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Growth Retarding Effects of the Herbicide Difenzoquat

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Abstract. The growth-regulating properties of the herbicide Difenzoquat (1,2-dimethyl-3,5-diphenyl-1-*H*-pyrazolium methyl sulfate) were investigated in seedlings and cell suspension cultures of sunflower (*Helianthus annuus* L.). Application of 10 μg or more Difenzoquat to the apex of seedlings resulted in a transient inhibition of internode elongation. Application of GA_3 to treated seedlings resulted in enhanced internode elongation but did not reverse the degree of growth inhibition elicited by Difenzoquat. Endogenous gibberellin levels were estimated by bioassay and were qualitatively and quantitatively similar in extracts from control and treated seedlings. Treatment of suspension cultures of sunflower cells with 1 μM or more Difenzoquat resulted in an inhibition of cell division (dry-matter accumulation). Neither GA_3 nor a mixture of sterols (cholesterol, β-sitosterol, and stigmasterol) alone or in combination was able to overcome this inhibition of cell division. It was concluded that the growth-retarding activity of Difenzoquat was the result of its action at the cellular level and was not mediated by inhibition of gibberellin biosynthesis.

The potential utility of many herbicides and other agricultural chemicals often extends beyond their initial or principal application in crop husbandry. Many currently registered plant bioregulators were initially employed as pesticides. Their effectiveness as growth regulators was discovered only after the systematic examination of their biological effects.

Difenzoquat (Fig. 1; trade name Avenge) is a post-emergence herbicide used to control wild oats in wheat and barley. Although it is effective in this regard,

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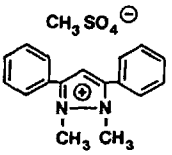

DIFENZOQUAT
(Methyl Sulfate)

Fig. 1. The chemical structure of Difenzoquat: 1,2-dimethyl-3,5-diphenyl-1*H*-pyrazolium methyl sulfate.

its mechanism of action has not been clearly established (Shaner 1984). During crop tolerance trials, we observed that sunflowers treated with field rates of Difenzoquat exhibited a conspicuous reduction in stature. Closer examination of these treated plants revealed no inhibition of internode number, but instead internode length was reduced.

It has been proposed that, under certain agricultural situations, oilseed sunflowers could benefit from stature reduction through the use of a growth retardant (Bayliss and Dicks 1978). The previously undocumented growth-retarding action of Difenzoquat coupled with the paucity of data concerning the physiology of its herbicidal action prompted us to explore its effects on sunflower seedlings. Portions of this research have appeared previously (Suttle and Hultstrand 1984).

Materials and Methods

Plant Material and Chemicals

Seeds of sunflower (*Helianthus annuus* L.) were sown directly in 12-cm-wide plastic pots containing vermiculite or were initially germinated in vertically oriented cylinders of moist paper toweling and after 5–6 days of growth transferred to glass jars (500 ml) containing nutrient solution (Blankendaal et al. 1972). Seedlings were grown in a growth chamber under a 14-h photoperiod provided by a mixture of cool-white fluorescent and incandescent bulbs (light intensity at plant height: 300 $\mu Em^{-2}s^{-1}$, PAR). Day/night temperatures were 25/23°C, respectively, and a relative humidity of approximately 50% was maintained. Unless otherwise stated, seedlings were treated when they were 6 days old. All chemicals used in this study were of the highest purity commercially available and were purchased from supply houses.

Seedling Dose-Response Studies

Six-day-old vermiculite-grown seedlings were treated by placing a 50-μl droplet of water containing various amounts of Difenzoquat directly on the apex. Following treatment, seedlings were returned to the growth chamber. Internode length was determined 22 days after treatment. Studies examining the effects of Difenzoquat treatment on primary root growth were conducted using hydroponically grown seedlings. Six-day-old seedlings (germinated on paper toweling) were transferred to glass jars containing nutrient solution with

various concentrations of Difenzoquat. Prior to transfer, roots were marked with indelible ink 1 cm behind the root tip. Root elongation was then determined 1, 3, and 5 days after exposure to Difenzoquat, using the ink mark as a reference point.

