



Genetic determinations in winter wheat *Triticum aestivum* L. em. Thell. aneuploids using serological techniques

by Raymond Bradford Volin

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
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Abstract:

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Antibodies were produced in rabbits by injecting them with leaf extract antigens from 2 sources: (1) a disomic which was derived from segregating monosomic and disomic lines of the variety Kharkoff MC22 and (2) a single plant disomic selection. Two lots of crude sap and two lots treated with ammonium sulfate saturation methods were used.

Ouchterlony's gel diffusion techniques revealed no differences between the different inject antigens or between the monosomic and disomic antigens when reacted against any one antiserum. Precipitate lines of partial or nonidentity were not present. All antigens were absorbed by any one of the 4 antisera sources. A soluble precipitate complex occurred when crude antigen or absorbed antisera was reacted against the purified antigen but was not antigenically important.

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ABSTRACT

Antigenic components from the leaves of several monosomic ($2n-1$) and disomic ($2n$) plants from each of eighteen chromosome substitution lines of winter wheat, Triticum aestivum L. variety Kharkoff MC22 ($2n=42$) were compared serologically.

Antibodies were produced in rabbits by injecting them with leaf extract antigens from 2 sources: (1) a disomic which was derived from segregating monosomic and disomic lines of the variety Kharkoff MC22 and (2) a single plant disomic selection. Two lots of crude sap and two lots treated with ammonium sulfate saturation methods were used.

Ouchterlony's gel diffusion techniques revealed no differences between the different inject antigens or between the monosomic and disomic antigens when reacted against any one antiserum. Precipitate lines of partial or nonidentity were not present. All antigens were absorbed by any one of the 4 antisera sources. A soluble precipitate complex occurred when crude antigen or absorbed antisera was reacted against the purified antigen but was not antigenically important.

No differences could be found between antigens inherent in the monosomic and disomic genetic condition when compared serologically.

INTRODUCTION

Plant breeders and cytogeneticists have long searched for workable methods to economically improve cereal crop varieties genetically.

With the development of monosomic ($2n-1$) and nulisomic ($2n-2$) lines in hexaploid wheat, a new technique of genetic improvement became available. Through use of this technique, complete chromosomes containing genes of desirable cereal quality or disease resistance may be substituted into an otherwise acceptable genetic complex. The resultant goal is a variety engineered to meet the plant breeder's specifications.

A major problem in this technique lies in positive cytological identification of the aneuploid condition. While microscopic analysis reveals the monosomic condition, it may be seasonally untimely, as well as laborious. An accurate, less time consuming, method is needed.

Serology, the study of blood serum, is used as a method in bio-systematic and plant taxonomic comparisons. By reacting immune sera with homogenous antigenic material, and relying upon the specificity of the immune sera, corresponding relationships may be quantitated and evaluated.

The nature of the biochemical components of the plant are determined by chromosomes. These units of heredity direct processes including protein synthesis; the composition of such proteins being determined by the nucleotide "code" inherent within the chromosomes.

Welsh and Hehn (1964) launched a study to determine, through the use of monosomic ($2n-1$) lines, the chromosome or chromosomes which carry factors influencing bread flour quality. Tests indicated that chromosome 1D (XVII) was very important in the determination of bread flour charac-

teristics. The monosomic condition of this chromosome resulted in drastic reduction in gluten strength of the flour. It was postulated that some structural change in protein had occurred allowing for the alteration of various physical properties of the endosperm.

The objective of this investigation was to determine if there are detectable serological differences between a disomic ($2n$) line of hard red winter wheat Triticum aestivum L. em. Thell variety Kharkof MC22 and several derived monosomic ($2n-1$) lines of the same variety. Meaningful differences could then be useful in identifying monosomic individuals.

REVIEW OF LITERATURE

I. Serology

Bordet (Boyden 1942) initiated interest in the use of serology for studying organism relationships in 1895 when he recognized the agglutinin reaction as a "distinct phenomenon of immune serum". Bordet indicated by further study that an injected animal will not respond to the injections of proteins from closely related species and that this inability to respond may serve as a guide to close relationships.

The discovery of the precipitin reaction is generally accredited to Kraus (Boyden 1942). In 1897 he mixed antiserum obtained from goats with filtrates used for injection and observed the presence of a precipitate.

Nuttall (1901) pioneered in zoosystematics by publishing on the phylogenetic relationships of many animals using the precipitin reaction. The intensity of the antiserum-antigen reaction was found to parallel the systematic position of the species tested. The application of these same techniques to plant systematics was undertaken in the same year by Kowarski (Chester 1937). Kowarski injected heat-resistant wheat albuminose into rabbits and found that the induced precipitins reacted strongly with wheat albumin extracts and weakly, or not at all, with the extracts of rye, barley, oats or peas.

One year later, Bertarelli (Chester 1937) successfully applied the precipitin method in the detection of wheat flour contamination by Vicia sativa.

Ballner and Burow (Chester 1937) working with grains in 1911 showed that rye is serologically more closely related to wheat than to barley and oats. They also found that rice and maize were still more distantly related to rye. In 1911 Relander (Chester 1937), after working with varieties of barley, oats, Vicia, clover and lupine, succeeded in serologically separating the various species and, in some cases, the varieties of a single species.

Applying serology to the study of grape hybrids Rives (Baldwin et al., 1927) found in 1923 that closely related grape hybrids could be grafted upon each other. Those which did not show close serological relationships could not be grafted together. Green (1926) used similar techniques with Rutaceae, Rosaceae, and Solanaceae and came to the same conclusion as Rives.

Baldwin, Fred, and Hastings (1927) made a serological comparison of cultivated legumes. Their results showed, "all members of any cross-inoculation group are closely related with respect to the protein characteristic of their seeds and, in the majority of cases, all legumes which possess closely related seed protein complexes cross inoculate".

Early application of serological methods was undertaken by Nelson and Birkeland (1929) to associate serological correspondence and stem rust resistance. *Triticum durum* ($2n=28$) and four common wheat varieties ($2n=42$) - Marquis, Hard Federation, North Dakota Selection 1656-81, and Hope - were used. Generally, it was found that the varieties of wheat possessing the greatest number of genetic factors

in common showed the closest serological relationship. For example, close relationship was shown between Hope, selection 1656-81, and the common parent, Marquis. In conclusion it was stated "since the genetic composition of the variety is responsible for its morphological as well as physiological characteristics, the serological reactions may be used, within limits, to ascertain these characteristics. The apparent parallelism in serological relationship and rust resistance seems to offer data more hopeful for study than any that genetic or cytological research such as chromosome determination has so far produced".

During the period 1920-1930 many botanical and zoological investigators felt that the serological technique would provide an objective approach to systematics. A noted group of plant serologists emerged at Königsberg, Germany headed by Gohlke in 1913 and later by Mez (Chester 1937). After extensive research Mez and Ziegenspeck in 1926 (Chester 1937) published the book "Serodiagnostische Stammbaum" showing a phylogenetic tree derived almost entirely from comparative serological investigations of the plant kingdom. Much criticism of the system came from classical morphological systematists whose data showed little correlation. Gilg and Schurhoff (1927), serological investigators in Berlin, attempted to duplicate results of the Stammbaum using modified techniques. Being relatively unsuccessful they stated, "The serodiagnostic method is, for investigation of plant relationships, completely useless". Others, however, felt that serological techniques could be used if supplemented by morphologic

and cytologic data.

