CELLULASE ACTIVITY IN CORN STALKS INFECTED WITH *FUSARIUM MONILIFORME* SHELD. AND ITS RELATION TO STALK ROT

by

Richard William Ikenberry

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major Subject: Plant Pathology

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1964

TABLE OF CONTENTS

INTRODUCTION

Stalk rot, a disease of corn prevalent in most corn-growing areas, may be incited by a diversity of organisms, both fungal and bacterial. While conditions may be favorable in a given area for development of a certain stalk-rotting pathogen, some other pathogen might be favored by conditions elsewhere.

The relationship between stalk rotting and actual stalk strength or "standability" has been studied. However, the data reported from various sources regarding what is universally termed "stalk rot" are confusing because of the diverse methods used in evaluating the condition.

Very little has been reported on the particular processes involved in rotting, i.e., the avenues of degradation involved in microbial deterioration of the stalk. The limited data available suggest that rotting of cellulosic components of stalk tissue is accomplished through action of cellulolytic enzymes secreted by fungal pathogens.

Among the organisms in the list of stalk-rotting pathogens is the controversial Fusarium moniliforme Sheld. While this fungus appears to have an ubiquitous distribution in corn-growing areas, its actual pathogenic capabilities appear to be questionable. Some have attributed to F. moniliforme a low disease-inducing capacity, while others cite it as an active, destructive pathogen. The former point of view has been reached in many cases after observations of mild effects in the host

following artificial inoculation.

The work reported here was undertaken with the primary objective of studying prevalence of cellulolytic enzymes in corn stalk tissue as related to observed rotting and strength of the stalk. These studies have been performed by sampling, in July, August, September, and October, stalks of four single-cross hybrids of varying resistance to stalk rot, acquiring data regarding strength, pith deterioration, and cellulase content of the samples, and making appropriate comparisons of such data.

The presence of F. moniliforme in stalk tissue has also been investigated in an attempt to discern the role of this entity in stalk rotting.

REVIEW OF PERTINENT LITERATURE

Stalk Rotting and Lodging

Stalk rot generally does not affect plants until after pollination. Up to this time plants appear to remain healthy even after being inoculated (10, 28, 35, 46). With approaching senescence, pith parenchyma tissue is readily disintegrated. Only the vascular elements and rind tissue remain intact in severely rotted stalks (1, 10, 41). While fungal mycelium is present in internodal areas (3) nodal tissue appears to retard mycelial growth (4).

Several fungal and bacterial organisms have been cited as pathogens causing rotting (31). Many of these are also found as incitants of ear rots, kernel rots, root rots and seedling blights of corn (41, 42, 65, 66).

Several aspects of plant resistance in the stalk rot syndrome have been reported in literature. It appears that carbohydrate content of the plant influences severity of the disease. When green leaf area has been reduced, either by clipping or through leaf blights, stalk rot severity has been observed to increase substantially (6, 23, 32, 34, 46, 52). Furthermore, DeTurk *et al.* (6) showed that clipping of leaves significantly decreased the total active carbohydrates and total sugars and that resistance to Diplodia stalk rot was definitely associated with high total active carbohydrates and total sugars. Pappelis

and Smith (54) found that resistance in plants was associated with well-hydrated, high-density tissue composed of living cells, whereas susceptibility appeared to be conferred in low-density tissue containing a high proportion of dead parenchyma cells. They reported that mycelium of Diplodia zeae (Schw.) Lev. and Gibberella zeae (Schw.) Petch. was not observed in living tissue and concluded that inherent susceptibility was related to presence of many dead cells in the stalk.

Juices and extracts of stalks have been found to retard growth of G. zeae and D. zeae (28) and Whitney and Mortimore (69, 70) isolated from young stalks an antifungal compound which inhibited growth of Fusarium moniliforme and G. zeae.

Pappelis (53) suggested that resistance is afforded in certain varieties through substances believed to be phenolic compounds, continuously formed in stalk tissue and released, on wounding, to accumulate to toxic levels against certain fungi. Oxidation and condensation of such compounds to pigments would then account for discoloration often associated with rotting.

Differing views appear in connection with the relationship of lodging in corn to resistance or susceptibility of the stalk to rotting. Durrell (7, 8) found that stronger stalks showed less infection by various fungi than weaker stalks, and concluded that since cultures of D. zeae were able to utilize a cellulosic medium, this organism weakened nodes of stalks through cellulolytic action. Pammel et al. (51) found in broken stalks that

the pith was destroyed and discolored and that vascular bundles were softened. In studies of stalk rotting caused by Pythium butleri Subr., Elliott (12) observed that the outer rind, epidermis, lignified cells of the hypodermis and parenchyma cells were softened, disorganized and destroyed, leaving only the separated vascular bundles which could not support the plant. Foley (15) concluded that activity of microorganisms caused weakening of stalks, eventually resulting in stalk breakage and disintegration.

