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The Effect of Some Environmental Influences in Bulk Hybridization of Grass

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A thesis submitted in partial fulfillment of the requirements for the degree of

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John W. Clark

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INTRODUCTION

For decades man has been interested in selection and hybridization of crop plants, but it has not been until in comparatively recent years that anything of importance has been done toward the improvement of perennial forage grasses.

Replies to questionnaries on grass breeding submitted to various agricultural institutions in 1936 revealed the fact that selection for improvement was then under way with a large number of grasses. Limited activities in this field have been in progress for 16 years or more, but organized and intensive grass breeding activities, for the most part, have been inaugurated only within the last ten years.

Many improved strains of grasses have been developed by selective breeding in foreign countries but very few of these have shown outstanding value in the United States. In fact, they are generally inferior.

There is a great deal of interest at the present time in this country in the field of grass breeding, but the smallness of the floral parts of some grasses makes the work of controlled hybridization tedious and slow. A simple, inexpensive, accurate method for bulk hybridization would be useful and would speed up the work. The study herein reported was designed to throw further light on the possibility of bulk hybridization. The

technic involves immersion of inflorescences in water heated to specific temperatures to effect enactivation of pollen. As a necessary corollary, the desired pollen is then applied by any of several methods.

Several investigators, mentioned below, have shown that controlled heat treatments applied to inflorescenses near the time of anthesis are effective in seriously reducing the viability of pollen without having a similar effect on the female gametophyte. However, none has demonstrated good female fertility following complete male sterilization. It has become apparent that influences of the plants' environment other than the heat of the emasculation treatment must be taken into consideration. In the present study, which was conducted on three important forage grasses, smooth bromegrass (Bromus inermis), crested wheatgrass (Agropyron cristatum), and bluestem wheatgrass (Agropyron smithii), the general findings of earlier investigators are substantiated and in addition evidence is presented to show (1) the sterilizing effect of the isolation bag and (2) the existence of a daily cycle in the efficiency of emasculation of treatments at critical temperatures.

REVIEW OF LITERATURE

The literature on bulk emasculation and bulk pollination has been adequately reviewed by Domingo (2) and will be mentioned only briefly here. Stephens and Quinby (4) reported the effectiveness of hot water in the emasculat on of sorghum. Jodon (3) reported that both hot and cold water were effective in the emasculation of rice. Suneson (5) demonstrated that wheat was partially emasculated by prolonged low air temperatures. Bulk pollen transfer has long been useful in corn breeding, Webber (6), Coulter (1). It has also been effective in obtaining hybrids among cereals, as reported by several investigators. Perhaps the most critical and extensive study reported in the literature, covering both bulk emasculation and pollination, is that of Domingo (2). He attempted bulk emasculation of smooth bromegrass (Bromus inermis) by the use of hot water, hot air, and cold air. The plants used were selected on the basis of their relatively high self-fertility. The success of emasculation was measured by the difference in seed set of treated panicles which were selfed and those which were exposed to atmospheric pollen. Hot water treatments were made for 5 minutes at one-degree intervals from 37-51 degrees centigrade by immersing the panicles in water contained in a one-gallon thermos jug. Several treatments prevented the formation of selfed seed yet permitted the formation of seeds on similarly treated panicles which were exposed to

atmospheric pollen, but the results were not altogether consistent. The most effective temperatures appeared to be 46° and 47° C. Hot air was found to be effective in emasculating smooth bromegrass but the results were less consistent than with hot water. Several methods of bulk pollination tried by Domingo all yielded some hybrid seed. However, in a preliminary study conducted on the bulk pollination of emasculated panicles, little seed was produced.

