A Germination Inhibitor from Sugar-Beet

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SUMMARY

There is present in the water extract of sugar-beet seed balls an unsaturated yellow oil which is capable of inhibiting germination of various seeds. It also inhibits salt uptake and the respiration of sugar-beet tissue disks. The activity of the polyphenolase enzyme present in beet tissue is also depressed. The role of the oil in inhibition of sugar-beet germination is discussed.

A recent comprehensive review of our knowledge of germination inhibitors (Evenari, 1949) obviates any need for a preliminary survey of the literature in this article.

That sugar-beet seeds do not readily germinate is well known to growers and seedsmen. The actual cause of the delayed germination has been variously attributed. Henley and Woodman (1930) found that treatment with dilute sulphuric acid, which in their opinion increased the permeability of the seed-coat, increased the rate of germination. According to Froeschel (1940) and Tolman and Stout (1940) the seed-coat contains substances which inhibit the germination of sugar-beet and other seeds. Stout and Tolman (1941) claim that, when beet seeds germinate, free ammonia is liberated from nitrogenous substances present in the seed-coat. They consider that the ammonia, liberated by a hydrolytic enzyme, is produced in quantities sufficient to exert a toxic action on the growth of the young embryo. Ammonia liberation in plant tissue can only be caused by a limited number of enzymes: it may, for instance, occur through the medium of an ortho-quinone produced by a polyphenolase acting on free amino-acids (James, Roberts, Beevers, and de Kock, 1948). Stout and Tolman (1941) themselves suggested urease and asparaginase, but could only find slight indications of urease. It is questionable that such enzymes exist in the dead tissue forming the pericarp round the true seed, and yet to support the view that ammonia is liberated from the seed-coat by enzyme activity the enzymes would have to occur in situ as it is unlikely that they would be secreted from the embryo.

Duym, Komen, Ultee, and van der Weide (1947) have shown that the osmotic effects of substances contained in the seed ball are important in relation to germination, but they failed to demonstrate a specific inhibitory substance.

In a previous note (de Kock and Hunter, 1950) we reported the separation from the water extract of sugar-beet seeds of an unsaturated yellow oil which inhibits the germination of both sugar-beet and other seeds and has a marked

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Effect on root growth. Subsequent to this communication, Rees and Skelding (1950) claim to have demonstrated in the tissue of red beet an unidentified inhibitor which prevents salt absorption and is removed on prolonged washing.

MATERIALS AND METHODS

The sugar-beet (*Beta vulgaris* L.) seed balls used in these studies were obtained from five different seedsmen, two of them local. No attempt was made to secure seed of one variety only, but significant differences were not found between different samples.

Water extracts were made simply by steeping the seeds in sufficient glass distilled water to cover them completely, leaving them overnight at room temperature, and filtering off the liquid through a Buchner funnel. This extract, containing the water-soluble substances from the beet seeds, was then employed in germination tests.

In attempting to find an ether-soluble inhibitor in the water extract the extract was acidified with dilute sulphuric acid and repeatedly treated with peroxide-free ether. The ether used was Analar peroxide-free ether, thrice distilled through an all-glass apparatus, as it became evident that commercial ether contained substances which would inhibit germination and ether itself would extract inhibiting substances from rubber bungs. As a check therefore the last 3 ml. of the ether from the final distillation were allowed to evaporate from a small filter-paper which was then placed in the inner ring of a Conway Unit moistened with 1 ml. water and containing 25 cress (*Lepidium sativum*) seeds set out to germinate on it in the dark at 16·5°. If germination agreed with a control containing distilled water only, the ether was deemed suitable.

Extraction was carried out in 1·5-l. separating funnels, using 600-ml. portions of water extract and three successive 50-ml. portions of ether. The ether fractions were bulked, allowed to stand overnight till no more water separated, dried with anhydrous sodium sulphate, and the ether distilled off. The resulting sweet-smelling oil was freed from ether by blowing over it a slow current of air. The oils extracted from different samples varied in colour from pale yellow to brown and were more viscous in some batches. On prolonged standing the odour became somewhat rancid and the colour paler. This affects the potency, the lighter being more potent, and the fresher the seed the more potent the oil yielded.