Gibberellin Reversal Studies

Sunflower seedlings were grown in rolled paper toweling. After 6 days, these seedlings were transferred to jars containing nutrient solution fortified with various concentrations of GA_3. The seedlings were treated apically with Difenzoquat (30 μg/seedling). The lengths of the first and second internodes were measured 12 days after treatment.

Endogenous Gibberellin Levels

Vermiculite-grown seedlings were treated with Difenzoquat (100 μg/seedling) when they were 6 days old. Nine days after treatment the seedlings were harvested and the roots were excised and discarded. The apical portions were quickly frozen in liquid nitrogen and freeze-dried. The dried plant material was weighed and homogenized in 80% aqueous methanol. The subsequent fractionation and purification procedures (solvent-solvent partitioning, charcoal column chromatography, etc.) have been described previously (Metzger 1983). The resulting acidic, ethylacetate fraction was dried under a stream of nitrogen and redissolved in 20% aqueous methanol containing 1% (v/v) acetic acid. After filtering, this material was fractionated by reverse-phase HPLC on a C_{18} column using a 20–100% linear gradient (2 ml/min) of methanol in water (all solvents contained 1% acetic acid). After drying, each fraction was assayed for GA-like activity using a modification of the d_5 corn bioassay of B. O. Phinney (Kende and Lang 1964).

Cell Suspension Studies

Freely suspended callus cells from *H. annuus* L. were provided by Dr. D. G. Davis, Fargo, North Dakota, and were cultivated in a B-5 medium (Gamborg et al. 1976) supplemented with the following growth regulators: 2,4-D, 6 mg/l; NAA, 0.4 mg/l; and kinetin 0.2 mg/l. The cells had been grown in suspension culture for roughly 1 year and were subcultured at intervals of 7–10 days (while in exponential growth phase). The culture conditions were as follows: a 250-ml Erlenmeyer flask containing 60 ml of cell suspension was shaken on a rotary shaker at 100 rpm in darkness at 25°C. Stock solutions of Difenzoquat and GA_3 were prepared in DMSO and added to previously autoclaved flasks to the final concentrations indicated. All flasks contained 0.1% DMSO (final concentration). Sterols were prepared in chloroform and were added to the medium prior to autoclaving (controls received chloroform only). Equivalent aliquots (10 ml) of cell suspensions were added to the culture flasks. The flasks

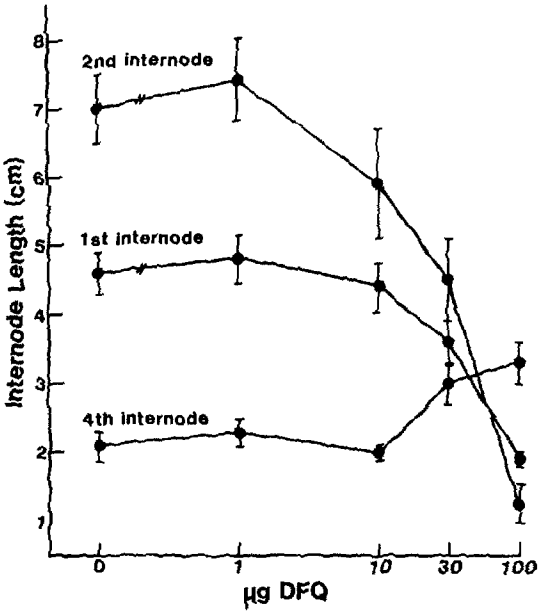

Fig. 2. Effect of increasing doses of Difenzoquat applied directly to the apex on subsequent internode elongation in sunflower seedlings. Seedlings were 6 days old at the time of treatment, and final internode length was determined 22 days after treatment. Average ± SEM ($n = 9$).

were returned to the growth cabinet. At various times thereafter, cells were harvested by filtration, freeze-dried, and finally weighed to obtain dry weights. Because in many instances cells grew in various-size aggregates, no attempt was made to determine viability following treatment. Reversal experiments with GA_3 or the sterol mix (1:1:1 molar ratio of cholesterol, β-sitosterol, and stigmasterol) were conducted similarly.