EXPERIMENTAL METHODS

Bulk emasculation and bulk pollination was attempted on three species of grasses by treating the inflorescences with hot water at specific temperatures 1 to 5 days prior to anthesis. During anthesis, pollen was transferred from male to female plants by several different methods listed below. To insure against damage to the plants when inserting the inflorescences into the thermos jug, a large handkerchief was wrapped firmly around the group of stems to be treated, starting at the base and extending upward. Plants were selected for treatment which had previously produced considerable selfed seed when isolated in kraft bags. The success of the technic was evaluated by a difference in the amount of seed produced following hot water treatments and specific pollination practices relative to seed produced by controls. Several inflorescences were treated simultaneously and bagged immediately or left exposed, according to the methods which follow:

1. Selfed.
2. Bagged, but having strained pollen introduced into the bag on five days favorable for atmospheric pollen.
3. Bagged, but having severed pollen bearing inflorescences introduced into the bag on five successive pollen shedding days.
4. Bagged, with severed pollen bearing inflorescences standing in water and inserted in the bag at the beginning of anthesis.

5. Bagged, but in association with intact inflorescences of another genotype (untreated) which provided pollen.

6. Bagged, but exposed 1 hour on each of five successive days favorable for natural pollination.

7. Bagged without treatment, then being subjected to various numbers of daily exposures at hourly intervals on days favorable for atmospheric pollen.

8. Exposed to natural pollination following emasculation treatment.

9. Three untreated and unbagged inflorescences from each of the several genotypes were harvested and seed counts obtained. This gave a check on the efficiency of the various methods of controlled pollination.

Of the three grasses experimented with, methods 4 and 7 were not applied to smooth bromegrass; methods 5, 6 and 7 were not applied to crested wheatgrass, and methods 2 and 5 were not applied to bluestem wheatgrass.

Treatments which effected emasculation without appreciable injury to the female organs were detected by lack of seed set on the selfed inflorescences, accompanied by formation of seed on those which received the same treatment simultaneously but remained exposed to natural pollination. The pollen transfer methods were used to produce controlled hybrids on the inflorescences that had been emasculated. The also served as a check on the viability of the stigmas. Inflorescences not treated and unbagged gave a measure of the effectiveness of atmospheric pollen, and apparent injury to the stigma by treatment.

Equipment for emasculation. - Emasculation equipment consisted of a wide-mouth thermos jug, an accurate thermometer, and a wire loop for stirring the water in the thermos jug. A one-burner gasoline stove was very useful for heating water in the field, and it, together with a water bag filled with cold water, provided a means of making temperature changes rapidly.

Equipment for isolation. - Isolation of spikes or panicles was made possible by the use of 3" x 26" kraft paper bags. The bags were supported by a number 9 galvanized wire that was forced into the ground at the base of the plant. A 1 3/4" loop in the upper end of the wire prevented the bag from collapsing and thus injuring the infloresence.

Equipment for pollination. - A large light reflector was used to gather pollen for the isolation bags that were to receive strained pollen. The pollen was separated from the anthers by a fine mesh screen. A small metal container was used to carry the pollen from one genotype to another. This container was wrapped with cloth and the cloth was soaked in water. Evaporation cooled the container and helped prevent the pollen from forming aggregates. A teaspoon was used to transfer the pollen from the metal container into the isolation bags. In one of the several other bulk pollination methods employed, stems were severed near their bases and placed in water contained in one-quart mason jars, in order to extend the period during which their inflorescences

would shed viable pollen. None of the other methods of pollination required equipment.

EXPERIMENTAL RESULTS

Demonstration of differential and comparison of several methods of bulk pollination. - A differential in the thermal death point of male and female gametophytes is demonstrated if it can be proved that, following an emasculation treatment self pollen is nonfunctional while untreated, introduced pollen leads to seed formation. This relationship is most easily demonstrated with relatively self fertile genotypes. Tables 1 and 2 contain data supporting the differential for the respective grasses involved. The most appropriate critical temperature appears to be 47° C. However, good results were also secured at 48° C. on bluestem wheatgrass. It is shown that 3 relatively self-fertile genotypes of B. inermis (mean self-fertility under bags per 100 spikelets 106 seeds (table 1)), were restricted to .1 seeds per 100 spikelets when treated at 47° C. for 5 minutes and then bagged. These same 3 genotypes, on panicles treated simultaneously with those selfed, yielded 10.3 seeds per 100 spikelets when untreated pollen was introduced during anthesis, and 26.8 seeds per 100 spikelets were obtained when the isolation bag was removed for 1 hour during anthesis on 5 days favorable for pollination.

