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Abstract. Bleaching (interference with the production and/or accumulation of potentially green tissues) of *Arabidopsis thaliana* (L.) Heynh. by Streptomycin (SM) is followed by severe inhibition of expansion of leaves, elongation of stem internodes, coleoptiles and roots. Arabidopsis shows no enchancement of growth when sugar is supplied to the medium, and it was found that Gibberellic acid has an antagonistic effect on growth inhibition of SM.

Seeds germinated in darkness in SM-containing medium when subcultured on SM-free medium in darkness and transferred to light, turned green normally. Seeds germinated in light in SM-containing medium stay bleached permanently, even after subculturing in SM-free medium in light.

The naturally occurring antibiotic, Streptomycin (SM), has a number of interesting biological effects in addition to its well known bacterial action. In higher plants it acts as a powerful growth inhibitor (1). In chlorophyllous organisms generally, be they algal flagellates or angiosperms, it produces a disruption of the photosynthetic pigment system (2). This interference with the production and/or accumulation of chlorophyll in potentially green tissue has been referred to as bleaching (2).

The cellular control of development and inheritance of the photosynthetic apparatus is far from understood. Particularly, our knowledge of the mechanisms controlling chloroplast replication, development and inheritance is extremely fragmentary. The roles of nuclear and cytoplasmic regulators of chloroplast development, are insufficiently understood.

SM-bleaching in *Euglena* is permanent, but shows no retardation of growth if supplied with sugar (2). But because *Euglena* is not known to reproduce sexually, this organism is not suitable for exploration of the problems mentioned above. In flowering plants, studies of SM-bleaching have been restricted to vegetative tissue and no reports are known of observation on sexual progeny wholly or in part from SM-bleached plants.

The present study was an attempt to treat a flowering plant with SM in a manner which would permit crosses to be made between gametes derived from SM-bleached and normal green parents. This in turn, necessitated a search for methods of exposure to SM which would maximize the bleaching effect of SM while minimizing its growth inhibiting action.

Arabidopsis thaliana (L.) Heynh. was chosen because it has a very short life cycle, it can complete its life cycle *in vitro* on a

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fully defined medium (3), and its genetics is fairly well known.

It is hoped that studies of factors influencing the bleaching action of SM on a flowering plant will contribute to our knowledge of the mechanism of the bleaching action. Observations on the gametes of the bleached plants and on the progeny derived therefrom should contribute to a better understanding not only of SM action but also of normal chloroplast development and inheritance.

There is no agreement as to how SM bleaches. The question arises whether the bleached cells have lost their plastids permanently or whether the plastids continue to duplicate, but no longer differentiate into chloroplast. Lwoff (4) held that plastids did not disappear. Pringsheim (5) maintained that the plastids were lost from the bleached cells. Provasoli (2) suggested SM caused fragmentation and loss of plastids. Rosen (6) suggested that SM interfered with a Mn requiring step in chlorophyll synthesis. The destruction of chloroplast was considered to be a secondary effect.

Materials and Methods

Arabidopsis thaliana, family Cruciferae, was used throughout the investigation. Major mineral requirements were provided as Knop solution. The trace elements supplied were iron, maganese, zinc, copper, boron and molybdenum as recommended by Langridge (3). Unless otherwist stated the medium had 2 per cent sucrose. The water used was pyrex glass distilled. The mineral solution was adjusted to pH 6.2.

The nutrient solution was solidified with 0.75 percent agar. The melted medium was dispensed in 5 ml aliquots to 16 X 150 mm pyrex test tubes, which were then plugged with non-absorbant cotton wool and autoclaved at 15 pounds pressure for 10 minutes.

The seeds were sterilized by immersion in a solution of absolute ethanol and hydrogen peroxide (1:1) for 10 minutes and planted with a platinum loop. When the seeds were planted, care was taken to assure that the seeds were laid on the surface of the agar; for if they were placed even slightly below the surface, the plants failed to orient properly.

Immediately after they were planted, the cultures were placed in darkness at a low temperature (0°-5°C) for 48 hours to insure uniform germination. They were then transferred to the growth chamber which was maintained at 26°C and supplied with continuous illumination of 800 foot candles from Sylvania 'grow-lux' fluorescent lamps.

