EFFECT OF STREPTOMYCIN ON THE CHLOROPHYLL FORMATION IN THE TIMOTHY

II. INFLUENCES OF INORGANIC SALTS UPON THE STREPTOMYCIN EFFECT ON THE TIMOTHY SEEDS (UNDER A RESTING CONDITION)

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In the previous paper ⁽¹⁾ the author reported that streptomycin (SM) had a checking effect on the chlorophyll formation in the timothy (*Phleum pratense*). In this report are presented the results of studies carried out on the effect of SM on the timothy seeds which were in resting condition and also on the counter-active influences, upon the action of SM, of inorganic salts with whose solutions the SM-treated seeds were rinsed under resting condition likewise.

MATERIALS AND METHODS

The timothy seeds used here were those offered by Sakata Seeds Inc. in Yokohama, Japan (Catalogue No. 2709), and the SM (dihydrostreptomycin sulfate) was the product of Daiichi Seiyaku Inc. in Tokyo, Japan.

The seeds were put into 200γ/cc of SM solution at 5°C for 4 days and then rinsed for 24 hours at 5°C in various kinds of solutions prior to their cultivation, namely the seeds were put into contact with SM under resting condition and rinsed in rinsing solution also under resting condition. The method of cultivation was the same as reported in the previous paper⁽¹⁾.

In order to observe both the effect of SM upon the seeds and the influences of inorganic salts on the SM activity, the rate of chlorophyll formation in the leaves developed from above treated seeds was measured and compared. For these comparative observations it was indispensable to select only the leaves which had germinated within 4-5 culture days⁽¹⁾. According to the rate of chlorophyll formation, leaves were classified into following three classes: white, partial green, and normal green. Among "partial green" leaves were also included those in which the chlorophyll formation was so minute as to stain only the tips of leaves.

EXPERIMENTAL RESULTS

1. Chlorophyll formation when the SM-treated seeds were rinsed with pure water

The seeds which had been soaked in SM solution (200 γ /cc) for 4 days at 5°C, hardly brought forth green leaves when they were planted on water medium after having been rinsed for 24 hours at 5°C with 100 cc of desalted water which had been treated with ion exchange resin. Almost all the leaves were colorless (white) without any conceivable chloroplasts in them and found to be dwarf with incidentally shrunken roots as reported previously⁽¹⁾. Some seasonal influence was acknowledged by the fact that inspite of using the same batch, the seeds which were soaked in SM solution from April to June formed chloroplasts a little stronger than those which were soaked in winter under the same rinsing condition. But the amount of chloroplasts in this case was very small, with pale green maculae appearing only in the leaf tip.

The SM-treated seeds in this test had been in fluid for 5 days,—that is, for 4 days in SM solution and for 24 hours in rinsing water. Then as a control measure, normal seeds which were rinsed for 5 days in SM-free desalted water were cultivated on water medium. They germinated green leaves irrespectively of seasons (with exceptional natural white ones appearing at such a low per-

Table 1
*Chlorophyll Formation in the Leaves and its Seasonal Difference;
the SM-treated Seeds were Rinsed in Desalted Water*

Treatments of seeds	Season (month)	Color of leaves developed on water medium		
		White %	Partial green %	Green %
Soaked in SM for 4 days at 5°C and rinsed in desalted water for 24 hours at 5°C	Jan.	97	3*	0
	Feb.	98	2*	0
	Mar.	97	3*	0
	Apr.	88	12*	0
	May	89	11*	0
	Jun.	88	12*	0
	Jul.	95	5*	0
	Aug.	97	3*	0
Control measure: Soaked in desalted water (SM-free) for 5 days at 5°C	Jan.~Aug.	0	0	100

* In these partial green leaves the amount of chloroplasts was so small as to stain only leaf tip, so that these leaves might be classified into white leaves.

Note: (1) Desalted water means water highly purified by passing through ion exchange resin.

(2) Methods of rinsing—Approximately two hundred seeds were rinsed in 100 cc of desalted water and left for 24 hours at 5°C.

centage of 0.2). This comparative experiment seemed to show that the cause of the failure in chlorophyll formation was not attributed to the nutrients in seeds necessary for the chlorophyll formation which might be washed away with water in which the seeds were soaked for a certain period of time, but that the cause was SM itself. The results are summarized in table 1.

