# テンサイ種球中の生長阻害物質 I.

| | |
|---|---|
| 誌名 | 日本作物學會紀事 |
| ISSN | 00111848 |
| 著者 | 井上, 和雄<br>山本, 良三 |
| 巻/号 | 43巻3号 |
| 掲載ページ | p. 349-444 |
| 発行年月 | 1974年9月 |

Proc. Crop Sci. Soc. Japan 43(3) : 439-444 (1974)

# The Growth Inhibitors in Sugar Beet Seed Balls

## I. Isolation of mono-sodium oxalate as a root growth inhibitor*

Kazuo INOUE and Ryôzô YAMAMOTO

*(Faculty of Agriculture, University of Nagoya, Nagoya)*

### INTRODUCTION

It is well known to many workers that water soluble inhibitory substances are present in sugar beet (*Beta vulgaris*) seed balls and they delay the seed germination of sugar beet and inhibit subsequent growth of seedlings. Various inhibitory substances have been suggested to have a strong connection with the seed germination and subsequent growth of seedlings of sugar beet. Stout *et al.*[16] claimed that free ammonia caused inhibition of beet seed germination and the ammonia was liberated from the nitrogenous substances present in the perianth when beet seeds germinated. They considered that the ammonia was liberated by such hydrolytic enzymes as urease and asparaginase. Rehm[14] agreed that free ammonia acted as a germination inhibitor but stated that this was formed mainly by bacteria and the development of bacteria could be prevented by disinfection. De Kock *et al.*[4,5], however, suggested that the appropriate enzymes to release ammonia from the perianth were not present and demonstrated the existence of an unsaturated yellow oil in the water extract of sugar beet seed balls, which could inhibit seed-germination of various plants. This oil inhibits also salt uptake, respiration of tissue disk and polyphenolase activity of sugar beet. Besides many workers reported on unidentified inhibitors in sugar beet seed balls[6,9,13]. Later Makino *et al.*[10] isolated the toxic substance from the water extract of sugar beet seed balls and identified it as oxalic acid. Snyder *et al.*[15] reported that oxalate as a germination inhibitor was present in the corky material of sugar beet seed balls and at least one other inhibitor besides oxalate also affected the rate of germination. Massart[11] identified vanillic, *p*-hydroxybenzoic, ferulic and *p*-coumaric acid in the ether extract from an acidified water extract of sugar beet seed balls. Koves *et al.*[2] suggested the existence of *p*-hydroxybenzoic, ferulic, *p*-coumaric, caffeic and salicylic acid. Van Sumere[17] detected *p*-hydroxybenzoic, ferulic, *p*-coumaric, vanillic, sinapic acid and coumarin and suggested the beet seed extract contained enough amounts of ferulic acid to act as a strong germination inhibitor. Moreover Battle *et al.*[1,2] confirmed that ferulic, vanillic, *p*-coumaric and *p*-hydroxybenzoic acid were present in the ether extract from an acidified water extract using thin layer chromatography. Two other inhibitory substances had been suggested in the water extract from sugar beet seed balls. Wheeler[18] reported that, when the water extract was acidified and partitioned with ethyl acetate, betaine was retained in the aqueous layer and it inhibited the growth of dwarf French bean (*Phaseolus vulgaris*) leaf disks and cress (*Lepidium sativum*) roots. Mitchell *et al.*[12] identified cis-4-cyclohexene-1,2-dicarboximide as one of germination inhibitors present in sugar beet seed balls. Moreover Chetram and Heydecker[3,7,8] suggested that germination of sugar beet seed balls was impeded by excess moisture.

Thus various results were published about growth or germination inhibitors contained in sugar beet seed balls but they are not consistent. In order to clarify the growth inhibitors, especially a root growth inhibitor in sugar beet seed balls, this study was undertaken.

* Received for publication on February 22, 1974. Outline of this report was presented at the 155th meeting of the Crop Science Society of Japan, April, 1973.

## Material and Methods

Sugar beet seed balls (variety, Dônyu No. 2) used in this study were kindly gifted by Hokkaido National Agricultural Experiment Station.

