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DIFFERENT GROWTH RESPONSES OF THE AXIAL AND COTYLEDONARY TISSUES OF SUNFLOWER SEEDS TO C_2H_4 UNDER WATER AND SALT STRESSES

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Abstract

Germination of non-dormant sunflower *(Helianthus annuus L.)* seeds and the growth of their axial and cotyledonary tissues in response to exogenously applied C_2H_4 were examined under water and salt stresses. Seed germination was slightly promoted by exogenous C_2H_4 under both normal and water stressed conditions. However, the promoting effect of C_2H_4 on germination was not detected under salt stress. Under normal conditions, C_2H_4 was slightly effective in stimulating both axial and cotyledonary growth. The higher concentrations of KNO_3 gave some stress to the axial growth, in which C_2H_4 was promotive. However, the cotyledonary growth was rather enhanced with higher concentrations of KNO_3 (0.3M). Nevertheless, the KNO_3 - stimulated cotyledonary growth was completely inhibited by C_2H_4, and this phenomenon was also observed with other salts. In the cotyledons, C_2H_4 production was higher under the water-stressed condition than it was under normal conditions, and ACC (1.5mM) action was similar to C_2H_4, suggesting the involvement of endogenous C_2H_4 in the cotyledonary growth under water stress. This possibility was supported by the inhibition of cotyledonary growth with both NBD and Ag_2SO_4 as the inhibitors of C_2H_4 action. It thus became apparent that the axial tissue of the sunflower seed is quite different from that of the cotyledonary one in growth response to salt and water stresses and the salt stress is different from the water stress in function.

Introduction

C_2H_4 is a regulator of seed germination [1]. In a few species, such as *Plantago* [2] and *Potentilla* [3], C_2H_4 inhibits seed germination. On the contrary, in most species, including lettuce [4] and cocklebur [5], C_2H_4 promotes seed germination. Moreover, there are many kinds of C_2H_4 non-responsive seeds. However, response to C_2H_4 in purslane [6] and lamb's-quarters [7] seeds was induced or restored by nitrate addition. In other words, seeds responded to C_2H_4 only when they contained some amounts of nitrate. On the other hand, it has been reported [8] that the

Keywords: *Helianthus annuus L.*; 1-aminocyclopropane-1-carboxylic acid (ACC); Axial growth; Ethylene; Cotyledonary growth; Germination; Nitrate; 2, 5-norbornadiene (NBD); Salt stress; Water stress

germination promoting action of C_2H_4 in cocklebur seeds is enhanced under water-stressed conditions. Meanwhile, there are reports that the responsiveness of sunflower seeds to C_2H_4 appears when they are primarily [9, 10] and secondarily [11] dormant. The above-mentioned facts suggest that, even when sunflower seeds are nondormant, they may exhibit some responsiveness to C_2H_4 in the presence of nitrate or under water-stressed conditions. The present study was designed to investigate this possibility.

Experimental Section

Seed Source

Commercial sunflower (*Helianthus annuus L.*) seeds of Russian origin were obtained from a local dealer in Sendai, Japan. The preliminary test revealed that 98% of the seeds were nondormant and germinable at 25°C.

Germination Experiment

Twenty seeds were sown on two layers of filter paper wetted with 4 ml distilled water (control), KNO_3 or mannitol solution in 125 ml Erlenmeyer flasks. All flasks contained a small glass tube filled with 0.5 M NaOH solution as CO_2 absorbent in which a narrow tape of absorbent paper was inserted to increase the absorbing surface. On the other hand, control (C_2H_4 free) flasks contained another tube filled with 0.2 ml 0.25 M Hg $(ClO_4)_2$ solution as a C_2H_4 absorbent. The flasks were sealed with a skirted rubber stopper and 0.6 ml of 10 µl/l C_2H_4 was injected with a syringe to make a final concentration of 50 µl/l C_2H_4. Holes, produced by a needle on the rubber stopper, were sealed with a piece of adhesive vinyl tape. Triplicates of 20 seeds for each treatment were incubated at 25°C in the dark, and germinated seeds were counted at desired time intervals. After 24 hours, the flasks were opened and the germinated seeds removed. The flasks were sealed again, and C_2H_4 was re-injected as explained.