In the germination experiments the test solution used was either the water extract or a solution of the oil at varying concentrations. The oil solution was similarly used in the respiration and salt absorption experiments. Germination tests were carried out in Petri dishes into which a circle of Whatman's No. 1 filter-paper was introduced. Six ml. of the test solution were added after 50 or 100 seeds had been set out on the filter-paper. The Petri dishes were kept in the dark in a constant temperature room at 16·5°. To obtain beet seeds free from inhibiting substances, a number of seeds were secured in a muslin bag under a tap and water allowed to run over them for 12 hours. They were then dried over slight heat and left in a constant temperature room for 3 days until
they had attained constant weight. These were then used in germination experiments as controls.

Respiration and salt absorption experiments were carried out with disks of tissue cut from the beet. In early experiments disks were obtained by striking cylinders of tissue from a mature sugar-beet with a cork borer of 1 cm. diameter and sectioning these into disks of 1 mm. thickness, using a sharp razor. In later experiments on salt absorption in which large numbers of disks were required a sugar-beet was sliced by means of a bacon slicer and disks were cut from 5 to 6 slices at a time, using a sharp cork borer. It has been suggested by Turner (1938) that slicing the roots first with subsequent cutting of disks from the slices minimizes the bruising of the tissue.

Prior to use, the disks were kept in a reservoir of aerated tap-water (Fig. 1). The reservoir was a 1.5-l. separating flask into which tap-water was continually running, a constant level being maintained by the operation of an intermittent siphon. The siphon consisted of a Y-piece, the descending limb of which was attached to a 20-cm. long glass tube bent through 360° at its upper end. To one arm was attached a capillary siphon and to the other a siphon of wide glass tubing, the end of which determined the level of water in the container. The capillary siphon would operate automatically and would thus fill the 360° bend, which would itself siphon over and so provide sufficient pull to set off the wide glass siphon. This siphon arrangement operated over extended periods even when a strong stream of air was blown into the container.

The water was aerated by a current of air supplied by a Proctor Heavy Duty air pump. By permitting the air supply to enter the reservoir through
the stem of the flask the air current served the dual purpose of aerating the water and of keeping the disks in constant circulation entirely free from one another, thus preventing the development of anaerobic conditions. It also served to ensure that no bacteria could long remain on the disks. In this apparatus it is possible to keep up to 1,200 disks in a condition which will permit their being used for investigations such as those described in this paper from 1 to 600 hours.

Respiration rates were determined by standard Warburg techniques. In these estimations ten disks of uniform size and in a firm healthy condition were used per vessel and after being carefully blotted dry were immersed in a solution of volume 2·5 ml. contained in the annulus, there being 0·2 ml. 5 per cent. KOH absorbed on a fluted filter-paper in the centre cup. The substance, the effect of which was under investigation, was added to the side arm and, at zero time, was tipped into the main compartment of the vessel. The vessels were immersed in a water-bath kept at 20°C by a sensitive thermostat. Readings were taken every 30 minutes for a period of 2 hours after equilibration. Respiration readings are expressed as $\mu_l$ oxygen absorbed per 100 mg. dry weight.

The experimental fluid used in the phosphate uptake experiments was a suitably diluted Sorensen's phosphate buffer solution of pH 6·5, a pH which approximated to the pH of the tissue. The phosphate estimations were usually carried out every 30 minutes on the solution from the Warburg vessels in which the disks were respiring by adding 1 ml. ammonium molybdate to 1 ml. test solution followed by 1 ml. hydrazine hydrochloride, warming after suitable dilution to 100°C, and estimating the colour intensity on a 'Spekker' photo-electric absorptiometer. This method of following phosphate absorption has the advantage that the ratio of tissue to solution is always constant: 10 disks to 2·5 ml. solution.

To identify amino-acids present in the seed-coat one-dimensional paper chromatograms were run on a concentrated sugar-beet seed extract in the usual manner (Consden, Gordon, and Martin, 1944), using phenol-water as solvent. Later two-dimensional chromatograms with butanol-pyridine-water as second solvent were employed. De-salting was carried out electrically or by using pyridine.

**EXPERIMENTAL RESULTS**

(a) Germination

Germination tests using sugar-beet in the presence of other seeds showed conclusively that the beet seeds have an inhibiting action on the germination and growth of sugar-beet and other seeds. As this has been demonstrated by a number of earlier investigators (Froeschel, 1940; Stout and Tolman, 1941), further results are not presented here.