As root and hypocotyl growth showed the largest increment during 2–3 days after germination, 2 days old seedlings were used for the seedling test to measure inhibitory action.

**Preparation of the water extract:** The water extract was prepared by shaking 100 g of seed balls with 400 ml of distilled water for 3 hours at room temperature and then filtrated. The filtrate thus obtained was named as "original water extract". The seed balls recovered from the above extracting procedure were further washed by immersing them in running water for 6 hours, subsequently were dried at room temperature for 2–3 days. They were the "washed seed balls" and used for the seedling growth tests. The various "organic solvent extracts" were prepared by immersing 20 g of seed balls in 80 ml of each organic solvent cited in fig. 2 for 24 hours at room temparature and then the filtrates were evaporated to dryness and the residues were re-extracted with distilled water. The concentration of the every "organic solvent extract" was adjusted as correspond to that of the "original water extract".

**Seedling growth test:** The extracts were assayed on sugar beet seedlings that germinated from the "washed seed balls". Two days old seedlings showing uniform growth were selected and placed on a Tôyô No. 2 filter paper sheet of 11 cm diameter in a Petri dish of 12 cm diameter added with 10 ml of the extract. Each dish contained 20 seedlings and the test was duplicated. The cultivations of seedlings were carried out at 25°C in the dark, and the increments in length of hypocotyls and roots were measured after 24 hours cultivation.

**Paper chromatography:** Two hundred milliliters of the "original water extract" was evaporated and the residue was dissolved in 5 ml of distilled water. One milliliter of this "concentrated water extract" was charged in a line on four sheets of filter paper (Tôyô No. 50 filter paper, 40×40 cm) and chromatographed by ascending development using ethanol—acetic acid—water (8:2:5 v/v). Paper chromatogram was divided into several zones according to the fluorescence pattern, and every zone belonging to the same pattern was collected from the four sheets of chromatogram respectively and eluted with each 20 ml of distilled water. The each eluate was assayed on sugar beet seedlings. The blank test was undertaken using the eluate from segments of paper obtained from the region under the starting line in the chromatogram.

## Results and Discussion

Variation in the growth of seedlings at different concentrations of the water extract was shown by length of hypocotyls and roots representing the increment of elongation for 24 hours (fig. 1). Root growth was inhibited greatly by high concentration solutions and the root tips turned black, a symptom of toxicity found by Stout and Tolman. Hypocotyl growth was depressed by "original water extract" but was stimulated by low concentration solutions.

The water extract was assayed also after boiling and the results were shown in table 1. This "boiled

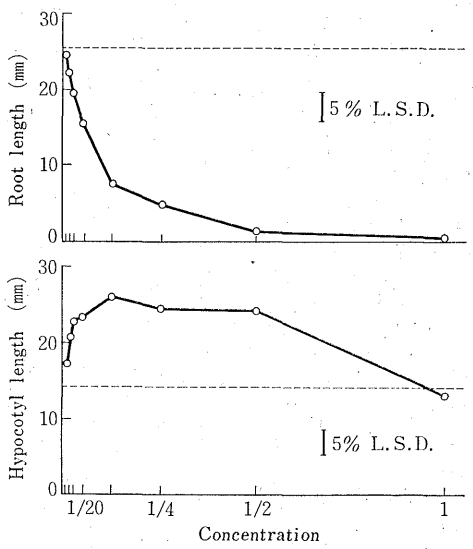


Fig. 1 Effects of the the water extract on the growth of seedling roots (top) and hypocotyls (bottom) at different concentrations. Concentrations were represented by ratio to the concentration of the "original water extract". Broken line indicates control.