Growth Experiments

Three millimetre long axial segments were separated from the cotyledons of sunflower seeds. To determine tissue growth, the cotyledons and axial segments were pre-soaked in water for 5 hours at room temperature. Seed coats were removed, and the cotyledons were split into each part by hand. After the segments had been washed with tap water several times and rinsed with deionized water, they were blotted with paper towels. The initial fresh weight of 20 split cotyledonary or 50 axial segments were determined. The segments were placed on two layers of filter paper in 125 ml Erlenmeyer flasks, wetted with 3 ml of H_2O, KNO_3 or mannitol solution of a known concentration. Control flasks had tubes containing NaOH and $Hg(ClO_4)_2$, while other flasks had only the former. The flasks were sealed with rubber stoppers and sufficient volumes of C_2H_4 were injected by a syringe to make a final concentration of 50 µl/l. Holes produced by needles were similarly sealed. The flasks were incubated at 25°C in the dark. After 48 and 72 hours, the flasks were opened and fresh air was pumped into them with a 100 ml glass syringe. Sufficient amounts of solutions were supplemented at 48 hour operations. The flasks were stoppered, and then sealed after C_2H_4 was re-injected. At the end of the predetermined times, the fresh weight of the segments were determined again, and the gain in weight was calculated on a percentage basis. Axial growth was similarly measured, but terminated 20 hours after incubation. Therefore, the flasks were not opened during incubation of the axial tissues.

Cotyledonary Growth in the Presence of Some Chemicals

Twenty split cotyledons were prepared as before; they were weighed and placed on two layers of filter paper in each flask, wetted with 3 ml of H_2O, ACC and Ag_2SO_4 solutions of known concentrations under stressed or normal conditions. For treatment with NBD, a small glass tube with 10 µl NBD was placed in each flask. Glass tubes containing NaOH solution were included in all flasks in order to absorb CO_2. They were sealed and incubated at 25°C in the dark. After 48 and 72 hours, the flasks were opened for air replenishment, when NBD was again added. The final weights were determined after 96 hours.

C_2H_4 Production

Lots of twenty split cotyledons were soaked for 5 hours and placed separately on two layers of filter paper wetted with 3 ml distilled water, KNO_3 or mannitol solution in 125 ml Erlenmeyer flasks containing a test tube of 0.5 ml 2.5 M NaOH solution. The flasks were sealed with skirted rubber stoppers. At given times, after the start of incubation, 1 ml gas sample was withdrawn from the flask with a syringe and the C_2H_4 content was assayed with a gas chromatograph (Hitachi Ltd., Model 063) equipped with an alumina column and a flame ionization detector [5]. Then the fresh weights were measured, and the C_2H_4 production was calculated on the basis of nl/gram fresh weight/day.

Statistics

A completely randomized design was used in each experiment. Three or four replicates were used for each treatment, and the data were shown by the mean ± SE. Each experiment was repeated twice.

Results

Germination Response of Sunflower Seeds to C_2H_4 under Stressed Conditions

Under normal conditions in water, C_2H_4 significantly accelerated the germination of sunflower seeds at the beginning, but its effect disappeared gradually (Fig. 1). Similarly, C_2H_4 accelerated germination under a mannitol imposed water stress, although germination was delayed for about 24 hours (Fig. 1). Under a KNO_3 - imposed salt stress, not only a promotive C_2H_4 effect was not observed but also C_2H_4 caused a slight inhibition and the germination was further delayed.

