J Plant Growth Regul (1991) 10:79–83

Journal of
Plant
Growth
Regulation

Induction of Flower Bud Formation In Vitro by Dihydrozeatin

W. M. van der Krieken*, A. F. Croes, M. Hermans, and G. J. Wullems

Molecular Plant Physiology Research Group, Department of Experimental Botany, University of Nijmegen, Toernooiveld, NL-6525 ED Nijmegen, The Netherlands

Received February 25, 1990; accepted November 9, 1990

Abstract. Flower stalk explants of tobacco cultured on a medium with an auxin and cytokinin regenerate flower buds within 14 days. The optimal medium concentrations of dihydrozeatin (DHZ) and benzyladenine (BA) were both 1 μM. The presence of DHZ in the culture medium was only essential during an initiation period of 7 days, whereas BA was needed only during the first 4 days. The difference in length of the initiation period is neither explained by the unequal uptake rates of the cytokinins nor by differences in their conjugation. At the medium concentration optimal for bud formation, the internal concentration of DHZ was two to three times the internal concentration of BA, which could be attributed to faster uptake of DHZ. It is concluded from the combined data that DHZ is less active in inducing flower bud formation than BA and that the exogenous cytokinins play only a role during the initiation phase of bud regeneration.

The role of auxin and cytokinin in organ regeneration in vitro is commonly studied by relating the hormone concentrations in the medium to the magnitude of the response (Skoog and Miller 1957, Tran Thanh Van 1981). For a detailed analysis of the relation between hormone concentration and response, however, it is necessary to determine the concentrations of the physiologically active hormone or hormone derivatives *inside* the tissue. Cytokinins are converted into a number of derivatives (Barendse et al. 1985, Horgan 1987, Laloue and Pethe 1982, Letham and Palni 1983, Van der Krieken et al. 1988). Most likely, only the free base is active (Laloue and Pethe 1982, Van der Krieken et al. 1990).

* Present address: Center for Agrobiological Research, P.O. Box 14, 6700 AA Wageningen, The Netherlands.

In thin-layer explants of tobacco pedicels (Tran Thanh Van 1973) various cytokinins show different activities in promoting flower bud formation. Benzyladenine (BA) and dihydrozeatin (DHZ) induce maximal bud formation at the same medium concentration. Isopentenyladenine is required at a 20 to 40 times higher concentration, whereas zeatin is almost noninductive (Van der Krieken et al. 1990).

The cytokinin does not need to be present in the medium during the entire culture period. BA has been found to initiate maximal flower bud formation in the first 2–4 days of culture (Van den Ende et al. 1984, Van der Krieken et al. 1990). This could mean that the physiological response is related to the dose of the hormone accumulated in the tissue. A dose-dependent response of the same tissue culture system to auxin has recently been reported (Smulders et al. 1990). Such a relationship implies a dependency of the number of buds regenerated on the rate of hormone uptake measured during a fixed period as has actually been found for BA (Van der Krieken et al. 1988). The physiological effect of an accumulation of BA, in combination with processes such as conjugation and distribution of hormone over the tissue, would be the establishment of an inductive cytokinin concentration in the responsive cells.

That DHZ and BA induce the same maximum number of flower buds at the same medium concentration in explants continuously exposed to the hormone, does not necessarily mean that both cytokinins are equally active in bud induction, because the minimum exposure times to the two compounds and their uptake rates might differ. Differences in the rates of conjugation of the two cytokinins could also contribute to unequal concentrations within the tissue.

In this study the activities of DHZ and BA were compared by determining the lengths of the initiation period for maximal flower bud formation and by measuring the internal cytokinin concentrations resulting from uptake. To this end, uptake and con-

jugation of these cytokinins were quantified at various intervals during culturing.

