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1. Diplontic selection and its modification by gamma radiation in a colchicine-treated grass hybrid.
2. (Proposed) Comparative radiosensitivity studies in the Gramineae.

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Summary of Completed and Planned Work

The principal objectives of the research conducted in 1964-1965 were: (1) To determine whether gamma radiation could inhibit the growth of triploid tissue in shoot meristems of an inter-specific hybrid, while colchicine-produced hexaploid tissue in the same meristem continued to develop because of the differential radiosensitivity of the tissues. (2) Calculate the average interphase chromosome volume for Agropyron cristatum and A. subsecundum, the presumed parents of the hybrid. (3) Produce a controlled hybrid between A. cristatum and A. subsecundum. (4) Prepare a karyotype analysis of the natural triploid hybrid between the above Agropyron species which would permit us to determine whether total chromosome length is a reliable index of radiosensitivity.

Objectives (2) and (4) were achieved. The data from (2) will be utilized in our investigations of comparative radiosensitivity. The data from (4) clearly indicate that total chromosome length is not useful in predicting radiosensitivity.

The primary objective (1) was not achieved, despite this second year of concentrated effort. We are, however, by no means ready to abandon our idea since the hypothesis has not yet been fully tested. We have had extraordinary difficulty in producing hexaploid tissue, and, in addition, we have only recently been able to determine with certainty that our gamma dosages have been too high.

Objective (3) also was not achieved. We have no reasonable explanation for this other than the intrinsic difficulties associated with such wide crosses.

The scope and objectives of the proposed 1966-1967 investigations are:

1. Gaining predictable control of diplontic selection of triploid tissue through interaction of colchicine and ionizing radiation remains an important objective. More efficacious methods of colchicine treatment have been devised, and we now have a better understanding of the appropriate dosages to apply.
2. Additional attempts will be made to produce a controlled hybrid between A. cristatum and A. subsecundum.
3. A new program of study entitled, "Comparative radiosensitivity studies in the Gramineae", will be initiated. Using a unique assemblage of diploid and polyploid representatives of different species, we will attempt to resolve the following questions:
 - (1) Are the effects of X-rays on polyploid plants independent of the type of polyploidy present, when interphase chromosome volume and energy absorbed per chromosome are used as the bases of comparison?
 - (2) What is the effect of hybridity on radiosensitivity in a representative of the Gramineae?

Introduction

The differential response of organisms, tissues and cells to ionizing radiation continues to be one of the most interesting and baffling aspects of radiobiology. We were initially intrigued with the possibility of controlling ploidy levels in colchicine-treated meristems through differential sensitivity to gamma radiation. The production of polyploids through colchicine treatment has from the beginning been an unpredictable, strictly empirical, procedure. It occurred to us that, in view of the relative radioresistance of polyploid tissue it might be entirely possible to inhibit the growth of diploid or triploid tissue in a chimeric meristem, and permit the doubled tissue, produced by colchicine, to form new shoots in a predictable fashion. We have not yet fully achieved our objectives, and some of the difficulties we've experienced are described in the report.

During 1965 several investigators made extremely important advances in the field of comparative radiosensitivity. Sparrow (1965); and Sparrow et al. (1965) presented important evidence that the single most significant factor in radiosensitivity is the amount of energy absorbed per chromosome. This hypothesis suggests, among other things, that the higher radioresistance shown by most polyploids is simply a reflection of their smaller interphase chromosome volume and does not have its origin in the protective effect of genetic redundancy. Evans (1965) presented evidence that chromosome fracture and the subsequent loss of acentric fragments is not the prime cause of radiation injury.

Entirely new concepts of the bases of differential radiosensitivity are therefore being formulated. We have assembled and synthesized unique experimental materials during the last 10 years. The financial support given us by the A.E.C. for 2 of those years has been instrumental in allowing us to move as close as we have to our goal. We believe that our past experience as well as the data and materials we've developed, will now permit us to make a significant contribution to an understanding of what effects polyploidy and hybridity have on sensitivity to ionizing radiation.