It was apparent, however, when using cress seeds that the growth of the radicle was much inhibited while the hypocotyls were only slightly affected. This is shown by the data given in Table I, based upon an experiment in
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which beet seeds were placed at the centre of the Petri dish and cress seeds arranged around the perimeter. The root-tips of the cress seedlings were characteristically blackened, as were those of the beet seeds. This fact was observed by Stout and Tolman (1941) and attributed to the liberation of ammonia by enzymes present in the seed-coat acting on nitrogenous substances.

**Table I**

Cress-seedling measurements (inches \( \times 10^{-1} \)) when placed round perimeter of Petri dishes with beet-seed clusters in centre. Means and standard errors of 25 seedlings.

<table>
<thead>
<tr>
<th>Seed clusters per dish</th>
<th>Inner ring of seedlings</th>
<th>Outer ring of seedlings</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Radicle length</td>
<td>Hypocotyl length</td>
</tr>
<tr>
<td></td>
<td>Radicle length</td>
<td>Hypocotyl length</td>
</tr>
<tr>
<td>5</td>
<td>11.54 ± 0.95</td>
<td>8.96 ± 0.68</td>
</tr>
<tr>
<td>10</td>
<td>7.99 ± 0.63</td>
<td>6.63 ± 0.60</td>
</tr>
<tr>
<td>0 (control)</td>
<td>18.9 ± 1.01</td>
<td>7.6 ± 0.3</td>
</tr>
</tbody>
</table>

Experiments using the water extract from beet seeds described earlier showed that it was as effective as the beet seeds themselves in preventing the germination of cress and other seeds. Seeds of cress, beet, and Italian rye grass (*Lolium italicum*) were germinated in Petri dishes in the dark at 16.5° using the water extract above and also dilutions of half and two-thirds, using 6 ml. per Petri dish. Controls had glass distilled water. The germination was noted daily for 11 days and is presented in Table II.

**Table II**

Percentage germination of *Lepidium sativum*, *Lolium italicum*, and *Beta saccharifera* in water extract of beet-seeds at three dilutions

<table>
<thead>
<tr>
<th>Concentration of extract</th>
<th>Days from sowing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>Lepidium</strong></td>
<td></td>
</tr>
<tr>
<td>Full strength o 0.0233</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>0.33</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td><strong>Lolium</strong></td>
<td></td>
</tr>
<tr>
<td>Full strength o 0.0236</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>0.33</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td><strong>Beta</strong></td>
<td></td>
</tr>
<tr>
<td>Full strength o 0.0236</td>
<td>0</td>
</tr>
<tr>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>0.33</td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
</tr>
</tbody>
</table>

If cress seeds which have been inhibited by the sugar-beet seed water extract are washed and set out for germination, the rate at which they germinate is remarkably rapid and the hypocotyls are much stouter than the controls. It can be concluded from this that some part of the growth process is being retarded whereas other processes may proceed normally or be stimulated.
To test the effect of the oil on germination of cress seeds 50 seeds were set out in a Conway unit and water containing oil at various concentrations was added. Table III shows the germination figures obtained and gives germination percentages and weights of radicle and hypocotyl. It is apparent that both the oil and the original water extract markedly affect the length of the radicle.

**Table III**

*Fresh weights (g.) per 100 seeds of hypocotyls and radicles after 6 days germination in water with added ether extract*

<table>
<thead>
<tr>
<th>Ether extract (p.p.m.)</th>
<th>Hypocotyl</th>
<th>Radicle</th>
<th>Total wt.</th>
<th>Ratio radicle/hypocotyl</th>
<th>% Germination number days shown in brackets</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,000</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>23 (4)</td>
</tr>
<tr>
<td>100</td>
<td>0.396</td>
<td>0.126</td>
<td>0.522</td>
<td>0.31</td>
<td>82 (6)</td>
</tr>
<tr>
<td>50</td>
<td>0.619</td>
<td>0.170</td>
<td>0.789</td>
<td>0.26</td>
<td>90 (6)</td>
</tr>
<tr>
<td>10</td>
<td>0.735</td>
<td>0.277</td>
<td>1.012</td>
<td>0.37</td>
<td>100 (6)</td>
</tr>
<tr>
<td>Control</td>
<td>0.793</td>
<td>0.391</td>
<td>1.184</td>
<td>0.49</td>
<td>100 (4)</td>
</tr>
</tbody>
</table>

(b) *Respiration.* In earlier respiration experiments 14 disks suspended in pH 5.5 Sorensen's phosphate buffer were used per Warburg vessel. The inhibitor oil was weighed directly into each vessel to give 1 part per 1,000 final concentration. Fig. 2 gives the result of such an experiment.

