Etiology

Relationships of Planting Density and Competition to Growth Characteristics and Internal Crown Breakdown in Arrowleaf Clover

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ABSTRACT


Arrowleaf clover was grown in field plots at intervals of 5-40 cm between plants in pure stands, and at 40 cm between plants overseeded with ryegrass, during two growing seasons. Growth characteristics and internal crown breakdown (ICB) were evaluated near the end of each season. Numbers of flowering stems, crown and root diameters, and the incidence and severity of ICB increased with greater planting intervals in pure stands and decreased in plants grown with ryegrass. ICB scores were positively and closely correlated with values for all of the plant characteristics and with various ratios between them. ICB also was more frequent and severe following a harsh winter growing season than after a mild one. Fifteen species of fungi were represented among 41 isolates obtained from crowns of arrowleaf clover before and after ICB symptoms developed. Fusarium acuminatum, F. oxysporum, and Chaetomium trilaterale were isolated most frequently and occasionally caused symptoms in crowns following artificial inoculations; all other fungi were nonpathogenic. Results suggest that ICB in arrowleaf clover is a noninfectious disease that is related to morphological characteristics of plants grown at low densities or with insufficient aboveground competition. However, the incidence and severity of ICB may be affected by winter injury and by invasion of crowns by weakly pathogenic fungi.

Additional key words: Trifolium incarnatum, Trifolium pratense, Trifolium subterraneum, Trifolium vesiculosum.

Internal breakdown of crown tissue (ICB) in red clover (Trifolium pratense L.) was first described in greenhouse-grown plants by Graham and Newton (5) in 1959. They observed small necrotic areas in pith tissue of crowns of plants ≥ 3 mo old that enlarged, became corky and dry, and eventually resulted in necrosis and decomposition of most of the internal tissue in crowns and upper taproots (5, 6). Similar symptoms were observed in field-grown red clover throughout the northeastern USA (6) and in the states of Washington (2) and West Virginia (4), in Prince Edward Island (Canada) (11), and in Finland (12). ICB occurred at high frequencies in stands after 1 yr and was suggested to be a major cause for the failure of red clover to persist or remain economically productive after 2 yr (2, 6, 7).

No pathogenic fungi, bacteria, or viruses have been demonstrated or implicated as causes of ICB. Species of Fusarium, Phoma, and Chaetomium usually were isolated most frequently from symptomatic crowns (2, 6, 11, 13). However, these and other fungi were not consistently associated with ICB, especially in its early stages (2), and they did not induce symptoms similar to ICB when inoculated into crowns and roots (6, 13). Often no organisms were isolated from or observed in symptomatic crown tissue (2, 6, 13). In one instance, ICB developed in plants grown aseptically in isolation chambers (13). Infection of plants by red clover vein mosaic virus and bean yellow mosaic virus also was not related to the presence of ICB (6, 13).

Graham et al (6) suggested that ICB was a "physiologic" disease. This opinion was supported by Zeiders et al (13). Cressman (2) similarly suggested that the cellular breakdown in ICB resulted from a "malignant cytological disturbance."

ICB in red clover is positively correlated with crown and root diameters (2, 6, 11, 13). Most other factors or experimental treatments have not been clearly correlated with ICB or shown to affect its incidence, including macro- and micronutrients, fungicides and insecticides, temperature and light regimes, soil types and organic matter, or growth regulators (2, 6, 13). Frequent clipping of foliage resulted in less ICB, but this effect was attributed to reduced crown diameters (13). Ylimaki (2) stated that excessive applications of boron caused hollowing-out and subsequent browning of crowns along with foliar symptoms, but this sequence is opposite to that which occurs in ICB. One instance of differences in incidence of ICB between cultivars of red clover was reported (13).

