DEPARTMENT OF AGRICULTURE, SOUTH AUSTRALIA

Agronomy Branch Report

KABATIELLA CAULIVORA (KIRCHN) KARAK-THE CAUSAL ORGANISM OF NORTHERN ANTHRACNOSE OR CLOVER SCORCH

This summary of literature was compiled in 1972 as a background for a research project on the biology of Kabatiella caulivora.

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Research Officer (Plant Pathology)

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1. OCCURRENCE & HOST RANGE:

1.1 World Distribution

The first records of northern anthracnose were those of Mehner[33] (1901) and Kirchner[25] (1902), both in Germany, with Trifolium pratense (red clover) recorded as the host. It was first reported in America on red clover by Sheldon (1906) in West Virginia[22]. In 1915 in the Riazan province in the Republic of Russia, Kabatiella was the only serious disease of red clover with a widespread distribution[29]. Scorch was first recorded in the British Isles by Petherbridge[46] in 1920 near Cambridge and in the same year, it was reported from Buckinghamshire, Shropshire, Norfolk, Lincolnshire and Cardiganshire; the attack sometimes being very severe. Between 1920 and 1928, the disease was recorded in Kent, Hampshire, Glamorgan Yorkshire, Derbyshire, but the damage was not as serious as in 1920[46].

It is now one of the major diseases of red clover in the cool moist regions of North America, Europe, Asia and Australia. The disease has been collected only in higher latitudes, e.g. it occurs in the U.S.A. from Maine to Minnesota and south to Missouri and North Carolina[5, 22, 32, 41, 51], hence the name "northern anthracnose". The Commonwealth Mycological Institute published a map in 1958 which summarised the world distribution at the time[2].

In 1955, Walker[48] recorded clover scorch on the Trifolium subterraneum cultivars Bacchus Marsh and Mount Barker in the Berrigan irrigation area in New South Wales and it was seen in South Australia in the same season[53].

Leach[27] reported in 1962 that Kabatiella had become a serious disease of Trifolium incarnatum (crimson clover) in Western Oregon. The disease had been most severe during cool moist springs and complete

collapse of clover stands had occurred. It was a virulent pathogen of crimson clover but innocuous on red clover under field conditions in Oregon. There have been no reports of severe attacks on crimson clover in any other country.

1.2 Distribution in Australia

Walker[48] recorded the first losses of pasture due to Kabatiella attack in Australia (as mentioned above). During the spring in 1955 several hundred acres of subterranean clover were severely damaged and grazing was considerably reduced over a large area. Scorch had been observed by growers in the previous season but it had not caused much damage.

Since 1955 in South Australia, clover scorch has been seen in subterranean clover throughout the higher rainfall areas but only occurring in isolated patches that were of no economic significance. However, following the wet winter in 1968, a severe outbreak of the disease occurred on Kangaroo Island, mainly affecting the cultivar, Yarloop. The incidence of the disease increased in following years and affected other cultivars, Woogenellup, Seaton Park and Mount Barker. In 1971, serious outbreaks of Kabatiella occurred in the Adelaide Hills and the South Eastern area of the State causing most damage to Yarloop[3].

The main areas affected in Victoria are in the north-central region and to the west of Geelong. Although Yarloop is the most susceptible cultivar, severe losses have occurred in other subterranean clovers. Field observations have shown that Bacchus Marsh is more tolerant; red clover is also susceptible but white clover (T. repens) is resistant[23].

In Western Australia, the disease has been obvious since 1966 and the severity varies within districts, possibly due to differences in local weather patterns. In 1971 in the Esperance area, all pad-

docks inspected had the disease irrespective of the variety of clover. Yarloop was also badly affected in the Mount Barker-Albany area[6].

1.3 Losses Caused by Kabatiella infection

1.3.1 Perennial pastures

In the perennial red clover stands of the northern hemisphere, Kabatiella causes reduction in both the quantity and quality of hay cut from diseased stands. Secondary shoots are produced by badly affected plants before the first hay crop has been taken and the second cut is thereby reduced even if it escapes an attack of the disease[46].

Heavily infected plants produce few or no seeds as flowers on infected stems wilt or detach so seed production becomes unprofitable in areas where anthracnose is found[9].

Leaf area is greatly reduced by the fungus attack impairing photosynthesis and carbohydrate metabolism. Continuous production of shoots by infected plants throughout the growing season utilises large amounts of food materials. Plants over-winter with insufficient root reserves for survival. Population levels are reduced and the planting is of little further value[7].

Sampson[46] noted that farm crops of red clover were sometimes attacked in the seedling year necessitating resowing.

1.3.2 Annual pastures

In the annual pasture situation occurring in Southern Australia, there are similar losses in hay production with many pastures completely losing their sub. clover component before the hay has been cut. As a result, paddocks are either not closed for hay or are reopened to grazing as no hay of worthwhile nutritive value can be cut. In Victoria, 173 bales/hectare would be expected so a complete loss due to Kabatiella would cost the farmer $69/20/ha (at 40 cents/bale) or $103.80/ha (at 60 cents/bale)[23].

Spring pastures can be severely reduced. Pastures showing only moderate infection are reported to have had their stocking capacity halved. In some severe cases, stock had to be agisted or grain purchased to offset the loss in pasture production[3].

There is also a drastic loss of seed production. Many infected pastures set no seed at all, particularly Yarloop and Woogenellup which mature earlier than other cultivars. Such failures mean that potential seed yields in older paddocks are reduced by as much as 70-80%, the only seed present being hard seed buried in the soil in previous years. In many cases paddocks set aside for seed production are not worth harvesting, despite attractive prices for seed[6].

1.4 Differences in susceptibility of cultivars of *Trifolium pratense*

Early workers recording the incidence of *Kabatiella caulivora* on various cultivars of red clover noted that different nationalities of red clover varied in their relative resistance to the disease in the field. Kirchner found in Germany in 1902[25] that Northern French red clover suffered more severely than any other and suspected that the fungus might have been introduced by seed from this district. Linhart[28], however, in a more extensive tour of Germany and Bohemia in the same year found the fungus widely distributed on both European and American clover. Malkoff (1902)[31] recorded a severe attack on red clover plots of Italian, South French and North American origin, a slight attack on those from the west of North America, while lots from Bohemia, South Russia, Poland and Canada were entirely resistant. Gran and Rostrup (1923)[16] recorded the disease in Denmark where it occurred only on red clover from Switzerland and Czechoslavakia. Ware (1923)[50] found in England that the most susceptible variety in a severe attack of the disease in that year was English broad red, while Chilean and English cowgrass and English late flowering were less

susceptible and perennialised broad red and Danish hersnap were almost immune. Pieters[39] (1924), in Tennessee, reported that clover from southern Europe was more resistant than that from America or northern Europe. Monteith[35] found the opposite that red clover of Italian and southern European origin was most susceptible to Kabatiella injury while some strains of American red clovers were the most resistant. Mains[30] in Indiana had similar results; a moderate to slight infection in French, Italian, Altaswede, Indiana, Oregon, Hungarian, Rumanian and Polish strains while Chilean and most North American strains proved nearly immune.

Sampson[46] found in Wales, for the conditions prevailing during the seasons under review, the attack of Kabatiella was relatively more severe on the early flowering red clovers. She continued to make observations on the late clovers up to the date of cutting which confirmed the difference shown some weeks earlier and indicated that in the field, these strains suffered less than those which flowered earlier in the season.

