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ABSTRACT

In the improvement of forage crop varieties knowledge of the extent of cross-fertilization is of value for *(1)* determining the extent and randomness of crossing in breeding procedures, *(2)* estimating varietal changes due to contamination and mechanical mixtures, and *(3)* evaluating effects of cross-fertilization in the development and production of synthetic and hybrid combinations. Genetic markers can aid greatly in the evaluation of cross-fertilization, and thus in the development and production of new varieties. Ideally, markers should be distinctive and readily detected in the seedling stage, conditioned by a single dominant gene with complete penetrance, and have no adverse effects on plant growth, zygote viability, or randomness of cross-pollination.

Additional index words: Genetic markers, Hybrid forages, Varietal contamination, Forage breeding.

EVALUATION of the extent of cross-fertilization can be a simple task, or a very difficult one, depending upon the genetic markers available. The value of a marker is related to the complexities of its inheritance. In some cases, quantitative characters such as yield, height, maturity, and regrowth have been used as markers, and elaborate replicated tests were needed to estimate the extent of cross-fertilization. Such tests are expensive, laborious, and very time-consuming. The best markers for measuring cross-fertilization are those that are distinctive, readily detected, and simply inherited. However, a review of the literature suggests that few markers with these characteristics have been used in the improvement of forage varieties.

Many of the special problems encountered in breeding and producing seed of cross-fertilizing grasses and legumes are due to the presence of a high degree of inherent heterogeneity. Variations in breeding behavior, caused by fluctuations in the extent and randomness of cross-pollination, can markedly influence the effectiveness of many of the commonly used breeding procedures. Varietal contamination can result from outcrossing or mechanical mixing of seeds. In addition, cross-fertilization, although indispensable in the development and production of synthetic and hybrid combinations, is often highly variable. The use of good genetic markers that permit rapid evaluation of cross-fertilization can simplify the evaluation and solution of each of these problems.

Cross-Pollination in Forage Breeding Procedures

In forage crop breeding variations in randomness of pollination and in self-fertility complicate the evaluation of breeding behavior of clones and lines. Tests for estimating general combining ability, such as the polycross, top-cross, or open-pollination progeny, are based on the assumption that each line or clone is subjected to equal and random cross-pollination. For example, in a polycross test differences in the performance of progeny of selected clones are considered to result from variations in the ability of maternal clones to transmit high performance to their progeny. However, if the sample of pollen that produced the polycross seed was not the same for each of the clones, the differences in performance between the polycross progenies could be due in part to nonrandom pollination.

Several studies have shown that the assumption of equal and random cross-pollination is not entirely valid. In perennial ryegrass (*Lolium perenne* L.), Wit (18) used a dominant marker that produced roughness of the culms and upper leaf sheaths to demonstrate a localization of pollination, such that clonal rows were fertilized 40% by the two adjacent rows and 74% by the four adjacent rows. Clones represented by single clonal pieces in 10 replications of a polycross resulted in uniform progenies in some instances but not in others. For bromegrass (*Bromus inermis* Leyss.) Hittle (12) evaluated several agronomic characters in a polycross progeny test and concluded that pollination was nonuniform. In a more recent study of bromegrass Knowles (14) also demonstrated nonrandom pollination by use of a dominant, yellow-leaved marker. In a polycross test involving green and yellow-leaved clones Knowles found that some green clones had two to three times more crossing with the yellow marker plants than other green clones.

Similarly, in insect-pollinated legumes, several studies have shown that pollination is not completely random. In alfalfa Boren et al. (2) demonstrated a decided clonal preference by individual honey bees that could result in considerable self-pollination. The bees differentiated among very closely related clones, even those having identical parents. Odor was assumed to be the major factor in this preference. Hanson et al. (9) observed individual bee preference for alfalfa clones differing in flower color, but there was no direct evidence that flower color in itself was a factor in attraction. Reciprocal differences for flower color, in the progeny of these clones, indicated that as high as 85 to 90% selfing had occurred. Wilt reaction of reciprocals indicated that the selfing problem was not restricted to those clones that differed in flower color but that it was general for the experiment.

Pedersen (15) found that leafcutter bees preferred the flowers of colored-flower alfalfa to those of a recessive white-flowered synthetic when collecting pollen. However, both types of flowers were equally attractive to nectar-collecting honey bees. Pedersen also reported a highly significant bee-by-variety interaction for cross-pollination. The extent of crossing be-

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tween 'Lahontan' or 'Ranger' alfalfa and a white-flowered line was similar when pollination was effected by leafcutter or honey bees. But, when the variety 'Uinta' was the male, the crossing was low when pollination was done by leafcutter bees and high when done by honey bees.