Serological results and techniques of the many zoological investigations have been helpful to certain aspects of botanical serology. Levit et al. (1936) after applying serological techniques in a genetic study of Drosophila melanogaster concluded that such methods could be used to detect the presence of the Y chromosome in males and the attached X chromosome in females. Cumley (1940) in evaluation of Levit's work felt it possible to apply immunological techniques to the study of the expression of individual chromosomes or even genes. Irwin and Cumley (1943), working with several species of doves, their hybrids and backcrosses, concluded that the species specific antigens of the serum are controlled by genes. Thus, a genetic basis of classification is given the systematist who uses serology in his work.

Hyun in 1949 investigated the serological relationship of different species of the genus Quercus. Proteins were extracted from seeds in the first stage of germination and injected into rabbits to stimulate antibody production. He found that his results agreed with the treatment of systematic botanists.

Recent botanical investigations in systematic serology at Rutgers University began with the work of Johnson (1954) on the Magnoliaceae. Several genera were compared with Magnolia and then several species of Magnolia were compared to establish intrageneric, serological relationships. Intergeneric serological comparisons confirmed the classical taxonomy groupings. Other data presented by Johnson

indicated that inter-specific differences in Magnolia surpass the inter-generic differences in certain cases. No explanation of this apparent paradox is presented.

Lewis (1952) applied the precipitin ring test in an attempt to determine the nature of certain competitive interactions which occur between incompatibility alleles of Oenothera organensis and also to give a clue to the nature of the incompatibility system. Pollen extracts of four different incompatibility genotypes were used as antisera sources. The results of twenty-five different combination tests, with the exception of two cross reactions, were found to agree with the theory that the "S" alleles produce specific substances in the pollen each of which is antigenically different. It was also shown that the stylar incompatibility substance is preformed and is not the result of an antigenic stimulus from the pollen tubes.

Hammond (1955) compared a number of genera in the Ranunculaceae on the basis of their serological interactions and this criterion, together with cytological and morphological data, was used to produce a new systematic treatment of the genera.

Considerable disagreement arose among earlier investigators as to whether individual plants were serologically homogenous or whether different organs or tissues from the same plant had different antigenic complements. Mez believed that plants were homogenous but the Berlin group disagreed. Chester (1937) noted, however, that comparisons between seed proteins and proteins from other plant parts were distinctively different.

Gell et al. (1960) with the application of immunological methods studied the taxonomy of 15 Mexican species and 22 South American species of the genus Solanum. Extracts were prepared from the tubers and the crude juices were adjusted to yield a protein concentration of 0.5 per cent. The gell-diffusion technique developed by Elek (1948) and Ouchterlony (1948, 1958, 1964) was used as modified by immuno-electrophoresis developed by Grabar and Williams (1953). Their scheme of relationships between species of the Mexican species was similar to that already postulated on the basis of morphological and cytogenetical studies. The technique was ineffective in separating the South American species. The researchers maintained that the serological method regardless of its limitations in this study could be used, together with other data, in taxonomical or evolutionary appraisals.

Wright (1960) has refined further investigation of organ specific antigens. By combining ultracentrifugation and immunodiffusion he was able to demonstrate an antigen in the microsome fraction of three-day old coleoptile tissue of wheat. In order to exclude non-microsomal antigens, the antiserum was first absorbed with the supernatant of the microsome fraction. The precipitin band associated with the microsome fraction of the three-day old coleoptile tissue was absent from coleoptile tissue of a younger age and from root and leaf tissue. Wright indicated that "a non-organ specific meristematic pattern of antigens has superimposed upon it, during

differentiation, a combination of proteins characteristic of differentiated cells".

The serological distinctness of different organs of a single plant was proven by Kloz et al. (1960). By comparing the antigenic substances from cotyledons, seedling roots, and mature leaves of Phaseolus, it was found that differences in organ reactions often exceeded differences in species reactions. As an example, a serological comparison of the individual organs of the same species (P. vulgaris) as against different species reactions showed the following precipitin results:

(1) Organ interactions

(Antiserum against cotyledons tested against sera from the following sources):

<u>Source</u>	<u>Reaction (Per cent)</u>
Cotyledons	100.0
Seedling roots	8.9
Mature leaves	5.2

(2) Species interactions

Phaseolus vulgaris (Antiserum of leaves against sera from leaves of following species):

<u>P. vulgaris</u>	100.0
<u>P. coccineus</u>	89.7
<u>Glycine soja</u>	41.7
<u>Vicia faba</u>	19.9

Tucker (1963) working with two subspecies of Marchantia polymorpha was able to show a serological difference between them using double-diffusion techniques where antiserum-antigen and absorbed

antiserum-antigen methods both were used. He concluded that "results show antigenic proteins may be extracted from green tissues and used in study of plant relationships".

Serological studies were conducted within families Cornaceae and Nyssaceae by Fairbrothers and Johnson (1964). Seeds were ground and the colloidal precipitate after protein extraction was injected into rabbits. The photorefractometer and Ouchterlony methods were used and it was generally concluded that serological groupings obtained for the taxa studied were in agreement with those presently employed by some taxonomists.

Creel (1964)^{1/} compared the species of Agropyron serologically and suggested that not all four sections of the genus Agropyron (Goulardia H., Holopyron H., Agropyron and Eremopyrum), as proposed by Holmberg (1926), were valid.

Serological relationships were determined by Kleese and Frey (1964) between Avena sativa var. Cherokee and six other oat varieties used as parents in crosses. Relationships were also studied between 4 corn inbreds used in single crosses. Reactivity was tested quantitatively by photoelectrically measuring the turbidity (after Boyden and Defalco, 1943) from each antigen-antibody system. The researchers concluded that the serological relationships of the parents used in the oat crosses agreed with the within-cross variance components for

^{1/} Creel, G.C. 1964. Biosystematic comparison of species in the genus Agropyron Gaertn. with particular emphasis on serological studies. Ph.D. Thesis. Montana State University.

grain yield whereas comparisons to heading date and height corresponded only in isolated cases. Similar methods used in comparison and predictions of the corn inbreds yielded less conclusive results.

Vaughn et al. (1965) used immunodiffusion, double-diffusion (method by Ouchterlony) and electrophoretic techniques in taxonomic analysis of three species of the genera Brassica. Evidence based on seed proteins supported the classification of the three plants into three taxa and also supports classification by Schultz (1919) on morphological grounds.

Lester et al. (1965) compared 14 species of Baptisia by means of double-diffusion and immunoelectrophoretic techniques. A few definite and repeatable differences could be detected; however, in general, the serological differences appeared "conservative" when compared to morphological criteria. The investigators felt that "serological data should be regarded as adjuncts to other systematic knowledge only on the basis of empirical manifestations of their utility. There is no clear justification for regarding serological data as intrinsically either superior or inferior to other systematic criteria".

The Ouchterlony double-diffusion plate method was used by Esposito et al. (1966) for the successful identification and differentiation of nine Medicago sativa L varieties. Specific serological tests were reported as "sufficiently definite" for the identification of seed protein phenotypes. The results indicated that it is possible

to identify the nine varieties and to distinguish one from another. The researchers indicated, however, that a combination of genetic, as well as environmental components, may have influenced seed protein content; thus, either or both components may have been responsible for varietal diversifications displayed.