Horsfall[22] postulated in 1930 from the evidence above that southern European varieties seemed most susceptible while the northern ones were resistant. If the disease was a northern one, then clovers grown constantly in its presence were more likely to be resistant, whereas clovers not normally subjected to it should have been more highly susceptible. The fungus had a relatively low optimum temperature 20°C (with a minimum temperature 4°C) for causing disease.

1.5 Host Range of Kabatiella caulivora

The first reports that Kabatiella infected species other than T. pratense in the field were from Walker[48] in Australia who found an irrigated stand of T. subterraneum var. Bacchus Marsh with scorch, and Leach[27] who reported that in Western Oregon, unlike other temperate

regions, northern anthracnose was not an important disease of red clover but a virulent pathogen of crimson clover (T. incarnatum).

Many workers have artificially infected a range of Trifolium species as well as species from other genera that are important pasture legumes. Their records do not always agree and are summarised in Table 1.

Walker[48] tested a range of subterranean clover cultivars for susceptibility and found that lines of the same cultivar from different sources may react differently, so he concluded that it was not possible to generalise about the behaviour of a cultivar towards the disease.

Sampson[46] found similar differences with the cross pollinated red clover. Although within a strain, individual plants of red clover showed marked resistance to the disease, an immune plant was not found. She also conducted an experiment which suggested that the resistance shown by clover plants in the field to the disease was not comparable with that shown by rust-immune varieties of wheat (which are found to be immune also under optimum conditions for infection), but was similar to those varieties which were rust-escaping under certain field conditions.

1.6 Physiologic specialisation of Kabatiella caulivora

Hanson[20] compared isolates from the northern clover areas of the U.S.A. and found that Kabatiella caulivora was comprised of an indefinite number of physiologic races. All isolates were started from single spores. On synthetic media, isolates differed in colour and topography of colonies, in ability to bud or produce mycelium, in tendency to sector and in rate of growth. All of the isolates grew within the temperature range 4-28°C but grew best at 20-24°C. Two of the Wisconsin isolates grew approximately twice as fast at optimum temperature as most other isolates. One isolate differed from all others in

Table 1: Host Range of Kabatiella caulivora

Species	Author (1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	Days to Symptom Expression (9)	Site of Infection (9)
T. alexandrinum						S	SL		17	L&P
T. angustifolium									10	L&P
T. dubium							SL		17	L&P
T. fragiferum							M-SL		10	L&P
T. hirtum									16	L&P
T. hybridum			✓		FR	M-SL		R	R	
T. incarnatum	S				R	M-SL	S-M	✓	15	L&P
T. glomeratum									8	L&P
T. lappaceum						S			R	
T. medium		✓			R	M-SL			10	L&P
T. pallidum									10	L&P
T. pratense	M-SL	✓	✓	✓	S	S	S-SL		8	L&P
T. purpureum		✓			FR					
T. repens				✓	SL	M-SL	SL	SL	19 R	L
T. resupinatum						M-SL			R	
T. striatum						M-SL			R	
T. suaveolens				✓	✓					
T. subterraneum						M-SL	S-SL-R		R	
T. wormskjoldii						M-SL				
Medicago lupulina					R			SL	R	
M. sativa					R			SL	20	L
Melilotus alba									19	L
Onobrychis sativa								✓		

Authors:

(1) Leach[27]
(2) Malkoff[21]
(3) Fulton[15]
(4) Stapledon
(5) Sampson[46]
(6) Hanson[21]
(7) Walker[48]
(8) Wellensiek[52]
(9) Cole[7]

Key:

S = severe M = moderate SL = slight FR = field resistant
R = resistant ✓ = infection obtained l = leaf P = petiole

Resistant Species in Cole's test: (Table 1 Contd.)

Lotus corniculatus	T. phleoides
Melilotus indica	T. tembense
Melilotus officinalis	T. subratundum
Medicago falcata	T. amobile
Medicago minima	T. warinskoldii
Medicago scutellata	T. carolinianium
T. nigrescens	T. corniculatus
T. variegatum	T. apellianum
T. ambiguum	T. semipillosum
T. pamonicum	T. agrarium
T. scutatum	T reflexum
T. arvense	T. ismocarpum

that it grew well at 28°, the rate of growth of the other isolates decreased rapidly when the temperature exceeded 24°C. At least several strains could be distinguished on the basis of pathogenicity tests on differential strains of red clover.

Darunday[13] in 1967 also observed distinct differences in pathogenicity and virulence among isolates. Clones of red clover differed greatly in their reaction to given isolates. He concluded that plants within most cultivars of red clover differed greatly in reaction to Kabatiella so it was possible to improve the resistance of almost any cultivar of red clover by eliminating the more susceptible plants. Highly resistant new cultivars could be developed by the procedure. He warned, however, that since the fungus was composed of many races which differed in their ability to infect a given plant, it was imperative that breeding lines should be tested against a composite of isolates.

Martin[32] suggested a detached leaflet method for studying the differences in susceptibility of clover varieties to Kabatiella, as well as determining the differential pathogenicity of isolates. The advantages of this method over methods in which whole plants were

inoculated, were that many treatments could be imposed on the leaves of a single plant, less greenhouse and moisture chamber space were required and less labour was involved. He made preliminary studies of this method by inoculating plants with an atomiser either before or after detachment, floating them on a 2% cerelose solution at 21-24°C subjected to the daily cycle of light and dark. Symptoms appeared 7-14 days later and excellent agreement was obtained between the results with detached and attached leaves. Differences between isolates were consistent.

1.7 Genetics of Resistance

Athow and Davies[1] in 1957 attempted to determine the genetics of resistance to both northern and southern anthracnose (Colletotrichum trifolii). They selected red clover plants of the varieties Dollard and Purdue which were resistant to Kabatiella caulivora and plants of the variety Kenland which were resistant to Colletotrichum trifolii, after artificial inoculation in the glasshouse. An attempt to combine the resistances gave 9% of the F_2 progeny with resistance to both diseases indicating that resistance to each disease is recessive. The F_2 segregation in crosses of plants resistant to Kabatiella with plants susceptible to this disease did not fit a 3:1 ratio. However, the F_2 progeny from 13 of 15 F_1 plants in one cross and from 13 of 16 F_1 plants in another cross did segregate in a ratio of three susceptible to one resistant, indicating a single factor segregation.

2. EPIDEMIOLOGY:

2.1 The Influence of Weather Conditions on Disease Severity

The amount of damage caused by northern anthracnose is very dependent on weather conditions. Niemczyk and Kiesling[37] reported that in the 1959 and 1960 growing seasons in Michigan, Kabatiella was found in most fields examined but caused only minor damage. They concluded that the inoculum was present but the weather conditions were

not suitable for an epidemic. In ordinary years, K. caulivora caused little damage in Illinois clover fields as it is a cool, wet weather disease. But when weather conditions were favourable to its development, as they were in the springs of 1945 and 1946, severe epidemics occurred[5]. Sigrianski and Potapova[47] found that hot and dry weather in the summer of 1936 arrested the spread of the disease which developed fully only from September when there was an average temperature of 9-16.2°C and an average relative humidity of 79-85% till the onset of snowy weather.

Boewe in 1946[5] reported a survey in Illinois that showed K. caulivora to be widespread and destructive in some fields, being very severe in central Illinois, light in the south and less severe in the north. The losses varied from a trace to 75% of the hay crop. By examining mean temperatures and rainfall, he found that average temperatures for May were below and the rainfall was above the state averages when the attacks were severe.

Other people have reported severe attacks of Kabatiella only when seasonal conditions were abnormal, as in 1956 in New England and New York[24]. The spring was wet and late, the humidity high and temperatures cool during June, while July and August were dry. Northern anthracnose occurred on red clover with up to 90% defoliation. The least injury among all the strains planted was 20% defoliation in the variety New Wisconsin. The disease was still developing in September and differences between host strains were still obvious.

