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Effect of Certain Dicarboxylic Acid Monoesters on Growth, Chlorophyll Content, Chlorophyllase and Peroxidase Activities, and Gas-Exchange of Young Maize Plants

D. Todorov, V. Alexieva, E. Karanov, D. Velichkov, and V. Velikova

Acad. M. Popov Institute of Plant Physiology, Bulgarian Academy of Sciences, Acad. G. Bonchev Str., Bl. 21, Sofia 1113, Bulgaria

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Abstract. The effect of some dicarboxylic acid monoesters on growth, chlorophyll content, chlorophyllase (EC 3.1.1.14), and total peroxidase (EC 1.11.1.7.) activities was examined in detached and intact leaves of maize (*Zea mays*) plants grown in a greenhouse. The β-monomethyl ester of itaconic acid (MEIA) at 1250 ppm had no effect on growth. However, application of the monoethyl ester of succinic (MESA) and monoethyl ester of adipic (MEAdA) acids (1250 ppm) resulted in an increased leaf area, fresh and dry weight of leaves and stems. These compounds retarded chlorophyll degradation in both detached and intact leaves. Chlorophyllase activity of the control and treated leaves was measured and related to chlorophyll content. Delaying of senescence by treatment with monoesters resulted in greater chlorophyll and protein content, compared with the control. However, the chlorophyllase activity/chlorophyll *a* ratio in the treated plants decreased. Total peroxidase activity was higher in senescent leaves, but all treatments inhibited the increase of this enzyme activity. Prolonged carbon assimilative activity and enhanced leaf water use efficiency in treated plants was noted.

The process of plant aging after plant growth regulator (PGR) application is a problem of exceptional theoretical and practical interest because of the possibility of preserving leaf functional activity. Practically, increasing plant productivity and prolonging the storage period for cut flowers and vegetables is important (Karanov et al. 1988). Cytokinins are, indisputably, the most active of all PGRs used for this purpose. However, their application is relatively limited for two reasons: first, results obtained from cytokinin application in isolated leaf explants do not always correlate with results from intact plants (Al-Khatib and Paulsen 1985, Mishra and Gaur 1985); and, second, cytokinin used in plant products may be limited because of structural analogy between cytokinin molecule and purine bases of nucleic acids (Armstrong et al. 1976, Karanov and Iliev 1985).

Recently, it was shown that mono- and disubstituted esters of dicarboxylic acids retarded growth and chlorophyll degradation in leaf explants from mono- and dicotyledonous plants incubated in the dark (Karanov and Alexieva 1985, Alexieva 1987), monoesters being generally more effective than diesters. Some of the monoethyl esters also manifested senescence-delaying activity when applied on intact plants. Barley plants, treated when completion of expansion of the flag leaf had occurred with monoethyl esters of succinic and adipic acids, increased productivity because of continued leaf function. This led to better feeding of the spike (Alexieva 1987). In addition, the effects of several dicarboxylic acid derivatives on photosynthetic and transpiration rates have been reported. This includes the partial closing of the stomata and decreased transpiration rate in sugar beet (Kudrev and Petrova 1978), soybean leaves (Georgiev and Karanov 1989), and maize and barley seedlings (Georgiev et al. 1991). That is, they act as antitranspirants. They also increase photosynthetic rate and RuBP carboxylase activity in soybean plants (Velichkov et al. 1989, Vasileva and Dimitrova 1990).

We now present data showing the senescence-retarding effect of some dicarboxylic acid monoesters on growth, chlorophyll degradation, chlorophyllase and peroxidase activities, and some gas-exchange parameters in aging maize leaves.

Materials and Methods

Plant Material and Treatment

Plants from maize hybrid "Kneija"-650 were grown hydroponically in a greenhouse using Hellriegel solution and trace elements. Aerial plant parts were treated by spraying with water solutions of the β-monomethyl ester of itaconic acid (MEIA), monoethyl ester of succinic acid (MESA), or monoethyl ester of adipic acid (MEAdA) when the 4th leaves were well-developed. The concentration used was 1250 ppm. Leaf area was determined as the sum of the 4th to 9th leaf areas, calculated by the formula: leaf area = length × maximum width × 0.75 (Lasarov 1965). Fresh weight was measured 18 days after treatment (before 12th leaf appearance). Chlorophyll content of 4th and 5th leaves was measured before treatment and on day 7 and after day 14. Discs (8 mm) were isolated 24 h postspraying from 4th and 5th leaves of all variants and were placed in the dark on moistened filter paper for 96 h. Discs taken from 4th leaf of the control plants were placed on the test solution and identically incubated.