More recently, Damirgi et al. (1967) reported applying a serological technique in a study which was conducted to determine the distribution of rhizobial strains of Rhizobium japonicum in nodules of soybeans grown on certain soils. Homogenized nodule suspensions were used as antigens and the agglutination test was used to compare the groups serologically. The results were then used to distinguish specific strains of rhizobia found in different areas and in various soils.

Rewarding contributions in the research of hybrids has been brought about by serological determinations. One of the classical papers on the serological study of plant hybrids was that of Zade (Alston and Turner 1963) who published a study including a serological comparison of three species of clover: Trifolium repens, T. pratense, and T. hybridum. At that time, T. hybridum was regarded as a hybrid of T. pratense and T. repens (this is questioned by recent investigators). Zade reported that T. hybridum serum reacted more strongly with the supposed parents than did reciprocal tests with serum from the parents. Chester (1937) who discussed this work mentioned "Zade with the precipitin test showed that Trifolium pratense and T. repens are related but serologically distinct. Their hybrid T. hybridum

reacts so strongly with both as to demonstrate its hybrid nature".

Had there been no previous assumption of the hybrid nature of T.

hybridum it may have been concluded that this species was the closest of the three to some primitive Trifolium stock. The results are open to various interpretations.

Hall (1959) when serologically investigating rye-wheat crosses and parents conclusively indicated all proteins immunoelectrophoretically identified in the wheat could be traced in the rye-wheat hybrid and specific rye-wheat proteins could not be detected.

Irwin and Cumley (1943) studied serological relations in the Pearlneck dove, the ring dove, and their hybrids. They found in the hybrids all the antigens shared by the parental species and most of the characters specific to each parent. In contrast to Hall's report, all of the hybrids possessed a "hybrid substance not found in either parent".

Commenting on techniques to disclose hybrids through serological methods, Alston and Turner (1963) wrote: "Suppose, for example, that species A contains antigen complement $a+b$ and species B contains antigen complement $b+c$. Thus, b represents the common antigenic substances. The hybrid should, therefore, possess a complement $a+b+c$, and a hybrid antiserum, if absorbed with serum type A and then with serum type B, should be completely neutralized. It should then give a negative response to hybrid serum. Presumably then, if a residual activity remained in the antiserum after absorption with

sera A and B, one of three explanations might hold:

- (1) The plant was not a hybrid.
- (2) New "hybrid-type" antigenic substances were present.
- (3) Genetic heterozygosity in one or both parents led to individual differences in antigenic complement.

However, if serum of the "hybrid" completely neutralized antisera of type A and type B, this would offer strong support for the true hybrid nature of the plant in question.

II. Cytogenetics - Aneuploidy

The first extensive aneuploid genetic program in wheat was initiated by Sears (1939) when he observed two haploid plants in a field culture of 105 plants grown from seed of Triticum vulgare variety Chinese Spring ($n=21$). Both of the haploids were completely male sterile. One was female sterile but the other set 14 seeds from florets pollinated by normal wheat pollen. The 14 seeds yielded 13 mature plants which ranged from 40-42 in somatic chromosome numbers.

Each of these plants was investigated cytologically at meiosis. Five of the 41 chromosome plants showed 1 univalent and 20 bivalents, and 2 showed 2 univalents and 1 trivalent each. Of the two 40 chromosome individuals one had 17 bivalents, 2 univalents and 1 ring of four and the other had 19 bivalents and 2 univalents. Of the four 42 chromosome plants, 2 were normal with 21 bivalents each, 1 had 15 bivalents, 2 univalents, 2 trivalents, and a ring of four, and the other had 19 bivalents, 1 trivalent and 1 univalent. Sears hypothesized that the different meiotic configurations in the progeny

were the result of abnormal division or segregation of the 21 univalent chromosomes at meiosis of the original haploid plant. The resulting gametes which carried duplications or deficiencies for one or more of the chromosomes when combined with the normal male gametes, would result in progeny which were abnormal in their chromosome numbers and pairing configurations. Sears proposed that the occurrence of the rings of four was the result of segmental interchanges which occurred in the haploid as a result of crossovers between paired homologous chromosomes. The tetrasomic condition was an unlikely contribution.

Sears (1939) discussed several possibilities for the use of chromosome aberrants in a broad genetic analysis of T. vulgare. An effort was initiated to determine homologies among the monosomic individuals by using T. durum (n=14) as a common parent. Another area of study is the immediate effects on the plant resulting from duplications and deficiencies of chromosomes and parts of chromosomes. Monosomics and trisomics may also be used to locate genes on specific chromosomes of common wheat.

By 1944 Sears (1944) had obtained seventeen of the 21 possible nullisomics and monosomics. From observations of the nullisomic individuals, the factors governing seed color, pubescent nodes, squareheadedness and suppression of speltoidy, and awn development, were assigned to specific chromosomes. Sears' observation of the frequency of transmission of the nullisomic and monosomic conditions

from selfing monosomic plants indicates the nullisomics are produced in frequencies ranging from 3 to 10 percent while monosomic frequency ranged from 75 to 85 percent. Burnham (1962) points out that in the production of female gametes there is approximately 50 percent chance of the univalent being lost in the first meiotic division and a 50 percent chance of it being lost in the second meiotic division. The final percentage of cells that receive the univalent may be about 25 percent. Deficient female gametes, against which there is no apparent selection, occur on an average of 75 percent of the time resulting from this transmission - not as a result of there being a 50-50 chance of the univalent passing to one pole or the other at both divisions.

Male transmission of $(n-1)$ is reported by Sears (1944) as ranging from 1 to 15 percent depending on the chromosome. This low number of functioning deficient male gametes is presumably due to the elimination of deficient pollen through competition with normal pollen.

From these studies of transmission frequency, Sears (1958) was able to predict breeding behavior of a typical monosomic plant for the common wheat variety Chinese Spring ($n=21$).

Table I. Breeding behavior of a typical monosomic plant.

<u>Female</u>	<u>Male</u>	
	<u>21 Chromosome Pollen 96%</u>	<u>20 Chromosome Pollen 4%</u>
21 chromosome eggs 25%	21 _{II} plants 24%	20 _{II} + 1 _I plants 1%
20 chromosome eggs 75%	20 _{II} + 1 _I plants 72%	21 _{II} plants 3%

By 1958 Sears reported the final complete grouping of all 21 chromosomes into 7 homologous groups and also designated the genome to which most chromosomes belonged.

MATERIALS AND METHODS

I. Cytogenetics

In the present study, twenty-one monosomic lines, each monosomic for a different chromosome, of the hard red winter wheat Triticum aestivum L. variety Kharkoff MC22 (subsequently referred to as KMC22), were obtained as seed from Dr. B. C. Jenkins, Department of Plant Science, University of Manitoba, Winnipeg, Canada. These lines had been produced by a backcrossing program with KMC22 as the recurrent parent on original crosses with Chinese Spring monosomics. The backcrosses had been completed for 8 or 9 generations to insure relatively pure genetic recovery of the Kharkoff complement. One exception to this was the line monosomic for chromosome 6D which had a history of only 3 backcrosses.

The chromosome numbering system used in this study is explained as follows: The first numeral and capital letter refer to the specific chromosome involved, the subscript letter refers to one of two families developed for each substitution line and the final numeral designates the original plant in this line from which the progenies for this study were propagated. As an example, 1Da-2 would designate substitution line 1D, family a, and the second plant.