In West Virginia, the rainfall during the 1953 season was abnormal, involving an extended period of uniformly wet weather until June, followed by drought for the remainder of the season. In these conditions northern anthracnose was a major disease on red clover[14].

Since the disease was first recorded in New South Wales, there have been three or four seasons in which it has caused quite severe

damage to subterranean clover pastures. In each case, the disease has been associated with damper and cooler weather conditions in winter and early spring. In most years, the disease is not recorded as a problem[49].

Roberts[42] examined the possibilities of a disease forecasting system when weather conditions occurring as long as a month or more before the first harvest of hay, affected the severity of disease. Horsfall[22] determined that rainfall and temperature conditions in the autumn and spring preceding harvest were the environmental factors that most strongly influenced the severeity of the disease. Roberts[42] recorded the number of days during which there was measurable rainfall and also the total rainfall occurring in October and May before harvest. The mean autumn temperatures from 1951 to 1955 did not vary sufficiently in any one of three widely separated areas to have accounted for important differences in the amounts of over-wintering structures of the fungus. Mean temperatures for the spring months were variable from year to year and were recorded because they were considered important in the production of inocula for both primary and secondary cycles of the disease. K. caulivora was more severe in 1953 than in any other year and the number of days with measurable rainfall during the preceding October and May had been 17 and 18 (Table 2). This was apparently more important to the disease severity than was total rainfall. The disease did not become an epiphytotic, regardless of total rainfall, unless there had been at least 18 days with measurable rainfall during the May before the harvest. Further, when the number of rainy days did not limit disease development as in 1952 and 1956, the number of rainy days during the October before harvest was more nearly correlated with disease severity than was total rainfall. Rainy weather in October preceding harvest would favour infections that would provide an abundance of over-wintering inoculum as

Kabatiella survives winters on diseased stems and leaves. Kabatiella develops in the spring as a consequence of secondary cycles of the disease when periods of wetting favour production and dissemination of secondary inoculum.

Table 2: Estimated Percentage Hay Loss Due to Northern Anthracnose on Red Clover in Central New York 1952-56 and Rainfall & Temperature Data (Roberts[42]).

Year	% Hay Loss	Rainfall				Mean Temperature (°F)		
		Total (in)		Number of Days				
		October	May	October	May	March	April	May
1951-52	1.2	1.0	4.7	8	20	32	48	53
1952-53	6.0	3.1	3.4	17	18	36	44	58
1953-54	1.5	1.5	3.3	10	7	33	47	55
1954-55	2.0	1.6	1.7	6	7	35	50	58
1955-56	1.3	8.2	1.9	9	12	28	41	51

2.2 Conditions Required for Infection

The weather conditions described as inciting severe attacks of scorch correlate with the needs of the pathogen to infect host tissue. Many workers have studied the temperature range and optimum temperature for Kabatiella infection as well as the high relative humidity requirement of the pathogen. The early workers[20, 36, 46] reported 20°C as the optimum temperature for growth and infection. The first critical studies of the moisture and temperature requirements for the production of artificial epiphytotics were made by Cole and Couch[8]. They found 32% of plants at 20-25°C were infected, with symptoms appearing 8 days after inoculation, 9% of plants at 15-17° were infected with symptoms appearing within 12 days while only 1% were infected at 25-28°C. At 20°C, the minimum exposure to a saturated atmosphere required for infection was 3-5 hours and the percentage of plants infected increased with increased exposure up to 60 hours, where 33% were infected.

Martin[32] found that scorch was most severe at temperatures between 20° and 24°C, with three or four days of high humidity following inoculation. Disease severity at 16°C in 3 or 4 days of high humidity compared favourably with disease development at 20-24° but development was slower at the lower temperature. No disease occurred at any temperature without exposure to high humidity. At all temperatures, disease severity was greater when the plants were subjected to 3 or 4 days of high humidity than when this period was for 1 or 2 days.

Darunday[13] in 1967 found the optimum temperature was near 24°C. The disease ratings of inoculated clover plants were 5.7 at 20°C, 6.6 at 24°C and 1.0 at 28°C. However, only one isolate and one clone of red clover was used. Further studies showed that isolates differed in their temperature optima. The disease developed well at 20-24°C. It could also cause considerable damage at 16° but it developed slowly at this temperature. High temperatures completely stopped the disease.

The disease develops over a wide range of light intensities but is favoured by low light intensity, particularly during the first few days after incoulation[13].

The density of inoculum influences disease severity. Cole and Couch[9] found no infection with spore concentrations of 63,000 spores per ml or lower, 3% plants infected with 125,000 spores/ml and 30-48% infected with 4×10^6 spores/ml. Martin[32] found that inoculum should contain a minimum of 122,500 spores/ml and managed to infect 93% of his plants with 4×10^6 spores/ml. Darunday[13] obtained infection with 2,000 spores/ml and found no significant increases in disease level by using inoculum containing more than 1,250,000 spores/ml on a less susceptible clone and more than 442,000 spores/ml on a more susceptible clone. He suggested that it was important not to dilute inoculum below 500,000 spores/ml

and 1 x 10^6 spores/ml was better for testing breeding materials with some resistance. The addition to the inoculum of a sticker to reduce runoff of the spores while the plants were in the moisture chamber was also recommended.

2.3 Symptoms of Kabatiella caulivora

The attack of K. caulivora on red clover is almost entirely confined to the stems and petioles of the plant. The general effect of an attack in the field is blackened and broken stems, withered petioles and brown dead leaves so the name "scorch" has been aptly used to describe the disease. Plots of badly affected clover, at an early stage of the attack, have a lighter coloured appearance than plots of healthy clover. This is due to the large number of leaves which expose their lighter coloured under-surfaces because they are hanging from wilted petioles and petiolules. The effect might at first be attributed to damage by wind. Injured leaves soon wilt, become dry and brown and remain attached to the plant until mechanically broken off[2, 46].

Petiolules are extremely vulnerable to attack by Kabatiella, and when infected, they turn dark brown and shrivel causing the leaflets to twist and expose their undersides. The fungus may spread from the petiolules along the midrib and in the ground tissue at the base of the lamina which may then be covered with acervuli[21].

Infections of the lamina (independently of the pulvinus) also occur when plants are heavily infected both in the field and under experimental conditions. Symptoms on the leaflets vary considerably on different clover strains. Usually, the veins become dark and prominent, small to large elongate lesions with dark margins and light coloured centres develop or large areas of the leaves turn olive grey and later light to dark brown as if they had been scorched. Any part of the leaf may be affected. Acervuli develop on both surfaces of the lesion when the leaves are kept moist[21, 46].

Stem lesions are usually quite characteristic. The first sign of infection is a slightly depressed, dark brown streak or a small dark spot which spreads chiefly in the longitudinal direction. The blackened cells form an area slightly sunken below the surrounding green healthy tissue. When the diseased area increases, the central part becomes lighter in colour, and as the stem grows in length and thickness, a slit often appears in the centre of the lesion. In dry weather especially, the margins of the slit roll inwards, enlarging the opening which finally coalesces with the pith cavity present in some stems of red clover. Greyish-white masses of conidia and conidiophores become conspicuous within the central depressed area of the lesions after 10-20 days. The weakened stem or petiole often bends at the lesion site producing a typical "shepherd's crook" symptom. If a bend occurs near the base of an otherwise healthy shoot with several branches, these show negative geotropic curvatures and for a time continue their growth, but eventually the stem may be completely girdled and the death of the whole shoot above the lesion follows.