Chlorophyll Content

Chlorophyll content was determined in all cases by extraction with 80% acetone, followed by measurement of the optical density at 663 and 645 nm.

Chlorophyllase Activity

Chlorophyllase (Chlase) (chlorophyll-chlorophyllidohydrolase, EC 3.1.1.14) activity in maize leaf tissues was determined by Holden's method (1961) and a modification of Sudyina et al. (1972). The acetone preparations from the 5th leaf of controls and treated plants were used as a source of Chlase enzyme. Approximately 1 g fresh material was ground with 15 ml acetone and the suspension was filtered through a Büchner funnel. The residue washed with acetone to remove as much of the pigment as possible (full bleaching), dried in a vacuum for 1 hr at room temperature, and powdered. One hundred milligrams of this powder was incubated with 1.5 ml 100 mM phosphate buffer (pH 7.2) and 1 ml of chlorophyll solution (131 μg chl *a*). These conditions were established as the most appropriate for the plant material used (data not shown). Several sources of chlorophyll were found to be equally satisfactory as a substrate, and, in these experiments, *Aspidistra* sp. chlorophyll was used. Leaves were extracted with cold acetone and extracts were kept at 0–4°C. The reaction was carried out in the dark at 25°C for 10 min, and was shaken in a water bath. After filtration, the filtrate was partitioned against diethyl ether and 0.2 N KOH and the chlorophyll retained in the ether (upper) phase was estimated at 663 and 645 nm.

Total Peroxidase Activity

Total peroxidase (TPOX) (EC 1.11.1.7) activity was determined according to Boyarkin (1951), using a modification, measuring the optical density at 600 nm (Dencheva and Klisurska 1974). Approximately 0.5 g fresh material was homogenized with 5 ml of 100 mM borate buffer (pH 8.5). The crude enzyme extract obtained after centrifugation (15,000 *g*, 30 min). The assay medium contained 0.5 ml acetate buffer (pH 4.7); 0.7 ml, 5 mM benzidine dihydrochloride; 0.3 ml, 40 mM H_2O_2; and 0.1 ml crude enzyme. The assay was carried out in a series of cuvettes by adding reaction medium, enzyme extract, and H_2O_2. The cuvettes were then covered, mixed, and allowed to incubate for 1 min. The reaction was initiated immediately by adding H_2O_2. A cuvette containing all components except H_2O_2 was used as a control. All operations were carried out at 0–4°C. Enzyme activity is expressed as units mg protein^{-1} · min^{-1}, where one unit of activity is the amount of enzyme extract producing a ΔA of 0.01 in 1 min.

Gas Exchange

Net photosynthetic rate (*A*), transpiration rate (*Tr*), and stomatal resistance (R_s) of 5th leaf were determined on days 1, 3, 7, 11, and 15 after the treatment by using a portable gas-exchange monitor LI-6000 (LI-Cor, USA) and under natural conditions (leaf temperature 28–34°C, and irradiance 1200–1600 μmol · m^{-2} · s^{-1}).

Protein Content

Total soluble protein content was estimated by the method of Lowry et al. (1951).

The results were statistically analyzed using Fisher's criteria.

Results and Discussion

Retardants are synthetic chemical substances that inhibit plant growth without inducing formative effects (Cathey 1964). As previously described, dicarboxylic acid derivatives possess growth-retarding activity (Karanov et al. 1975, Alexieva and Karanov 1987a,b). The application of monoesters of succinic and adipic acid resulted in statistically significant increases in area, and in fresh and dry weight of leaves and stems (Table 1). A trend toward increasing the root weight was observed. MEAdA was the most effective compound on growth, while MEIA was least effective. These results suggest that the retardant effect is plant- and chemical-specific.