The twenty-one monosomic lines, perpetuated by selection, were grown on an individual plant basis during the 1965-66 season at the Agricultural Research Station, Bozeman, Montana. Head samples for pollen mother cell cytological analysis were taken on plants of

monosomic lines about 3 days prior to emergence from the boot. Head samples were preserved and refrigerated in Newcomer's fixative (Newcomer 1953). Slide preparations were made according to Belling's (1926) method of staining. Chromosome counts were made when possible, otherwise the occurrence of a univalent in the cell was taken as an indication of a monosomic condition.

Absolute identification of monosomic lines of chromosome 4Aa-3 and chromosome 1Db-2 was made uncertain due to the presence of unusual chromosome configurations at meiosis. These two lines were not included in the study. Line 7Ba-1 was not included because nonsegregating progeny indicated the original parent was a disomic.

The correct meiotic stage for cytological identification was unavoidably missed in certain plants. These plants were allowed to self pollinate and mature. Five plants from each of the unidentified parents were grown in the greenhouse and examined cytologically. If the parent was a monosomic the progeny would segregate for the aneuploid condition in an approximate ratio of 3 monosomic to 1 disomic. (Witnessed deviation from this ratio may be explained by small sample size and irregularities of univalent transmission inherent in the monosomic line.) If an examined plant was monosomic, indications are that the parent was monosomic, therefore, no other samples were observed from the other 4 plants. On the other hand, if a plant was disomic the other 4 plants were examined also. If they were all disomic the parent was considered a disomic; if all

were not $2n$ the parent was considered a monosomic. The possibility of misclassifying a parent as a nullisomic or a monosomic was disregarded for this study because morphological identification of the nullisomic plant is possible, and cross pollination in a nullisomic is an infrequent occurrence.

II. Extraction, Antigen Treatment, and Protein Determination

Concurrent with cytological sampling, the leaves were clipped from field plants in about the 6 leaf stage. The plant tissue, after being washed and blotted dry, was confined within a stainless steel cylinder, semipulverized and subjected to 3,000 pounds per square inch of pressure. The crude plant sap was drawn off and frozen within 20 minutes. All plant samples of expressed sap were identified and kept separate throughout the study.

The antibody sources were the Single plant selection and the selection 1Da-2. Selection 1Da-2 is a derived disomic from segregating monosomic and disomic lines of KMC22. The selection referred to as a "Single" plant line is a propagation of a single plant selection of the variety KMC22 from the Montana State Experiment Station breeding program. These two selections were grown under greenhouse conditions, vernalized and allowed to reach the 4-6 leaf stage before procedures were repeated for sap expression. Five grams of green tissue was found to yield approximately 2 ml. of plant sap.

The expressed sap from each selection was divided into 2 lots. One lot of each selection was retained in the native crude state and the other lot was treated by ammonium sulfate protein saturation

methods (Colowick and Kaplan, 1955). The frozen sap was thawed and centrifuged at 4,000 xg. for 5 minutes. The supernatant liquid was diluted to twice its volume with 0.05 Molar phosphate buffer pH 7.0 and saturated to 20 percent with ammonium sulfate. After twenty minutes of constant agitation under refrigeration, the mixture was centrifuged at 15,000 xg. for 10 minutes. The supernatant portion was then made to 60 percent saturated with respect to ammonium sulfate and, after being agitated as before, was centrifuged at 12,000 xg. for 10 minutes. The supernatant liquid from this fraction was discarded, the precipitate was taken up in 10 mls. of the phosphate buffer, and dialyzed overnight at 2° centigrade in 1,500 mls. of the buffer. After 2 hours of dialysis, the buffer was replaced by fresh phosphate buffer. The prepared material was then placed in closed vials and frozen.

The biuret method of protein determination outlined by Cambell et al. (1964) was used for quantitation of the treated preparations. The treated extracts were diluted to measure 1 milligram of protein per ml. for injection.

III. Serology

To produce the antiserum, four rabbits, all litter mates, were maintained. The animals were bled previous to any injection and the resulting non-immune serum was frozen. Included is the injection schedule (Table II).

Table II. Injection schedule.

Rabbit Number				No. Days From Initial Injection (0)				
Treated		Crude						
1Da-2	Single	1Da-2	Single	0	14	16	18	20
01				1 ml	2 ml	2 ml	2 ml	2 ml
	02			1 ml	2 ml	2 ml	2 ml	2 ml
		03		1 ml	2 ml	2 ml	2 ml	2 ml
			04	1 ml	2 ml	2 ml	2 ml	2 ml

The thawed crude sap was centrifuged to remove chloroplasts before injection. Subcutaneous injections were administered in 4 places at each injection, over the shoulders and in the lumbar region.

A suitable antibody level was generally obtained the seventh day after the fifth injection at which time the animals were bled following the method of Creel, et al. (1965). Immediately following each bleeding a 2 ml. booster injection was given to maintain antibody titer.

A modification of the gel-diffusion method described by Ouchterlony (1948) was used. The gel-diffusion plates were 90 mm. in diameter with agar poured to a depth of approximately 2 mm. The Ionagar No. 2 was prepared as a 0.6 percent (w/v) solution. The solvent for the agar was prepared according to Creel, et al. (1965). A preservative of sodium azide, 1 part in 2000, was added. After dissolving the agar in the solvent, 11 ml. were pipetted into the sterile petri dishes. The plates were allowed to stand several hours before the well and trenches were made.

Gel-diffusion plates were constructed reacting each antiserum with its homologous antigen. Comparisons could then be made involving serum specificity differences between animals as well as differences, if any, between crude or treated and lDa-2 or Single antigen preparations.

Experiment I. Unabsorbed antisera with selected monosomic and disomic extract preparations.

Wells and trenches were made in the agar plates with a template made according to Fig. 1. Wells were cut in the cooled agar with a number 3 cork borer (8 mm. in diameter) 0.8 mm. apart and 7.5 mm. from the trench edge. The trench measured 5 x 76 mm.

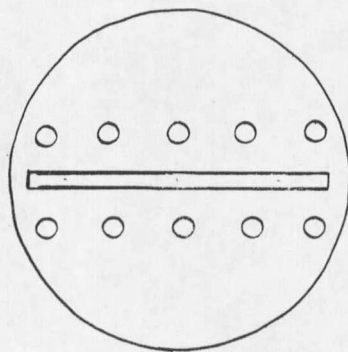


Fig. 1 Template Design for Experiment I.

A total of ten extract samples from monosomic and disomic plants within each of the eighteen monosomic lines were chosen for study (Table III). The crude sap was allowed to thaw, then each well was filled with appropriate crude extract. One plate accommodated plant samples from 1 monosomic line. The trench was filled with antiserum

Table III. Numbers of monosomic and disomic plants selected for serological investigation.

Monosomic Line	Number of Plants	
	Monosomic	Disomic
1Ab-1	6	4
2Aa-1	7	3
3Aa-2	2	8
4Aa-3 ^{2/}		
5Ab-1	6	4
6Aa-1	6	4
7Aa-1	7	3
1Ba-1	6	4
2Ba-2	5	5
3Bb-1	3	7
4Ba-1	5	5
5Ba-1	8	2
6Ba-1	6	4
7Ba-1 ^{3/}	0	10
1Db-2 ^{2/}		
2Db-1	6	4
3Db-2	6	4
4Da-1	7	3
5Db-2	6	4
6Db-1	3	7
7Da-1	6	4

^{2/} Lines 4Aa-3 and 1Db-2 were not studied serologically due to the presence of unusual chromosome configurations at meiosis.

