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Effects of Mefluidide and Dicamba on In Vitro Growth and Embryogenesis of *Dactylis glomerata* (Orchard Grass)

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Abstract. The effects of N-(2,4-dimethyl-5-(((trifluoromethyl)sulfonyl)amino)phenyl)acetamide (mefluidide) and 3,6-dichloro-o-anisic acid (dicamba) on in vitro growth and somatic embryogenesis of *Dactylis glomerata* L. (orchard grass) were studied using suspension cultures and explanted leaf bases. All experiments employed modified Schenk and Hildebrandt medium amended with concentrations of dicamba ranging from 15 to 120 μM (SH-15 to SH-120) and of mefluidide ranging from 1 to 100 μM. SH medium without either growth regulator was used for embryo germination. Embyro production in suspension cultures with SH-30 medium plus 3 g/L casein hydrolysate was significantly reduced by 1 μM mefluidide. Only 15% of these embryos germinated and produced plants compared to 84% from controls. Growth, as measured by dry weight, was significantly reduced by 50 or 100 μM mefluidide. The number of embryos formed on leaf sections was significantly reduced by 20 or 25 μM mefluidide. Embryos that formed with 10 μM or more mefluidide were callused on both SH-15 and SH-30 media. Shoot formation was inhibited from individual embryos and embryo/callus masses that developed on either SH-15 or SH-30 medium containing 5 μM or more mefluidide. Radicle emergence was significantly reduced with 10 μM mefluidide regardless of 15 or 30 μM dicamba. Histological examination revealed that mefluidide inhibited both shoot and root meristem development with shoot development being the more sensitive. Inhibition of both was independent of dicamba concentrations. Shoot formation was also reduced from embryos that had developed on SH-30 medium without mefluidide when transferred to medium containing mefluidide without dicamba.

Mefluidide (N-(2,4-dimethyl-5-(((trifluoromethyl)sulfonyl)amino)phenyl)acetamide) (Embark) is a plant growth regulator that suppresses vegetative growth

(Truelove et al. 1977, Field and Whitford 1982, Watschke 1976) and inhibits reproductive development of some cool-season forage and turf grasses (Field and Whitford 1982, Chappel et al. 1977, Glenn et al. 1980, Dernoeden 1984). Mefluidide has also been reported to enhance the forage quality of tall fescue (*Festuca arundinacea* Schreb.) during late spring and early summer (Glenn et al. 1981). Sheaffer and Marten (1986) reported that mefluidide applied to *Dactylis glomerata* L. (orchard grass) in the early spring increased leafiness and forage quality but did not improve summer production or quality of the grass. Seedhead suppression was also noted.

The mode of action of mefluidide is not known. However, it was shown to induce morphological changes in plants similar to those produced by 2,4-dichlorophenoxyacetic acid (2,4-D) (McWhorter and Barrentine 1979). It caused disorganization of reproductive apices of grasses by initiating uncontrolled cell divisions (Field and Whitford 1982). Mefluidide was also reported to promote polar transport of indoleacetic acid (IAA) through corn (*Zea mays* L.) coleoptiles and soybean (*Glycine max* L. Merr.) hypocotyls. Mefluidide was also shown to have auxinlike activity; 10^{-8} to 10^{-6} M stimulated corn coleoptile elongation equal to that of 10^{-6} M IAA (Glenn and Rieck 1985).

Somatic embryogenesis in in vitro cultures of orchard grass is well characterized (Gray and Conger 1985, Gray et al. 1984, Conger et al. 1983, Hanning and Conger, 1982, 1986, McDaniel et al. 1982) and ideally suited to study the effects of growth regulators on growth and differentiation. Conversely, we were also interested in the identification and study of compounds that can be used to regulate and control embryogenesis in our system. Therefore, the objective of this investigation was to study the effects of mefluidide and the synthetic auxin, 3,6-dichloro-o-anisic acid (dicamba) on in vitro growth and embryo development, differentiation, and germination in orchard grass leaf and suspension cultures.