Materials and Methods

Culture In Vitro

Flowers of tobacco (*Nicotiana tabacum* L. cv. Samsun) were picked with pedicels attached at anthesis when the inflorescence was in full bloom (stage IV, Croes et al. 1985). Small explants (8 × 1 mm) were cut from the flower stalks and cultured on Murashige and Skoog medium (Murashige and Skoog 1962) with 125 mM glucose and 1% agar (Difco) as described previously (Van den Ende et al. 1984). 1-Naphthaleneacetic acid (NAA) was added at 1 μM, and DHZ and BA were supplied as indicated for each experiment. Flower buds were counted after 14 days of culture. The data were entered in the figure after the logarithmic transformation X = ln (bud number + 2). The least significant difference (LSD) at p = 0.05 was calculated from the transformed values and presented in the figure as an error bar. To study uptake and metabolism, [2,3(n)-^{3}H]DHZ (specific activity 1520 TBq mol^{-1}, Amersham) or [2,8-^{3}H]BA (specific activity 155 TBq mol^{-1}, Amersham) was included in the medium. The explants were weighed prior to extraction of the cytokinins and their metabolites.

Separation of DHZ Metabolites by Thin-Layer Chromatography

DHZ and its metabolites were extracted from groups of 10 explants after incubation on radioactive DHZ by the procedure of Laloue and Pethe (1982). The tissue was homogenized in 3 ml modified Bieleski's fixative. Uptake was measured by counting the radioactivity in 0.3 ml of the homogenate after mixing with 4 ml Lumagel (Lumac) in a liquid scintillation analyzer. The rest of the extract was centrifuged and the pellet was washed in 1 ml fixative. The pooled supernatants were dried at 40°C under nitrogen and the residue was dissolved in 50% ethanol. The extracts were analyzed by thin-layer chromatography (TLC) (Tao et al. 1983) on silica gel 60 PF_{254} plates (Merck) with authentic DHZ-7-glucoside (7G-DHZ), DHZ-9-glucoside (9G-DHZ), O-glucoside of DHZ [(OG)-DHZ], DHZ-riboside (9R-DHZ), DHZ-riboside monophosphate [9R(P)DHZ], and DHZ as the standard (Apex Organics). In the first dimension, the plates were developed twice with *n*-butanol:14 M NH_4OH:water (6:1:2 vol/vol/vol, upper phase) as the solvent. The solvent in the second dimension was *n*-butanol:acetic acid:water (12:3:5, vol/vol/vol). The fraction banding at the position of 9R(P)DHZ is referred to as the nucleotides. No attempt was made to separate the mono-, di-, and triphosphates of 9R-DHZ. The ultraviolet-absorbing spots were scraped off and extracted with 0.5 ml water for 2 h. After addition of 4 ml Lumagel, the radioactivity was measured by liquid scintillation counting. In some experiments TLC in only the first dimension was used to separate DHZ from its derivatives.

Results

Flower Bud Induction by DHZ and BA

The same number of flower buds were formed after 14 days of continuous culture on medium supplemented with either 1 μM BA or DHZ (Fig. 1; Van der Krieken et al. 1990). The effect of exposure time to the hormone on the number of buds regenerated was determined by transferring explants after different culture periods on inductive medium to medium without cytokinin (Fig. 1). The most striking difference was observed when the cytokinins were present for 2–4 days. After a pulse of 3 days, for example, there was almost no effect of DHZ, whereas an incubation of the same length on BA led to 70% of the maximal bud number. The number of buds did not increase after 4 days on BA or 7 days on DHZ.

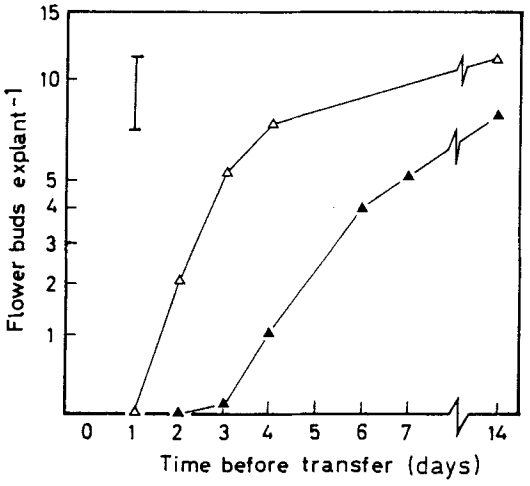

Fig. 1. Time during which the presence of DHZ and BA in the culture medium is required to induce bud formation. Explants were first cultured on media containing 1 μM BA or DHZ. At the times indicated, explants were shifted to media without DHZ (▲——▲), or without BA (△——△). Vertical bar: LSD at p = 0.05.

