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EFFECT OF PHYTOHORMONES ON PLANT REGENERATION FROM CALLUS OF *GLADIOLUS* CULTIVAR "JENNY LEE"

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Summary

Callus capable of plant regeneration was initiated at a higher frequency from the basal leaves of in vitro plants (70% explants) as compared to cormel slices (30% explants) when cultured on medium containing various concentrations of auxin. The greatest number of plants were regenerated from 4-mo.-old callus (112 plants/g fresh weight callus) cultured on medium containing 10 mg/liter (53.8 μM) 1-napthaleneacetic acid. The addition of 2 mg/liter (9.3 μM) kinetin to a Murashige and Skoog's basal salts regeneration medium resulted in an average two- to three-fold increase in the number of plants regenerated compared to regeneration on medium without hormones. Ten months after callus initiation, all callus maintained on auxin-supplemented media showed a drastic reduction in its capacity to regenerate plants. Ten-month-old callus maintained on dicamba regenerated the greatest number of plants (14 to 23 plants regenerated per gram fresh weight callus) as compared to callus maintained 10 mo. on medium containing 1-napthaleneacetic acid or 2,4-dichlorophenoxyacetic acid. Cormel slices cultured on cytokinin-supplemented media formed small amounts of callus which regenerated up to 19 plants per cormel slice within 1 to 2 mo. after the cormel slice had been placed on either 10 mg/liter (49.2 μM) N^6-2-isopentenyladenosine or 1 mg/liter (4.4 μM) 6-benzylaminopurine.

Key words: Gladiolus; plant regeneration; callus; monocots; bulb crops.

Introduction

Monocots have been more difficult to regenerate in vitro than dicots. Most of the tissue culture research on monocots has involved the agronomically important cereals such as corn, wheat, barley, rice, and sorghum. Generally the potent auxin 2,4-dichlorophenoxyacetic acid (2,4-D) has been used to induce embryonic callus in the cereals, and its removal has resulted in subsequent differentiation of the callus into plants (Bhaskaran and Smith, 1990). For wheat, plant regeneration was enhanced when the auxin 3,6-dichloro-o-anisic acid (dicamba) rather than 2,4-D was used (Pappenfuss and Carman, 1987), and dicamba is routinely used for maintaining embryogenic callus of orchardgrass (Hanning and Conger, 1982; Gray et al., 1984). Variable results have been reported for the role of cytokinins in enhancing plant regeneration from embryogenic callus of the cereals (Bhaskaran and Smith, 1990).

The hormonal requirements for callus initiation and subsequent plant regeneration from the monocotyledonous bulb crops in the floral industry are largely unknown. The research done so far indicated that there was a wide difference in the hormonal requirements. The initiation of lily (*Lilium*) callus required auxin; however, once the callus was established hormones were not required for its continued proliferation (Sheridan, 1968 and 1974; Simmonds and Cumming, 1976; Stimart et al., 1980). Plant regeneration occurred on a medium supplemented with 1-napthaleneacetic acid (NAA) (Simmonds and Cumming, 1976). Embryogenic callus of daylily, *Hemerocallis*, was initiated from flower buds cultured on a medium supplemented with either hormonal combination: kinetin, 6-benzylaminopurine (BAP), NAA, or BAP and 2,4-D (Krikorian and Kann, 1981). Cell suspensions of *Hemerocallis* initiated from this callus regenerated plants after the removal of 2,4-D from the medium.

The callus of *Crocus sativus* was induced by culturing bulbs on a medium supplemented with 2,4-D and zeatin, and plants were regenerated after the callus was cultured on a medium containing NAA and BAP (Isa and Ogasawara, 1988). Embryogenic callus of *Iris* was initiated from zygotic embryos placed on a medium supplemented with 2,4-D and kinetin, and plants were regenerated when the callus was transferred to medium containing gibberellic acid (GA_3) and BAP (Reuther, 1977; Radojevic et al., 1985; Radojevic and Subotic, 1992). As with the monocotyledonous cereals, the flower bulbs studied so far, *Hemerocallis, Crocus, Iris*, formed callus capable of plant regeneration when tissue explants were placed on MS medium supplemented with 2,4-D. The exceptions were *Lilium* species which required only the weak auxin, indole-3-acetic acid, for callus induction and no auxin for callus maintenance. Dicamba has not yet been tested on flower bulb crops for initiation of regenerable callus. As with the cereals, the role of cytokinins in plant regeneration and callus induction is unclear.