Results and Discussion

Initially, in an attempt to mimic a postemergence treatment under laboratory conditions, Difenzoquat was applied in aqueous solution directly to the seedling apex. Using this methodology, the application of 10 µg or more Difenzoquat per seedling resulted in the marked suppression of subsequent internode elongation (Fig. 2). Although the first internode initiated elongation immediately following treatment (i.e., by 8 days after sowing), extension of the second internode was more sensitive toward Difenzoquat treatment. The inhibition of internode elongation was transient. Extension of the third (data not shown) and fourth internodes was in no way inhibited by Difenzoquat treatment, and in some instances elongation of this internode was actually enhanced (Fig. 2). Chlorosis was observed only in those tissues (i.e., the first true leaves) that were in direct contact with the treatment solution. Later-developing leaves exhibited no such symptoms. This contact toxicity towards Difenzoquat has been observed by others (Pallett and Caseley 1980). However, its relationship to either the herbicidal or growth-regulating activity of Difenzoquat is uncertain. When seedlings treated in this fashion were allowed to grow to maturity, no

Table 1. Effect of root-applied Difenzoquat on tap root elongation in sunflower seedlings.

Difenzoquat concentration (μM)	Root elongation (days posttreatment) (cm)		
	1	3	5
None	2.7 ± 0.6[a]	6.2 ± 0.4	13.1 ± 0.8
0.1	2.9 ± 0.2	7.5 ± 0.5	13.7 ± 0.9
1	2.9 ± 0.1	5.5 ± 0.4	7.2 ± 0.5
10	2.9 ± 0.1	3.4 ± 0.3	3.7 ± 0.9
100	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1

[a] Average ± SEM (n = 10)

Table 2. Effect of root-applied GA_3 (10 ppm) on Difenzoquat-mediated inhibition of internode elongation in sunflower seedlings.

Treatment	Internode elongation (cm)[a]	
	First internode	Second internode
Control	2.7 ± 0.2	1.5 ± 0.1
GA_3	6.9 ± 0.5	7.8 ± 0.4
Difenzoquat[b]	1.8 ± 0.1	0.7 ± 0.1
GA_3 and Difenzoquat[b]	3.4 ± 0.4	0.7 ± 0.1

[a] Average ± SEM (n = 6)
[b] 30 μg Difenzoquat/seedling.

loss of apical dominance was observed at Difenzoquat treatments of 30 μg or less, and fully functional and developmentally normal flowers formed (data not shown). These results indicated that Difenzoquat was not acting by killing or drastically injuring cells of the apical meristem.

The elongation of the seedling tap root was not affected by treatment of the shoots with Difenzoquat (data not shown). In contrast, direct exposure of the roots to Difenzoquat at concentrations equal to or greater than 1 μM resulted in an inhibition of tap root elongation (Table 1). Root treatment also resulted in the inhibition of shoot elongation (not shown). These results indicated that Difenzoquat moved primarily in an acropetal fashion in these seedlings. These results are in accordance with other studies that also demonstrated the limited basipetal movement of this xenobiotic (Sharma et al. 1976, Pallett and Caseley 1980).

The demonstrated ability of Difenzoquat to transiently inhibit both internode and tap root elongation fulfills the definition of a growth retardant as proposed by Cathey (1964). Many growth retardants have been shown to exert their physiological effects by interfering with gibberellin synthesis or action (for review see Dicks 1979). As a result, the action of many growth retardants can be alleviated by exogenous gibberellins (such as GA_3). When GA_3 was supplied to sunflower seedlings via the nutrient solution, the elongation of the first and second internodes was enhanced (Table 2). Application of GA_3 to Difenzoquat-treated seedlings resulted in a slight increase in first-internode elongation but