Table 1. Seeds produced by selfing and by controlled hybridization procedures by relatively self-fertile genotypes of Bromus inermis, Agropyron cristatum, and A. smithii following hot water emasculation treatments at specified temperatures. None of the controlled hybridization procedures differed significantly in seed production. 47° C. appears to be the most suitable emasculation temperature. Each cell in the table is based on 2150 to 4160 spikelets (B. inermis); 11 to 20 spikes (A. cristatum); 26 to 43 spikes (A. smithii).

5-minute treatment temperature C.	Bromus inermis* (means of 5 genotypes)		Agropyron cristatum (means of 4 genotypes)				Agropyron smithii (means of 9 genotypes)		
	Selfing	1**	Selfing	2	3	4	Selfing	3	4
48			0	2.3	4.6	0	1.9	17.6	12.0
47	.1	10.3	.6	23.2	6.0	12.8	2.2	11.5	20.0
46	8.2	39.8	4.8	17.2	28.0	16.0	5.5	25.0	23.6
45	17.3	36.5	31.6	41.2	15.6	14.8	14.0	18.8	19.8
44			20.8	18.0	42.4	35.6	26.2	30.9	24.9
43			14.0	33.6	34.8	28.0	24.9	30.0	64.0
Untreated	106.0	99.0	51.6				30.5		
Mean of treated				22.6	21.9	17.9		22.3	27.4

* Seeds per 100 spikilets. A. cristatum and A. smithii based on seeds per 4 spikes.

** Description of hybridization procedure.

1 - Plants were grown in pairs, 2 genotypes bagged together, the one providing pollen was untreated.

2 - Freshly strained pollen from a different genotype was introduced into bag on 5 successive pollen shedding days.

3 - 2 severed pollen bearing spikes suspended in top of isolation bag on each of 5 successive pollen shedding days.

4 - 2 severed pollen bearing spikes standing in water, at beginning of anthesis.

Table 2. Seeds produced by selfing and by intermittant and continuous exposure by relatively self-fertile genotypes of B. inermis, A. cristatum and A. smithii following hot water emasculation treatments at specified temperatures. Intermittant exposure consisted of removing the isolation bag for 1 hour during anthesis on each of 5 pollen shedding days. Each cell in the table is based on 2150 to 4160 spikelets, (B. inermus); 11 to 20 spikes (A. cristatum); 26 to 43 spikes (A. smithii).

	Bromus inermis* (means of 3 genotypes)			A. cristatum (means of 4 genotypes)		A. smithii (means of 9 genotypes)		
Treatment temperature C.	Selfing	Exposed 1 hr. on 5 days	Continuously exposed (unbagged)	Selfing	Continuously exposed (unbagged)	Selfing	Exposed 1 hr. on 5 days	Continuously exposed (unbagged)
48				0	33.4	1.9	28.4	79.0
47	1	26.8	86.6	.6	91.6	2.2	28.7	91.5
46	8.2	56.3	145.0	4.8	132.8	5.5	42.0	99.4
45	17.3	88.0	206.0	31.6	136.0	14.0	53.6	119.9
44				20.8	126.8	26.2	59.0	150.6
43				14.0	175.2	24.9	62.4	160.2
Untreated	106.0		288.0	51.6	126.8	30.5		140.9
Mean of treated					116.0		59.0	116.8

* Seeds per 100 spikelets: A. cristatum and A. smithii based on seeds per 4 spikes.

