INFLUENCE OF SHADING AND GIBBERELLIC ACID ON GROWTH AND SEX OF THE PROTHALLIA OF *CERATOPTERIS THALICTROIDES*(1)

by

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ABSTRACT

The growth of prothallia of *Ceratopteris thalictroides* in area was seriously restricted under the full intensity of sun light in the greenhouse. By shading, however, the growth was improved. In the early period of their growth, the growth was better under 1/8–1/4 sun light. As the prothallia grew older, the better growth was made under 1/2 sun light. It is thought that these results are related to the "smallness" of the prothallium; the prothallium has to be small in order to get a suitable shading with ease in a small hollow of the ground. The best growth was made when the prothallia were placed in continuous daylight fluorescent light in culture room. All concentrations of gibberellic acid (GA) ranging from 0.01 to 100 ppm inhibited the growth of prothallia during the early stage of their growth under all intensities of sun light. As the prothallia grew older, the lower concentrations (*e. g.*, 0.01 ppm) became effective to promote the growth of prothallia which showed less growth under weak sun light. It thus appears that the lower concentrations of GA are effective to reverse the growth limitation imposed by the lower intensities of sun light. The effect of GA and light conditions on sexuality was indirect; primarily, GA and light conditions affected greatly on growth. More sex organs were formed on those prothallia which showed better growth in area. Both unisexual and bisexual prothallia were obtained in the present study. It is suggested that a large heterogeneity sexuality in this fern is presumably related to antheridium-inducing substances. The larger prothallia with antheridia less than five were always found forming the sporophyte. On rare occasions, two sporophytes were developed from two different archegonia on the same prothallium.

INTRODUCTION

The *Ceratopteris thalictroides* is an annual, aquatic fern usually found growing in the tropics in rice-fields, pools and marshes (Ford, 1902; Pal and Pal. 1962). Although the systematic position of this fern is still a matter of dispute it is ordinarily classified under the Parkeriaceae (Brongniart, 1821; Hooker, 1825, 1858; Kny, 1875; Diels, 1898, 1902; Benedict, 1909; Bower, 1928; Ching, 1940; Christensen, 1938; Copeland, 1947; Holttum, 1949; Wettstein, 1935).

As pointed out by Miller and Miller (1961), fern gametophytes have many advantages as research materials for experimental plant morphology. Some studies on

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the growth and sex of the fern prothallia have been made by Wuist (1910), Mottier (1910, 1925, 1927), Albaum (1938a, 1938b), Kato (1955), Mohr (1956a, 1956b, 1964), Näf (1956, 1958, 1963), Hotta and Osawa (1958), Miller and Miller (1961) and Momose (1964). The prothallia of *Ceratopteris thalictroides* have been previously studied by several workers. There is, however, a difference of opinion concerning the sexuality of the prothallia of this fern. According to the classic description made by Kny (1875) and Leitgeb (1879), the prothallia are dioecious, since the cultural materials studied by each of them showed a marked tendency toward dioecism. This view, however, was not accepted by Yabe and Yasui (1913) who also studied the material from culture. Although they could observe the one-lobed, small male prothallia, like those described by Kny (1875), they did not agree that those prothallia were true male, since they considered that these prothallia would continue to grow and finally produce archegonia on them if grown under suitable circumstances. Thus, Yabe and Yasui described the prothallia of *Ceratopteris thalictroides* as monoecious. Mahabale (1948) studied the prothallia of this fern found growing in its natural habitat in India and reported that all the prothallia he could harvest were bisexual. Later, this fern were again studied by Javalgekar (1960) in India who concluded that the prothallia were hermaphroditic in culture as well as in nature. Although he could find extremely small proportions of male prothallia, like those observed by Kny (1875) but he seems to disregard this small exception to his theory regarding hermaphroditism of *Ceratopteris*. The prothallia bearing only archegonia were not found by him.