DHZ Uptake and Conjugation

The uptake rate of DHZ was greater than the corresponding BA uptake at all medium concentrations tested ($p < 0.05$) (Fig. 2). The metabolites formed from DHZ were tentatively identified by cochromatographing a labeled extract of the explants with a mixture of unlabeled metabolite standards (Table 1). Radioactive spots were found at the positions of DHZ, 7G-DHZ, 9R-DHZ, and the nucleotides. Only few counts cochromatographed with the O-glucoside of DHZ and with 9G-DHZ. In the following experiments, only DHZ and the three most abundant metabolites were quantified. One-dimensional TLC proved to be sufficient.

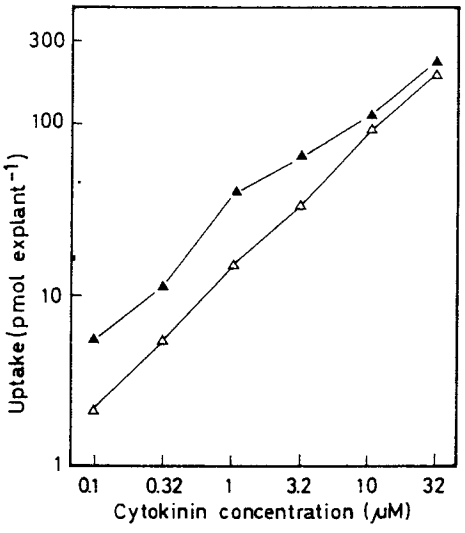

Fig. 2. Uptake of DHZ (▲——▲) and BA (△——△) in the first 24 h of culture in relation to their concentrations in the medium.

Table 1. Metabolites formed from DHZ after a 24-h incubation on Murashige and Skoog medium with 2.2 μM [^{3}H]DHZ (6.7 kBq ml^{-1}).

	Radioactivity in derivatives (%)	
Compound	DHZ	BA
Free base	10	8
9-Riboside	12	2
Nucleotides	13	8
7-Glucoside	61	83
9-Glucoside	3	—
O-Glucoside	1	—
Total	100	101

An extract of 10 explants was cochromatographed by two-dimensional TLC with standards of DHZ metabolites. After the standards had been visualized under ultraviolet light, the spots were scraped off and the radioactivity was determined. Data on BA conjugation obtained from a similar experiment (Van der Krieken et al. 1988) are given for reference.

Accumulation and Metabolism of DHZ During Flower Bud Initiation

Uptake and metabolism of DHZ were determined during the 7-day period of bud initiation (Fig. 3). The total amount of DHZ accumulated increased almost linearly with time (Fig. 3A). Only a small fraction was recovered in DHZ from the first day on, which indicates that conjugation is a fast process. The concentrations of most compounds when expressed on a fresh weight basis (Fig. 3B) peaked at 3 days after the start of incubation and declined thereafter as a result of the increase in fresh weight of the explants. The concentration of DHZ itself remained at a level of 1–2 μM throughout the 7 days of culture.

Changes in DHZ Uptake and Metabolism During Culture

The rate of uptake of DHZ and the proportions in which the various metabolites were formed changed during culture. This was inferred from an experiment in which we placed explants on radiolabeled DHZ either at the onset of culture or after a 3-day preincubation on medium with 1 μM DHZ. Whereas during the first day less nucleotide than 7G-DHZ was synthesized, the reverse was true at day 4 (Table 2). Indications for an extensive leakage was also found. The amount of DHZ taken up during the fourth day of culture (Table 2) is much greater than the amount of DHZ accumulated between the third and fifth day (Fig. 3A).

Discussion

Pedicel explants of tobacco regenerate the same number of flower buds in response to equal concentrations of BA or DHZ in the medium. However, BA proves to be much more active than DHZ when the comparison is made on basis of the internal hormone concentration and the minimum exposure time required.

DHZ is taken up two to three times faster than BA at the optimal medium concentration of 1 μM (Fig. 2). Approximately 90% of both cytokinins is converted into conjugates (Table 1). Uptake and conjugation thus lead to an internal concentration which is two- to threefold higher for DHZ than for BA. These levels are fairly stable throughout the initiation period (Fig. 3B; Van der Krieken et al., submitted for publication).