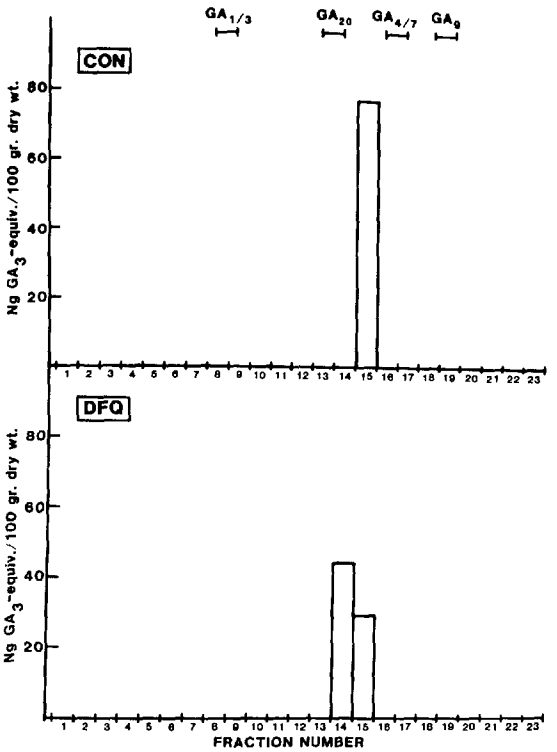

Fig. 3. Effect of Difenzoquat (100 μg/seedling) on endogenous gibberellin-like activity in extracts of sunflower seedlings. Plants were harvested 10 days after treatment. Gibberellins were fractionated by HPLC and were assayed with the d_5 corn bioassay. Upper panel: control extracts. Lower panel: Difenzoquat-treated seedlings. Brackets on upper panel indicate elution patterns of gibberellin standards.

had no effect on the second internode. However, seedlings treated with Difenzoquat and GA_3 still exhibited pronounced growth inhibition relative to those that received GA_3 alone. In addition exogenous GA_3 was unable to reverse the Difenzoquat-mediated inhibition of tap root elongation (not shown).

Seedlings were treated apically with 100 μg of Difenzoquat and were harvested after 10 days (the period of maximum growth inhibition). Extracts were fractionated by HPLC and GA-like activity was assayed by the d_5 maize bioassay. Extracts prepared from both control and treated seedlings were found to possess a single zone of GA-like activity (Fig. 3). This single zone of biological activity was of intermediate polarity (as indicated by retention time), and it eluted in a fashion similar to GA_{19} or GA_{20}. Both GA_{19} and GA_{20} are among the eight gibberellins known to exist in seeds of sunflower (MacMillan 1983). The endogenous level of this zone of GA-like activity was not influenced by Difenzoquat treatment. Because bioassays were used to estimate endogenous GA levels, it could be argued that the presence of interfering substances obscured the effects of Difenzoquat treatment. However, millimolar concentrations of Difenzoquat were found to have no effect on the ability of the GA-producing fungus *Fusarium moniliforme* to synthesize and secrete gibberellins (not shown). Together these results suggest that Difenzoquat-mediated growth inhibition in these seedlings was not the result of changes in GA biosynthesis or metabolism.

At the cellular level, both sustained cell division and cell expansion contribute to overall tissue elongation. Previous studies using wheat seedlings have found that Difenzoquat can affect both of these processes (Pallett and Caseley 1980). Suspension cultures have proved to be reliable assay systems for examining the effects of bioregulators on a range of cellular processes, including cell division (Gressel 1984). The effects of Difenzoquat on cell division were examined using suspension cultures of sunflower cells. Exposure of sunflower cell suspension cultures to concentrations of Difenzoquat ≤ 1 μM resulted in an inhibition of cell proliferation, as evidenced by decreases in final culture dry weight (Table 3). From these data, an I_{50} value of approximately 30 μM was obtained. Even in the presence of 100 μM Difenzoquat, some cell division occurred.

Tetcyclasis, a novel norbornenodiazentine-type plant growth retardant, has been found to be one of the most potent growth retardants examined to date. Tetcyclasis effectively inhibited internode elongation in sunflower seedlings at sub-micromolar treatment concentrations, and it inhibited cell division in sunflower suspension cultures at slightly higher concentrations (Nitsche et al. 1985). From the data presented in their study, an I_{50} value of slightly less than 10 μM can be derived. Thus, Difenzoquat was only slightly less potent in its growth-retarding activities in suspension cultures.