It is now well known that gibberellins cause many physiological responses in many plants (Stowe and Yamaki, 1957; Phinney and West, 1960). Knoblock (1957) has published a short note on the effect of GA on spore germination and early growth of the gametophyte in *Dryopteris filix-mas* and said that the treated spores produced more and vigorous prothallia. Kato (1955) has worked on two species of ferns, *Dryopteris erythrosa* and *Cyathea* sp. and reported that the germination of spores was hastened and the growth of rhizoid cells were promoted by gibberellin treatment. Voeller (1964) reported that the gametophytes of some ferns were responded to gibberellins by forming antheridia. Recently, an interesting phenomenon concerning the role of antheridia-inducing substances in ferns in successful development of sex organs has been known (Näf, 1963; Galston, 1964).

The present investigation consists of the effect of the various light conditions and gilbberellic acid concentrations on growth and sex of the gametophyte of *Ceratopteris thalictroides*.

MATERIALS AND METHODS

Ceratopteris thalictroides (L.) Brongn. from Chiai, Taiwan was used as material for the present investigation. The spores used were collected on October 7, 1960 in the greenhouse of the Department of Botany, National Taiwan University where we had let the plants grow by themselves in an aquarium. Spores were sieved through the lens paper which retained the debris of sporangial wall, and then stored in desiccator. Prothallia were grown from these spores. Petri-dishes used for culture were of pyrex glass, 10 cm in diameter and 2 cm in depth. A layer of 0.5 gm cotton was paved in each Petri-dish, on which 1 mg of spores suspended in 20 ml distilled water were uniformly sown on March 23, 1962. Thirty six cultural Petri-dishes were set up and placed under continuous daylight fluorescent light (about 2,500 lux) in culture room. Spores germinated within about one week. Two weeks after sowing, *i.e.*, on April 6, 1962, distilled water in Petri-dishes were replaced by 1/2 strength of Hoagland's inorganic nutrient solution containing various concentrations of gibberellic acid (=gibberellin A_3) (GA). Thirty six Petri-dishes were divided into six groups and placed under six different light conditions: full, 1/2, 1/4, or 1/8 sun light in greenhouse, natural diffuse light in laboratory by the window (about 20,000 lux at noon), and continuous daylight fluorescent light in culture room (about 2, 500 lux). The intensity of full sun light was approximately 60,000 lux during middle of the fine day. The concentrations of GA in six Petri-dishes, in each group were 0, 10^{-2}, 10^{-1}, 10^{0}, 10^{1} and 10^{2} ppm respectively. Light intensity was adjusted by covering with sheets of perforated PVC black clothes on some of the Petri-dishes. By varying the number of holes (5 mm in diameter) uniformly perforated in the sheet the intensity of solar radiation was reduced to 1/2, 1/4 and 1/8 full sun light. The composition of Hoagland's solution was: distilled water 2 litters Ca $(NO_3)_2 \cdot 4H_2O$, 1.18 gm; KNO_3, 0.51 gm; $MgSO_4 \cdot 7H_2O$, 0.49 gm; KH_2PO_4 0.14 gm; FeEDTA, 0.005 gm; $MnCl_4 \cdot 4H_2O$, 1.81 mg; H_3BO_3, 2.86 mg; $ZnSO_4 \cdot 7H_2O$, 0.22 mg; $CuSO_4 \cdot 5H_2O$, 0.08 mg; $H_2MoO_4 \cdot H_2O$, 0.09 mg. Cultural solutions containing various concentrations of GA were renewed every week up to the third week after exposure to various conditions of light. Twenty prothallia were harvested from each Petri-dish at the dates of renewal of solutions, *i.e.*, on April 13 (1st week), April 20 (2nd week), and April 27 (3rd week), and fixed immediately in 5% $CuSO_4$ F. A. A. solution for microscopic study. The prothallia were mounted in distilled water on a slide and covered with a cover glass. The drawings of prothallia were made under the microscope by use of an Abbe's camera lucida. The area of prothallia, number of antheridia and archegonia and size of sporophyte were studied from these drawings. More than 2,000 prothallia were studied during this investigation. The average minimum and maximum temperature in ℃ during the last week of this experiment (from April 20 to April 27, 1962) were respectively: 19.5 and 27.7 in greenhouse, 26.2 and 26.5 in laboratory, and 21.5 and 22.5 in culture room.