Materials and Methods

All cultures of *D. glomerata* were grown on modified Schenk and Hildebrandt (1972) basal medium (SH) containing 5 mg/L thiamine, as the only vitamin, and 1 g/L inositol. The basal medium was supplemented with various concentrations of dicamba ranging from 0 to 120 μM. Analytical-grade mefluidide (3M lot 40—B1161F5) was dissolved in a small amount of absolute ethanol, diluted to appropriate concentrations, and filter-sterilized into autoclaved medium. The pH of all media was adjusted to 5.4 with 0.1 N KOH before autoclaving for 20 min at 121°C.

Growth and Embryogenesis Studies in Liquid Medium

Orchard grass suspension cultures were initiated from somatic embryos that were formed on leaf sections as described by Gray et al. (1984). Cell suspensions were grown in SH-30 (30 μM dicamba) medium amended with 3 g/L

filter-sterilized casein hydrolysate (Sigma lot 44F-0403) (SH-30-C) and subcultured every week. Embryos were observed after 12 weeks of culture. Cultures were then resuspended with 25 ml SH-30 in 125-ml Erlenmeyer flasks to inhibit embryogenesis (Gray and Conger 1985) and transferred thereafter at weekly intervals for 5 weeks. At this time very few embryos were observed. One day after the final transfer, the contents of four flasks were homogenized in a blender using high-speed pulses (1 pulse s^{-1}) for 30 s. Two milliliters of suspension (~150 mg tissue fresh weight) was pipetted into each 125-ml flask containing 8 ml SH-30-C amended with mefluidide. The final concentrations of mefluidide were 0, 1, 10, 25, 50, and 100 μM. Six flasks for each mefluidide concentration were inoculated and arranged in a randomized complete block design. Flasks were incubated at 23°C in the darkness on a rotary shaker at 80 rpm. Ten milliliters of the appropriate concentration of mefluidide in SH-30-C was filter-sterilized into flasks after 2 and 4 weeks. The number of embryos per flask was determined 6 weeks after inoculation according to the method of Gray et al. (1984). Twenty embryos for each replicate that produced embryos were transferred to SH-0 (SH medium without dicamba) solidified with 8 g agar/L. Dishes were incubated in a growth chamber at 25°C and a 16 h/8 h day/night regime. Light intensity from cool white fluorescent bulbs was 50 μE m^{-2} s^{-1}. Germination and shoot formation were assessed after 3 weeks.

Fresh weight of the cultures was determined by collecting the contents of each flask on Whatman No. 2 9-cm diameter qualitative filter paper using a Buchner funnel and a side-arm flask. Suction was applied until all free medium was removed from the tissue and filter paper. The average weights of five filter papers moistened with SH-30-C medium and treated in a similar manner as above, was subtracted from the weight of each treatment. Dry weights were obtained by oven-drying the tissue and filter paper at 60°C for 24 h. The average dry weight of the five SH-30-C filter papers was subtracted from the weights of the treatments. Data from all parameters measured were evaluated by analysis of variance, and significant differences between means were determined by Duncan's New Multiple-Range Test.

Embryogenesis from Leaf Bases and Plant Regeneration

All plants used in these experiments were regenerated from somatic embryos of a highly embryogenic genotype (Hanning and Conger 1982). Plants were maintained under greenhouse conditions, and tillers were selected according to criteria previously outlined (Conger et al. 1983). The two innermost leaves were split lengthwise along the midvein, and the basal 50 mm surface sterilized for 1.5 min in 2.62% NaOHCl solution containing 0.1% w/v Triton X. This solution was removed from leaves with three changes of sterile, distilled water. The basal 30 mm of the leaf halves were transversely cut into six equal sections and serially explanted to 9-cm diameter Petri dishes containing ~25 ml solidified SH medium amended with either 0, 15, or 30 μM dicamba in combination with either 1, 5, 10, 15, 20, 25, 50, or 100 μM mefluidide. Corresponding sections of the sister half leaves were explanted onto SH-30 without mefluidide to serve as controls. In addition, similar experiments were con-

ducted using media supplemented with 15, 60, and 120 μM dicamba as treatments and SH-30 as controls.

