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Research was conducted to determine the distance and amount of outcrossing between varieties of highly fluorescent annual ryegrass (Lolium multiflorum Lam.) and low-fluorescing perennial ryegrass (Lolium perenne L.). Studies were conducted under both spaced-plant and seed production field conditions.

Fluorescence tests on seed collected from low-fluorescing perennial ryegrass at successive distances from the fluorescent annual ryegrass pollen source revealed that outcrossing was considerably less than that reflected by present certification isolation distances.

Outcrossing under spaced plant situations decreased rapidly and became minimal at a distance of 100 feet. Crossing between adjacent high-density fields was substantially below that revealed between space-planted ryegrass. Under field conditions, outcrossing

became negligible after about ten feet, and no evidence of crossing was observed at distances greater than 40 feet.

Measurement of Outcrossing in *Lolium* Spp. as Determined by Fluorescence Tests

by

Lawrence Odell Copeland

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INTRODUCTION

The fluorescence test of ryegrass has been used for many years to distinguish between fluorescent annual ryegrass (Lolium multiflorum Lam.) and nonfluorescent perennial ryegrass (Lolium perenne L.) seedlings. These two species of ryegrass cross readily, and F_1 hybrids also have the capacity to fluoresce. The possibility of using the gene controlling seedling fluorescence to detect outcrossing between annual and perennial ryegrass arose during routine observations on a small group of fluorescent plants, with two rows of nonfluorescent plants on each of two sides. The pattern of fluorescence results revealed by individually-tested plants suggested that outcrossing between wind pollinated grass varieties might be substantially less than that reflected by present certification isolation requirements.

It has long been recognized that outcrossing between varieties can occur, but experimental data about the extent and distance of such crossing has not been available. It is known that isolation is necessary, so pollen dispersal studies have been used to arbitrarily establish isolation distances. Information concerning pollen dispersal is abundant, but actual outcrossing data has been contingent on the discovery and use of appropriate genetic markers which can be used

to distinguish F_1 hybrids of two parent strains. The fluorescence test of ryegrass and the gene controlling fluorescence provides an ideal marker for this purpose.

Knowledge of realistic varietal isolation requirements is critical in today's agriculture which demands that plant breeders continue to develop crop varieties with superior and highly specific agronomic characteristics. Once developed, it is important that seed of such varieties be maintained, increased, and made available to farmers. Programs for maintenance and increase of these varieties must provide measures which will insure the retention of varietal genetic purity. This is accomplished primarily by the isolation between pedigreed seed producing fields of different varieties. In general, similar isolation has been used for crops of the same general nature, such as grasses, legumes, and horticultural crops.

The purpose of this research was to investigate outcrossing patterns in ryegrass under controlled spaced-plant situations and between adjacent seed production fields. Adjacent highly fluorescent and low fluorescing varietal situations used in these investigations provided an ideal laboratory to test for crossing between varieties with different levels of fluorescence.

LITERATURE REVIEW

Contamination by pollen, spores, etc., generally decrease as one proceeds further away from the source. This was recognized as far back as 1806 when rust on rye was reported to be more severe near barberry bushes, which were known to be the alternate host for the fungus (40). Presence of air-borne spores and pollen has been known to occur hundreds of miles from the source of origin. However, it was not known how far such contamination would present a serious threat to fertilization by pollen at various distances.

Erdtman (14) analyzed air samples in mid-ocean during the early summer with the aid of a vacuum cleaner and found the concentration of pollen to be 0.1 grain per 100 cubic meters. A comparable figure for the Swedish mainland was 18,000 grain per cubic meter. Erdtman (15) later stated that pollen grains of certain sedges and grasses may be carried in great quantities in the air for distances of more than 625 miles into the middle of the ocean.

Although Erdtman established the possibility of pollen dispersal for great distances, isolation distances used to aid in maintenance of varietal integrity in certified seed production have been less because of practical considerations. Haskell (21) reported that distances up to one mile or more are commonly recommended for isolation between varieties. A comparison of reports by Pederson (36) and the

standards of the International Crop Improvement Association (24) reveals that the isolation distances between varieties are greater in some countries than those required in the United States and Canada.

Theoretical Aspects of Pollen Dispersal

Gregory (18) reported that dispersal of passively air-borne particles such as seed, spores, or pollen, results in a scatter around the point of liberation. Nageli (cited from Gregory (18) stated that the density of dust particles at various distances from the point of liberation should fall off inversely as the square of the distances. Kursanov (cited from Gregory, 18) concluded that in the absence of wind, the number of fungus spores would fall off inversely with the cube of the distance from the source.

Stokes Law (46) describes the mathematical relationship between the size and the velocity of a smooth sphere in a viscous fluid. Many investigators have since attempted to describe spore dispersal in terms of Stokes Law.

Buller (8) found that the spores of Amintopsis vaginata fell at velocities of about 50% greater than expected from Stokes Law. Erdtman (15) also observed that terminal velocities of air-dry pollen are usually somewhat higher than expected values according to Stokes Law. The effect of density of the pollen of various species on the distance of dispersal was suggested by Pohl (cited from Erdtman,

15) who reported that specific gravity of pollen grains varied from 1.161 for Typha latifolia to 0.391 for Pinus sylvestris.

Zeleny and McKeehan (52) observed that spores of Lycopodium, Bovista, and Polytrichum fell only about one-half as fast as predicted by Stokes Law and attributed this difference to surface irregularities setting up eddies in the immediate neighborhood of the particles, while Stokes Law applied only to non-turbulent conditions.

Several authors (5, 7, 47, 52) have presented calculations on spore deposition based on the theory of diffusion-eddy. Gregory (18) reviewed all these reports and showed data which proved that spore deposition at various distances could be predicted in terms of diffusion-eddy. He presented evidence which showed it appropriate to consider spores or pollen as cloud-suspensions in air rather than a continuous shower of pollen falling under gravity. Spores are deposited from the suspension by turbulence (diffusion-eddy) which breaks away part of the suspension and causes spores to be brought down to the relatively still ground layer where gravity can prevail, and deposition occur. Gregory stated that turbulence which causes spore dispersion was both mechanical turbulence caused by friction between the wind and roughness of the ground over which it passes, and thermal turbulence caused by re-radiated heat lost from the earth's surface.

Bateman (5) also found better correlation between theoretical

and actual pollen dispersal if diffusion-eddy (turbulency) was considered instrumental in pollen dispersal. He found equally good correlation regardless of high or low turbulency conditions. Bateman failed to note any practical difference between upwind and downwind dispersal or between bee and wind pollen dispersal. He discussed formulae presented by Sutton (47) attaching atmospheric turbulence to pollen deposition from both a point and a line source at two different turbulencies and one presented by Bosanquet and Pearson (6) discounting turbulence as important--for both wind pollinated and insect pollinated crops. Using these formulae, and contamination observed at two distances, he predicted contamination at all distances from the pollen source. He reported that all previously available data on pollen dispersal for various crops correspond very well with those predicted by his formula.

Brunt (7) explained atmospheric turbulence as it influenced pollen dispersal. He reported that there is a large number of small-scale eddies occurring for durations of about one second, about every five seconds. This action on the numerous spores produced from plant sources makes possible a regularity in the dispersal of pollen.

Gregory (18) reported that while the problem of a single spore in a wind eddy was inscrutable, the distribution of vast numbers of spores over a uniform area and over a period of time offers hope

for rational treatment. Rempe (41) implied that this treatment might be different depending on the species considered since surface irregularities of pollen grains caused their effective diameter to become greater due to a coating of stationary air surface. This was given support by Bateman (4) who observed that maize has excessively large pollen grains which are consequently less buoyant and would not be carried so widely by a given wind. He reported that grasses having smaller pollen would have the same shape of the dispersal curve, but a more extended slope.

Pollen Dispersal and Outcrossing

In 1941, Jensen and Bogh (26) showed that ryegrass and orchardgrass pollen contamination at a distance of 500 to 600 meters was about 1/20th of that at the source. Bateman (4) used glycerine-coated slides to trap pollen around maize plots and showed that about 60 feet was sufficient to reduce atmospheric pollen to 1% of that at the source. In a very extensive study of 16 cool season and 13 warm season grasses, Jones and Newell (28) reported that most pollen dispersal followed general wind directions but concluded that isolation distances should be the same for all wind directions. The effect of gravity on the pollen rapidly decreased the pollen load

as distance from the pollen source increased. Approximately 31% of the amount of pollen observed at the source was caught per unit area at five rods, 10% at 15 rods, 4.4% at 25 rods, 1.2% at 40 rods, and 0.8% at 60 rods. They concluded that the chance of maintaining genetic identity of improved strains of cross-pollinated grasses are much greater at 60 rods of isolations and less at smaller distances. Even the low percentages of pollen caught at 60 rods was considered to be an omnipresent source of contamination of pure seed stock.

Using a marker gene controlling the early plant character in corn to determine crossing into Canada Gold corn, Bateman (4) found that the rate of decrease in contamination per unit distance proceeds very rapidly at first from the pollen source; but at greater distances, the contamination, though small, becomes persistent and decreases very slowly. He found that in beets, which are pollinated primarily by wind-blown pollen, the results were similar. Crane and Mather (11) worked with radish and turnips, both insect pollinated crops, and also found similar results. Bateman (5) attempted to derive a mathematical expression to predict the variation in contamination with distance under any condition regardless of the crop being grown. He concluded (4) that although significant differences between wind pollinated and insect pollinated crops might be

disclosed later, the evidence then available did not justify different isolation distances for them.