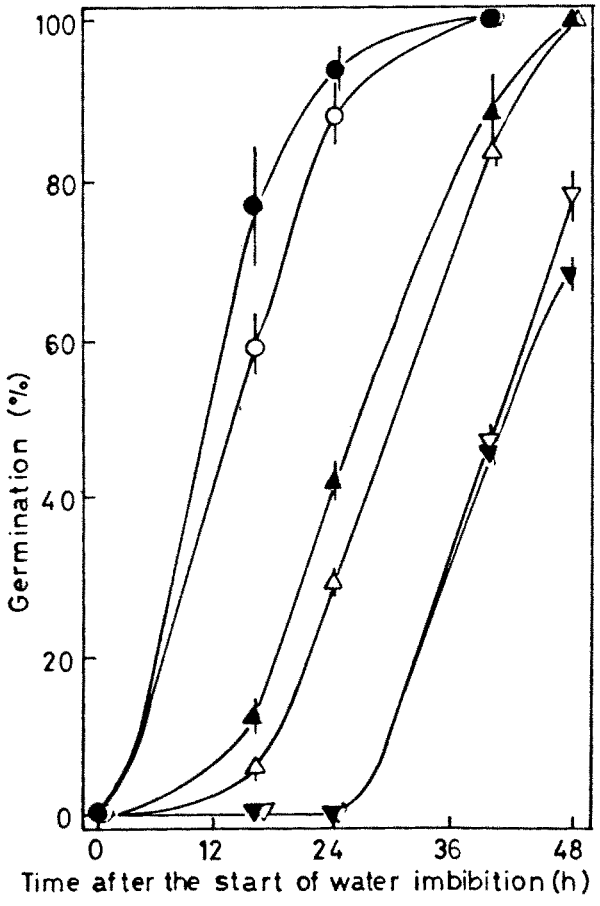

Figure 1. Germination responses of sunflower seeds to 50 μ l/l C_2H_4 (●, ▲, ▼) under normal conditions in H_2O (○, ●), under water stress imposed with 0.4 M mannitol (△ ▲) or under salt stress imposed with 0.3 M KNO_3 (▽, ▼). Data are shown by the means of 4 replicates with the vertical bars for SE.

Axial Growth in Response to C_2H_4 under Stressed Conditions

Axial growth decreased linearly with increasing water stress, i. e. by raising mannitol concentrations under the conditions employed. C_2H_4 was always promotive to the axial growth under these conditions (Fig. 2). On the other hand, KNO_3 had a slight promotive effect up to 0.1 M and only at 0.3 M did it strongly inhibit axial growth. In every case, however, C_2H_4 was significantly promotive to axial growth. (Fig.3).

Cotyledonary Growth in Response to C_2H_4 under Stressed Conditions

As in the case of axial tissue growth (Fig. 2), cotyledonary growth decreased proportionally with increasing water stress, however, in the presence of C_2H_4

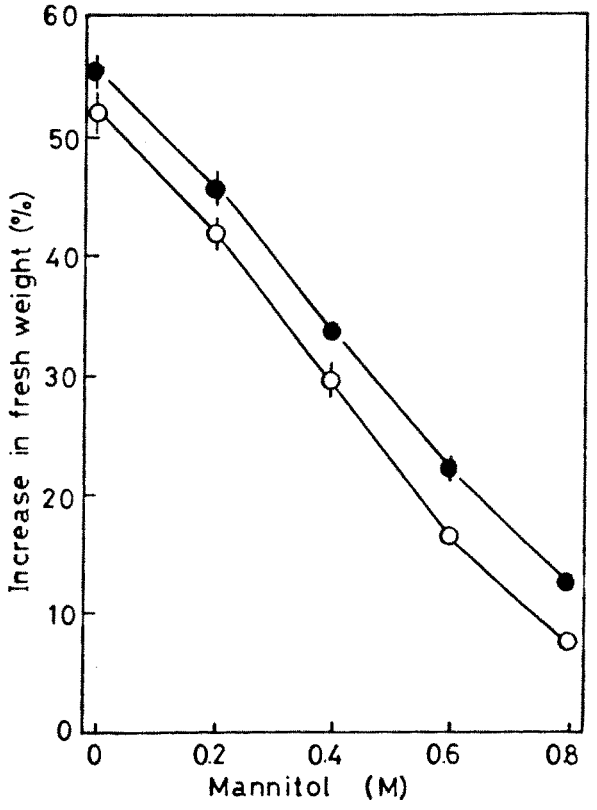

Figure 2. Growth response of axial tissues of sunflower seeds to 50 μ l/l C_2H_4 (●) under mannitol-imposed water stress. C_2H_4-free control flasks are shown with hollow (○) circles. The data are shown as in Figure 1.

some of the stress effects were relieved (Fig. 4).