Ten to twenty paired replications for each dicamba–mefluidide combinations were explanted, and the Petri dishes were incubated at 25°C in the darkness. If one of the paired replications (leaf sections) became contaminated or if the control explants did not produce embryos, both dishes were discarded. After 5 weeks, 15 individual embryos (IE) from each replicate of all treatments were transferred to SH-0 and incubated at 25°C in the light as described above. These were assessed for germination by radicle emergence and shoot formation. At the same time, leaf sections with callus and remaining embryos were transferred in toto to SH-0 and incubated identically. Shoots from leaf explants (LE) were counted after 3 weeks. The number of embryos per experimental unit was estimated by dividing the number of shoots formed from LE by the average shoot formation rate of IE from the same treatment, i.e., (number of embryos per experimental unit) = (number of shoots per LE) (shoot formation rate of IE). All data were analyzed using a *t*-test for paired variates.

Histology

Ten to thirty embryos for each mefluidide–dicamba combination and controls were prepared for histological examination. These were oriented for longitudinal sectioning and immobilized in 0.8% agarose. The preparations were fixed with 3% glutaraldehyde in 0.067 M phosphate buffer, pH 7.0, at 4°C for 24 h. Samples were dehydrated in a graded ethanol-t-butanol series and embedded in Paraplast Plus. Ten-micrometer serial sections were cut on a rotary microtome and mounted on glass slides with Haupt's reagent. Sections were stained with safranin, crystal violet, and fast green (Johansen 1940) and examined for the presence and quality of apical meristems and other embryonic tissues.

Germination of Embryos on Mefluidide Containing Media

Embryos were initiated from leaf bases on SH-30 as previously described. After 5 weeks, 20 embryos were transferred individually to SH-0 solid medium containing 0, 1, 10, 25, 50, or 100 μM mefluidide. Seven replications for each treatment were plated and arranged as a randomized complete block design. Embryos were incubated as previously described, and radicle emergence and shoot formation were assessed after 3 weeks. Data were evaluated by analysis of variance, and significant differences between means were determined by Duncan's New Multiple-Range Test.

Results

Growth and Embryogenesis in Liquid Media

Slight tissue browning (phytotoxicity) was evident in cultures grown in 1 μM

Table 1. Effects of mefluidide concentration on the growth and somatic embryogenesis in *Dactlyis glomerata* suspension cultures

Mefluidide (μM)	Replications	Means per replication		
		Fresh weight (g)	Dry weight (mg)	Somatic embryos (number)
0	5	3.02b	349a	769a
1	4	4.72a	380a	188b
10	6	3.70ab	336a	0c
25	6	2.57bc	310ab	0c
50	6	1.51cd	231b	0c
100	6	0.30d	75c	0c

Means followed by the same letter are not significantly different at $\alpha = 0.05$ according to Duncan's New Multiple-Range Test.

mefluidide–SH-30-C medium. Browning became progressively darker with increasing concentrations of mefluidide. Data from this experiment are presented in Table 1. Growth as measured by fresh weight was significantly enenhanced by the addition of 1 μM mefluidide to SH-30-C medium. Mefluidide concentrations of 50 or 100 μM significantly inhibited growth as measured by both fresh and dry weight.

Embryogenesis was drastically reduced by 1 μM mefluidide and completely inhibited by higher concentrations. Of the embryos formed in 1 μM mefluidide and transferred to SH-0 medium, 64% and 15% formed roots and shoots, respectively, whereas 96% and 84% of the embryos from control treatments formed roots and shoots, respectively. Plants from the 1-μM mefluidide treatment were approximately one-half the size of those from controls after 3 weeks of growth (data not shown).

Embryogenesis from Leaf Bases

Treatments including mefluidide but not dicamba did not support the growth of callus or the development of embryos from leaf bases. The tissue became brown and exuded water. Tissue explanted on medium containing both dicamba and mefluidide became increasingly brown proportional to the concentration of mefluidide, whereas tissue on medium amended with dicamba only exhibited little or no discoloration.

Data for these experiments are presented in Figs. 1–3. Embryogenesis was significantly inhibited by 20 or 25 μM mefluidide at both 15 and 30 μM dicamba (Figs. 1, 2). When used without mefluidide, dicamba at 15 μM initiated a similar level of embryogenesis as 30 μM, whereas 60 and 120 μM were inhibitory (Fig. 3).