Table 1 The growth of seedling roots and hypocotyls at different concentrations of the water extract and the boiled water extract

| Concentration* | Root length (mm) | | Hypocotyl length (mm) | |
|---|---|---|---|---|
| | Water extract | Boiled Water extract | Water extract | Boiled Water extract |
| 0 | 27.5 | | 15.1 | |
| 1/8 | 13.9 | 13.2 | 20.5 | 20.1 |
| 1/4 | 7.6 | 5.3 | 21.9 | 21.6 |
| 1/2 | 1.4 | 1.8 | 21.0 | 20.3 |
| 1 | 0.6 | 0.2 | 12.4 | 11.6 |

* Concentration was represented by ratio to the concentration of the "original water extract". Ratio 0 means the control test using distilled water.

water extract" inhibited root growth and stimulated hypocotyl growth as same as the non-boiled water extract did, so it could be said that the water extractable inhibitors were heat-stable.

To examine the solubility of inhibitor in various organic solvents, the "organic solvent extract"s were prepared from sugar beet seed balls. The bioassay results were shown in fig. 2. The root growth inhibitors were extracted with acetic acid easily and with methanol and ethanol considerably, but with isopropanol, *n*-butanol, acetone, ethyl acetate, chloroform, ether, petroleum ether, benzene and *n*-hexane hardly. The hypocotyl growth stimulators were extracted with methanol most extensively.

Paper chromatography was carried out on the "concentrated water extract" and the chromatogram was divided into several regions according to the fluorescence pattern in U.V. light. The bioassay revealed that the strong root inhibitory substance(s) located at Rf 0.47 to Rf 0.55 and the hypocotyl stimulating substance(s) at Rf 0.55 to Rf 0.62 (fig. 3). As the region, the main inhibitory substance located, did not fluoresce in U.V. light, some color reagents were examined to the paper chromatogram. Diazotized *p*-nitroaniline or ferric chloride reagent revealed various phenolic compounds on the paper chromatogram as shown in fig. 4, but the objective region was negative to these reagents. When a Bromphenol Blue solution, pH indicator, was applied,

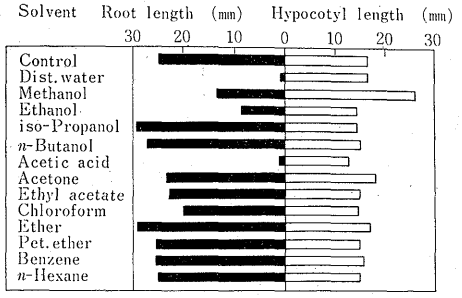


Fig. 2. Effects of extracts with various organic solvents on the growth of seedlings during 24 hours, showing length.

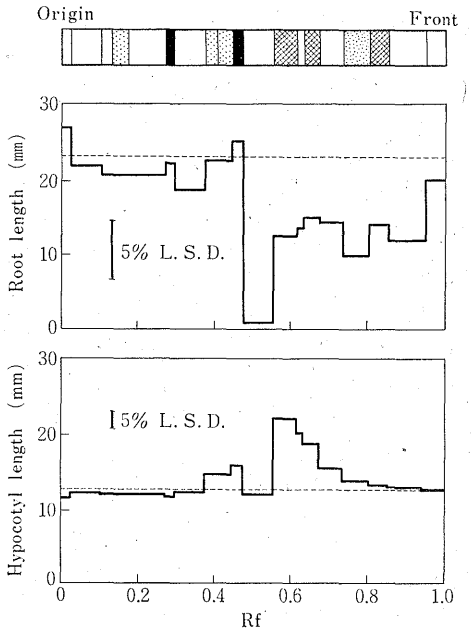


Fig. 3. Chromatogram and histograms of the water extract of sugar beet seed balls, using ethanol-acetic acid-water (8 : 2 : 5 v/v) as developing solvent. Broken line indicates control.
Fluorescence pattern ▨ Untreated ▨ After ammonia

the objective region became yellow. So, the main root inhibitory substance might be carboxylic but not phenolic.

To isolated the main inhibitory substance, the corresponding region of chromatogram (Rf 0.47—0.55, detected by fluorescence pattern) was collected from preparative paper chromatograms and the main inhibitory substance was extracted with distilled water. The eluate gave precipitates by adding of much amounts of acetone. The supernatant liquid had

not an inhibitory action on root growth. The precipitates were dissolved again in a small volume of water and concentrated in a desiccator to give colorless rhombic crystals. The infrared spectrum of the crystal resembled that of oxalic acid, but was not identical. An aqueous solution of this crystal was weakly acidic, however, the crystal left a white salt after combustion which was basic to a moistened litmus paper.

