# Effect of Acid Detergent Lignin Concentration in Alfalfa Leaves on Three Components of Resistance to Alfalfa Rust

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**ABSTRACT**

Webb, D. H., Nutter, F. W., Jr., and Buxton, D. R. 1996. Effect of acid detergent lignin concentration in alfalfa leaves on three components of resistance to alfalfa rust. Plant Dis. 80:1184-1188.

As plant breeders select alfalfa (*Medicago sativa*) genotypes for improved digestibility by ruminants, there may be an increased risk of yield losses due to plant disease. This is because increases in digestibility are often associated with a decrease in lignin content and lignin has been shown to play an important role in plant defense mechanisms against disease and pests. The method most often employed by public and private alfalfa-breeding programs to assess digestibility is acid detergent lignin (ADL) analysis. ADL concentration was determined for individual alfalfa plants from two different alfalfa populations. Plants representing a range of ADL concentrations within each population were arbitrarily selected, cloned, and used in experiments to quantify the relationship between leaf ADL concentration and components of resistance to *Uromyces striatus*, the causal agent of alfalfa rust. Three components of resistance were quantified: infection efficiency (pustules per $cm^2$ leaf area), latent period (the time from inoculation to when 50% of the pustules were visible), and sporulation capacity (the number of urediniospores produced per pustule). Although analysis of variance found significant differences among clones for infection efficiency, latent period, and sporulation capacity, regression analysis revealed little or no relationship between ADL concentration and components of alfalfa rust resistance. *F* statistics for regression equations and *t* statistics for slope parameters generally were not statistically significant and when these statistics were significant, coefficients of determination ($r^2$) values indicated that ADL concentration explained only 23% or less of the variation in resistance components.

Additional keyword: disease components

One of the most important characteristics of alfalfa (*Medicago sativa* L.) as a feed source is its digestibility (13,20). An important fraction of the potential energy from alfalfa forage is unusable by ruminants due to the limited digestibility of cell walls (4,5,7–10,23). The plant cell wall is a complex matrix composed of polysaccharides, proteins, phenolics (including lignin), water, and minerals (7). The chemical component of forages most commonly associated with reduced digestibility is lignin (8,20,21).

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Research was supported by State and Hatch funds allocated to the Iowa Agricultural and Hone Economics Experiment Station and, in part, by the Leopold Center for Sustainable Agriculture and Agripro Biosciences, Inc.

Journal Paper No. J-16478 of the Iowa Agriculture and Home Economics Experiment Station, Ames 50010. Project No. 3116.

Accepted for publication 8 July 1996.

Publication no. D-1996-0805-06R


In recent years, public and private alfalfa breeders have developed screening programs to select for improved digestibility, but improved digestibility is often associated with reduced lignin concentration in herbage. Reductions in the concentration of lignin and/or phenolic compounds through plant breeding could potentially impair resistance mechanisms critical for protection against abiotic and biotic stresses (4). Although numerous studies and reviews have suggested that lignin has a role in preventing or reducing plant injury caused by pathogens (2,15,18,22), nearly all of these reports have focused on lignification as an active plant defense mechanism as opposed to determining the effect of innate leaf-lignin content on host plant resistance.