Cressman (2) observed that lethality due to ICB was related to root characteristics of red clover; plants with a tendency to produce numerous adventitious and lateral roots from taproots, crowns, and lower stems tolerated severe ICB, whereas plants with few such roots were killed. Zeiders et al (13) successfully selected for both greater and lesser ICB in red clover in the greenhouse; however, they did not describe morphological characteristics of selections.

ICB also occurs in alfalfa (T. hybridum L.) (13), a biennial species. It has not been reported in any of the annual species that are primarily grown in the southeastern USA. These clovers are fall-planted for grazing or cover crops, and they flower, seed, and senesce the following spring or summer.

From 1978-1982, ICB symptoms were observed in arrowleaf (T. vesiculosum Savi), crimson (T. incarnatum L.), and subterranean (T. subterraneum L.) clovers at Mississippi State. Symptoms were most frequent and severe in arrowleaf and were observed in both field- and greenhouse-grown plants. No likely pathogens were consistently isolated from ICB-affected tissue (R. Pratt, unpublished). Symptoms were nearly universal in space-planted nurseries and appeared to contribute to death of plants prior to
flowering or seed set (8). Symptoms were less severe in volunteer stands, where growth characteristics of plants differed from those in nurseries.

The purpose of this investigation was to determine relationships of planting density and grass competition to growth characteristics and ICB in arrowleaf clover. The identity and pathogenicity of fungi associated with ICB were also evaluated.

**MATERIALS AND METHODS**

**Establishment of plots.** Seed of arrowleaf clover cultivar Yuchi was germinated on water agar and planted in a soil mixture (8) in peat pots (70-cc capacity) (one plant per pot) in flats. Commercial Rhizobium inoculum was applied to soil after 1 wk, and plants were grown for 6–7 additional weeks in the greenhouse.

Plots were established on the Animal Research Center of Mississippi State University by transplanting plants on 14 October in 1980 and 1981. Soil was a Kispi Klip silty clay loam (fine, montmorillonitic, thermic Vertic Hapludalf) on a well-drained upland site that had never previously been planted to clovers. The site was limed to pH 6.5–6.9 and disked prior to transplanting. Treatments were 5-, 10-, 20-, and 40-cm planting intervals between individual plants within plots, and a 40-cm interval overseeded with annual ryegrass, during both years. A 15-cm planting interval was also included in 1981.

Plants were transplanted at the specified intervals in square plots with uniform sampling areas (1.2 × 1.2 m) surrounded by one or two border rows of additional plants at the same intervals. Sampling areas of each plot at the 5-, 10-, 15-, 20-, and 40-cm planting intervals contained 625, 169, 81, 49, and 16 plants, respectively. Four replicate plots of each treatment were arranged in a completely random design in 1980 and 1981. In a randomized complete block in 1981 with 1.2-m alleles between plots. Following transplanting, seed of annual ryegrass (Lolium multiflorum L. ‘Gulf’) was broadcast over four 40-cm plots and tall fescue (Festuca arundinacea) Schreb. ‘Kentucky 31’ was seeded in alleys. Pots were sprinkler-irrigated following establishment in 1980.

**Sampling and evaluation of plants.** Whole-plant samples were collected from 28 April–11 May 1981, and from 17–20 May 1982. In 1981, samples were collected from 5-, 10-, and 20-cm plots by a stratified random-sampling procedure: the sampling area of each plot was divided into 16 equal parts and a single plant was selected from each one by random drawings of position numbers. All plants in the sampling areas of each 40-cm plot were collected. In 1982, following death of some plants in most plots during a severe winter, samples were collected only among surviving plants, which were bordered by at least two others on all sides.

Plants were excavated and roots were washed free of soil. The number of flowering stems originating within 2 cm of the crown, the maximum crown diameter, and the diameter of the taproot or largest root 1.5 cm below uppermost lateral roots were recorded for each plant.