On the other hand, Zuber et al. (71) found that lodging was not associated with a high incidence of stalk rot by either D. zeae or G. zeae. They concluded that hardness of rind and other anatomical features exerted considerable influence on stalk strength. In fields displaying up to 85% prevalence of stalk rot, Hooker and Britton (25) found only 5% of the plants lodged. They stated, however, that had winds and rains occurred during harvesting season, the percentage of lodged stalks might have been much greater. Loesch et al. (39) observed that stalk rot ratings for lodging-resistant crosses were nearly as great as for lodging-susceptible varieties. According to Johann and Dickson (28) strong stalks do not necessarily appear to be those with high physiological resistance to D. zeae.

Perhaps Wernham (67) has best summed up the situation concerning stalk lodging and rotting. He states, "The interpretation of stalk rot reaction in hybrid corn is further confused

by the methods that various agencies use in accumulating the data. To be truthful, stalk rot has not been strictly defined. The plant pathologist thinks of it in terms of breakdown of fundamental tissues. The agronomist thinks of the disease in terms of stalk lodging, that is, breakage below the ear, as opposed to root lodging. Often there is little distinction made between stalk lodging due to stalk rot and lodging due to corn borer infestation and activity. Evaluation of stalk lodging is not a standardized procedure. Many agronomists count broken stalks; others use a pushing technique to which all standing stalks at harvest time are subjected. A more severe test is one wherein a lusty kick is given the corn plant just above ground level. All these tests are accomplished at the time of harvest, usually 8 to 12 weeks after anthesis. There is little wonder, then, that stalk rot data on corn hybrids in the Northeast provide a controversial issue. On the one hand pathologists are interested in the extent of tissue damage, while on the other hand agronomists are interested in what may be spoken of as 'standability,' that is, the ability of the corn to stand erect. Even though agronomic results vary from station to station, the concept of 'standability' appears to be the one best suited for protection of the farmer against stalk rot losses."

Nature of the actual process of rotting has been fairly well ignored by most workers. Durrell (7) recognized that D. zeae was able to utilize cellulose and from this concluded that a cellulose-decomposing phenomenon was operative in the disease.

Cellulase has recently been isolated from corn stalk tissue by Foley (14); enzyme activity was demonstrated in both resistant and susceptible plants and was found to be higher in nodes than in internodes. The extracted enzyme has also been found capable of dissolving cell walls of corn pith parenchyma tissue and of softening excised rind tissue.[1] Cellulase was also isolated from cultures of F. moniliforme by Foley[1] and by Fagle (13). Fagle was able to demonstrate the decomposition of parenchyma cells of corn pith tissue by this enzyme preparation.

Cellulolytic Enzymes

Research with cellulase has shown this enzyme to be a complex substance. In analyzing the makeup of cellulase, Reese and his co-workers (56, 57, 58) have postulated that the complete enzyme is actually made up of more than one constituent. "Native" cellulose is attacked only by a "C_1" component, usually found only in the actual presence of a cellulolytic organism, and is broken down to linear, anhydroglucose chain fragments. A second component, referred to as "Cx," then hydrolyzes these chains at the beta-1,4 linkages to yield the disaccharide cellobiose. Cellobiose in turn is broken down to glucose units through the action of the enzyme beta-glucosidase. As many as

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[1]Foley, D. C. Assoc. Prof. of Botany and Plant Pathology, Iowa State University, Ames, Iowa. Presence of cellulase in corn stalks. Private communication. 1963.

eight components have been separated in cellulase preparations (56) on the basis of electrophoretic mobility, hence the makeup of the enzyme may be even more complex than just outlined. Marsh (40) has isolated an enzymic fraction which causes swelling of cotton fibers and has referred to this as an "S factor." The relationship of this factor to C_1 and Cx has not been definitely established, however it is thought that it acts primarily in fracturing the primary wall of cotton fibers, thus allowing swelling of the fibers (5).

Through electrophoretic studies of cellulase recovered from Myrothecium verrucaria (Albertini and Schweinitz) Ditmar, Whitaker (68) has claimed that there is a single active component in the enzyme capable of breaking down "native" cellulose to the final end product, glucose. Other workers, however, have demonstrated through a number of procedures, the existence of several separate entities in cellulase of this organism (18, 47, 57). Thomas (62) has also found at least three protein components, using paper electrophoresis, in dried cellulase from Stachybotrys atra Corda.

Inhibition of hydrolysis of cellulosic compounds has been investigated by many workers. Reese et al. (59), Greathouse (20) and Goldthwait et al. (19) have demonstrated that various substitutions on the cellulose molecule, in both qualitative and quantitative aspects, appear to protect cellulose from enzymic attack. Reese et al. state that the presence of a single

constituent on every anhydroglucose unit appears to confer immunity to hydrolysis, whereas a relatively low degree of partial saponification may detract from protection markedly. According to Basu and Whitaker (2), hydrolysis of cellulose by cellulase from M. verrucaria is inhibited, to varying extents, by salts of heavy metals, by oxidizing and reducing agents, by iodoacetate and by para-chloromercuribenzoate. Basic and acid dyes were found to be inhibitory or stimulatory, depending upon pH. King (30), in view of the findings that crystalline cellulose is more resistant to degradation than the amorphous regions, has suggested that in addition to the prevention of entry of enzyme molecules into the crystals due to spatial integrity of the cellulose molecule, resistance might also occur due to a rigid configuration of glucosyl units such that a molecular configuration exists with which the enzyme simply can't fit. More complete literature reviews on enzymic degradation of cellulose by microorganisms have been compiled by Siu (61), Cowling (5), Gascoigne and Gascoigne (17) and King (30).