Plant regeneration occurred from callus of *Gladiolus* derived from either inflorescence stalks (Ziv et al., 1970), cormel stem tips (Simonsen and Hildebrandt, 1971), or suspension cells (Kamo et al., 1990). Inflorescence stalks cultured on medium supplemented with 10 mg/liter NAA and 0.5 mg/liter kinetin formed a thin callus layer followed quickly by differentiation into buds and cormels (Ziv

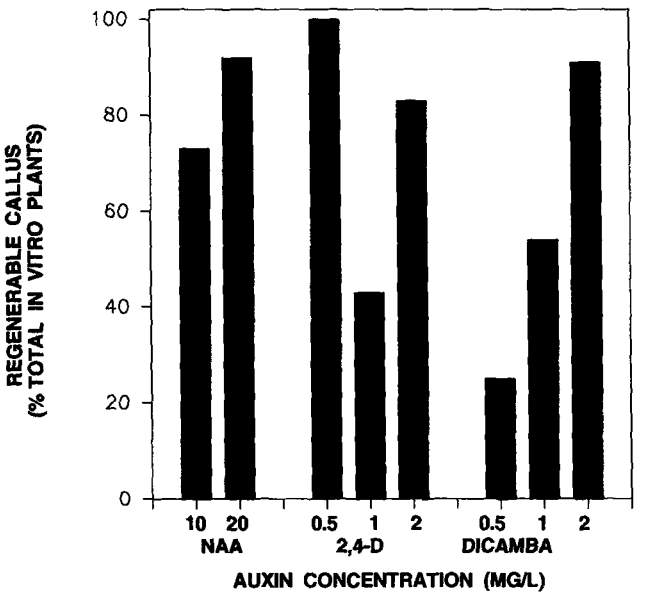

FIG. 1. Effect of auxin on the initiation of regenerable callus from in vitro plants of *Gladiolus* "Jenny Lee." There were 12 explants of in vitro plants, each explant consisting of 6 stems, used for callus initiation on each auxin concentration.

et al., 1970). Callus was initiated from the main bud of a cormel when cultured on medium supplemented with 5 mg/liter kinetin, and the callus was maintained on medium containing 0.5 mg/liter

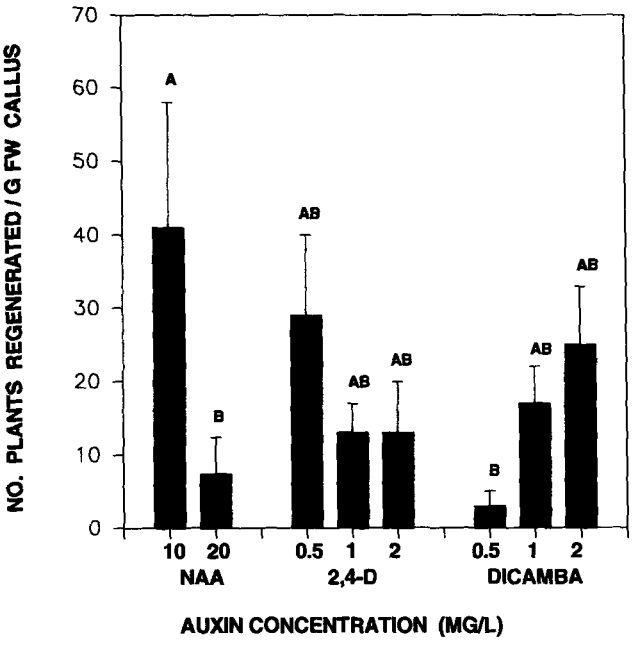

FIG. 2. Plant regeneration from callus that was initiated from in vitro plants and maintained 4 mo. on MS basal salts medium supplemented with NAA, 2,4-D, or dicamba. Twelve calli were grown on each auxin for regeneration. *Standard error bars* shown. Means with different letters are significantly different at $P \leq 0.05$ according to Duncan's new multiple range test (Steel and Torrie, 1980).

