Influence of cytokinin on morphogenetic competence in non-blooming and blooming *Nicotiana* thin layer and leaf tissue cultures

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Phytohormonal control of the transition to *de novo* morphogenesis was demonstrated in cultures of thin layer tissues obtained from stem and leaf discs of non-blooming *Nicotiana tabacum* L. and from flower stalk tissues of blooming *Nicotiana alata* L. Neither organs nor somatic embryos occurred when explants were grown on a medium supplemented with 1 μM auxin (IAA) in the absence of cytokinin (BA). Only vegetative buds formed in non-flowering tobacco stem thin layer tissues irrespective of auxin-to-cytokinin ratio. BA used in four doses (1, 2.5, 5, and 10 μM) with the amount of the auxin constant, appeared to be an important effector of both organogenesis and somatic embryogenesis. Vegetative organogenesis in stem thin layer tissues of non-blooming plants was most significant at 2.5 and 5 μM BA. However, inhibition of the vegetative and promotion of the generative development were observed under the same conditions in flower stalk thin layer tissues of blooming *Nicotiana*. The realisation of the flowering stimulus in isolated flower stalk tissues was considerably dependent on BA in the range from 1 to 5 μM. An increase of BA up to 10 μM greatly stimulated the vegetative development and inhibited the formation of reproductive organs. The elimination of IAA from the media of leaf explant cultivation revealed the effectiveness of BA in direct somatic embryogenesis. The data confirmed the fact that both vegetative organ neoformation or reproductive development significantly depend on the amount of BA when the floral stimulus in inflorescence tissues is realised and somatic embryogenesis is performed only at certain BA concentrations.

Key words: cytokinin, flower, vegetative buds, somatic embryos, thin layer tissues
Abbreviations: CK = cytokinin, BA = 6-benzylaminopurine, IAA = indole-3-acetic acid, IBA = indole-3-butyric acid, NAA = α-naphthaleneacetic acid, MS = Murashige and Skoog's salts

INTRODUCTION

The complexity of phytohormonal control is revealed by interactions among different classes of phytohormones in the regulation of different developmental and physiological processes. It is becoming increasingly evident that these interactions also include changes in one phytohormone level caused by another [1]. Natural CKs are the N^6-adenines and their riboside, ribotide and glycoside conjugates. BA is one of the most frequently used exogenous CKs in *in vitro* research. The pathways of organogenesis and somatic embryogenesis in tissue cultures are under evident phytohormonal control. The ability of CKs to realise the apical dominance is widely applied in micropropagation. Recent researches have shown that morphogenetic control performed by CKs can depend on the genotype, physiological features of the tissue, cultivation conditions, other plant growth regulators, etc. [2]. Despite considerable data on CK effects, the role of CKs in such processes of generative development as flowering or embryogenesis has not yet been proved and is still under discussion [3, 4]. The flowering is controlled by the leaves that produce physiological promoters and inhibitors [5, 6]. There is no doubt that when the floral signal is realised in the flowering plant, a physiological response concerning flowering can be defined in different organs. Since tissues of flower stalks are on the pathway of the transference of the floral stimulus, the vegetative growth or flower formation can be induced only under certain experimental conditions. Therefore, taking into consideration the assumption that isolated thin layer tissues of the flower stalk have the ability to realise the floral stimulus,

the influence of CK on the formation of flowers and vegetative buds in this model system was investigated. The ratio of generative and vegetative buds was tested in non-blooming and blooming *Nicotiana* tissue cultures, and morphogenetic competence of the applied CK on direct somatic embryogenesis in *Nicotiana* leaf tissues was analysed.

MATERIALS AND METHODS

The experiments were carried out with thin layer and leaf tissues of non-blooming and blooming plants of tobacco (*Nicotiana tabacum* L. and *Nicotiana alata* L.). *Nicotiana tabacum* L. plants were grown under sterile conditions. Intact *Nicotiana alata* plants were cultivated in a greenhouse. All plants grew under a 16-h light photoperiod. Flower stalk, stem and leaf tissue cultures were used in this research. Thin cell layer tissues (7 x 2 mm in size) composed of epidermis and 3–6 subepidermal cell layers were excised from flower stalks or from non-blooming plant stems (2nd and 4th internodes). The initial biomass of the used thin layer tissues explant was 14 ± 3 mg in weight. Leaf disc explants of equal size were taken from the leaf of the 3rd or 4th upper internodes. The beginning of evocation was determined by histological analysis. The average number of structures and their weight were estimated after 30 days of cultivation. The medium of tissue cultivation contained MS mineral salts [7], 3% sucrose, and 0.8% bactoagar. MS was supplemented with 100 mg/l myoinositol and 0.1 mg/l thiamine·HCl. pH was adjusted to 5.7 ± 0.1 before autoclaving. Phytohormones (auxin IAA and citokinin BA) were added to the medium after autoclavation. The used IAA concentration was constant (1 µM), while that of BA was as follows: 0, 1, 2.5, 5, and 10 µM. In the cases when the media were supplemented with 1 µM IAA, the ratios of auxin and CK in the media were 1/0, 1/1, 1/5, and 1/10. The effect of 1 µM auxin without BA was tested, too. Each experiment was carried out twice and at least 10 explants were used per treatment. The tissue cultures were culivated under 16 h/d light (40 µM $m^{-2}s^{-1}$ from cool white fluorescent tubes) at 25 °C.