Currence and Jenkins (12) studied the effect of distance on contamination in tomato (wind pollinated) by designing an experiment with a central square of one variety and two rows in opposite directions of the other variety. Contamination in the rows decreased to a minimum at the furtherest isolation distance, which was 72 feet.

Thompson (48) grew lettuce plants just far enough apart so that the lateral branches did not touch and found only 1.33% to 6.22% outcrossing. He concluded that under normal field conditions the value would be much less.

Haskell (21) reports that Tedin, working in Sweden, investigated the danger of crossing in turnip seed production and found that only 1% contamination occurred at 27 yards between crops and no perceptible decrease occurred after that. Crane and Mather (11), working with closely planted experiments using Icicle and Scarlet-Globe radish varieties, found the amount of intercrossing ranged from 30% to 40% at a distance of nine inches to an average of 1% at 15 feet. But when single plants of Icicle were extended out from plots of Scarlet-Globe, crossing was about 100% at nine inches, dropping to 1% after 20 feet. They concluded that if only small numbers of tester-plants are used, intercrossing is likely to occur over greater distances. Bateman (3) used two insect pollinated

self-compatible species, radish and turnip and a large amount of plants of the contaminating and contaminated varieties to test for outcrossing. He also found a progressively smaller rate of decrease in contamination as isolation distances became greater.

Factors Influencing Amount of Outcrossing

Tracy (49) reported that a grove of trees four rods wide acted as an effective intervarietal barrier to outcrossing between fields. Haskell (21) stated that hedges definitely acted as screens against contamination and that a definite relationship existed between the height of the hedges and the reduction in outcrossing.

Bateman (4) reported that time isolation was effective in both corn and beets in preventing outcrossing between varieties. Rampton (38) made extensive study on the pollination dates of grass seed crops grown in the Willamette Valley of Oregon. He presented five years of data which showed consistent differences in periods of anthesis between several different varieties of the grass seed crops. Rampton concluded that for many varieties, time isolation could be used very effectively instead of spatial isolation to prevent crossing between different seed crop varieties. The Oregon Seed Certification Standards (34) show that time isolation instead of spatial isolation is used for prevention of outcrossing between Oregon annual ryegrass and Linn perennial ryegrass.

Hayes and Immer (22) report that the Minnesota Crop Improvement Association isolation standards may be reduced $2\frac{1}{2}$ rods in certain cases for each additional border row of the male parent planted around the plot. In this way, outcrossing would be prevented by intra-varietal pollen acting as a screen against invading intervarietal pollen. Knowles (29), working with a marker gene in bromegrass in Canada, showed that outcrossing could be decreased considerably if border rows of seed producing fields were allowed to pollinate with the remainder of the field and then cut subsequent to pollination and kept separately. Seed from plants subsequent to the border strips could be certified as genetically pure due to their being protected from intervarietal pollen by the intra-varietal pollen of the border strips. Based on Knowles' work, the International Crop Improvement Standards (25) were changed in 1966, to allow for shorter isolation distances if border strips of varying widths were left uncertified along the border of certified seed fields.

There are numerous reports in the literature that pollen viability decreases with pollen age and that loss of viability may be considerably different, depending on the type of pollen involved. Pfundt (37) reported that air-dry pollen remained viable only for one day. For most grasses the length of pollen viability was less than three hours, but for Hordeum spp. and Zea pollen remained viable 48 and 32 days respectively. He reported that pollen of the grass family

remained viable for a shorter period than pollen of any other plant family. Holman and Brubaker (23) attributed the very short viability period of grass pollen to a very rapid water loss.

Stephens and Quinby (45) reported that pollen of sorghum decreased in viability very rapidly and pollen more than five hours old was not capable of producing seed set. Working with annual and perennial ryegrass, Gregor (17) found that in no case did flowers of Lolium italicum set seed when pollinated by 24- and 48-hour old pollen. In the case of Lolium perenne, 33% of the flowers pollinated with 24-hour old pollen produced seed, while for other plants, fertilization did not take place.

Jones and Newell (27) found that a high percentage of pollen of buffalograss and corn stored in beakers at 40°F and 90% relative humidity remained viable for seven and eight days, respectively. When pollen of corn was stored in pollinating bags, viability was lost in only three hours in direct sunlight, and in 30 hours in the shade, although the viability was lost gradually throughout these 30 hours.

Cooper and Burton (9) reported that pollen of pearl millet could be stored 24 hours at 80°F and up to four days at 40°F with less than 50% loss in viability.

Dean (13) observed that tobacco pollen stored at room conditions lost viability in nine weeks; when either temperature or

humidity was lowered, viability was retained longer.

Sonchez and Smeltzer (45) reported that sorghum pollen, when stored in the shade at normal daytime temperatures, decreased from 100% to 30% viability in about four hours and completely in about 24 hours; when stored at 4° C and 75% relative humidity, it suffered the same decrease after about 70 hours.

Sandsten (43) states that not all pollen capable of germination is capable of effecting fertilization. Knowlton (30) also reports that pollen may be able to germinate and yet be incapable of functioning in fertilization. On the other hand, Nohora (32) reports relative to certain Salix pollen that pollen can be used for fertilization as long as it is able to germinate; and Roemer (42) even maintains that in Streptocarpus, pollen grains which had lost germination power in sugar solutions were nevertheless capable of fertilization.

The study of the length of the pollen tube growth appears to give no additional information about viability over that gained by determining percent germination according to Warnock and Hagedorn (50). Working with pea pollen, they found a significant correlation between pollen germination percent and ability to set seed. Lehman and Puri (31) studied grades of germination and pollen tube growth of stored and fresh alfalfa on agar medium. They found a high correlation between pollen germination and seed set. There was, however, no correlation between the total length of the pollen tube

growth and the capacity of the pollen to effect seed set; that is, pollen tube growth of freshly collected pollen was the same as pollen which had been stored for several hours even though the total pollen germination decreased considerably.

Outcrossing in Ryegrass

In 1950, Griffiths (20) reported on extensive experiments in which he used a simply-inherited dominant gene controlling the expression of a red pigmented culm base of ryegrass to trace the effective pollination pattern into spaced non-red ryegrass plants. The progeny from different non-red plants at successive intervals from the source of contamination showed that there was a rapid initial decrease in outcrossing with increasing distance; however, a progressive lessening in the rate of decrease occurred at greater distances from the contamination source. The actual rate of decrease depended on the plant density and arrangement of the experimental plots. A later paper by Griffiths (19) gave similar results. He concluded that intra-varietal pollen from plants within the same field was the most effective factor for prevention of outcrossing from adjacent fields which pose a potential problem.

Wit (51) reported on similar work conducted on ryegrass outcrossing between breeding trials by use of a dominant gene controlling the presence of downwardly-directed teeth on the culm and upper

leaf sheaths. Inter-clonal pollination of breeding blocks showed that the first few rows of adjacent clones were highly effective in reducing the effect of the contamination pollen; but when a clone was bordered on two sides by other clones, from 40% to 74% of the clonal fertilization occurred from adjacent clones.

Both Wit (51) and Griffiths (19, 20) found that outcrossing was considerably less than that indicated by previous pollen dispersal studies (26, 28). Plant density seems to be a significant contributing factor to this discrepancy since actual plant densities present in seed production conditions were not used. However, in each of these studies (19, 20), crossing was reduced as plant density increased. This trend is highly consistent with results observed by Crane and Mather (11) and Bateman (3) who reported that as plant densities increased, the decrease in outcrossing became progressively smaller.

Characteristics of the Fluorescence Expression

In 1929, Gentner (16) discovered that seedlings of annual ryegrass could be distinguished from those of perennial ryegrass by the fluorescence reaction of the former when subjected to ultraviolet light, and the lack of such a fluorescence response in the latter. This process soon became widely used because of its simplicity and because separation of the two species based on seed morphology is

difficult. The usefulness of the fluorescence test has long been recognized but only recently has the chemical nature of the fluorescence substance been determined. In 1958, Axelrod and Belzille (2) reported that they were able to extract and isolate a substance from the roots of annual ryegrass which fluoresced under ultra-violet light. The substance had a formula of $C_{20}H_{19}NO_4$ and was named annuloline. Their results were later confirmed by Ching[1].

By natural selection, the ryegrasses have evolved in such a way that the annual characteristic has become genetically closely associated with the fluorescence characteristics. Thus, Rampton (39) in 1931, was able to report that most annual ryegrasses were highly fluorescent while most perennial ryegrasses were highly non-fluorescent.

The gene controlling fluorescence in ryegrasses was reported by Nyquist (33) in 1963 to be a simply-inherited dominant gene which caused this expression in either the homozygous dominant or heterozygous form, and was quite easily transferred from one variety to another by crossing. Nyquist proved this quite conclusively when he transferred the fluorescent gene from annual to perennial ryegrass through a series of backcrosses which resulted in a completely perennial-like plant having the fluorescent capacity.

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[1] Personnel correspondence to O. L. Justice from T. M. Ching, dated March 26, 1963.