Kehr (13) found that natural crossing in alfalfa by honey bees, as measured in white- and yellow-flowered populations, varied from 32 to 96%. Variables that influenced crossing percentage were marker populations, planting methods, and environments.

The above review of reports of nonrandom cross-pollination, although incomplete, is sufficient to demonstrate that serious departures from random pollination have been observed in breeding procedures presently used with both grasses and legumes. Thus, one of the basic assumptions underlying these breeding techniques is partially invalidated. A larger supply of more usable markers in a wider variety of forages is needed in order that the assumptions underlying forage breeding procedures can be evaluated adequately.

Genetic Change and Variety Contamination

In cross-fertilizing forage crops the genetic composition of strains or varieties can be altered by intravarietal or extravarietal causes. Supplies of reliable seed are dependent on systems of seed production that will maintain the genetic integrity of these varieties, thereby insuring variety performance equivalent to that expected from previous test results. The main cause for intravarietal modifications is a genotype × environment interaction that induces variations in the contribution of certain clones or segregating genotypes to the total germplasm at different locations. These genetic shifts occur particularly when varieties are grown outside their region of adaptation, or subjected to unusual systems of management. At present, the extent of these modifications can be measured only by quantitative evaluation of several important agronomic characters. Such complex evaluations, requiring considerable precision, are costly and time-consuming.

Variety modification brought about by extravarietal factors is commonly called contamination. Little information is available on this type of modification, apparently because the inherent heterogeneity in forages and the scarcity of genetic markers make it difficult to identify. Rules for the production of certified seed originally were established on an empirical basis. This procedure has worked very well, but critical investigations are needed of some of the variables affecting these rules in each forage crop. Variables such as isolation distance, borders, field size, field shape, and method of pollination should be studied.

Stevenson (17) conducted a verification test of different seed lots of 'Climax' timothy (*Phleum pratense* L.) and reported an increase in contamination with successive generations of seed production. Data used to estimate the amount of contamination were obtained from spaced-plant, replicated tests that were evaluated for yield, height, and frequency of undesirable plants and early-flowering plants. Contamination studies that depend upon such quantitative characters are expensive, laborious, and are often difficult to interpret. However, simply inherited and easily identifiable marker genes have been used for contamination studies in Ladino clover, lupines, and sweetclover, and are available in several other forages.

In Ladino clover Portz and Jackobs (16) used the presence of the cyanogenetic glucoside and its hydrolyzing enzyme, linamarase, both simply inherited genetic markers, to study variations in 235 certified seed lots. Wide differences were found in the frequency of cyanophoric plants.

The work of Forbes and Wells (3) in breeding improved varieties of blue lupine is a classic example of what can be done when a conscious effort is made to incorporate markers into new varieties. This effort was made because the low-alkaloid variety 'Borre,' released in 1955, soon became mechanically contaminated with seeds of high-alkaloid strains used for cover crops. Small percentages of high-alkaloid plants (5 to 10%) caused cattle to reject lupine pastures. The seeds of the two types were indistinguishable and both types had blue flowers. Contamination by high-alkaloid plants, detected by qualitative tests with Dragendorf's reagent, was found to increase rapidly in successive years because high-alkaloid plants produced more seeds per plant than low-alkaloid plants.

To insure that new low-alkaloid varieties could be easily separated from contaminants Forbes and Wells incorporated several distinctive and simply inherited markers. One of these was the *leucospermus* gene that conditions a white seed coat, white flowers, an absence of purple pigment in the cotyledons and a light green foliage. Other characters incorporated into new low-alkaloid varieties were soft seeds, anthracnose resistance, and gray-leaf-spot resistance. Lupines are thought to be 100% self-pollinated; thus, the contamination problem in the variety Borre appeared to be due to the mechanical mixture of seeds. However, the importance of suitable genetic markers for detecting this type of contamination is fully as great as their importance for detecting contamination due to outcrossing.

Biochemical markers have been used to detect contamination in varieties of sweetclover that are low in content of *o*-hydroxycinnamic acid. Two simply inherited, independent gene pairs were used. The *Cu/cu* alleles influence the content of glucosidically bound *cis*- and *trans*-*o*-hydroxycinnamic acid, while the *B/b* alleles determine the presence or absence of β-glucosidase activity. Under suitable conditions this enzyme hydrolyzes the *cis*-glucoside, yielding free coumarin. Plants of the *CuCu* genotype are high in content of *o*-hydroxycinnamic acid glucosides, and preparations of the *BB* plants possess β-glucosidase activity. The *Cu* and *B* genes are both lacking in dominance (6, 10). A simple, rapid method of testing for both *o*-hydroxycinnamic acid and β-glucosidase activity permits differentiation of the four phenotypes conditioned by these two gene pairs (11).