On red clover, lesions are often found just below the flower head and the disease may seriously affect seed production. Almost total losses of seed associated with an attack of this disease have been recorded[46].

2.4 Infection & Development Within the Host

In studies of the infection process of Kabatiella on a susceptible clone of red clover, it was noted that the number of spores which germinated compared to the number of spores present on the surface of the host tissue was very low and less than 1%[9]. Germination, as evidenced by the formation of germ tubes has been observed 12-48 hours after inoculation. Swelling at the tip of the spore was the first indication that a germ tube was about to be formed. Within 48

hours, many spores had germ tubes approximately half the length of the spores and occasionally longer. From 48-72 hours, the hyaline conidia produced germ tubes which were four times the length of the spore. Sometimes the hyaline conidia changed their appearance prior to or during germination by becoming darker in colour, one or more septate and different in shape. Germ tubes were relatively wide and produced at random from all parts of the spore surface, frequently two or more at a time. Germ tubes were often observed adjacent to stomata but were never seen to enter stomata[9, 32, 45, 46].

Appressoria were formed occasionally after 48 hours and direct penetration occurred 48-96 hours after inoculation although Sakuma and Shimanuki[45] saw no appressoria. Cole and Couch[9] stated that penetration appeared mechanical.

The first indication of penetration was a distinct brown colour in the wall of the epidermal cell upon which the spore rested. Germ tubes were seen inside the host cell wall which was distinctly swollen at the point of penetration. The entering hyphae appeared to travel for some distance in the cell wall immediately below the cuticle and were never seen to penetrate the cell. The protoplasm of epidermal cells thus infected was contracted and slightly discoloured. The cell walls were distinctly swollen for some distance in front of the germ tubes as was also the case where advancing hyphae were observed in the host tissues on the margin of an older lesion. The mycelium was scanty and difficult to trace until the tissues were completely disorganised[46].

Fungal hyphae remained intercellular and adjacent to the epidermis in the cortex during the early stages of the disease development while the contents of adjacent epidermal cells became brown and discoloured. After 72 hours, little mycelial proliferation had occurred; the most striking evidence of the fungus advance was the browning of cell walls and ultimate collapse of cells with their con-

tents turning black. The fungus was present in the cortical cells 7-8 days after inoculation and later was not restricted to any specific tissue. Fungal hyphae appeared to be both intra- and intercellular throughout the tissue and spread laterally through the cell walls of the cortex into the vascular tissue. Cole and Couch[9] reported that the vascular bundles remained relatively intact although the collapse of surrounding parenchymatous tissue caused them to be displaced. Sakuma and Shimanuki[45] found hyphae penetrated the phloem in which abundant branches and septa were formed. Complete disruption of the plant tissue resulted from the mass of fungus hyphae which was present prior to the formation of acervuli. Acervuli were formed sub-epidermally and the conidiophores and conidia were seen after the acervulus had broken through the epidermis. Conidiophores and fungal hyphae could also be seen growing from stomata on the leaflets.

In the leaves, the hyphae were both intercellular and intracellular and were not restricted to any specific tissue. Infection resulted ultimately in the almost complete destruction of the attacked tissue. Only the xylem elements in the vascular bundles retained their characteristic appearance and often they were displaced as a result of the collapse of the surrounding tissue[32].

Sakuma and Shimanuki[45] report a hypersensitive reaction to Kabatiella infection in resistant clones of red clover. Cells at the infection site were not killed in the early stages of infection although minute black flecks appeared after 7 days. Degeneration of phloem was limited to only 2-3 cell layers which were coagulated.

2.5 Production of Conidia in Nature

The minute white acervuli occurring on diseased areas consist of short conidiophores, not unlike basidia in shape, growing parallel to each other and pushing through the epidermis. Conidia are produced from the apex by a process of budding and are usually in groups of 3-5

but as many as 9 may be found at one time on a single conidiophore. The conidia are apparently sessile but after dispersion, very minute papillae can be seen occasionally on the conidiophore. The conidia when free from the conidiophore are held together to some extent by their mucilaginous walls. The coating of mucilage around each spore may be seen by staining with very dilute gentian violet and mounting in water[46].

Kirchner[25], who originally described the species, reported that conidia ranged in size from 12-22μ x 3.5-5.3μ. Sampson[46], working with isolates from the British Isles, described mature spores as being hyaline, oblong, slightly bent and tapering at each end, some being slightly wider at the end remote from the point of attachment with the average measurement 14.6 x 3.5μ and the range 8-24 x 2.5-3.5μ. No other spore form was observed on the host and setae were never found in or near the acervulus. Martin[32], is the only worker to report measurements of the spores of American isolates. They ranged in size from 3.9-30μx 1.1-6.1μ. Most of the spores were in the range 11-20μx 3-4μ.

2.6 Dispersal of the Pathogen in Nature

In general, fungi of the order Melanconiales are not adapted to wind dissemination because of the production of spores in sticky masses. Sampson[46] has shown that the conidia of Kabatiella are easily dislodged by water or falling water droplets. Cole and Couch[9] tried to determine if the conidia were wind-borne by placing stem sections with mature lesions upstream from healthy clover two feet away, using both dry and moist lesions. No infection of the healthy plants occurred. Martin[32] attempted to catch spores of K. caulivora in the field using vaseline slides on stakes but was unsuccessful.

Kabatiella did not germinate on agar. Howed plants in front of a spray nozzle in a moist

chamber and healthy plants at the opposite end, symptoms were obtained on the healthy plants indicating that the combination of wind and rain play at least some role in the movement of Kabatiella spores.

Poos[40] (et al 1955) noted a close association of the clover root borer, Hylastinus obscurus, with diseased plants of red clover. He allowed borers to crawl over a culture of Kabatiella, then transferred these to soil at the base of red clover plants. Two out of eight plants developed symptoms. Martin[32] carried this idea further. He suggested that sticky or moist spores adhere well to the hairy bodies of insects so he swept foliage of a diseased field of red clover with an insect net (collecting Miridae, Coccinellidae, Ceropidae, Nabidae) and placed these on susceptible plants for 24 hours but obtained no symptoms. Insects in the family Coccinellidae were sprayed with a conidial suspension of Kabatiella and transferred to the soil at the base of susceptible clover plants but no transmission of the disease occurred. The failure to observe symptoms would seem to indicate that insect transmission plays a minor role in the spread of Kabatiella. However, the temperature used in this experiment (26°C) was close to the upper limit for scorch development.

2.7 Seed Transmission

Kirchner[29] in 1902 suspected that northern anthracnose was introduced into Germany on seed of red clover. Fulton[15] in 1913 noted that "dry spores retain their vitality for a number of months and it is likely that such dust-like spores lodged on the outside of seeds may serve to carry infection".

Sampson[46] examined and tested 52 samples of seed from 12 countries and found no positive evidence for the transmission of the disease by naturally contaminated seed. She did not observe infection of the calyx and corolla and thought the chance of such a method of transmission was small. Flowering shoots infected just below the head

usually suffered severely, the stem was quickly girdled so the head drooped and withered before seed was formed.

Minyaeva[34] found that seed from infected plants bore spots characteristic of anthracnose infection on the seed coat and were usually poorly developed. On spotted seeds, however, no spore germination occurred either in a damp room or on media, and no mycelia were observed. Less than 2% of poorly developed seeds on nutrient media produced fungus colonies. Wellansiek[52] was unable to find Kabatiella in over 3,000 plants grown from seed harvested from 32 diseased fields.

