Development of Kabatiella caulivora in Plants of Trifolium subterraneum 'Yarloop' of Different Ages

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ABSTRACT

Time-course development of infections of *Kabatiella caulivora,* the incitant of scorch or northern anthracnose of clover, was examined under specified environmental conditions in plants inoculated 4 weeks after sowing. Following inoculation, 90-100% of the leaves that were expanded at the time of inoculation, developed lesions. The disease did not spread to leaves which expanded after inoculation. A maximum of 25-30% of leaves present at the time of harvest, were recorded as diseased 9-13 days after inoculation. Evidence suggested that slight injury promoted disease development.

The effect of plant age on susceptibility was examined in plants inoculated 1-5 and 8-10 weeks after sowing. In plants inoculated one week after sowing, 89% of cotyledons and 73% of unifoliolate leaves became diseased. Susceptibility of these organs decreased rapidly with seedling age. The percentage of trifoliolate leaves recorded as diseased increased from 19% for plants inoculated 2 weeks after sowing, to 33% for plants inoculated at 5 weeks. It was only 15% for plants inoculated at 8-10 weeks after sowing. The percentage of inflorescences recorded as diseased increased from 3% for plants inoculated at 8 weeks to 9% for plants inoculated at 10 weeks. The percentage of inflorescences recorded as dead either due to the disease, or to environmental conditions being unfavorable for development of inflorescences, increased from 20% for plants inoculated at 8 weeks to 32% for plants inoculated at 10 weeks.

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Kabatiella caulivora (Kirchn.) Karak, causes an important disease of *Trifolium pratense* L. (red clover) in the cool, moist regions of Europe and North America (6, 10) and of *T. incarnatum* L. (crimson clover) in western Oregon (9). The occurrence of the pathogen in *T. subterraneum* (subterranean clover) in Australia was first reported in 1956 (11, 12). During the past few years, the disease has become prevalent in cool, wet, southern parts of Australia (1, 2, 8). It can be severe in grazed pastures, and can cause considerable reduction in hay and seed production. Plants of several cultivars may die following experimental infection (5, 12).

In preliminary experiments, using an isolate of the pathogen from Western Australia, 4-week-old plants of *T. subterraneum* 'Yarloop', and environmental conditions satisfactory for experimental infection of *T. pratense* (7), the disease developed well in relatively old leaves but did not markedly retard plant growth. It was clear that previous methods of assessing severity of symptoms of *K. caulivora,* which made use of whole-plant rating systems (7, 12), would be impracticable in the present experiments. Therefore, assessment of disease severity was made by determining the percentages of specific organs (e.g., leaves or inflorescences) with symptoms. This allowed more detailed information to be obtained on development of infections within plants.

This paper reports studies on the kinetics of disease development, and the effects of age on susceptibility of plants grown under specified environmental conditions.

MATERIALS AND METHODS.—All experiments were done in the Canberra phytotron. Plants grown from seeds of *Trifolium subterraneum* 'Yarloop' were used in all experiments. Seeds were germinated on filter paper in petri dishes in the dark at 20 C for 48 hours and uniform seedlings were transplanted into 10-cm-diameter pots containing a mixture of vermiculite and perlite (2:1) which was watered daily, once with nutrient solution and once with demineralized water. The plants were grown in a naturally lit glasshouse area of the phytotron maintained at 21 C during the day and at 16 C during the night. Initially, there were 6-7 seedlings per pot; later these were reduced to five. Plants were inoculated at specified ages.

A single-spore culture was made from an isolate of *K. caulivora* derived from the Margaret River area of Western Australia, and this was maintained at 20 C on potato-dextrose agar. Seven-day cultures were transferred to potato-dextrose broth in Erlenmeyer flasks and kept at 20 C on a rotary shaker for 4 days before being used as inoculum. Inoculum was applied to plants by means of a hand atomizer, at a concentration of 4×10^6 conidia per ml, together with a wetting agent (Span 20 : Tween 20; 4:1) at a concentration of 0.01%. Inoculated plants received 100% humidity for 4 days. This was achieved by standing the pots in trays of water (6 pots per tray) and covering the plants in each tray with an open box made of clear, 3 mm Plexiglas, the open end of which was submerged in the water. Plants were placed in a cabinet kept at 20 C and were illuminated with fluorescent lights of 753-861 lx (70-80 ft-c) for 8 hours daily. Afterwards, the plants were put in a glass cabinet with uncontrolled humidity and with natural light supplied 8 hours daily. In this environment, plants were watered from above.

