Necessity of Replicated Measurements for Selection of Alfalfa Plants Resistant or Susceptible to Stem Inoculation by *Sclerotinia trifoliorum*

MYINT AUNG, Applied Research Division, Gyogone, Insein, Rangoon, MYANMAR (Burma); and DENNIS E. ROWE and ROBERT G. PRATT, USDA-ARS, Forage Research Unit, Mississippi State, MS 39762-5367

ABSTRACT

Aung, M., Rowe, D. E., and Pratt, R. G. 1994. Necessity of replicated measurements for selection of alfalfa plants resistant or susceptible to stem inoculation by *Sclerotinia trifoliorum*. Plant Dis. 78:14-17.

The variation in lengths of necrotic tissue following replicated stem inoculations with *Sclerotinia trifoliorum* was characterized on 25 plants in each of four alfalfa populations. The plants were ranked for resistance based on measurements in 12 replications, and the five most resistant and the five most susceptible (selection pressure of 20%) plants in each population were identified. Subsets consisting of 11, 9, 7, and 5 replicates were systematically or randomly selected by a computer program from the original 12 replicates, and the plants were ranked again and selected for resistance. The probability of reselecting plants previously identified as either one of the five most susceptible or resistant plants in each population was determined for each size subset. With five replicated inoculations, there was an 83% probability of reselecting three to five of the most resistant or most susceptible plants, and a 55% probability of reselecting four or five of the most resistant or susceptible plants. These results indicated that at least five replicated measurements of each plant with the stem inoculation method are needed for selection of resistance to *S. trifoliorum*.

Sclerotinia crown and stem rot, caused by *Sclerotinia trifoliorum* Eriks., is often a serious disease of fall-planted alfalfa (*Medicago sativa* L.) in temperate climates during the winter and spring seasons (4). Alfalfa cultivars with resistance to *S. trifoliorum* have not been developed, in part because no screening technique has been proven effective at identifying resistance.

Evaluations of seedlings using infested grain inoculum (6) have shown significant differences among alfalfa cultivars and germ plasms (9,15), while other studies using the same type of inoculum have shown no significant differences among cultivars (7,11). Field tests with natural infections showed significant differences among cultivars and germ plasms, which in one test were highly correlated between years ($r = 0.87$) (3,7,15); but the field results were not correlated with tests in the greenhouse using infested grain as inoculum (15). The infested grain technique was criticized for either developing so rapidly that both resistant and susceptible plants were killed or allowing too many escapes (5,9). Other seedling inoculation techniques used to test resistance to *S. trifoliorum* utilize inoculum of mycelial fragments, which showed significant differences in disease among plant introductions (10), and inoculations of plant crown with agar plugs containing mycelium, which showed significant differences among cultivars and between plants within cultivars (14).

A stem inoculation method developed by Pratt and Rowe (8) showed significant differences among 19 randomly selected alfalfa clones for lengths of necrotic

Accepted for publication 22 September 1993.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1994.

tissue following replicated inoculations on ramets of each plant. Unlike most other techniques, which measured differences among populations, this procedure measured a response on the individual plant with single or multiple measurements, was nondestructive to the plant, and could be used to estimate incomplete resistance. The variability in responses of different stems inoculated on the same plant required the use of replicated measurements to show statistically significant differences among clones (8).

Multiple evaluations of a plant for any trait should increase the precision of the measurement, but they also increase the time and cost, and reduce the utility of a procedure for practical selection in plant breeding. A critical assessment of the variation found with the stem inoculation method of Pratt and Rowe (8) is needed to determine the number of replications needed for effective screening for resistance.

This research was undertaken to characterize the variation occurring with different numbers of replications on hypothetical selection for resistance and susceptibility to *S. trifoliorum* by a stem inoculation technique in four genetically divergent alfalfa populations.

MATERIALS AND METHODS

Alfalfa populations and fungal isolate. Twenty-five plants were randomly chosen from cultivars Arc, Apollo, and Delta, and from one germ plasm, BIC-6 CLS_5 (13). Hereafter, these cultivars and this germ plasm are referred to as "populations." The plants were grown in a greenhouse in pots (ca. 1,900 cm^3) with potting mix (peat moss and vermiculite, 5:2, v/v) and sand (1:1, v/v). Plants were inoculated with the appropriate strain of *Rhizobium meliloti* Dang., fertilized with balanced liquid fertilizer at 14-day intervals, and pruned twice when at 10% flowering to promote crown development and rapidly growing vegetative stems.