MATERIALS AND METHODS

Three separate investigations were conducted during the period of 1964 to 1967. The investigations were designed to measure outcrossing in ryegrass under both spaced-plant conditions and high-density seed production conditions. Seed collections were made as described below for each experiment, hand threshed, cleaned, and subjected to germination and fluorescence tests. The procedure consisted of counting and positioning the seeds on the germination media by means of a vacuum counter head. Four hundred seeds from each sample were germinated in transparent plastic petri dishes containing Whatman chromatographic paper moistened with 0.2% potassium nitrate solution, and stratified for seven days at a temperature of 5°C to break any remaining dormancy. After stratification, the containers were placed in a slanting position in a germinator. The germinator was maintained at a temperature of ~25°C and a light intensity of about 100 foot candles during a $9\frac{1}{2}$-hour day (cool white fluorescent). The $14\frac{1}{2}$-hour night-time conditions consisted of high humidity, absence of light, and a temperature of ~15°C. After three days, the seed was transferred to another germinator having similar conditions, except that the light intensity was reduced to approximately 30 foot candles. The initial light intensity of 100 foot candles was an additional factor employed

in breaking dormancy. The 30 foot candle intensity provided enough light for substantial seedling growth, and seemed to have less destructive effect on the fluorescent material exuded by the seedling roots.

Seven days after removal from stratification, a preliminary count was made for both germination and fluorescence. Normal seedlings were determined according to rules of the Association of Offical Seed Analysts (1). All normal, fluorescent seedlings were removed at this time and recorded. Fluorescence determinations were made in a darkroom by use of a near-ultraviolet-emitting lamp (300-400 mμ, Sylvania, Blacklite, E15T8-BLB). The seedlings were then replaced in the germinators for seven additional days at which time final counts were made. Data concerning each test were recorded according to the percentage of normal, germinated seedlings which exhibited fluorescence. This procedure was repeated for each of the 1,200 samples collected in these investigations.

Five different ryegrass varieties were selected for these investigations, on the basis of their fluorescence levels. Highly fluorescent varieties of Gulf and Oregon annual ryegrass, with fluorescence levels of 95% to 100%, were selected as sources of contaminating pollen. The perennial ryegrass varieties of Linn, Norlea, and Sceempter, with fluorescence levels of 1-8%, 0-2%, and 0-4% respectively (35), were selected for measuring the amount of crossing

from the annual ryegrass varieties.

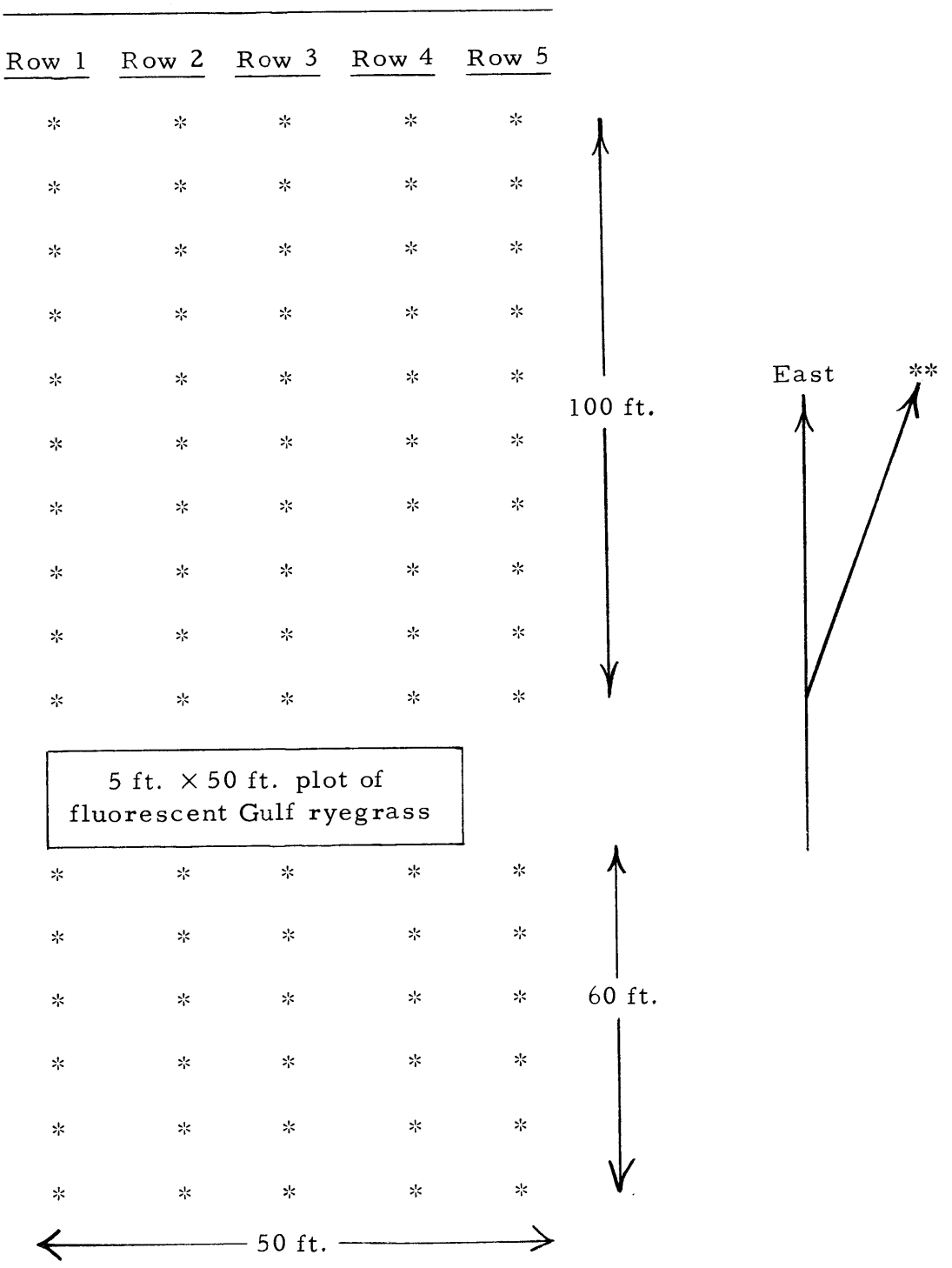
Experiment I

Two parallel rows of perennial ryegrass plants, 18 inches apart, were established in the spring of 1964 on the Central Oregon Experiment Station, in an area free of other ryegrass contamination. These rows were spaced 30 inches apart and included a group of fluorescent plants bordered on the east and west sides by nonfluorescent plants. During the summer of 1964 and 1965, seed was harvested from each individual plant. All seed collections were then threshed, cleaned, and subjected to germination and fluorescence tests.

Experiment II

A five foot by 50 foot plot of highly fluorescent Gulf annual ryegrass was established in 1965 on the Central Oregon Experiment Station, well isolated from other ryegrass contamination. Adjoining this, both into and away from the prevailing wind direction, five rows of non-fluorescent Linn perennial ryegrass plants were established as shown in Figure 1. Seedlings which had previously exhibited a negative reaction to the fluorescence tests tests were transplanted to the field. The rows were spaced ten feet apart and the plants within a row were spaced at ten feet intervals. The rows

Figure 1. Rows of spaced (10 ft.) nonfluorescent Linn perennial ryegrass plants extending in two directions from a pollen source of Gulf annual ryegrass.



** Indicates prevailing wind direction

extended 100 feet windward and 60 feet leeward. No seed was produced in 1965 by the Linn perennial plants; consequently, the plants were retained through the winter of 1965-66 to promote vernalization in order that seed would be produced the following year. Although the annual plants produced seed during the summer of 1965, they were not harvested, but were permitted to reseed the plot in order to provide annual plants the following summer. In addition, two rows of Gulf were seeded at three two-week intervals throughout the 1966 spring planting period. This was done to insure plants of different maturity and provide greater opportunity for concurrent pollination of Gulf and Linn during the following summer.

When the fluorescent annual plants began to show anthesis during the spring of 1966, all tillers from the non-fluorescent perennial plants which previously produced flowers were removed. This was done to make certain that all flowers subsequently produced by the non-fluorescent perennial plants were exposed to, and had the opportunity to be pollinated by, an abundance of pollen produced by the fluorescent annual plants.

One-fourth of each plant was harvested at four one-week intervals, allowing later maturing seed the opportunity to have resulted from concurrent anthesis periods of Gulf and Linn.

Material taken at each harvest was hand-threshed, cleaned separately, and subjected to germination and fluorescence tests

according to plant number and harvest date. This involved a total of 640 samples for the four harvests.

Experiment III

This study was conducted by utilizing the unique ryegrass varietal situation existing in the Willamette Valley grass seed growing area where many situations exist with low-fluorescent perennial fields situated adjacent to highly fluorescent annual ryegrass fields. These situations involved several different ryegrass varieties with flowering dates which coincide to varying degrees.

Ryegrass is harvested by first windrowing the seed crop into swaths approximately ten feet apart in a circular arrangement within the fields. These are later picked up by a combine and threshed. In this study, during harvest, hand samples were taken at random from a 30-foot section of any given swath, beginning at the edge of the field which bordered the annual pollen source. All samples were hand-threshed and the inert matter was separated from the pure seed. The pure seed was then subjected to germination and fluorescence tests as described for the previous study.

Summer 1965: A large field of highly fluorescent (95-100%) annual ryegrass was selected which was bordered on the east (downwind), south, and west sides by low-fluorescing (1-8%) Linn perennial ryegrass. Samples from the perennial field were taken from

swaths number 1, 2, 3, 4, 5, 10, 15, 20, 30, and 66 on the east side; 1, 3, 5, 7, and 14 on the south side; and 1, 2, 3, and 5 on the north side.