RESULTS AND DISCUSSION

It is generally accepted that *in vitro* morphogenesis is controlled by the dynamics of both exogenous and endogenous phytohormones [2]. Using blooming and non-blooming plant tissues, the influence of BA was revealed and different development pathways were determined. The course of the appearance of flower and vegetative buds or shoots in thin layer tissue cultures obtained from flower stalks or stems of *Nicotiana alata* L. under the influence of BA was shown (Fig. 1). Auxin used without CK was unable to induce differentiation in either of applied tissues. However, BA with auxin or alone acted as an effective inductor of organogenesis, and direct somatic embryogenesis initiated in leaf disc tissues (Fig. 2). Only vegetative buds in non-flowering tobacco stem thin layer tissues appeared irrespective of BA concentrations. The number of organs formed per ex-

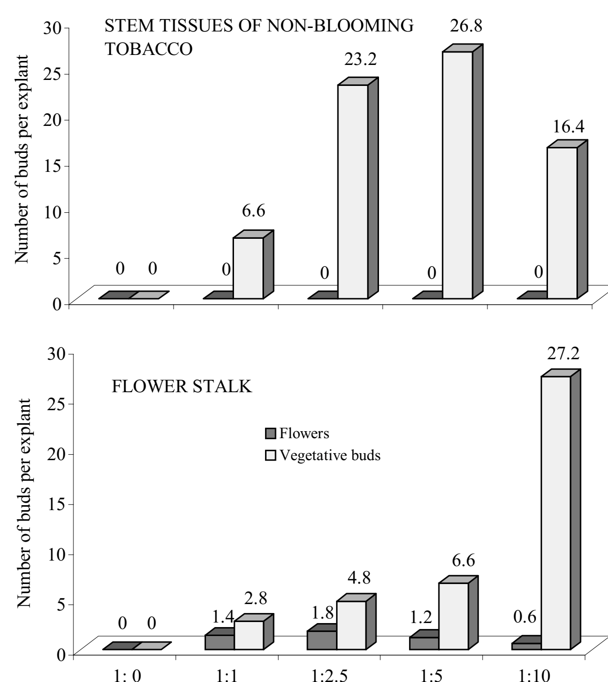

Fig. 1. The number of vegetative and floral buds formed in thin layer tissues of flower stalks under the influence of BA. Cytokinin doses were used with constant (1 µM) IAA amount

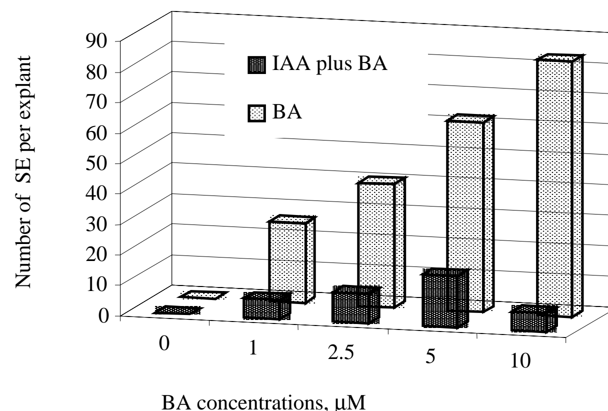

Fig. 2. Influence of IAA/BA and BA on direct somatic embryogenesis in leaf tissues of *Nicotiana*