Each plant was bisected longitudinally to evaluate ICB. Symptoms were compared by assigning scores (Fig. 1) as follows: 0 = no ICB symptoms; 1 = red-brown discolored in centers of crowns but with no disintegration of tissue; 2 = discoloration plus disintegration of 1–20% of crown tissue in longitudinal section; 3 = discoloration plus disintegration of 21–50% of tissue; and 4 = discoloration plus disintegration of 51–100% of tissue. Plants were scored according to the most severe ICB symptoms observed in either half of the crown.

Significant differences between mean plot values for plant characteristics and ICB were determined by analysis of variance and by use of the Student–Newman–Keuls’ test (9).

**Isolation, identification, and pathogenicity of fungi from crowns.** In October 1981, additional plants were transplanted at 40-cm intervals in four rows 6 m apart (45 plants per row). Five plants were randomly selected from each row on 18 November, 17 December, 20 January, and 19 February. Crowns were bisected, ICB symptoms were recorded, and two pieces of tissue (3–4 mm in diameter) were aseptically excised from inner crowns of each plant, surface-disinfested in aqueous 1% NaOCl for 1 min, rinsed in sterile distilled water, blotted on sterile filter paper, and plated on Difco cornmeal agar (CMA). After 4–11 days, one fungal colony from each plant (where present) was selected and transferred to an additional plate. Attempts were made to select as many different-appearing colonies as were present. Cultures were transferred subsequently to tubes of CMA and stored at 4 C.

Fungi were identified according to texts and original and secondary species descriptions (1, 3, 10).

Pathogenicity of fungi was evaluated by inoculating crowns of Yuchi arrowleaf clover with infected toothpicks. Pieces (1 cm) of wooden toothpicks were autoclaved in Difco nutrient broth and transferred to plates of Difco potato-dextrose agar (four per plate). Plates were inoculated with the fungi and incubated for 2 wk. Arrowleaf clover was grown in clay pots (15-cm top diameter, 1.75-L capacity, four plants per pot) in the soil mixture for 9–10 wk in the greenhouse. Each plant was inoculated by boring a hole 0.5-cm deep into the crown with a flamed needle and inserting an infected piece of toothpick into the hole. Seven plants were inoculated with each of the 41 isolates tested. Ten control plants received simulated inoculations with noninfested toothpicks.

Twenty-four days after inoculation, plants were removed from pots, crowns were bisected longitudinally through inoculation holes, and symptoms were recorded. When symptoms in plants inoculated with fungi were different from those in controls, three pieces of internal symptomatic tissue were aseptically excised from crowns and upper taproots, surface-disinfested, rinsed, blotted, and

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**Fig. 1.** Representative scores and symptoms of internal crown breakdown (ICB) in longitudinally bisected crowns of arrowleaf clover: Score 0 = no ICB symptoms; 1 = internal discoloration in crown with no disruption of tissue; 2 = discoloration plus disintegration of 1–20% of crown tissue; 3 = discoloration plus disintegration of 21–50% of tissue; 4 = discoloration plus disintegration of 51–100% of crown tissue.
Planted on CMA. All fungal colonies originating from tissue within 5-8 days were identified.

RESULTS

Plant growth habit and ICB, 1980-1981. Growth habits of plants differed greatly between the five treatments (Table 1). Plants grown at 5-cm intervals usually developed only one flowering stem, and crowns were unenlarged and tapered evenly into taproots. In contrast, plants grown at 40-cm intervals had up to 56 flowering stems originating from near crowns. Crowns were greatly enlarged and abruptly narrowed at the crown-root interface. Plants grown at 10- and 20-cm intervals had intermediate numbers of stems and crown and root diameters (Table 1).

