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Effect of Putrescine, 4-PU-30, and Abscisic Acid on Maize Plants Grown under Normal, Drought, and Rewatering Conditions

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Abstract. The experiments were carried out with maize (*Zea mays* L.) seedlings, hybrid Kneja 530, grown hydroponically in a growth chamber. Twelve-day-old plants were foliar treated with putrescine, N^1-(2-chloro-4-pyridyl)-N^2-phenylurea (4-PU-30), and abscisic acid (ABA) at concentrations of 10^{-5} M. Twenty-four hours later the plants were subjected to a water deficit program, induced by 15% polyethylene glycol (PEG; molecular weight, 6,000). Three days after drought stress half of the plants were transferred to nutrient solution for the next 3 days. The effects of the water shortage, rewatering, and plant growth regulator (PGR) treatment on the fresh and dry weights, leaf pigment content, proline level, relative water content (RWC), transpiration rate, activities of catalase and guaiacol peroxidase, hydrogen peroxide content, and level of the products of lipid peroxidation were studied. It was established that the application of PGRs alleviated to some extent the plant damage provoked by PEG stress. At the end of the water shortage program the plants treated with these PGRs possessed higher fresh weight than drought-subjected control seedlings. It was found also that putrescine increased the dry weight of plants. Under drought, the RWC and transpiration rate of seedlings declined, but PGR treatment reduced these effects. The accumulation of free proline, malondialdehyde, and hydrogen peroxide was prevented in PGR-treated plants compared with the water stress control. The results provided further information about the influence of putrescine, 4-PU-30, and ABA on maize plants grown under normal, drought, and rewatering conditions.

Key Words. Maize—Putrescine—4-PU-30—ABA—Drought

More than any other single environmental factor the shortage of water limits plant growth and crop productivity in many regions (Boyer 1982). Genetic modification of plants by breeding to allow growth and yield under unfavorable conditions is a solution to problems of environmental stress. However, this approach is time consuming and demands sustained efforts. There is much evidence that plant responses to unfavorable environments can be modulated by various plant growth regulators (PGRs). Undoubtedly, abscisic acid (ABA) plays a central role for improvement of plant drought resistance by its effect on stomata. The endogenous level of ABA rises markedly in response to water stress (Bradford and Hsiao 1982, Mizrahi et al. 1971, Zabadal 1974), and exogenous application of ABA and its analogs reduced the damage caused by drought and other stresses (Dorffling et al. 1990). Similarly, water shortage provoked sharp increases in endogenous levels of another group of PGR, polyamines (Turner and Stewart 1986). Polyamine titers also increased after many other abiotic stresses (Flores 1991). Moreover, numerous effects of the exogenous application of polyamines have been cited. Many reports refer to the capability of polyamines to retard senescence-linked processes (Kaur-Sawhney and Galston 1991) and to ameliorate osmotic stress (Capell et al. 1993) and herbicide stress (Zheleva et al. 1994). Although it is difficult to ascribe a single mechanism for the polyamine protective action, it is assumed that because of their unique properties as biological poly-

Abbreviations: PGR(s), plant growth regulator(s); ABA, abscisic acid; 4-PU-30, N^1-(2-chloro-4-pyridyl)-N^2-phenylurea; putrescine, 1,4-diaminobutane; PEG, polyethylene glycol; RWC, relative water content; FW, fresh weight; GDHP, guaiacol-dehydrogenated product; MDA, malondialdehyde; LSD, least significant difference; DW, dry weight; SOD, superoxide dismutase.

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cations, polyamines bind to acidic sites of nucleic acids and cell membrane phospholipids, thereby stabilizing their structure and preventing the breakdown of macromolecules under stress conditions (Altman et al. 1982). However, polyamine functioning as free radical scavengers could not be excluded (Bors et al. 1989).