A similar interpretation is appropriate to A. cristatum and A. smithii. In no case do the several methods of controlled hybridization differ significantly in effectiveness, but exposure to random natural pollination gives increases in seed over controlled hybridization as shown by table 2; whereas treated inflorescenses, which are not bagged at all, invariably yielded several times more seed than was obtained from bagged inflorescences (table 2). Two important questions are here raised: (1) Are any methods of pollination of bagged inflorescences adequate (including 1-hour exposures on 5 successive pollen shedding days)? and if so, (2) are the reduced seed yields under bags an indication that the bag itself has an effect which is additive to that of the emasculation temperature treatment? The limited information at hand does not allow a satisfactory answer.

Successive daily exposures. - In order to determine if possible, the adequacy for cross fertilization of 5 daily exposures, as reported in table 2, an experiment was set up with bluestem wheatgrass in which different groups of untreated but bagged spikes were exposed on 1 to 4 successive pollen shedding days. The plants were growing in proximity to many other plants which assured a good pollen supply. Nine genotypes were employed and each contributed one bag of 4 spikes to each exposure treatment. The data are summarized in table 3. Examination of the table reveals that four daily exposures yielded only half as

Table 3. Effect on seed production of different numbers of successive daily exposures of one hour during anthesis in relation to seed production by selfing and from continuously exposed (not bagged) spikes of bluestem wheatgrass (not emasculated).

Number of successive daily exposures during anthesis					
None (selfed)	One	Two	Three	Four	Continuously exposed
30.5	49.7	47.5	40.6	73.4	140.9

much seed as continuous exposure (not bagged). One to three exposures yielded little more seed than selfing but, although pollen was shed on each day that bags were removed, the atmospheric temperature during the course of this study was considerably below normal and the data have probably been influenced accordingly.

Daily temperature fluctuations inside and outside the isolation bag during anthesis. - In recognition of the possibility that high temperatures developed inside the isolation bags, readings were taken simultaneously inside and outside the bags 4 or 5 times daily.

A small hole was made in the top of each isolation bag just large enough for a thermometer to be inserted. When the temperature was not being taken a small piece of adhesive tape covered the opening. Two accurate thermometers were used, one inside the bag, and the other in the shade of the bag for recording the atmospheric temperature. When temperature readings were being taken the thermometers were left in place long enough to fully adjust to the temperature encountered. The data are presented in figure 1. Each point in this figure is the mean of nine readings, each taken in a different bag.

It is apparent that during the daytime, temperatures within the bags ranged approximately two degrees above those taken simultaneously in the shade of the bags. It is considered highly

