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Studies were conducted to determine the effectiveness of a biological pollen barrier of rye, Secale cereale, in preventing outcrossing between Lolium multiflorum genotypes in each of the 300 crossing blocks established in this experiment.

Fluorescent tests of seeds of open-pollinated and single-crossed progeny of four annual ryegrass sources were used to determine the effectiveness of the rye barrier in preventing crossing between crossing blocks. The average percentage of fluorescence of open-pollinated progeny in 30 crossing blocks containing supposedly only non-fluorescing plants was 16.3 percent. The open-pollinated and single-crossed progeny in 46 other crossing blocks were highly correlated and did not differ significantly from each other in percent fluorescence. The percent fluorescence of each crossing block was mainly due to intra-crossing block pollen

rather than inter-crossing block or foreign pollen. The results indicate that an eight foot barrier of rye is effective in preventing crossing between the annual ryegrass crossing blocks.

Self-fertility of Lolium multiflorum was generally low, but self fertility estimates ranged from 0 to 87 percent. The results revealed that self-fertility data obtained by germination rather than seed count may give more accurate self-fertility estimates, especially when individual plants and their differences are being investigated.

The average seed set under white parchment bags was not significantly lower than the average seed set obtained with bag-type dialysis tubing. The use of dialysis tubing as a substitute for white parchment bags in bagging plants would be of little, if any, benefit to improve seed set under bag in Lolium multiflorum.

Studies on the Methodology of Crossing and Selfing in Lolium Multiflorum

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INTRODUCTION

Forage breeders should have a knowledge of the cross- and self-fertility of potentially useful basic plant material in order to make use of the most efficient selection procedures. High self-fertility, for example, would make selection work within selfed progenies possible.

The amount of selfed and crossed seed obtained from each basic plant of a synthetic variety will determine, to some extent, the proportion of progeny from each basic plant in the synthetic variety in future generations of seed-multiplication. Therefore, cross- and self-fertility estimates of potentially useful plants should be a prerequisite to the development of synthetic varieties.

The maintenance of varietal purity in forage crops is a major problem in forage grass breeding and for the seed industry. Maintenance of varietal purity has been accomplished primarily by space isolation and by various types of artificial barriers. Since space isolation is not usually possible when hundreds or thousands of plants are to be investigated, forage breeders continue to adopt various types of barriers to isolate grasses for controlled pollination.

The objectives of this study were:

(a) To use the marker gene fluorescence to test the effectiveness of a biological barrier of Secale cereale in preventing outcrossing between Lolium multiflorum crossing blocks.

(b) To compare self-fertility estimates for Lolium multiflorum on the basis of self-fertility data obtained by seed germination and on the basis of self-fertility data obtained by seed count.

(c) To determine the feasibility of substituting bag-type dialysis tubing for white parchment bags in bagging Lolium multiflorum plants.

LITERATURE REVIEW

Marker Genes in Ryegrass

The use of several suitable markers in a forage grass species to investigate outcrossing patterns would greatly facilitate solution of some of the problems in forage breeding. An examination of the literature shows that there are several marker genes available in the Lolium species.

Wit (34) used a simple dominate character, roughness versus smoothness of culm and upper leaf sheath to determine the amount of outcrossing between breeding trials in Lolium perenne. The dominant gene controlled the presence of downwardly-directed teeth on the culm and upper leaf sheaths. Nyquist (26) and Nyquist and Schulke (27) reported that several mutants have been found in Lolium multiflorum and Lolium perenne. The mutants branched culm and deformed spikelet in Lolium multiflorum and Lolium perenne were each due to a single recessive mutant gene. Branched culm and rudimentary spikelet in Lolium multiflorum were found to be allelic. Nyquist and Schulke (27) reported that both branched culm and rudimentary spikelet mutants have limitations as marker genes but each would be useful in some studies. One plant in Lolium perenne deviated from a 3:1 ratio in selfed generations.

Jenkin (14, 16) reported on the inheritance of two non-surviving green seedling lethals and a yellow-tipped albino, y. Both seedling

characters were due to a single recessive gene. In a later paper, Jenkin (15) found that the non-red base color factor in Lolium perenne was controlled by two recessive genes, c and r with dominant complimentary interaction. A dwarf was controlled by a single recessive gene, d. Aberrant ratios were found upon selfing. He (17) further reported that y, r, and d were linked. In addition, brittle stem was found to be controlled by a single recessive gene in Lolium multiflorum.

Griffiths (11) studied pollination dispersal in Lolium perenne by spacing non-red recessive base plants at successive intervals from dominant red-based plants. The non-red base plants were recessive for the two factors controlling anthocyanin color.

The inheritance of the fluorescence reaction of Lolium seedlings has been reported by several workers. The fluorescent test of ryegrass has been widely used to differentiate Lolium multiflorum from Lolium perenne. Rampton (28) reported that most annual ryegrasses are highly fluorescent while most perennial ryegrasses are highly non-fluorescent. In 1958, the fluorescence response of annual ryegrass seedling roots under ultraviolet light was reported to be due to a chemical, $C_{20}H_{19}NO_4$, named annuloline by Axelrod and Belzile (2).

Cokill (5) studied the inheritance of fluorescence of Lolium multiflorum roots under ultraviolet light. His results indicated that

fluorescence of Lolium multiflorum seedling roots is controlled by a single dominant gene.

Justice (22) reported that the fluorescence reaction of ryegrass seedlings when subjected to ultraviolet light is dominant over non-fluorescence in the ratio of 3:1 and the gene for this character is not linked with genes for the annual habit in Lolium multiflorum.

Nyquist (26) concluded that the dominant gene for fluorescence was transferred from Lolium multiflorum to Lolium perenne and the gene for this character was not linked with genes for annual characteristics in Lolium multiflorum. However, three of the six populations studied deviated from a 3:1 ratio. Nyquist reported that apparently some mechanism is operative on a single gene, giving a confounded ratio.

Trumble and Phipps (33) concluded that fluorescence is inherited as a dominant character, dependent on one, and possibly two, genetic factors giving 3:1 and 9:7 ratios respectively. Two of the selfed progenies of the ten hybrid plants studied gave 9:7 ratios while the remaining eight segregated 3:1. The single factor inheritance of fluorescence is thus brought into question although Trumble and Phipps did not eliminate the alternative hypothesis that some mechanism operative on a single gene results in a confounded 3:1 ratio.

Fejer (9) studying the effects of seed quality in genetical

experiments with forage plants reported that the fluorescent gene in Lolium showed confounded segregation ratios after seed storage.

Outcrossing and Pollen Dispersal

Maintenance of varietal purity in cross-pollinated species is a major problem for the seed industry and in forage grass breeding. The principal means of maintaining varietal purity is by establishing minimum isolation distances between varieties. Knowledge of realistic varietal isolation requirements is very important in developing, maintaining and increasing, superior crop varieties. By the use of suitable genetic markers, the extent and distance of outcrossing between varieties has been studied in many species to establish these minimum isolation requirements.

Griffiths (11) stated that the four main factors affecting outcrossing between varieties are (a) mode of pollen dispersal, (b) isolation distance, (c) population size and (d) varietal differences in time of flowering.