Reduced innate (pre-formed) lignin content in plants has been reported to be associated with increased insect feeding. Buendgen et al. (3) found that low concentrations of lignin in corn (*Zea mays* L.) resulted in increased feeding by the European corn borer *Ostrinia nubilalis* (Hubner). Larsson et al. (13) reported that consumption of willow leaf tissue by the leaf beetle (*Galerucella lineole* F.) was five times greater for plants grown under low irradiance, which resulted in plants with lower levels of starch, tannin, and lignin, compared with plants grown under higher irradiance. Buxton and Casler (4) evaluated divergent selections for high or low lignin concentration in alfalfa cv. Saranac (10) in field experiments conducted at Ames, IA. After 2 years, only 34% of low lignin plants had survived, compared with 64% of the high lignin selections (4). To determine if plant diseases played a role in the poorer survival of the low acid detergent lignin (ADL) divergent line, plots were assessed for disease severity (diseased leaf area/total leaf area) in the fall of 1992. There were four replications per treatment and all leaves at the primary nodes of 10 stems/plot were assessed for disease severity of alfalfa rust (*Uromyces striatus* J. Schröt.) and phoma spring blackstem and leaf spot (*Phoma medicaginis* Malbr. & Roum. in Roum.). Alfalfa rust severity in the low ADL selection was more than twice as high as rust severity in the high ADL selection (Table 1). Disease severity of leaves due to spring blackstem and leaf spot was also significantly higher in low ADL plants than in high ADL selections (Table 1). Since alfalfa is a perennial crop, even small differences in resistance can lead to higher levels of inoculum and infection over time, thereby creating relatively greater biological risks.

Associated risks need to be thoroughly assessed before new cultivars and/or new crop production practices are recommended for use by growers. The effect of innate lignin content of plants on disease resistance has been little studied. Moreover, the disease risks associated with reduced ADL

**Table 1.** Effect of divergent selection for high and low acid detergent lignin (ADL) concentration on disease severity of alfalfa rust and spring blackstem assessed during the fall of 1992 in Ames, IA[y]

| Disease/relative ADL content | Disease severity (%)[z] |
|---|---|
| Alfalfa rust | |
| High | 3.0 a |
| Low | 6.9 b |
| Spring blackstem | |
| High | 6.3 a |
| Low | 9.3 b |

[y] Divergent selections from a population of Saranac.
[z] Means followed by a different letter were significantly different ($P < 0.05$).

concentrations in alfalfa have not been investigated. In light of the field results presented in Table 1, we felt it was necessary to assess the epidemiological consequences of selecting for increased forage digestibility based on ADL analyses. Although more sensitive and quantitative methods can be used to assess the amount of lignin in specific plant tissues, these other methods are not routinely used to select alfalfa genotypes for improved digestibility (lower lignin content). Both public and private alfalfa-breeding programs employ ADL analysis as an overall indicator of improved digestibility (6,21). Therefore, the objective of this study was to investigate the effect of ADL concentrations in alfalfa (using methods currently employed by alfalfa breeders) on three components of resistance (infection efficiency, latent period, and sporulation) to *Uromyces striatus*.

## MATERIALS AND METHODS

**Selections and production of host populations.** Alfalfa clones from two different sources were used to obtain two ranges of ADL concentrations. The first population was derived from divergent selections from alfalfa cv. Saranac based on ADL analyses for high and low values (11). Plants from this population were grown from seed in 10-cm-diameter pots and later transplanted to 15-cm pots. The second population was acquired from Agripro Biosciences Incorporated (ABI), Ames, IA. Sixty-one clones were selected based on their leaf and stem lignin concentrations as determined by ADL analysis (4–6) and near-infrared spectrophotometry (8,9). Plants selected from this population were propagated from stem cuttings and planted in flats containing a 3:1:3:1 mix of sand/peat/Perlite/Vermiculite, and maintained in a mist chamber for 4 weeks to encourage root development. Plants were then transplanted to 15-cm-diameter pots containing a steam-sterilized 2:2:4 mix of Webster silty clay loam/peat/Perlite. Plants from both populations were maintained in a greenhouse under a 16-h photoperiod and 23 ± 4°C. Plants were fertilized weekly with a solution of 20-20-10 Peters.

**ADL analysis.** After two complete growth cycles in the greenhouse, leaves from individual plants from each population were harvested following 4 weeks of regrowth. Leaves were separated from stems at the base of the petioles, placed in paper bags, and dried at 54°C for 72 h. Dried leaf samples were ground to pass through a 1-mm screen of a Cyclone Sample Mill (UDY Corporation, Fort Collins, CO) and then analyzed for ADL concentration with the procedure described by Goering and Van Soest (6) as modified by Van Soest and Robertson (21). Each clone was replicated three times and ADL analyses were performed twice for each replication.

