Influence of DB-cAMP, Adenosine, Forskolin, and Traumatic Acid on in Vitro Bulbing of *Lilium longiflorum*

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Abstract. Both dibutyryl-cAMP and forskolin, with and without a pretreatment with traumatic acid, stimulated bulbing in vitro of *Lilium longiflorum* Thunb. by up to 65% over the controls. Dibutyryl-cAMP, forskolin, their combinations with traumatic acid, and adenosine significantly increased the micropropagation yield. Traumatic acid alone produced similar results on bulblet number per explant, but with increased fresh mass per bulblet. Chemical names used: 9- β -D-ribofuranosyladenine (adenosine); adenosine 3', 5'-cyclic monophosphate (cAMP); dibutyryl adenosine 3', 5'-cyclic monophosphate (DB-cAMP); 9- β -L (+) adenosine or 9- β -L (+) ribofuranosyladenine; forskolin; 10(E) dodeca-1,12-dicarboxylic acid (traumatic acid).

Cyclic AMP (cAMP) can act as a second messenger mediating the intracellular transfer of several environmental signals that trigger physiological processes (Martin, 1983). Brown and Newton (1981) reviewed several studies that reported the existence of cAMP in plants, and aroused expectations about its putative role in physiological processes. Evidence of the existence of the adenylate cyclase system and of phosphodiesterase in plant cells regulating the endogenous content of cAMP appeared later (Brown and Newton, 1992; Curvetto et al., 1994; Curvetto and Delmastro, 1990; Darjania et al., 1994; Morsucci et al., 1991, 1992). Tanimoto and Ishioka (1991, 1992) reported increased bulblet differentiation in apical explants of *Lilium longiflorum* after adding cAMP and calcium ionophore A 23187 to the culture medium. Similar effects were found on addition of forskolin, a diterpene extracted from the roots of *Coleus forskohlii* Brig. (Seamon and Daly, 1981) and known to affect different physiological processes through its interaction with the adenylate cyclase system in animals (Hoshi et al., 1988; Metzger and Lindner, 1981; Seamon et al., 1981) and plants (Curvetto and Delmastro, 1990). Tezuka et al. (1993) reported a stimulatory effect of cAMP and forskolin on the development of the self-incompatible pollen tubes of *Lilium*. Tanimoto and Harada (1984) found an increase in bud proliferation in stem explants of *Torenia fournieri* Lindl. after applying cAMP to the culture medium. In tobacco calli, externally applied cAMP and guanosine 3',5'-cyclic monophosphate promoted growth and organogenesis (Mangat and Janjua, 1987). The

effects on sections of lily scales cultured in vitro of DB-cAMP and some effectors of the adenylate cyclase system, such as adenosine (Ade) and 9- β -L (+) adenosine [L (+) Ade], have not been studied.

Traumatic acid (TA), isolated from the mesocarp of wounded beans, reportedly plays a role in enhancing cell proliferation in wounded plant tissue (English et al., 1939).

The objective of our study was to obtain evidence of the influence of DB-cAMP and effectors of the adenylate cyclase system (forskolin, Ade and L (+) Ade) on in vitro bulblet differentiation in explants from *Lilium* scales. The goal was to identify the concentrations required for each substance, alone or in combination with TA, which maximized bulblet differentiation.

Materials and Methods

The basic in vitro culture methods used were described by Stimart and Ascher (1978). Scales from bulbs of *L. longiflorum* Thunb. cv. Snow Queen, 14–16 cm in diameter, that had been stored at 2 °C for at least 6 weeks, were removed and washed with tap water. They were disinfected by immersion in 70% ethanol for 30 s followed by immersion in a sodium hypochloride solution (1.6% active chlorine) for 20 min. After three rinses in sterile water, the scales were cut into 3-mm sections. These explants were randomly distributed, maintaining the polarity, in 50 tubes per treatment. Basal medium was MS (Murashige and Skoog, 1962) containing 100 mg·L⁻¹ inositol, 0.03 mg·L⁻¹ naphthalene acetic acid, 0.4 mg·L⁻¹ thiamine, 30 g·L⁻¹ sucrose, and 8 g·L⁻¹ agar, pH 5.7.

Experiment 1. DB-cAMP, Ade (Sigma Chemical Co., St. Louis) and L (+) Ade (Moravsek Biochemicals, Brea, Calif.) were filter-sterilized and separately added to the basal medium using 0.05, 0.5, 5, and 50 mg·L⁻¹ DB-cAMP or 0.1, 1, 10, and 100 μ g·L⁻¹ Ade and L (+) Ade. The tubes were kept in darkness for

8 weeks at 25 $\pm$ 2 °C. Subsequently, bulblets differentiated at the abaxial basal part of the explant were counted.