Copeland (7) used the fluorescent marker gene to investigate outcrossing patterns in ryegrass under controlled spaced-plant situations and between adjacent seed production fields. He reported that outcrossing under spaced plant situations decreased rapidly and became minimal at a distance of 100 feet. Outcrossing between adjacent seed production fields was reported to be negligible after

approximately ten feet with no evidence of crossing at distances greater than 40 feet.

Griffiths (11) studied outcrossing in Lolium by spacing plants recessive in respect for anthocyanin color of the basal sheaths at successive intervals from red-based plants. He reported that pollination within a population occurs chiefly between neighboring plants and that intervarietal pollen is much more effective in reducing contamination than isolation distance within short distances from the contaminant source. Intervarietal crossing was considerably reduced when the varieties differed in the date of anthesis although there was some overlap in flowering between all strains of Lolium studied.

Rampton (29) showed consistent differences in periods of flowering between varieties of grass seed crops in the Willamette Valley of Oregon and reported that time isolation could be used to effectively prevent crossing between some grass seed varieties.

Hayes and Immer (12) reported that the isolation of maize may be provided by actual distance, natural barriers, male border rows, or a combination of these methods. Two-hundred and twenty yards was suggested as the minimum isolation distance. However, for each additional male border row this distance may be reduced approximately 14 yards as intra-varietal pollen acts as a screen against incoming inter-varietal pollen to prevent outcrossing.

Jensen and Bogh (20) as cited by Griffiths (11) showed that the quantity of pollen decreased rapidly over the first 400 to 500 meters from the field, and after that only very slowly. On the basis of calculations of the amount of pollen dispersed in the air at different distances from the seed fields, the ryegrass and orchardgrass contamination at a distance of 500 to 600 meters was about 1/20th of that at the source.

Jones and Newell (21) found a similar pollen dispersal pattern in experiments conducted with seven species of cross-pollinated grasses and cereals. They concluded that the chance of maintaining genetic purity of varieties or improved strains of cross-pollinated grasses is much greater at a distance of 60 rods and less at smaller distances. Only 0.8 percent of the amount of pollen observed at the source was caught at 60 rods, however, even this low percentage of pollen was considered to endanger the genetic purity of seed stocks. Crane and Mather (8) concluded from their experiments with Icicle and Scarlet-Globe radish varieties that intercrossing is likely to occur over greater distances if only small numbers of tester plants are used.

Wit (34) conducted pollination studies of Lolium perenne in clonal plantations of polycross fields. A single factor character, roughness versus smoothness of culm and upper leaf sheaths, was

used to detect ryegrass outcrossing. Heterozygous rough clones were used as the source of contaminating pollen and homozygous recessive smooth clones were used as testers. Wit reported that the percentages of cross-pollination decreased rapidly at distances of 48 to 64 inches from the source but at greater distances the rate of decrease in cross-pollination was much slower. Pollination within the populations studied occurred chiefly between neighbor plants as the clones were fertilized by the two adjacent clones by an average of 90 percent. When a clone was bordered on two sides, this clone was fertilized by the three neighboring plants on both sides by an average of 74 percent. Wit further reported that simultaneousness of flowering often had a greater effect on the percent of crossing than vicinity.

Methods Used to Ensure Selfing and/or Measuring Self Fertility

Hayes (13) reported on two general methods for the exclusion of foreign pollen in controlling self-pollination. The one method is the use of space isolation to ensure selfing from other plants with which they may cross while the other method involves the use of some type of artificial barrier to enclose the inflorescence and exclude all foreign pollen thereby ensuring self-pollination.

Space isolation, of course, is the spacing of single plants far

enough from other plants with which they may intercross to ensure selfing. The distance needed for isolation depends largely on the species, on maturity differences among strains, and on the number of plants in each isolation block. The distance required is also affected by the direction of prevailing winds, weather conditions, and natural barriers.

Brewbaker and Majumder (4) showed that pollen grains have a mutually stimulating effect in germination, so that, at low concentrations their germination is low. Therefore, the possibility of an ovule being fertilized is likely to be less, the fewer the pollen grains falling on the stigma.

Since space isolation does lower the concentrations of pollen, it is possible that a shortage of pollen in the air may well reduce the percentage of florets setting seed and subsequently reduce seed yield of single isolated plants.

Space isolation is not possible when hundreds or thousands of plants are to be investigated and as a result the plant breeder has adopted various types of artificial barriers to ensure self-pollination. Various types of cloth cages, bags, or tents and various types of paper bags have been used to isolate grasses for controlled pollination.

Bagging may have a detrimental effect upon seed setting ability. Stapledon (31) suggested the use of small greenhouses as a substitute

for bagging in crossing large numbers of grass plants. The houses would be kept tight during pollination and the panicles agitated by means of cords pulled from the outside.

Polythene - terylene tents were used by Foster (10) for large-scale artificial isolation in ryegrass breeding. Using this method of isolation, seed setting was lower than under field conditions but seed yield, quality, and germination compared favorably with that of field sown crops.

Myers (24) studied the nature of variations both between and within years in number of seeds per panicle set under bag among clonal lines of Dactylis glomerata. He listed four factors that may contribute to random variation in seed set under bag as follows: (a) variations in panicle size and subsequently in number of florets per panicle, (b) injury to the culms as a result of bagging, (c) variations in the number of panicles enclosed in each bag, and (d) differences in date of flowering among panicles on the same plant. The earlier panicles set significantly more seeds than the later panicles on the same plant. Meyers concluded that the number of panicles enclosed per bag may be a serious factor in contributing to the large amount of variation that occurred in seed set under bag, especially if as many as eight panicles were used per bag.

In an earlier paper (25), Meyers concluded from the results of his experiment that a general reduction in seed-setting ability,

either from reduced fertility or reduced number of florets per panicle or both, accounted for a considerable proportion of the reduction in seed set under bag. The average number of seeds per panicle set under bag was 40.2 for the 46 parent clones and 14.9 for their inbred progenies. Selfed seed set of the parent clones, expressed as a percentage of open seed set, ranged from 0.8 to 54.5 percent.

Since bagging may have a deleterious effect upon seed setting and random variation in seed set under bag has also been reported, ability to set seed under bag may not be an accurate measure of self-fertility.

Fertility has been measured or expressed differently by several workers. Thrupp and Slinkard (32) reported that fertility evaluation falls into one of three categories; (a) number or weight of seeds per inflorescence, (b) weight of seed produced per inflorescence expressed as a percent of the gross weight of inflorescence, or (c) percent of florets setting seed. The authors suggest that seed set rating be substituted for the percent fertile basal florets method as a measure of fertility in grasses. Their results were very similar for both methods but the seed set rating required considerably less time for determination than did the percent fertile basal florets method. Seed set rating was determined by dividing the unthreshed weight of ten randomly chosen spikes per plant into the weight of clean seed from these ten spikes and was expressed as

a percentage.

Beddows (3) reported that if the structure of the florets or the form of seed make it impossible to decide whether a caryopsis is present or not, then germination is usually the only satisfactory method of determining self-fertility. He further reported that the number of caryopses per inflorescence and per 100 spikelets is less tedious than counting florets.