Using only the *Cu/cu* alleles as markers, Goplen and Weber (4) determined contamination levels through four generations of seed increase in the sweetclover variety, 'Cumino,' which has a low content of *o*-hydroxycinnamic acid. Plants high in *o*-hydroxycinnamic acid (contaminants), which made up 0.27% of the breeder's seed, increased to 1.11% in foundation seed, 4.21% in registered seed, and 19.80% in certified seed. Cross-pollination from volunteer plants high in *o*-hydroxycinnamic acid in nearby areas was

suspected as the major reason for the rapid increase in contamination, although contributing factors may have been the lower inherent seedling vigor and seed yield of Cumino compared to the contaminants. Detailed studies revealed that volunteer plants within the seed field, admixtures of seed in cleaning and handling, and differential fertilization had little or no effect on the contamination level. In a similar study of contamination in the sweetclover variety, 'Denta,' which is also low in *o*-hydroxycinnamic acid content, Gorz and Haskins (7) used both the *Cu/cu* and *B/b* gene pairs as markers and obtained results for the first three generations of seed increase that were essentially similar to those of Goplen and Weber.

The importance of controlling contamination and genetic shift in varieties cannot be overemphasized when consideration is given to possible consequences from the use of contaminated seed lots. For example, as has already been mentioned in lupines, a 5 to 10% contamination results in rejection of the forage and loss of variety usefulness. Similarly, in low-coumarin varieties of sweetclover, more than 10% contamination with high-coumarin plants can result in the death of cattle due to sweetclover bleeding disease (5). In such crops contamination must not exceed established tolerance limits and a purity test of all seed lots should be required for seed certification.

Hanson (8) lists four basic elements that are essential if modern varieties are to represent stable and definitive products. For each new variety these include *(1)* name assignment and demonstration of distinctiveness, *(2)* accurate description, *(3)* definition and maintenance of breeder seed, and *(4)* description of seed increase procedures. These procedures, which are designed to hold changes in genetic structure to a minimum, include designation of (a) number of seed generations, (b) area(s) of seed increase, and (c) isolation requirements.

Cross-Pollination in Hybrid Forages

Information on pollination controls will increase in importance as additional varieties of hybrid forages are developed. Several types of pollen control, such as genetic male sterility, cytoplasmic male sterility, and self-incompatibility, are available for the production of these hybrids. However, pollen control is not absolute in any of these systems. Therefore, it will be necessary to determine the actual percentage of cross-fertilization that has occurred.

The Federal Seed Act defines the term "hybrid" and specifies how hybrid seed is to be labeled. For example, in a hybrid variety, the hybrid seed must constitute at least 95% of the pure seed. If less than 95% but more than 75% of the seed is hybrid, the actual percentage of hybrid seed is to be designated on the label, or a statement can be included that says "contains 75% to 95% hybrid seed." Seed lots containing less than 75% hybrid seed cannot be labeled as "hybrid." The above classes of hybrid seed have been defined on the assumption that percentage of cross-fertilization is known or can be approximated. To accomplish this determination with the greatest accuracy when pollen control is not complete, appropriate marker genes should be incorporated into the clones or lines used in the hybrid combination.

CONCLUSIONS

The limited evidence presented above is sufficient to indicate that plant breeders should be aware of the potential benefits that can accrue from the utilization of good genetic markers in the development and production of new forage varieties. It is recognized that incorporation of markers into polyploid species may not be economically feasible because of the genetic complexity involved, but one or more markers should be included in most diploids, and in those polyploids where it is possible to do so. Useful new markers can undoubtedly be found if a concerted effort is made to search for them However, new markers could also be generated, if necessary, by the treatment of suitable germplasm with chemical mutagens, such as ethyl methanesulfonate, or irradiation with X-rays or thermal neutrons. Markers should be distinctive and readily detected in the early seedling stage, be conditioned by a single dominant gene with complete penetrance, but should have no adverse effect on plant growth, zygote viability, or randomness of cross-pollination (1). The widespread use of such markers will result in more rapid progress from breeding, in the maintenance of maximum varietal purity, and in the development of hybrid varieties having known percentages of hybrid seed.

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