A field of low-fluorescing (0-4%) Sceempter perennial ryegrass which was joined on the east side by a field of highly fluorescent Oregon annual ryegrass, was sampled from swaths number 1, 3, 5, 10, 20, 30, 40, and 50. Samples were also taken from swaths number 1, 2, 3, 4, 5, 10, 15, 20, and 25 in a field of low-fluorescing (0-2%) Norlea perennial ryegrass which was joined on the south side to a field of annual ryegrass.

Summer 1966 and 1967: In 1966, eight situations were selected, utilizing Oregon Certification records, where highly fluorescent, Oregon annual ryegrass was growing adjacent to low-fluorescing Linn perennial ryegrass. Thirteen situations were also selected where Linn grew adjacent to highly fluorescent (95-100%) Gulf annual ryegrass. The following summer (1967), ten additional situations of adjacent seed fields of Gulf and Linn were selected. Each of these situations was observed for directional relationship between the low-fluorescing and high-fluorescing fields, the distance between them, and the type of barrier, if any, which separated them. This sampling information from each of the fields is given in Table 1.

Table 1. Sampling procedure for detection of crossing between highly fluorescent annual ryegrass varieties (Oregon annual and Gulf annual) and low-fluorescing Linn perennial ryegrass.

Linn-Oregon (1966)		Linn-Gulf (1966)		Linn-Gulf (1967)	
Field numbers	Legend	Field numbers	Legend	Field numbers	Legend
1	S-F	1	S-F	1	N-F
2	S-F	2	S-F	2	S-F
3	E-F	3	S-F	3	S-F
4	E-F	4	S-F	4	E-F
5	E-F	5	S-F	5	E-F
6	E-R	6	E-F	6	E-F
7	E-R	7	E-F	7	W-F
8	E-R	8	W-F	8	W-F
9	E-R	9	E-R	9	S-R
		10	E-R	10	N-R
		11	E-R		
		12	S-R		
		13	W-R		

N, S, E, and W indicates direction of perennial from annual ryegrass fields.

F indicates the perennial and annual fields were separated by a fence row approximately ten feet wide.

R indicates the perennial and annual fields were separated by a roadway approximately 60 feet wide.

EXPERIMENTAL RESULTS

Fluorescence data obtained in these investigations are presented graphically in Figures 2 through 6. Figures 2 and 3 show fluorescence trends obtained in spaced-plant studies, and Figures 4 through 6 show fluorescence trends observed under seed production conditions. In few cases did fluorescence values beyond the first swath of seed production fields exceed those characteristic of the varieties investigated. In all cases the fluorescence values observed in seed production conditions were substantially less than those observed under spaced-plant conditions at comparable distances. Detailed production conditions are included in Appendix Tables 1 through 11.

Experiment I

The pattern of fluorescence values (Figure 2) on seed collected from individual plants along two parallel rows was very similar for both 1964 and 1965. Generally, the fluorescence values were very low; however, there was a group of plants near the west end of the rows in which the fluorescent seed content was very high. On each side of the highly fluorescent group of plants, fluorescence dropped off very rapidly.

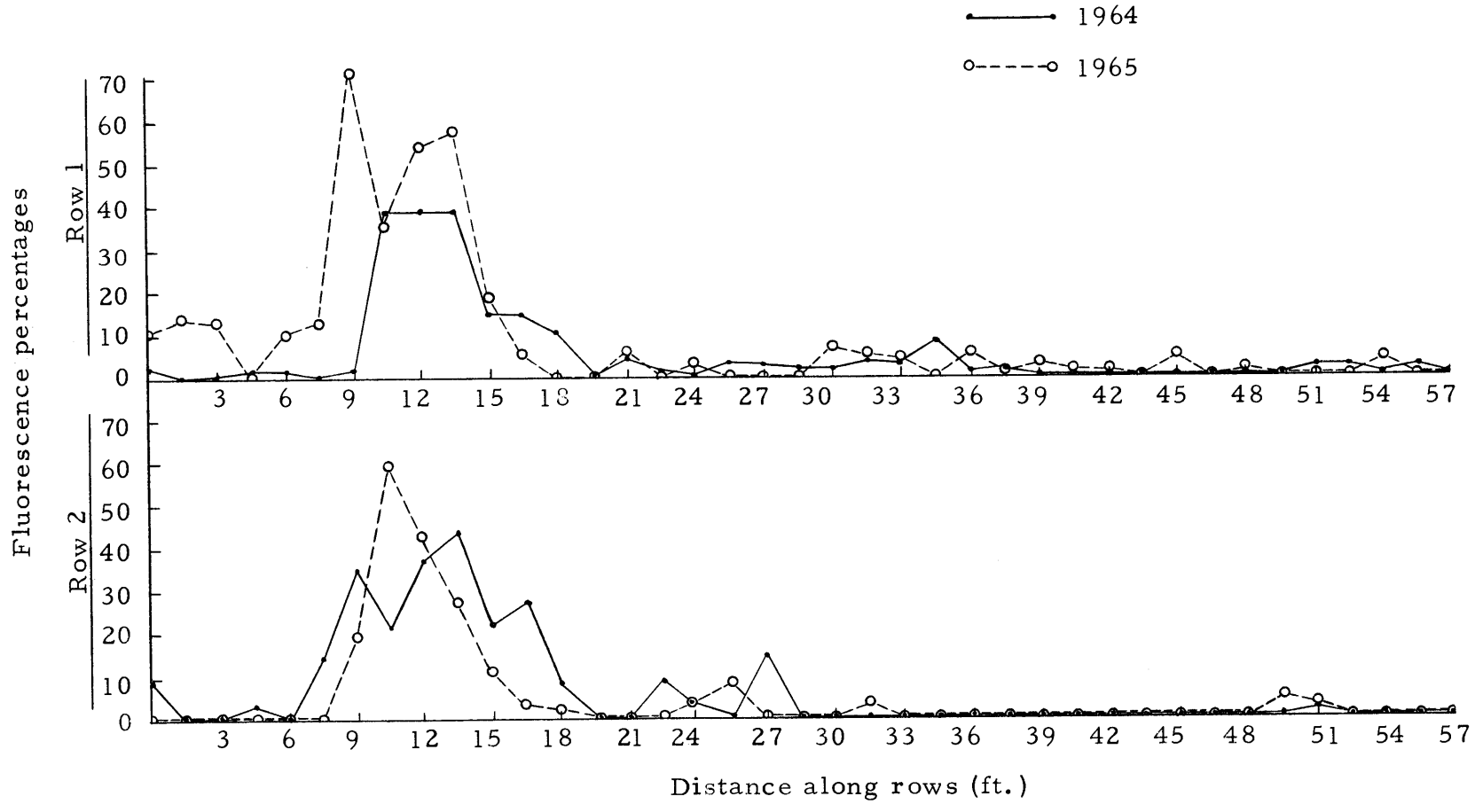


Figure 2. Graph showing the pattern of fluorescence values of seed from individual plants at various distances along two parallel rows of ryegrass (1964 and 1965 data).

Experiment II

The average fluorescence content of the individual Linn ryegrass plants in this study was 36% at a distance of ten feet (Figure 3). With increasing distance from the Gulf pollen source, the fluorescence content decreased, until at 100 feet, only 4% fluorescence occurred. Decrease in fluorescence followed the same trend both upwind and downwind. Appendix Table 2 shows the average amount of fluorescence for all four dates of harvest. Only small differences in fluorescence occurred among the four dates observed; results for individual harvest dates are given in Appendix Tables 3, 4, 5, and 6.

Experiment III

Summer, 1965: Samples taken from successive swaths in Linn ryegrass fields bordered by Oregon annual ryegrass showed only small fluorescence values and no decrease in fluorescence with greater distances from the annual pollen source (Figure 4). This was true for all directions sampled.