An isolate of *S. trifoliorum* (AF-4) from alfalfa at Starkville, Mississippi, was maintained on Difco cornmeal agar (1.7%) at 17 C and subcultured every fourteenth day. This isolate was characterized in a prior study as highly virulent (8).

Stem inoculations. The stem inoculations were performed using a modification of the procedure described by Pratt and Rowe (8). The major change was the use of excised stems instead of attached stems. For preparing inoculum, pieces of absorbent cotton (ca. 0.05 g) were hand rolled into loose balls, autoclaved, and flooded with sterile V8 juice (20%). These balls were placed at the margins of 5-day-old fungal colonies growing on cornmeal agar in petri dishes. After 3 days, just prior to inoculation, the mycelium-infested cotton balls were spread with tweezers to a rectangular shape, ca. 18 × 12 mm, and placed at the center of a piece of masking tape (27 × 38 mm).

One day before inoculation, vegetative stems of plants were excised, cut to 25-cm lengths, placed in sterile water in an Erlenmeyer flask, and secured with cotton at the neck of the flask so that stem bases were submerged in 5 cm of water. For inoculation, a piece of tape with mycelium-infested cotton was sealed around each stem tip. A transparent plastic bag (40 × 20 cm) was placed over the stems and taped to the flask to maintain a humid atmosphere. The flasks with stems were incubated in a growth chamber with ambient air temperature of 18 C and 12 hr of light. Four days after inoculation, the plastic bags were removed. Water was added to the flasks to keep the level constant; and 14 days after inoculation, the length of stem from the base to the edge of necrotic tissue was measured (8). The length of necrotic tissue was determined by subtraction (8).

Experimental design. The experimental design was a 5 × 5 balanced lattice design (design plan 10.3 from Cochran and Cox [2]) with six replications. One replication was a single stem from each of the 25 plants in a population where five stems were assigned to a flask (the blocks). The experiment was repeated twice to get 12 measurements of the length of necrotic tissue for each plant. Appropriate statistical models were fitted for analysis of variance using programs of Statistical Analysis Systems (12).

Replication effects. The plants in each population were ranked by mean length of necrotic tissue with measurements on 12 stems. Assuming 20% selection pressure in each population, five plants were identified as most susceptible (longest necrotic regions), and five were identified as most resistant (shortest necrotic regions) and hereafter are referred to as the "select" plants.

Replication was reduced by using subsets of the 12 original measurements. The subsets were generated from the original data set by deleting one, three, five, or seven of the replications. For each subset, the mean length of necrotic tissue for each plant was calculated, the plants were ranked, and the five most susceptible and most resistant plants were identified. A counting was then made of which select plants were reselected using fewer than 12 replicated measurements on the tissue necrosis. The frequency of reselection was calculated as the number of select plants reselected in all subsets divided by the total number of plants selected.

From the original 12 replications, many different subsets of a given size may occur. There are 12 different subsets using 11 replications (one replication eliminated), 220 different subsets using nine replications, and 792 subsets using seven or five replications. For subsets of 11 or nine replications, every possible subset was generated; and the means and rankings were calculated. For subsets with seven or five replications, the means and rankings were calculated for a random sample of 250 of the 792 possible subsets. The identification of which replications were included in each subset, the calculation of mean length of necrosis for each plant, the ranking of plants by length of necrotic region, and the calculation of frequencies for select plants re-