<table>
<thead>
<tr>
<th>Season</th>
<th>Planting interval (cm)</th>
<th>Flowering stems</th>
<th>Crown diameters (cm)</th>
<th>Root diameters (cm)</th>
<th>ICB scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>1980-1981</td>
<td>5</td>
<td>1.0 a</td>
<td>0.7 a</td>
<td>0.2 a</td>
<td>0.1 a</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.3 b</td>
<td>1.2 b</td>
<td>0.4 b</td>
<td>0.1 a</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>10.5 c</td>
<td>1.8 c</td>
<td>0.6 c</td>
<td>2.3 c</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>32.4 d</td>
<td>2.7 d</td>
<td>0.6 c</td>
<td>2.9 d</td>
</tr>
<tr>
<td></td>
<td>40+ RG</td>
<td>12.7 d</td>
<td>1.6 c</td>
<td>0.5 c</td>
<td>0.8 b</td>
</tr>
<tr>
<td>1981-1982</td>
<td>5</td>
<td>1.7 a</td>
<td>0.8 a</td>
<td>0.2 a</td>
<td>0.9 a</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.6 a</td>
<td>1.3 b</td>
<td>0.4 b</td>
<td>1.5 b</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>8.6 b</td>
<td>1.6 b</td>
<td>0.5 c</td>
<td>3.0 c</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>14.5 c</td>
<td>2.2 c</td>
<td>0.6 d</td>
<td>3.4 cd</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>26.2 d</td>
<td>3.3 d</td>
<td>0.9 e</td>
<td>3.8 d</td>
</tr>
<tr>
<td></td>
<td>40+ RG</td>
<td>11.1 b</td>
<td>1.4 b</td>
<td>0.5 c</td>
<td>0.7 a</td>
</tr>
</tbody>
</table>

Values for 1980-1981 are means for 64 total plants from four replicate plots. Values for 1981-1982 are means for 41-62 total plants from four replicate plots. Numbers within a column for each season not followed by the same letter differ significantly (P = 0.05) according to the Student-Newman-Kuels' test.

Intervals between individual plants within each plot. A uniform sampling area (1.2 x 1.2 m) was used for all plots.

Based on numbers of stems 2 cm above crowns, maximum diameters of crowns, and maximum diameters of taproots 1.5 cm below the uppermost lateral roots.

Scores were assigned to symptoms as follows: 0 = no ICB symptoms; 1 = internal discoloration but no disintegration of tissue in crowns; 2 = discoloration plus disintegration of 1-20% of crown tissue; 3 = discoloration plus disintegration of 21-50% of crown tissue; and 4 = discoloration plus disintegration of 51-100% of crown tissue.

Plants grown at the 40-cm interval with ryegrass had greatly reduced numbers of stems and crown diameters. These plants appeared generally similar to those grown at 20-cm intervals (Table 1); however, stems grew more vertically from crowns of plants grown in the presence of ryegrass.

ICB symptoms occurred in 5% and 8% of sampled plants grown at 5- and 10-cm intervals, in 97% and 100% of plants grown at 20- and 40-cm intervals, and in 41% of plants grown at 40 cm with ryegrass. Differences in mean scores of ICB were significant (P = 0.05) between all treatments except the 5- and 10-cm planting intervals (Table 1). Plot means of ICB scores for all treatments were significantly correlated (P = 0.05) with means of numbers of stems, crown diameters, root diameters, and with certain ratios of values for these plant characteristics (Table 2).

Plant growth habit and ICB, 1981-1982. Plants were subjected to unusually severe winter growing conditions. Numerous plants died in some plots following low temperatures (down to -18°C), ice storms, and frost-heaving of soil. Among surviving plants in treatments without ryegrass, numbers of stems, crown diameters, root diameters, and ICB scores were nearly always higher than in 1980-1981, but differences between treatments were generally similar (Table 1). ICB symptoms occurred in 59% and 91% of plants at 5- and 10-cm intervals, in 100% of plants at 15-, 20-, and 40-cm intervals, and in 33% of plants at the 40-cm interval with ryegrass. Plot means of ICB scores for all treatments were significantly correlated with means of values for plant characteristics and with most ratios as in 1980-1981 (Table 2).

