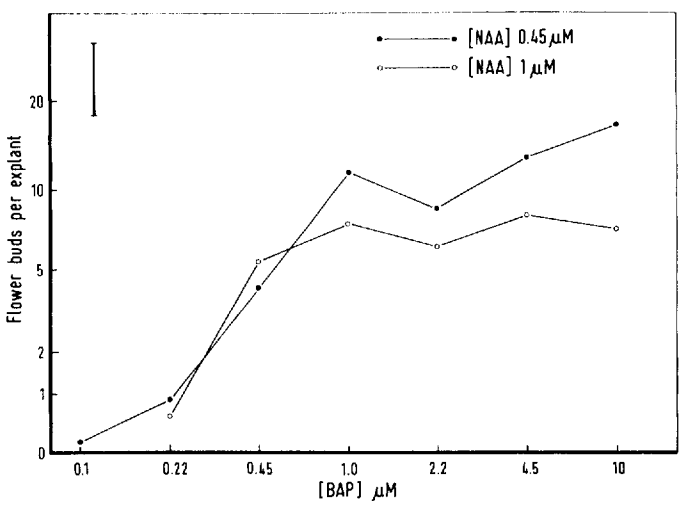

Fig. 2. Effect of BAP and NAA on *in vitro* flower bud formation.

concentrations of free NAA and BAP, the explants were cultured for 24 h on a medium containing radioactive hormones and subsequently extracted, and the hormone content was analyzed by HPLC (Barendse et al. 1986).

An example of HPLC analysis of NAA and BAP metabolism is shown in

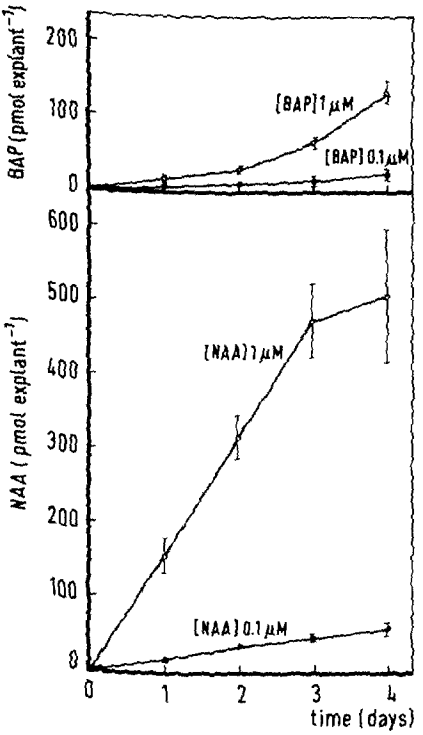

Fig. 3. The uptake of BAP and NAA during the first 4 days of culture.

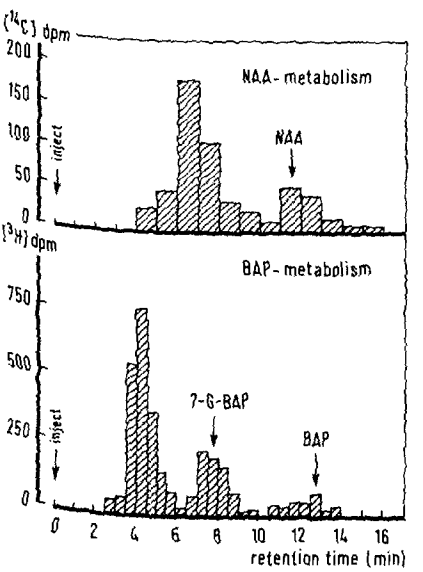

Fig. 4. Reversed-phase HPLC of extracts from tobacco explants after 24 h of culture. NAA, BAP, and their metabolites were separated by isocratic elution with appropriate mixtures of methanol/0.02 M acetic acid (see Materials and Methods). 7-G-BAP: 7-glucosyl BAP.

Fig. 4. Simple isocratic elution was employed to separate the free NAA or BAP from their metabolites after 24 h of culture. It was not considered necessary to separate all the individual metabolites, which requires a more elaborate gradient elution system, as has been used for identification purposes. The

Table 1. Uptake and metabolism of [^{14}C]NAA and [^{3}H]BAP by pedicel explants of tobacco in 24 h.