Materials and Methods (1964-1965)

I. Colchicine and radiation treatments.

1. Forty clones of the hybrid: Agropyron cristatum X A. subsecundum were removed from pots April 9, soil washed off, roots clipped, and placed in containers of aerated tap water overnight.
2. The roots of each clone were immersed in 0.2% colchicine for 12 hours and then in tap water for 12 hours, alternately, for 4 days. The colchicine solution and the water in which the roots were immersed were aerated in order to increase the number of cells entering mitosis.
3. All clones were repotted and kept in the greenhouse for 3 days.
4. The 40 clones were then divided into 4 lots of 10 plants each. Three lots were subjected to the following radiation doses on April 17: Lot I: 2000 R; Lot II: 3000 R; Lot IV: 4000R. The fourth lot of 10 plants served as controls. The radiation was given with a Picker C 3000, model 581A cobalt⁶⁰ gamma therapy

instrument. The dose rate was 72.3 R per minute. The source activity was approximately 3175 curies. The instrument is located in the Radiology Department of St. Marks Hospital, Salt Lake City.

5. Following irradiation the clones were repotted in larger containers and returned to the nursery.

II. Completion of analysis of 1964 treatments.

Numerous new culms were formed following last year's treatments. Because these did not flower in 1964, completion of analysis was delayed until anthesis in June, 1965. Each spike produced was tested for ploidy level by preparing an acetocarmine smear of an anther of one spikelet from each spike produced. The triploid condition was identified by (a) direct observation of PMC divisions, (b) presence of sterile pollen, or (c) aborted anthers. A total of 1027 spikes were examined.

III. Average interphase chromosome volumes of A. cristatum and A. subsecundum.

Vegetative meristems were dissected from clones of control plants, fixed in Craff III, dehydrated with tertiary butyl alcohol, sectioned in paraffin, stained with warm safranin and fast green, and mounted in balsam. Measurements of nuclear diameters were made from median longitudinal sections cut at 10 microns. Interphase nuclear diameters were calculated from the mean diameter obtained by measuring two diameters at right angles to each other under the oil immersion lens. The nuclei measured were in the corpus region, chiefly above the two youngest primordia. Nuclei in the tunica were avoided because of their distorted shape.

One hundred interphase nuclei from 6 meristems from each species were measured. No attempt was made to distinguish between G1, S, and G2 stages. The formula for the volume of a sphere was applied and the average interphase chromosome volume was obtained by dividing the nuclear volume by the somatic chromosome number.

IV. Hybridization studies between A. cristatum and A. subsecundum.

In view of the reported self sterility of A. cristatum (Dewey, 1964) synthesis of the hybrid was attempted without emasculation with this species serving as the female parent. Two systems of pollination were utilized: (1) hand pollination, where pollen from A. subsecundum was brushed on the stigmas of A. cristatum, and (2) pollinator culms of A. subsecundum were placed in containers of water attached to a stake placed adjacent to the culms of A. cristatum. Five spikes of A. cristatum were utilized in each system. In all cases the inflorescences were subsequently covered with a Kraft paper bag. Seven flowering culms were used in the first method and 76 in the second. A total of 415 spikes of A. cristatum were utilized.

Two difficulties attend the crossing of these species: A. subsecundum flowers early in June and the florets open in the morning hours. A. cristatum flowers in late June, and the florets open in the afternoon hours. The overlap time is short indeed.

Seeds were collected and planted in compartmented flats. The success or failure of hybridization could be detected early by examining mitotic divisions in the root tips of the seedlings. The hybrids would have 21 chromosomes and the selfs, 14, since the latter of course are derived from diploid A. cristatum used as the female parent.

The chromosome counts were facilitated by pre-treatment of the excised root tips in 0.1% colchicine for 4 hours.