Cytokinins also take part in the regulation of plant response to stress (Georgiev et al. 1992, Iliev et al. 1995), which may be the result of their possible function as antioxidants (Leshem et al. 1981). Georgiev et al. (1992) reported a strong protective effect of phenylurea cytokinin 4-PU-30 on marrow (*Cucurbita pepo* var. girimontia L.) after chilling stress. Puneva and Iliev (1995) and Iliev et al. (1995) demonstrated that 4-PU-30 added to the nutrient medium of tobacco callus showed higher antistress activity than kinetin. According to Stoyanov et al. (1994), this compound alleviated to some extent the injuries caused by high salinity.

On the other hand, increasing evidence suggests that many damaging environmental stressors (including water shortage) have their effects directly or indirectly through the formation of activated oxygen after impairment of electron transport systems (Smirnoff 1993). Plants subjected to water stress undergo increased exposure to activated forms of oxygen and accumulation of free radicals associated with damage to membranes and buildup of lipid peroxides (Smirnoff 1993, Zhang and Kirkham 1994); so, exogenous application of some free radical scavengers could decrease the membrane damage and increase the enzymatic defense systems against active oxygen forms.

The aim of our investigation was to compare the effects of PGRs of differing chemical structure and physiological action on growth and on some stress markers and stress defense systems of young maize plants subjected to water stress and rewatering.

Materials and Methods

Plants and Treatment

The experiments were carried out with maize (*Zea mays* L.) seedlings, hybrid Kneja 530, grown hydroponically on Hoagland-Arnon solution and trace elements in a growth chamber (12/12-h photoperiod, 25°C, 65% relative humidity, 160 $\mu mol \cdot m^{-2} \cdot s^{-1}$ photon flux density). Ten-day-old plants were foliar treated with 1,4-diaminobutane (putrescine), 4-PU-30, and ABA at concentrations of 10^{-5} M. We applied putrescine at this concentration because in previous experiments we found that concentrations of 10^{-3}, 10^{-4}, and 10^{-5} M caused similar effects in polyethylene glycol (PEG)-stressed maize seedlings (Todorov et al. 1995). Twenty-four hours later some of the plants were subjected to a water deficit program, induced by 15% PEG, MW 6,000 (water potential −0.5 MPa) in the nutrient solution. Three days after drought, half of the stressed plants were transferred to nutrient solution for the next 4 days (rewatering period), and the other half remained in PEG. Thus, at the end of experimental period there were PGR-treated and water-treated plants, grown at normal, drought, and rewatering conditions. The final treatments were in four replications of two plants each.

Relative Water Content and Transpiration Rate

The leaves were weighed and floated in 50-mL bottles with bidistilled water. The weights of leaves at full turgor were measured 24 h later. The relative water content (RWC) of the leaves was calculated by the formula of Turner (1986). The transpiration rate was determined as the difference in weight of turgid shoots and those subjected to light (160 $\mu mol \cdot m^{-2} \cdot s^{-1}$) and temperature (30–32°C) for 10 min. The transpiration rate was expressed as percent of the fresh weight (FW) of the plants.

Enzyme Extraction and Assay

Enzymes were extracted at 4°C from about 0.5 g of fresh leaf material, using a mortar and pestle, with 100 mM potassium phosphate buffer (pH 7.0). Extracts were centrifuged (15,000 × g, for 30 min), and the supernatants were used for the assays. Catalase (EC 1.11.1.6) was determined according to Brennan and Frenkel (1977) by measuring the rate of hydrogen peroxide (H_2O_2) disappearance in the enzyme reaction mixture. The reaction was stopped by the addition of 20% titanium tetrachloride in concentrated HCl, v/v. The absorbance was read at 415 nm against a water blank. Peroxidase (EC 1.11.1.7) was measured by the method described by Dias and Costa (1983). The optical density of the reaction mixture at 470 nm was recorded. The linear initial reaction rate was used to estimate the activity, which is expressed in μmol of guaiacol-dehydrogenated product (GDHP) formed per mg of protein per min, using the extinction coefficient of 26.6 $mM^{-1}\ cm^{-1}$ for GDHP.