Scutella of embryos that developed on media containing 10 or 25 μM mefluidide were callused. The extent of callusing increased with increasing concentrations of mefluidide but was independent of 15 or 30 μM dicamba (Tables 2,

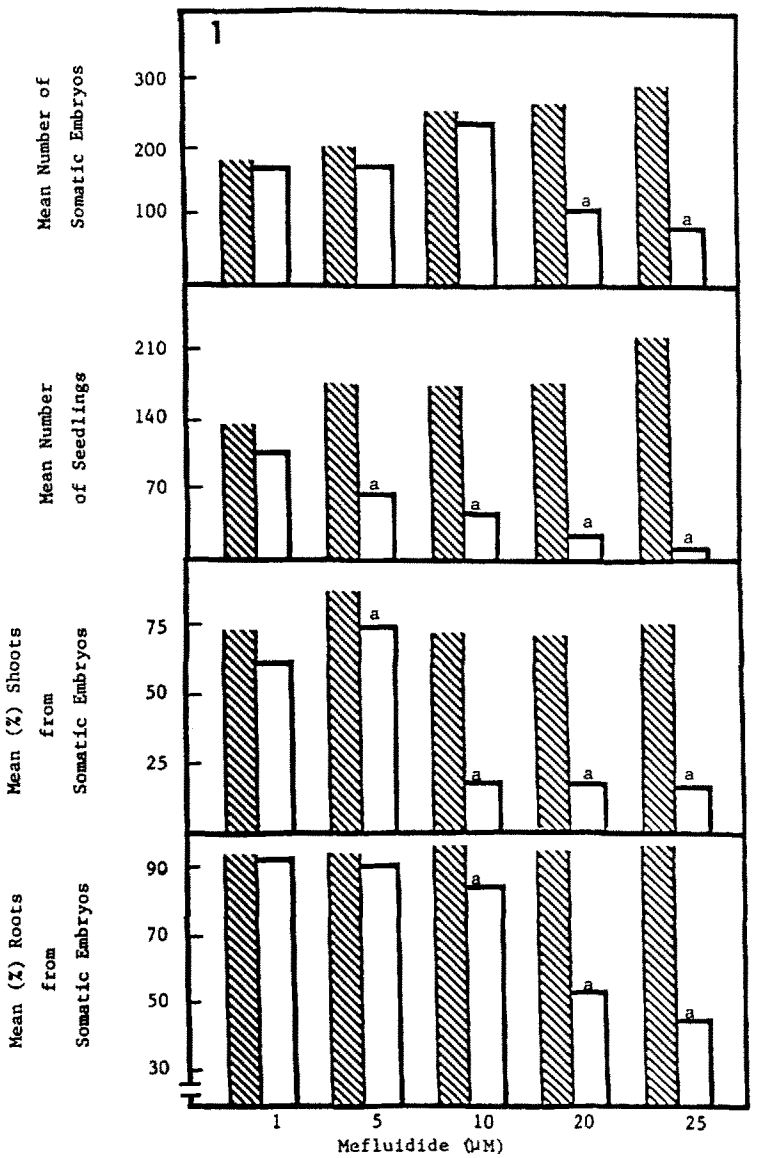


Fig. 1. Influence of mefluidide concentration on somatic embryogenesis, number of seedlings, and shoot and root formation. Slashed bars represent means for controls grown on SH-30 without mefluidide, and open bars represent means for mefluidide treatments on SH-30. The letter *a* indicates that the means are significantly different at $\alpha = 0.05$ according to a *t*-test for paired variates.

3). A visual assessment of embryos produced on media containing 15, 30, or 60 μM dicamba without mefluidide indicated they were not callused; embryos developed on 120 μM dicamba were only slightly callused.