These results suggested that the inhibitory substance might be the alkali metal half salt of oxalic acid. Flame reaction suggested the alkali metal would be sodium. This was further confirmed as follows. Each 100 ml of 0.1 mol aqueous solutions of oxalic acid and sodium oxalate were mixed and the mixed solution was evaporated to a small volume to give the crystals of mono-sodium oxalate. The infrared spectrum of the isolated crystal was identical with that of the synthesized mono-sodium oxalate (fig. 5). Thus the main growth inhibitory substance contained in sugar beet seed balls was identified as mono-sodium oxalate.

The biological effect of mono-sodium oxalate on sugar beet seedlings was shown in fig. 6. At a concentration of 400 ppm, root growth was inhibited completely and the root tips turned black. However there was not stimulating effects on hypocotyls to every concentration. As the amount of crystals obtained from 600 ml of "original water extract" was 0.276 g, the water extract contained enough amount of mono-sodium oxalate to act as the root growth inhibitor. Makino *et al.*[10] isolated oxalic acid from water extracts and suggested[15] that the oxalic acid was present in sugar beet seed balls as oxalate but did not state on its salt form. When a aqueous solution of oxalic acid was used directly, without pH adjustment, on the seedlings test, the physiological responces (growth and/or symptom) of sugar beet seedlings were quite different from that shown by the natural water extract of seed balls.

From all above results, it is concluded that the inhibitory behaviours of water extract of sugar beet seed balls were caused mainly by mono-sodium oxalate and several phenolic compounds would be acting as minor inhibitors. The amount of the

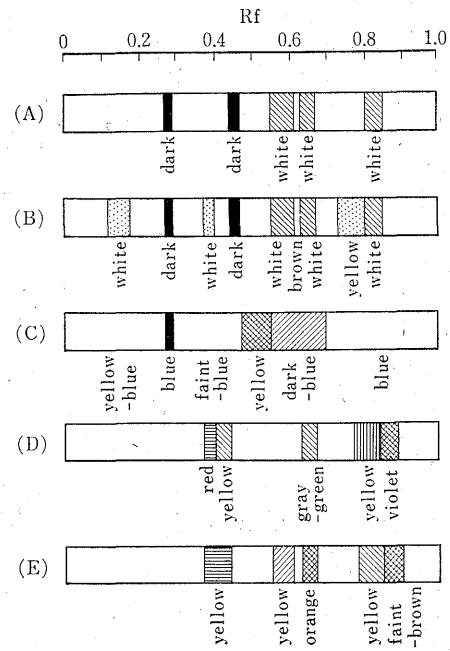


Fig. 4. Chromatograms of the water extract of sugar beet seed balls, using ethanol—acetic acid—water (8:2:5 v/v) as solvent. Fluorescence in U.V. light: (A) Untreated, (B) After ammonia. Color by detectors: (C) 1% Bromphenol Blue, (D) Diazotized *p*-nitroaniline—$Na_2CO_3$, (E) 2% $FeCl_3$.

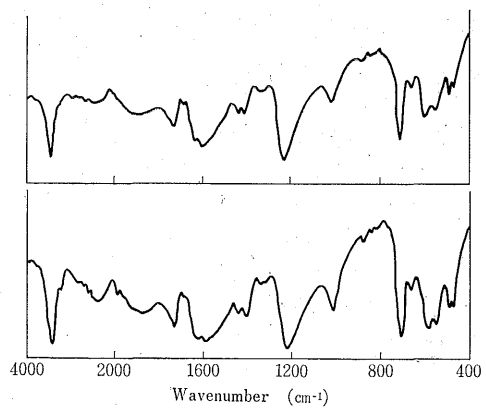


Fig. 5. Infrared spectra of the isolated crystal from the water extract of sugar beet seed balls (top) and synthesized mono-sodium oxalate (bottom).