Cole[9] found positive evidence of floral infection and transmission of the pathogen through uncleaned seed. In Pennsylvania during the 1956 growing season he observed lesions on all above ground parts of red clover, including leaves, stipules, petioles, main stems and floral heads. The disease on the floral heads included infection of individual flowers. Infected floral heads generally produced shrivelled seed or plump seed with dark brown spots. If the supporting petiole was attacked early in the floral ontogeny, no seeds were produced. If infection occurred later but before maturity, shrivelled seeds were produced. He collected a seed sample from severely diseased red clover of which 75% of the seed was shrivelled or discoloured and the sample also contained foreign matter including stem pieces, shattered leaves, petioles and dried floral parts. When planted in sterile flats with a saturated atmosphere for four days at 20°C, 15 out of 4,000 seedlings were diseased. Infection was typified by a darkening of the leaf bases and a shrivelling of the petioles of the primary and trifoliate leaves.

Leach[27] examined seed transmission because of sporadic outbreaks of scorch in isolated crimson clover fields never previously sown to clovers. Dr. Noble[27] observed K. caulivora to be seed-borne in Oregon-produced crimson clover during the 1959 international

referee tests conducted by the Plant Disease Committee of the International Seed Testing Association. Leach confirmed this in 1960 and found the disease only associated with crimson clover seeds in Oregon, not on red, alsike or ladino clover tested at the same time.

To determine the incidence of seed infection, Leach obtained 1,000 seeds from a crimson clover field with 30-50% peduncles infected at the time of flowering and plated these on malt agar - only two infected seeds were found. However, by examining 60 seed lots from Marion County in which scorch was widespread, he found 30% of the seed lots infected at levels from 0.4-1.3%. Because the levels of infestation of seed lots was low, there was no visual way of differentiating between infected and healthy seeds. So he used surface disinfection methods to determine whether the pathogen was superficially borne or if it had penetrated deeply into the seed. Surface disinfection for 10 minutes with chlorox:ethanol:water (4:1:8) had no appreciable effect on the incidence of viable colonies obtained from the seeds. However, 10 minutes disinfection completely eradicated the disease from artificially inoculated seeds, 52% of which were infested. He assumed therefore that the pathogen associated with naturally infected seed survived as mycelium that had penetrated the seed coat.

By examining infested seedlings, he found that all seed coats showed invasion and production of sporodochia. No three day old seedling showed evidence of infection except one which had a few intercellular hyphae in the upper epidermis of one cotyledon. In older seedlings, invasion of the cotyledons was general. The hyphae appeared to ramify through the cotyledons and then grow down the cotyledonary stalks into the hypocotyls. In younger seedlings, hyphae were mainly intercellular but in older seedlings, intracellular hyphae were evident. Initial invasion of the cotyledonary stalks was apparently through the cortical parenchyma. In the older plants

(12-15 days) there was a dense concentration of hyphae in the phloem on both cotyledons and cotyledonary stalks. The initial invasion of the hypocotyl was also largely through the cortical parenchyma. Fifteen day old seedlings showed general invasion of cotyledons, cotyledonary stalks and hypocotyls but not radicals. Sporulation was first observed on the cotyledons and, to a lesser extent, on the cotyledonary stalks of 12 day old seedlings. Many of the conidiophores emerged through stomata but some also emerged directly through the epidermal cell walls. After about 9 days, seedlings became moribund with hyphae ramifying profusely through most tissues. An increase in temperature to 30°C slowed down the death rate of seedlings.

These results suggested that seed-borne mycelium penetrates into the testa but not into the embryo of the ungerminated seed. Under field conditions, it is possible that infected seedlings might emerge through the soil surface and thereby act as centres of infection. Under unduly warm conditions, the invasion of diseased plants might well be retarded and the subsequent spread of the disease reduced.

2.8 Survival in Infected Seed

Sampson[46] artificially contaminated seed and stored it for 18 months. At various intervals, she placed some of the seed in a moist atmosphere and examined it for the presence of acervuli. The conidia were still viable after 18 months storage though viability was reduced (Table 3). She also planted 100 artificially infected seeds, and one month after sowing, 27 leaves showed symptoms and developed characteristic conidia. Three days later, 37 more leaves possessed wilted petioles. After 2 months, there were 28 infected plants and 13 healthy. Minyaeva[34] found that the viability of conidia on artificially inoculated seed was significantly decreased as the storage period lengthened. She obtained 92.5% survival at the beginning of the experiment but only 0.28% after 9 months storage. Clover seedlings sown in sterile

soil immediately after inoculation gave 16.9% infected seedlings and 30.7% infected when grown on damp filter paper. Cole and Couch[9] found that conidia sprayed on red clover seed kept at 23-30°C infected healthy plants after storage periods up to 240 days but not after 270 days. When sown, infested seed that had been stored for periods up to 210 days produced diseased seedlings. Symptoms were readily apparent on the seedlings 9 days after germination. The parts attacked included cotyledons, the unifoliate leaf and petiole and the first trifoliate leaves.

Table 3: The longevity of K. caulivora on the surface of red clover seed artificially contaminated (Sampson[46])

Storage Period	% Seed Showing Viable Conidia
3 days	100
6 months	84
8 months	45
12 months	35
18 months	5

These investigations have apparently assumed that survival of Kabatiella associated with seed is by means of conidia. Leach[27] shook a number of infested seed samples with water and detergent, centrifuged the washings and examined these for conidia but none were observed. He stored naturally infected seed at room temperature 18.3-37.8°C at RH 5-50% and at 4, 17, 28 months 0.2, 0.55, 0.18% of seed was infected.

Leach gives several reasons why seed transmission has never been proved convincingly:-

* Infected seeds present at low levels of infestation are easily overlooked when seeds are planted on media or blotters.

* The pathogen grows quite slowly in culture and is quickly obscured by the more rapidly growing saprophytes associated with untreated seed.
* In culture, the macroscopic appearance of K. caulivora can be confused with certain saprophytic fungi associated with clover seed.
* The morphology of the fruiting structures and mycelium in culture are dissimilar to those exhibited on the host plant.

Though the importance of seed dissemination has not been shown under field conditions other than by circumstantial evidence, the infection studies conducted in the laboratory indicated infected seedlings might emerge through the soil in the field and there act as centres of infection. If a sample of seed with 0.5% infected seed was sown at 13.75 kg/ha (325,600 seeds/kg) there would be 2.15 infected seeds per square metre. If only a fraction of these infected seeds emerged, there would still be many centres of infection scattered throughout the field.

2.9 Survival of Free Conidia

Wellensiek[52] reported that clover plants became severely diseased if grown in soil that had been previously contaminated by spraying with a conidial suspension. Cole and Couch[9] stored soil samples, inoculated with a spore suspension, in airtight containers at 18-20°C. Portions were removed at monthly intervals, wetted and brushed on clover petioles and leaves. Infection was obtained from inoculated sterilised and unsterilised soil stored for periods up to 300 days. Martin[32] mixed a spore suspension into non-sterile soil and placed it on top of the ground in December where it was frozen for 3 months. In March it was thawed, mixed with sterile water and flooded onto potato cerelose agar and also sprinkled on the surface of moist red clover foliage. The pathogen was viable and pathogenic after 3 months

freezing. Naturally infested soil, collected in May, was sprinkled on foliage and it also produced northern anthracnose symptoms. It appears that the pathogen can survive for at least several months as conidia in the soil.