Shoot formation from embryos grown on 1 μM or more mefluidide was in-

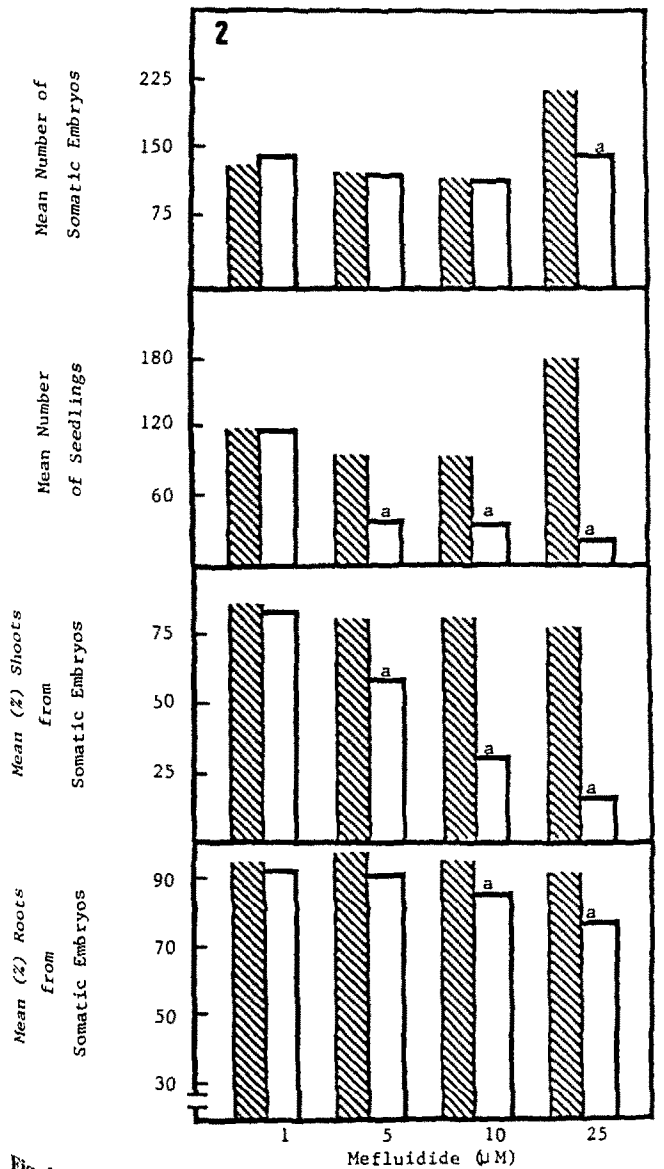


Fig. 2. Influence of mefluidide concentration on somatic embryogenesis, number of seedlings, and shoot and root formation. Slashed bars represent means for controls grown on SH-30 without mefluidide, and open bars represent means for mefluidide on SH-15. The letter *a* indicates that the means are significantly different at $\alpha = 0.05$ according to a *t*-test for paired variates.

hibited regardless of dicamba concentration (Figs. 1, 2). Radicle emergence was significantly inhibited from embryos grown on medium combinations of 10 μM or more mefluidide regardless of the dicamba concentration (Figs. 1, 2). Germination and shoot development rates were not adversely affected by di-