Keller (23) emphasized that self-fertility data should be obtained by the simplest possible method and that the self-fertility data be presented in frequency distributions. He used histograms to show the number of selfed seeds produced per panicle or spike for 21 species of grasses. Species considered to be low in fertility had only a fraction of the right side of the curve produced. Species which were considered to be relatively self-fertile yielded almost normal curves. Keller concluded that a histogram based upon self seeds per inflorescence or on weight of seeds per inflorescence is just as satisfactory as one based on seeds per floret or any other tedious method. The method described, however, was not intended for detailed studies of individual plants and their differences.

An examination of the literature shows there is a wide diversity of methods used to obtain self-fertility data and also in presenting the data once it is obtained. Cross-pollinated species have been shown to have varying levels of general and self-fertility.

Anslow (1) reported on the general fertility level in Lolium perenne. He found that two-thirds of all the florets set seed but the proportion was lower in late heads than earlier heads. There was a slight fall in floret fertility in the upper spikelets, as compared with the spikelets in the middle and at the base of the spike. The outer florets of each spikelet showed a marked reduction in fertility and the maximum percentage of florets setting seed was approximately 75 percent. Since empty or sterile florets tend to remain attached to normal florets, quality of seed as well as seed yield may be lowered at low levels of fertility. Any improvement in the general fertility level of a species may well result in increased yield and seed quality.

Cooper (6) found that it was impossible to establish early and late lines from within the selfed progenies of Lolium. The failure to maintain the inbred material was due partly to self-sterility and partly to low vigor and poor survival value. Only four of the eight initial parents of Irish ryegrass and five of the eight initial parents of Kent ryegrass produced more than 20 seedlings.

Nyquist and Schulke (27) reported that self-sterility in Lolium was high as only 22 of the 53 plants bagged produced ten or more mature individuals. Jenkin (18) concluded that self-fertility in Lolium multiflorum is low and in selection work, plants heterozygous for defective seedling characters are frequently encountered. His

results showed that only one plant of 20 gave more than one seed per spikelet. Assuming the number of florets per spikelet to be five, only one plant out of 20 gave over 20 percent of the possible number of seeds. The average for all the 20 plants self-pollinated was 28.6 seeds per 100 spikelets. Assuming five florets per spikelet, less than six percent of the possible number of seeds was obtained.

High self-fertility would make selection work within selfed progenies possible. Jenkin (19) concluded that it would not be difficult to produce a line of Lolium perenne of very high or perhaps full self-fertility since his data showed that the self-fertility of a parent plant exerts a profound influence upon the self-fertility of the progeny. The individual plants, however, varied considerably in self-fertility although in most cases, the same plant had been tested only once or only a few times. Jenkin stated that a single result or even a few results can be regarded as only an approximate representation of the potential self-fertility of the plant concerned. Assuming that the average number of florets per spikelet capable of setting seed was five, he further stated that 50 percent of the Lolium perenne plants studied set less than one percent of the possible number of seeds, 75 percent of the plants set less than 3 percent and only in two plants per hundred was seed setting greater than 20 percent, or one seed per spikelet. The results, therefore, showed that Lolium perenne has a low degree of self-fertility.

METHODS AND MATERIALS

Source of Materials

Four sources of Lolium multiflorum L. obtained from the Oregon State Seed Laboratory were used to establish 300 crossing blocks in this experiment. The sources were Redmond, Leda, Oregon Common, fluorescing type and Oregon Common, non-fluorescing type. Leda Daehnfeldt is a Danish Lolium multiflorum variety. Oregon Common refers to an ecotype of Lolium multiflorum commonly found in the Willamette Valley of Oregon while Redmond refers to a seed source of Lolium multiflorum that the Oregon State Seed Laboratory obtained from prior experimental material used at Redmond, Oregon.

Seed from each seed source was germinated in transparent plastic petri dishes containing Whatman number two chromatographic filter paper moistened with 0.2 percent potassium nitrate solution. The seed was stratified for seven days at 5°C. to relieve any possible dormancy. After stratification, the petri dishes were transferred to another germinator and tilted at approximately a 45 degree angle to allow the roots of the seedlings to grow without the roots of one seedling crossing those of another. The germinator was maintained at a temperature of 25°C. during a 9-1/2-hour day and at a temperature of 15°C. during the 14-1/2-hour night.

Seven days after the petri dishes were transferred,

approximately 400 fluorescent seedlings were selected from each of the Lolium multiflorum sources Redmond, Leda and Oregon Common and transferred to plant-bands in the greenhouse. Seedlings which showed no fluorescence were gently pulled off while the ultraviolet lamp was still on and the path of each root observed since sometimes a faint fluorescence will show on the paper as the root is pulled off.

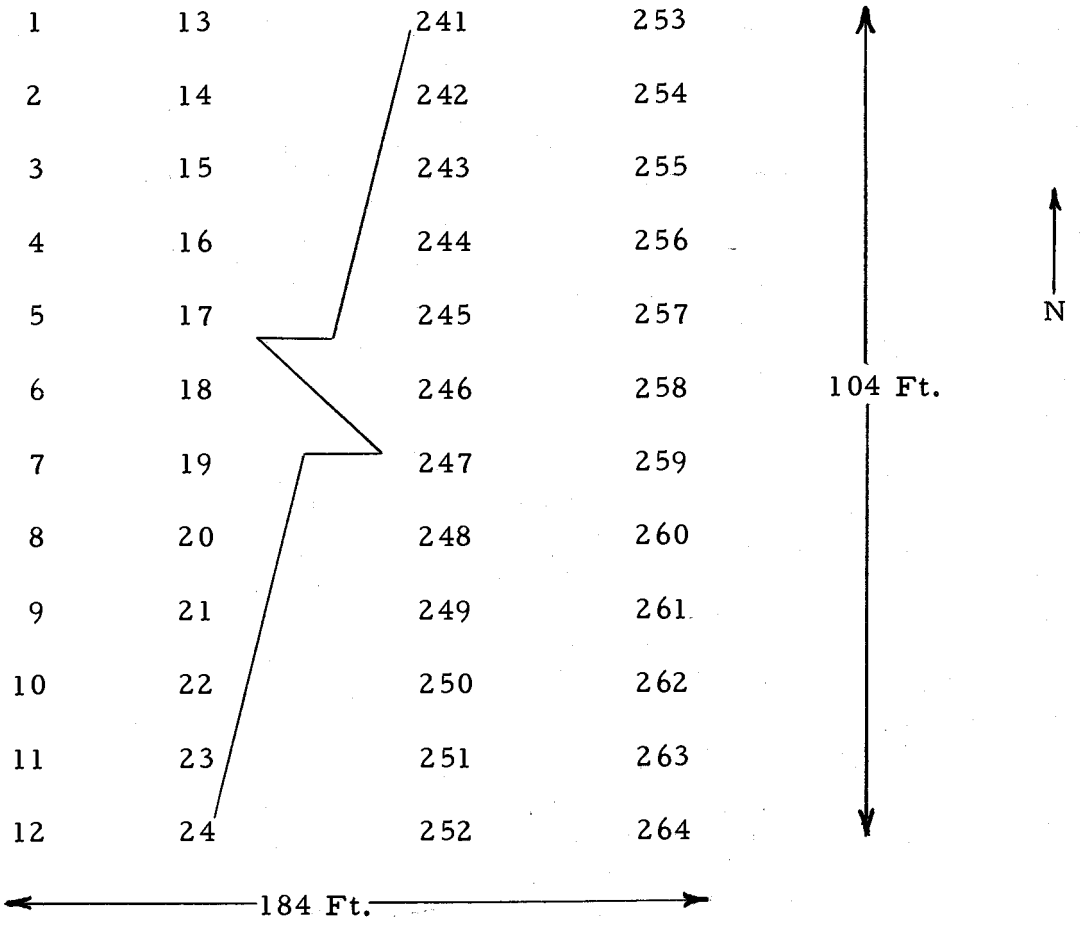
Non-fluorescent seedlings of Oregon Common removed under fluorescent light in a darkroom were replaced on fresh filter paper media in petri dishes for seven additional days in the germinator. Seedlings which showed no fluorescence at the end of the seven additional days of germination were transferred to plant-bands in the greenhouse. Approximately 400 Oregon Common, non-fluorescing type seedlings were selected.