To confirm that lesions on inoculated plants were correctly identified, numerous reisolations of the fungus were made from lesions on cotyledons, primary leaves, and trifoliolate leaves. During the 11 days that plants were examined, no lesions characteristic of *K. caulivora* were found on uninoculated plants grown in the same cabinet as inoculated plants. Only rarely was a lesion found in uninoculated plants kept for a longer period.

Records were made of numbers of expanded and folded leaves per plant at the time of inoculation. Plants

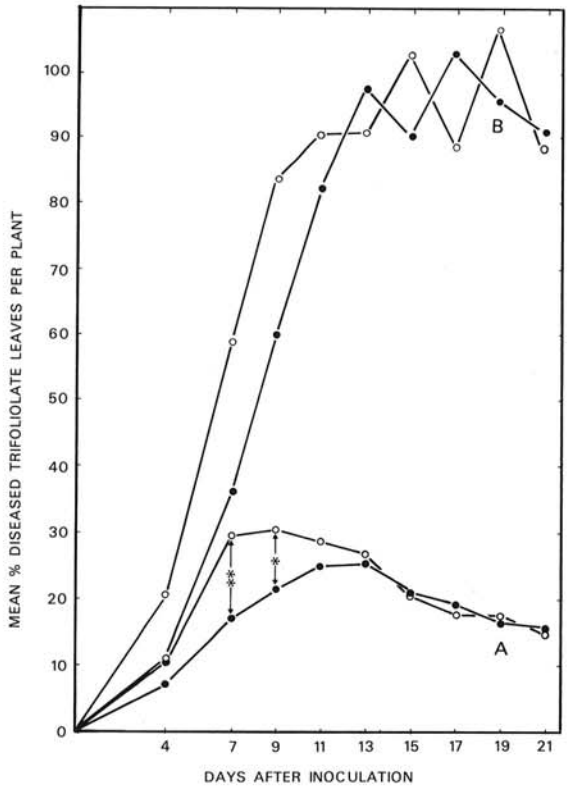

Fig. 1. Time-course development of clover scorch (caused by *Kabatiella caulivora*) in nontagged (●—●) and tagged plants (○—○) (To identify leaves which were expanded at the time of inoculation, in each plant, just before inoculation, a tag was placed on the petiole of the youngest leaf with an expanded lamina) of *Trifolium subterraneum*. Disease was assessed A: as the mean number of diseased leaves per plant relative to the mean of the total number of leaves per plant present at the time of harvest × 100 (%) and B: as the mean number of diseased leaves per plant relative to the mean of the total number of leaves per plant expanded at the time of inoculation × 100 (%). Differences between tagged and nontagged plants were significant at 7 days (**; $P = 0.01$) and at 9 days (*; $P = 0.05$).

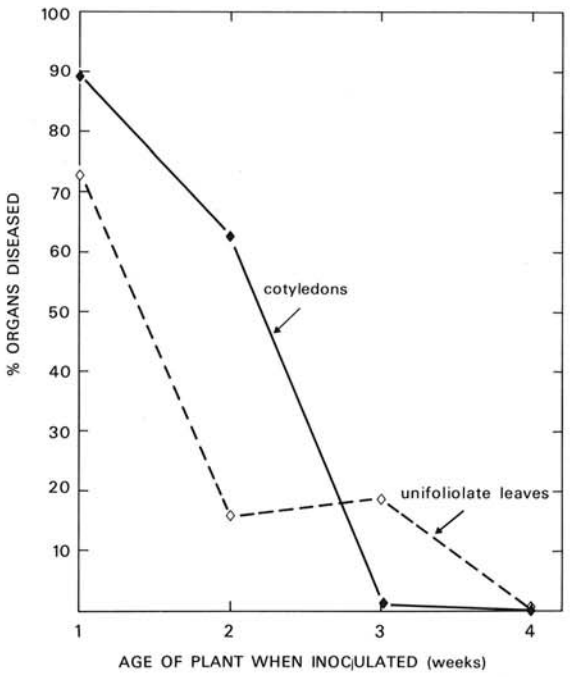

Fig. 2. Susceptibility of cotyledons and unifoliolate leaves of *Trifolium subterraneum* to clover scorch (caused by *Kabatiella caulivora*) in relation to plant age. Data recorded 11 days after inoculation. For cotyledons, differences between 1 and 2 weeks and between 2 and 3 weeks were highly significant ($P = 0.01$). For unifoliolate leaves, differences between 1 and 2 weeks were highly significant ($P = 0.01$).

were harvested at specific intervals after inoculation and attached leaves were rated as diseased, dead, or normal (expanded and folded leaves). Leaves were rated as diseased when one or more lesions were identified, at a magnification of ×2, on the petioles or petiolules, or occasionally on the leaflets. Some leaves senesced during the course of the experiment; leaves rated as dead included attached dead leaves with lesions due to *K. caulivora,* and attached dead leaves without lesions. The total number of leaves per plant is the sum of the attached leaves.