As shown in Figure 5, however, the cotyledonary growth was enhanced with an increase in KNO_3 concentrations which is in contrast to both cases of mannitol (Fig. 4) and KNO_3 (Fig. 3) effects on axial growth. At 0.01 M KNO_3, C_2H_4 was promotive, but in the presence of 0.1 and 0.3 M KNO_3, cotyledonary growth was strongly inhibited. That is, the C_2H_4 action was completely reversed by higher KNO_3 concentrations.

Effect of C_2H_4 on Axial and Cotyledonary Growth under Various Salt Stresses

To test whether or not the reversal of C_2H_4 action, as shown in Figure 5, is also induced by other salts, both axial and cotyledonary segments were subjected to C_2H_4 under different salt solutions at 0.2 M (Table 1). The axial growth was greatly reduced by all salts, suggesting that 0.2 M salt solutions impose some stress on axial growth. However, the effect of C_2H_4 on axial growth was slightly promotive. In contrast, the cotyledonary growth was significantly stimulated with all salt solutions which suggests that 0.2 M salt solution is not a stress to cotyledonary growth. Nevertheless, C_2H_4 severely inhibited the salt-enhanced cotyledonary growth, regardless of the kinds of salts. In other words, the different kinds of salts imposed some

Table 1. Effects of C_2H_4 on the axial and cotyledonary growth of sunflower seeds under the salt stresses imposed by various salts

Salt (0.2M)	C_2H_4 (50 μ 1/1)	Increase in fresh weight (%)	
		Axis	Cotyledon
H_2O	-	94.0 ± 3.3	95.0 ± 1.8
	+	98.0 ± 2.4	98.7 ± 3.6
KNO_3	-	54.1 ± 2.0	161.4 ± 3.3
	+	60.1 ± 1.8	86.5 ± 4.8
KCL	-	58.3 ± 5.0	144.6 ± 4.0
	+	56.9 ± 2.7	112.7 ± 4.7
$NaNO_3$	-	36.7 ± 3.8	118.4 ± 7.2
	+	44.4 ± 0.7	73.8 ± 3.7
NaCl	-	41.3 ± 4.0	114.8 ± 6.4
	+	46.1 ± 3.9	88.1 ± 6.8

Axial and cotyledonary segments were pre-soaked for 5 hours and then incubated for 20 and 96 hours, respectively, at 25°C. Data are the mean ± SE of 4 replicates.

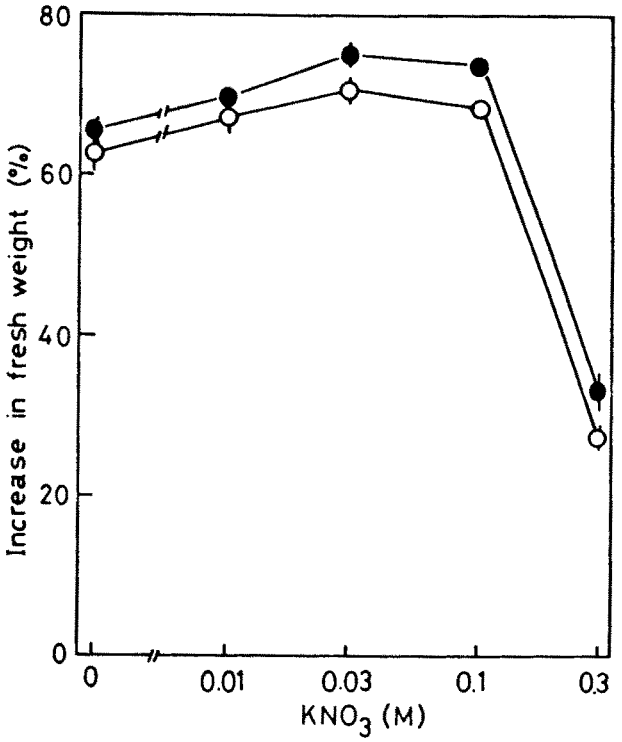

Figure 3. Growth response of axial tissues of sunflower seeds to 50 μ 1/1 C_2H_4 under KNO_3 - imposed salt stress. The symbols are the same as those in Figure 2.