Establishment

The seedlings were transplanted from the plant-bands to the Hyslop Agronomy Farm, Corvallis, Oregon on July 20, 1966. Three hundred crossing blocks as presented in Tables I and II were established. In field one there were 22 parallel rows with 12 crossing blocks per row. In field two there were four parallel rows. Three of the four rows had 11 crossing blocks per row while the fourth row had only three crossing blocks. The crossing blocks were four foot by four foot and each crossing block contained two plants centered

Table I. Twenty-two parallel rows with 12 crossing blocks[1] of Lolium multiflorum per row, crossing blocks are spaced eight feet apart.

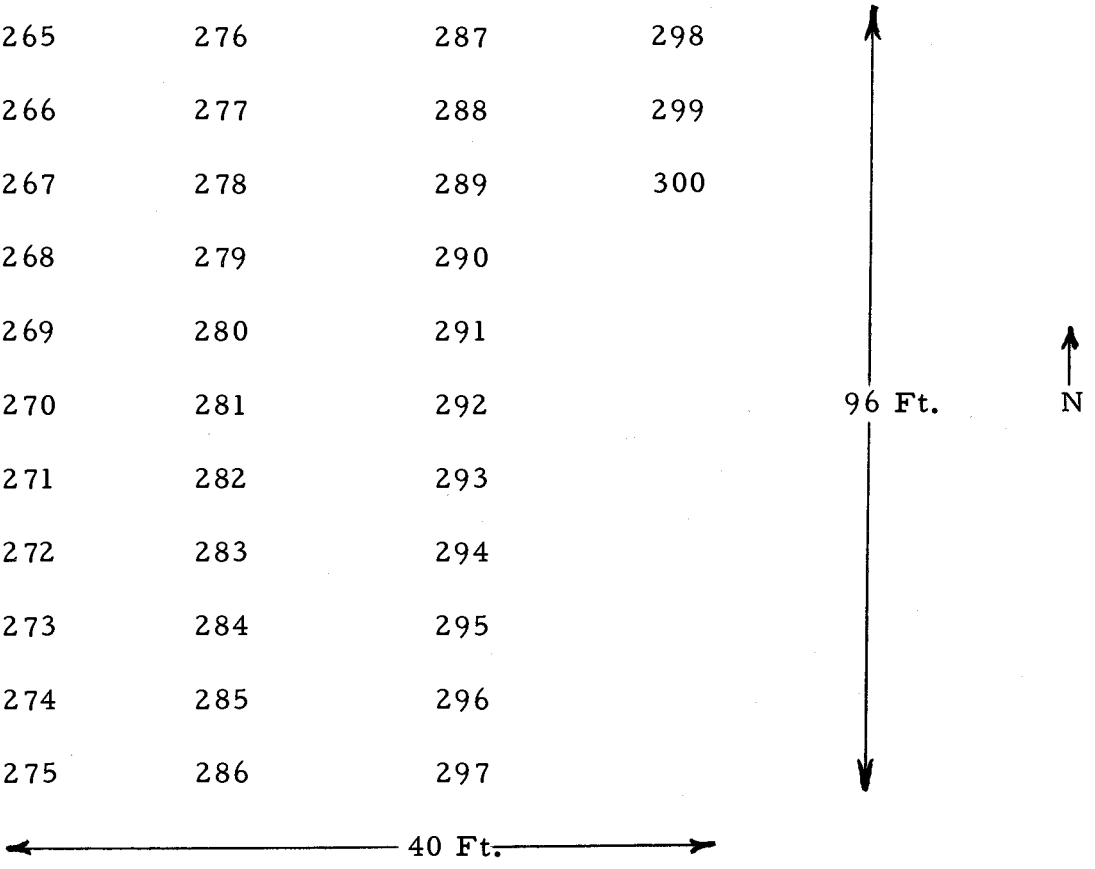
Row 1	Row 2	Row 21	Row 22
1	13	241	253
2	14	242	254
3	15	243	255
4	16	244	256
5	17	245	257
6	18	246	258
7	19	247	259
8	20	248	260
9	21	249	261
10	22	250	262
11	23	251	263
12	24	252	264



[1] Crossing blocks were four foot by four foot.

Table II. Three parallel rows with 11 crossing blocks[1] spaced eight feet apart, and one parallel row with three crossing blocks spaced eight feet apart.

Row 1	Row 2	Row 3	Row 4
265	276	287	298
266	277	288	299
267	278	289	300
268	279	290	
269	280	291	
270	281	292	
271	282	293	
272	283	294	
273	284	295	
274	285	296	
275	286	297	



[1] Crossing blocks were four foot by four foot.

approximately 12 inches apart as presented in Figure 1. Plant sources in each crossing block are shown in Table III.

The crossing blocks were located on 12 foot centers so that each block could be enclosed within an eight foot biological pollen barrier of cereal rye, Secale cereale. An example of the isolation of the crossing blocks by use of eight foot biological barriers of cereal rye is shown in Figure 1. The cereal rye was seeded at the rate of 100 pounds per acre in October of 1966.

A total of 600 plants of Lolium were used in the crossing blocks. Any dead plants were replaced July 29 or August 26, 1966. Weeds were controlled by hoeing within crossing blocks and tillage between crossing blocks before the fall planting of the cereal rye. The crossing blocks were again hoed in the spring of 1967.

The experiment was irrigated by use of a portable water tank on July 20 and July 25, 1966. On July 26 and August 3, 1966 the experiment was irrigated by use of sprinklers.

Measurements

Data were collected on annual habit, fluorescence, germination, seed set under bag when selfed and single-crossed, and seed set under two bag types during the summer and fall of 1967.