There were no necrotic inflorescences in plants examined immediately before inoculation. Inflorescences of inoculated plants were rated as diseased, dead, or normal. Those rated as diseased were relatively mature with typical lesions on the peduncle just below the flower head. Those rated as dead were relatively young; they were either newly formed and completely necrotic, or the distal end of the peduncle and the developing inflorescence were necrotic. The total number of inflorescences per plant is the sum of the inflorescences in all three classes.

Time-course development of symptoms.—Plants were inoculated 4 weeks after sowing and leaves were examined for disease development at successive intervals. At the time of inoculation, there was a mean of 5.4 expanded leaves per plant. To distinguish these leaves from those which expanded during the course of the experiment, half of the plants were tagged just prior to inoculation, on the partially expanded petiole of the youngest leaf with expanded leaflets. At intervals between 4 and 21 days after inoculation, individual leaves of 18 tagged plants (6 plants in each of 3 pots) and 18 nontagged plants were detached and rated as described above. In tagged plants, records were made of leaves which were younger than the tagged leaf and also infected. The experiment was replicated twice on different occasions.

Age of plants in relation to susceptibility.—Plants were inoculated at different stages of growth, and were examined for disease development 11 days after inoculation. Cotyledons, unifoliolate and trifoliolate leaves, and inflorescences were assessed for disease development. The experiment was done in two parts. Part A: plants were inoculated 1, 2, 3, 4, and 5 weeks after sowing. For each age group, data were recorded from five plants in each of six pots. This part of the experiment was replicated four times on different occasions. Part B: plants were inoculated 8, 9, and 10 weeks after sowing. For each age group, data were recorded from five plants

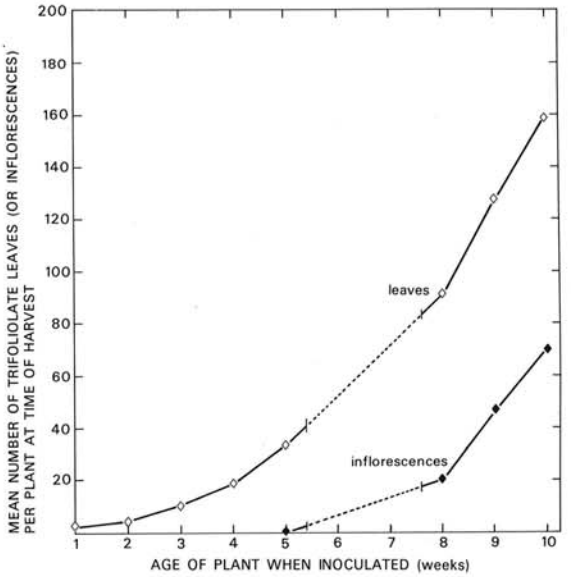

Fig. 3. Numbers of leaves and inflorescences in plants of *Trifolium subterraneum* of different ages inoculated with *Kabatiella caulivora*. Data recorded 11 days after inoculation. All differences between data for pairs of successive ages were highly significant ($P = 0.01$).

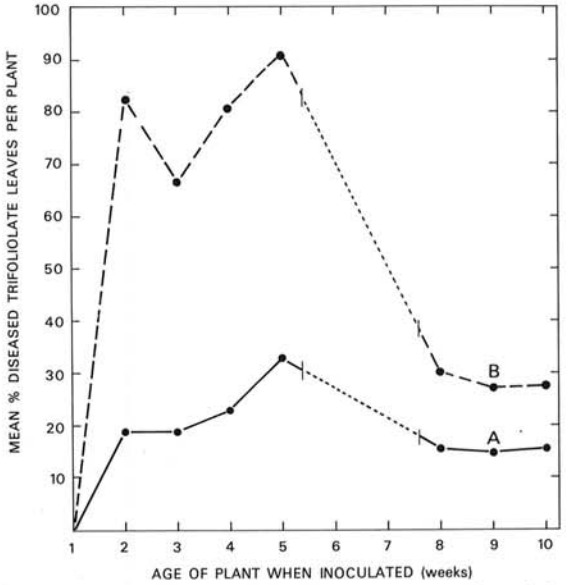

Fig. 4. Susceptibility of trifoliolate leaves of *Trifolium subterraneum* to clover scorch (caused by *Kabatiella caulivora*) in relation to plant age. Data recorded 11 days after inoculation. Disease was assessed A: as the mean number of diseased leaves per plant relative to the mean of the total number of leaves per plant present at the time of harvest × 100 (%) and B: as the mean number of diseased leaves per plant relative to the mean of the total number of leaves per plant expanded at the time of inoculation × 100 (%).

in each of three pots. This part of the experiment was replicated three times on different occasions.

