Relation of Water Soluble Substances in Fruits of Sugar Beet to Speed of Germination of Sugar Beet Seeds[1]

F. W. SNYDER, JOHN M. SEBESON AND J. L. FAIRLEY[2]

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At least 10 substances (9 are organic compounds) which may be potentially inhibitory to germination have been isolated from the fruits of sugar beets (2,5,6,7,8,11)[3]. Some of the compounds have not been identified as to chemical structure. Also, only limited data on the quantity of inhibitors in sugar beet fruits are presently available. The concentration of each of these substances which will adversely affect germination of sugar beet seeds remains to be determined critically. Of greater fundamental and practical importance, the causal relation between concentration of specific inhibitors in the fruit and speed of germination must be established.

Duym et al. (3) have concluded that the osmotic pressure exerted by inorganic substances in the seedball of the sugar beet is responsible for the inhibitory effect on germination. Specific conductance values of different seedball extracts may vary in the amount of electrolytes and these differences are reflected by the wheat growth test (10). Fröschel (4) observed that demineralized extracts of sugar beet fruits considerably inhibited germination of *Lepidium* and other seeds and that the extracts also emitted volatile substances which caused inhibition. He indicated the presence of specific organic substance(s) as the cause of the inhibition. According to Makino and Miyamoto (6,8), water soluble oxalate, which may exceed two percent in the fruits of some sugar beet varieties, may be causally related to germination performance.

This paper reports (a) results of physiological and chemical tests which indicate that at least one of the mechanisms suggested as the cause of delayed germination is untenable, (b) a significant correlation between water-soluble oxalate in the fruit and speed of germination of sugar beet seeds, (c) evidence that at least one other inhibitor besides oxalate also affects speed of

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[2] Research Plant Physiologist, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture; Professor, Lansing Community College; and Professor of Biochemistry, Michigan State University, East Lansing, Michigan respectively.

[3] Numbers in parentheses refer to literature cited.

germination in variety US 401, and (d) data on the distribution of water-soluble oxalate in the fruits of sugar beet variety US 401.

Methods and Materials

To ascertain some of the characteristics of the inhibitory substances in the water extract of sugar beet fruits (seedballs) of lot 4401, variety US 401, exploratory investigations were conducted involving specific conductance measurements, cryoscopic determinations, ashing, dialysis, vacuum distillation, ether extraction, treatment with lead acetate, calcium chloride, activated charcoal, and osmotic additives; addition of suggested inhibitors; and the use of a bioassay technique. The bioassay, designated as the wheat test, has been described previously (10). Briefly, the test compares the growth of wheat on aqueous extracts of seedballs with the growth of wheat on distilled water in 96 hours.

Thirty-six samples of seed from the variety US 401, each of which was harvested from a single plant grown in the field at East Lansing, Michigan, were used in the study of oxalate content. The speed of germination of each sample was determined by the blotter method.

The seedball extracts used for the physiological studies and chemical analysis were prepared by soaking air-dried seedballs in distilled water (usually 1 gm per 10 ml of water) for 18 hours in a refrigerator at approximately 4 C. The extract was decanted and filtered.

Oxalate was isolated from the extract according to a modification of the procedure suggested by Suzuki (6) and was as follows: A 25 ml sample of extract was adjusted to a pH between 5 and 6 with acetic acid. Fifteen ml of hot (80 C) 15% calcium chloride (calcium acetate may also be used) was added to the extract. The mixture was heated for an hour and a half at approximately 80 C to promote crystal enlargement. After cooling to room temperature, the solution was filtered on No. 2 Whatman paper and the precipitate was washed sparingly with distilled water. The precipitate was dissolved in 2 N hydrochloric acid heated to 80 C and the solution made alkaline with ammonium hydroxide diluted 1:1.