Selfed seed was obtained by enclosing four spikes in a single paper bag per plant. The bags used were four inch by four inch by

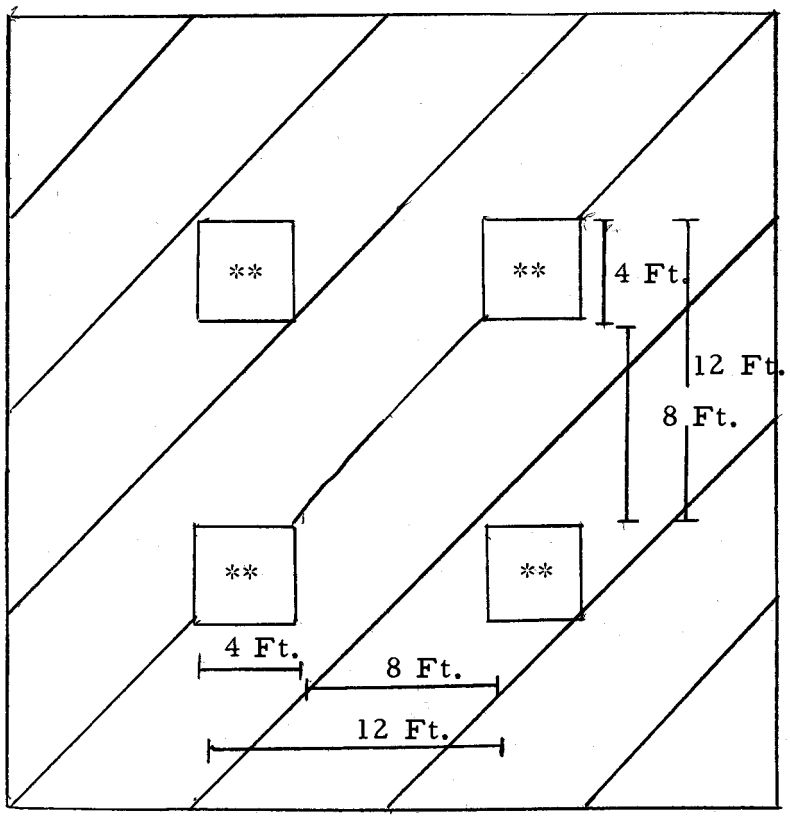


Figure 1. An example of the isolation of the crossing blocks within eight foot pollen biological barriers of rye. Rye was seeded between the four foot by four foot crossing blocks which contained two plants centered 12 inches apart.

Table III. A list of the Lolium multiflorum sources used in each of the 300 crossing blocks established.

Crossing Block Entries[1]		Crossing Block Entries[1]	
1-15	F-F	151-165	L-N
16-30	F-R	166-180	N-L
31-45	R-F	181-210	L-L
46-60	R-R	211-225	R-L
61-75	F-N	226-240	L-R
76-90	N-F	241-255	F-L
91-105	R-N	256-270	L-F
106-120	N-R	271-285	F-F
121-150	N-N	286-300	R-R

[1] F indicates the annual Oregon Common, fluorescing source.
N indicates the annual Oregon Common, non-fluorescing source.
L indicates the annual Leda source.
R indicates the annual Redmond source.

sixteen inch white parchment bags. The bags were supported by passing a wire through an eyelet which had been punched in an upper corner of the bag and tying it around a wooden stake. The bottom end of the bag was tied loosely to the stake and the upper end tied tightly to the same wooden stake to allow for elongation of the stems and inflorescences. The wires attached to the bag were also occasionally slid up the stake to prevent stem and inflorescence elongation from pulling the spikes out of the bags.

Single-crossed seed was obtained in a similar manner with two spikes from each parent being enclosed in the bag. Thus, four spikes were enclosed per single paper bag, Yellow wired labels were used for selfings while blue wired labels were used on the single crosses. The plant number, date of bagging, and type of cross were written on the wired label, the wire of which was used for closing the bottom of the bag. The bags were agitated daily during the flowering period by shaking the wooden stakes. The bags were left on until harvest.

Open-pollinated seed was obtained by bagging the spikes after the flowering period but just before harvest. At harvest time, the spikes were left in the bag with the label attached until threshing.

The spikes were threshed with a head thresher. After the spikes from a bag were threshed, the head thresher was cleaned with forced air. The threshed seed was cleaned by the use of a

South Dakota blower and hand separation. The total number of seeds produced per bag were counted for the single-crosses and selfings. Self-fertility estimates were obtained by two methods. One method used the number of seeds obtained when selfed as compared to the number of seeds obtained when single-crossed. This was expressed as a percentage. The other method was similar except number of germinated seeds was used rather than total number of seeds obtained. All seed collections were subjected to germination and fluorescence tests.

Fluorescence tests were made to test the effectiveness of the biological pollen barrier of cereal rye.

The following laboratory technique was used in testing the annual ryegrass seed for fluorescence and germination in the fall of 1967.

Two layers of Whatman number two white filter paper moistened with 0.2 percent potassium nitrate solution were placed on top of plastic handi-wrap strips twice as wide as the filter paper. Handi-wrap was used as it is non-toxic to ryegrass seedlings. The length of the filter paper strips was 20 inches; the width was approximately 6-2/3 inches. Two rows of 50 seeds each were planted across the filter paper strips unless the sample being tested contained less than 100 seeds. The first row was placed about one-half inch from the top of the paper while the second row was placed about three inches

below the first row. The seeds were planted with the embryo end down to prevent curling of the roots as they emerge from the seed. Each filter paper was marked with a test number.

After the seeds were planted the handi-wrap strips were folded over the seeds, enclosing the filter paper and seeds in the wrapping material except at the upper edge. The rolls were placed in an upright position for testing so that the plumules would grow straight up and the roots straight down with the force of gravity.

The filter paper rolls were stratified at 5° C. for seven days. After stratification, the rolls were transferred to a germinator maintained at a temperature of 25° C. during a 9-1/2-hour day and a temperature of 15° C. during the 14-1/2-hour night. Seven days after the filter paper rolls were transferred to the new germination conditions, fluorescent readings were taken in the darkroom by use of a near-ultraviolet-emitting lamp (300-400 mμ) (Sylvania, Black-lite, F15T8-BLB). The fluorescent seedlings were removed at this time and recorded. The remaining seedlings were then replaced in the germinators for seven additional days and final counts were taken at that time. This technique was used on all the samples studied in this experiment.

Two types of bags were also used to study the influence of bag type on seed set. One bag of each type was placed on about 50 identical plants. Four spikes were enclosed per bag. White

parchment bags and dialysis tubing were the two bag types used. The seamless regenerated cellulose dialysis tubing (viscose process) was 1.729 inches in flat width with a wall thickness of 0.0010 inches. The tubing was cut to a length of 16 inches. The white parchment bags were four inch by four inch by sixteen inch.

RESULTS AND DISCUSSION

Effectiveness of the Biological Pollen Barrier, Secale Cereale

The fluorescent marker gene was used to test the effectivness of a biological pollen barrier of rye in reducing or preventing crossing between the 300 crossing blocks established in this experiment.