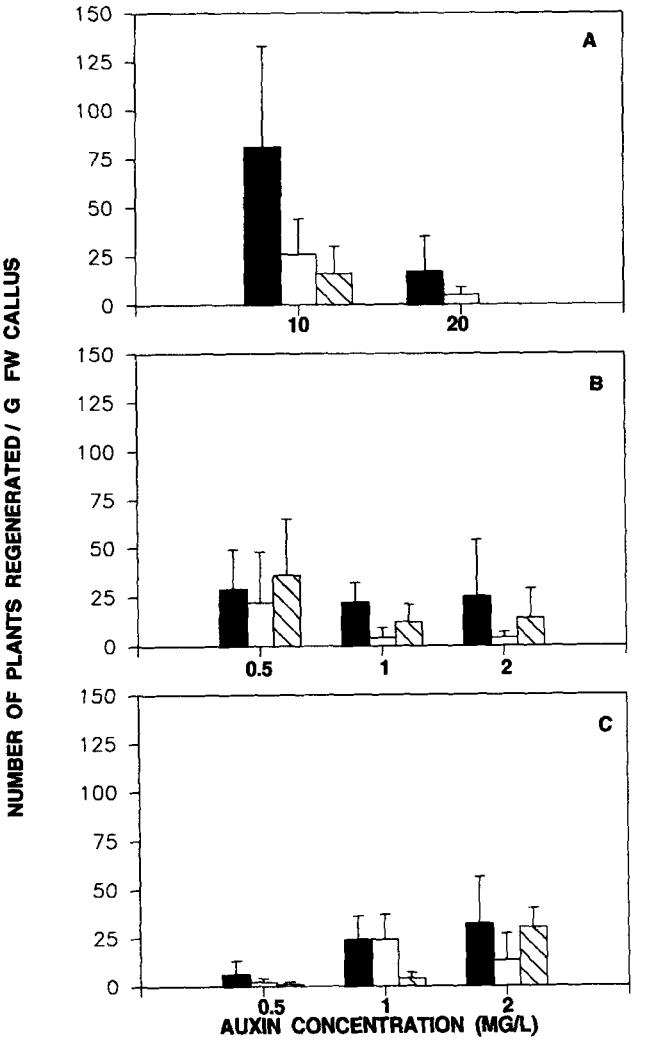

FIG. 3. Plant regeneration from callus that was derived from in vitro plants. Callus was initiated and maintained 4 mo. on MS basal salts medium supplemented with either *A*, NAA, *B*, 2,4-D, or *C*, dicamba before regeneration. For regeneration, callus was placed on MS basal salts medium with either 2 mg/liter (9.3 μM) kinetin for 3 mo. (*solid bar*), 10 mg/liter (46.5 μM) kinetin for 7 days followed by a transfer to hormone-free medium for 3 months (*open bar*), or no hormones for 3 mo. (*hatched bar*). Four callus samples were grown on each auxin and used for each regeneration treatment. *Standard error bars* shown.

kinetin and 0.1 mg/liter NAA (Simonsen and Hildebrandt, 1971). Although these two studies used kinetin and NAA, *Gladiolus* callus and suspension cells capable of plant regeneration have been initiated on 2,4-D (Kamo et al., 1990).

The purpose of this study was to optimize the frequency of plant regeneration from callus of the *Gladiolus* cultivar "Jenny Lee". The effect on plant regeneration of various auxins, cytokinins, explants, and time in culture was compared. Regenerable callus would provide a source of cells for creating transgenic plants using the particle gun. The number of transgenic plants recovered would depend greatly on the regeneration capacity of the callus. A well-characterized system of plant regeneration would also be useful in developmental studies.