**Selection and increase of plants for experimentation.** Twelve plants representing a wide range of ADL concentrations were arbitrarily selected from the Saranac population, and 10 plants representing a wide range of ADL concentrations were selected from the ABI population (Table 2). The Saranac population had a twofold range in ADL concentration while the ABI population had a nearly threefold range. Twelve daughter plants were cloned from each selection by taking stem cuttings as previously described. Clones were first grown in flats in a mist chamber and later transplanted to 10-cm-diameter pots. All plants used in experiments were obtained by stem cuttings from these daughter clones. After rooting, individual plants were transplanted to 10-cc Cone-Tainers (Stuewe & Sons, Inc., Corvallis, OR) and placed on a greenhouse bench with a 16-h photoperiod at 23 ± 4°C.

**Production of inoculum.** Urediniospores of *U. striatus* were obtained in 1992 from diseased alfalfa (CV Pioneer 5262) from a field established in 1991 in Ankeny, IA. This collection was increased and maintained on cv. Ranger (susceptible to rust) for use in all experiments. Urediniospores were collected weekly from infected alfalfa plants with a cyclone spore collector (Instrument Shop, Iowa State University, Ames, IA) attached to a vacuum pump, and stored in a refrigerator in the dark at 3°C. Two-week-old urediniospores were used for all experiments.

**Inoculation procedure.** Following 3 weeks of regrowth, alfalfa plants were inoculated with a spore settling tower (Instrument Shop, Iowa State University, Ames). The tower consisted of a cylinder 119 cm in diameter and 128 cm tall with a turntable at the bottom of the cylinder. An atomizer was inserted through the side of the chamber at a height of 35 cm above the top of the plants.

Spore suspensions were produced by mixing urediniospores with Isopar M (Exxon Company, U.S.A., Houston, TX), determining the number of spores per ml with a hemacytometer, and diluting to the desired concentration. Plants were inoculated by placing one replication on the turntable (five revolutions per minute) and then injecting 10 ml of a spore suspension via an aerosol into the tower at the rate of 1.25 ml/min at 138 kPa.

**Quantification of infection efficiency and latent period.** Plants were arranged in a randomized complete block design with two plants per ADL level for each of six replications. Each replication was inoculated separately with 22 urediniospores/cm$^2$ leaf area (75,000 spores/ml). Following inoculation, plants were misted with deionized, distilled water and placed in a dew chamber (Percival Manufacturing Co., Boone, IA) in darkness at 19°C for 24 h. These environmental conditions were previously found to optimize infection efficiency of alfalfa rust on cv. Ranger (D. H. Webb and F. W. Nutter, Jr., *unpublished*). Plants were then placed in a growth chamber at 21°C with a 24-h photoperiod.

For each plant, pustules were counted on the adaxial surface of the three leaves, located immediately below the whorl that was fully expanded at the time of inoculation. Pustule counts began on the day pustules were first visible and continued each day until there was no further increase in pustule number for two consecutive days. Total leaf area (cm$^2$) of the three sampled leaves per plant was measured with a leaf area meter (Decagon Devices, Inc., Pullman, WA). Infection efficiency (pustules per cm$^2$ leaf area) was determined by dividing the final pustule count for each sample by the total area of those leaves. The number of pustules per cm$^2$ for each of two plants was averaged for each replication. Latent period, defined as the time from inoculation to when 50% of the pustules were visible ($T_{50}$), was measured by determining the number of pustules per cm$^2$ per day as described above, transforming cumulative daily pustule per cm$^2$ values to gompits, regressing gompits versus days after inoculation with the computer program EPIMODEL (16), and solving for $T_{50}$. Experiments were conducted separately for the ABI and Saranac populations and all experiments were repeated.