Biochemical Analyses

Chlorophyll was extracted with 80% cold acetone and was estimated spectrophotometrically (Arnon 1949). Potassium phosphate buffer (pH 7.0, 100 mM) extractable protein was estimated by the method of Bradford (1976), using bovine serum albumin as a standard. Proline was extracted with 3% sulfosalicylic acid and was measured according to Bates et al. (1973). Malondialdehyde (MDA) was estimated as the thiobarbituric acid-reactive material using the molar extinction coefficient 155 $mM^{-1}\ cm^{-1}$. The endogenous level of hydrogen peroxide was measured spectrophotometrically (λ = 390 nm) after incubation of 0.1% trichloroacetic acid leaf extract with 1 M KI. The H_2O_2 content was calculated using a standard curve.

The results presented are the mean of at least three independent experiments. The data were analyzed statistically, and the least significant difference (LSD) was used to evaluate differences between the treatments.

Results

The application of 4-PU-30 and ABA caused a decrease of the FW of plants grown at optimal conditions 4 days after the treatment, but later the FW of plants reached the level of the control (Fig. 1). When the maize plants were subjected to water stress by incubation with PEG solutions, their growth was inhibited strongly. At the end of the experimental period the FW of the drought-stressed control plants was 37% that of the nonstressed control. The plants treated with putrescine and subjected to water deficit possessed a higher FW than the stressed control at the end of the water shortage program. Although the

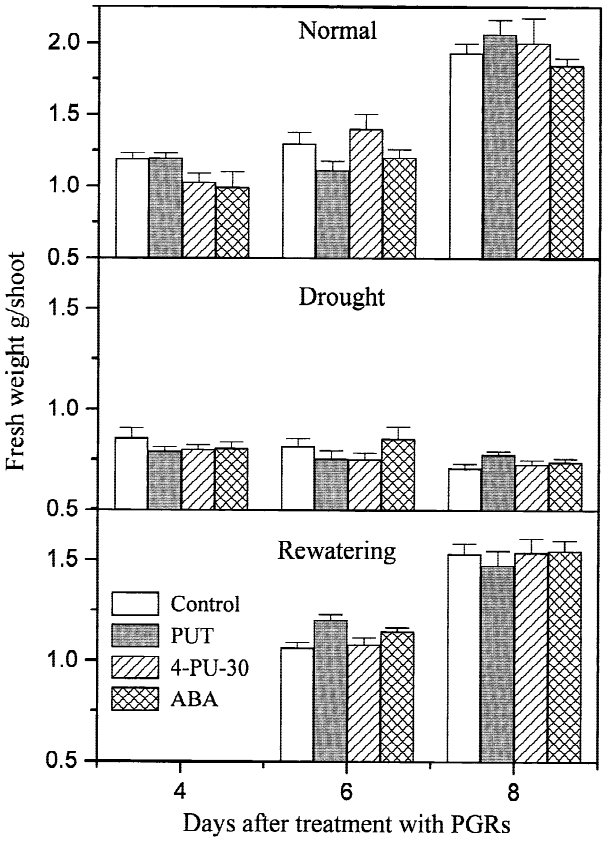

Fig. 1. Influence of putrescine, 4-PU-30, and ABA on the FW of maize seedlings grown at different conditions. The values are the means of three experiments each with four replications.