The delay of chlorophyll destruction is typical for some retardants. In our experiments, the 4th leaf contained 5.98 mg chl · *a* + *b*/dm^2 (initial state) when fully developed; however, after 14 days the chlorophyll content was only 15.4% of the initial state (Table 2). Treatment of the plants resulted in retarded chlorophyll degradation during aging. The line of activity was MEAdA (55.4%), > MEIA (43.5%), > MESA (31.5%) as compared to the control. Chlorophyll decrease in the 5th leaf was less pronounced (Table 2). Seven days posttreatment the control plants retained 79.6% of the initial chlorophyll content; this fell to 37.7% on day 14. Initially, leaf aging was expressed as less (day 7 post-

Table 1. Influence of MEIA, MESA, and MEAdA on the growth of young maize plants.

Treatment	Leaf area (cm^2)	Fresh weight (g/plant)				Dry weight (g/plant)			
		Leaves	Stems	Roots	Total	Leaves	Stems	Roots	Total
Control	966	27.5	43.3	29.6	100	4.07	3.97	2.08	10.1
MEIA 1250 ppm	944	26.5	44.5	29.7	101	4.04	4.07	2.09	10.2
MESA 1250 ppm	1017	29.8	50.0	30.3	110	4.59	4.68	2.13	11.4
MEAdA 1250 ppm	1045	30.7	52.4	30.8	114	4.77	4.81	2.16	11.7
LSD 5%	72	1.9	4.4	NS	5.5	0.3	0.6	NS	0.6
LSD 1%	103	2.8	6.4	NS	7.9	0.4	0.8	NS	0.8

treatment) and the effect of monoesters varied from 6.8–14.7% relative to controls. After intensification of the degradative processes (day 14 posttreatment), monoethyl adipate retarded chlorophyll destruction by more than 50% and the monoesters of itaconic and succinic acids by 29% and 26%, respectively.

The chlorophyll content in dark incubated leaf discs from intact plants decreased sharply, and after 96 h was only 16% (for the 4th leaf) and 18% (for the 5th leaf) of the initial content. Under these conditions, however, the "antisenescence" effect of the compounds was lest evident (data not shown).

The compounds investigated, however, manifested a high activity in retarding aging when applied directly (Fig. 1); however, at a concentration of 10 mM, almost all monoesters were phytotoxic. Maximal effect in retarding chlorophyll degradation was noted using 0.1 mM monoethyl adipate. These results confirm data from other tests (radish and barley leaf explants) where the monoethyl ester of adipic acid was also the most active (Alexieva 1987).

The observed senescence-retarding effect we noted could be due to reduced chlorophyll degradation, increased chlorophyll synthesis, or a combination of both. Though some chlorophyll degradation in leaves may result from photooxidation of the pigments, the fact that leaves lost chlorophyll in the dark indicates that the degradation in vivo is, at least, partially enzymatic. The enzyme(s) responsible for chlorophyll degradation per se has not been clarified and the existing evidence is circumstantial (Kato and Shimizu 1985).

Two enzymes in vitro have been shown to be capable of degradation. Chlase, which cleaves the phytol side chain with the production of free phytol and chlorophyllide, has been demonstrated (Holden 1974, Ellsworth et al. 1976). Peroxidative degradation has been proposed as an alternative system (Matile 1980, Martinoia et al. 1982). Peroxidase bleaches chlorophyll in the presence of H_2O_2 and certain phenols. Crude preparation of peroxidase from flavedo of *Citrus sinensis* (Huff 1982) and *Nicotiana tabacum* (Kato and Shimizu 1985) can degrade chlorophyll in vitro. Another possible enzymatic degradative pathway for chlorophyll bleaching in vitro has been established. Lipoxygenase-mediated polyunsaturated fatty acid oxidation produces free fatty acid radicals, which react with chlorophyll and oxydate it (Hildebrand and Hymowitz 1982, Klein et al. 1984). However, this enzyme is present mainly in nongreen tissues (seeds and tubers). So, we examined the effects of dicarboxylic acid monoesters on the Chlase and TPOX activities in control and treated maize leaves. Results in Table 3 indicate that in all treated plants Chlase activity (μg chl.*a* hydrolyzed/10 min/mg protein) was lower, compared with the control. Through this action, monoesters retard the catabolism of the cell and cause a maintenance in chlorophyll content. However, the accessibility of the enzyme and substrate must also be taken into account as part of the mode of action. Therefore, Chlase activity/chl.*a* ratio was shown (Table 3).