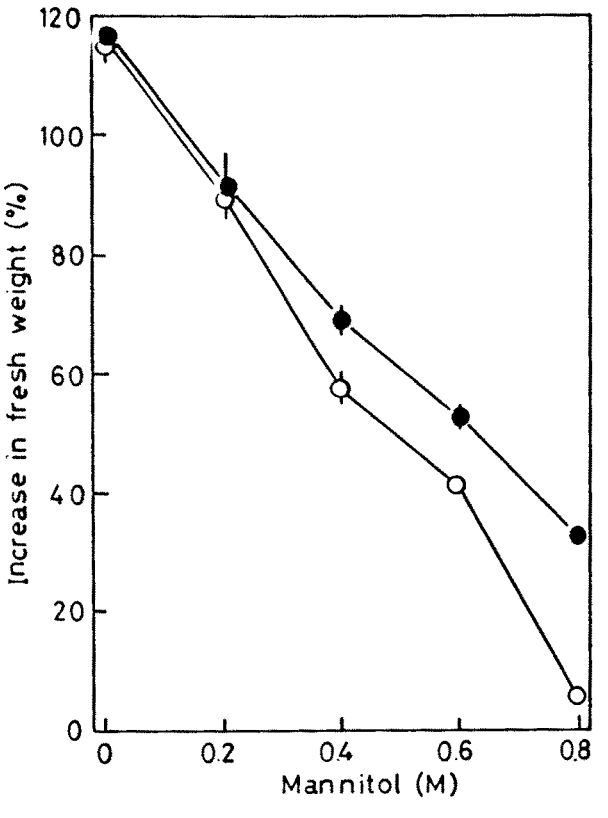

Figure 4. Growth response of cotyledonary tissues of sunflower seeds to 50 μ 1/1 C_2H_4 under mannitol-imposed water stress. The symbols are the same as those in Figure 2.

stress on the cotyledonary tissue when C_2H_4 was exogenously applied.

C_2H_4 Production in the Cotyledonary Tissues of Sunflower Seeds in Response to Water and Salt Stresses

It is well known that the various types of stresses stimulate C_2H_4 production in different tissues of many plant species [12]. In an experiment shown in Table 2, the C_2H_4 production from the cotyledonary tissues was examined at varying concentrations of mannitol or KNO_3.

As was expected, C_2H_4 production positively correlated with increasing mannitol concentrations. However, the salt stress imposed by 0.3 M KNO_3, unexpectedly strongly reduced C_2H_4 production, the rate of reduction was proportional to KNO_3 concentration. That is, the water stress which suppressed the cotyledonary growth enhanced C_2H_4 production, while KNO_3 which promoted growth, inhibited C_2H_4 production.

Effects of ACC, NBD and Ag_2SO_4 on Cotyledonary Growth under Water and Salt Stresses

Using ACC as a precursor of C_2H_4 production [13, 14], NBD as a competitive inhibitor of C_2H_4 production [15, 16], and Ag_2SO_4 as another inhibitor of C_2H_4 action [17], the growth behavior of sunflower cotyledons under water and salt stresses were further investigated. Similar to C_2H_4 effect, growth inhibition under mannitol-imposed water stress was less in the presence of ACC (Table 3). Moreover, ACC not only decreased the increment of KNO_3 - induced growth, but also reduced the cotyledonary growth below the level of non-stressed control, as C_2H_4 did (Table 3).

On the other hand, both NBD and Ag_2SO_4 decreased cotyledonary growth under water stress, suggesting the involvement of endogenous C_2H_4 in the cotyledonary growth under water stress. NBD significantly decreased the KNO_3 - induced cotyledonary growth. Ag_2SO_4 applied singly or combined with mannitol was also slightly inhibitory, but its combination with KNO_3 reduced cotyledonary growth drastically.