V. Karyotype analysis of the natural triploid hybrid between A. cristatum and A. subsecundum.

The structural features of each chromosome in the somatic complement of the hybrid were obtained chiefly from acetocarmine squash preparations of root tips. Low temperature pre-treatment of excised root tips provided the most useful material. Thirty-three complements were analyzed. Material from root tips sectioned in paraffin, and those pre-treated with colchicine and 8-hydroxyquinoline provided useful supplementary data.

VI. New colchicine treatments

The techniques (described earlier) that we successfully employed to induce chromosome doubling in other hybrids (Ashman and Boyle, 1955; Boyle, 1960), have been notably unsuccessful with the present hybrid. In October and November of 1965 we tried modifications of Sears' (1941) cotton pack and Bell's (1950) glass tube techniques.

Ten large potted plants of the hybrid were permitted to go dormant in the summer. On September 15 they were repotted and transferred to the greenhouse under optimum growing conditions. Abundant new tillers were initiated. On October 18 the tops of 5 of the plants were clipped and a cut-off section of a coffee can placed around the crown. Absorbent cotton was then tightly packed between the culms of the crown and around the crown. The cotton was kept moist with twice-daily applications of 0.5% colchicine for 5 days. The pots were covered with bell jars and kept well watered during the treatment period. The bell jars were kept over the plants for an additional 5 days.

The other 5 plants were subjected to a continuing glass-tube type of colchicine treatment from September 15 to December 6. As new tillers developed they were clipped to a length of approximately 2" and a glass tube of small diameter slipped over the shortened tiller. The tubes were sealed at the base with lanolin and supported by a wire extending into the soil of the pot. The tubes were kept filled with 0.1% colchicine for 5 days. A total of 110 tillers were treated in this fashion. These cut tillers were expected to act simply as conduits to transmit colchicine to meristematic tissue in the crown and not themselves to continue to grow. New tillers formed later will, hopefully, contain doubled sectors.

It is extremely difficult to detect doubled sectors in the somatic tissue of grass shoots. In order to obtain pollen mother cells in which the ploidy level can be readily detected, the treated plants were subjected to a lengthened photoperiod in January and February to induce anthesis. If the cotton pack or glass tube treatments are successful they will be used in another colchicine-radiation series of treatments in the spring of 1966.

Results and Discussion

I. Colchicine and radiation interaction.

The working hypothesis of this experiment is as follows: Induction of polyploidy by conventional treatments is at best an empirical, unpredictable procedure. It occurred to us that the production of polyploids might be placed on a predictable basis by utilizing a possible differential in radiosensitivity; in this case between triploid and hexaploid tissue. Accordingly, we have attempted to induce hexaploid tissue development by various kinds of colchicine treatment and then have irradiated treated plants with the expectation that the triploid tissue, probably being more radiosensitive, would be retarded or killed while the hexaploid tissue, probably being more radioresistant, would be able to grow and produce new tillers. "Diplontic" selection of triploid cells would thereby be blocked.

We have thus far been unable to substantiate the hypothesis. There are at least three possible reasons for this: (1) We have found it extraordinarily difficult to produce hexaploid tissue in the shoot system. There may simply not have been enough doubled tissue present to test the hypothesis. (2) Hexaploid tissue may not, in fact, be more sensitive to ionizing radiation than triploid tissue. Most, but not all polyploids are more resistant than diploids in terms of KR required to produce a comparable end point (Sparrow et al., 1965). (3) The doses of gamma employed may have been too heavy. Spike production in the radiated lots was profoundly affected (Table I and Figures 1,2).

The first and third alternative explanations appear to be most likely. Examination of root tips of colchicine treated plants (first series) disclosed abundant hexaploid plates. The effectiveness of the cotton-pack and glass tube treatments remains to be evaluated.

All of the spikes produced in the lot irradiated with 4000 R were produced three months later than normal. Seven of the nine came from one plant, from tillers arising from an underground rhizome near the edge of the pot. The parent plant died.

No seeds were produced from any lot.