For bleaching, dihydrostreptomycin sulfate (Nutritional Biochemical Corporation) was used. In an attempt to find a bleaching technique with maximum bleaching effect but with minimum

growth inhibition, the following bleaching techniques were tried:

SEED SOAKING. Provasoli (2) reported that SM bleaching is a function of exposure time and concentration. Attempts were made to give seeds short exposure time at high SM concentration. SM concentrations used were 0.01-10 per cent, and soaking time were 5-48 hours.

ADDING SM TO THE MEDIUM. Because it is liable to heating, SM cannot be autoclaved. The autoclaved medium in the test tubes was kept in a melted state by placing the tubes in a water bath. Seitz filtered SM, to give a final concentration of 100 mg per liter, was added to the medium aseptically.

ATTEMPTS TO OVERCOME THE GROWTH INHIBITION EFFECT OF SM. One way SM might act on an organism is as an enzyme inhibitor, blocking the synthesis of some nutrient or growth substance necessary for normal growth of the organism. Plant growth substances, sugar, amino acids, vitamins, coconut milk, soy hydrolysate, casein hydrolysate and yeast extract were added to the meddium separately as well as in different combinations (Table 1).

Table 1. Summary of materials tested for overcoming growth inhibition of SM

Material	concentration range
3-Indole acetic acid (IAA)	50 mg/liter - 0.1 mg/liter
Gibberellic acid (GA)	100 mg/liter - 0.1 mg/liter
GA and IAA	50 mg/liter and 2 mg/liter respectively
Kinetin	50 mg/liter - 0.1 mg/liter
Kinetin and GA	2.0 mg/liter and 50 mg/liter respectively
Kinetin and IAA	2.0 mg/liter and 2.0 mg/liter respectively
1-Naphthalene acetic acid (NAA)	50 mg/liter - 0.1 mg/liter
NAA and GA	2.0 mg/liter and 50 mg/liter respectively
2, 4-Dichlorophenoxyacetic acid	50 mg/liter - 0.1 mg/liter
2, 4-Dichlorophenoxyacetic acid and GA	2.0 mg/liter and 50 mg/liter respectively
Inositol	50 mg/liter - 2.0 mg/liter
Tryptophan	100 mg/liter - 0.1 mg/liter
Casein hydrolysate	1.0 g/liter - 0.1 mg/liter
Yeast extract	1.0 g/liter - 0.1 mg/liter
Peptone	1.0 g/liter - 0.1 mg/liter
Soy hydrolysate	1.0 g/liter - 0.1 mg/liter
Malt extract	1.0 g/liter - 0.1 mg/liter
Coconut milk	100 percent - 10 per cent
Ascorbic acid, riboflavin, nicotinic acid, pyridoxine HCl, biotin, thiamine HCl (vitamin solution)	2.0 mg/liter - 0.01 mg/liter of each
Vitamin solution and GA	50 mg/liter GA and 2mg/liter - 0.1 mg/liter of each vitamin

RESULTS

BLEACHING.

SEED *soaking in SM*. Short exposures to high SM concentrations were very toxic. On the other hand, long exposures to low

SM concentrations produced incompletely bleached plants. Seeds soaked in 0.1 per cent SM for 25 hours were completely bleached and exhibited the most growth. These plants when grown in minimal medium had a stem 2 mm long, a pair of leaves 1 mm each, and a primary root 1.5 mm long.

ADDITION OF SM TO THE MEDIUM. 100 mg of SM per liter produced a completely bleached plant, and the plants exhibited similar growth has in the case of seeds soaked in 0.1 per cent SM for 25 hours (Table 2). Concentrations below this concentration produced incompletely bleached plants; where as stronger concentrations produced extremely stunted plants.

OVERCOMING THE GROWTH INHIBITORY EFFECTS OF SM. Bleached plants grown in minimal medium with 2 percent sucrose did not exhibit any additional growth over those grown in the minimal medium (Table 2).