^{3/} Line 7Ba-1 was not studied serologically because the original parent was a disomic.

produced from one of the 4 antigen sources. For this experiment the eighteen monosomic lines were divided such that lines tested against the 2 antisera sources from crude extract antigens were not tested again against the 2 antisera sources from purified extract antigen. The plates were allowed to remain at room temperature for 48-60 hours after which they were at optimum stage for reading and recording. Reading was done by allowing rays from a fluorescent lamp to pass through the agar at an oblique angle, thus making the precipitate lines clearly visible. Plates were not run in duplicate because of the minimal supply of antisera. They were not recharged.

Precipitate lines that joined from one area of the plate to its adjacent area were interpreted as being formed by identical reactants. The formation of a spur, rather than a smooth curve, where the lines joined was interpreted to mean that non-identical but similar antigens were reacting with the same antibody. Lines which did not join, even though they were close enough to do so, were considered to be caused by different reacting antigens. The total number of common lines and the mode of joining of adjacent lines were included in the criterion of comparison.

Experiment II. Absorbed antisera with selected monosomic and disomic extract preparations.

Absorption is a serological process whereby antigens and their corresponding antibodies are allowed to react and be removed prior to their introduction on a gel-diffusion plate. (Homologous

reactions are evidenced by a precipitating complex which may be separated by centrifugation.) The system as used in this study may be illustrated in this manner: If we suppose extract from the disomic plant selections 1Da-2 and single consists of antigens F, G, H and I then the antisera would contain homologous antibodies f, g, h and i. A disomic plant selection would also contain antigens F, G, H and I. However, due to the absence of a chromosome, the monosomic may contain only antigens F, G and H. To detect this difference, chosen disomics and monosomics are allowed to absorb with antiserum f, g, h and i. The supernatant portion is then reacted on a gel plate with antigen 1Da-2 or Single. No precipitation reaction should be visible between antigen F, G, H and I and absorbed sera f, g, h, i: the homologous complexes Ff, Gg, Hh and Ii were precipitated. A reaction should, however, be visible between monosomic antigen F, G and H and absorbed sera f, g, h and i; the complexes Ff, Gg, Hh were precipitated but antibody i remained free to react with I from antigen 1Da-2 or Single.

Wells were cut in the agar according to the design of Fig. 2. Absorbed serum wells (hollow) were 13.5 mm. apart and were separated by 7.5 mm. from the antigen wells (solid).

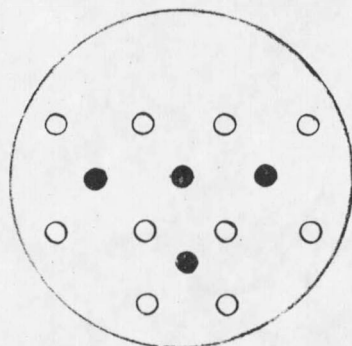


Fig. 2 Template Design for Experiment II.

Extract samples of the 10 monosomic and disomic plants from each of the eighteen monosomic lines was absorbed with the 4 different antisera. Antigen and antibody were mixed in equal volumes. The complex was incubated at 37 degrees centigrade for 2 hours, then at 5 degrees centigrade overnight. After centrifugation, the supernatant portion was placed in the outside wells (Fig. 2, hollow wells).

The 4 center wells (Fig. 2, solid wells) were filled with the antigen preparations corresponding to the absorbing antisera. A complete monosomic line containing 10 plants could be reacted with any one given antisera on one plate. Four plates per line were made each utilizing a different antisera. Plates were not replicated or recharged. Reaction times and reading was done in the same manner as for Experiment I.

For preservation purposes, certain of the gel plates from Experiment I were dried and stained. They were washed with distilled water and 0.85 percent saline. The agar gel could then be

mounted on lantern slides, dried and stained in a 0.5 percent solution of Buffalo Black NBR (amino schwartz) (Jutila, 1966).^{4/}

^{4/} Jutila, J.J. 1966. Personal communication. Dept. of Bot and Micro. Montana State University, Bozeman, Montana.

RESULTS AND DISCUSSION

I. Serology

Comparisons of purified protein extract from sources 1Da-2 and Single plant line plated against all 4 antisera sources indicated there were no detectable differences between the two purified antigen sources. Differences in the number of common lines among such antigens were apparent only as sera were changed. Antisera 01 and 04 (Fig. 3) produced 3 common precipitate lines against all purified antigen sources, whereas, serum 02 (Fig. 3) and 03 only produced 1 common line with the treated antigens. Comparisons of crude primary antigens from the above sources were also tested against the 4 antisera.^{5/} Antisera 01 and 03 produced 3 common lines, 02 produced 1 common line (Fig. 4) and 04 produced 4 common lines (Fig. 4).

Combined comparisons of crude and purified Single and 1Da-2 plant lined were tested against all 4 antisera sources. Fluorescent light recordings indicated sera 01 (Fig. 5), 03 and 04 (Fig. 6) all produced 3 lines in common against all primary antigen sources. Serum 02 (Fig. 5) produced only 1 common line against all primary antigen sources. Differences between the primary antigen sources were not detectable by observing the number of precipitate lines formed against any one serum.

^{5/} Primary antigen refers to those antigen preparations which were injected as opposed to the term secondary antigens which are extracts from monosomic and disomic plants.