Examination of the plants 6 months after irradiation disclosed the following mortality: Lot I (2000R) - 20%; Lot II (3000R) - 60%; Lot III (4000R) - 80%. The two survivors in Lot III endured only by virtue of laterally arising tillers from rhizomes well buried in the soil at the time of radiation. The original crowns in both cases were killed.

We are aware that in terms of the absorbed energy in eV per chromosome, Sparrow, et al (1965) found no statistically significant difference in sensitivity between diploids and polyploids. However, this of course does not mean that polyploid plants or tissues may not tolerate larger doses than diploids in terms of total R delivered. The difference may indeed be due to a smaller interphase chromosome volume in the polyploid. The question of the influence of genetic redundancy is not applicable here because in a strict allopolyploid no duplication (beyond homologous pairs) is present.

II. Completion of analysis of 1964 treatments.

As explained in our previous report, numerous new culms that formed following last year's treatments did not flower in 1964. In June of 1965 all spikes produced were checked for ploidy level. The results are shown in Table 2. The additional data further confirmed the failure of the 1964 treatments.

III. Average interphase chromosome volumes of A. cristatum and A. subsecundum:

These species are almost certainly the parents of the hybrid currently being studied. The nuclear volume and interphase chromosome volume data for these species, compared to those of their hybrid, are summarized in Table 3.

It is of interest to note that the addition of one complement in the triploid hybrid did not result in an increased nuclear volume compared to the diploid A. cristatum. The decrease in average interphase chromosome volume in the polyploids is noteworthy. It will be of considerable interest to find out if A. cristatum and the hybrid are equally radiosensitive as would be predicted by Sparrow (1965) on the basis of comparable interphase chromosome volume.

IV. Hybridization studies between A. cristatum and A. subsecundum.

Results of the attempted hybridization are shown in Table 4.

Examination of root tip divisions in the 125 seedlings disclosed that all were selfs from A. cristatum, possessing 14 chromosomes. Obviously, under the conditions of this experiment, A. cristatum is not as self sterile as reported (Dewey, 1963). Approximately 42% of the seeds did not germinate or produced short-lived seedlings possessing varying degrees of albinism.

Additional plantings of both A. cristatum and A. subsecundum have been made which will permit much more overlap in anthesis. This will facilitate renewed attempts to hybridize these species next year.

Despite the virtually conclusive circumstantial evidence that A. cristatum and A. subsecundum are the parents of the hybrid, positive proof demands synthesis of the hybrid. Our planned studies in comparative radiosensitivity involving these taxons require unequivocal establishment of the genetic relationships involved.

V. Karyotype analysis of the natural triploid hybrid between A. cristatum and A. subsecundum.

Figures 3-8 are representative of the camera-lucida drawings and photomicrographs from which the data for construction of the karyotype was obtained. An ideogram summarizing the structural features of the triploid somatic complement is portrayed in Fig. 9. The total chromosome length at metaphase is calculated to be approximately 186 microns.

Total chromosome length is closely related to total DNA content per nucleus in wheat and barley (Bhaskaran and Swaminathan, 1960, 1961). Sparrow (1965) has shown that as metaphase chromosome length increases, the interphase chromosome volume will also increase and the latter is unquestionably of major significance as an index of radiosensitivity. In light of these relationships, we expected that total chromosome length would prove to be a useful index of radiosensitivity.

The evidence, summarized in Table 5, indicates, however, that total chromosome length at metaphase, is not a useful index of radiosensitivity. The taxa have similar lethal levels but are strikingly different in total chromosome length.

Chromosome length at metaphase has some puzzling relationships with other data (Marshak and Bradley, 1944). These workers observed that while the inhibitory effects of X-rays on mitosis in *Triticum* and *Bromus* were inversely proportional to chromosome number, the effects were not proportional to chromosome length at metaphase.

The relative amounts of heterochromatic segments, the role of the centromere, and possible differential condensation at metaphase are probably significant but poorly understood factors that account for this lack of correlation between species.