mono-sodium oxalate per unit weight of sugar beet seed balls was not determined. A detailed study of the action of mono-sodium oxalate is undertaken and several minor inhibitory substances will be

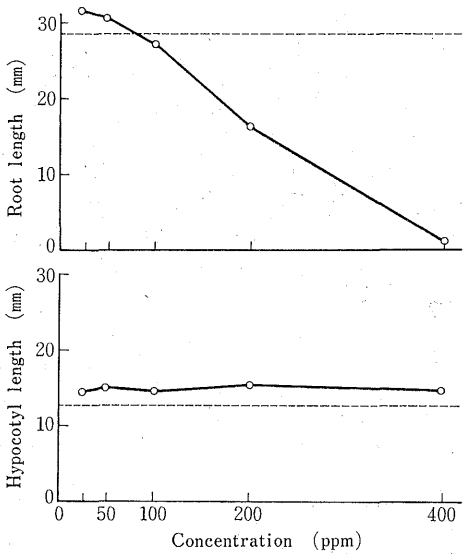


Fig. 6. Effects on the growth of roots (top) and hypocotyls (bottom) at different concentrations of the aq. solution of the crystal isolated from the water extract. Broken line indicates control.

clarified in following papers.

## SUMMARY

Sugar beet seed balls contain the water soluble growth inhibitory substances and these inhibitors are easily removed from seed balls by washing with water. The water extract of sugar beet seed balls inhibited root growth and made the root tips black at high concentrations. The hypocotyl growth was not inhibited even by high concentration extract but stimulated by diluted extract. Paper chromatography was carried out on the water extract. By using of ethanol—acetic acid—water (8:2:5 v/v) as the developing solvent, main root inhibitory substance came to the region of Rf 0.47—0.55, and hypocotyl stimulating substance to the region of Rf 0.55—0.62. Phenolic compounds came to the several Rf regions but the above main root inhibitory substance was not phenolic compound. The water eluate from the Rf 0.47—0.55 region afforded precipitates by adding of acetone. From the aqueous solution of this precipitates, the main root inhibitory substance crystallized out. The infrared spectrum of the isolated crystal was identical with that of the synthesized mono-sodium oxalate. It could be said that the main inhibitory substance contained in sugar beet seed balls was mono-sodium oxalate. The mono-sodium oxalate solution inhibited at 200—400 ppm the root growth and made the root tips black as did the water extract of sugar beet seed balls. The mono-sodium oxalate solution did not stimulate hypocotyl growth in range of 0—400 ppm.

## ACKNOWLEDGEMENT

The authors are grateful to Dr. H. Aoki of this Faculty for valuable discussions and suggestions.

## LITERATURE CITED

1. BATTLE, J. P. and W. J. WHITTINGTON 1969. The influence of genetic and environmental factors on the germination of sugar beet seed. J. agric. Sci., Camb. **73**: 329—335.
2. ———— and ———— 1969. The relation between inhibitory substances and variability in time to germination of sugar beet clusters. J. agric. Sci., Camb. **73**: 337—346.
3. CHETRAM, R. S. and W. HEYDECKER 1967. Moisture sensitivity, mechanical injury and gibberellin treatment of *Beta vulgaris* seeds. Nature **215**: 210—211.
4. DE KOCK, P. C. and R. F. HUNTER 1950. A germination inhibitor from sugar beet. Nature **166**: 440—441.
5. ————, ———— and I. R. MACDONALD 1953. A germination inhibitor from sugar beet. J. Exp. Bot. **4**: 272—282.
6. DE ROUBAIX, J. and O. LAZAR 1960. The inhibitory substances contained in sugar beet glomerules. Phenolics in Plants in Health and Disease (ed. J. B. Pridham): 35—41.
7. HEYDECKER, W. and R. S. CHETRAM 1971. Water relations of beetroot seed germination I. Microbial factors, with special reference to laboratory germination. Ann. Bot. **35**: 17—29.
8. ———— and ———— 1971. Water relations of beetroot seed germination II. Effects of the ovary cap and of the endogenous inhibitors. Ann. Bot. **35**: 31—42.
9. ISIKAWA, S. 1951. On the growth inhibitor

present in the pericarp of spinach fruit (*Spinacia oleracea*). Bot. Mag. Tokyo **64**: 120—125.