Besides interfering with gibberellin levels, certain growth retardants including tetcyclasis have been found to alter sterol metabolism in treated tissues (Douglas and Paleg 1974, Grossman et al. 1983). Being integral components of all biological membranes, changes in sterol levels would be expected to alter membrane function, thereby resulting in cell dysfunction. Difenzoquat has been shown to inhibit ion uptake by roots, an effect possibly related to membrane perturbation (Cohen and Morrison 1982). Exogenous sterols have been found to reverse the inhibition of cell division caused by tetcyclasis (Grossmann et al. 1985). Exposure of sunflower suspension culture to Difenzoquat (30 μM) resulted in a pronounced inhibition of cell proliferation (Table 4). The inclusion of both GA_3 (10 μM) and the sterol mix (all 5 μM) did not reverse this inhibition. This was also true when GA_3 and the sterols were administered separately (not shown).

The data presented herein have clearly demonstrated the growth-retarding properties of Difenzoquat. The question arises as to the relationship between this type of activity and the herbicidal action of Difenzoquat. Difenzoquat has been shown to inhibit the following processes: cell elongation, cell division, thymidine uptake and incorporation, oxidative phosphorylation by isolated mitochondria, and ion uptake by roots (Pallett and Caseley 1980, Cohen and Morrison 1982, Halling and Behrens 1983). A major difficulty in interpreting these studies arises from both the experimental systems studied and the concentrations of Difenzoquat employed. Thus, both cell division and thymidine incorporation were inhibited in susceptible seedlings, but which came first? Similarly, how does the inhibition of ion uptake by roots relate to the herbicidal (or in this case growth-regulating) action of a foliarly applied compound with little to no basipetal translocatability? The demonstrated action of Difenzoquat on oxidative phosphorylation in isolated mitochondria could, in concept, account for many of the diverse actions of this compound. Yet no other

Table 3. Effect of Difenzoquat on growth (dry-matter accumulation) in sunflower cell suspension cultures.

Difenzoquat concentration (−log M)	Dry weight of culture[a] (g)
Control	0.286 ± 0.007[b]
7	0.272 ± 0.024
6	0.241 ± 0.020
5	0.197 ± 0.020
4	0.084 ± 0.003

[a] Measured after 7 days of growth in the presence or absence of Difenzoquat. Initial dry weight: 0.063 g.
[b] Average = SEM (n = 3).

Table 4. Effect of GA_3 and sterols (1:1:1 molar ratio, stigmasterol, β-sitosterol, cholesterol) on growth (dry-matter accumulation) in control and Difenzoquat-treated sunflower cell suspension cultures.

Treatment	Dry weight of culture[a] (g)
None	0.432 ± 0.063[b]
GA_3/sterol mix[c]	0.398 ± 0.048
Difenzoquat[d]	0.104 ± 0.004
Difenzoquat + GA_3/sterol mix	0.089 ± 0.008

[a] Measured after 8 days of growth. Initial dry weight 0.044 ± 0.007 g.
[b] Average ± SEM (n = 4).
[c] [GA_3] = 10 μM, [sterol] = 5 μM (each).
[d] [Difenzoquat] = 30 μM.

herbicide known to affect energy balance has, to our knowledge, been reported to elicit growth-retarding activity. Another interesting question concerns the demonstrated (Fig. 1) transience of the response. This effect was also noted in wheat (Pallett and Caseley 1980). It is tempting to ascribe this phenomenon to metabolic inactivation of Difenzoquat, but, where studied, Difenzoquat is not appreciably metabolized (Sharma et al. 1976).

Thus, although Difenzoquat elicits a wide range of effects ranging from growth retardation to herbicidal activity, the underlying biochemical mechanism(s) have yet to be identified with certainty. Undoubtedly, further studies will shed more light on this matter and may, in addition, uncover other interesting biological actions of this compound.

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