It is virtually certain that A. cristatum and A. subsecundum are the parents of the hybrid. The several lines of circumstantial evidence supporting this conclusion have been previously described in earlier reports. Our 1964-65 karyotype analysis data add additional evidence. Three chromosomes from in A. cristatum can be positively identified in the hybrid. Cytological studies on A. subsecundum are expected to further confirm this parentage.

Treatment	No. plants	No. spikes produced
colchicine + 2000 R	10	2
colchicine + 3000 R	10	2
colchicine + 4000 R	10	9*
colchicine only	10	568

* produced laterally and very late from one underground rhizome;
none from original crown.

Table I. Spike production in irradiated and control plants.

Treatment	Number of spikes produced	Ploidy level
<u>Lot II.</u> One immersion of roots in colchicine (0.1% for 24 hrs.) followed a week later by one gamma irradiation (1000R).	222	3 n
<u>Lot III.</u> Colchicine spray on lower culms (0.1% for 8 hours each week for 5 weeks). Gamma irradiations (1000R) were applied at 5 weekly intervals following colchicine treatment, for a total of 5000R.	2	3 n
<u>Lot IV.</u> Colchicine spray as in Lot III. No radiation.	94	3 n
<u>Lot V.</u> Immersion of roots in colchicine (0.1% for 24 hours) each week for 5 weeks. No radiation.	36	3 n
<u>Lot VI.</u> Colchicine immersion as in Lot V and gamma radiations (500R) applied at 5 weekly intervals following each colchicine treatment for a total of 2500R.	105	3 n

Table 2. Effects of 1964 treatments as analyzed from plants flowering in 1965.

Species or hybrid	Average nuclear volume (μ^3)	Somatic chromosome number	Average interphase chromosome volume (μ^3)
<u>A. cristatum</u>	295.5	14	21.1
<u>A. subsecundum</u>	442.5	28	15.8
hybrid	294.5	21	14.0

Table 3. Nuclear volume and interphase chromosome volume of A. cristatum, A. subsecundum, and their hybrid.

Pollination system	No. spikes of <u>A. cristatum</u> used	No. seeds produced	No. seedlings established	% selfs in seedlings
Hand pollination	35	56	29	100%
Pollinator culms	<u>380</u>	<u>156</u>	<u>96</u>	100%
	412	212	125	

Table 4. Results of hybridization studies between A. cristatum and A. subsecundum.

Plant	Total chromosome length (microns)	Lethal level (R)
<u>Crepis capillaris</u>	81.62 (1)	3750 (3)
<u>A. cristatum</u> X <u>A. subsecundum</u>	185.96	4000 (4)
<u>Triticum aestivum</u>	353.1 (2)	4022 estim. (3)

Table 5. Lack of relationship between total chromosome length and lethal level of radiation. (1- Hollingshead, 1930; 2- Marshak & Bradley, 1944; 3- Sparrow, et al., 1965; 4- Bottino, 1965).

Figure 1. Effects of various levels of gamma irradiation on colchicine-treated plants approximately 4 months after irradiation.

Figure 2. Lateral tillers arising from underground rhizome in a plant irradiated with 4000R.

10 μ

Figure 3. Camera-lucida illustration of somatic metaphase from root tips of the hybrid A. cristatum X A. subsecundum.

Figure 4. Camera-lucida illustration of somatic metaphase from root tips of the hybrid A. cristatum X A. subsecundum.

10 μ

Figure 5. Camera-lucida illustration of somatic metaphase from root tips of the hybrid A. cristatum X A. subsecundum.

Figure 6. Photomicrograph of somatic metaphase from root tips of the hybrid A. cristatum X A. subsecundum.

Figure 7. Photomicrograph of somatic metaphase from root tips of the hybrid A. cristatum X A. subsecundum.

Figure 8. Photomicrograph of somatic metaphase from root tips of the hybrid A. cristatum X A. subsecundum.

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Figure 9. Composite ideogram of structural features of the triploid somatic complement of the hybrid A. cristatum X. A. subsecundum.