The solution was again heated in a water bath for one and a half hours at 80 C. After cooling to room temperature, it was filtered. The precipitate was washed twice with hot (80 C) 30% acetic acid and then dissolved in hot (80 C) 1 N sulfuric acid. The solution was increased to a volume of 50 ml. This procedure differs from that of Suzuki with respect to the addition of the acetic acid wash. The wash was found to be necessary because

determinations of the quantity of oxalate by the potassium permanganate method and by a colorimetric method involving indole failed to agree.

Apparently substances other than oxalate were co-precipitated with the calcium oxalate or adsorbed to it. The values for oxalate by the indole method were as much as double those for the permanganate method, which indicates that the impurities in the precipitate were intensifying the indole colorimetric test much more than the permanganate test. By washing the precipitate with 30% acetic acid (calcium oxalate is essentially insoluble in acetic acid), the impurities were removed from the calcium oxalate precipitate to the extent that the determinations of oxalate by both methods agree very closely.

The quantity of oxalate was determined by titration with potassium permanganate (9) and by means of a colorimetric method using indole (1). Twenty-five ml of the final solution were titrated with potassium permanganate. For the indole method, the reagent was prepared by dissolving 100 mg of very pure indole in 100 ml of concentrated sulfuric acid. A standardization curve was prepared from 1 N sulfuric acid containing from 0.1 to 1.0 mg of oxalic acid per ml. To 5 ml of oxalate solution in a test tube were added 5 ml of indole reagent by means of a pipette. After waiting 60 seconds the contents were mixed thoroughly. The test tube was placed in a water bath and heated to 80-90 C for 45 minutes. The solution was cooled to room temperature, transferred to a cuvette, and its absorbance measured in a colorimeter at a wave length of 525 mμ. A blank, consisting of 5 ml of 1 N sulfuric acid and 5 ml of indole reagent, was prepared with each set of samples to determine the zero reading of the instrument.

The proportion of the inhibitory substances removed from the sugar beet fruits during processing was determined for a sample from 4 different plants of variety US 401. These samples appeared to contain oxalate as the chief inhibitor. A water extract was prepared from (a) whole seedballs, (b) processed seedballs, and (c) the material removed by the hand-processing operation. A portion of each extract was analyzed for oxalate content and another portion was used for the wheat test.

Results

The data (Table 1) do not support the conclusion of Duym et al. (3) that the inhibitory effect of the sugar beet seedball on germination is largely osmotic. If the effect was osmotic and largely caused by the inorganic constituents, then the growth of

Table 1.—Characteristics of a variously treated seedball extract from bulked seed of lot 4401. US 400 sugar beet.

Treat No.	Treatment of extract*	Specific conductance (Mhos $\times 10^{-5}$)	Moles of solute by cryoscopy	pH	Growth of wheat on extract, as percent growth on distilled water
1	No treatment	98	0.086	6.9	58
2	Evaporated, ashed, reconstituted with distilled water	104	0.065	11.2	99
3	Dialyzed exhaustively under refrigeration	0	0.011	6.7	85
4	Hydrolyzed with conc. HCl, neutralized, dialyzed	0	....	6.6	96
5	Vacuum distillation, reconstituted with distilled water	95	....	7.3	57

* One gram of seedballs per 10 ml distilled water.

wheat in treatment 2 should have been approximately the same as in treatment 1. Treatments 3 and 4 indicate that the water-soluble substances are not completely dialyzable, but they are hydrolyzable. In another experiment in which treatment 4 was included, the growth of the wheat also was not significantly different from that on distilled water. The toxic material was not volatile, as indicated by treatment 5 and also by boiling the extract. Cryoscopic data indicated that the organic solutes constituted approximately a fourth of the total solutes. Additional evidence that the inhibitory action is more complex than an osmotic pressure effect is indicated by the fact that the more concentrated sodium chloride solution failed to suppress germination and growth of wheat as much as the seedball extract (Table 2).

Table 2.—Effect of osmotic pressure on growth of wheat in the wheat test.