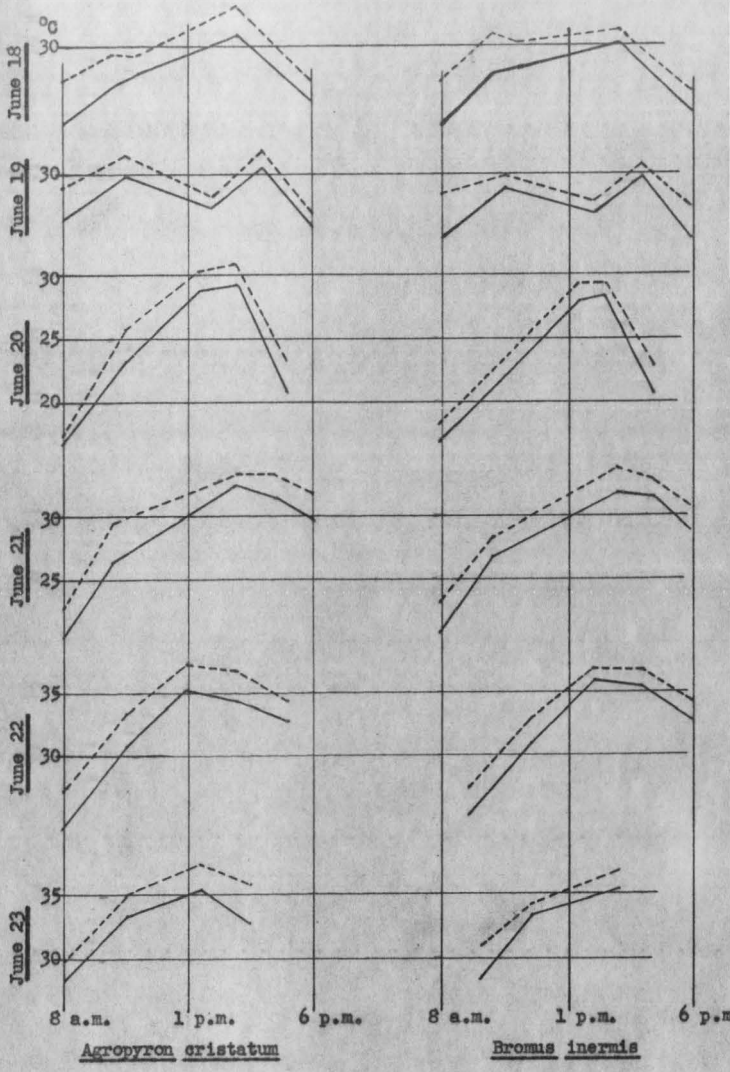

Figure 1. Air temperatures inside (dotted lines) and outside the isolation bags (solid lines) for Agropyron cristatum and Bromus inermis for a period of six days during anthesis.

improbable that a difference in temperature of this magnitude would be responsible for the very significant reduction in seeds produced under bags. It is probable, however, that light, humidity, air composition or other factors which the bag might influence, or a combination of any of these might contribute to the effect noted. As mentioned above, it is also probable that part of this effect relates to inadequate pollination, even when exposures were made for one hour during anthesis on each of 5 successive pollen shedding days.

The effect of time of treatment on seed production. - One genotype of smooth bromegrass was selected to receive treatments at hourly intervals throughout the day from 6:00 A.M. to 6:00 P.M. Treatments were made at 45°, 46° and 47° C. At each temperature 16 panicles were treated simultaneously and then separated into 6 groups, for pollination by different methods.

These were as follows:

(1) Two panicles bagged in association with two intact panicles of another genotype (untreated) which provided pollen.

(2) Same as (1) but in addition a cotton plug was placed in the top of each bag to serve as a ventilator.

(3) Three panicles bagged and pollinated on five successive pollen shedding days by introducing strained pollen into the top of the isolation bag.

(4) Three panicles bagged and pollinated by suspending two panicles in early anthesis in the top of the isolation bag on each of 5 pollen shedding days.

(5) Three panicles were bagged but the bags were removed for one hour during anthesis on each of 5 successive pollen shedding days.

(6) Three panicles left unbagged for natural open pollination.

Results from the first 5 groups, in which bags were involved, are presented in table 4. The data represent means for the three treatment temperatures. It is evident that seed production is very low. The data suggest injury to the stigmas since 4 of the 5 methods averaged out approximately equal in efficiency. Examination of the means of different treatment intervals reveals a very pronounced daily cycle, many more seeds resulted from the treatments made during the warmer part of the day, than during the cooler part.

The data for the 6th method of pollination listed above are presented in table 5. Approximately half as many seeds were produced by open pollination of panicles treated at 47° as by those treated at 46° C. Likewise only half as many seeds were produced following treatment at 46° as at 45° C. Since the only differential was the treatment temperature, it appears to be a reasonable conclusion that at the higher temperatures an increasing amount of injury was suffered by the stigmas. The means for time-of-treatment classes point clearly to the daily cycle which characterized the data presented in table 4 for bagged plants. It is evident that this cycle is not an effect of the bag. It is also evident that the cycle is most clearly

Table 4. Effect of time of treatment on seed production by five methods of cross pollination in a self-sterile genotype of smooth bromegrass. Figures are seeds per 100 spikelets. Means of treatment at 45°, 46° and 47° C. (43,930 spikelets went to make up table, each value averaged 1351).