10. MAKINO, I. and T. MIYAMOTO 1954. On the growth inhibiting substance in the germinating spinach seeds. Jap. J. Breeding **4**: 158—160.
11. MASSART, L. 1957. Inhibitors of germination in the glomerules of sugar beet and other dried fruits and seeds. Biochimia **22**: 417—420.
12. MITCHELL, E. D. and N. E. TOLBERT 1968. Isolation from sugar beet fruit and characterization of cis-4-cyclohexene-1,2-dicarboximide as a germination inhibitor. Biochemistry **7**: 1019—1025.
13. REES, W. J. and A. D. SKELDING 1950. An inhibitor of salt absorption in root tissue of red beet. Nature **166**: 823—824.
14. REHM, S. 1951. The origin of toxic ammonia in germinating garden beet seed. J. Hort. Sci. **28**: 1—13.
15. SNYDER, F. W., J. M. SEBESON and J. L. FAIRLEY 1965. Relation of water soluble substances in fruits of sugar beet to speed of germination of sugar beet seeds. J. Am. Soc. Sugar Beet Technol. **13**: 379—388.
16. 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. Agric. Res. **63**: 687—713.
17. VAN SUMERE, C. F. 1960. Germination inhibitors in plant material. Phenolics in Plants in Health and Disease (ed. J. B. Pridham): 25—34.
18. WHEELER, A. W. 1963. Betaine: A plant-growth substance from sugar beet (*Beta vulgaris*). J. Exp. Bot. **14**: 265—271.

〔和 文 摘 要〕

## テンサイ種球中の生長阻害物質

### I. 根の生長を阻害する物質としてのシュウ酸一ナトリウムの単離

井 上 和 雄・山 本 良 三
(名古屋大学農学部)

テンサイ種球中には生長阻害物質が含まれており，発芽時において発芽および実生の生長を阻害するが，種球を水で洗うことによって容易にその生長阻害物質を除くことができる．しかしその生長阻害物質については多くの報告があるけれども，それぞれの意見は一致していない．そこで本研究は実生の根の生長を阻害する物質を明らかにするために行なわれた．

1) テンサイ種球の水抽出液は根の生長を阻害し根端を黒変させた．この抽出液ははい軸に対して生長阻害を示さなかったが，希釈した時にはかえってはい軸の生長を促進させた．

2) 水抽出液からエタノール—氷酢酸—水 (8：2：5 v/v) を展開剤としたペーパークロマトグラフィーにより阻害物質を分離した．根の生長の阻害効果は $R_F$ 0.47 から $R_F$ 0.95 の部分でみられ，最も著しい部分は $R_F$ 0.47 から $R_F$ 0.55 であった．またはい軸の生長促進効果は $R_F$ 0.55 から $R_F$ 0.73 の部分でみられ，最も著しい部分は $R_F$ 0.55 から $R_F$ 0.62 であった．$R_F$ 0.47 から $R_F$ 0.55 の部分からの水溶出液をアセトンで可溶部と沈殿部に分け，阻害効果のある沈殿部から無色透明の斜方状の結晶を得た．この結晶は酸性塩であることが確認され，その赤外線吸収スペクトルがシュウ酸一ナトリウムのそれと一致した．したがってテンサイ種球中の主要な阻害物質はこれまでシュウ酸塩として存在していると報告されていたが，その塩はシュウ酸一ナトリウムであることが明らかにされた．シュウ酸一ナトリウムは水抽出液中に根の生長を阻害するのに充分量存在しており，その阻害は水抽出液と同様に根を黒変させる．しかしはい軸の促進効果はもっていなかった．

3) ペーパークロマトグラムから阻害効果をもつ他の部分に種々のフェノール化合物の存在が確認されたが量的に主要な生長阻害物質ではないと考えられる．