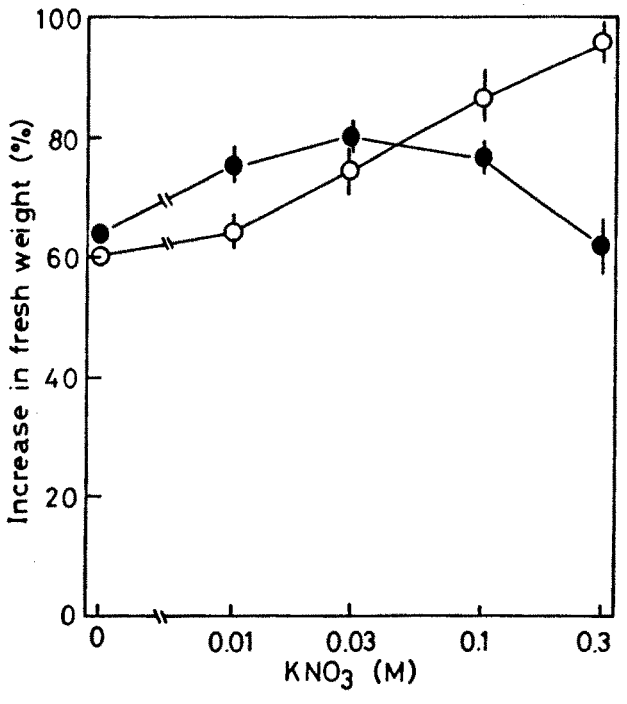

Figure 5. Growth response of cotyledonary tissues of sunflower seeds to 50 µ 1/1 C_2H_4 at varying concentrations of KNO_3. The symbols are the same as those in Figure 2.

Table 2. Promotion and inhibition of growth and C_2H_4 production in sunflower cotyledons under different degrees of water or salt stresses

Treatment	Growth (%)	C_2H_4 Production (%)
0.2 M Mannitol	- 23.9	19.2
0.4 M Mannitol	- 39.1	39.7
0.6 M Mannitol	- 64.1	120.6
0.03 M KNO_3	9.5	- 9.2
0.1 M KNO_3	32.0	- 13.1
0.3 M KNO_3	36.7	- 57.7

Cotyledonary segments pre-soaked for 5 hours were incubated in the presence (for growth assay) or absence (for C_2H_4 assay) of Hg $(ClO_4)_2$ solution for 3 days at 25°C in the flasks including a glass tube with NaOH solution. Control segments under H_2O showed 18.2%. Day $^{-1}$ in fresh weight increase and 13.0 nl. g fr. wt^{-1}. day^{-1} in C_2H_4 production.

Table 3. Effect of some chemicals on the cotyledonary growth in sunflower under water and salt stresses

Chemical	Increase in fresh weight (%)		
	H_2O	Mannitol (0.6 M)	KNO_3 (0.3 M)
H_2O (control)	99.4 ± 3.5	39.3 ± 0.7	174.3 ± 6.2
10 µl NBD	97.0 ± 2.4	29.6 ± 1.9	129.0 ± 3.0
1.5 mM ACC	102.7 ± 1.7	50.1 ± 0.5	62.7 ± 9.3
1.0 mM Ag_2SO_4	89.3 ± 3.6	34.8 ± 3.0	1.3 ± 0.6

Cotyledonary segments pre-soaked for 5 hours were incubated for 96 hours at 25°C. Data are the mean ± SE of 3 replicate determinations.

Discussion

According to some reports [9, 10, 11], sunflower seeds are very responsive to C_2H_4 only when they are primarily or secondarily dormant. In the present study, however, the germination of non-dormant sunflower seeds was also promoted by the application of C_2H_4 under normal and water-stressed conditions. This promotive effect of C_2H_4 was not enhanced by the addition of KNO_3 (Fig. 1). This slight germination promotion by C_2H_4 during the earlier stages of seed growth under both normal and water-stressed conditions seems to be due to the C_2H_4 enhancement of axial growth (Fig. 2). Interestingly, the germination of sunflower seeds in the presence of 0.3 M KNO_3 was slightly inhibited by C_2H_4 (Fig. 1). This may arise from the fact that the major thrust necessary to rupture the seed coat of sunflower seeds is generated by the axial growth on which 0.3 M KNO_3 caused a salt stress that was not recovered with C_2H_4 (Fig. 3).