The reduced number of buds formed when the cytokinin is withdrawn from the medium during the induction period, suggests that a continuous influx is required to maintain a physiologically active hormone level in the tissue. Once the influx is interrupted, this level drops due to continuing conjugation and leakage from the tissue. The occurrence of leakage was inferred from the observation that DHZ uptake during the fourth day of culture surpassed the increase in the accumulated dose (Fig. 3A; Table 2). For BA, the concentration of the free base has been found to decrease by a factor of 50 during the first 10 h after removal of the hormone (Van der Krieken et al., submitted for publication). A high permeability of the membrane to the free

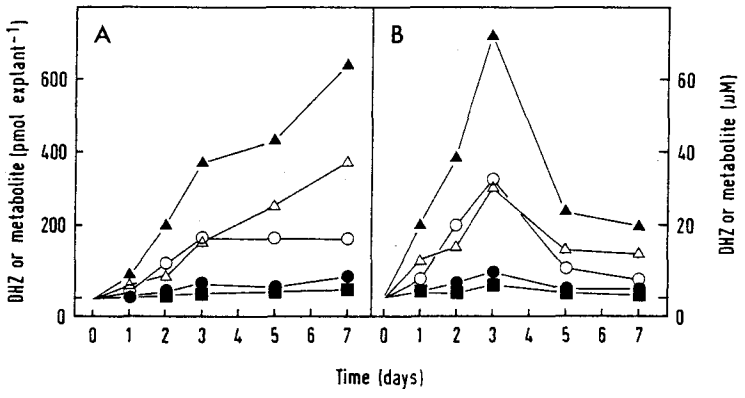

Fig. 3. Uptake and metabolism of DHZ during continuous incubation. Explants were incubated on Murashige and Skoog medium with 2.2 μM DHZ and 1.7 kBq ml^{-1} [^{3}H]DHZ. Uptake and levels of the various compounds are expressed in absolute amounts (**A**) and on a fresh weight basis (**B**). ▲——▲, uptake; △——△, 7G-DHZ; ○——○, nucleotides; ■——■, DHZ; ●——●, 9R-DHZ. Twenty explants were used per treatment.

Table 2. Changes in DHZ uptake and metabolism during culture: Uptake and conversion of DHZ were measured in explants cultured for 24 h on medium with 2.2 μM [^{3}H]DHZ (3.3 kBq ml^{-1}) after preincubation for different times on the same medium without the radiolabeled DHZ.

	Preincubation time (days)	
Compound	0	3
DHZ	7 (13)	13 (3)
9R-DHZ	7 (12)	70 (18)
Nucleotides	13 (25)	220 (57)
7G-DHZ	27 (50)	82 (21)
Total uptake	54 (100)	385 (99)

Values are expressed in pmole explant^{-1} and are means of duplicate experiments. Percentages of total uptake are given in parentheses.

cytokinin bases has been reported for cultured tobacco cells (Laloue and Pethe 1982).

Maximum bud formation thus requires DHZ to be present at a higher internal concentration for a longer period of time as compared to BA. There is no easy explanation for the difference in response of the tissue to the two cytokinins. One possibility is that DHZ is more efficiently ribosylphosphorylated than BA by the cells in contact with the medium. This might lead to a steep DHZ gradient over the medium–explant interface and, by consequence, to a relatively low DHZ concentration near the upper cell layers from which the buds arise. Such a fast ribosylphosphorylation of DHZ would also account for the difference in the uptake rate between DHZ and BA. Alternatively, the concentration of DHZ in the vicinity of the hypothetical receptor might be locally lowered by side-chain cleavage to a degree that is not reflected in the bulk DHZ level. A third possibility is that the hypothetical cytokinin receptor is less sensitive to DHZ than to BA. Since the DHZ concentration is stable from the first day on (Fig. 3B), this alternative would not explain the differences in the length of the initiation period for BA and DHZ.

The existence of a well-defined initiation period (Fig. 1), in combination with the data on leakage and conjugation, suggests that the exogenously supplied cytokinin only functions during the induction phase of bud development. The accumulation of endogenously synthesized DHZ in explants cultured in the absence of a cytokinin (Van der Krieken et al. 1991) provides circumstantial evidence for this conclusion.

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