Martin placed eight isolates out of doors in November and tested these in January, March and April. All the isolates were viable and pathogenic. The results show that Kabatiella had no difficulty in surviving the wide range of temperature conditions characteristic of Wisconsin winters, regardless of the origin of the isolate.

Cole and Couch[9] studied the length of survival on glass coverslips. Conidia survived 380 days at 3, 6, 9°C (at 0 vapour pressure deficit (VPD) and 5 mm Hg VPD); 360 days at 12, 15, 18°C (5 mm Hg VPD); 150 days at 0 VPD and 150 days at 24°C (5 mm Hg VPD and 0VPD). Conidia were still viable after 20 months at -10°C.

2.10 Survival in Plant Parts

Sampson[46] reported that in Wales, Kabatiella persisted during the winter months in the petioles of leaves infected in the late autumn. A careful search in February revealed a few petiole lesions from which the fungus was isolated after a short period of incubation. A number of old stems covered with lesions were kept over the winter and examined for resting organs of the fungus but none were found. The fungus had apparently perished with the cortical layers of the stem of which little beyond the bleached vascular cylinder tissues was left. Wellensiek[52] also thought that the fungus hibernated in the leaf stalk. The young growing organs of the plant were infected by over-wintered diseased stalks in spring and spores formed on newly infected parts aided the dissemination of the fungus during the growing season of the host. Kabatiella survived at least 2 weeks in leaf stalks buried in sterile soil at -22°C. Horsfall[22] observed

lesions in the early spring and assumed that the fungus over-wintered in the perennial green stems. Cole and Couch[9] collected debris in September and stored it at room temperature 23-30°C. Kabatiella was recovered after 320 days but not 350 days. Debris stored outside in an exposed location survived 400 days but not 450 days. Martin[32] placed diseased hay in the open and in metal cans on the surface of the soil and buried cans at 76 mm and 152 mm. Some were removed in January, March and May, dried, hammer-milled and powdered onto foliage. All samples produced typical symptoms and the pathogen was recovered from them. The experiments established that K. caulivora could over-winter under Wisconsin conditions in infected leaves, petioles and stems of red clover. In the autumn of 1958, 50 plants of red clover showing symptoms of scorch were staked. By the following spring the plants were dead as a result of winter injury but the crown tissue was dried, powdered and sprinkled on susceptible red clover plants. Northern anthracnose developed confirming observations that the fungus could over-winter in dead plant parts.

2.11 Control of Clover Scorch

Wellensiek[52] in 1926 recommended the following control measures:- mowing of fields when infection began to spread, rotation of crops, wide spacing of plants mixing with resistant crops, use of clean seed free from debris, cultivation of resistant varieties.

Martin[32] believed that resistant varieties offered the only practical means of controlling Kabatiella. Resistant plants were present in most red clover varieties and the aim of his work was to supply the information needed to create artificial epiphytotics to screed red clover populations for resistance to Kabatiella.

Other workers have investigated the use of fungicides. Horsfall[22] in 1930 reported that two seasons of field dusting with sulphur gave no control. Ryazantsev[43] tested six fungicides and found zineb

(zinc ethylene bis dithiocarbamate) most promising and most economical. Colloidal sulphur with dinitrorhodane benzene and colloidal sulphur alone were also effective. Shooting, budding and flowering were the most favourable times for treatment. Kudryasheva[26] found removal of plant debris from the field and a spring application of K_2O (90 and 120 kg/ha) gave complete control.

A spray trial in Western Australia in 1971 included Maneb (329, 658, 1316 g/ha), benlate and thiabendazole (1316, 2634, 5187 gm/ha). There was a good control of scorch on Seaton Park at all rates of thiabendazole and the higher rate of benlate[6].

Leach treated naturally infected seed with Spergon (98% tetrachloroparabenzoquinone), Ceresan NI (5% ethyl mercuric phosphate) and Captan (8-% N-trichlomethyl mercapto-4-cyclohexene - 1, 2 dicarboxamide) Kabatiella was completely eradicated with captan and ceresan and only survived on 1 in 2,000 seeds treated with Spergon. Thirteen of 2,000 seeds in the control were infected. The fungicidal treatment of legume seed is complicated by the possible effect of the chemicals on nodule bacteria. Leach considered the possibility of testing and certifying seed for freedom from Kabatiella in the laboratory and field. The pathogen could be readily detected in surface-disinfected seed within 3-6 days by the MA plating method but unfortunately because of the low levels of infestation, it would be necessary to plate very large numbers of seed to obtain accurate reproducible results. Attempting to control the disease through extending the storage of the seed is precluded because Kabatiella can survive at least 28 months in stored seed.

3. CULTURAL CHARACTERISTICS:

3.1 Growth of Kabatiella in Culture

The microscopic features of Kabatiella in culture are not the same as those on infected plant tissue. On agar, the colonies tend

to grow slowly increasing by yeast-like budding and conidiophores are not present. In host tissue, the conidia, though quite similar to those produced in culture, do not show budding. A characteristic feature, however, both in culture and on the host is the scanty development of mycelium[27].

Wellensiek[52] found typical Phoma-like pycnidia in cultures of Kabatiella. The conidia produced in culture were smaller than those formed naturally (8 x 2-4μcf. 18.3 x 4.3μ) and were cylindrical instead of sickle-shaped. No perfect stage was observed. The pycnidia often produced very slender spore tendrils while in other cases the spores were forcibly ejected as a powder. Light did not appear to influence the rapidity of mycelial growth but promoted more aerial hyphae. In some cases, sectioning was observed with a white saltant produced.

Sampson[46] described the growth of Kabatiella in culture. On agar slants, the growth after 4 days was an opaque cream coloured pasty mass with a smooth glossy surface resembling the growth of certain bacteria. A flesh coloured tint developed after about one week, the surface becoming corrugated and dull. Sooner or later, olive green flecks appeared and spread over the culture which became dark and finally black. The time taken for cultures to turn black depended, among other factors, on the age of the fungus. The interval was shorter when the organism had been in culture for some months. With recent isolations, conidia formation was more abundant and extended over a longer period. A coarse white aerial mycelium was sometimes observed in cultures three or more weeks old, especially if the medium became dry.

The blackening of the cultures was due mainly to a dark pigment formed in cells of the mycelium and in the germinating conidia, which became compacted together to form a kind of stromatic mass. The dark appearance was due also to the formation of pycnidia. On prune agar, these were not usually present before a month or 6 weeks from

the time of transfer but on cooked clover stems, they were found in two weeks. They were small, measuring 100-120μ in diameter but irregular compound pycnidia sometimes developed on agar. New pycnidia appeared to develop within the hollow interior of older ones. There was no indication in old or young cultures of ascus formation[46].

Different isolates may exhibit variations on the pattern of growth in culture. Colotelo and Grinchenko[11] used two isolates that differed considerably in appearance when growing on malt-yeast agar. One isolate readily became black and leathery in appearance whereas the other remained pink with only a slight darkening of the surface. Underlying the black leathery layer and on the surface of the mycelium of the former culture was a layer of a hyaline to pink conidia. Sixteen days after inoculation, tufts of coarse grey black mycelium protruded from the surface. The latter culture appeared black 30 days after inoculation and no mycelium was observed.

Grinchenko and Colotelo[17] reported that they obtained the perfect stage of Kabatiella by cultural methods. Mature ascocarps were produced on autoclaved kernels of Zea mays and stem and leaf pieces of T. pratense embedded in Sach's agar. It was necessary to maintain the cultures at 10°C for 60-90 days. The inoculum consisted of a conidial suspension of two morphologically distinct strains of K. caulivora. In the greenhouse, only sterile ascocarps were obtained on inoculated plants of T. pratense cv. Siberian.