The effectivness of the rye barrier was detected in one of two ways. The first method employed was to determine the approximate amount of outcrossing in 30 crossing blocks on the basis of the fluorescence test. By determining the average percentage of fluorescence for all the open-pollinated progency in these crossing blocks, the effectiveness of the rye barrier in excluding foreign pollen from other crossing blocks was indicated. In using this method, it was presumed that all the selected plants in the 30 crossing blocks were truly non-fluorescing types. It was also presumed that fluorescence was due to a single dominant gene. The data presented in Table IV shows the segregation of fluorescence of 34 selfed plants. The observed ratio of 337:203 did not fit in a 3:1 ratio, however, this ratio also did not fit a 9:7 ratio. Although considerable evidence indicates that the fluorescent reaction of seedlings is controlled by a single dominant gene, fluorescence may be governed by two genes. These data are inconclusive.

Table IV. The segregation for fluorescence in the selfed progeny of Lolium multiflorum genotypes.[1]

Entries	Fluorescent seedlings	Non-Fluorescent seedlings	Entries	Fluorescent seedlings	Non-Fluorescent seedlings
1	14	11	18	38	10
2	7	1	19	42	2
3	15	1	20	1	1
4	29	2	21	7	1
5	3	6	22	3	2
6	2	1	23	10	2
7	8	2	24	1	38
8	2	11	25	1	2
9	24	5	26	6	1
10	46	18	27	1	2
11	1	16	28	2	17
12	7	1	29	19	8
13	2	1	30	1	6
14	11	1	31	3	6
15	1	13	32	4	5
16	16	2	33	2	2
17	1	1	34	7	5
Sub-total	189	93	Total	337	203

[1] The ratio of 337:203 did not fit a 3:1 or 9:7 ratio.

The second method used to indicate the effectiveness of the rye barrier was to compare the percentage of fluorescence between the single-crossed and open-pollinated progeny from 46 crossing blocks. If the single-crossed and open-pollinated progeny were highly correlated and did not differ significantly in percentage of fluorescence, it would indicate the rye barrier was effective. If the rye barrier was not effective in excluding foreign pollen, the percentage of fluorescence of the open-pollinated progeny should be significantly greater than that of the single-crossed progeny.

The results of the two methods are presented in Table V and Table VI. The means in percent fluorescence of the open-pollinated progeny of individual plants in crossing blocks 121 through 150 are shown in Table V. The average of all 30 crossing blocks was 16.3 percent. The mean fluorescence of the open-pollinated progeny of several plants was very high and only 11 of the 38 means were above the average, leading one to suspect that the parents of these open-pollinated progeny may not have been non-fluorescing types. Thus, the rye barrier may be even more effective than the average of 16.3 percent fluorescence for the 30 crossing blocks studied indicates.

The results of the second method as shown in Table VI support the above statement. On the basis of a paired "t" test, it was determined that the average percentage of fluorescence of the open-pollinated progeny was not significantly greater than the average

Table V. Table of means in percent fluorescence for the open-pollinated progeny of plants in crossing blocks 121 through 150. [1]

Plant Identification [2]	Percent Fluorescence	Plant Identification	Percent Fluorescence	Plant Identification	Percent Fluorescence
121a	9.43	131a	----	141a	----
121b	----	131b	----	141b	----
122a	----	132a	3.23	142a	38.84
122b	1.27	132b	58.55	142b	----
123a	2.83	133a	----	143a	15.04
123b	----	133b	1.32	143b	8.27
124a	----	134a	39.35	144a	61.93
124b	2.04	134b	7.96	144b	5.92
125a	4.98	135a	0.00	145a	----
125b	2.92	135b	----	145b	7.07
126a	19.51	136a	----	146a	39.57
126b	15.38	136b	2.56	146b	4.65
127a	11.97	137a	----	147a	5.19
127b	52.99	137b	24.54	147b	2.01
128a	----	138a	23.96	148a	47.77
128b	1.67	138b	----	148b	17.04
129a	----	139a	----	149a	----
129b	2.21	139b	7.52	149b	----
130a	4.96	140a	----	150a	50.57
130b	13.46	140b	0.76	150b	----

[1] The mean percent fluorescence for all plants studied in crossing blocks 121 through 150 was 16.30 percent.

[2] a and b represent the two plants in each crossing block.

Table VI. The means in percent fluorescence of the open-pollinated and single-crossed progeny for 46 crossing blocks.[1]

Crossing Block Entries	Open Pollinated Progeny	Single Crossed Progeny	Crossing Block Entries	Open Pollinated Progeny	Single Crossed Progeny
70	47.5	50.0	116	72.4	0.0
71	79.5	62.5	117	58.3	44.0
73	61.2	62.5	118	32.3	0.0
75	72.6	94.3	119	72.2	100.0
77	89.2	92.2	120	74.3	49.5
78	88.4	100.0	152	68.9	89.3
81	5.5	0.0	154	73.4	90.4
82	82.5	100.0	156	92.7	100.0
85	89.9	50.0	158	94.7	97.5
86	83.3	83.8	160	73.5	64.7
87	69.9	50.0	162	88.3	100.0
94	94.9	46.8	164	96.9	100.0
97	72.0	76.2	165	98.5	97.5
98	97.3	86.9	166	76.4	55.4
99	85.8	100.0	167	76.6	96.6
103	89.2	92.2	168	90.5	81.5
104	86.3	60.0	169	94.9	66.7
106	91.2	50.0	170	53.3	53.9
107	96.2	92.3	172	90.2	88.5
109	84.6	100.0	174	74.2	47.4
110	88.6	82.4	175	89.5	82.4
114	87.4	86.1	176	66.1	61.6
115	47.2	100.0	177	77.1	67.3

[1] The mean percent fluorescence for the open-pollinated progeny was 72.88 percent. The mean percent fluorescence for the single-crossed progeny was 78.81 percent. A paired "t" test of significance showed that the two means did not differ significantly at the five percent level as the calculated "t" value of 0.6289 did not exceed the critical "t" $_{.05}$ value. The correlation coefficient between the percent fluorescence of the open-pollinated and single-crossed progeny was 0.672 and significant at the one percent level.

percentage of fluorescence of the single-crossed progeny in the 46 crossing blocks studied. The calculated "t" value of 0.6289 did not exceed the critical "t"$_{.05}$ value at 45 degrees of freedom. The correlation coefficient between open-pollinated and single-crossed progeny of 0.672 was significant at the one percent level. Since the two variables are highly correlated and did not differ significantly from each other in percentage of fluorescence, this method of testing the effectiveness of the rye barrier indicates that an eight foot barrier of rye was effective in preventing outcrossing between crossing blocks.

Self-Fertility Estimates

Self-fertility data on 110 plants were obtained by seed count and by germination. Self-fertility estimates based upon these two methods of obtaining self-fertility data were calculated and are presented in Table VII. Self-fertility estimates based on seed count data compared the number of seeds obtained when selfed to the number of seeds obtained when single-crossed and was expressed as a percentage. The self-fertility estimates based upon self-fertility data obtained by germination were calculated in the same manner.