EXPERIMENTAL RESULTS

Growth of prothallia under different light conditions.

As shown in Figs. 1 and 2, the growth of prothallia in area was remarkably influenced by various light conditions. The growth was seriously restricted under the full intensity of sun light in the greenhouse. By shading, the growth was improved. The prothallia required different degrees of shading at different stages of growth (Fig. 1). In the early period of their growth, *i.e.*, during the first week after exposed to different degrees of shading, the growth was better under 1/4–1/8 sun light. As the prothallia grew older they needed more light, *i.e.*, the better growth was made under 1/2 sun light, whereas 1/8 sun light was not sufficient during

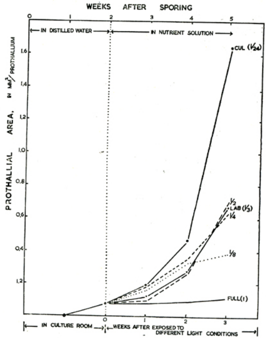


Fig. 1. Growth of prothallia in area under various light conditions. Cul. (1/24), under continuous daylight fluorescent light in culture room where the intensity of light was 1/24 that of full sun light; Lab. (1/3), under, natural diffuse light in laboratory where the light intensity was 1/3 that of full sun light; Full, 1/2, 1/4, 1/8, under indicated amounts of sun light in greenhouse.

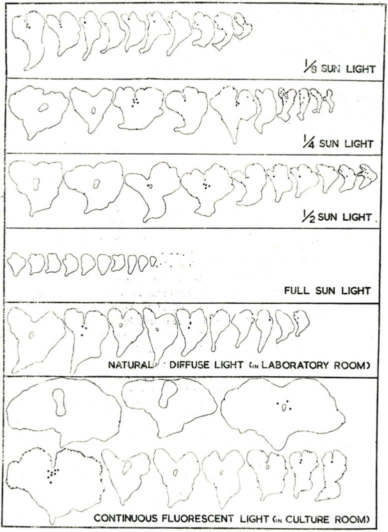


Fig. 2. Camera lucida drawings of prothallia, showing their growth and development in 1/2 Hoagland's solutions without GA under various light conditions for 3 weeks. Prothallia were 2 weeks old at the the time of beginning of the experiment. • indicates antheridium; ⊗ indicaties archegonium; ℊ indicates embryo-sporophyte. All × 13.

the third week. The prothallia grown in the natural diffuse light in laboratory by the window where the light intensity was approximately equal to 1/3 of full sun light of the greenhouse showed about equal growth as those prothallia grown under between 1/2 and 1/4 sun light in the greenhouse. The prothallia grow also in artificial light. Among all cultures, the prothallia showed the best growth in area when they were placed under the continuous daylight fluorescent light in culture room. Fig. 1 also shows that a slight delay of the growth of prothallia happened in the earlier stage will be recovered if they get a suitable light intensity in the later stage. Fig. 2 is camera lucida drawings of prothallia grown under various light conditions for three weeks. It is difficult to describe the effect of shading on the shapes of prothallia because of their great irregularity in appearance. In general, however, the prothallia were more or less elongated under the weak light whereas they were roughly circular in shape under the stronger light. (Compare the prothallia grown in 1/8 sun light with those in 1/4 or 1/2 sun light in Fig. 2). Some of the large prothallia grown under continuous artificial daylight fluorescent light were nearly elliptical or fun-shaped rather than wedge-shaped as seen in the other cultures. It is intereting to note that the prothallia were greatly variable in size even grown in the same Petri-dish (Fig. 2).

Growth of prothallia in different concentrations of GA under different light conditons.

The growth responses of prothallia to different concentrations of GA under different conditions of light vary as the prothallia grow older (Figs. 3A, 3B, 3C). All concentrations of GA inhibited the growth of prothallia in area during the first week of treatment under all intensities of sun light in the greenhouse (Fig. 3A). As the prothallia grew older (in the third week), the lower concentrations of GA, *i.e.*, 0.01–10 ppm, especially 0.01 ppm, became effective to promote the growth of prothallia which showed less growth under the weak sun light, *i.e.*, 1/4–1/8 sun light (Fig. 3C). It thus appears that the lower concentrations of GA are effective to reverse the growth limitation imposed by the lower intensities of sun light. As a whole, the effect of GA on growth of the prothallia in *C. thallictroides* was more or less complex, non-effective, promotive or inhibitory, depending upon the age of the prothallia, light conditions, and GA concentrations.