Hormone	Glucose	Concentration (μmol l^{-1})	Uptake (pmol explant^{-1})	Free hormone	
				pmol explant^{-1}	μmol kg^{-1}
NAA	+	0.1	29 ± 2.8	3.2	1.6
	−	0.1	31 ± 0.6	3.4	1.7
	+	1	266 ± 19	31.9	16.0
	−	1	203 ± 4	24.4	12.2
BAP	+	0.1	5.7 ± 0.5	0.4	0.2
	−	0.1	5.2 ± 0.2	0.4	0.2
	+	1	33.1 ± 10	2.5	1.3
	−	1	25.9 ± 1.9	2.2	1.1

major conjugate of BAP was tentatively identified as 7-glucosyl BAP (Fig. 4.).

On the basis of these HPLC analyses, the concentrations of free and conjugated NAA and BAP were determined (Table 1). Only a minor part, between 7% and 12%, occurs as free hormone inside the explants.

In spite of the fact that the major part of the hormones is metabolized, the concentrations of the free hormones are still high. Particularly, the free NAA concentration reaches as much as 16 times the concentration supplied in the medium. Also the free BAP concentration that is taken up much more slowly than NAA (Fig. 3) exceeds the BAP concentration at both concentrations supplied in the medium. In addition, the results of Table 1 demonstrate that during the first 24 h of culture, no significant differences occur in the uptake and metabolism of hormones in the tissues cultured either in the presence or in the absence of glucose.

Discussion

The number of flower buds formed is mainly determined by the BAP concentration in the medium (Fig. 2), provided the NAA concentration is not too low. The lowest effective BAP concentration is 0.22 μM (Fig. 2). This hormone concentration roughly corresponds to 0.4 μM free BAP in the explant after 24 h of uptake (Table 1). A linear relationship between uptake rate of NAA and concentration in the medium (Fig. 3) has been described for a number of systems (Zenk 1962, Vijayaraghavan and Pengelly 1986).

It seems that in our experiments, diffusion of the undissociated acid (Rubery and Sheldrake 1973) is a more important component of NAA uptake than carrier-mediated anion transport (Rubery 1978, Rubery and Sheldrake 1974). The rate of uptake of both hormones appeared to be relatively high but different for BAP and NAA, the latter being taken up at a considerable higher rate. As a result, the ratio of hormones inside the cultured explants is different from the ratio supplied in the medium. In agreement with previous observations (Bar-

endse et al. 1986, Caboche et al. 1984, Cohen and Bandurski 1982, Goren and Bukovac 1973, Vijayaraghavan and Pengelly 1986, Zenk 1962), NAA was found to be rapidly metabolized to more polar components, presumably conjugates (Table 1). Because of the reported biological inactivity of these conjugates (Kazemie and Klämbt 1969, Vijayaraghavan and Pengelly 1986), the concentration of the free hormone is of prime importance. Only a minor part of the hormones taken up occur in the free form inside the explants. However, in spite of the rapid metabolism, the concentrations of the free hormones in the explants reach relatively high levels, which even in the case of BAP exceed the concentration supplied in the medium within 24 h of uptake.

The percentage of free NAA relative to the total amount taken up is independent of the NAA concentration in the medium. The same result has been described for a tumor line of tobacco (Vijayaraghaven and Pengelly 1986). In combination with the uptake data, this means that the NAA level inside the tissue is strictly proportional to the concentration in the medium.

It has been shown previously (Van den Ende et al. 1984b,c) that a continuous supply of NAA and BAP at sufficiently high concentrations is required for at least 10 days for optimal bud initiation and development. After the 4-day initiation period, the rate of uptake levels off when the uptake is expressed per unit of fresh weight of the tissue (unpublished data). Nevertheless, the explants attain high to very high levels of (free) hormones.

Explants on mannitol medium maintain their capacity for flower bud formation, as has been shown in transfer experiments (Van den Ende et al. 1984a).

No significant differences in uptake and rate of metabolism were observed in explants cultured either in the presence or absence of glucose during the first 24 h of culture (Table 1). Also, no qualitative differences occur in the metabolites formed from NAA and BAP on media with and without glucose up to 7 days after the onset of culture (Barendse et al. 1986). It thus appears that the modalities of bud formation on explants of tobacco are mainly controlled by the relatively high levels of free BAP and NAA in the tissue. Hormone metabolism seems to play a minor role in the regulation of bud formation, because equilibrium between hormones and their metabolites is affected neither by the hormone concentration nor by conditions that are otherwise prohibitive to bud initiation, like glucose deprivation. However, the possibility that some of the metabolites may directly affect flower bud formation cannot be excluded and is under investigation.

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