Treatment	Time of treatment							Mean for each cross pollination method
	A.M.				P.M.			
	6-7	8-9	10-11	12-1	2-3	4-5	6*	
A X B Bagged together	.2	0	5.3	6.7	3.0	0	0	1.88
A X B (cotton plug) Bagged together	.2	1.0	2.2	2.4	3.1	3.3	0	1.76
A Selfed plus pollen 5 days	.0	2.3	4.4	2.5	3.6	2.6	0	2.21
A Selfed plus severed stems 5 days	.1	.1	1.1	.9	.4	0	0	.38
A Selfed plus 1 hour exposure 5 days	.2	1.0	8.7	.3	1.3	1.9	.1	1.93
All methods two-hour means	.14	.88	3.94	2.56	2.28	1.56	.02	

* Actual values obtained from panicles treated at 6:00 P.M. have been doubled in order to make this column more comparable to the others.

Table 5. Seeds produced per 100 spikelets of a self-sterile genotype of smooth bromegrass. The plants were emasculated at hourly intervals throughout the day but remained unbagged. Treatments were either 45° 46° or 47° C. for an interval of 5 minutes. (7,545 spikelets went to make up table, each value averaged 255-571).

	Hour of treatment							
	A.M.				P.M.			
Treatment	6-7	8-9	10-11	12-1	2-3	4-5	6*	
47°C	3.6	12.2	0.6	108.0	136.2	25.7	44.5	47.3
46°C	39.8	10.5	102.2	116.5	105.6	87.7	96.5	79.8
45°C	139.0	104.2	184.0	170.0	212.5	179.5	114.0	157.5
Mean	57.3	40.2	95.5	151.2	151.0	93.6	84.8	

* Actual values obtained from panicles treated at 6:00 P.M. have been doubled in order to make this column more comparable to the others.

expressed at the higher treatment temperatures. Table 6 presents the data of table 4 rearranged according to treatment temperatures. The striking feature of these data is the relationship of treatment temperature to the range in time of day through which treatment led to relatively good seed sets. Relatively high seed yields were obtained from treatments at 47°C. only when these treatments were made in mid-day (12 noon to 1:00 P.M.). At 46°C. relatively high seed yields were obtained when treatments were made between 10:00 A.M. and 3:00 P.M. while at 45°C. this time range was extended from 8:00 A.M. to 5:00 P.M. Although the data do not so indicate, it is suspected that emasculation at 45°C. may not have been highly effective at mid-day. In a broad sense these conclusions drawn from table 6 are born out by the data presented in table 5.

Table 6. Seeds produced per 100 spikelets in a self-sterile genotype of smooth bromegrass by cross pollination following emasculation at hourly intervals throughout the day. Each value in the table is a mean of 5 methods of cross pollination. 43,950 spikelets went to make up table, each value averaged 2252.

	Time of treatment							Means for each emasculation temperature
	A.M.				P.M.			
	6-7	8-9	10-11	12-1	2-3	4-5	6*	
47°C. treatment-5 min.	.1	.6	.5	4.0	.6	.0	.0	.84
46°C. treatment-5 min.	.2	.2	3.6	1.9	4.1	.5	.1	1.47
45°C. treatment-5 min.	.1	1.8	7.5	1.8	2.2	4.2	.0	2.52
Two-hour means	.15	.87	3.93	2.57	2.30	1.57	.03	

* Actual values obtained from panicles treated at 6:00 P.M. have been doubled in order to make this column more comparable to the others.