TABLE 1

EFFECT OF MEDIUM CONTAINING VARIOUS AUXINS ON 10-MO.-OLD CALLUS INITIATED FROM IN VITRO PLANTS AND SUBSEQUENT PLANT REGENERATION ON MEDIUM CONTAINING KINETIN

	No. Plants Regenerated/gFW Callus Regeneration Media—Kinetin, mg/liter		
Auxin, mg/liter	0	2	10
NAA 10 (53.8 μM)	0	0	0
NAA 20 (107.6 μM)	0	0	0
2,4-D 0.5 (2.2 μM)	1 ± 1	6 ± 4	2 ± 2
2,4-D 1 (4.5 μM)	0	0	0
2,4-D 2 (9.0 μM)	0	0	0
Dicamba 0.5 (2.2 μM)	1 ± 1	0	1 ± 1
Dicamba 1 (4.5 μM)	5 ± 4	14 ± 1	0
Dicamba 2 (9.0 μM)	0	0	0

[a] For regeneration the callus was placed on MS basal salts medium supplemented with either no hormones for 3 mo., 2 mg/liter (9.3 μM) kinetin for 3 mo., or 10 mg/liter (46.5 μM) kinetin for 7 days followed by no hormones for 3 mo. Four callus samples were grown for 10 mo. on each auxin concentration and used for each regeneration treatment. Mean ± standard error shown.

Materials and Methods

Plant stocks. Virus-indexed plants of the *Gladiolus* cultivar "Jenny Lee" were a gift (Oglesby Plant Laboratories, Altha. FL) and maintained in vitro by propagation on Murashige and Skoog (1962 (MS) basal salts medium supplemented with 2 mg/liter (9.3 μM) kinetin (Logan and Zettler, 1985). In vitro plants were grown in Magenta jars under a 16-h light photoperiod (75 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 25° C.

Callus cultures. Callus was initiated from either in vitro plants or from cormels grown in vitro. Each cormel was sliced into 2-mm transverse sections that were placed with a cut surface in contact with the solid medium and oriented so that the side of the cormel slice which had been closest to the apical meristem was facing away from the medium. The medium for callus initiation was MS basal salts, 3% (wt/vol) sucrose, 0.2% Gelrite (Merck & Company, Fiskeville, RI), and the following in mg/liter: glycine, 2.0; thiamine; 1.0; pyridoxine, 0.5; nicotinic acid, 0.5; myoinositol, 100.0; and various hormones as indicated. The hormones 2,4-D, NAA, dicamba, kinetin, and BAP were autoclaved, and filter-sterilized N^6-2-isopentenyladenosine (iPA) was added after autoclaving. For each hormone concentration, either 20 cormel slices or 12 samples of in vitro plants were cultured. Each sample of in vitro plants consisted of six stems of plantlets per Magenta jar. All media were adjusted to pH 5.8 and autoclaved at 121° C for 20 psi for 20 min. Callus cultures were grown in the dark at 25° C. The number of cultures forming regenerable callus was determined by viewing with a dissecting microscope 2, 3, and 4 mo. after callus initiation.

Plant regeneration. Approximately 1 *g* fresh weight (FW) samples of callus were weighed and then placed on regeneration medium. Each treatment consisted of four callus samples. Medium for plant regeneration was MS basal salts medium as described above supplemented with either a) no hormones for 3 mo., b) 2 mg/liter (9.3 μM) kinetin for 3 mo., or c) 10 mg/liter (46.5 μM) kinetin for 7 days followed by a transfer to MS medium containing no hormones for 3 mo. Regenerating callus was maintained under a 16-h light photoperiod (75 $\mu E \cdot m^{-2} \cdot s^{-1}$) at 25° C. When the regenerated plantlets were 1- to 2-cm tall, they were excised and counted. All cultures were transferred every 4 wk to fresh media.