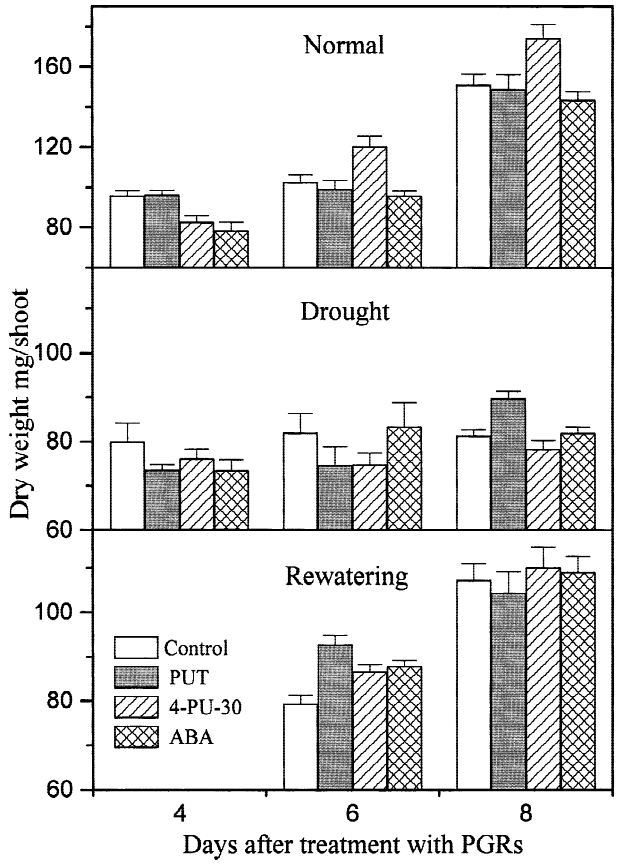

Fig. 2. Influence of putrescine, 4-PU-30, and ABA on the DW of maize seedlings grown at different conditions. The values are the means of three experiments each with four replications.

leaves were wilted, they recovered turgor and survived after rewatering. It was found that putrescine and ABA increased the FW of the plantlets 2 days after rewatering, but the effects disappeared at the end of the period. Among the PGRs used, only 4-PU-30 increased the dry weight (DW) of the seedlings grown at optimal conditions at the 6th and 8th days after treatment (Fig. 2). At the end of the drought period putrescine-treated plants possessed the greatest DW among plants subjected to osmotic stress.

The RWC has been used to determine the water status of tissues (Turner 1986). In our experiments, the RWC of the drought-treated control seedlings declined gradually from 97.85 to 70.48% (Table 1). The PGRs used reduced the water loss, and the most significant effects were observed at the end of the experimental period.

The plants treated with putrescine possessed the highest transpiration rate of the treatments under all growth conditions (Fig. 3). The well watered, ABA-treated plants showed lower transpiration rates at day 4, but in the next stages they were not significantly different from the control transpiration value. 4-PU-30 caused a significant increase in the transpiration rate relative to non-

Table 1. Relative water content (%) of maize seedlings during a drought stress program.

	Days after plant growth regulator application		
Treatment	4	6	8
Control	89.95	84.17	70.48
Putrescine	89.07	89.05	75.32
4-PU-30	89.92	86.60	80.10
ABA	92.27	88.85	76.15
LSD at 0.05	N.S.	1.70	2.00

Nonstressed control plants, 97.85%; rewatered control plants, 98.88%. The values are the means of three experiments each with four replications. N.S., not significant.

stressed control plants at the 4th and 6th days after treatment. As drought progressed, the transpiration rate of control plants decreased, and at the end of the experimental period it was only 29%. Upon rehydration, transpiration recovered to the initial rate. At the end of the experimental period (under water stress and recovered conditions) the transpiration rate of plants treated with