Analyses of variance were made using a $\log_{10}$ transformation for the data expressed as numbers of leaves and inflorescences, and an arcsine transformation for data expressed as percentages. Data for cotyledons and unifoliolate leaves were analysed for plants inoculated 1-3 weeks after sowing. Separate analyses of data for trifoliolate leaves were made for plants inoculated at 2-5 weeks, and at 8-10 weeks. In Fig. 1 and 4, only data for graph A were analysed, because graph B represents a different presentation of the same data.

RESULTS.—*Time-course development of symptoms.*—The purpose of this experiment was to determine the optimum time after inoculation for assessment of infection, and to identify the leaves within a plant which became infected during the course of the experiment. During the 21 days after inoculation that plants were observed, the mean number of leaves per plant increased about 4-fold (from 9.1 - 35.1). There were no differences between tagged and nontagged plants in the time-course patterns for leaf development.

Within individual plants, symptoms usually developed earlier in tagged than in nontagged leaves. Almost all tagged petioles were infected 7 days after inoculation. Disease development occurred only rarely in a leaf younger than the tagged leaf. This indicated that folded leaves present at the time of inoculation either were "resistant" to, or escaped from, infection and that during the course of the experiment, spread of the disease beyond the expanded inoculated leaves did not occur. Therefore, as well as comparing numbers of diseased leaves in

TABLE 1. Incidence of dead inflorescences in clover plants inoculated with *Kabatiella caulivora* and in uninoculated plants which received the same environmental treatment

	Dead inflorescences (%)[a] of plants treated at age		
	8 wk	9 wk	10 wk
Inoculated plants[b]	20.26 (23.22)	26.42 (28.95)	31.97 (34.41)
Uninoculated plants[c]	3.09 (8.85)	2.28 (6.26)	6.94 (14.20)

[a]Arcsin-transformed values in brackets.
[b]Three replicates, each with 15 plants for each age group.
[c]One replicate, with 30 plants for each age group.
LSD's (based on arcsin-transformed values:
For row of inoculated plants: ($P = 0.05$) = 4.20
($P = 0.01$) = 5.75
For columns: ($P = 0.05$) = 5.61
($P = 0.01$) = 7.54

relation to the total numbers of leaves present at the time of harvest (%; Fig. 1-A), it was of interest to compare numbers of diseased leaves in relation to the numbers of leaves expanded at the time of inoculation (%; Fig. 1-B). At the time of harvest, 25-30% of leaves present had developed symptoms, while 90-100% of leaves expanded at the time of inoculation developed symptoms.

For both tagged and nontagged plants, the percentage of diseased leaves increased significantly ($P = 0.01$) between days 4 and 7 (Fig. 1-A). For tagged plants, a maximum of 30% was reached on day 9, and this was followed by a significant decrease ($P=0.01$) between days 9 and 15. For nontagged plants, a maximum of 25% was reached on day 13, and this was followed by a significant ($P=0.01$) decrease between days 13 and 21. The decrease was associated with increases in numbers of leaves, and with loss of some leaves due primarily to the disease or to senescence. Differences between tagged and nontagged plants were significant on day 7 ($P=0.01$) and day 9 ($P=0.05$) (Fig. 1-A).

Age of plants in relation to susceptibility.—1) Cotyledons and unifoliolate leaves.—When inoculated one week after sowing, seedlings possessed cotyledons, one unifoliolate leaf, and no trifoliolate leaves; two weeks after sowing, the mean number of trifoliolate leaves was 1.5. Young cotyledons and young unifoliolate leaves were highly susceptible and susceptibility decreased markedly with age (Fig. 2).

—2) Trifoliolate leaves.—Numbers of trifoliolate leaves per plant (as observed at the time of harvest), for plants inoculated at 1-5 and 8-10 weeks after sowing, are shown in Fig. 3. The percentages of these leaves which were diseased are shown in Fig. 4-A. Leaves of plants inoculated 5 weeks after sowing were significantly more susceptible ($P = 0.01$) than were those inoculated at 2, 3, and 4 weeks. Differences for plants inoculated at 2, 3, and 4 weeks were not significant. The susceptibility of leaves of plants inoculated 8-10 weeks after sowing was relatively low, and no differences were significant.