Fluorescence tests of samples collected from Sceempter and Norlea ryegrass varieties showed little evidence of a decreasing trend beyond ten feet (Figure 5). Although the Sceempter field was downwind from the contaminating pollen source, the seed collected from the first swath showed a mean fluorescence of 8% (Appendix

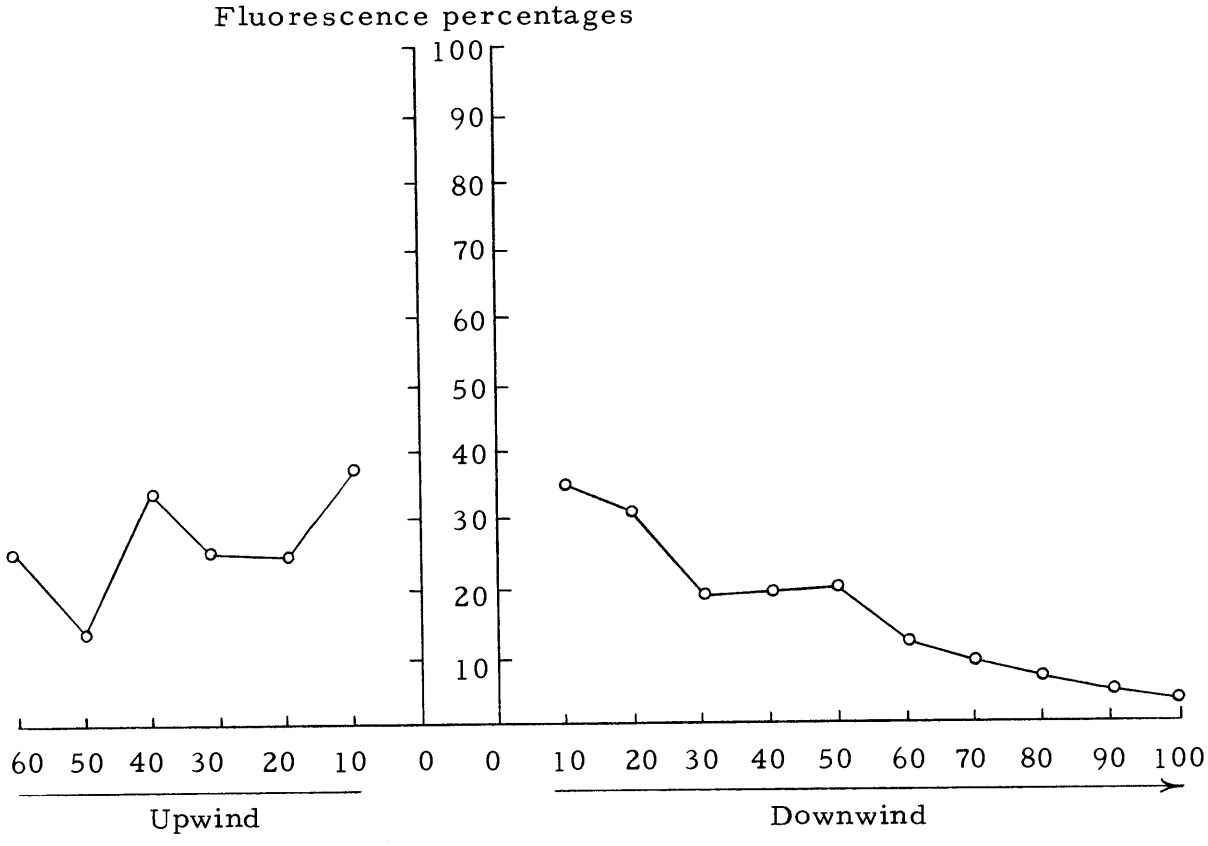


Figure 3. Fluorescence percentages of seed collected from nonfluorescent Linn ryegrass plants at successive distances from highly fluorescent Gulf ryegrass pollen source (mean of five plants).

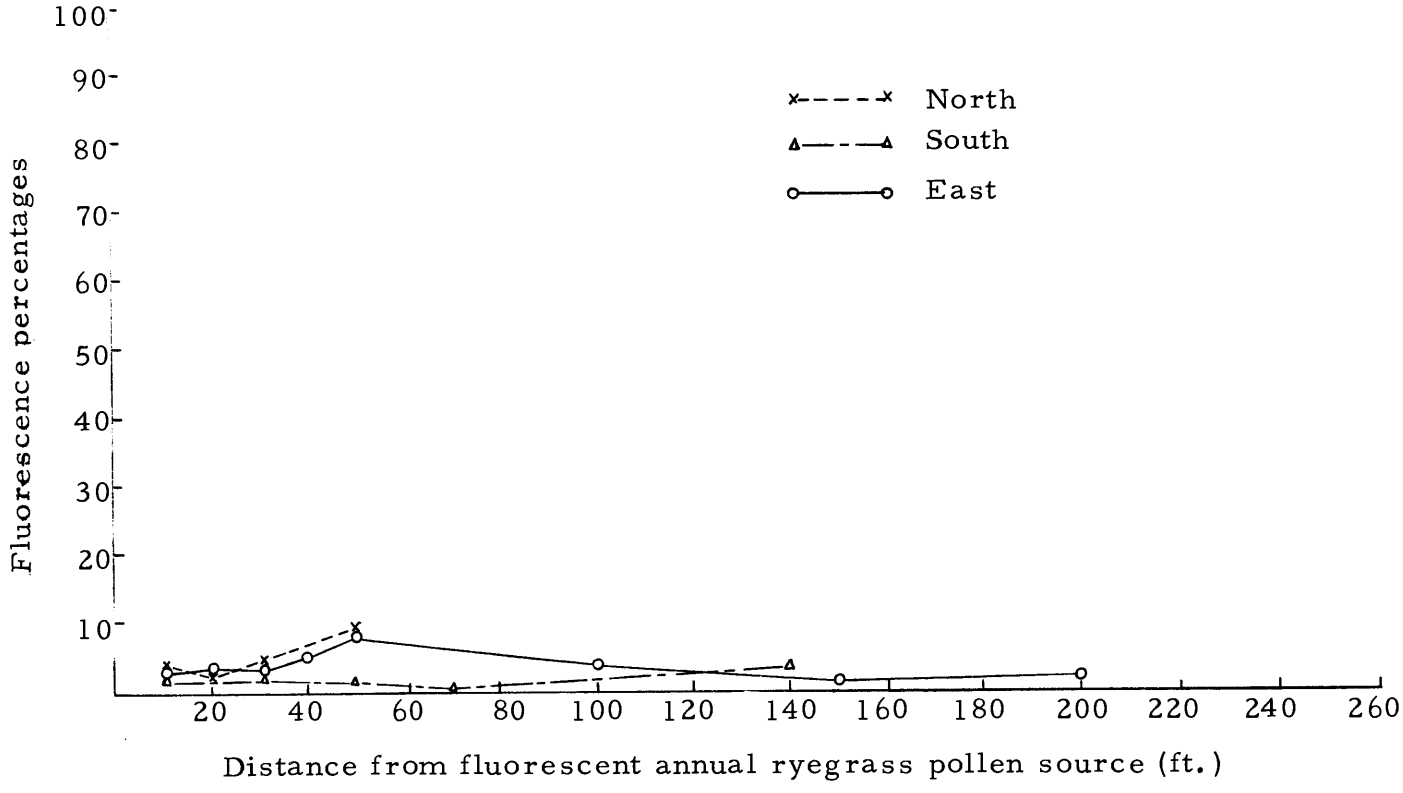


Figure 4. Graph showing pattern of mean fluorescence values of seed collected at successive distances into Linn ryegrass fields which bordered a large field of Oregon annual ryegrass on the north, south, and east sides.

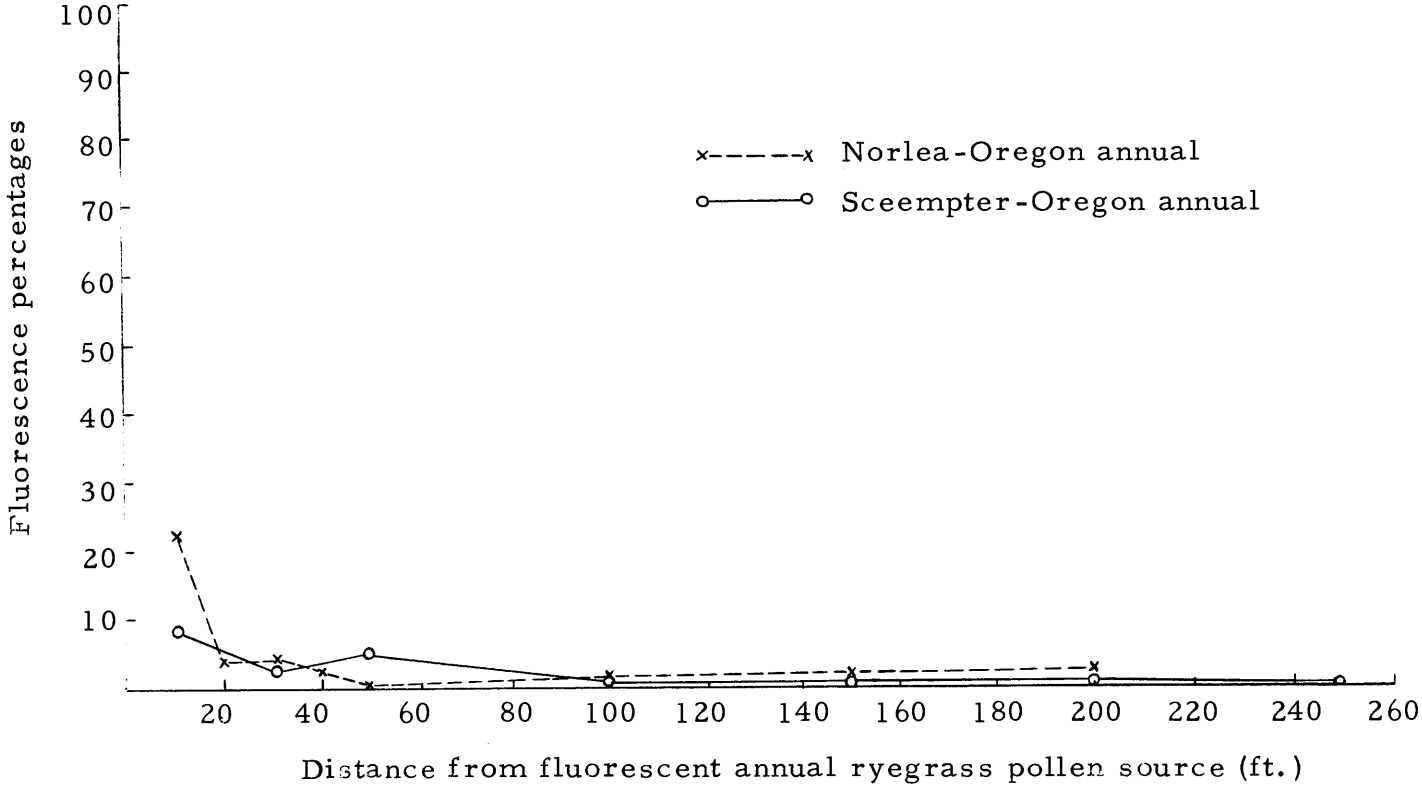


Figure 5. Graph showing pattern of mean fluorescence values of seed collected at successive distances into Norlea and Sceempter ryegrass fields which bordered fields of Oregon annual ryegrass.