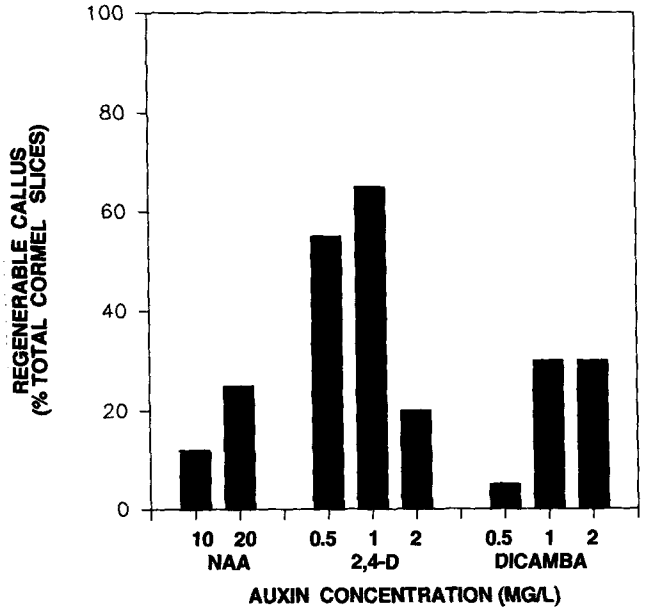

Fig. 4. Effect of auxin on initiation of regenerable callus from cormel slices of *Gladiolus* "Jenny Lee". Twenty cormel slices were cultured on each auxin concentration.

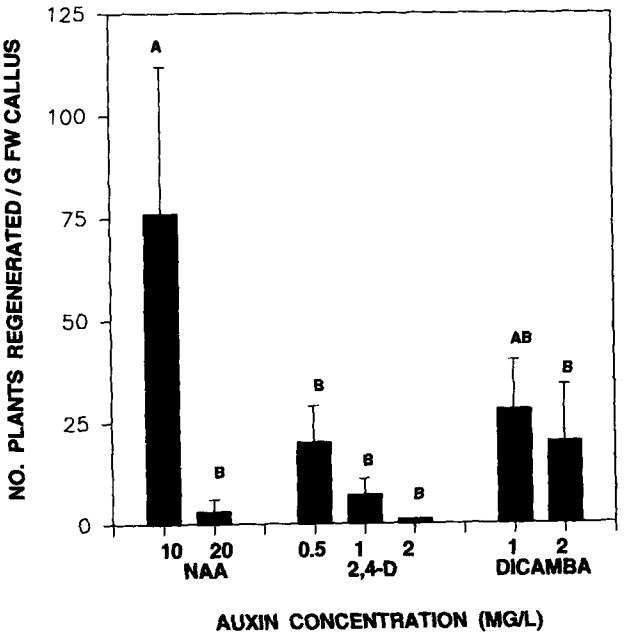

Fig. 5. Plant regeneration from callus that was initiated from cormel slices and maintained 4 mo. on MS basal salts medium supplemented with NAA, 2,4-D, or dicamba. Twelve calli were grown on each auxin for regeneration. *Standard error bars* shown. Means with different letters are significantly different at $P \leq 0.05$ according to Duncan's new multiple range test.

Results and Discussion

Callus initiated from in vitro plants. Regenerable callus formed from the basal leaf region of in vitro plants approximately 2 mo. after the plants were placed on hormones for callus induction. A high percentage (75 to 100%) of the in vitro plants formed regenerable callus when cultured on either NAA (10 or 20 mg/liter), 2,4,-D (0.5 or 2 mg/liter), or dicamba (2 mg/liter) (Fig. 1). Regenerable callus was previously initiated from in vitro plants cultured on 1 and 2 mg/liter 2,4-D, but not 10 mg/liter NAA or 0.5 mg/