When numbers of diseased trifoliolate leaves were examined relative to numbers of leaves expanded at the time of inoculation, they reached a maximum of 91% in plants inoculated 5 weeks after sowing (Fig. 4-B) and decreased to about 30% in plants inoculated 8-10 weeks after sowing. The data indicate that expanded leaves of young plants are more susceptible than are those of relatively old plants.

—3) Inflorescences.—Numbers of inflorescences per plant, as observed at the time of harvest, are shown in Fig. 3. The percentages of these which showed typical symptoms of disease were 3.0, 8.5, and 9.4% for plants inoculated 8, 9, and 10 weeks, respectively, after sowing. The differences between 8 and 9 weeks was significant ($P = 0.01$), but the difference between 9 and 10 weeks was not significant. With similar calculations, the mean percentages of dead inflorescences were considerably higher (inoculated plants; Table 1). The combined totals of diseased and dead inflorescences in plants inoculated 8, 9, and 10 weeks after sowing were 23, 35, and 41%, respectively, per plant.

Since dead inflorescences were not present in plants before they were inoculated, inflorescences could have died primarily as a result of infection by the pathogen, and/or primarily as a result of the environmental treatment. These possibilities were examined by comparing the percentages of dead inflorescences in inoculated plants of different ages, with those in control, uninoculated plants which received the same treatments as the inoculated plants, except that they were sprayed with water and a wetting agent instead of inoculum and a wetting agent. In a preliminary experiment, inflorescences died in uninoculated plants as well as in inoculated plants, but there were fewer dead inflorescences in the former than in the latter. To avoid the possibility that death of inflorescences in uninoculated plants could have been associated with infection by the pathogen, another experiment was made with uninoculated plants only; i.e., there was no possibility of contamination. Again, some inflorescences of uninoculated plants died. The data, which were quantitatively similar to those in the preliminary experiment, are compared with those for inoculated plants in Table 1. For all three ages, differences between the percentages of dead inflorescences in inoculated and uninoculated plants were highly significant. It is evident that in experiments in which plants were inoculated with *K. caulivora,* a significant number of inflorescences died as a result of the disease, whereas others died because environmental conditions were unfavorable for development of inflorescences. It follows that the percentages of diseased inflorescences recorded in the text above are an underestimation of the percentages of inflorescences which were in fact diseased.

DISCUSSION.—Leaves expanded at the time of inoculation were the sites of primary infection. In plants inoculated 4-5 weeks after sowing, but not in plants inoculated after 8-10 weeks, almost all leaves expanded at the time of inoculation became diseased. Since plants were watered from above (after the 4-day incubation period), spread of the pathogen by splash from initially formed lesions to leaves which expanded during the course of the experiments could have occurred, but for this there was no evidence. Even if spread of conidia did occur, the environmental conditions were probably unsuitable for development of secondary infections.

The clover scorch disease generally attacks red clover plants in the first harvest year, but it is capable of attacking cotyledons and leaves of seedlings with only 2-3 trifoliolate leaves (3, 10). With subterranean clover, which is annual, the disease is most obvious in vigorous, ungrazed stands kept for hay or seed production (2, 5, 8) and it has been suggested that plants are more susceptible close to the time of flowering than earlier (5). In the present experiments, plants up to 5 weeks old were highly susceptible. In seedlings inoculated one week after sowing, 89% of cotyledons and 73% of unifoliolate leaves became infected. Trifoliolate leaves became susceptible as they unfolded, and in 5-week-old plants up to 90% of leaves which were expanded at the time of inoculation became infected. At the time of flowering, leaves were less susceptible than were those of younger plants.

Subsequent to inoculation, peduncles of older inflorescences may develop typical symptoms of infection, whereas younger inflorescences may become completely necrotic. The association of necrosis of young inflorescences with infection by the disease does not

appear to have been reported before. It is of interest that environmental conditions which promoted death of young inflorescences in inoculated plants, also promoted death of young inflorescences in uninoculated plants. A 4-day treatment at high humidity and low light appears to be unfavourable for development of inflorescences.

Symptoms developed earlier in tagged than in nontagged plants and also tended to be more severe in tagged petioles than in nontagged petioles. Perhaps, slight injury of hairs caused by handling the plants during placement of tags resulted in the exudation of sap which in turn promoted initiation of more infections or perhaps, displacement of epicuticular wax permitted easier entry of the pathogen. These possibilities are in accord with field observations which suggest that the disease is favored by plant damage, which may result from grazing, insects, hail, or vehicle movement (4).

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