Identity and pathogenicity of fungi from crowns. Fifteen species of fungi were represented among 41 isolates from crown tissue (Table 3). No obvious differences were apparent between numbers of species or identities of fungi isolated before and after ICB symptoms became prevalent in January and February. Fusarium acuminatum, F. oxysporum, and Chaetomium trilaterale were isolated most frequently.

All control plants that received noninfested toothpicks developed dark discoloration 1-3 mm deep surrounding the wounds, and occasional light- or red-brown discoloration extending 2-3 mm farther into crowns and down the centers of crown roots.

<table>
<thead>
<tr>
<th>Sampling time (1981-1982) and no. of isolates</th>
<th>Pathogenicity of fungi to crowns</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of plants with ICB symptoms</td>
<td>Nov</td>
</tr>
<tr>
<td>Fungi isolated</td>
<td>Acremonium strictum</td>
</tr>
<tr>
<td></td>
<td>Alternaria sp.</td>
</tr>
<tr>
<td></td>
<td>Chaetomium trilaterale</td>
</tr>
<tr>
<td></td>
<td>Cephalosporium sp.</td>
</tr>
<tr>
<td></td>
<td>Cladosporium cladosporioides</td>
</tr>
<tr>
<td></td>
<td>Eupenicillium brefeldianum</td>
</tr>
<tr>
<td></td>
<td>Fusarium acuminatum</td>
</tr>
<tr>
<td></td>
<td>Fusarium dimerum</td>
</tr>
<tr>
<td></td>
<td>Fusarium oxysporum</td>
</tr>
<tr>
<td></td>
<td>Penicillium sp.</td>
</tr>
<tr>
<td></td>
<td>Phoma sp.</td>
</tr>
<tr>
<td></td>
<td>Rhizoctonia solani</td>
</tr>
<tr>
<td></td>
<td>Stemphylium botryosum</td>
</tr>
<tr>
<td></td>
<td>Verticillium nigrum</td>
</tr>
</tbody>
</table>

Values twenty plants from rows space-planted in October were randomly selected at each sampling time. ICB symptoms were observed in bisectioned crowns, and fungi were isolated by plating two pieces of tissue from each crown on Difco CMA. One isolate, where present, was collected from each plant.

Data are: (number of plants with symptoms in crowns following inoculation)/total number of plants inoculated. Plants were inoculated by inserting toothpick pieces infested with the fungi (one per plant) into holes in crowns. Seven plants were inoculated with each isolate.

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roots. Isolations were not attempted from these plants. Inoculations with most fungi did not result in any additional or more extensive symptoms. However, in 20 of 189 plants inoculated with F. acuminatum, F. oxysporum, or C. trilaterale, symptoms were more pronounced than in controls (Table 3). Red-brown discoloration extended up to 1.5 cm down the centers of roots, often in streaks, and portions or all of crows were pale brown. All three species were reisolated from crown or root tissue of one or more plants.

**DISCUSSION**

Results of this study demonstrate that ICB, previously known only in perennial clovers, also occurs in annual species, and that its occurrence and severity in arrowleaf clover are closely related to plant growth characteristics. These characteristics, in turn, are related to planting density and the presence or absence of grass competition. The results also demonstrate for the first time that ICB can be controlled or manipulated in the field under favorable growing conditions.

The relationships of ICB to growth characteristics in arrowleaf clover, and the inconsistent association of pathogenic fungi with it, provide evidence for the concept that ICB is a noninfectious disease. The similar condition in red clover was described as a physiogenic disease (6,13). For arrowleaf clover, the term “physiogenic disease” appears to be more descriptive. This indicates that ICB is related to plant morphology in general, and specifically to morphological features of plants grown at low stand densities with little or no aboveground competition. However, the term is not intended to preclude “physiogenic” or to deny that the ultimate cause for ICB is physiological.