2. Chlorophyll formation and the length of time for rinsing

The length of time for rinsing the SM-treated seeds was found to have a noticeable influence upon the chlorophyll formation in their leaves, when they were rinsed with running tap water preceding their cultivation on water medium. Table 2 shows the results. This being tested in the coldest season, more exactly between Dec. 1958 and Feb. 1959, it was considered that those seeds were in resting condition when they were in rinsing water. As shown in the table, if rinsing was done only in a short period of time more than 90% of the leaves appeared white with no chloroplasts in them, and with lengthening of rinsing time chlorophyll formation became more active. But when the SM-treated seeds were left without rinsing in a refrigerator just after their release from SM-contact and subsequently kept for 24-48 hours, chlorophyll formation hardly occurred in their leaves. In short the mere lapse of time without rinsing did not make SM inactive which had been taken in the seeds; chloroplasts could be formed in leaves only when seeds were rinsed for a long period of time. Thus it was deduced that rinsing had something to do with the recovery of chlorophyll formation.

Table 2
*Chlorophyll Formation in the Leaves when the SM-treated Seeds
were Rinsed in Running Tap Water*

Methods of rinsing	Color of leaves developed on water medium		
	White %	Partial green %	Green %
Several minutes in running tap water	98	2	0
3 hours in running tap water	94	6	0
24 hours in running tap water	16	39	45
48 hours in running tap water	4	3	93
Rinsed for several minutes and then preserved at 5°C			
1. for 24 hours	96	4	0
2. for 48 hours	94	6	0

Note: These experiments were made in the coldest season, that is, between Dec. 1958 and Feb. 1959.

3. Chlorophyll formation and purity of rinsing water

Table 3 shows the difference in chlorophyll formation in the leaves when desalted water, tap water, and well water were used for rinsing the SM-treated

seeds prior to the cultivation on water medium. In these tests the limitations were as follows: quantity of rinsing water 1.2 litre each, temperature 5°C and the length of time for rinsing 24 hours. In case of desalted water almost no chloroplasts were formed as stated before, while well water was found to have a remarkable effect on the recovery of chlorophyll formation as compared with the other two. Tap water, when its quantity was limited in 1.2 litre, left 88% of the leaves colorless, in discrepancy to the experiment shown in table 2, in which a great amount of water was used in running state with a remarkable recovery of chlorophyll formation. The evaporated residues of tap water and well water, were respectively 80 ppm and 329 ppm, showing the higher purity of rinsing water resulted in the lesser recovery of chlorophyll formation. Along with these experiments another test was done by using the suspension of kaolin as an adsorbent only to fail in finding its rinsing effect.

It is thus reasonable to think that the recovery of chlorophyll formation was not attained by mechanically rinsing away the SM present in the seeds but by the action of some ingredient in rinsing water which had a certain influence upon the seeds.

Table 3
The Difference in Chlorophyll Formation Potency of the Leaves when the SM-treated Seeds were Rinsed in Four Kinds of Solutions of the Same Amount

Kinds of solutions (Rinsed with 1.2 litre of solutions for 24 hours at 5°C)	Color of leaves developed on water medium		
	White %	Partial green %	Green %
Water desalted by passing through ion exchange resin	97	3	0
Tap water (evaporated residues 80 ppm)	88	12	0
Well Water (evaporated residues 329 ppm)	10	32	58
0.1 % kaolin suspension	90	10	0

Note: These experiments were made during Jan. and Mar.

4. Influence of pH of rinsing water on the chlorophyll formation

The rate of chlorophyll formation differed with the change of pH of rinsing water as shown in table 4. In this test the SM-treated seeds were soaked in 100 cc of rinsing water for 24 hours at 5°C preceding the cultivation on the water medium. At pH 4.0 chlorophyll formation was observed in approximately 85% of the leaves and at pH 4.5 in nearly 50%. These percentages and the quantity of chloroplasts in each leaf highly surpassed the recovery tendency in chlorophyll formation which was urged by seasonal influence. This fact shows that H⁺ has

something to do with this kind of recovery. But such a conspicuous result was not recognizable at pH values greater than 5.