Formation of sex organs and embryo-sporophytes.

As shown in Table 1, the formation of sex organs and embryo-sporophytes is positively correlated with the size of prothallium in area. Generally speaking, the larger prothallium has a higher possibility to have sex organs and the sporophyte. The larger prothallium shows a tendency to be monoecious or bisexual. This implies that a dioecious or unisexual prothallium may develop to monoecious if its growth progresses under a suitable condition. Those which are cultivated in the greenhouse the percentage of male and monoecious prothallia increases as the prothallia increase in area. The percentage of female prothallia, on the other hand, decreases as the

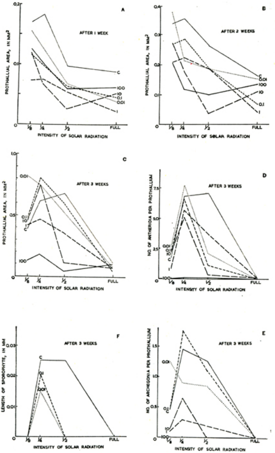


Fig. 3. Effects of GA and solar radiation on growth of prothallia and formation of sex organs and sporophytes. A-C, growth of prothallia in area in nutrient solutions containing various concentrations of GA (in ppm) under various intensities of solar radiation for one (Fig. 3A), two (Fig. 3B) and three (Fig. 3C) weeks in greenhouse. D, average number of antheridia per prothallium. E, average number of archegonia per prothallium. F, average length of embryo-sporophytes.

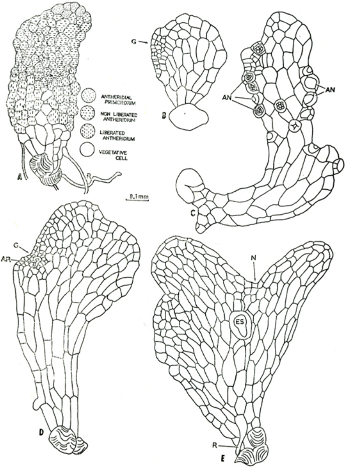
Table 1. Formation of sex organs and embryo-sporophytes on the prothallia grown under various light conditions*.

Light conditions	Size of prothallium in area in mm² per prothallium	% of prothallia						
		bearing no sex organs (immature)	bearing sex organ	dioecious prothallia ♂	dioecious prothallia ♀	dioecious prothallia tatal	monoecious prothallia ☿	bearing embryo-sporophytes
Greenhouse								
Full sun light (1)	0.12	95	5	5	0	5	0	0
1/8 sun light	0.39	45	15	20	25	45	10	0
1/4 sun light	0.62	0	100	25	10	35	65	15
1/2 sun light	0.65	0	100	30	0	30	70	25
Laboratory (1/3)								
Natural diffuse light	0.67	10	90	25	30	55	35	15
Culture room (1/24)								
Continuous daylight fluorescent light	1.66	0	100	0	10	10	90	65

*Average of 20 prothallia examined at the end of 3rd week under experimental conditions.

prothallia increase in area. The prothallia showed the best growth in area under continuous daylight fluorescent light and all of them bore archegonia (Table 1). Unisexual prothallia (Figs. 2, 4A, 4C, 4D, 4E, 5A, 5D), were obtained from some of the cultures. The percentages of male and female prothallia were 22.2% and 4.4% respectively (Table 2). The majority of prothallia were monoecious or hermaphroditic (42.6%) (Figs. 2, 4F, 4G, 5C). The male prothallium is usually smaller in size and generally spatulate or elongated in form (Figs. 2, 4A, 4A, 4C, 5A). Many antheridia are produced on the whole area of some male prothallia in a single layer except the smaller regions of the prothallium at the base near spore where cells remained vegetative (Fig. 4A). The maturation of antheridia on the male prothallium is accomplished basipetally, *i.e.*, the immature antheridia are found at the tip and mature antheridia near the base of the prothallium. Remaining 30.8%