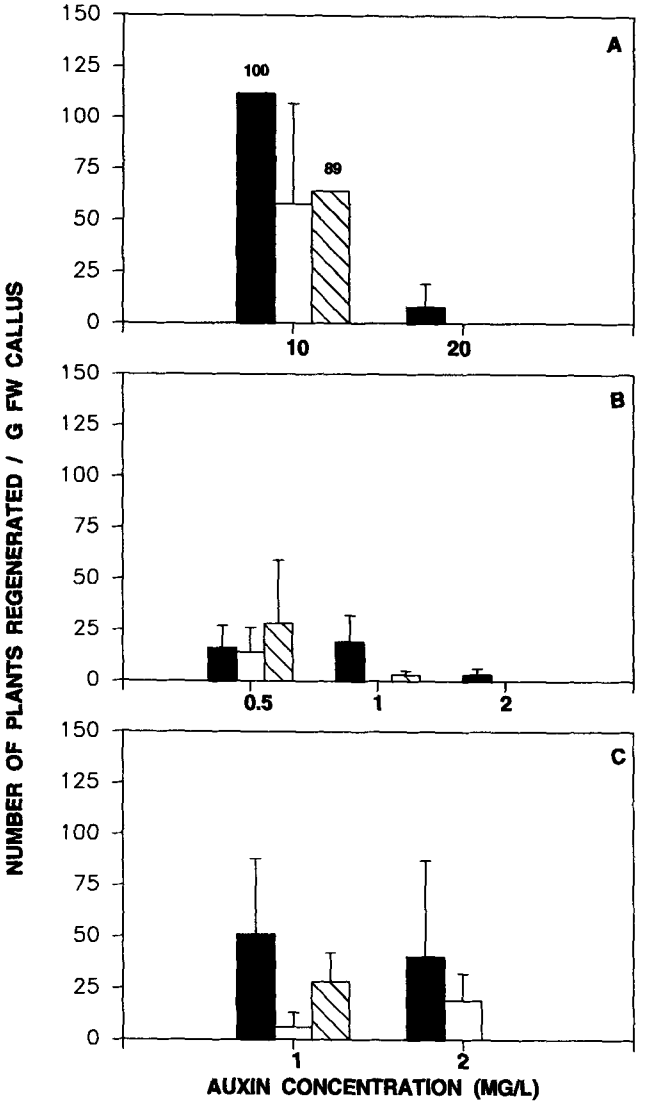

FIG. 6. Plant regeneration from callus that was initiated from cormel slices. Callus was initiated and maintained 4 mo. on MS basal salts medium supplemented with either *A*, NAA, *B*, 2,4-D, or *C*, dicamba before regeneration. For regeneration, callus was placed on MS basal salts medium with either 2 mg/liter (9.3 μM) kinetin for 3 mo. (*solid bar*), 10 mg/liter (46.5 μM) kinetin for 7 days followed by a transfer to hormone-free medium for 3 mo. (*open bar*), or no hormones for 3 mo. (*hatched bar*). Four calli were grown on each auxin concentration and used for each regeneration treatment. *Standard error bars* shown or *numbers shown* when standard error bar was off scale.

liter 2,4-D (Kamo et al., 1990). Only actively growing in vitro plants of *Gladiolus* formed regenerable callus, and this has contributed to the different responses observed in callus formation. Callus initiated on the various concentrations of these three auxins was friable and increased 2- to 3-fold in FW over a 30-day period (data not shown). In comparison, callus initiated from in vitro plants cultured on medium containing the cytokinins, kinetin (2 mg/liter), BAP (0.1 or 1 mg/liter), or iPA (10 or 100 mg/liter) was difficult to maintain as callus because it regenerated plants.