Table 8). No subsequent swath produced seed with a fluorescence greater than 5%. Seed from the first swath of the Norlea field showed a fluorescence of 23%. The next three swaths showed fluorescence values from 2% to 3% and subsequent swaths produced only minute fluorescence values.

Summer, 1966: Samples taken from successive swaths in Linn ryegrass fields, bordered by fields of Oregon annual ryegrass, revealed no decreasing trend in fluorescence (Figure 6). Field 7 showed a fluorescence of 14% in the first swath (Appendix Table 9) but no decreasing trend occurred subsequently. The first two swaths of Field 2 showed fluorescence values of 9% but no subsequent decreasing trend. The mean of all eight fields showed no consistent decrease in fluorescence.

The 1966 mean fluorescence results (Figure 6) of samples from adjacent Linn-Gulf situations shows a slight decreasing trend in fluorescence. However, beyond the first swath in nine of the 13 fields sampled, no subsequent decrease occurred (Appendix Table 10). In fields number 4, 5, and 11, there was some decrease in fluorescence across successive swaths from the field border. Field number 7 showed considerable variability among fluorescence values obtained.

Summer, 1967: Fluorescence values (Figure 6) of seed collected from Linn versus Gulf situations in 1967, showed lower initial fluorescence and less subsequent decrease than was observed

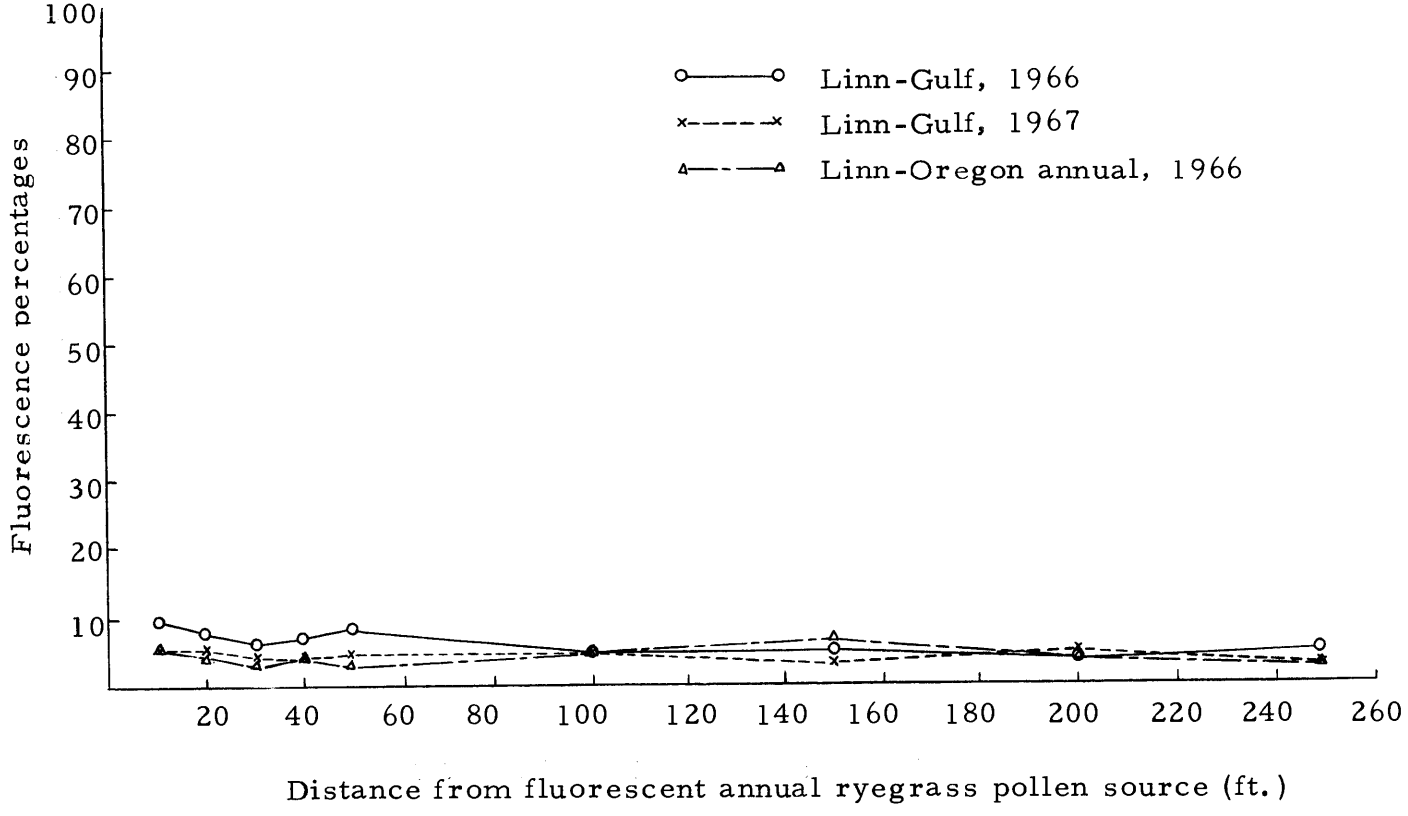


Figure 6. Graph showing pattern of mean fluorescence values of seed collected at successive distances into Linn ryegrass fields which bordered fields of Gulf and Oregon annual ryegrass.

in 1966. Seven of the ten fields exhibited no decreasing fluorescence trend beyond the second swath (Appendix Table 11). One of these seven fields (number 6) exhibited uniformly higher fluorescence values among swaths than the other six. Fields number 1, 2, and 4 did show some decrease in fluorescence with greater distance from the Gulf pollen source.

DISCUSSION

Results in Experiment I first suggested the possibility that the fluorescence test might provide an ideal marker for the detection of outcrossing between fluorescent and non-fluorescent strains of ryegrass. This led to the establishment of Experiment II which provided conclusive evidence of the suitability of the fluorescence test as such a marker and showed that the amount of outcrossing, even in spaced plant conditions, was considerably less than that reflected by pollen dispersal studies and certification isolation requirements. Information gained in controlled Experiments I and II provided an excellent basis for the establishment of the field investigations included in Experiment III.

The observed fluorescence pattern in Appendix Table I could not be exaplained on the basis that interplant pollination was occurring freely, regardless of distance. A gradual decline in fluorescence would have been expected if pollen from individual plants traveled considerable distance from the source and was effective in fertilization.

The sharp decline in fluorescence is attributed to a limited amount of fertilization by pollen from plants more than a very short distance away. Apparently, the gene for fluorescence was present in the heterozygous dominant condition. Such plants would produce both pollen and embryo sacs with and without the dominant allele,

which would explain the lack of 100% fluorescent seeds from these plants.

The decreasing trend in fluorescence in Figure 3 (Experiment II) validates the fluorescence technique in detecting genetic crossing between fluorescent and non-fluorescent ryegrass. All parental plants of the Linn variety had been previously determined to be non-fluorescent, so fluorescence of the resulting seed must be attributed to fertilization by pollen carrying the fluorescent gene. It also shows conclusively that crossing between different varieties of annual and perennial ryegrass can occur under natural conditions if given the opportunity.

Experiment II was arranged in such a manner that there was a preponderance of pollen available from the annual contaminating block in comparison to that of the spaced perennial plants; consequently, there was a greater opportunity for the spaced perennial plants to be pollinated from the annual pollen block than if the mass of the tester and contaminating plants had been equal. However, only 36% outcrossing occurred at a distance of ten feet. This steadily decreased with distance until only 4% occurred at 100 feet. This amount of crossing agrees closely with that observed by Griffiths (20) who also worked with space-planted ryegrass.

Outcrossing did not seem to be affected by wind direction, since the mean fluorescence values were as great upwind as

downwind from the annual pollen source. The lack of wind effect corresponds with results reported by Bateman (5).

Fluorescence results presented in Figures 4 through 6 indicate that very little crossing occurs between adjacent fields of annual and perennial ryegrass. This was quite unexpected, based on results observed in the spaced-plant study (Figure 3). Outcrossing was considerably less than that observed by Griffiths (20) who also worked with spaced-plant conditions. The results are especially surprising compared to the great distances that pollen has been observed to be dispersed. This reveals that there is a great discrepancy between the distance that pollen may be carried by the wind and the distance that it is effective in fertilization.

The discrepancy between outcrossing under spaced-plant conditions and that occurring under actual field conditions emphasizes the inadequacy of such spaced-plant data for a basis in establishing isolation distances. Several of the workers cited earlier recognized that as plant density increased, the amount of outcrossing dropped off more sharply as distance from the field border increased; however, none were able to use the actual density of plant populations existing under seed production conditions. Under the high plant populations of the ryegrass seed fields, intra-varietal plants near the border evidently produce such a large pollen mass that the probability of crossing with inter-varietal pollen from adjacent fields is very low.