Growth substrate	Specific conductance* (Mhos $\times 10^{-5}$)	Growth of wheat on substrate, as percent growth on distilled water
Seedball extract (1 : 7)	172	40
Sodium chloride solution	191	89
Sodium chloride solution	355	76

* Diluted 1 : 1

The data (Table 3) indicate some of the chemical characteristics of the inhibitory material. The toxicity of the extract is reduced only slightly by treating it with activated charcoal (treatment 2). Dialysis removed much more of the toxic materials (treatment 3). A combination of dialysis and activated charcoal

Table 3.—Effect of various treatments on the toxicity of the sugar beet seedball extract (lot 4401, US 400) in the wheat test.

Treatment number	Treatment of extract*	Specific conductance** (Mhos $\times 10^{-5}$)	Growth of wheat on extract, as percent of growth on distilled water
1	No treatment	550	25
2	Added activated charcoal, filtered	510	27
3	Dialyzed	14	70
4	Added activated charcoal, filtered, dialyzed	10	95
5	Dialyzed, added activated charcoal, filtered	18	98

* One gram seedballs per 4 ml distilled water.
** Undiluted extract.

removed the toxic materials. Since dialysis removed only a portion of the toxicity, some of the molecules were so large that they failed to pass through the pores of the cellophane tube. A combination of precipitation with basic lead acetate followed by filtering, precipitating the excess lead with monobasic potassium phosphate, and dialysis effectively removed the toxic substances. In one test, precipitation with calcium chloride followed by dialysis was not as effective as the lead acetate in removing the toxic substances. In summary, the inhibitory action seems to be caused by a combination of dialyzable and non-dialyzable substances. The most potent of the substances appear to be organic compounds which are dialyzable. Osmotic stress cannot account for the degree of inhibition observed in the wheat test.

In the procedures employing precipitation, the precipitability of given organic anions must be established. The differences in growth of wheat on a seedball extract treated with either calcium or lead suggest that some inhibitors may be removed by lead (but not by calcium) precipitation. The inhibitory substances, vanillic, caffeic, and ferulic acids, previously isolated from sugar beet fruits (5,7), are not precipitated by calcium but are precipitated by lead. The presence of these inhibitors and others could account for some of the inconsistency observed between lead and calcium precipitation.

Relation of soluble oxalate to speed of germination

Because of the large quantity of soluble oxalate in the fruits as compared with any of the other inhibitors, the influence of water-soluble oxalate on speed of germination seemed appropriate to investigate. For the 36 samples examined, the rapid-germinating samples generally contained lower amounts of water-soluble oxalate than the slow-germinating samples (Table 4). The correlation coefficients for speed of germination versus soluble oxalate in the seedball extract are –0.66 for the permanganate

Table 4.—Relation of speed of germination of sugar beet seeds to water-soluble oxalate in seedball extract of sugar beet variety US 401.

Seed number	Percentage germination after 2 days	Milligram-percent of oxalate in extract as determined by	
		Indole	Permanganate
581	95	21	18
469	90	18	16
401	88	19	16
514	88	22	19
368	85	16	16
528	85	22	22
547	78	29	21
524	73	21	19
318	70	42	31
266	65	19	23
553	63	34	30
236	60	41	36
584	48	34	31
568	48	32	34
560	48	70	63
426	43	37	34
288	43	69	69
372	35	20	17
358	35	18	17
204	33	41	31
493	30	18	18
617	28	24	24
577	25	37	37
525	23	36	38
477	18	34	32
305	15	89	83
211	10	33	34
257	10	95	84
433	8	20	17
549	8	67	67
457	0	68	53
517	0	63	58
312	0	69	59
362	0	61	66
194	0	81	81
128	0	92	94

methods and -0.64 for the indole method. These are significant at the 1% level. Approximately 7 samples do not fit the correlation. Sample 433 had a very low oxalate content but still germinated very slowly. When the wheat test was also used to evaluate these exceptions, the speed of germination of the sugar beet seeds related more closely to the growth of wheat than to the oxalate content. This behavior suggests that the samples which failed to fit the pattern probably contained an additional substance in sufficient concentration to cause the slower germination.