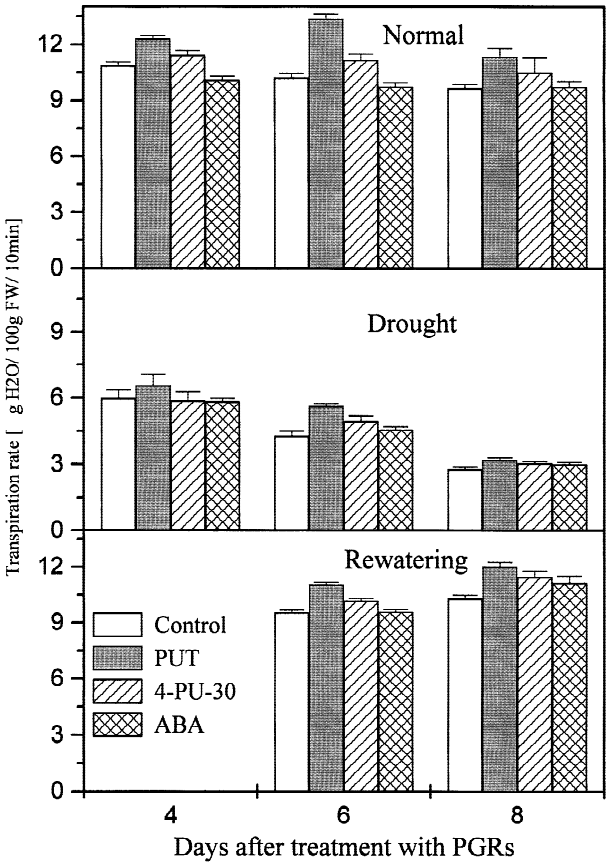

Fig. 3. Influence of putrescine, 4-PU-30, and ABA on the transpiration rate of maize seedlings grown at different conditions. The values are means of three experiments each with four replications.

Table 2. Influence of putrescine, 4-PU-30, and ABA on the pigment content (mg/leaf) of the first maize leaf at the end of the drought stress program.

Treatment	Chlorophyll *a*	Chlorophyll *b*	*a* + *b*	Carotenoids
Control	0.0527	0.0214	0.0742	0.0089
Putrescine	0.0738	0.0282	0.1020	0.0103
4-PU-30	0.0853	0.0337	0.1190	0.0121
ABA	0.0667	0.0260	0.0927	0.0100
LSD at 0.05	0.0162	0.0055	0.0215	N.S.

Nonstressed control plants (in mg/leaf): chlorophyll *a*, 0.07134; chlorophyll *b*, 0.0255; carotenoids, 0.0107. Rewatered control plants (in mg/leaf): chlorophyll *a*, 0.06220; chlorophyll *b*, 0.02354; carotenoids, 0.0096. The values are the means of three experiments each with four replications. N.S., not significant.

Table 3. Influence of putrescine, 4-PU-30, and ABA on the soluble protein content of leaves (mg/g FW), peroxidase (μmol GDHP/mg protein/min), and catalase (mmol H_2O_2 degraded/mg protein/min) activities in drought-stressed maize plants. The measurements were made at the end of the drought stress period.

Treatment	Soluble protein	Peroxidase activity	Catalase activity
Control	7.034	1.490	0.192
Putrescine	7.380	1.257	0.193
4-PU-30	7.470	1.332	0.307
ABA	7.919	1.248	0.248
LSD at 0.05	N.S.	0.088	0.024

Nonstressed control plants: protein, 7.323; peroxidase, 1.105; catalase, 0.415; rewatered control plants: protein, 8.191; peroxidase, 0.960; catalase, 0.220. The values are the means of three experiments each with four replications. N.S., not significant.

Table 4. Influence of putrescine, 4-PU-30, and ABA on the free proline (μmol/g FW), MDA content (nmol/g FW), and level of H_2O_2 (μmol/g FW) in maize leaves at the end of drought treatment.

Treatment	Free proline	MDA	H_2O_2
Control	2.42	82.30	0.423
Putrescine	1.89	58.35	0.245
4-PU-30	1.91	77.91	0.236
ABA	1.62	52.20	0.332
LSD at 0.05	0.25	8.46	0.016

Nonstressed control plants: free proline, 1.59; MDA, 49.81; H_2O_2, 0.180. Rewatered control plants: free proline, 2.04; MDA, 67.84; H_2O_2, 0.206. The values are the means of three experiments each with four replications.

PGRs was higher than those of the stressed and recovered control plants, respectively.