Fig. 4. Camera lucida drawings of some representative prothallia at various levels of growth and development under various conditions. A, a male prothallium grown on moist sand in a small flower pot under diffuse natural daylight in laboratory for four weeks from spore. B, an immature prothallium found in 0.01 ppm GA under full sun light in greenhouse for three weeks. C, a male porthallium found under 1/8 sun light without GA in greenhouse. D, a growing prothallium found in 1 ppm GA under natural diffuse light in laboratory for three weeks. Note that the archegoium was produced on the prothallium prior to antheridium. E, a female prothallium grown in 1/2 Hoagland's solution containing 0.01 ppm GA under continuous fluorescent light in culture room for three weeks. F, a monoecious prothallium with many antheridia and archegonia found growing in nutrient solution without GA added, under continuous daylight fluorescent lamp in culture room for three weeks. G, a monoecious prothallium with only a few antheridia and an embryo-sporophyte found growing under same condition as in Fig. 6F.


ANTHERIDIAL PRIMORDIUM
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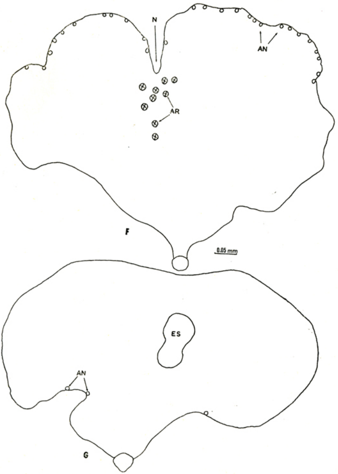

Fig. 5. Photographs of some representative prothallia obtained in the present work. A, a male prothallium with about thirty marginal and sub-marginal antheridia. B, a female prothallium with four archegonia, only one of which developed into an embryo-sporophyte. C, monoecious prothallium with one archegonium and nine marginal antheridia. D, a prothallium with twin-sporophytes.

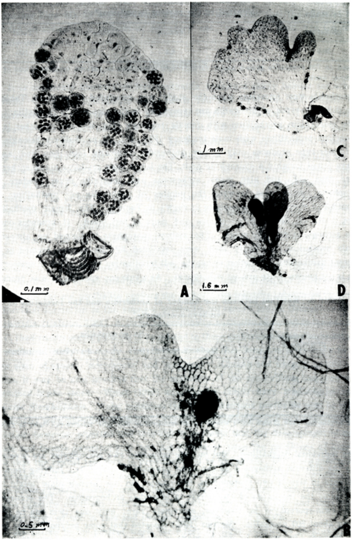
0.1 mm
A
1 mm
C
1.6 mm
D
0.5 mm
B

Table 2. Average % of immature, male, female and monoecious prothallia obtained in the present experiment*

Immature	Male	Female	Monoecious	Total
30.8	22.2	4.4	42.6	100

*Based on 720 prothallia grown under various light conditions in various concentrations of GA for three weeks.

of prothallia were immature when the study was concluded (Figs. 2, 4B, Table 2). Two different types of prothallia are recognized in the monoecious prothallia: one is the monoecious prothallium with female tendency (Fig. 4G) and the other is the monoecious prothallium with male tendency (Fig. 4F). A prothallium belonging to the former type produces only a few antheridia (typically less than five) and is always found forming a sporophyte on it. The latter type produces many antheridia (more than six) and also many archegonia and generally never produce any sporophyte. A relation between the number of antheridia per prothallium and formation of the embryo-sporophyte is shown in Table 3. The female prothallium (Figs. 2, 4D, 4E, 5B) is larger in size and more or less lobed. It produces one or more archegonia near the notch. The embryo-sporophyte is formed on almost all female prothallia. Evidently they were fertilized by the sperms formed in the different prothallium. Whether or not the self-fertilization takes place in monoecious ones is not known in the present experiment. As a rule one sporophyte was produced

Table 3. Relation between the number of antheridia and the formation of embryo-sporophyte*