Of importance is that the response of the cotyledonary tissues of sunflower seeds to C_2H_4 is completely different from that of the axial tissues (Figs. 4 and 5). Under mannitol-imposed water stress, the growth of the cotyledonary tissues, like the axial growth, declined gradually with increasing mannitol concentrations, but this decline was less in the presence of exogenous C_2H_4, similar to cocklebur [8]. However, KNO_3 solution at 0.2 or 0.3 M significantly increased the cotyledonary growth and reversed the action of C_2H_4 on its growth. In other words, KNO_3 at those concentrations was a stress factor to cotyledonary growth only when C_2H_4 was present (Fig. 5).

Moreover, similar results were obtained with other salts (Table 1), suggesting that C_2H_4 action on cotyledonary growth is reduced in the presence of many salts and it could be accepted as a general phenomenon. The question is why do the salt solutions become a stress factor against the cotyledonary tissue only in the presence of C_2H_4? One possibility for the different response to salt stress between the axial and cotyledonary tissues may result from the different permeabilities to salt solutions. Only when C_2H_4 is present, does the lower permeability of the cotyledonary tissues reach a level similar to that of the axial ones, and, as a result, C_2H_4 enables the cotyledonary tissues to accumulate toxic levels of salts, as in the axial tissues. These facts clearly indicate that the water stress imposed with mannitol differed completely from the salt stress in action. In other words, the action of C_2H_4 in cotyledons of sunflower seeds was reversed by the application of KNO_3 (Fig. 5).

Similar reversal of C_2H_4 action has been observed in the germination of cocklebur seeds by increasing the temperature [18]. In cocklebur seeds, high temperatures not only increase the rate of respiration but also the ratio of CN-resistant to CN-sensitive respiration [19, 20]. The inhibitory action of C_2H_4 at high temperatures is reported to come from a high temperature- induced, abnormally high engagement of the CN-resistant path which is inadequate for germination because of decreased energy conversion [21]. In CN-resistant respiration, the P/O ratio is one while in CN-sensitive respiration it is 3. Furthermore, it has been found that the O_2 consumption in germinating *Spergula arvensis* seeds is greater in a combination of C_2H_4 and KNO_3 than in C_2H_4 alone [22]. Accordingly, if KNO_3 greatly increases the electron flow via the CN-resistant path, C_2H_4 seems to further increase it and causes an abnormally high ratio between the two fluxes at high temperatures as it did in cocklebur seeds.

The C_2H_4 production from the cotyledonary tissues of sunflower is increased under the mannitol-imposed water stress (Table 2), which is in accordance with that in the broad bean [23]. The growth-stimulating effect of C_2H_4 in cotyledonary tissue increased with increased concentrations of mannitol (Fig. 4). Furthermore, the cotyledonary growth was promoted by ACC and inhibited by both NBD and Ag_2SO_4, the degree being greater under water stress than under normal conditions (Table 3). These results suggest that cotyledonary growth under water stress is dependent upon the endogenous C_2H_4, and its contribution to the rupture of the seed coat of sunflower seeds is greater under water stress than under normal conditions. It is thus likely that the regulation of C_2H_4 production in seeds may be of prime importance for plant survival under water stress and salt stress conditions, by increasing the role of their cotyledonary tissues and thereby permitting their adaptation to arid and saline environments. Under water stress, C_2H_4 increases the membrane permeability to H_2O so that enough turgor pressure is produced for axial tissues to rupture the seed coat. However, under salt stress low C_2H_4 production will lower the cell permeability to ions and prevent the toxic accumulation of salts. So, in saline environments lower C_2H_4 production, while in arid environments higher C_2H_4 production would result in a vigorous cotyledonary growth.

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