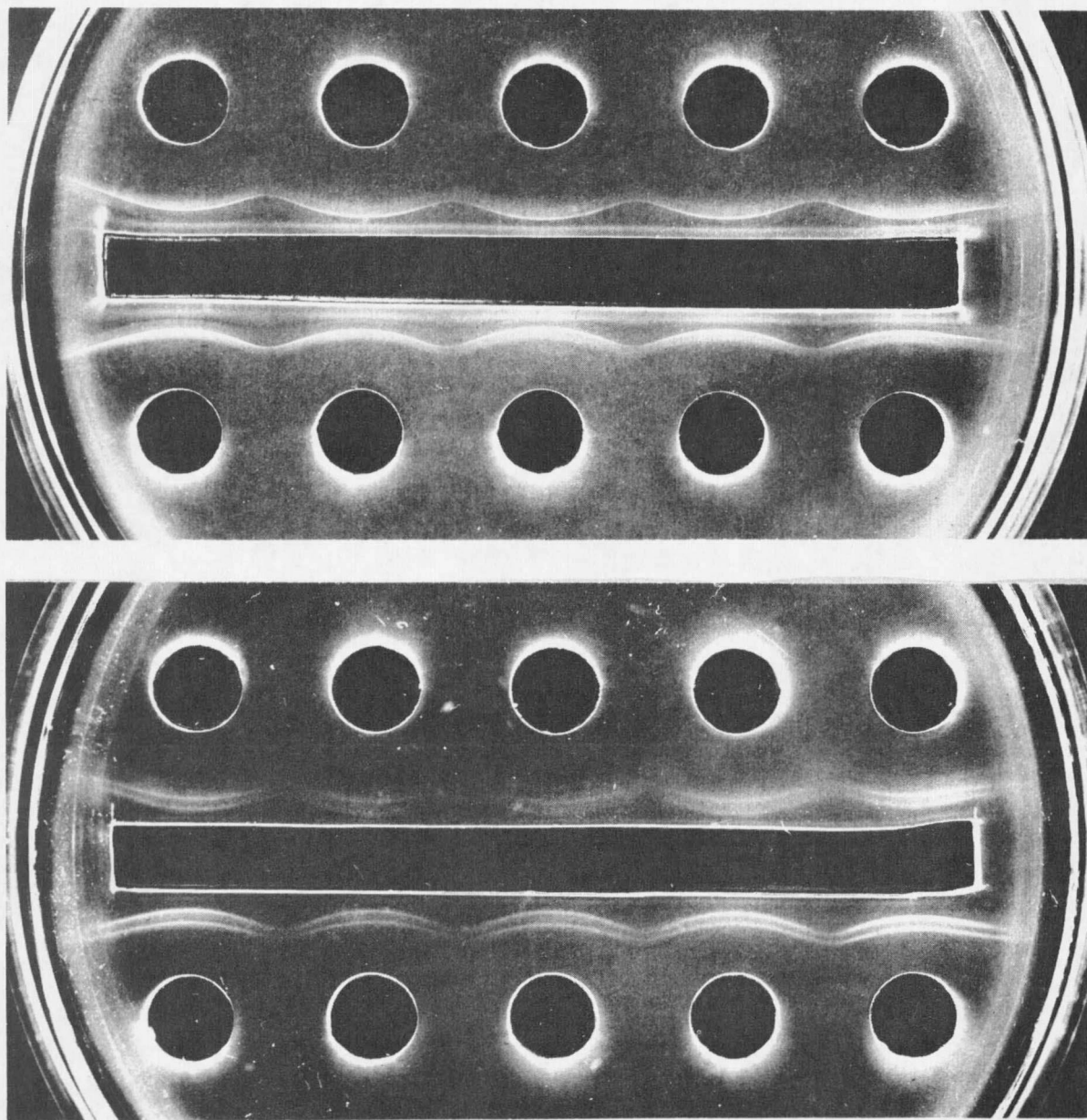


Fig. 3 Gel diffusion plates with antisera in trenches and antigen in wells. Wells of each plate are numbered 1-10 from left to right and top to bottom. Antiserum 02 (top) and 04 (bottom) reacted against treated antigen 1 Da-2 (wells 1, 3, 5, 7, 9) and Single (wells 2, 4, 6, 8, 10).

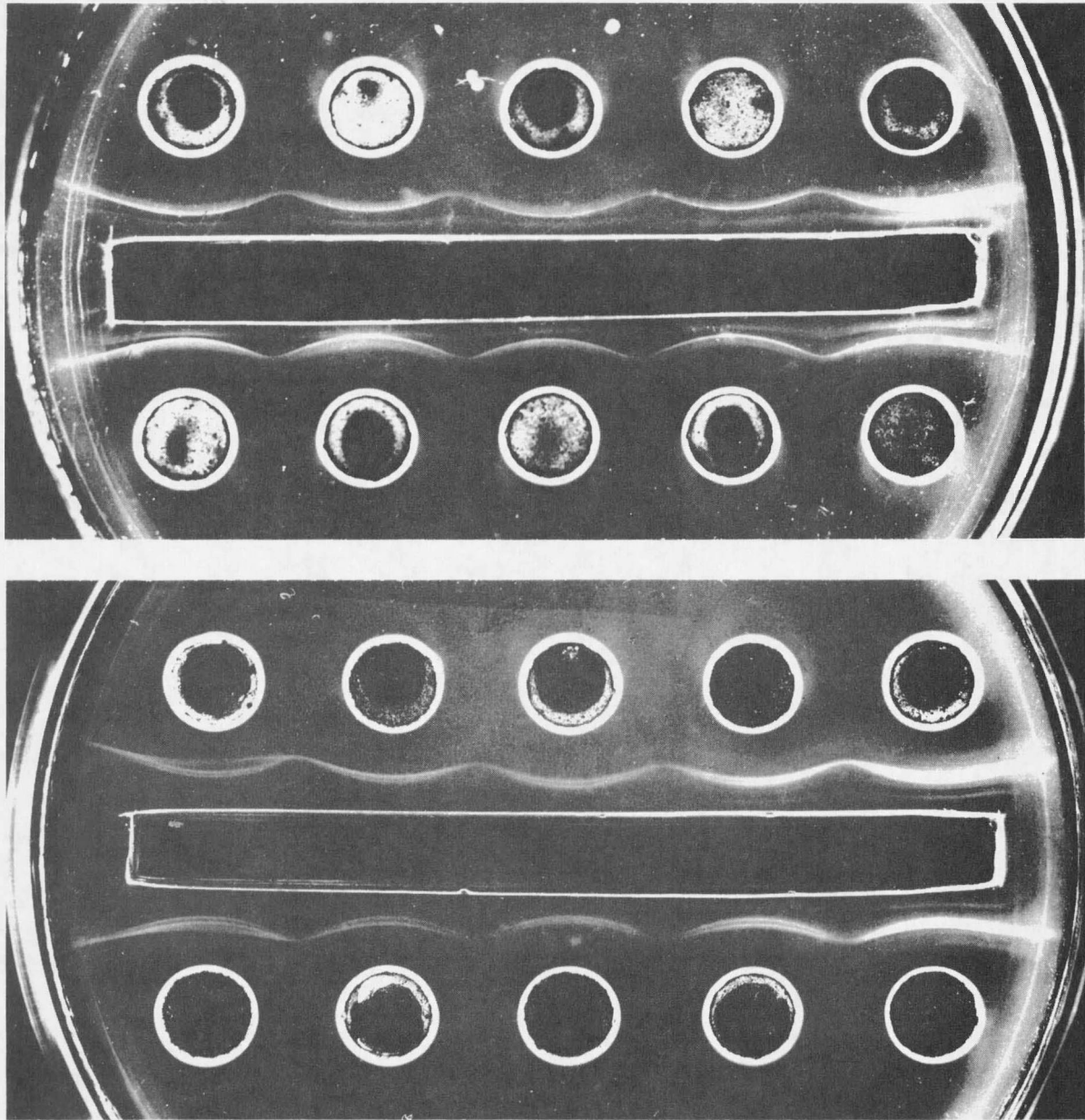


Fig. 4 Gel diffusion plates with antisera in trenches and antigen in wells. Wells of each plate are numbered 1-10 from left to right and top to bottom. Antiserum 02 (top) and 04 (bottom) reacted against crude antigen 1 Da-2 (wells 1, 3, 5, 7, 9) and Single (wells 2, 4, 6, 8, 10).