When nutrient solutions were inoculated with conidia of the yeast-like type, they became cloudy and viscous, much the same as bacterial suspensions. Ten days or more following inoculation, black specks of mycelium were visible floating on the culture surface, attached to the walls of the flask and also throughout the solution. These bits of mycelium enlarged and gradually became the dominant

growth within the flask after 15-30 days[7].

3.2 Nuclear Characteristics

Cole and Couch[10] studied the nuclear characteristics of the mycelial and conidial growth forms by the use of differentially stained spores and by using phase contrast microscopy to study living conidia. Under these conditions, the nuclei were approximately 2-3μ in diameter and the spores possessed spherical vacuoles containing oil droplets. They were coated with a thick transparent gelatinous matrix easily discernible because it diffracted light.

The mechanism of spore germination and budding was traced by examination of conidia at one hour intervals. At 20-24^{o} germination was first evidenced by the enlargement and projection of a portion of the conidial wall. Nuclear migration and cyclosis were not observed prior to this expansion. In the mycelial type, enlargement of the projection continued, culminating in the production of a germ tube, while in the yeast-like type, a daughter conidium was produced. Prior to septation, one or more nuclei migrated into the newly formed portion. In some instances, mitotic nuclear divisions within the conidium occurred for periods up to 24 hours without accompanying budding or the production of a germ tube. The germination and budding processes usually required 15-24 hours. During metaphase, the equatorial plate was oriented perpendicularly to the long axis of the conidium with the spindle fibres parallel to the long axis. Although not distinct, chromosomes were evident in the interphase nucleus. In several spores, as many as 4 nuclei were observed dividing simultaneously. Chromosomes were most distinct from prophase to metaphase. Owing to the small size of the nucleus and nuclear components, acrocentric and metacentric chromosomes could not be distinguished and therefore a definite chromosome count was not established. However, the chromosome complement of the conidial and mycelial forms appeared to be similar. All the nuclei observed seemed to have the same chromosome number, this being 3-5 de-

pending on interpretation.

The variations in cultural growth form led these workers to determine whether this fungus existed as two separate entities - mycelial and conidial forms. Previously Hansen[18] examined 900 isolates from 30 genera of Imperfect fungi by single spore series methods and found that two or more culturally distinct growth forms could be obtained from over 50% of them. He felt that this variability was not due to a high degree of genetic instability but rather that it could be attributed to the fact that although many of these fungi existed in nature as definite entities, they were actually composed of two distinct entities or individuals. This concept was referred to as the dual phenomenon.

Cole and Couch employed a successive single spore transfer series to study the pattern of variation of the cultural growth forms (Table 4). Germination and incubation procedures were conducted at 20°C in the absence of light on PDY agar. Colour varied among the colonies and within colonies ranging from white to pink to olive green, brown and black in many graduations. In all single spore series transfers, segregation of the mycelial type from the yeast-like type was observed. As high as 2% of the spores from any yeast-like colony produced germ tubes and initiated a mycelial growth form while in no instance was reversion of the mycelial to yeast type observed. In this investigation no pycnidia or setae were formed in culture or on infected plants. The conidia borne on conidiophores was the only spore form observed.

Many members of the Fungi Imperfecti including genera of the Melanconiales (of which Kabatiella is one) are multinucleate. Through mutation, hyphal anastomosis and conidial production, varying combinations of genetically dissimilar nuclei may occur, each combination expressing different morphologic, physiologic and pathogenic characters. In the light of cultural variability and occurrence

Table 4

Kabatiella caulivora single spore transfer series

(Cole and Couch[10])

conidial culture isolated from host

portion of colony suspended in water and conidia plated out

germination of 500 single conidia checked

12 produced germ tubes nd formed mycelial growth colonies

10 colonies isolated. After 14 days growth, the conidia produced by each colony were collected and plated

germination of 500 conidia from each colony checked

all 5,000 conidia examined produced germ tubes and formed mycelial colonies

No reversion of mycelial to conidial type of growth was observed

488 budded and produced colonies composed solely of conidia

10 colonies were isolated. After 14 days growth the conidia produced by each were collected and plated out. Germination of 500 from each colony checked.

47 produced germ tubes and formed mycelial colonies

10 colonies isolated. After 14 days, conidia plated out

germination of 500 conidia checked

all 5,000 conidia produced mycelial colonies

4953 budded and formed conidial colonies

10 colonies isolated. conidia plated out at 14 days

germination of 500 conidia checked

17 produced germ tubes

4983 budded

of the mycelial type within K. caulivora, it was believed that heterocaryosis might also be the basis for these phenomena. Using a technique involving Feulgen's reagent where nuclei appeared red and the rest of the spore colourless or grey, the nuclear numbers of both growth types were compared. The nuclear number ranged from 1-8 for both growth types and the mean conidial nuclear number was 2.8.

They decided that the cultural variability exhibited by K. caulivora could not be reconciled with Hansen's concept of dual phenomenon. The mean nuclear number for both conidial and mycelial types was found to be the same. The cultural instability of the conidial type coupled with the absence of an intermediate mycelial/conidial type would seem to preclude the possibility that variability in this case was due to the association or dissociation of unlike nuclei as described for the dual phenomenon. This does not eliminate the possibility that the reported variability in cultural types, production of pycnidia or the occurrence of physiologic races could result from the ultimate segregation of genetically dissimilar nuclei. In attempting to explain variability of a multinucleate organism of this type, the nuclear behaviour pattern is only a final step in the sequence that leads to the biological expression under consideration. The real basis for the variability rests with the process that gave rise to the original genic alteration that in turn produced the population of dissimilar nuclei.

With K. caulivora, the overall pattern of change from conidial to mycelial types with mycelial types failing to revert to conidial types closely paralleled the situation described by Hansen and Snyder[19] for Hypomyces solani f. cucurbitae. It was their conclusion that this cultural variation arose as the result of a true mutation. Cole and Couch[10] decided that the mycelial homotype in

Kabatiella also arose as the result of a unidirectional mutation within the conidial form.

3.3 Nutrition

Sampson[46] found that over 24 hours, spores in distilled water elongated considerably and occasionally branched but rarely formed cross septa or budded off conidia. If a strip of epidermis was added, the conidia increased in size and budded off conidia from the ends, usually in ones and twos. Subsequently long thin germ tubes were formed sometimes measuring 20x the length of the spore.

Sakuma and Sakai[44] found that 1-2% of the conidia germinated in water while 100% germinated in red clover extracts. 1/600 dilution of the extract caused budding on germ tubes and the lengths of the germ tubes were reduced to 47% of that of the tubes at 1/60 dilution. There appeared no different between effects of extracts from resistant and susceptible varieties.

Berkenkamp[4] examined spores produced on lesions from red clover and found that the viability ranged from 70-92% alive, averaging 82%. After 7 days growth on preboiled red clover extracts, samples averaged 1125 spores/ml and had 74% live spores whereas samples grown for 7 days on freshly ground red clover extracts had 260 spores/ml with 50% alive. Changes which occurred during blending in the fresh sample before boiling suppressed growth and viability.

Colotelo and Grinchenko[11] used two different isolates whose growth on malt-yeast-dextrose agar had been described previously (page 29). No cultural differences between the two isolates were evident on synthetic minimal media. It appeared that extracts of malt and yeast contained certain factors which promoted the growth of mycelium of one isolate but restricted the other to the conidial stage. On medium containing glycine the fungus produced large conidia which had twice as many nuclei as were developed on other media. Asparagine

promoted the production of typical conidia.