The results showed that self-fertility of the parent plants ranged from 0 to 87 percent. The average percent self-fertility

Table VII. Comparison of self-fertility estimates of Lolium multiforum based upon self-fertility data obtained by germination and by seed count.[1]

Entries	Percent Self-Fertility		Entries	Percent Self-Fertility		Entries	Percent Self-Fertility	
	By Germination	By Seed Count		By Germination	By Seed Count		By Germination	By Seed Count
1	20.33	30.89	27	2.05	4.43	53	0.00	83.33
2	33.33	11.36	28	54.54	64.28	54	13.66	18.99
3	22.36	34.37	29	4.70	6.79	55	0.00	3.76
4	60.00	57.43	30	18.39	29.11	56	1.10	1.73
5	46.96	47.50	31	28.00	26.66	57	2.54	6.73
6	4.47	4.40	32	33.33	51.11	58	3.33	8.81
7	0.81	1.88	33	42.85	35.71	59	4.40	2.76
8	76.47	77.41	34	0.00	46.15	60	55.00	52.85
9	42.85	45.45	35	0.00	36.36	61	72.73	50.00
10	35.71	34.48	36	11.76	13.33	62	86.95	79.48
11	50.00	28.57	37	2.60	4.10	63	20.00	30.83
12	16.04	16.28	38	7.14	18.64	64	26.21	24.59
13	29.90	31.43	39	11.11	28.57	65	6.80	7.20
14	0.00	21.43	40	75.00	77.27	66	16.36	13.15
15	33.33	28.57	41	33.54	25.11	67	12.31	14.45
16	36.57	22.11	42	30.57	25.79	68	13.64	16.47
17	3.48	5.15	43	44.00	41.86	69	19.05	25.00
18	13.22	18.54	44	0.00	2.60	70	59.52	57.14
19	35.04	34.12	45	29.41	33.33	71	0.00	0.74
20	57.44	59.18	46	7.94	18.40	72	4.55	4.46
21	3.14	4.94	47	3.53	4.95	73	41.37	43.33
22	24.24	24.44	48	4.00	5.96	74	10.00	17.86
23	3.85	10.53	49	41.37	38.46	75	7.70	6.67
24	54.55	52.94	50	69.64	71.42	76	53.17	49.34
25	80.00	81.73	51	77.77	60.27	77	10.81	9.46
26	0.00	13.64	52	50.00	83.33			

[1] Thirty-three additional entries showed estimates of 0 percent self-fertility on the basis of self-fertility data collected by germination and seed

Continued on next page.

Table VII. Continued.

count. The average percent self-fertility based on germination was 18.23 percent. The average percent self-fertility based on seed count was 20.74 percent. A paired "t" test of significance showed that the two treatment means differed at the five percent level of probability as the calculated "t" value of 2.205 exceeded the critical "t" $_{.05}$ value. The sample linear correlation coefficient between the percent self-fertility based on germination and that based on seed count of 0.872 was highly significant at the one percent level.

based on self-fertility data obtained by seed count was 20.74 percent. Based on self-fertility data obtained by germination, the average self-fertility was 18.23 percent.

A paired "t" test showed that the average self-fertility based on seed count differed from the average self-fertility based on germination at the five percent level of probability. The correlation coefficient between self fertility estimates based on germination data and on seed count data of 0.872 was significant at the one percent level. The results show that average self-fertility in Lolium multiflorum is low, although some plants were very high in self-fertility. Low self-fertility may be due partly to (a) low vigor and poor survival value, (b) self-sterility, and/or (c) the fact that plants heterozygous for defective seedlings are frequently encountered.

The results further indicate that self-fertility estimates based on self-fertility data obtained by germination may be a more satisfactory method of determining self-fertility in detailed fertility studies of individual plants and their differences than self-fertility estimates based on data obtained by seed count. Several plants that showed 0 percent self-fertility based on germination showed over 35 percent self-fertility based on seed count. It would be very difficult, perhaps impossible, to develop a line of Lolium multiflorum of very high self-fertility for selection work within selfed progeny if these plants had been used.

Influence of Bag Type on Seed Set

Two types of bags were used to study the influence of bag type on seed set. The bag types used were white parchment bags four inch by four inch by sixteen inch and seamless regenerated cellulose dialysis tubing 1.729 inches in flat width with a wall thickness of 0.0010 inch. The tubing was cut to a length of 16 inches. Forty-seven plants were used in this investigation with four spikes being enclosed per bag type on each plant. Seed count per bag type was made for each plant and a paired "t" test was used to determine any significant differences in seed set under the two bag types.

The results as presented in Table VIII showed that the two bag types did not differ at the five percent level of significance in number of seeds set.

The correlation coefficient between the bag types of 0.6561 was significant at the one percent level. The average number of seeds set under the white parchment bag was 185.79 while the average number of seeds set under dialysis tubing was 201.26. The use of dialysis tubing as a substitute for white parchment bags in bagging plants is therefore questionable.

Table VIII. Comparison of seed set under two bag types of 47 plants.[1]

Entries	Dialysis Tubing[2]	White Parchment Bags	Entries	Dialysis Tubing	White Parchment Bags
1	128	153	25	411	212
2	262	223	26	164	146
3	203	119	27	265	318
4	67	41	28	62	4
5	144	160	29	394	294
6	180	115	30	168	174
7	123	50	31	177	159
8	179	102	32	225	171
9	40	32	33	250	133
10	392	306	34	109	121
11	136	232	35	118	16
12	0	143	36	216	144
13	196	163	37	67	96
14	195	188	38	298	195
15	66	32	39	141	170
16	129	230	40	77	136
17	56	267	41	213	114
18	239	102	42	117	115
19	317	276	43	249	202
20	449	332	44	276	262
21	244	150	45	256	413
22	107	256	46	389	362
23	258	372	47	400	306
24	307	325			

[1] The average seed set under white parchment bag was 185.79 while the average seed set under dialysis tubing was 201.26. The paired "t" test of significance showed that seed set did not differ under the two bag types at the five percent level of probability. The correlation coefficient of 0.6561 was highly significant.

[2] Number of seeds set per four spikes.

SUMMARY AND CONCLUSIONS

Studies were conducted on the methodology of crossing and selfing in Lolium multiflorum. Four foot by four foot crossing blocks containing two plants centered twelve inches apart were located on 12 foot centers so that each block was enclosed within an eight foot biological barrier of rye, Secale cereale.

Fluorescent and non-fluorescent plants of several Lolium multiflorum sources were used to investigate outcrossing in Lolium multiflorum to test the effectiveness of the rye barrier in preventing crossing between the 300 crossing blocks established. It was presumed that fluorescence was due to a single dominant gene. The results revealed the average percentage of fluorescence of open-pollinated progeny in crossing blocks containing supposedly only non-fluorescing plants to be 16.3 percent.

The average percent fluorescence of open-pollinated and single-crossed progeny in 46 additional crossing blocks studied were highly correlated and did not differ from each other in percent fluorescence. This showed that the percent fluorescence of these crossing blocks was mainly due to intra-crossing block pollen rather than inter-crossing block (foreign) pollen. The results indicate that an eight foot barrier of rye is effective in preventing outcrossing between Lolium multiflorum crossing blocks.

Self-fertility estimates ranged from 0 to 87 percent. Several individual plants were very high in self-fertility although self-fertility was generally low.

Based on self-fertility data obtained by germination, average self-fertility was 18.23 percent. Based on seed count, average self-fertility was 20.74 percent. A paired "t" test of significance showed that the two differed at the five percent level.