Table 2. Influence of MEIA, MESA, and MEAdA on the chlorophyll content (*a* + *b*) of aging leaves of intact maize plants (mg/dm^2).[a]

Treatment	Days after treatment			
	4	5	4	5
Control	2.84	4.83	0.92	2.29
MEIA 1250 ppm	3.18	5.34	1.32	2.96
MESA 1250 ppm	3.00	5.16	1.21	2.89
MEAdA 1250 ppm	3.05	5.54	1.43	3.51
LSD 5%	NS	0.56	0.21	0.33
LSD 1%	NS	0.81	0.31	0.47

[a] Initial state chl. *a* + *b*: 4th leaf, 5.98: 5th leaf, 6.07.

Before spraying, the TPOX activity was 7.12 U/mg protein, and 15 days later it was 9.98 U/mg protein in control leaves. Similar results were shown by Grill et al. (1980) and Kato and Shimizu (1987), who noted a sharp increase of enzyme activity of senescing leaves. Day 15 after spraying, the TPOX activity in treated leaves was inhibited with 15–30%, compared to control (Table 3). Conse-

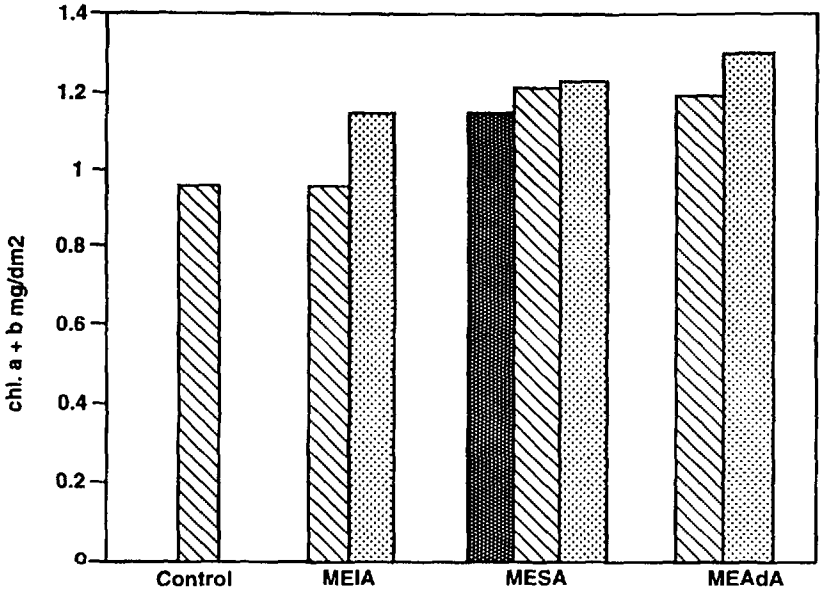

Fig. 1. Chlorophyll content in maize leaf explants from control plants incubated on test solutions. Concentrations: ■ 10 mM, ▨ 1 mM, ▩ 0.1 mM; *, phytotoxicity. Control: Chl.*a* + *b*, initial state 5.92 mg/dm^2. LSD: 5% = 0.20, 1% = 0.27.

Table 3. Influence of MEIA, MESA, and MEAdA on protein, chlorophyllase, and total peroxidase activities of 5th maize leaves.

Treatment	Soluble protein (mg/g)	Chlase activity[a]	Chlase act/chl.*a* ratio[b]	TPOX activity (U 600 nm/mg protein)
Control	4.21	6.20	5.96	9.98
MEIA				
1250 ppm	5.28	5.74	4.53	8.55
MESA				
1250 ppm	5.34	5.96	4.52	8.08
MEAdA				
1250 ppm	6.31	5.57	3.30	7.06
LSD 5%	0.54	0.37	0.8	0.91
LSD 1%	0.78	0.53	1.2	1.31

[a] Chlase activity (μg chl.*a* hydrolyzed/10 min/mg protein).
[b] Chl.*a* mg/g fresh weight.

Table 4. Influence of MEIA, MESA, and MEAdA on the photosynthetic rate (*A*) (mg $CO_2 \cdot m^{-2} \cdot s^{-1}$) of 5th maize leaf.