Table 4
The Difference in Chlorophyll Formation Potency in the Leaves According to the Change in the pH of Water Used for Rinsing

pH of rinsing water (in 100 cc of water at 5°C for 24 hours)	Color of leaves developed on water medium		
	White %	Partial green %	Green %
4.0	15	66	19
4.5	50	39	11
5.0	83	17*	0
5.2	87	13*	0
5.4	90	10*	0
5.6	90	10*	0
6.0	90	10*	0
7.0	89	11*	0
7.5	87	13*	0
8.5	88	12*	0

* In these leaves the amount of chloroplasts was so minute, with pale green maculae appearing only in leaf tip, that these leaves might be classified into white ones.

Note:

- (1) The pH values were adjusted with 0.1 N HCl which was added to desalted water (acid side), or with 0.1 N KOH (alkaline side).
- (2) These experiments were made during April and June.

5. Rinsing with phosphate buffers of the same pH value but of different phosphate content

In this test the SM-treated seeds were rinsed for 24 hours at 5°C with 100 cc

Table 5
Relationship between Chlorophyll Formation in the Leaves and the Phosphate Salt Content in Phosphate Buffer by which SM-treated Seeds were Rinsed

Rinsing solutions (24 hours rinsing in 100 cc of solution at 5°C)	Color of leaves developed on water medium		
	White %	Partial green %	Green %
1/15 M Phosphate buffer, pH 7.0	10	40	50
1/30 M " " " "	28	47	25
1/60 M " " " "	57	40	3
1/15 M Phosphate buffer, pH 6.2	9	38	53
1/30 M " " " "	25	45	30
1/60 M " " " "	59	37	4
1/15 M Phosphate buffer, pH 5.6	10	33	57
1/30 M " " " "	36	49	15
1/60 M " " " "	57	43	0

- Note 1. Phosphate buffer is made of KH_2PO_4 and Na_2HPO_4 .
2. These experiments were made during May and June.

of phosphate buffers as described in table 5, and then planted on water medium. As shown in the table, the recovery of chlorophyll formation always became more active with increase in phosphate content of buffers. Now it becomes clear that inorganic salts have influence on the chlorophyll formation for its recovery as well as H^+ .

6. Chlorophyll formation when the SM-treated seeds were rinsed with the solutions of monovalent metal salts

In order to obtain a more detailed knowledge concerning the action of inorganic salts, solutions containing Na-, K-, and Li-salt respectively were used for rinsing which were done at $5^{\circ}C$ for 24 hours prior to the cultivation on water medium. The quantity of each solution was 100 cc and their concentration was 0.07 N. As shown in table 6, over 70% of the leaves recovered chlorophyll formation. In these recovery rates similarity was observed among Na-, K-, and Li-salts, when they had the same kind of anion. But among the salts of the same kind of metal these recovery rates could show considerable difference when they had different kind of anion. These recoveries in chlorophyll formation were far more active than that caused by seasonal influence.

Table 6
*Chlorophyll Formation in the Leaves when the SM-treated Seeds
were Rinsed in the Solutions of Monovalent Metal Salts*

Rinsing solutions (24 hours rinsing in 100 cc of solution at $5^{\circ}C$)		Color of leaves developed on water medium		
		White %	Partial green %	Green %
NaCl	0.07 N	25	55	20
KCl	"	20	56	24
LiCl	"	26	51	23
NaNO ₃	0.07 N	24	56	20
KNO ₃	"	21	55	24
LiNO ₃	"	24	52	24
Na ₂ SO ₄	0.07 N	15	44	41
K ₂ SO ₄	"	15	36	49
Li ₂ SO ₄	"	18	50	32
NaHCO ₃	0.07 N	19	53	28
Na ₂ HPO ₄	"	12	45	43

Note: These experiments were made during April and June.