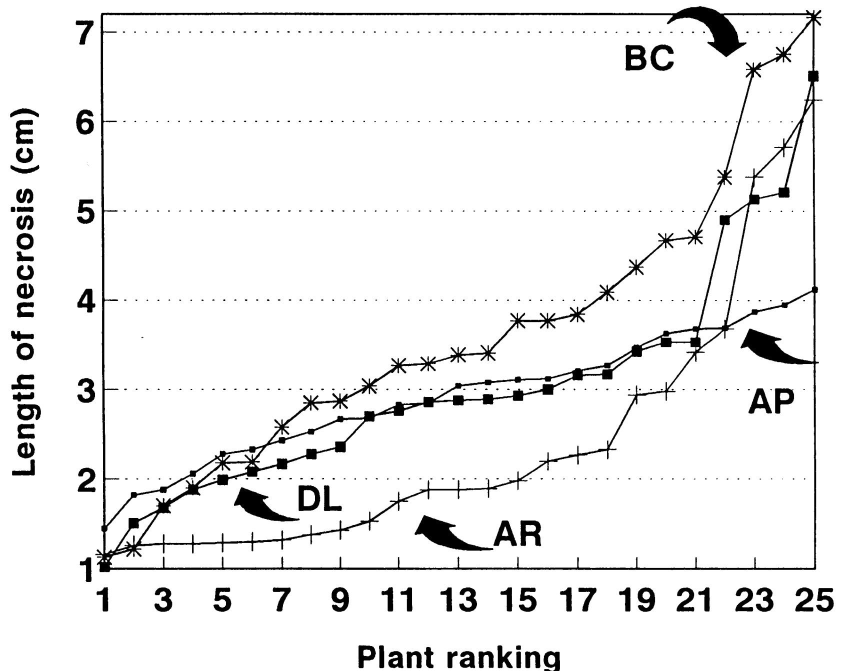

Fig. 1. Distributions for length of necrotic tissue after inoculations with *Sclerotinia trifoliorum* on 25 plants each of BIC-6 CLS_5 (BC), Arc (AR), Apollo (AP), and Delta (DL) alfalfa populations.

selected in each subset were made using programs written in the GAUSS computer language (1).

RESULTS AND DISCUSSION

In each population, an analysis of variance using 12 replications showed that the differences among the plants were significant ($P = 0.01$). The interblock error was insignificant, which indicated that within flasks there was no influence of cuttings from one plant on the response of cuttings from other plants.

The distributions of mean length of necrosis for the 25 plants were not the same in each population (Fig. 1). Plants from Apollo had the least variation, and the necrosis exceeded 4 cm in length in only one plant. In Arc, 60% of the plants had necrotic tissue less than 2 cm long.

With reduced replication, the means and rankings may be altered; but for plant breeding, the only important changes in rankings are those that change which plants are selected for resistance or for susceptibility. A simple correlation between plant means based on 12 measurements and means based on a subset of 11 or fewer replications is not informative because many changes in the means and ranks do not affect selection. Thus, it was necessary to systematically count the number of times select plants, either resistant or susceptible, were reselected in each subset.

As the number of replications was reduced from 12 to 11, 9, 7, and 5, the frequency of reselecting the select plants for resistance or susceptibility was usually reduced (Table 1). The fact that the frequency was not always reduced was attributed to variation caused by random sampling of 31% of the possible subsets (250 of 795) rather than measuring of all subsets. The frequency of reselecting the resistant select plants was reduced, on average, from 87% with 11 replications to 68% with five replications (Table 1). Similarly, for reselection of susceptible select plants, the frequency was reduced from 89 to 70% (Table 1). The actual decrease in frequency of reselection depended on the plant, population, and direction of selection. For instance, the most resistant plant of Delta, DL190, was selected in 100% of the screenings, even with five replications per subset, and the least resistant selected plant of Delta, DL187, was selected only 50% of the time with 11 replications.

On average, the probability of reselecting select plants had a regression of $Y = 91.3 - 3.17s$ ($r^2 = 0.42$), where Y is the probability of success (selection of select plant) and s is the number of replicates deleted. Extrapolating this regression to the case of a single replicate, the predicted probability of success approached 0.5; i.e., for an unreplicated measurement, about half of the five selected plants would be select plants.

The probabilities averaged over all populations of selecting exactly zero, one, two, three, four, or all five of the select plants, either resistant or susceptible, are shown in Figure 2. These probabilities were calculated directly for 11, 9, 7, and 5 replications and predicted for the single replicate (with $Y = 0.5$). This figure shows that with selection based on five replications, there is a 16% probability of having all five of the select plants, a 36% probability of selecting four of the five select plants, and a 31% probability of selecting three of the five select plants. Thus, the probability of selecting three or more of the select plants based on means of five replicated measurements is 83% (16 + 36 + 31), and the probability of selecting either four or five of the select plants is 52%.