No. of antheridia per prothallium	No. of prothallia	
	produced sporophyte	not produced sporophyte
0 – 9	15	0
10 – 19	0	1
20 – 29	0	1
30 – 39	0	3

*The prothallia were grown for 3 weeks under continuous daylight fluorescent light in culture room.

on an individual prothallium. But on rare occasions, two sporophytes are developed from the different archegonia on the same prothallium (Fig. 5D). Judging from the difference in size of two sporophytes, fertilization in one archegonium might take place after the formation of sporophyte in the other archegonium. The occurrence of the twin-sporophyte is, however, extremely rare; only one twin can be found among about 500 prothallia. Mottier (1925) reported that some polypodiaceous ferns and *Osmunda Claytoniana* L. may be made to produce two to many sporophytes on the individual prothallium under experimental conditions. In Table 1 and also by comparing Figs. 3D, 3E and 3F with Fig. 3C, it is evident that the effect of light and GA on sexuality is indirect or secondary one; primarily, light and GA affect greatly on growth.

DISCUSSION

It is commonly known that fern prothallia require a shady habitat for a better growth. Unlike the sporophyte which needs full sun light, the prothallium of *C. thalictroides* can grow only under a suitable degree of shading. The prothallium shows the best growth under 1/4 sun light in the early stage of its growth and then under 1/2 sun light as the prothallium grows older. This experimental result may help us to understand the fact that the prothallium of this fern is so tiny as compared with the sporophyte. The prothallium has to be small in order to get a suitable degree of shading with ease in the small hollow of the ground or under the other plants in nature. The prothallia showed a great deviation in size even they were cultivated under the same environmental condition. This is presumably also related to the "smallness" of the prothallia, since every prothallium is so small that it is not easy to get exactly the same environmental condition on the wet cotton, *i. e.*, some prothallia were found submerged under the cotton fibers and the others were exposed to the air. Recently, it has been known that the formation of antheridia in ferns is dependent on "togetherness" or the simulaneous presence of many prothallia of heterogeneous ages, since the old prothallia produce the antheridium-inducing substances that induce antheridial formation in the younger prothallia but cannot induce themselves to form antheridia (Galston, 1964). A large heterogeneity sexuality observed in the prothallia of *C. thalictroides* seems possible to be related to these antheridium-inducing substances. A statistical study on spore size indicated that this fern has a tendency to have two sizes of spores, 'microspore' and 'megaspore' (Chiang and Chiang, unpublished). Thus, it may be also possible that the sexuality of the prothallium has been predetermined in the spore. Wuist (1910) and Mottier (1910) have studied the sex of *Onoclea Struthiopteris*. Wuist considers that the monoecious prothallia may be induced artificially from dioecious prothallia. She could induce antheridia on female prothallia. Mottier, however, reported that it is highly probable that the development of purely male and female gametophytes are not dependent upon the conditions of nutrition, but that the sexual tendency is predetermined in the spore. The environmental determination of sexuality is known in numerous plants. According to Swanson (1957) *Equisetum* exhibits marked female tendencies in strong sun light and male tendencies under opposite conditons. He said that whether there is a subtle and unrecognized genetic basis of sex in this and similar organisms is not known.

As known in the present work, the effect of light conditions on growth of the prothallia is certainly striking. Miller and Miller (1961) reported that the light is important in at least two roles in determining the growth and morphology of the gametophyte of *Onoclea sensibilis*. One is the requirement for photosynthesis; and the other is a non-photosynthetic, low intensity light requirement which is known to be a red-far-red phenomenon. He said that the increase of growth of the gametophytes in area grown on sucrose in red light is almost completely reversed in the gametophytes treated with far-red. In the present experiment the growth inhibition of prothallia under strong sun light may be due to the strong far-red light which is a component of the solar spectrum. By increasing the degree of shading, this inhibiting effect of far-red light decreases, and consequently results in increase of growth of the prothallia. On the contrary, the growth retardation in lower intensities of sun light is presumably due to the deficiency of solar energy for photosynthesis. The best growth of the prothallia is made under continuous daylgiht fluorescent light in which far-red light is weak.

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