**Quantification of sporulation capacity.** Plants were arranged in a randomized complete block design with three replications. Due to the labor-intensive nature of these experiments, eight clones differing in ADL concentration were selected from each population. ADL concentrations of clones selected from the Saranac population were 16.2, 17.5, 19.3, 23.2, 25.6, 29.5,

**Table 2.** Acid detergent lignin (ADL) concentration of alfalfa clones selected from two populations of alfalfa

| Clone no. | ADL[z] |
|---|---|
| Saranac clones | |
| 1 | 16.2 |
| 2 | 17.5 |
| 3 | 19.3 |
| 4 | 22.2 |
| 5 | 23.2 |
| 6 | 24.9 |
| 7 | 25.6 |
| 8 | 26.6 |
| 9 | 29.5 |
| 10 | 30.9 |
| 11 | 31.8 |
| 12 | 34.7 |
| ABI clones | |
| 1 | 9.9 |
| 2 | 15.5 |
| 3 | 17.1 |
| 4 | 18.3 |
| 5 | 20.5 |
| 6 | 22.3 |
| 7 | 24.6 |
| 8 | 27.9 |
| 9 | 29.2 |
| 10 | 32.9 |

[z] Grams of ADL per kilogram of dry leaf matter.

31.8, and 34.7 g of ADL per kg of dry leaf matter. ADL concentrations of clones selected from the ABI population were 9.9, 15.5, 18.3, 22.3, 24.6, 27.9, 29.2, and 32.9 g/kg of dry matter. Plants were inoculated with 9 urediniospores/cm$^2$ leaf area, as described earlier. This lower spore concentration was used to obtain a sufficiently low density of pustules per cm$^2$ to avoid competition among pustules. Inoculated flats were misted with deionized, distilled water and placed in dew chambers at 19°C for 24 h of darkness. Plants were then placed in a growth chamber at 24°C with a 16-h photoperiod for 18 days.

Urediniospores were collected from all pustules into a No. 00 gelatin capsule (Eli Lilly and Co., Indianapolis, IN) with a cyclone spore collector attached to a vacuum pump. Spore collections began the first day pustules appeared and continued every 48 h thereafter for a total of five collection times. A 0.5-ml aliquot of Isopar M was added to each capsule and the number of urediniospores per ml was determined with a hemacytometer. Sporulation capacity (number of urediniospores produced per pustule) was determined by counting the number of pustules on the adaxial surface of three leaves per plant immediately following the collection of spores. The number of spores per pustule was determined by dividing the number of spores per capsule by the number of pustules on the three sampled leaves. Spores per pustule were counted for each collection time; the numbers were added to obtain the total number of spores produced per pustule (sporulation capacity).

**Data analysis.** Analysis of variance and the Waller-Duncan *k*-ratio *t* test ($k = 100$; $P \leq 0.05$) were used to determine if there were significant differences among clones for infection efficiency, latent period, and sporulation capacity. All experiments were repeated and the data combined prior to analysis of variance. Simple linear regression was used to characterize stimulus-response relationships between ADL concentration ($x$) with three response variables: infection efficiency, latent period, and sporulation capacity. The SAS PROC GLM procedure (SAS Institute, Cary, NC) was used for all regression analyses. Individual experiments were analyzed by regression and the slopes, if significantly different from zero, were compared by means of a *t* test (12). The parameters used to evaluate regression models were the *F* statistic for the overall model to test if there was a significant linear relationship between ADL concentration ($x$) and resistance components ($y$), the *t* test for the slope of the regression line, the coefficient of determination ($r^2$), and the standard error of the estimate for $y$ ($SE_y$).