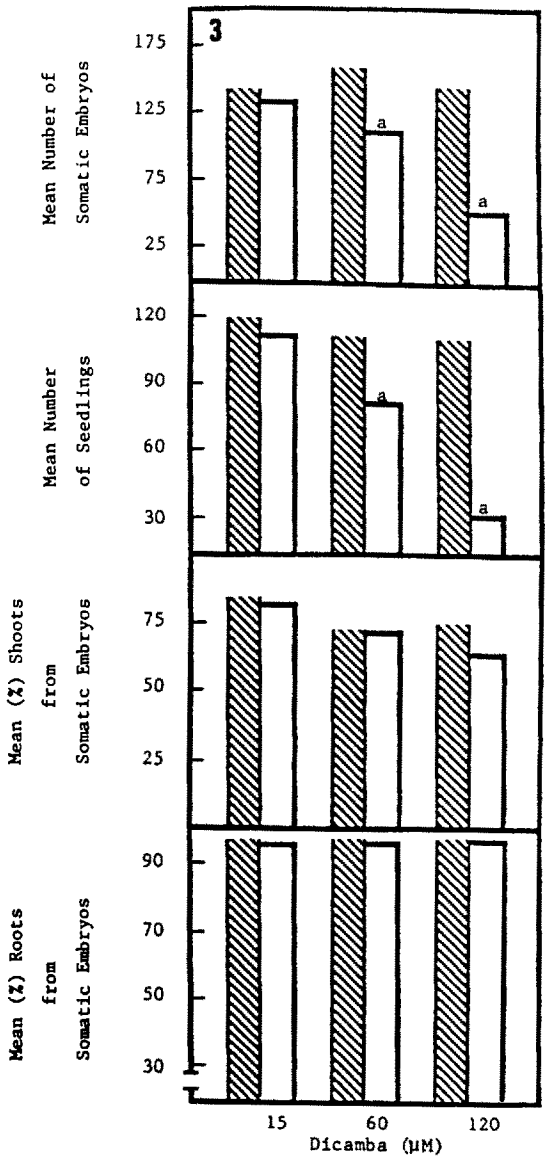


Fig. 3. Influence of dicamba concentration of somatic embryogenesis, seedling development, and shoot and root formation. Slashed bars represent means for controls grown on SH-30 medium, and open bars represent means from dicamba treatments. The letter *a* indicates that the means are significantly different at $\alpha = 0.05$ according to a *t*-test for paired variates.

camba at any concentration when used as the sole regulator (Fig. 3). Leaf lengths from embryos grown on mefluidide were much less than those from embryos that developed on medium lacking mefluidide after 3 weeks (data not shown).

Table 2. Histological data for the influence of different mefluidide concentrations with SH-15 medium on the development of root and shoot apical meristems and callusing of somatic embryos of *Dactylis glomerata*

Mefluidide (μM)	No. of observations	Somatic embryos with meristems (%)		Embryos callused (%)
		Shoot	Root	
0	16	96.3	100.0	0
1	18	88.9	94.4	0
10	16	43.8	82.5	44.0
25	19	15.8	68.4	100.0

Histology

Histological data are presented in Tables 2 and 3. Embryos from leaf explants on medium containing 0 or 1 μM mefluidide in combination with 15 or 30 μM dicamba were typically well developed (Fig. 4). Nearly all the embryos had well-formed, recognizable shoot and root meristems. There were orderly divisions of cells in shoot-root axes, and cells of scutellar and coleorhizal tissues were of uniform size and shape. The protoderm consisted of a discrete single layer of cells, and scutellar and coleorhizal tissues were not callused. Less than 50% of the embryos that developed on medium amended with 10 μM mefluidide, regardless of dicamba concentration (15 or 30 μM), had shoot meristems (Tables 2, 3). The other half had abnormal shoot and root meristem development. The area of the shoot-root axis consisted of small cells that had divided in random planes to produce differentiated but atypical tissue (Fig. 5). One-half of the embryos exhibited scutellar callusing. Most embryos from 25-μM mefluidide–dicamba treatments lacked shoot meristems, and root meristems were typically enlarged and appeared disorganized (Fig. 6). Scutellar tissues were callused, and protoderm tissue was absent. Sections of embryo-like structures produced in treatments on 25 or 50 μM mefluidide revealed highly compacted cell masses with the other layers of cells arranged in tiers. Meristematic areas (probably root) were present in about one-half of the samples and located in the center of the embryolike masses (Fig. 7).

Germination and Shoot Formation of Embryos on Media Containing Mefluidide

Shoot formation was significantly reduced by the addition of 1 μM mefluidide to SH-0 medium (Table 4). Radicle emergence was not significantly reduced by concentrations up to 10 μM. Embryos did not callus on mefluidide medium but did exhibit browning, especially on medium containing 50 or 100 μM mefluidide. Leaf growth was reduced to approximately one-third to one-half of that from leaves of embryos plated on medium without mefluidide.

Table 3. Histological data for the influence of different mefluidide concentrations with SH-30 medium on the development of root and shoot apical meristems and callusing of somatic embryos of *Dactylis glomerata*