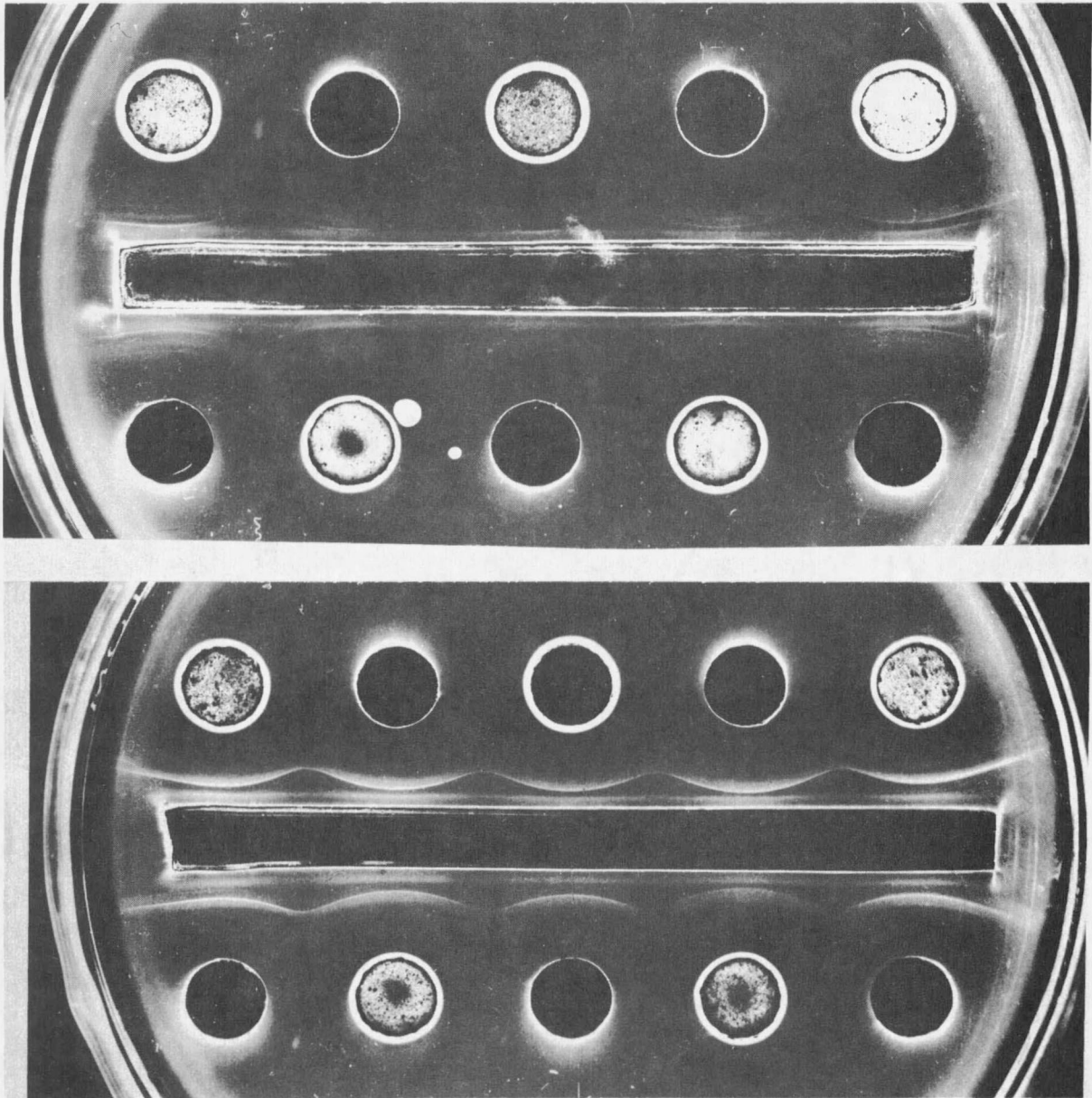


Fig. 5 Gel diffusion plates with antisera in trenches and antigen in wells. Wells of each plate are numbered 1-10 from left to right and top to bottom. Antiserum 01 (top) and 02 (bottom) reacted against crude antigen 1 Da-2 (wells 1, 3, 5) and Single (wells 7, 9); treated antigen 1 Da-2 (wells 2, 4) and Single (wells 6, 8, 10).

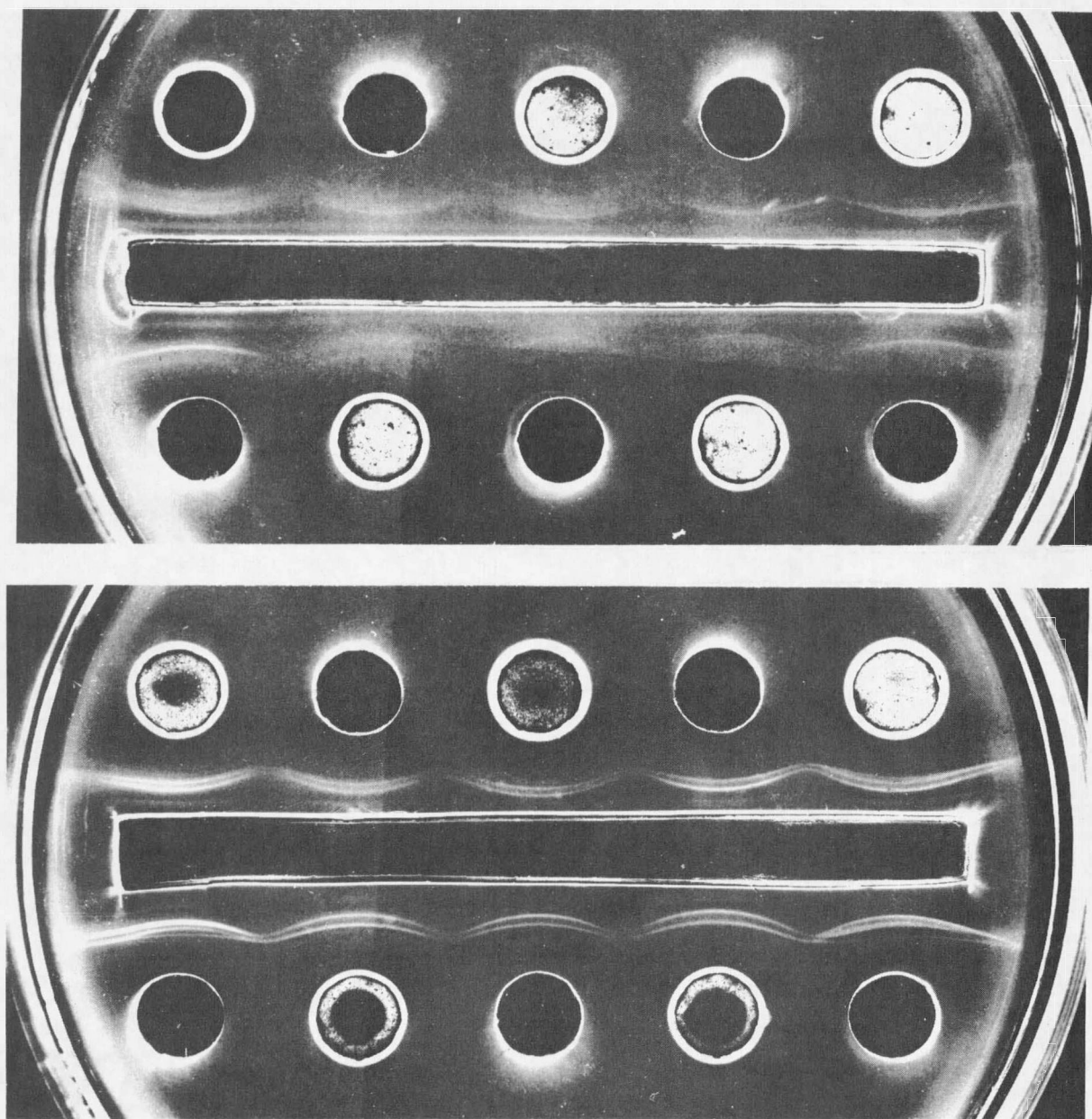


Fig. 6 Gel diffusion plates with antisera in trenches and antigen in wells. Wells of each plate are numbered 1-10 from left to right and top to bottom. Antiserum 03 (top) and 04 (bottom) reacted against crude antigen 1 Da-2 (wells 1, 3, 5) and Single (wells 7, 9); treated antigen 1 Da-2 (wells 2, 4) and Single (wells 6, 8, 10).