Experiment 2. An experiment with DB-cAMP, forskolin, and TA was carried out using bulbs of the same cultivar stored at 2 °C, but from a different commercial source. These bulbs were 16–18 cm in diameter. Forskolin and TA were filter-sterilized. The following eight treatments were applied: control (basal medium only), 5 mg·L⁻¹ DB-cAMP, 0.4 mg·L⁻¹ forskolin, and DB-cAMP plus forskolin, each with and without preincubation with TA. This preincubation consisted of 1 h treatment of explants with 0.25 mg·L⁻¹ TA, with the corresponding control explants in distilled water. Cultures were kept in darkness at 25 $\pm$ 2 °C for 8 weeks before the differentiated bulblets were counted and weighed.

Statistical analysis. A completely randomized design was used for both experiments. One-way ANOVA was used for Expt. 1, a two-way ANOVA was used for the second experiment; the Fisher's protected LSD test at 5% was used for bulblet yield and biomass comparisons.

Results and Discussion

Experiment 1. Addition of DB-cAMP or Ade to the culture medium markedly promoted bulbing over a wide range of concentrations, i.e., 0.05 to 50 mg·L⁻¹ for DB-cAMP and 0.1 to 100 μ g·L⁻¹ for Ade, with maximum response at 5 mg·L⁻¹ DB-cAMP and 10 μ g·L⁻¹ Ade (Fig. 1). In contrast, increasing L (+) Ade concentrations from 0.1 to 100 μ g·L⁻¹ reduced bulbing (Fig. 1).

Adenosine has two opposite effects on adenylate cyclase activity; at low concentrations it activates the enzyme, at higher concentrations it inhibits activity (Katada and Ui, 1981; Morsucci et al., 1991; 1992). Our results suggest that Ade, within the range used, can produce an activation of adenylate cyclase, which in turn increases the intracellular cAMP level and stimulates bulblet formation.

The stereoisomer L (+) Ade is a natural compound in plants. When applied at very low concentrations (picomolar range) it can induce rapid physiological responses (Curvetto and Darjania, 1995) and stimulate growth (Ries et al., 1990). Dry mass of rice plantlets increased following in vivo spray application of L (+) Ade concentrations up to 1 μ g·L⁻¹, but higher concentrations decreased dry mass (Ries et al., 1990).

Experiment 2. Pretreatment of explants with TA increased bulblet formation, regardless of subsequent treatment (Table 1). All subsequent treatments also increased bulblet formation, but the effects were significant only in scale sections not pretreated with TA.

Fresh mass per bulblet was also significantly increased by pretreatment with TA in two of the four comparisons. In contrast, DB-cAMP and forskolin reduced mass per bulblet, although their individual effects were non-significant in the presence of TA.

The influence of TA on various in vitro cultured tissues has been reported. In vitro

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formation of adventitious buds in *Brassica oleracea* L. var. *botrytis* (Margara, 1977), in *Torenia fournieri* Lindl. (Tanimoto and Harada, 1984), and in *L. longiflorum* Thunb. (Ishioka and Tanimoto, 1990) was stimulated by TA. Marinangeli et al. (1995) also reported in vitro stimulation of bulblet differentiation by TA in bulb-scale segments of the Asiatic hybrid *Lilium* 'Connecticut King'. These previous results provide indirect evidence of the involvement of TA in inducing adventitious organs by influencing cellular division and differentiation (Ishioka and Tanimoto, 1990; Marinangeli et al., 1995).

Our results suggest that either TA or cAMP, both reported as intracellular messengers (Brown and Newton, 1992; Farmer, 1994), affect bulb differentiation in *L. longiflorum*. Supplementing the culture medium with (1) DB-cAMP and Ade, and (2) DB-cAMP and forskolin and their combinations with TA, significantly increased *Lilium* micropropagation yield. When used alone TA increased both bulblet number and fresh mass. We conclude that the use of TA alone or in combination with cAMP and/or activators of the cAMP synthesis system, may contribute to improved in vitro propagation of *L. longiflorum*.

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Fig. 1. Effects of DB-cAMP, Ade and L (+) Ade on bulblet formation on bulb scale sections of *Lilium longiflorum* Thunb. in vitro.

Table 1. Effects of DB-cAMP (5 mg·L⁻¹), forskolin, FK, (0.4 mg·L⁻¹) and their combination, with and without one hour pretreatment with traumatic acid (TA) (0.25 mg·L⁻¹) on mean number of bulblets differentiated per explant and mean fresh mass per bulblet of *Lilium longiflorum* Thunb.

Pretreatment	Treatment	Bulbets per explant	Fresh mass per bulblet (mg)
None	Control	2.0 c ^z	107 a
	DB-cAMP	2.6 b	85 b
	Forskolin	2.5 b	91 b
	DB-cAMP + FK	3.2 a	82 b
TA	Control	2.9 a	124 a
	DB-cAMP	3.3 a	102 a
	Forskolin	3.4 a	107 a
	DB-cAMP + FK	3.4 a	87 b
F significance			
TA pretreatment		**	*
Treatments		*	*
TA × treatments		NS	NS

^zMean separation within columns by LSD, *P* < 0.05.

NS, *, **Nonsignificant or significant at *P* ≤ 0.05 or 0.01, respectively.

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