A persistent and complex problem in plant breeding, which is not addressed without actually knowing the genotype of each plant, is the effect on population improvement of substituting one plant for another in selection. Results indicated the effect of substituting one plant for another in the selected population was dependent on the alfalfa population and the direction of selection. For instance, selection for resistance in the Arc population might be little affected by replacement of select plants by any of 11 other plants which did not have over 2 cm of necrosis, assuming a close relationship between length of stem necrosis and genes conferring resistance. In the other populations, such changes in ranking might greatly increase the probability of selecting much less desirable genotypes.

The ranking of plants in each population often changed as replications were deleted or added, but identification of extreme genotypes for resistance or susceptibility appeared much more stable than expected by the authors who have been using this stem inoculation procedure for several years. Fortunately, changes in ranking were greatest in Arc (for resistance) and Apollo (for susceptibility), where many differences among plants were minimal for each respective selection criteria, and where concurrently

Table 1. Percentage of times an alfalfa plant ranked as one of the five most resistant or susceptible to *Sclerotinia trifoliorum* with 12 replicated measurements is similarly ranked using fewer replicated measurements

Population	Five most resistant plants	Replications				Five most susceptible plants	Replications			
		11	9	7	5		11	9	7	5
Apollo	AP287	100	100	100	98	AP216	100	90	84	68
	AP145	100	100	88	83	AP365	92	88	70	60
	AP121	100	100	88	70	AP69	83	74	68	59
	AP96	100	80	70	56	AP80	67	64	60	62
	AP259	90	86	77	70	AP41	83	56	45	60
Average		98	93.2	84.6	75.4		85.0	74.4	65.4	61.8
Arc	AR407	100	92	74	65	AR429	100	100	99	97
	AR290	92	70	56	52	AR329	100	100	98	96
	AR280	58	66	56	50	AR86	100	100	99	88
	AR187	67	46	50	46	AR154	92	84	74	68
	AR282	50	50	48	53	AR414	92	90	65	60
Average		73.4	64.8	56.8	53.2		96.8	94.8	87.0	81.8
BIC-6 CLS_5	BC140	100	100	100	100	BC270	100	100	95	87
	BC215	100	100	100	92	BC29	100	100	100	97
	BC274	100	100	95	82	BC256	100	84	95	78
	BC44	92	90	73	64	BC369	100	84	62	56
	BC344	50	38	47	34	BC313	25	30	31	30
Average		88.4	85.6	83	74.4		85.0	79.6	76.6	69.6
Delta	DL190	100	100	100	100	DL60	100	100	98	88
	DL301	100	100	100	92	DL174	100	98	92	72
	DL382	100	100	85	72	DL78	100	94	89	77
	DL159	92	62	51	54	DL203	100	94	82	74
	DL187	50	42	36	41	DL50	50	30	18	27
Average		88.4	80.8	74.0	69.6		89.2	83.0	76.2	70.2
Overall average		87.0	81.0	74.6	68.1		89.0	83.0	76.3	70.9