This probability appears to decrease with increasing intra-varietal plant density.

Reports cited earlier on pollen viability may provide some insight into the reason for the minimal degree of outcrossing under field conditions. Pollen from adjacent fields must travel through an environment which is quite adverse for maintenance of viability. It is usually subjected to considerable wind and very high temperatures; these conditions are most effective in causing pollination but they are quite unfavorable for maintenance of pollen viability according to reports by several investigators (37, 23, 46). Jones and Newell (27) found that grass pollen viability could be completely lost in about three hours under such conditions. When considering the density of intra-varietal pollen plus the decreasing viability of pollen from adjacent fields, the small amount of crossing observed in these studies can be better understood.

The results seem to discount wind direction as a factor of practical importance in influencing outcrossing under seed production conditions. There was no obvious difference in apparent outcrossing due to direction for any of the varietal combinations. More crossing had been expected in an eastern or southern direction because of the prevailing southeast wind in the Willamette Valley. Rampton shows that the period of anthesis usually lasts about three to four weeks. During this period, variable winds occur, including

both high and low turbulency conditions. Consequently, it appears that wind velocity and turbulency do not significantly influence degree of outcrossing. This agrees with reports cited previously discounting the importance of these factors in establishing isolation distances. It also agrees with data presented in the spaced-plant study where outcrossing upwind was as great as that observed downwind.

The degree of overlapping in period of anthesis did appear to influence the amount of outcrossing between varieties of annual and perennial ryegrass. Rampton shows that pollination periods of different ryegrass varieties overlapped to varying degrees. The amount of apparent outcrossing observed in these studies appears to be directly associated to the extent that the pollination periods correspond. Regardless of this association, however, outcrossing was extremely small compared to that previously reported.

The inherent fluorescent values of Linn is 1-8% and 0-2% for Norlea and 0-4% for Sceempter (10, 35). These values must be considered when comparing the amount of crossing between varieties, since only values above these could be considered outcrossing possibilities.

The fluorescence patterns in Norlea, Sceempter, and Linn versus Oregon annual (Figures 4, 5 and 6) provide little evidence of outcrossing beyond the first swath (ten feet). The higher value

of the first swath may be from outcrossing near the field border, or due to border effects of volunteer annual plants.

The data for the Linn versus Gulf suggest that no appreciable amount of crossing occurs beyond ten feet from the field border. This is suggested both by the lack of a consistent decreasing trend in fluorescence throughout successive swaths and by the natural occurrence of about 4% fluorescence of Linn ryegrass. Sampling variation alone could produce fluorescence results in Linn from 1% to 8%; consequently, the mean fluorescence magnitudes found in successive swaths appears insignificant.

There were three fields (Appendix Table 10) which showed seeds with considerable fluorescence for a distance of 40 feet into the field which could have been outcrossing. If isolation distances were established to prevent inter-varietal crossing based on the most crossing observed in these studies, no more than 40 feet would appear to be needed. This distance should be adequate for maintenance of genetic purity of stock seed which is intended for further certified seed production. However, only about ten feet of isolation would generally appear enough in order to maintain adequate varietal integrity of the certified seed class which is not to be replanted for further seed production. Removal of a border strip from the field after pollination would seem to be adequate for preventing excessive outcrossing, as well as physical contamination from volunteer plants.

The small degree of further outcrossing, if any, should have negligible effect on the genetic purity of the entire field; after the seed is harvested and blended, seed from areas of the field near the border (the potential crossing area) would represent only a small portion of the total amount.

This study would suggest, as discussed earlier, that isolation distances presently required between varieties of pedigreed seed fields are unjustifiably stringent and could be decreased without excessive outcrossing. The first experiment of this study indicated this, the second experiment substantiated it, and the third experiment confirmed it.

Some reasons for the limited amount of crossing observed at these studies have been suggested. More work needs to be conducted on pollen viability and pollen tube vigor under field conditions, the border effect of the intra-varietal pollen screen, the effect of plant density, and the effect of varietal incompatability on the degree of outcrossing between adjacent fields.

SUMMARY AND CONCLUSIONS

Studies were conducted to determine the amount and distance of outcrossing between fluorescent and non-fluorescent strains of ryegrass. Both spaced-plant conditions and actual field situations were investigated.

Results from experiment I involving artificially-spaced plants first suggested that the fluorescence test might be used as an effective marker to detect genetic crossing between fluorescent and non-fluorescent strains. Experiment II validated the fluorescence test for detecting crossing, and showed that even with a preponderance of contaminating pollen, the distance at which pollen was effective in fertilizing nearby plants is considerably less than that reflected by present certification isolation standards.

Crossing between adjacent fluorescent and non-fluorescent varieties under seed production conditions in experiment III revealed substantially less crossing than that found under spaced-plant conditions. The observed amount of crossing under field conditions was also less than that reported by other investigators, working with lower plant densities.

There was a slight increase in apparent amount of outcrossing as more complete concordance occurred between periods of anthesis in the varieties tested. Gulf and Linn exhibited a small degree of

outcrossing, Oregon annual and Linn, a somewhat lesser amount, and the Sceempter versus annual and Norles versus annual situations exhibited negligible outcrossing.

Distance between borders of fluorescent and non-fluorescent fields had little or no effect on outcrossing. Beyond ten feet from the border, apparent outcrossing became negligible between all varieties tested. Beyond 40 feet from the border, there was no evidence of crossing.

Wind direction had no apparent effect on the amount of outcrossing observed in these studies.

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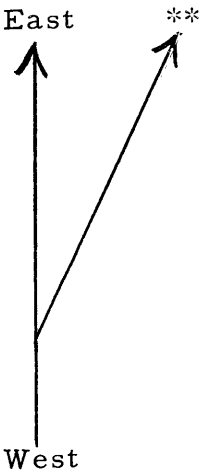
APPENDIX

Appendix Table 1. Fluorescence percentages of seed collected from individual Linn perennial ryegrass plants located in parallel rows 30 inches apart (1964 and 1965 data).

1964		1965	
Row 1	Row 2	Row 1	Row 2
1.5	10.5	8.1	0
0	13.0	0	0
0	12.0	0	0
1.1	0	2.9	0
1.3	10.0	0	0
0	13.3	14.4	0
1.1	71.5	35.1	19.4
39.5	37.9	21.7	59.3
39.7	54.8	37.0	42.6
39.2	57.1	43.9	27.9
14.8	19.0	22.2	11.1
13.7	6.1	27.9	3.2
10.3	0	8.1	2.3
1.4	0	0	0
4.1	5.9	0	0
1.2	0	8.7	0
0	2.5	3.2	3.2
2.4	0	0	8.1
2.3	0	14.1	0
1.4	0	0	0
1.6	7.1	0	0
2.5	5.9	0	2.9
2.6	3.1	0	1.5
7.9	0	0	0
0	6.3	0	0
1.2	1.7	0	0
0	3.9	0	0
0	1.7	0	-
0	1.3	0	0
0	0	0	0
0	4.3	0	0
0	0	0	0
0	1.3	0	0
0	0	0	4.5
1.4	0	1.8	3.8
1.7	0	0	0
0	4.0	0	0
1.6	0	0	0
0	0	0	0
-	0.	0	-
1.4	3.8	0	0

Appendix Table 2. Fluorescence percentages of seed collected from individual spaced plants of Linn perennial ryegrass at ten-foot intervals from a fluorescent Gulf annual pollen source (mean of four harvests).

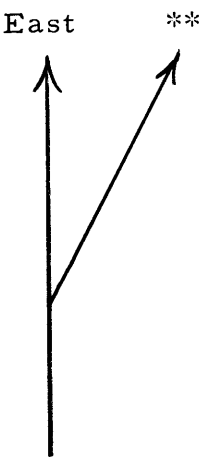
Row 1	Row 2	Row 3	Row 4	Row 5	Mean
6	4	6	-	1	4
13	4	5	3	2	5
-	10	6	5	5	6
9	14	4	3	8	8
28	7	18	8	6	12
14	33	28	21	10	21
-	1	29	30	-	20
-	-	29	17	11	19
-	35	41	26	26	32
58	37	33	34	20	36
5 ft. × 50 ft. plot of fluorescent Gulf ryegrass					Pollen Source
42	40	40	41	31	38
24	14	-	25	31	25
50	-	23	-	32	26
20	9	-	13	7	35
11	32	7	3	-	13
39	-	7	-	-	23



** Indicates prevailing wind direction

Appendix Table 3. Fluorescence percentages of seed collected from individual spaced plants of Linn perennial ryegrass at ten-foot intervals from a fluorescent Gulf annual pollen source (First harvest).