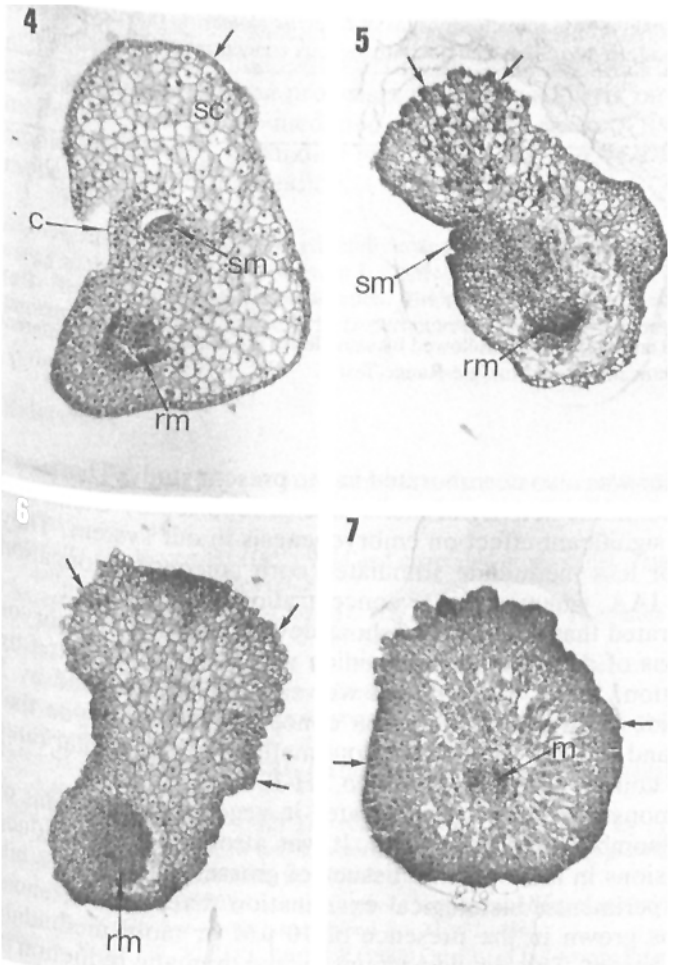
Mefluidide (μM)	No. of observations	Somatic embryos with meristems (%)		Embryos callused (%)
		Shoot	Root	
0	29	93.1	100.0	0
1	14	85.7	92.9	0
10	20	45.0	70.0	55.0
25	18	5.6	44.4	100.0

Discussion

Mefluidide was phytotoxic to orchard grass tissues grown in liquid medium or explanted to solid medium regardless of dicamba concentration. Browning was evident at 1 μM and became progressively darker with increasing concentration of mefluidide. Discoloration of *Poa pratensis* L. (Kentucky bluegrass) (Watschke 1976), *Festuca rubra* L. (red fescue) (Chappel et al. 1977), *Agrostis tenus* Sibth (browntop) (Field and Whitford 1982), and *Panicum miliaceum* (wild proso millet) (Carpenter and Hopen 1985) foliage was also observed in field applications of mefluidide.

Phytotoxicity expression at the low concentrations of mefluidide employed in liquid cultures was probably related to the constant exposure of cells to the regulator over 6 weeks. Growth of cultures, as measured by dry weight, was not affected by 25 μM or less mefluidide. Although not directly comparable to in vitro studies, growth of *Pennisetum americanum* (L.) Leeke (pearl millet) in pots was significantly increased by mefluidide treatments equivalent to 0.14 kg ha^{-1} (Fales and Wilkinson 1984). However, in field studies, dry-weight yield of orchard grass (Sheaffer and Marten 1986) and tall fescue (Glenn et al. 1980) was reduced by mefluidide treatments of 0.42 and 0.56 ha^{-1}, respectively. The high sensitivity of embryo development in suspension cultures compared to that from leaf sections explanted onto solid medium was probably due to the cells being in continuous contact with the regulator. Additionally, mefluidide was added to the liquid cultures every 2 weeks, whereas concentrations of mefluidide available to leaf explants on the solid medium for 5 weeks would diminish over time.

Mefluidide concentrations of 20 or 25 μM were required to significantly reduce embryogenesis on solidified medium and were independent of the level of dicamba (15 or 30 μM) employed. Glenn and Rieck (1985) demonstrated that mefluidide at 1 and 10 μM stimulated IAA transport in corn coleoptiles measurably but not significantly. However, 100 μM did significantly increase auxin transport in that system. In our experiments, 20 μM or more mefluidide may have reduced embryogenesis from leaf tissue by increasing transport of dicamba. We previously demonstrated that elevated levels of dicamba depressed embryogenesis in suspension cultures (Gray and Conger 1985) and 60 or 120 μM dicamba reduced embryogenesis in cultured leaf explants (Hanning and



Figs. 4–7. Light microscopy of *Dactylis glomerata* somatic embryos formed on media with and without mefluidide 5 weeks after initiation.