The best demonstration of the relationship of ICB to plant growth characteristics, and the best evidence for its noninfectious nature, was obtained in 1980–1981, when mortality and stress from winter injury and other causes were minimal. With generally favorable growing conditions that season, ICB was nearly universal in plants grown at 20- and 40-cm intervals in pure stands, but it scarcely occurred in plants grown at 5- and 10-cm intervals at randomly chosen locations within the same area. It is difficult to conceive that any pathogen, either soilborne or aerial, could so selectively infect only plants grown at the larger intervals. It appears equally dubious that either a deficiency or excess of a macro- or micronutrient, or any other physical factor, would only affect plants grown at the larger planting intervals. In contrast, the fact that numbers of flowering stems, crown diameters, and root diameters differed consistently between plants at the large and small intervals, and were positively correlated with ICB severity, suggests that ICB originated during development of these organs in some plants.

A general explanation we propose for ICB in arrowleaf clover is that it results from overproduction of stems by plants grown with little or no aboveground competition. We suggest that excessive stem production causes enlargement of crows and creates an imbalance between amounts of tissue in stems, crows, and the taproot. This imbalance causes internal stress during plant growth, which is manifested by necrosis and breakdown of tissue in the crows. The significant correlation of ICB scores with the composite ratios of numbers of stems to crown diameters to root diameters in 1980–1981 supports the concept that an imbalance between these organs either causes ICB or predisposes plants to it.

Incidence and severity of ICB were much greater in 1981–1982, when plants were subjected to extreme winter stress and many were injured and killed. The increases in ICB scores over the previous year, however, appeared to be disproportionate to the smaller increases in number of stems, crown diameters, and root diameters in 5-, 10-, and 20-cm plots (Table 1). This indicates that external stress factors can also predispose plants to ICB or increase its severity. Nevertheless, all of the plant growth characteristics were still significantly correlated with ICB during this severe growing season. This supports the premise that the primary cause of ICB is internal and results from development of unbalanced morphological features in plants, rather than from external stress factors.

Fungi such as F. acuminatum, F. oxysporum, and C. trilaterale are not considered to be primary causal organisms of ICB in arrowleaf clover, because symptoms developed in only a small minority of inoculated plants, and they seldom were severe. Nevertheless, that some necrosis did occur in crown tissue following inoculations suggests that these weak pathogens might accelerate development of ICB symptoms if crows are invaded. The more severe ICB observed in 1981–1982 may have resulted in part from greater invasion of crown and root tissue by fungi following the severe winter damage.

The symptoms of ICB in arrowleaf clover were similar or identical to those previously described in red clover (Fig. 1) (2,6,12,13). Relationships of crown and root diameters to ICB in arrowleaf clover also correspond to those reported for red clover (2,6,11,13). These similarities suggest that the nature and cause of ICB may be the same for perennial and annual clovers, and factors that contribute to lessening or avoidance of ICB in arrowleaf clover may also be applicable to red clover. One treatment in this study, which lessened ICB, was the overseeding of ryegrass with plants grown at wide intervals. Numbers of stems and crown diameters of plants grown at 40-cm intervals with ryegrass were often similar to those of plants grown at 20-cm intervals without it, but the incidence and severity of ICB were greatly reduced. Competition provided by the grass caused more erect development of stems from crows, and this in turn may have caused less internal stress during growth of the clover. Possibly overseeding red clover stands with grass at some time during the first two years might also lessen the incidence and severity of ICB and increase longevity and productivity of stands.

The importance of ICB in arrowleaf clover grown in pastures for grazing is not known. The condition appears to be potentially important in cover crops or seed-production fields, where plants compensate for stand thinning due to diseases or injury by producing additional stems from or near crowns. ICB is very important in space-planted breeding nurseries, where it can cause premature death of plants alone or in combination with viruses and fungal root diseases, which also are known to interact in causing death of plants (8). These results indicate that ICB can be avoided or lessened in nurseries by use of close spacings or by overseeding nurseries with an annual grass.

**LITERATURE CITED**