Water stress reduced the chlorophyll content of the first maize leaf, the effect being most dramatic on the chlorophyll *a* level (Table 2). Chlorophyll *b* and carotenoids were influenced to a lesser degree. There was a regreening after rewatering of the plants. Seven days after water deprivation the chlorophyll content of the first leaf was considerably greater in the PGR-treated plants, especially after the application of 4-PU-30. However, growth substances did not influence significantly the pigment content of nonstressed and recovered plants (data not shown). Moreover, the water shortage and PGRs had no effect on the leaf soluble protein content when protein was expressed as mg/g FW (Table 3). After 7 days of withholding water, catalase activity decreased by more than 50% and partly recovered after rewatering (Table 4). However, at the end of the experimental period 4-PU-30 and ABA increased catalase activity significantly in drought-stressed plants (160 and 129%, respectively, in relation to the PEG-stressed control), whereas putrescine had no effect. Unlike catalase activity, guaiacol peroxidase was increased greatly by water stress and then decreased after rewatering. In plants treated with growth substances, peroxidase had lower activity. However, these treatments did not influence peroxidase and catalase activities in either nonstressed or recovered plants (data not shown).

The levels of all stress markers studied (free proline,

MDA, and H_2O_2) were found to shift with drought conditions (Table 4), but the PGRs used prevented their accumulation compared with the water-stressed control. A lack of effect of 4-PU-30 on MDA content was observed.

Discussion

The results presented here show that the effects of the studied PGRs on the growth of maize seedlings depended on the duration of their influence as well as on the water supply. The nonstressed plants treated with 4-PU-30 and ABA had reduced FW, and DW at the 4th day, but at the 8th day the application of 4-PU-30 increased the DW considerably in relation to nonstressed control (Figs. 1 and 2). Stefanov et al. (1994) also reported a shift in the DW and a drop in the FW of maize seedlings after the application of 4-PU-30. Zheleva et al. (1994) observed a stimulating effect of putrescine (1 mM) on the FW, DW, and leaf area of pea (*Pisum sativum* L.) seedlings, grown hydroponically. In our experiment, putrescine increased the FW and DW of maize seedlings at the end of the drought stress program. Reports on the effects of ABA on growth are contradictory because its exogenous application can inhibit or promote root growth (Mulkey et al. 1983, Pilet and Chanson 1981). According to Kutschera and Schopfer (1986a, 1968b), ABA decreased the elongation rate of maize coleoptiles, and this effect was caused by an inhibition of cell wall loosening. The opposite effects of ABA on the growth of roots and shoots may be advantageous for the survival of plants under stress conditions. In shoots, ABA induced growth inhibition (Kutschera and Schopfer 1986a). We found that ABA reduced significantly the weight of the plants grown under normal conditions 4 days after it application. However, in drought conditions, negative ABA effects were not observed. Two days after the rewatering, ABA, 4-PU-30, and putrescine increased the growth, but after that the differences between control and PGR-treated plants were not significant. Both the FW/DW ratio and RWC are considered as good indices of water deficit stress intensity (Gamboa et al. 1991, Svenningsson and Liljenberg 1986). In our model system, water deprivation caused a decrease of these parameters in plants. However, the PGRs used retarded water loss during the water stress program (Table 1), but they did not influence the FW/DW ratio significantly (data not shown). The changes in RWC were accompanied by a decrease in the transpiration rate during water depletion (Fig. 3). The RWC and intensity of the transpiration of leaves decrease, and these results are in accordance with Lawlor (1970), Jacomini et al. (1988), and Gamboa et al. (1991).

It is known that ABA introduced exogenously increases stomatal resistance and decreases the transpiration rate (Zeevaart and Creelman 1988), whereas cytokinins show the opposite effect (Livne and Vaadia 1965, Velichkov et al. 1991). Our data on the effect of 4-PU-30 on the transpiration rate support this. However, the transpiration rate of ABA-treated plants grown at optimal conditions decreased at day 4; but at day 7, after osmotic stress, ABA-treated plants showed a higher transpiration rate than the stressed control. This is probably because of the improved water balance of the leaves of the PGR-treated plants during the stress program, since the transpiration rate depends not only on the influence of the compounds on the stomata, but also on the leaf water content. In shoots, ABA maintained the turgor pressure (Kutschera and Schopfer 1986a). On the other hand, a stimulation of root growth by ABA would enlarge the root system and thus, combined with increased hydraulic conductance (Bradford 1983), would increase water uptake and transpiration rate.