## RESULTS

**Effect of leaf ADL concentration on infection efficiency.** Although there were significant differences ($P \leq 0.05$) among alfalfa clones for their effects on infection efficiency, latent period, and sporulation capacity, in both the Saranac (Table 3) and ABI (Table 4) populations, leaf ADL concentration had little or no effect on infection efficiency for either population. Although there was a significant positive relationship between lignin concentration and infection efficiency for the Saranac population in one experiment ($P > F = 0.001$, $SE_y = 1.51$, $P > t = 0.05$), the $r^2$ value of 0.14 indicated that only a small proportion of the variation in infection efficiency could be explained by leaf lignin concentration (Fig. 1). Moreover, a single point contributed heavily in producing a significant *F* statistic as the *F* statistic was not significant ($P \geq 0.05$) when this point was omitted. Similar results were obtained when the experiment was repeated ($P > F = 0.13$, $SE_y = 2.18$, $P > t = 0.154$, $r^2 = 0.12$). The *F* statistic was not significant, indicating there was not a linear relationship between ADL and infection efficiency. For the ABI population, there was a negative linear relationship between ADL concentration and infection efficiency, but the coefficient of determination ($r^2$) indicated that ADL concentration explained only 26% of the variation in infection efficiency ($P > F = 0.0001$, $SE_y = 2.06$, $P > t = 0.0004$). The repeated experiment gave similar results ($P > F = 0.0001$, $SE_y = 2.96$, $P > t = 0.0009$, $r^2 = 0.19$).

**Effect of leaf ADL concentration on latent period.** Leaf ADL concentration had little or no effect on latent period ($T_{50}$) in either population. Although there was a significant negative relationship between ADL concentration and $T_{50}$ for the Saranac

**Table 3.** Effect of alfalfa cv. Saranac clones differing in acid detergent lignin concentration on infection efficiency, latent period, and sporulation of alfalfa rust

| Acid detergent lignin (g/kg of dry matter) | Infection efficiency (pustules/cm$^2$) | Latent period ($T_{50}$) | Spores/pustule |
|---|---|---|---|
| 16.2 | 5.51 ab[y] | 12.58 def | 1,245 bc |
| 17.5 | 0.78 e | 14.37 a | 4,523 a |
| 19.3 | 3.80 c | 13.94 ab | 969 c |
| 22.2 | 5.23 abc | 12.82 c | NT[z] |
| 23.2 | 5.73 a | 13.45 bc | 1,256 bc |
| 24.9 | 3.97 bcd | 13.06 cde | NT[z] |
| 25.6 | 5.48 ab | 12.03 fg | 1,190 bc |
| 26.6 | 3.69 cd | 13.11 cd | NT[z] |
| 29.5 | 6.46 a | 11.38 g | 1,906 b |
| 30.9 | 5.07 abcd | 12.11 fg | NT[z] |
| 31.8 | 3.49 d | 12.25 ef | 549 c |
| 34.7 | 6.00 a | 13.35 bcd | 950 c |

[y] Means with the same letter within columns are not significantly different according to the Waller-Duncan *k*-ratio *t* test, $k = 100$ ($P \leq 0.05$).
[z] Spores/pustule were not measured for this alfalfa clone.

**Table 4.** Effect of alfalfa ABI clones differing in acid detergent lignin concentration on infection efficiency, latent period, and sporulation of alfalfa rust

| Acid detergent lignin (g/kg of dry matter) | Infection efficiency (pustules/cm$^2$) | Latent period ($T_{50}$) | Spores/pustule |
|---|---|---|---|
| 9.90 | 2.88 de[x] | 12.86 f | 807 ab |
| 15.5 | 3.43 cd | 13.87 de | 431 bc |
| 17.1 | 0.00 f | –[y] | NT[z] |
| 18.3 | 4.28 bc | 14.98 c | 731 ab |
| 20.5 | 0.06 f | 17.79 a | NT[z] |
| 22.3 | 4.82 b | 14.55 cd | 620 abc |
| 24.6 | 1.90 e | 16.09 b | 278 c |
| 27.9 | 6.19 a | 13.67 ef | 745 ab |
| 29.2 | 6.32 a | 12.88 f | 634 abc |
| 32.9 | 4.69 b | 12.83 f | 999 a |

[x] Means with the same letter within columns are not significantly different according to the Waller-Duncan *k*-ratio *t* test, ($k = 100$, $P \leq 0.05$).
[y] No pustules formed, therefore latent period could not be measured.
[z] Spores/pustule were not measured for this alfalfa clone.