DISCUSSION

Domingo (2) obtained evidence that smooth bromegrass (B. inermis) could be almost completely emasculated by treatment of panicles with hot water. The success of emasculation was determined by the number of seeds produced under bags by relatively self-fertile genotypes following treatment, in contrast to seeds produced by similarly treated panicles which were not bagged but remained exposed to open pollination. On this basis there appeared good evidence for a differential in the thermal death points of the male and female gametophytes of 2° or 5° C. He also obtained fairly satisfactory quantities of seed in his studies of bulk pollination made on untreated (self-sterile) panicles. However, the results were disappointing when attempts were made to apply bulk pollination technic to heat emasculated panicles. It was clearly evident that treatment had been too severe. (All treatments preceeding bulk pollination were at 47° C.).

In the present study all controlled pollination methods were applied to treated inflorescences. The data presented in table 1 reveal significant increases in seed production, and clearly demonstrate that a differential exists, but seed production is far below that of similarly treated but unbagged inflorescences (table 2). This may be interpreted in either of 2 ways, (1) all methods of controlled pollination were inefficient, or (2) the bag has a sterilizing effect. Critical evidence is

not at hand but the second interpretation is favored by the writer, because of the very strong evidence presented in table 2 when continuous exposure is compared with 5 daily exposures of one hour duration during anthesis. It appears very improbable that the 5 daily exposures failed to provide ample opportunity for pollination. This view accepted, the disparity must be charged largely against the isolation bag. However, this type of isolation bag has been used at this station for 5 years in inbreeding studies. Many highly self-fertile plants have been detected by its use. The bag alone, therefore, certainly does not sterilize the plants. Conclusive evidence is presented that temperatures within the bags are approximately 2° C. higher than those outside the bags. In view of the much greater daily variations this difference is not considered highly important. All the data thus far available can be harmonized if it is postulated that an unfavorable interaction results from the bagging of treated inflorescences. This interaction appears to operate at temperatures lower than those essential to emasculation as evidenced by the low seed yields obtained by the various pollination methods (table 1) following emasculation at 43° C. Clear-cut evidence is presented in tables 4, 5 and 6 to demonstrate the operation of a daily cycle in the reaction of the plant toward the emasculation treatment. It is clear that a given temperature has a more severe effect on the plant in the early morning and late afternoon than at mid-day but no explanation for the observed condition is at hand.

Although new difficulties have been brought to light the present study adds weight to the belief that bulk hybridization can be realized because data are presented supporting the basic requirement of the method, namely the existence of a differential in the thermal death points of male and female gametophytes.

SUMMARY

Evidence is presented supporting the findings of other investigators that there is a differential in the thermal death points of male and female gametophytes. The differential is best demonstrated by hot water treatment at 47° C. for five minutes just prior to normal anthesis.

Hot water emasculation at temperatures of 45°, 46°, and 47° C. at 1 hour intervals from 6:00 A.M. to 6:00 P.M. revealed that treatments at mid-day were less injurious to the plant than treatments in the early morning or late afternoon. A treatment of 45° C. at 6:00 A.M. appeared to be as effective an emasculation as a treatment of 47° C. at noon.

Five methods of controlled pollination were investigated. These methods are:

(1) Selfing.

(2) Bagged, but having strained pollen introduced into the bag on five days favorable for atmospheric pollen.

(3) Bagged, but having severed pollen bearing inflorescences introduced into the bag on five successive pollen shedding days.

(4) Bagged, with severed pollen bearing inflorescences standing in water and inserted in the bag at the beginning of anthesis.

(5) Bagged, but in association with intact panicles of another genotype (untreated) which provided pollen.

Natural pollination consisted of:

(1) Treated panicles exposed to wind borne pollen at hourly intervals on each of five days during anthesis.

(2) Panicles left unbagged and allowed free access to atmospheric pollen.

None of the methods of controlled pollinations gave satisfactory seed yields. The low seed yields obtained were the result of some factor or combination of factors other than emasculation treatment, since similarly treated inflorescences exposed to continuous pollination (no bagged) gave very satisfactory seed yields. Similarly treated panicles exposed for one hour on 5 successive pollen shedding days produced no more seed than controlled pollination, methods 2, 3, 4 and 5.

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