The retardation of chlorophyll degradation by putrescine and 4-PU-30 was established on plants grown under stress conditions (Table 2). A lack of effect of putrescine on the chlorophyll content was observed by Zheleva et al. (1994) using pea seedlings, and by Alexieva (1996) in experiments with wheat seedlings. 4-PU-30 elevated chlorophyll and total nitrogen content in maize leaves (Stefanov et al. 1994). However, the close relation between lipid peroxidation and chlorophyll level is well known (Dhindsa et al. 1981). Thus, the observed increase in the level of lipid peroxidation in control stressed plants (Table 4) could explain the decline in chlorophyll content in these plants (Table 2).

Drought stress induces oxidative events in plants (Moran et al. 1994, Sgherri and Navari-Izzo 1995). Normally plants are protected against such effects by a complex of antioxidant systems (Smirnoff 1993). As the formation of active oxygen species is favored under water stress conditions, one can expect some adaptive changes in the active oxygen-scavenging systems to mitigate the situation. Among the enzymes responsible for the scavenging of active oxygen species, superoxide dismutase (SOD) scavenges O_2^- during water stress, and enhanced capacity of this enzyme (Baisak et al. 1994) favors the formation of H_2O_2, the product of the SOD reaction. We could not determine the SOD activity; but because in our experiment, as well as in experiments of other authors with different plant species (Baisak et al. 1994, Quatracci and Navari-Izzo 1992, Zhang and Kirkham 1994), the catalase activity was found to be suppressed after water deprivation, we concluded that catalase had a reduced capacity to decompose H_2O_2. This suggestion was supported by the augmented level of H_2O_2 in the control plants (Table 4). However, other authors found that SOD activity was depressed with an increasing magnitude of water stress (Chowdhury and Chowdhuri 1985, Quatracci and Navari-Izzo 1992, Zhang and Kirkham 1994),

which could be the result of product inhibition of the SOD reaction by accumulated H_2O_2.

Peroxidases catalyze H_2O_2-dependent oxidation of substrates (RH_2). An increase (about 30%) in total peroxidase activity using guaiacol as an artificial substrate was brought about by water stress (Table 3). A rise of peroxidase activity under drought conditions was also observed in other studies (Badiani et al. 1990, Zhang and Kirkham 1994), which also indicates the formation of large amounts of H_2O_2 during water deprivation, and our data were consistent with this.

The results obtained about the activities of both enzymes studied as well as H_2O_2, proline, and MDA contents suggest that PGRs alleviated the water shortage to some extent. According to Chowdhury and Chowdhuri (1985), the H_2O_2 level can reliably be taken as an index for water stress tolerance. Increases in both free proline and the products of lipid peroxidation are considered as a drought-injury sensor (Aspinall and Paleg 1981, Baisak et al. 1994, Chowdhury and Chowdhuri 1985, Irigoyen et al. 1992). Proline, H_2O_2, and MDA did not accumulate in the plants treated with growth substances under the conditions of water depletion (Table 4). Additionally, after rewatering, the amounts of these stress sensors were normalized, supported their role as stress markers. It was noteworthy that in our model system putrescine was effective at 10^{-5} M, lower than the physiologically active concentrations described by other authors (Masse et al. 1985, Zheleva et al. 1994). In a previous study it was established that putrescine (10^{-3}, 10^{-4}, and 10^{-5} M) had similar effects on drought-stressed maize seedlings and was more effective than spermine and spermidine (Todorov et al. 1995). However, putrescine accumulation occurs in cereal leaf segments exposed to osmotic stress, whereas in dicotyledonous plants spermine and spermidine titers increase (Tiburcio et al. 1986). Probably, the effect of the putrescine applied exogenously is in connection with its protective function in cereal plants.

In conclusion, the application of putrescine, 4-PU-30, and ABA alleviated to some extent the plant damage provoked by drought stress in maize seedlings. Even though compounds we investigated differ in their chemical structure and plant growth-regulating properties, they provoked similar effects on RWC, transpiration rate, peroxidase and catalase activities and, stress markers in maize seedlings subjected to PEG-induced stress.

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