plant depended on the amount of BA (the number of vegetative buds per explant was highest at 2.5 and 5 μM BA and was 23.20 ± 2.74, 26.8 ± ± 3.44 buds/explant, respectively). A different situation was revealed in flower stalk thin layer tissue cultures taken from blooming *Nicotiana alata* L. plants. In this context, it is important to reveal some factors that can determine flowering. At first, the commitment to flowering is controlled by the leaves that produce promoters and inhibitors of flowering when exposed to favourable and unfavourable conditions [5, 6]. At this stage, the apical meristem of the shoot makes transition to reproductive development and produces flowers. Undoubtedly, *in vitro* organogenesis is controlled by both exogenous and endogenous CKs [2]. The used flower stalk explant perceives exogenous CK and becomes committed to flower and shoot neoformation. Thus, the realisation of the flowering stimulus in isolated flower stalk thin layer tissues of *Nicotiana* was significantly dependent on BA in the range from 1 to 5 μM. An increase of BA up to 10 μM caused a considerable stimulation of the vegetative development, at the same time inhibiting the formation of reproductive organs. The investigated flower stalk tissues formed 33.33, 27.27, 15.38, and 2.16% flowers under 1/1, 1/2.5, 1/5, and 1/10 influence of IAA/BA, respectively. BA, used in the doses up to 5 μM, showed no effect on the growth of vegetative structures (the average biomass of a vegetative bud was in the range from 19.65 ± 2.56 to 17.53 ± 0.39 in the used BA). However, both the number of flowers and their biomass significantly decreased under the influence of BA (10 μM). According to previous reports, CKs determine the number of flowers in explants, while auxin acts as a modifier of CK action that depends on the stage of development of the plant it was taken from [8]. The obtained results support the idea suggested while studying intact plants that CK is one of the components of the floral stimulus [5]. The experiments performed by bark ringing of plants *Sinapis alba* L. indicated that the inhibition of the floral response due to bark ringing was reversed by the application of BA at a concentration of 5 μM [4]. According to this report, BA showed an inhibitory effect on the floral response at 1 mM. However, it cannot be excluded that the signal controlling flowering is not CK but some unknown signal which stimulates the accumulation of CKs in the apex [9]. Thus, the experiments performed with flower stalk tissues *in vitro* imply that in certain concentrations BA is involved in the control of flowering.

BA was an initiator of direct somatic embryogenesis in investigated tobacco leaf explants (Fig. 2). In the case when explants were uninterruptedly (30 days) cultivated on MS with 1 μM IAA plus BA, the number of induced SE significantly decreased in comparison with cultivation under the influence of BA alone. One of the possible interpretations of the presented results can be related to the data that showed the importance of the elimination of the phytohormonal stimulus after determination of the processes of somatic embyogenesis [10]. It can also be connected with degradation of IAA in prolonged cultivation and a negative influence of derived compounds on regeneration processes. As reported by Kamínek et al. [1], the level of CK in plant cells depends on such factors as CK biosynthesis and/or metabolic interconversions, inactivation and degradation. Auxin can affect the biosynthesis of CK and/or promote its degradation. On the other hand, experiments *in vitro* performed with physiological analogues of IAA showed that IAA was a less effective SE inductor in comparison with IBA and NAA [10]. Thus, the elimination of IAA from the media of cultivation revealed the effect of BA on SE formation. SE frequence and growth intensity were in reverse correllation (the average biomass of SE was 48.19 ± 8.35, 30.15 ± 2.25, 22.58 ± 2.88, and 15.67 ± 2.29 mg under the influence of BA in 1, 2.5, 5, and 10 μM, respectively). So, the data presented demonstrate the significance of the amount of BA in determining the vegetative organ neoformation and reproductive development when the realisation of the floral stimulus in inflorescence tissues of an intact flowering plant can be fulfilled and organogenesis *de novo* can be performed only at certain BA concentrations. Also, these observations demonstrate different commitment of the used tissues in performing particular morphogenetic programs.

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CITOKININO REIKŠMĖ NEŽYDINČIŲ IR ŽYDINČIŲ *NICOTIANA* PLONASLUOKSNIŲ AUDINIŲ IR LAPO KULTŪRŲ MORFOGENETINĖMS SAVYBĖMS

S a n t r a u k a

Fitohormonų reikšmė morfogenezei *de novo* tirta nežydinčio *Nicotiana tabacum* L. stiebo plonasluoksnėse audinių kultūrose ir lapo diskuose bei *Nicotiana alata* L. žiedkočio audiniuose. Nei organai, nei somatiniai embrioidai nesivystė ant terpės su 1 μM auksino (IAR) be citokinino. Tik vegetatyviniai pumpurai formavosi nežydinčio tabako stiebo audiniuose nepriklausomai nuo naudotų fitohormonų (IAR/BAP) santykio. Eksperimentuose naudotos keturios BAP koncentracijos (1, 2,5, 5 ir 10 μM). Auksino kiekis terpėse buvo pastovus (1 μM). Vegetatyvinių pumpurų skaičius didėjo nežydinčių augalų stiebo audiniuose, kai BAP buvo 2,5 ir 5 μM. Analogiški BAP kiekiai *Nicotiana alata* L. žiedkočio kultūrose slopino vegetatyvinį ir skatino generatyvinį vystymąsi. 1–5 μM BAP stimuliavo žydėjimą žiedkočio kultūrose, o 10 μM BAP intensyvino vegetatyvinį vystymąsi ir slopino generatyvinių organų formavimąsi. Be IAR, BAP labiau skatino somatinę embriogenezę tabako lapų audiniuose. Nustatyta vegetatyvinių organų genezės ir reprodukcinio vystymosi priklausomybė nuo BAP kiekio, kai žydėjimo signalas žiedkočio audiniuose ir somatinė embriogenezė lapo audinių kultūrose realizavosi esant apibrėžtai BAP koncentracijai.