It is postulated that each homologous system of antigen and antibody forms a separate precipitate line in the agar gel. However, two or more antigen preparations may react with one particular antiserum if they possess either a common antigenic component, or if the antibody has various active groups corresponding to all antigens tested (Kwapinski 1965).

If two antigens examined by the diffusion precipitation test possess the same determinant group exclusively, the precipitin lines resulting from the reaction with an antibody coalesce in the space above or beneath the antiserum trench. This is called "the reaction of identity". The reaction of "partial identity" is evidenced by a single spur line being formed in addition to a common coalescent line. The spur formed on either side of the antiserum trench, apart from the common coalescent precipitation line, is produced by two different, heterologous antigen preparations, if each of them possess two determinants, eg. AB and Ac, of which one is common to the antiserum. If 2 antigen preparations do not possess any common determinant being serologically nonrelated, but the antiserum contains antibodies with active groups to both, crossing precipitate lines occur. This is termed "the reaction of nonidentity".

Only reaction lines of identity were observed in this study which serves to indicate the corresponding homology of all antigens tested.

Experiment I. Unabsorbed antisera with selected monosomic and disomic extract preparations.

Selected plant lines (secondary antigens) all produced precipitate lines when tested with the 4 antisera sources. Tests reacting each antiserum against several monosomics and disomics from each plant line yielded a minimum number of 2 common lines and a maximum of 5 common lines depending upon which serum was tested. The variation encountered may be attributed to three main reasons: (1) detectable differences in antigenic properties between plant lines, (2) differences in serum specificity and antibody titer due to varied animal response to injected antigens and (3) differences due to dates of antiserum sampling. Gross differences between plant lines were not measured directly. Conclusions regarding differences would be obscured by variation between antisera owing to animal difference or dates of sampling within a single serum source. Efforts were made to maintain the serum at uniform specificity by administering weekly booster injections throughout the study. This should minimize sample date variation in the sera. The fact that no differences were detected in the number of common lines when the primary antigens were tested against any one antisera indicated that the inject antigens were antigenically homogenous. Sera differences may then be attributed to between animal variation. No differences were detected in the number of common lines when monosomics and disomics within any chromosome line were compared.

Detectable precipitate lines varied on any one plate from those with narrow, easily defined margins to those with broad, widely diffusing fronts. Ouchterlony (1953) described the broad lines as being a possible result of unequal concentrations between antigen and antibodies. "If, after the establishment of the precipitate, the further diffusion of the reactants into the precipitation zone occurs at different rates, which might be the case when the concentrations in the basins are largely different, the picture will slowly change in that the precipitate will grow in the direction of diffusion of that component which is in excess. Sometimes this will manifest itself as a broadening precipitate with a sharply defined front in the direction of growth and a more diffuse back boundary." Any lines previously visible could also be obscured by the advancing front which contributes to the problem of line resolution and determination.

Experiment II. Absorbed antisera with selected monosomic and disomic extract preparations.

Each of the 4 antisera was reacted with selected monosomics and disomics in an absorption procedure. After incubation and centrifugation, the supernatant was plated against the primary antigen used to build the corresponding antibodies. There was no distinguishable reaction when crude primary antigen was used against O3 or O4 absorbed sera. Further studies reacting crude primary antigen against O1 and O2 sera produced no evidence of unabsorbed antibodies.

When purified primary antigen was used against any of the absorbed sera a heavy, broad short, precipitation complex was noted between absorbed and antigen wells. These single lines measured 4 to 8 mm. in length and 2 to 3 mm. in width; they formed adjacent to absorbed monosomic and disomic wells and did not form a coalescent pattern.

An effort to determine the source of the lines by absorbing secondary antigen material in antiserum excess, as well as in antigen excess, provided evidence indicating the precipitate was not an antigen-antibody complex. In either case, the lines were still produced.

The lines were formed when any of the unabsorbed crude antigen preparations were reacted against purified primary antigen. The purified or crude primary antigens did not react visibly when plated against 0.85 percent saline, 0.5 percent or 1.0 percent ammonium sulfate.

When the diffusion gel containing the precipitate was subjected to rinsing, drying and staining no lines were observed. Indications remain that such lines were not antigenically important and were not subject to protein specific staining techniques.

From absorption studies, it may be concluded that the sera, after absorption with any of the antigens, did not contain antibodies capable of forming a complex with homologous antigens. Detectable serological differences between the monosomic and disomic condition in winter wheat, variety KMC22, were not found.

The inability of this study to detect antigenic differences should hardly serve as an indictment against the usefulness of serobotanical tests. Certainly serological techniques remain useful when accompanied by other meaningful and related studies. The modified technique of immunoelectrophoresis could provide more specific data. The use of acrylamide gel electrophoresis is also a potentially promising technique which could yield differences that may result from chromosome deficiencies.

SUMMARY

Antigenic components from the leaves of several monosomic ($2n-1$) and disomic ($2n$) plants from eighteen chromosome substitution lines of winter wheat, variety Kharkoff MC22, were compared serologically. The monosomic and disomic condition arose as a result of segregation within each of the eighteen selfed monosomic lines. All lines were monosomic for a different chromosome.

Antibodies were produced in 4 rabbits as a result of injection with leaf extract antigens from 2 sources. Selection 1Da-2 is a derived disomic from segregating monosomic lines of KMC22. The selection "Single" plant line is a propagation of a single plant selection of the variety KMC22 from the Montana State Experiment Station breeding program. Each source was then divided into a crude sap component and a treated extract component. The 20 to 60 percent ammonium sulfate saturation fraction was used in the study. Protein was quantitated by the Biuret colorimetric method for the treated fractions.

Double diffusion reactions in gel plates were applied comparing all inject antigens in combinations against all 4 antisera. No differences were found between the inject antigens when reacted against any one antiserum. Observed differences between sera may be attributed mainly to differences in animal response.

Unabsorbed antisera from 4 sources was reacted with crude antigen extracts from several monosomic and disomic plants. Two to 5 common precipitate lines were produced depending upon which antiserum was used. Lines of nonidentity or partial identity were not present. From these results it is concluded that by use of this method there are no detectable

serological differences between the monosomic and disomic condition.

Antisera was absorbed with monosomic and disomic extract and the inject antigen preparation. When crude inject antigen was used, all reactive complexes were removed. When purified antigen was used a broad precipitate line formed adjacent to all absorbed sera wells. The line was visible in plates in which crude antigen was allowed to react against the purified antigen. Further study as to the nature of the complex indicated it was not important serologically. The absorption study provided evidence indicating that the absence of a chromosome in a hexaploid winter wheat plant of the variety Kharkoff MC22 does not contribute sufficient protein differences to be serologically detected when compared to the disomic counterpart.

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