Further evidence that oxalate may be one of the principal inhibitors in the majority of samples, but not the only inhibitor in the variety US 401, is provided by comparing the growth of wheat on synthetic solutions and on seedball extracts (Table 5).

Table 5.—Growth of wheat on suger beet seedball extracts and synthetic solutions.

Treatment number	Type of solution	Oxalate content (Mg %)	Specific conductance (Mhos × 10^{-5})	Growth of wheat*	
				Expt. 1 (Grams)	Expt. 2 (Grams)
1	Distilled water	0	0	2.28	2.44
2	Synthetic—KCl	0	135	2.31	
3	Synthetic—oxalate + KCl	16	177	2.39	
4	Synthetic—oxalate + KCl	36	140	2.27	
5	Extract—seedballs of 469	16	176	2.15	
6	Synthetic—KCl	0	510	2.01	2.10
7	Synthetic—KCl	0	1,005		1.61
8	Extract—seedballs of 581	18	140	1.46	
9	Synthetic—oxalate	128	235	1.00	
10	Extract—seedballs of 457	53	400	0.97	
11	Synthetic—oxalate	300	350		0.74
12	Synthetic—KCl	0	2.000		0.70
13	Extract—seedballs of 517	58	515	0.66	
14	Synthetic—oxalate + KCl	128	440	0.62	
15	Synthetic—oxalate + KCl	60	1.060	0.39	

* Averages of 4 replications

The synthetic solutions contained the same quantity of oxalate as found in the seedball extracts and the same specific conductance, as well as greater or lesser quantities. The growth of wheat on a synthetic solution of oxalate and the osmotic additive (KCl) was similar to growth on the extract of seedballs of sample 469, but synthetic solutions which had similar oxalate and osmotic values to the other seedball extracts permitted considerably better growth than the seedball extracts. Additional inhibitory substances may be present in these samples.

Distribution of water-soluble oxalate in the fruits

Although the data (Table 5) indicate that 3 of the 4 samples used in this part of the study probably have some inhibitor in addition to oxalate, the 4 were chosen because of large differences in speed of germination and content of oxalate in the seedball extract. The recovery of the corky material after processing and the weights of the materials used to prepare the extracts are given in Table 6. Each portion of the material was soaked in 100 ml of water. Therefore, the value of oxalate for the processed seedballs plus the value for the material removed in processing

Table 6.—Quantity of material (grams) from the hand-processed sugar beet seedballs used to prepare extracts for oxalate analysis and growth of wheat.

Plant	Weight of seedballs	Weights recovered after processing operation		
		Processed seedballs	Material removed	Total recovered
457	10.0	7.4	2.2	9.6
469	10.0	7.5	2.2	9.7
517	10.0	7.3	2.4	9.7
581	10.0	8.0	1.9	9.9

should equal the value for the whole seedballs (assuming no loss of material removed and equal solubility for all samples). The oxalate appears to be concentrated in the corky material of the seedball (Table 7). Processing would be particularly beneficial for seedballs that have a high concentration of inhibitors.

Table 7.—Speed of germination (blotter method) of sugar beet seeds and growth of wheat on the seedball extracts (1:10) in relation to the distribution of water-soluble oxalate in the seedballs of 4 plants of sugar beet variety US 401.