Four months after callus induction, the callus was placed on regeneration medium for 3 mo. The most plants (41 plants/g FW callus) were regenerated from callus initiated and maintained on medium supplemented with 10 mg/liter NAA (Fig. 2). Callus initiated and maintained on all auxins tested, except 0.5 mg/liter 2,4-D, regenerated an equal or higher number of plants when the regeneration medium contained 2 mg/liter kinetin during the 3-mo. regeneration period as compared to the other regeneration media (Fig. 3). A short 7-day exposure to a high (10 mg/liter) level of

TABLE 2

EFFECT OF MEDIUM CONTAINING VARIOUS AUXINS ON 10-MO.-OLD CALLUS INITIATED FROM CORMEL SLICES AND SUBSEQUENT PLANT REGENERATION ON MEDIUM CONTAINING KINETIN[a]

	Number of Plants Regenerated/g FW Callus Regeneration Media with Kinetin, mg/liter		
Auxin, mg/liter	0	2	10
NAA 10 (53.8 μM)	0	0	1 ± 1
NAA 20 (107.6 μM)	0	0	0
2,4-D 0.5 (2.2 μM)	1 ± 1	1 ± 1	2 ± 2
2,4-D 1 (4.5 μM)	1 ± 1	0	1 ± 1
2,4-D 2 (9.0 μM)	3 ± 3	0	0
Dicamba 0.5 (2.2 μM)	ND	ND	ND
Dicamba 1 (4.5 μM)	0	1 ± 1	0
Dicamba 2 (9.0 μM)	1 ± 1	23 ± 10	4 × 4

[a] For regeneration the callus was placed on MS basal salts medium supplemented with either no hormones for 3 mo., 2 mg/l (9.3 μM) kinetin for 3 mo., or 10 mg/liter (46.5 μM) kinetin for 7 days followed by no hormones for 3 mo. There were no data (ND) for samples that did not form any callus. Four callus samples were grown 10 mo. on each auxin concentration and used for each regeneration treatment. Mean ± standard error shown.

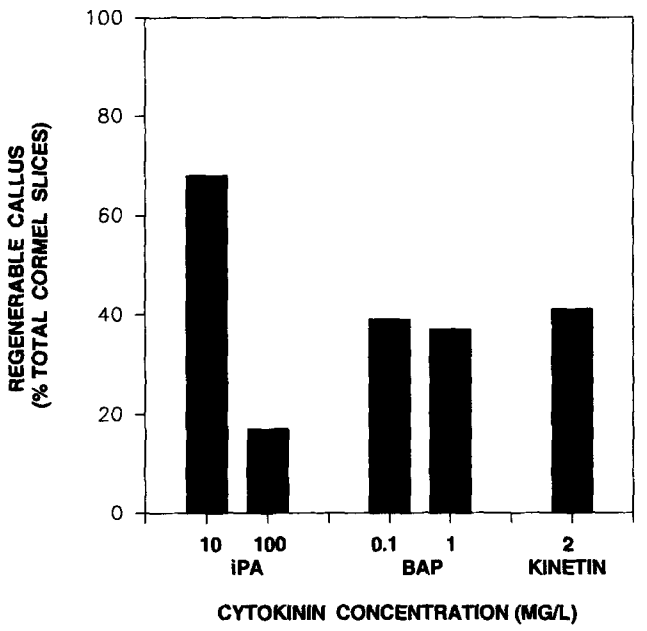

FIG. 7. Effect of cytokinin on initiation of regenerable callus from cormel slices of *Gladiolus* "Jenny Lee." There were 20 cormel slices for each cytokinin concentration.