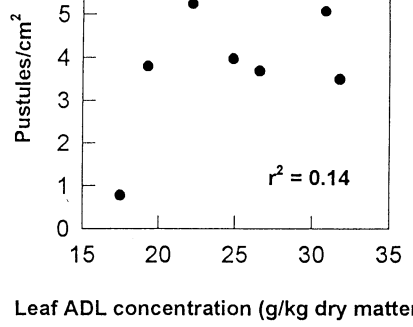


**Fig. 1.** Lack of a linear relationship between leaf acid detergent lignin (ADL) concentration (g per kg of dry matter) and infection efficiency (pustules per cm$^2$) of alfalfa rust.

population ($P > F = 0.0001$, $SE_y = 0.78$, $P > t = 0.001$), the low $r^2$ value (0.23) indicated that only a small proportion of the variation in latent period could be explained by ADL concentration (Fig. 2). In the repeated experiment, no effect of ADL concentration on $T_{50}$ was evident ($P > F = 0.07$, $SE_y = 0.88$, $P > t = 0.223$, $r^2 = 0.03$). No relationship between ADL concentration and $T_{50}$ was found for the ABI population in the initial experiment ($P > F = 0.08$, $SE_y = 1.78$, $P > t = 0.41$, $r^2 = 0.02$). There was a significant negative linear relationship between ADL concentration and $T_{50}$ for the repeated experiment ($P > F = 0.0001$, $SE_y = 0.86$, $P > t = 0.002$, but the $r^2$ (0.23) was quite low.

**Effect of leaf ADL concentration on sporulation capacity.** Leaf ADL concentration had little or no effect on sporulation capacity (spores per pustule) for either population. Although a significant negative linear relationship was found between ADL concentration and sporulation capacity for the ABI population ($P > F = 0.05$, $SE_y = 1209$, $P > t = 0.04$), a single data point contributed heavily toward significance of the regression coefficient. Moreover, the low $r^2$ (0.20) indicated only a small proportion of the variation in sporulation capacity could be explained by leaf ADL concentration (Fig. 3). Leaf ADL concentration and sporulation capacity were not related in the repeated experiment ($P > F = 0.86$, $SE_y = 721$, $P > t = 0.87$, $r^2 = 0.002$) and ADL concentration did not effect sporulation capacity of the ABI population in either experiment (experiment 1, $P > F = 0.39$, $SE_y = 236$, $P > t = 0.38$, $r^2 = 0.04$; experiment 2, $P > F = 0.83$, $SE_y = 654$, $P > t = 0.83$, $r^2 = 0.004$).

## DISCUSSION

Breeding alfalfa for increased digestibility is a promising means to increase the nutritive value of forages (4,10,11,14,20, 21,23). Both public and private alfalfa-breeding programs routinely employ ADL as a selection criterion that is related to digestibility. Reductions in innate ADL concentration, however, may present a potential disease risk, considering the numerous studies implicating the role of lignin and related compounds in disease resistance (2,15,18,22). To assess this risk epidemiologically, as a prelude to more elaborate physiological studies, we chose to establish a range of ADL concentrations within two alfalfa populations and then quantitatively measure the pathogen's response to decreasing levels of ADL.