Plant	Sugar beet germ. in 2 days (Percent)	Water extract prepared from	Oxalate in extract (Mg %)	Growth of wheat on extract expressed as % growth on water
581	95	Whole seedballs	14	68
		Processed seedballs	5	98
		Material removed	6	69
			11	
469	90	Whole seedballs	13	93
		Processed seedballs	5	92
		Material removed	4	91
			9	
457	0	Whole seedballs	62	33
		Processed seedballs	5	91
		Material removed	54	42
			59	
517	0	Whole seedballs	59	26
		Processed seedballs	5	87
		Material removed	40	33
			45	

Discussion

The complex nature of the inhibitory action of the sugar beet seedball extract on germination is indicated. Probably a number of effects interact to give the observed germination response. However, the data indicate that organic solutes are largely responsible for the inhibition. At least 2 organic substances seem to be involved. At present, only oxalate has been

causally related to speed of germination. If the additional substance(s) could be identified and causally related to the inhibitory action, further progress could be made in preparing synthetic solutions to determine the response to known combinations and concentrations of these substances. Identifications of all the substances in the seedballs which cause slower germination also would aid in formulating an empirical procedure for overcoming the inhibitory action.

The apparent tendency for high concentrations of oxalate to localize in the corky material of the fruit may indicate that other inhibitors are concentrated there.

The removal of inhibitory substances from the seedball extract by treating it with activated charcoal and dialyzing is visualized as a two-step process. (See Table 3). The activated charcoal removes the large molecules that cannot be removed by dialysis, while dialysis removes the smaller molecules or ions which cannot be absorbed on the charcoal.

Summary

A variety of simple chemical procedures were employed to partially characterize the inhibitory substances in a water extract of sugar beet fruits. The inhibition was not caused by inorganic substances in the fruit but largely by the presence of organic substances.

The speed of germination by the blotter method of 36 samples of seed of sugar beet variety US 401 was significantly correlated (−0.6**) with the amount of oxalate in a water extract of the fruits. Seven of the samples failed to correlate well, which suggests that an inhibitory substance other than oxalate was depressing speed of germination.

A study of the distribution of oxalate in the sugar beet fruit revealed that oxalate was concentrated in the corky material of the fruit.

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Literature Cited

(1) BERGERMAN, J. and J. S. ELLIOT. 1955. Method for direct colorimetric determination of oxalic acid. Anal. Chem. 27: 1014-1015.

(2) DEKOCK, P. C., R. F. HUNTER and I. R. MACDONALD. 1953. A germination inhibitor from sugar-beet. J. Exptl. Bot. 4: 272-282.

(3) DUYM, C. P. A., J. G. KOMEN, A. J. ULTEE and B. M. VAN DER WEIDE. 1947. The inhibition of germination, caused by extracts of seedballs of the sugar beet (Beta vulgaris). Proc. Kon. Ned. Akad. Wet. 50: 527-535.

(4) FROSCHEL, P. 1955. Over de remstoffen in de zaden van Beta sacchari fera. (On the inhibiting substances in the seedballs of Beta saccharifera). Natuurwetensch. Tijdschr. 37 (5/6) : 97-100. (Biol. Abs. 31: 9077. 1957).

(5) KOVES, E. and M. VARGA. 1959. Comparative examination of water- and ether-soluble inhibiting substances in dry fruits. Phyton 12: 93-99.

(6) MAKINO, I. and T. MIYAMOTO. 1954. On the growth inhibiting substance in the germinating spinach seeds. Jap. J. Breeding 4: 158-160.

(7) MASSART, L. 1957. Inhibiteurs de la germination dans les glomerules de la betterave a sucre et dans d'autres fruits secs et graines. Biokhimiia 22: 417-420.

(8) MIYAMOTO, T. 1957. The germination inhibitor in sugar beet seedballs. Quart. Bull. Mich. Agr. Expt. Sta. 39: 518-523.

(9) RIEMAN, W. and J. D. NEUSS. 1937. Quantitative Analysis. McGraw-Hill, New York. 425 pp.

(10) SNYDER, F. W. 1963. Some physico-chemical factors of the seedball influencing speed of germination in sugar beet. J. Am. Soc. Sugar Beet Technol. 12 (5) : 371-377.

(11) STOUT, M. and B. TOLMAN. 1941. Factors affecting the germination of sugar beet and other seeds, with special reference to the toxic effects of ammonia. J. Agr. Res. 63: 687-713.