7. Chlorophyll formation when the SM-treated seeds were rinsed with solutions of bivalent metal salts

Table 7 shows the state of recovery of chlorophyll formation when solutions of bivalent metal salts, such as Ca-, Mg-, Ba-, Sr-, and Zn-salt, were used for

rinsing the seeds prior to the cultivation on the water medium. These solutions gave rise to chlorophyll formation in about 80% of the leaves at 0.007 N concentration, which means 1/10 of the concentration in the case of solutions of monovalent metal salts, though MgSO_4 was somewhat behind in its effect. These recoveries in the chlorophyll formation were far more active than seasonally observed one, indicating clearly the presence of the favorable influences of inorganic salts. These solutions of bivalent metal salts had weak acidity (pH ranging from 5.4 to 5.6) but the rinsing with the water of pH 5.0, 5.2, 5.4, and 5.6 showed almost no efficacy in the recovery in the chlorophyll formation (see table 4). Therefore this fact may also show that not only H^+ but the very action of salt accounts for the experimental results.

Table 7
*Chlorophyll Formation in the Leaves when the SM-treated Seeds
were Rinsed in the Solutions of Bivalent Metal Salts*

Rinsing solutions (24 hours rinsing in 100 cc of solution at 5°C)		Color of leaves developed on water medium		
		White %	Partial green %	Green %
CaCl_2	0.007 N	18	52	30
MgSO_4	"	30	50	20
BaCl_2	"	17	49	34
$\text{Sr}(\text{NO}_3)_2$	"	18	51	31
ZnSO_4	"	17	50	33

Note: These experiments were made during April and June.

DISCUSSION

As stated above, when the seeds which had been soaked in SM solution (200 γ /cc) for 4 days at 5°C were rinsed with desalted water—highly purified by passing through ion exchange resin—for 24 hours at 5°C, they sprouted colorless leaves lacking in chlorophyll, and it was made clear what disturbed chlorophyll formation was due to the action of SM. However, when those seeds were rinsed with solutions having some special properties, they could recover chlorophyll formation. In order to see if the lapse of time had any effect in this case, the SM-treated seeds were left in refrigerator for 24–48 hours, but with such procedure SM activity was found intact. It was here demonstrated that some chemical attributes of rinsing solutions had something to do with the recovery of chlorophyll formation, one of which was its pH value. But when pH value reached to 5.0 and went farther to alkaline side, another factor had to explain the recovery. That was the action of inorganic salts which were contained in

rinsing solutions. In this action of salts, cations (metal ions) should be playing an important role but a certain kind of anion, for instance SO_4^{2-} and HPO_4^{2-} , seem to have been participating in this action. In the case of bivalent metal salts the influence of anion was not recognizable, because those bivalent salts were tested in lower concentration (1/10 of the concentration in the case of monovalent ones). Moreover, it was an interesting fact that in regard to this recovery of chlorophyll formation the efficacy rate of bivalent metal salts to monovalent ones was approximately 10:1 under the described condition. But why and how do these inorganic salts bring about such an effect? This may be explained in two lines—one in the interaction between SM and salts and the other in the effect of latter upon the protoplasm of seeds. These questions are left open in this stage and will be discussed in the next report.

SUMMARY

1) The seeds of Japanese timothy which had been treated with streptomycin solution (200 γ /cc) at 5°C for 4 days were rinsed with pure water for 24 hours at 5°C prior to the cultivation on water medium, and it was found that nearly 90% of the leaves were affected by streptomycin and became white without forming chlorophyll.

2) When streptomycin-treated seeds were rinsed, prior to the cultivation, in water of pH 4.0, about 85% of the leaves recovered chlorophyll formation, none of them recovered in water of pH values more alkaline than 5.0.

3) Chlorophyll formation was also recovered in the majority of the leaves when streptomycin-treated seeds were rinsed, prior to the cultivation, for 24 hours at 5°C in 0.07 N solutions of monovalent metal salts such as Na-, K-, and Li-salts. It was considered that metal ions should be playing an important role in this recovery but a certain kind of anion could have participated in this result.

4) Solutions of bivalent metal salts such as Ca-, Ba-, Mg-, Sr-, and Zn-salt presented almost the same results as those of monovalent ones at the concentration of 0.007 N, namely the efficacy rate of bivalent metal salts to monovalent ones was approximately 10:1.

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REFERENCE

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