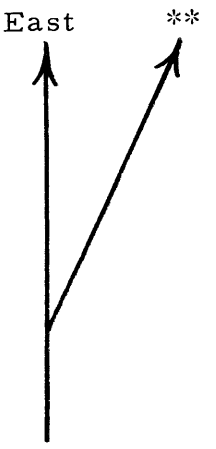
Row 1	Row 2	Row 3	Row 4	Row 5	Mean
-	3	-	-	-	3
7	-	5	25	-	12
-	-	5	50	-	28
-	-	3	67	-	35
-	11	18	17	1	12
-	-	25	12	22	20
-	-	-	-	-	-
-	-	14	-	5	10
-	20	26	10	67	31
20	-	20	25	-	22
Fluorescent Gulf Annual Pollen Source					
25	33	-	32	-	30
-	20	-	19	31	23
53	-	23	-	36	37
-	9	-	-	-	9
-	-	-	18	-	18
-	-	-	-	-	-



** Indicates prevailing wind direction

Appendix Table 4. Fluorescence percentages of seed collected from individual spaced plants of Linn perennial ryegrass at ten-feet intervals from a fluorescent Gulf annual pollen source (Second harvest).

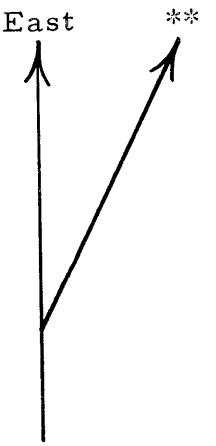
Row 1	Row 2	Row 3	Row 4	Row 5	Mean
5	-	-	-	-	5
14	5	5	4	50	15
-	9	8	9	-	9
-	11	2	-	9	7
58	-	15	42	6	30
-	31	18	18	11	19
-	-	28	2	-	15
-	-	33	8	11	14
-	29	42	24	27	30
61	50	29	30	15	37
Fluorescent Gulf Annual Pollen Source					
8	68	55	49	-	45
23	18	-	32	53	32
49	-	32	-	59	47
-	13	-	20	-	17
-	35	-	4	-	20
57	-	-	-	-	57



** Indicates prevailing wind direction

Appendix Table 5. Fluorescence percentages of seed collected from individual spaced plants of Linn perennial ryegrass at ten-foot intervals from a fluorescent Gulf annual pollen source (Third harvest).

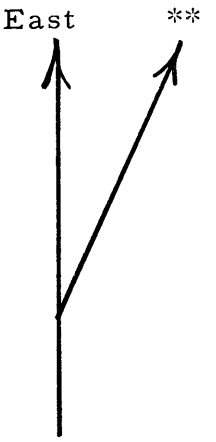
Row 1	Row 2	Row 3	Row 4	Row 5	Mean
7	4	8	-	3	5
12	3	5	4	12	7
-	8	8	9	6	8
23	13	6	10	9	12
23	11	21	8	0	16
10	24	33	25	-	23
-	-	29	3	-	16
-	-	27	20	11	19
-	38	40	26	19	30
57	31	32	41	20	36
Fluorescent Gulf Annual Pollen Source					
56	56	63	40	21	47
37	16	-	33	41	32
54	-	25	-	27	35
20	7	-	16	7	15
-	35	4	4	-	14
-	-	-	-	-	-



** Indicates prevailing wind direction

Appendix Table 6. Fluorescence percentages of seed collected from individual spaced plants of Linn perennial ryegrass at ten-foot intervals from a fluorescent Gulf annual pollen source (Fourth harvest).

Row 1	Row 2	Row 3	Row 4	Row 5	Mean
18	4	4	2	-	7
-	13	3	3	4	6
50	15	3	4	8	16
24	7	18	9	7	13
24	44	25	24	10	25
-	2	33	3	-	13
-	-	32	18	-	25
-	37	48	30	30	36
58	38	41	32	22	38
Fluorescent Gulf Annual Pollen Source					
42	30	21	5	4	20
11	8	-	15	15	12
44	-	19	-	29	31
-	10	-	9	8	9
11	26	11	14	-	16
34	-	7	-	-	21



** Indicates prevailing wind direction

Appendix Table 7. Fluorescence percentages of seed collected from swaths in Linn perennial ryegrass fields at successive distances, south, east, and north from the border of adjacent, highly fluorescent, annual ryegrass field.

South		East		North	
Swath	% Fl.	Swath	% Fl.	Swath	% Fl.
1	1.59	1	2.55	1	3.26
3	1.92	2	2.73	2	2.31
5	1.42	3	2.45	3	3.93
7	0.55	4	4.43	5	7.75
14	3.64	5	7.16	Mean	4.31
Mean	1.82	10	3.77		
		15	1.31		
		20	1.60		
		30	2.43		
		66	2.92		
		Mean	3.14		

Appendix Table 8. Fluorescence percentages of seed collected from swaths in low-fluorescing Sceempter and Norlea perennial ryegrass fields at successive distances from border of adjacent, highly fluorescent, annual ryegrass field.

SCEEMPTER (East from Annual)		NORLEA (South from Annual)	
Swath		Swath	
1	7.92	1	22.86
3	2.59	2	3.36
5	4.60	3	3.43
10	0.00	4	2.13
15	1.22	5	0.27
20	2.17	10	0.55
30	3.51	15	0.50
40	3.03	20	0.55
		25	0.00
Mean	3.13		3.74

Appendix Table 9. Fluorescence percentages of seed collected from swaths in low-fluorescing Linn ryegrass fields at successive distances from the border of an adjacent, highly fluorescent, annual ryegrass field (1966 data).

Swath number	Field Number 1	2	3	4	5	6	7	8	Mean
1	2.74	9.01	3.39	3.19	6.15	1.97	14.40	5.00	5.73
2	9.56	9.22	2.65	4.80	3.45	3.00	5.42	3.65	5.22
3	4.80	4.17	5.83	2.67	3.44	4.42	2.54	3.71	3.95
4	3.20	4.57	3.72	3.41	4.64	5.43	3.66	3.15	3.97
5	2.87	3.81	2.92	2.67	4.01	4.31	1.42	2.86	3.11
10	6.17	6.13	1.43	3.16	7.87	4.66	1.96	2.34	4.22
15	2.89	3.52	7.80	2.71	9.38	5.18	5.23	1.84	4.82
20	3.17	2.43	2.03	2.86	3.53	1.83	5.52	3.50	3.11
25	2.16	2.27	1.62	1.06	5.84	2.22	1.16	2.92	2.41
Mean	4.17	5.01	3.49	2.95	5.37	3.67	4.59	3.22	4.06

Appendix Table 10. Fluorescence percentages of seed collected from swaths in low-fluorescing Linn ryegrass fields at successive distances from the border of adjacent, highly fluorescent, Gulf ryegrass fields (1966 data).

Swath number	Field Numbers 1	2	3	4	5	6	7	8
1	3.24	3.22	15.13	23.56	10.67	7.73	9.55	22.13
2	5.33	2.15	7.78	4.96	12.50	3.75	24.03	5.30
3	3.71	4.57	7.80	9.01	13.97	5.13	7.79	2.67
4	3.76	4.08	5.43	6.87	9.01	4.21	12.30	4.30
5	3.79	1.38	6.03	6.67	7.76	5.47	28.33	7.16
10	2.82	3.85	2.37	3.46	7.18	4.62	9.19	4.26
15	4.16	3.59	14.17	3.83	5.14	3.20	1.96	5.26
20	3.83	3.53	5.10	2.07	3.66	1.59	3.08	4.66
25	2.69	4.21	11.73	2.82	2.55	2.59	3.02	5.62
Mean	3.70	3.40	8.39	7.03	8.05	4.25	11.03	6.82

(Continued)

Appendix Table 10. (Continued)

Swath number	Field Numbers 9	10	11	12	13	Mean
1	3.59	2.28	19.14	6.20	2.41	9.91
2	2.37	3.25	18.65	5.07	1.52	7.44
3	1.57	5.60	8.54	4.09	2.27	5.90
4	5.84	1.11	8.26	4.07	11.29	6.19
5	4.07	2.21	17.55	2.19	3.37	7.38
10	3.96	3.11	4.24	4.82	0.79	4.21
15	1.35	2.27	4.36	3.26	4.52	4.39
20	2.14	6.23	3.79	4.51	1.63	3.52
25	1.05	1.17	5.83	3.16	4.58	4.36
Mean	2.88	3.03	10.04	4.15	3.60	5.92

Appendix Table 11. Fluorescence percentages of seed collected from swaths in low-fluorescing Linn ryegrass fields at successive distances from the border of adjacent, highly fluorescent, Gulf ryegrass fields (1967 data).

Swath number	Field Numbers 1	2	3	4	5	6	7	8	9	10	Mean
1	4.54	8.40	6.53	8.60	5.00	10.66	1.59	2.41	5.25	2.56	5.54
2	8.16	10.48	3.66	4.76	3.68	14.14	1.38	1.38	5.78	3.64	5.70
3	6.58	7.45	2.32	7.94	3.34	9.40	2.20	2.35	0.52	3.64	4.57
4	3.57	7.85	1.29	3.71	4.66	9.02	2.16	1.34	2.11	2.34	3.81
5	5.76	8.29	3.98	3.91	5.40	6.22	0.54	2.16	2.34	2.31	4.09
10	5.91	7.22	2.85	5.32	3.44	9.46	0.78	1.37	3.89	1.29	4.15
15	6.70	5.64	2.86	5.21	3.61	3.11	3.88	0.51	1.04	1.83	3.44
20	2.56	5.23	1.58	1.59	3.32	20.53	1.29	2.07	1.81	5.85	4.58
25	1.07	4.45	3.31	2.81	1.56	8.05	1.58	1.03	1.72	2.81	2.84
Mean	4.98	7.22	3.15	4.86	3.78	10.07	1.71	1.62	2.71	2.92	4.30