In order to determine whether the inhibitor affected the rate of oxidation of catechol by the polyphenolase of sugar-beet, 100 disks were ground with sand in 10 ml. water, the extract filtered through muslin, centrifuged for 5 minutes at 3,000 r.p.m., and used as a source of the enzyme. Of this 0.5 ml. was added to each vessel together with 0.5 ml. pH 6 phosphate buffer and 0.5 ml. 0.05 M. catechol tipped in from the side arm. The oil was added to give a final concentration of 1 part per 250. The results obtained are presented in Fig. 3. Confirmation of the findings of the previous experiment was obtained by investigating the effect of the oil inhibitor on the oxidation of catechol by the polyphenolase of the mealworm (*Tenebrio mollitor*). Mealworms (2.13 g.) were ground in 30 ml. pH 6 phosphate buffer and then filtered through a Whatman's No. 1. The purplish clear liquid was used in Warburg vessels as above with 0.5 ml. 0.05 M. catechol added from the side arm and oil weighed in directly to give 1 part per 1,000 final concentration. The magnitude of the inhibition obtained is also shown in Fig. 3.

(c) *Phosphate absorption.* Experiments on the uptake of phosphate by sugar-beet disks in the presence of the oil were conducted as outlined in an earlier section. At the end of a 3-hour experiment in which 10 disks were allowed to respire in 2.5 ml. phosphate solution contained in a Warburg vessel, the percentage uptake of phosphate was determined; four controls had absorbed 85.0, 80.0, 83.5, and 81.4 and four series in presence of oil
absorbed 30.7, 28.5, 32.1, and 30.7. No exosmosis of phosphate occurred when disks were left in distilled water in presence of the inhibitor oil.

In a similar experiment phosphate determinations were made every 30 minutes. The oil was added to give a final concentration of 1 part per 1,000. Respiration readings were taken over a period of 2.5 hours (Fig. 4). As previously, the disks, in the absence of inhibitor, absorbed nearly 50 per cent. more phosphate than did inhibited disks. It is quite evident that the oil has a strong inhibitory effect on the absorption of phosphate by sugar-beet tissue.

(d) Biochemical investigations. To investigate the possibility of ammonia
liberation, urease experiments were carried out by soaking 50 g. seed in 150 ml. glass distilled water. The liquid was filtered off and 20 ml. urea (0.02 M.) added to a 20-ml. aliquot. Controls had no urea added. Ammonia estimations were made after 4 hours using the Conway technique (Conway, 1947). Ammonia produced in excess of controls was small. Even if seeds soaked in a phosphate-citrate buffer were used, very little ammonia liberation resulted. Similar experiments were performed with aspartic and glutamic acids over a 4-hour period, using a pH 7.8 phosphate buffer. No increase in free ammonia could be detected, a finding suggesting the absence of the appropriate enzymes.

Moreover, using Warburg manometers, no catechol oxidising enzyme was found in the water extract of sugar-beet seeds. Sugar-beet seeds ground in a hand-mill likewise showed no polyphenolase, although mature beet tissue contains this enzyme.

An analysis of the water extract for amino-acids indicated the presence of
leucine ++++; alanine, valine and glycine ++++; γ-amino-n-butyric and aspartic acids +++; glutamic acid, serine, and threonine +. The comparatively high proportion of glycine is probably significant in relation to biological inhibition.

Sugar-beet seeds store starch although starch is not found in the root. A water extract of the seed balls contains amylase in quantity. Investigations have so far failed to reveal the presence of other enzymes. The ether extractable oil from the beet seeds has a marked stimulating effect on the amylase as measured by the hydrolysis of soluble starch (Somogyi, 1937). It is hoped to present these experimental results in due course.

(e) Chemical investigations. Little information on the chemical nature of the oil inhibitor has so far been obtained, due to the low yield of oil (0.2 g. per kg.). No characteristic colour is given with aqueous ferric chloride. On treatment with hydrogen peroxide the inhibitory activity is only slightly lowered. Unsaturation is indicated by the fact that iodine water is decolorized by the oil and it also goes rancid on standing. It is quite possible that the oil is a mixture of substances which may act synergistically.