Preliminary experiments indicated that only certain amino compounds such as cysteine, glutathione and tyrosine caused the unicellular hyaline conidia to develop into dark heavy walled forms. The nitrogenous fractions from red clover and alfalfa likewise promoted the development of the dark multi-cellular conidia. The green multicellular conidia have been observed in living plant material and the dark brown conidia were found on detached sterilised red clover leaves and stems. The leaves and stems were sterilised by autoclaving and then inoculated with unicellular hyaline conidia and the whole incubated on a synthetic minimal medium. One of the factors leading to the development of the dark green multicellular conidia appears to be a lack of available nutrients. Multicellular conidia formed only from typical conidia located in the surface layer of rapidly growing conidial cultures. An increase in the amounts of available nutrients accelerated the rate of production of hyaline and dark green conidia. When nutrients were limited, the dark green, thick walled conidia and mycelium anastomosed. This would enable the fungus to withstand and survive adverse environmental conditions.

Cole and Couch[7] also looked at the effects of various carbon and nitrogen sources and vitamins on the mycelial growth of Kabatiella. It was able to utilise a wide range of carbon compounds (Table 5) with D glucose, sucrose, dextrin and maltose providing better sources. Yeast extract was the best nitrogen source but much of the growth increase may have been due to the B complex vitamins present. Of the non-vitamin materials tested, aspartic acid and glycine were the better nitrogen sources although they produced only 30% as much mycelium as the yeast extract. Thiamine produced a 200% increase in growth when used as a substrate supple-

ment. Biotin and inositol also increased the growth significantly.

Sakuma and Sakai[44] found that stimulatory action of sucrose, fructose and xylose was less than that of extracts containing large amounts of fructose, some sucrose, no xylose.

Isoflavones have been found in red clover and reported to be antifungal[4]. They occur in a bound form and are released by added glucosidase or naturally occurring enzymes during grinding. Boiling plant material before grinding inactivates enzymes which release isoflavones during grinding. This could explain the better growth and viability of Kabatiella in the preboiled plant sample (page 34).

Berkenkamp[4] tested the effect of a number of isoflavone compounds on the growth and viability of Kabatiella (Table 6). The reduction of growth and viability due to biochanin A to about half the control may account for the inhibition of Kabatiella when grown on freshly ground clover extract. The inhibitory effect of other isoflavones was not as prominent as biochanin A. Since biochanin A and formononetin are the major isoflavone components in red clover, these should have the greatest influence on Kabatiella.

Two workers have examined the effects of temperature and pH on the fungus in culture.

Cole and Couch[7] found the optimum temperature to be 20-21°C with a rapid decrease in growth above this optimum. Growth was meagre at 27°C and ceased at 33°C. At low temperatures, growth was retarded but occurred as low as 3°C. They also used a pH series from 1.5-11.5. Maximum growth occurred at pH 4.6-7.3 with no growth below pH 3.0 or above pH 8.8.

Berkenkamp[4] extended this type of study to include the viability of the cells present. He found no measurable growth at 30° and at 25° growth was depressed for the first 6 days. The greatest number of viable spores were found at 20°C after 10 days growth. At 10°C, the

Table 5: The influence of various carbon, nitrogen and vitamin sources on the growth of Kabatiella caulivora (Cole and Couch)

Source	Weight of Mycelium	Source		Weight of Mycelium
Carbon	mg	Nitrogen	%N	mg
D-glucose	179	Yeast	10.0	498
Sucrose	163	D-L-asparticacid	10.5	131
Dextrin	160	Glycine	18.7	114
Maltose	153	Peptone	16.0	84
Raffinose	132	Asparagine	21.2	83
Lactose	131	Urea	46.7	80
i-inositol	118	Ammoniumphosphate	21.2	79
Pectin	103	Potassium nitrate	17.3	76
Inulin	102	D-L-alanine	15.7	70
D-xylose	92	Casein hydrolysate	16.0	56
D-galactose	84	L-argenine	26.6	50
L-sorbose	78	L-glutamic acid	9.5	49
Sodium acetate	74	D-L-B-phenylalanine	8.5	28
D-fructose	66			
L-arabinose	65	Vitamin	Conc. mg/ml	
Cellobiose	36	Thiamine	100	399
D-mannose	34	Biotin	10	230
Salicin	32	i-inositol	1000	260
Sodium citrate	31	Pyridoxine	100	165
Cellulose	growth	Calcium pantothenate	100	58
Ammonium oxalate	0	Riboflavin	20	172
		No vitamin	0	155

Table 6: The effect of isoflavone compounds on the growth of Kabatiella caulivora (Berkenkamp[4])

Compound	Average No. Spores	% Viable
Control	1736	64
Coumestrol	1756	52
Formononetin	1509	46
Biochanin A	874	29
Pratensein	1449	34
Genistein	1563	50
Daidzein	1746	56

growth and viability curves were similar to 15°, except they showed somewhat lower values. No growth occurred at pH 1 and 2. The lowest pH at which growth occurred was 3 and the optimum for growth was 4. Poor growth was found at pH 8.

3.4 Maintenance of Cultures

Fungi differ in the length of time they can live in culture. Some persist almost indefinitely while others remain viable for relatively short periods. Temperature, type of substrate and various other factors greatly influence longevity. Moreover, when cultures remain viable, they may lose their pathogenicity rapidly under certain conditions. This is true for Kabatiella which frequently changes on culture media from a yeast-like budding type of growth, which is usually pathogenic, to a mycelial type, which is commonly nonpathogenic. Martin[32] described several methods for storing cultures. Autoclaved red clover petioles stored at 4°C and at 21-23°C for 6 months produced viable cultures in a sporulating condition and pathogenic. On agar slants under mineral oil, kept at 4° or room temperature, the cultures were still in excellent condition, viable and pathogenic after 6 months of storage. Cultures kept at 4° without oil were in fair condition and still viable and pathogenic after 6 months. Those kept at room temperature without oil were completely desiccated and dead after 6 months. After 3 months, they were in poor condition but still viable and pathogenic.

Martin[32] also examined the effect of time on infectivity of inoculum produced in shake culture and concluded that one week old inoculum is best. Berkenkamp[4] found that nutrient deficiency in flasks was not a factor affecting viability as dilutions to 1/4 showed very slight differences over 12 days. After about 10 days incubation, dead spores began to autolyse. The effect of autointoxication was examined by checking growth and viability inside arm flasks.

The viability of spores in flasks where the medium was changed daily average 53% for 11 days compared to 16% in the unchanged medium. It would appear that by-products build up in older cultures causing loss of viability.

3.5 Comparison of Kabatiella caulivora & Pullularia pullulans

Young colonies of Kabatiella caulivora growing on malt agar are creamy white to pale pink and yeast-like in appearance. They can be confused with certain bacteria, yeasts and P. pullulans[27]. The lack of lustre of Kabatiella colonies compared with these other organisms is a useful criterion for separating these species. When microscopically differentiating between young colonies of Kabatiella and Pullularia, in some isolates of the latter, conidia are more elongated than usual and may be confused with those of Kabatiella. The presence of hyphae in Pullularia colonies and their absence in young colonies of Kabatiella readily separates the two species. In addition, budding conidia are common in colonies of Kabatiella but rare or absent in P. pullulans. In older colonies of Kabatiella the mycelium becomes heavily pigmented, beginning as an olivaceous flecking which progresses to a jet black colour when the colonies are 10 days old. Macroscopically, there is little chance of confusing older colonies with either yeasts or bacteria but they may be confused with certain strains of Pullularia fairly commonly associated with clover seed.

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