Table 2. Growth of SM-bleached plants.

condition of plants	minimal medium with	stem length cm	leaf length cm	primary root length cm
wild type	minimal medium	10	2.0	3.3
wild type	2% sucrose	10.2	2.0	3.3
wild type	2% sucrose & 50 mg/ liter GA	10.5	2.0	3.4
bleached by soaking in 0.1 % SM for 25 hours	minimal medium	0.2	0.1	1.5
bleached by soaking in 0.1 % SM for 25 hours	2% sucrose	0.2	0.1	1.5
bleached by soaking in 0.1 % SM for 25 hours	2% sucrose & 50 mg/ liter GA	5.2	0.6	3.1
bleached by adding 100 mg/ liter SM to the medium	100mg/liter SM	0.2	0.1	1.7
bleached by adding 100 mg/ liter SM to the medium	2% sucrose & 100 mg/ liter SM	0.2	0.1	1.7
bleached by adding 100 mg/ liter SM to the medium	2% sucrose, 100 mg/ liter SM & 50 mg/liter GA	5.3	0.7	3.1

Gibberellic acid (50 mg/L) had a favorite effect on the growth of the bleached plants. These plants produced a stem 5.2 cm long, leaves 6 mm long, and a primary root 3.1 cm long (Table 2).

EFFECT OF LIGHT ON BLEACHING ACTION OF SM. SM bleached *Arabidopsis* only if both SM and light were present. Plans exposed to SM in darkness were not bleached. The results of effect of light on bleaching was summarized (Table 3).

DISCUSSION

Provasoli (2) postulated that SM acts on 2 sites in *Euglena*, one affecting the chloroplast, the bleaching site, and the other responsible for killing, the antibiotic site. The relative sensitivity of the sites determined whether an organism will be killed or

Table 3. Effect of light and darkness on the bleaching action of SM

growth in darkness	growth in darkness	growth in light	condition
SM-free medium	7 days →	transferred to light	2 days → green
subcultured on SM-free medium	7 days →	transferred to light	2 days → green
subcultured on SM-free medium	7 days →	transferred to light	2 days → green
planted on medium containing 100 mg/liter SM in darkness	7 days →	transferred to light	2 days → bleached
subcultured on SM-free medium	7 days →	transferred to light	2 days → green
subcultured on SM-free medium	7 days →	transferred to light	2 days → green

bleached by exposure to SM. The above worker also reported that the *Euglena* cells do not show any inhibition of growth if they are provided with sugar. The present investigation has failed to reveal the marked difference in sensitivity to the bleaching and toxic actions of SM. Attempts to reduce toxicity without reducing bleaching by shortening the exposure time or by reducing the concentration of SM have not been successful.

By growing a chlorophyll deficient mutant of *Arabidopsis* in Knop's medium wih different sugars, Langridge (7) found that 2 per cent sucrose was the most readily utilizable sugar. He also reported that the size of the plants was comparable to that of the wild type plants in culture. In the present investigation addition of 2 per cent sucrose to the medium did not cause any apparent promotion of growth of the bleached plants. The toxic effect of SM in *Arabidopsis* is not on the photosynthetic mechanism alone.

If the hypothesis that the toxic effect of SM on growth results from the necessity of some metabolites arising as a result of interference with the normal functioning of some enzyme is true, then by adding these substances normal growth should be obtained. Gibberellic acid anttagonized growth inhibition of SM. It is sug-

gested that SM interferes with Gibberellic acid metabolism of these plants.

It was observed that SM bleaching of *Arabidopsis* was permanent if both light and SM were present. Rosen (8) suggested that in *Euglena* the primary action of SM is inhibition of chlorophyll synthesis, with loss of chloroplasts, or failure of chloroplast synthesis, a secondary effect arising as a result of essentiality of chlorophyll for chloroplast synthesis.

It appears that proplastids are not affected by SM in darkness. In light, however, maturation of the proplastids is initiated but is blocked by SM from going to completion. The light activated, SM-inhibited conversion of the proplastid is irreversible; once the proplastids have been induced by light to a deranged form of chloroplasts, they can no longer form more proplastids and when all proplastids are converted to this form permanent bleaching results. These deranged forms of chloroplasts may be similar to the deranged forms of chloroplasts observed by Klein (9) in etiolated *Zea* leaves in which greening in light was blocked by low temperature or by dinitrophenol.

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