It is important to note that although there were significant differences among alfalfa clones for three quantitative components of resistance to alfalfa rust—infection efficiency, latent period, and sporulation capacity—these differences were largely unrelated to ADL concentration. ADL concentrations in two different alfalfa populations had little or no effect on infection efficiency, latent period, or sporulation capacity. Although there was a significant linear relationship between ADL concentration and latent period ($T_{50}$) and $r^2$ values were 0.23 in two out of four experiments, this relationship had a negative slope and, therefore, decreasing ADL concentration in alfalfa carries less risk of alfalfa rust epidemics because $T_{50}$ values would be longer as ADL concentrations are reduced, not shorter. In related work, we quantified the effect of temperature and leaf wetness duration on infection efficiency and latent period for alfalfa rust and demonstrated that these variables could be used to predict their effects on disease components of alfalfa rust (D. H. Webb and F. W. Nutter, Jr., *unpublished*). Therefore, we are confident that the lack of response of alfalfa rust to decreasing ADL levels in leaves indicates that there is little or no increased risk of alfalfa rust epidemics occurring in the field as a consequence of planting alfalfa cultivars selected for low ADL (increased digestibility). Other researchers have found that components of resistance were highly correlated to infection rates in the field and, therefore, components analysis can be used as one tool to evaluate the potential risks of disease epidemics in the field as a prelude to more expensive field studies (1,17, 9).

In legumes, lignin is believed to be found primarily in xylem and tracheary cells (7–9,23). To date, studies concerning the role of lignin in plant resistance to most pathogens have dealt almost exclusively with the deposition of lignin in response to pathogen invasion, rather than innate ADL concentration in plants (2,15,18,22). The composition of lignin and associated phenolics produced in response to infection has been shown to be different from that found in healthy plants. Although the mesophyll cells of leaves constitute most of the leaf tissue, the amount of lignin in mesophyll cells is much less relative to xylem tissues, which may explain why ADL concentration did not have a significant impact on infection efficiency, the latent period, or sporulation capacity of *Uromyces striatus*. While the results of this study are encouraging for alfalfa breeders, it cannot be assumed that innate ADL concentration of alfalfa is unrelated to resistance to other alfalfa pathogens and pests. For example, preliminary data suggest that pea aphids reproduce at a faster rate on reduced lignin lines, compared with divergent selections for high lignin content (J. J. O'brycki, F. W. Nutter, Jr., and D. R. Buxton, *unpublished*). Additional investigations on the effect of biotic and abiotic stresses on reduced ADL concentration in alfalfa cultivars is warranted before the widespread release of these cultivars.

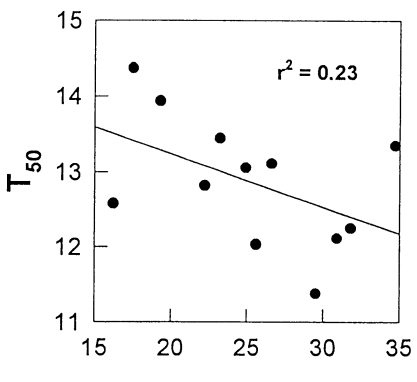


**Fig. 2.** Relationship between leaf acid detergent lignin (ADL) concentration (g per kg of dry matter) of selected alfalfa clones and latent period (time of 50% pustule appearance, $T_{50}$) of alfalfa.

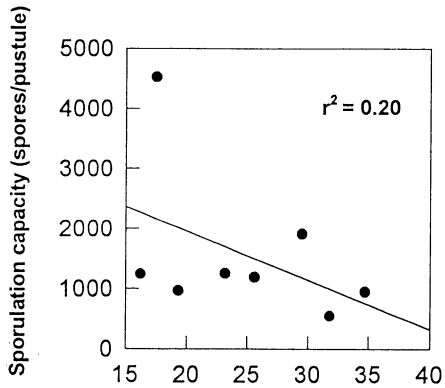


**Fig. 3.** Relationship between leaf acid detergent lignin (ADL) concentration (g per kg of dry matter) of selected alfalfa clones and sporulation capacity (spores per pustule) of alfalfa rust.

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