Discussion

Biological inhibitors are widespread in nature. Of these, bacterial inhibitors or antibiotics have received special prominence in recent years. Growth-inhibiting substances are, however, common among higher plants (Peyronel, 1947). Koeckemann (1934) was one of the first to draw attention to the existence of specific substances, the effect of which was to delay germination. He cited the case of tomato seeds which fail to germinate although present in an apparently ideal medium and suggested that this was due to the presence of inhibiting substances which he named blastocholine. These have been subsequently identified as a mixture of caffeic and ferulic acids which act synergistically (Akkerman and Veldstra, 1947). The seeds of Melilotus officinalis similarly fail to germinate because they contain coumarin in quantity (Zwenger and Bodenbrender, 1863). Others (Walger, Markus, and Nagymihaly, 1949) have shown that pumpkin (Cucurbita maxima) seeds are inhibited by the presence of volatile substances in the pumpkin.

Osmotic effects can also result in inhibition of germination. A high salinity prevents the germination of cress; the colloidal covering of the seed-coat fails to hydrate and the critical osmotic value of the embryo is probably exceeded. However, sugar-beet is a typical halophyte and is physiologically adapted to soils of high salinity. In fact yield of sugar is improved by adding common salt to agricultural soil. It is possible therefore that it is less seriously affected by high osmotic values.

The full significance of this inhibition of germination is not evident in the plant's economy. A possible ecological significance of the inhibiting substances present in sugar-beet has been demonstrated by Froeschel and Funke (1941). They noted the absence of the weed Agrostemma Githago from beet fields and showed that when Agrostemma and beet were sown together, the Agrostemma
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failed to germinate. The inhibitors may thus have a significance in relation to competition. The presence of a water-soluble germination inhibitor in the seed may be advantageous in that it would prevent germination and possible desiccation until the soil has attained a sufficient moisture content, by which time the inhibitor would be washed out. However, it may be the function of the inhibitor to maintain the seed only partially dormant. For instance, respiration may be suppressed while food reserves are made available by hydrolysing enzymes which continue to function, so that, when the inhibitor is finally removed, abundant energy is available for rapid growth. Evidence has been presented to show that in the presence of the extracted oil, respiration of the tissue is effectively inhibited, while amylase activity continues unchecked or at increased speed. The controlling mechanism is more likely to be endogenous and the result of a specific inhibitory substance. In this connexion it may be noted that Dufrénoy and Pratt (1948) have obtained similar responses with dithiobiuret on rice (Oryza sativa) seedlings. Stunting of the roots was obtained at 10 p.p.m. dithiobiuret, but elongation rapidly ensued on transfer to pure water. They suggested that the dithiobiuret was functioning by protecting phenolic substances from oxidation.

It is now generally agreed that there is a close relation between respiration and salt absorption (Robertson, 1950). From our results it will be evident that the inhibitor oil prevents respiration and salt uptake equally effectively. Rees and Skelding (1950) reported the presence in the storage tissue of red beet of a water-soluble substance which inhibited the absorption of salt by disks of beet tissue. That a similar substance occurs in sugar-beet is certainly feasible. In the preliminary work of the present investigation it became clear that the disks had to be washed in the storage apparatus for 4–5 days before it was possible to record an appreciable uptake of phosphate under our experimental conditions. This could suggest the presence in the tissues of an inhibiting substance which is removed on prolonged washing.

Our results would further seem to suggest that ammonia must occupy a minor role in any evaluation of the factors causing inhibition of germination in sugar-beet. It is at least questionable whether ammonia would be produced in quantity sufficient to cause inhibition. About 3.8 m.eq./NH₃/100 ml. water extract has been recorded (Duym et al., 1947), and it has not been possible to demonstrate the activity of enzymes able to liberate ammonia from nitrogenous substances in the seed-coat. The possibility is rendered less likely in acid soils, where the ammonia would be rapidly absorbed by the soil colloids. Of the nitrogenous substances present in the seed coat, glycine may exert some inhibitory activity, as has been shown by Audus and Quastel (1947).

That osmotic effects must have a considerable influence on the embryo is not to be denied. Other factors such as the presence of glycine or free ammonia may also make small contributions to the delayed germination which is characteristic of sugar-beet seeds. But the fact that we have been able to isolate from the seed balls an unsaturated oil which can be shown to cause a striking inhibition of seed germination and of respiration and salt uptake of
tissues would seem to suggest that the oil must play a decided role in the plant's metabolism and germination behaviour.

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