It is therefore suggested that self-fertility data be obtained by germination rather than seed count when self-fertility estimates of individual plants and their differences is desired. Low vigor and poor survival value, self sterility, and defective seedlings are frequently encountered in inbred material and if seed count is used, self-fertility may be overestimated.

There was no significant difference in seed set between two bag types used on the 47 plants studied. The average seed set under the white parchment bag was 185.79 seeds and the average seed set using dialysis tubing was 201.26 seeds. The use of dialysis tubing as a substitute for white parchment bags in bagging procedures would be of little benefit.

BIBLIOGRAPHY

1. Anslow, R. C. Seed formation in perennial ryegrass. I. Anther exsertion and seed set. Journal of the British Grassland Society 18:90-96. 1963.

2. Axelrod, B. and J. R. Belzile. Isolation of an alkaloid, annuloline, from the roots of Lolium multiflorum. Journal of Organic Chemistry 23:919-920. 1958.

3. Beddows, A. R. Seed setting and flowering in various grasses. In: Publication of the Welsh Plant Breeding Station, 1921 to 1939. Ser. H. Aberystwyth, University College of Wales, 1931. p. 5-99.

4. Brewbaker, J. L. and S. K. Majumder. Cultural studies of the pollen population effect and the self-incompatibility inhibition. American Journal of Botany 48:457-464. 1961.

5. Cokill, L. Inheritance of fluorescence in ryegrass. Nature 130:134. 1932.

6. Cooper, J. P. Selection and population structure in Lolium. III. Selection for date of ear emergence. Hereditary 13:461-479. 1959.

7. Copeland, Lawrence Odell. Measurement of outcrossing in Lolium spp. as determined by fluorescence tests. Ph.D. Thesis. Corvallis, Oregon State University, 1968. 59 numb. leaves.

8. Crane, M.B. and K. Mather. The natural cross-pollination of crop plants with particular reference to radish. Annales of Applied Biology 30:301-308. 1943.

9. Fejer, O. S. Effects of seed quality in genetical experiments with forage plants. (Abstract) Canadian Journal of Genetics and Cytology 5:99. 1963.

10. Foster, C. A. Polythene-terylene tents for large-scale artificial isolation in ryegrass breeding. Record of Agricultural Research. Northern Ireland 13:137-141. 1964. (Abstracted in Plant Breeding Abstracts 36:540. 1966)

11. Griffiths, D. J. The liability of seed crops of perennial ryegrass (Lolium perenne) to contamination by windborne pollen. Agricultural Science 40: 19-38. 1950.

12. Hayes, H. K. and F. R. Immer. Methods of plant breeding. New York, McGraw-Hill, 1942. 432p.

13. Hayes, H. K., F. R. Immer and C. Smith. Methods of plant breeding. 2d ed. New York, McGraw-Hill, 1955. 551p.

14. Jenkin, T. J. Inheritance in Lolium perenne L. II. A second pair of lethal factors. Journal of Genetics 19: 403-417. 1928.

15. _____ Inheritance in Lolium perenne L. III. Base colour factors C and R. Journal of Genetics 22: 389-394. 1930.

16. _____ Interspecific and intergeneric hybrids in herbage grasses. VII. Lolium perenne L. with other Lolium species. Journal of Genetics 52: 300-317. 1954.

17. _____ The ryegrasses (Lolium L.) In: Handbuch der Pflanzenzuchtung, ed. by H. Kappert and W. Rudorf. 2d ed. Paul Parley, Berlin, 1959. 4: 435-452.

18. _____ Self-fertility in Italian ryegrass (Lolium perenne var. multiflorum). In: Publication of the Welsh Plant Breeding Station, 1921 to 1939. Ser. H. Aberystwyth, University College of Wales, 1931. p. 120.

19. _____ Self-fertility in perennial ryegrass (Lolium perenne L.). In: Publications of the Welsh Plant Breeding Station, 1921 to 1939. Ser. H. Aberystwyth, University College of Wales, 1931. p. 110-119.

20. Jensen, I. and H. Bogh. On conditions influencing the danger of crossing in the case of wind pollinated cultivated plants. Tidsskrift Planteaul 46: 238. 1941.

21. Jones, M. D. and L. C. Newell. Pollination cycles and pollen dispersal in relation to grass improvement. Lincoln, 1946. 42p. (Nebraska, Agricultural Experiment Station. Research Bulletin No. 148).

22. Justice, O. L. A review of literature on the use of the fluorescence test for the classification of Lolium species and hybrids. Proceedings of the Association of Official Seed Analysts 39: 89-92. 1949.

23. Keller, W. Interpretation of self-fertility in grasses by frequency distributions. Journal of the American Society of Agronomy 40:894-900. 1948.

24. Meyers, W. M. Analysis of non-heritable variations in seed set under bag among plants of orchardgrass, Dactylis glomerata. Journal of the American Society of Agronomy 34: 1114-1124. 1942.

25. _____ Heritable variations in seed set under bag among plants of orchardgrass, Dactylis glomerata. Journal of the American Society of Agronomy 34:1042-1051. 1942.

26. Nyquist, W. E. Fluorescent perennial ryegrass. Crop Science 3:223-226. 1963.

27. Nyquist, W. E. and J. D. Schulke. Genetics of morphological characters in Lolium. I. Branched culm in L. multiflorum and rudimentary spikelet in L. perenne and L. multiflorum. Crop Science 1:441-445. 1961.

28. Rampton, H. H. The use of morphological characters as compared with fluorescence tests with ultraviolet light in classifying the ryegrasses (Lolium spp.) of Western Oregon. Journal of the American Society of Agronomy 30:915-922. 1938.

29. _____ Time isolation as a safeguard to varietal purity in perennial ryegrass, annual ryegrass, and orchardgrass. Corvallis, 1966. 12p. (Oregon, Agricultural Experiment Station. Circular of Information 623).

30. Schulke, J. D. and W. E. Nyquist. Inheritance of deformed spikelet in ryegrass (Lolium spp.). In: Crop Science Abstracts of the Annual Meeting of the Western Society of Crop Science, Davis, Calif., June 19-22, 1961. Davis, Calif., University of California, 1961. p. 39. (mimeographed)

31. Stapledon, R. G. The breeding of herbage plants; technique adopted at the Welsh Plant Breeding Station. 2. Methods as

applied to cocksfoot grass (Dactylis glomerata L.) and remarks as to technique in general. In: Herbage Plants. Aberystwyth, Wales, 1931. p. 35-45. (Imperial Bureau of Plant Genetics. Bulletin 3.)

32. Thrupp, C. R. and A. E. Slinkard. Seedset rating as a measure of self-fertility in grasses. Crop Science 6: 599-600. 1965.

33. Trumble, H. C. and I. F. Phipps. The inheritance of fluorescence in hybrids between perennial-ryegrass and Wimmera rye-grass. Australia Council for Industrial and Scientific Research Journal 6: 170-178. 1933.

34. Wit, F. The pollination of perennial ryegrass (Lolium perenne L.) in clonal plantations of polycross fields. Euphytica 1: 95-105. 1952.

35. Woodforde, A. H. Fluorescence in perennial x Italian ryegrass. Tasmanian Journal of Agriculture 4: 120-122. 1933.