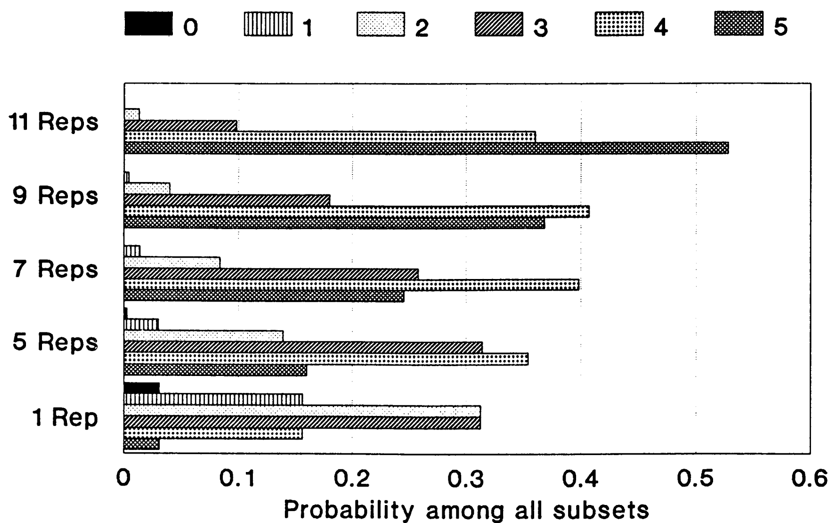

Fig. 2. The probability of reselecting exactly 0, 1, 2, 3, 4, and 5 of the alfalfa plants identified as most resistant or susceptible to *Sclerotinia trifoliorum* in a population of 25 plants. Five plants were selected based on rankings of mean length of necrotic region with 11, 9, 7, or 5 replicated measurements or predictions for a single measurement.

one may expect the many changes in ranking to have the little effect on frequency of desirable alleles in a selected population.

Although a single, accurate measurement of resistance is the ideal case, we suggest five measurements on a plant may be needed in selection for resistance to *S. trifoliorum* in the stem inoculation test. When resistant and susceptible check germ plasms are developed, possibly with application of the stem inoculation procedure of Pratt and Rowe (8), more efficient and cost-effective selection procedures may be developed.

LITERATURE CITED

1. Aptech Systems. 1988. The GAUSS System. Version 2.0. Aptech Systems, Kent, WA.
2. Cochran, W. G., and Cox, G. M. 1957. Experimental designs. John Wiley & Sons, New York.
3. Elgin, J. H., Jr., and Beyer, E. H. 1968. Evaluation of selected alfalfa clones for resistance to *Sclerotinia trifoliorum* Eriks. Crop Sci. 8:265-266.
4. Graham, J. H., Frosheiser, F. I., Stuteville, D. L., and Erwin, D. C. 1979. Compendium of Alfalfa Diseases. American Phytopathological Society, St. Paul, MN.
5. Hanson, A. A., Graham, J. H., and Kreitlow, K. W. 1953. The isolation of ladino clover plants resistant to *Sclerotinia trifoliorum*. Agron. J. 47:280-281.
6. Kreitlow, K. W. 1951. Infection studies with dried grain inoculum of *Sclerotinia trifoliorum*. Phytopathology 41:553-558.
7. Pierson, P. E., Anderson, T. H., and Rhodes, L. H. 1988. Screening for resistance to *Sclerotinia trifoliorum* in vivo and in vitro. Page 15 in: Rep. North Am. Alfalfa Improv. Conf. 31st.
8. Pratt, R. G., and Rowe, D. E. 1991. Differential responses of alfalfa genotypes to stem inoculations with *Sclerotinia sclerotiorum* and *S. trifoliorum*. Plant Dis. 75:188-191.
9. Rhodes, L. H. 1988. Screening for resistance to Sclerotinia crown and stem rot. Page 103 in: Rep North Am. Alfalfa Improv. Conf. 31st.
10. Rhodes, L. H., Pierson, P. E., Anderson, T. H., and Ellsworth, D. R. 1990. Resistance of Alfalfa plant introductions to *Sclerotinia trifoliorum*. Page 13 in: Rep. North Am. Alfalfa Improv. Conf. 31st.
11. Rowe, D. E., and Welty, R. E. 1984. Indirect selection for resistance to Sclerotinia crown and stem rot on alfalfa. Can. J. Plant Sci. 64:145-150.
12. SAS Institute. 1985. SAS User's Guide: Statistics. Version 5 ed. SAS Institute, Cary, NC.
13. Thyr, B. D., Leath, K. T., Hill, R. R., Jr., Hunt, O. J., Hartman, B. J., McCoy, T. J., and Knous, T. R. 1984. Registration of common leaf spot-resistant winter hardy alfalfa germplasms BIC-6 CLS_5, MSE6 CLS_6, MSF6 CLS_6, and Washington SNI CLS_4. Crop Sci. 24:388.
14. VanScoyoc, S. W., Pooranampillai, D. D., and Stromberg, E. L. 1988. Evaluation of a screen for selecting resistance to Sclerotinia crown and stem rot in alfalfa. Page 16 in: Rep. North Am. Alfalfa Improv. Conf. 31st.
15. Welty, R. E., and Busbice, T. H. 1978. Field tolerance in alfalfa to Sclerotinia crown and stem rot. Crop Sci. 18:508-509.