Treatment	Days after treatment				
	1	3	7	11	15
Control	2.00	1.84	1.06	0.41	0.32
MEIA 1250 ppm	2.02	1.71	1.11	0.45	0.40
MESA 1250 ppm	1.93	1.74	1.10	0.53	0.49
MEAdA 1250 ppm	1.95	1.83	1.12	0.59	0.55
LSD 5%	NS	NS	NS	0.08	0.12
LSD 1%	NS	NS	NS	0.11	0.17

quently, treatment of the plants caused depression of both enzyme activities.

Protein content in treated leaves was higher (Table 3). Probably dicarboxylic acid monoesters help in lowering protease activity.

Since photosynthesis is directly influenced by chlorophyll content, the photosynthetic rate may be preserved by delaying sensecence. A week following spraying there were no observed differences between *A* of control and treated plants. However, on days 11 and 15 the rate of CO_2 fixation in 5th leaf of treated maize plants indicated prolonged ability to fix significant amount of CO_2 (Table 4). The higher carbon assimilative activity in treated leaves resulted from retarded chlorophyll degradation, and suggested preservation of the photosynthetic apparatus.

The first three measurements of gas exchange showed that the stomata of treated plants were relatively more closed and the *Tr* was inhibited to a greater extent than *A* (Tables 5 and 6). Our data agree with those published (Georgiev and Karanov 1989, Velichkov et al. 1989). These data also confirm the standpiont for antitranspirant action of the retardants (Nickell 1982). Increased *A* at the end of the period observed correlated with the *Tr* augmentation, but to a smaller extent than for *A*, and the stomata were largely opened (Tables 4–6). The effect of PGRs could be estimated not only through their action on *A* and *Tr*, but also by the *A*/*Tr* ratio. In leaves, water use efficiency (WUE) is the ratio of carbon assimilated to water loss by transpiration (Turner 1986). The data showed that this ratio increased in treated plants (Table 7). Statistically significant differences were observed on day 7 post-treatment. All later measurements marked an en-

Table 5. Influence of MEIA, MESA, and MEAdA on stomatal resistance (R_s) (cm · s^{-1}) of 5th maize leaf.

	Days after treatment				
Treatment	1	3	7	11	15
Control	0.41	0.51	1.17	2.91	2.78
MEIA 1250 ppm	0.46	0.58	1.24	2.71	2.60
MESA 1250 ppm	0.45	0.57	1.22	2.44	2.34
MEAdA 1250 ppm	0.46	0.56	1.16	2.36	2.27
LSD 5%	NS	0.04	NS	0.32	0.41
LSD 1%	NS	0.06	NS	0.46	0.59

Table 6. Influence of MEIA, MESA, and MEAdA on the transpiration rate (*Tr*) (mg $H_2O \cdot m^{-2} \cdot s^{-1}$) of 5th maize leaf.

	Days after treatment				
Treatment	1	3	7	11	15
Control	335	270	99.1	62.2	60.1
MEIA 1250 ppm	333	243	89.1	61.7	65.7
MESA 1250 ppm	316	248	92.4	71.8	70.5
MEAdA 1250 ppm	321	249	96.6	70.7	72.1
LSD 5%	NS	18	9.8	7.0	8.1
LSD 1%	NS	26	14.0	10.1	11.7

Table 7. Influence of MEIA, MESA, and MEAdA on the water use efficiency (WUE, mg $CO_2 \cdot m^{-2} \cdot s^{-1}$/g $H_2O \cdot m^{-2} \cdot s^{-1}$) of 5th maize leaf.

	Days after treatment				
Treatment	1	3	7	11	15
Control	5.95	6.80	10.7	6.59	5.20
MEIA 1250 ppm	6.07	7.02	12.5	7.36	6.07
MESA 1250 ppm	6.10	6.99	11.9	7.39	6.88
MEAdA 1250 ppm	6.07	7.37	11.6	8.37	7.65
LSD 5%	NS	NS	1.64	0.92	1.33
LSD 1%	NS	NS	2.36	1.33	1.91

hanced WUE of the treated leaves. On day 15, MEAdA caused an increase of WUE by more than 45% compared to the control. These results confirmed results of certain dicarboxylic acid derivatives on soybean leaves (Velichkov et al. 1989).

Our data suggest that these PGRs should be field tested. Treatment should be the early development stage (4th to 5th leaf) because of logarithmic growth and tasseling and silking, when the needs for water are great and chlorophyll content begins to decrease, because of leaf senescence.

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