Fig. 4. Near median longitudinal section of an embryo formed on medium lacking mefluidide. The embryo has a single-cell layer protoderm (arrow), well-developed shoot (sm) and root (rm) meristems, a coleoptile (c), and a scutellum (sc) composed of uniform-size cells. Note that the division of cells in the shoot-root axis are oriented orthogonally to the long axis of the embryo. ×75.

Fig. 5. Oblique longitudinal section through the shoot (sm) and root (rm) meristems of an embryo formed on 10 μM mefluidide. The proximal area of the scutellum (arrows) is callused and is composed of nonuniform-size cells. There is no recognizable protoderm in this area. The shoot-axis is disorganized. ×56

Fig. 6. Longitudinal section through an embryo formed on 25 μM mefluidide. The entire protoderm (arrows) has callused, and the root meristem (rm) is enlarged. A shoot meristem was not found in any section of this embryo. ×60.

Fig. 7. Section parallel to the long axis of an embryolike structure formed on 25 μM mefluidide. The perimeter of the mass consists of tiers of cells (arrows), and a prominent meristematic region is centrally located. ×56.

Table 4. Shoot and root formation from somatic embryos of *Dactylis glomerata* that developed on SH-30 medium and then transferred to SH-0 medium with various concentrations of mefluidide

Mefluidide (μM)	Embryos producing shoots (%)	Embryos producing roots (%)
0	80.4a	98.6a
1	28.6b	91.4a
10	8.6c	80.0a
25	5.7c	42.8b
50	1.4c	21.4bc
100	0.0c	1.0c

Mean of 7 replications of 20 embryos. Means followed by same letter are not significantly different at $\alpha = 0.05$ according to Duncan's New Multiple-Range Test.

Conger 1986). The latter was also corroborated in the present study. There was no evidence that the auxinlike activity of mefluidide as reported by Glenn and Rieck (1985) had any significant effect on embryogenesis in our system. They reported that 1 μM or less mefluidide stimulated corn coleoptile elongation equal to that of 1 μM IAA, whereas higher concentrations had no effect.

Our study demonstrated that inhibition of shoot development from embryos grown on combinations of dicamba and mefluidide was primarily affected by mefluidide concentration. Shoot development was significantly reduced by 5 μM or more mefluidide regardless of dicamba concentration. Embryos that developed on 15, 60, and 120 μM dicamba without mefluidide had similar rates of shoot formation to control embryos grown on SH-30 medium.

Mefluidide was demonstrated to be concentrated in vegetative meristems of susceptible plants (Bloomberg and Wax 1978). It was also reported to induce uncontrolled cell divisions in meristematic tissues of grasses (Field and Whitford 1982). In our experiments, histological examination revealed that shoot meristems in embryos grown in the presence of 10 μM or more mefluidide were disorganized or absent. This would account for the dramatic reduction in shoot formation and would probably account for the suppression of seedhead formation in field applications of mefluidide to grasses (Field and Whitford 1982, Dernoeden 1984, Glenn et al. 1980, Sheaffer and Marten 1986). Media containing only mefluidide did not cause callusing of embryos, suggesting that it did not have auxinlike activity in our system. Hypotheses of enhanced auxin translocation and mefluidide toxicity would also account for the observed phenomenon of reduced radicle emergence from embryos grown on combinations of mefluidide and dicamba. However, compared to shoots, either root meristems were less sensitive to the toxic effects of mefluidide, less mefluidide was transported to those meristems, or less auxin was concentrated there than in shoots.

Mefluidide also inhibited germination and shoot formation from individual, isolated embryos and retarded the elongation of leaves. Suppression of leaf growth by mefluidide has been well documented for a number of grass species in field (Field and Whitford 1982, Moore and Tautvydas 1986) and hydroponic studies (Wilkinson 1982).

The physiological mechanism by which mefluidide influences the development growth and embryogenesis of orchard grass in vitro was unclear. However, inhibition of these processes may be dependent on the toxic properties of mefluidide, mefluidide-mediated elevation of auxin in tissues, or both. A study employing labeled mefluidide and dicamba would be useful for elucidating the mode of action of mefluidide.

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