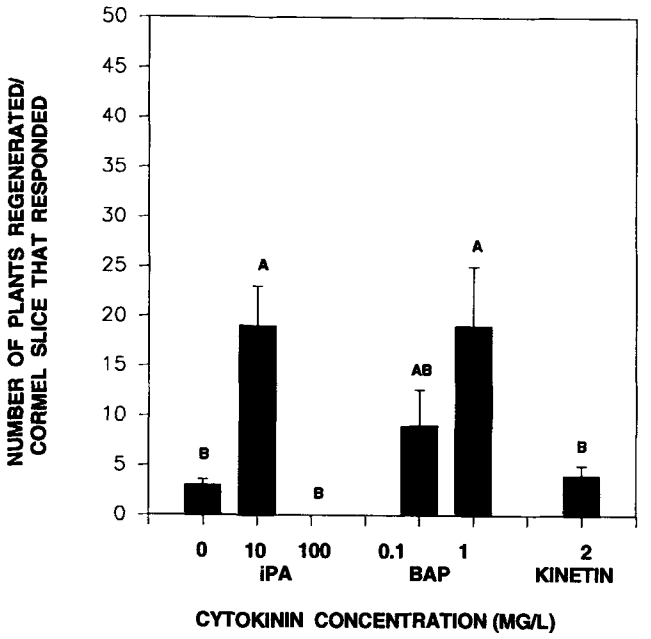

FIG. 8. Plant regeneration from cormel slice explants cultured on different concentrations of cytokinins. There were 20 cormel slices on each cytokinin for regeneration. *Standard error bars* shown. Means with different letters are significantly different at $P \leq 0.05$ according to Duncan's new multiple range test.

kinetin did not enhance plant regeneration from *Gladiolus* callus, although this treatment had improved regeneration frequencies from callus of other cereal monocots (Abe and Futsuhara, 1985; Lowe et al., 1985; Duncan and Widholm, 1988). Twenty-two to 32 plants regenerated/g FW callus from callus that was maintained on medium supplemented with 0.5, 1, or 2 mg/liter 2,4-D and 1 or 2 mg/liter dicamba, then transferred to regeneration medium containing 2 mg/liter kinetin.

Regeneration ranged from 6 to 17 plants regenerated/g FW callus when the callus had been grown on MS medium supplemented with either 20 mg/liter NAA or 0.5 mg/liter dicamba. The range in regeneration from callus that was initiated from different in vitro plants on a given auxin was very large (indicated by the standard error bars) because for each auxin concentration generally 25% of the callus did not regenerate plants (Figs. 2 and 3).

Ten months after callus initiation, the capacity of the callus to regenerate plants decreased dramatically (Table 1). Callus maintained 10 mo. on medium with NAA and 2,4-D regenerated no more than 6 plants/g FW callus. Callus that had been maintained on medium supplemented with 1 mg/liter dicamba regenerated only 14 plants/g FW callus. In comparison, 4-yr old suspensions of *Hemerocallis* regenerated plants "profusely" (Krikorian and Kann, 1981).

Callus initiated from cormel slices cultured on medium containing auxin. Regenerative callus capable of plant regeneration was initiated from 55 to 65% of the cormel slices cultured on medium supplemented with 0.5 or 1 mg/liter 2,4-D. NAA and dicamba were not as effective at inducing callus (Fig. 4). The callus initiated from cormel slices was generally friable and increased approximately twofold in FW over 30 days when grown on the various auxins (data not shown).

Optimal regeneration of plants occurred when 4 mo. old callus from cormel slices had been initiated and grown on medium supplemented with 10 mg/liter NAA (Fig. 5). Approximately twice as many plants regenerated from callus grown 4 mo. on dicamba (1 and 2 mg/liter) compared to 2,4-D (1 and 2 mg/liter) (Fig. 5). Roots, but not callus, were initiated from cormel slices cultured on 0.5 mg/liter dicamba. Callus maintained on the medium containing various auxin concentrations, except 0.5 mg/liter 2,4-D, regenerated more plants when the regeneration medium contained 2 mg/liter kinetin for 3 mo. compared to the other regeneration media (Fig. 6).

Callus maintained in culture for 10 mo. on medium containing various auxin concentrations regenerated no more than three plants/g FW callus, except for callus maintained on 2 mg/liter dicamba which regenerated 23 plants/g FW callus (Table 2).

Callus initiated from cormel slices cultured on medium containing cytokinin. The callus that formed from cormel slices cultured on cytokinin was compact and not friable. This compact callus was difficult to maintain because it immediately regenerated plants instead. Typically 40% and a maximum of 68% of the cormel slices cultured on a medium containing cytokinin formed callus that regenerated plants (Fig. 7). Cormel slices cultured on medium lacking a cytokinin did not form callus but regenerated plants directly from the cormel slice (Fig. 7). The highest number of plants regenerated from cormel slices cultured on medium containing 1 mg/liter BAP and 10 mg/liter iPA (Fig. 8).

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