The Role of Fusarium moniliforme in Stalk Rotting

The widespread occurrence of F. moniliforme in association with corn and its ability to produce cellulase is of interest in considerations of stalk rot. This organism was first described on corn in Nebraska by Sheldon (60) and since has been

isolated from corn stalks, roots and seed in Illinois (22, 33), Kentucky (65, 66), North Carolina (49), New Jersey (55, 64), Georgia (48), Iowa (14, 16, 21, 27), West Virginia (36), Ohio (38, 64), Kansas (43, 44), Louisiana (9), Ontario Canada (41, 42), The Philippines (45), and New South Wales (11).

Variable and somewhat conflicting evidence has led to contrasting conclusions regarding the actual pathogenicity of F. moniliforme to the corn plant. Edgerton and Kidder (9) concluded from three years' tests that this organism appeared to have no marked effect on stand or yield in Louisiana, even though approximately 50% of kernels tested showed infection by this fungus. In a study of root-rotting fungi in the soil, Ho and Melhus (21) classified F. moniliforme as possessing "slight to no disease-inducing capacity." Koehler et al. (33) cited common infection of seed by the fungus and noted slight reduction in stand and early vigor in corn grown from such seed, with resultant reduction in yield, over a three-year period, of 6.2%. They observed, however, that leaning of plants was not increased by occurrence of F. moniliforme on roots and concluded that the organism was evidently not operative as a root rotting organism to the extent of weakening anchorage. F. moniliforme was noted by Limber (38) to grow parasitically in the stalk and cause discoloration when young plants were inoculated. The limited development of the fungus resulting from various methods of stalk inoculation led him to conclude, however, that injury by the

fungus was mild,

McKeen (42) found F. moniliforme pathogenic to seedlings but decided that it was of little importance due to lack of conditions in the field suitable for marked development of seedling disease. Leonian (36) found pathogenicity to seedlings to be variable, while Ullstrup (64) noted distinct seedling pathogenicity by the fungus.

Mendiola (45) concluded from his observations that seed infected with F. moniliforme germinated poorly and produced plants characterized by limited vigor, stunting and poor yield. Valleau (65) concluded that this organism was an active parasite causing both root and stalk rots and noted that it seemed to be the more active parasite when present in association with Gibberella spp. in rotting stalks. He suggested, in 1920, that because of infection by the fungus in such a high percentage of seed, F. moniliforme would be the most common cause of root and stalk rots in the future. Peterson (55) found F. moniliforme to be as pathogenic as or more so than G. zeae in rotting stalks of a susceptible inbred. Some strains of corn were noted by Holbert et al. (22) to be very susceptible to ear, root and stalk rots caused by F. moniliforme. Edwards (11) inoculated stalks of corn with F. moniliforme and observed that extensive necrosis resulted. In studies of systemic infection of corn by the fungus, Foley (16) suspected F. moniliforme as an agent responsible for early tissue deterioration in the stalk resulting in a softened rind and consequently a weakened stalk.

FIELD PROCEDURES AND COLLECTIONS

1961

Untreated seed of each of four selected hybrids was obtained from the Agronomy Department of Iowa State University. The single-cross hybrids, selected according to average resistance to stalk rot, were: WF9 x W22, Os420 x 187-2, B14 x C103, and Hy x 38-11. B14 x C103 is generally resistant to stalk rot, Os420 x 187-2 is susceptible, and the remaining two intermediate in resistance.

Planting was done at the Agronomy Farm of Iowa State University on May 3, using a hand planter, at the rate of one kernel per hill, with hill spacing such as to provide populations of approximately 14,000 plants per acre. There were five replications, with five rows of each hybrid in each replication.

Collections of stalks were made four times: on July 18 and 19, August 14 and 15, September 15, 16 and 18, and October 14. Ten stalks of each hybrid were taken from each of the five replicates in every collection. In July the first node above the uppermost brace roots was saved in a stalk section approximately three inches in length and all such sections placed in a freezer (-20°C) immediately after being brought in from the field, for later cellulase activity determinations. In August, September, and October stalk sections including the first two nodes above the uppermost brace roots were brought into the laboratory. The second node of each stalk was used for stalk

strength determination after which the first node above the brace roots was saved in a three-inch length of stalk and immediately placed in the freezer for storage for future analysis.

1962

Certified seed of each of the four hybrids was obtained from the Clyde Black and Son Seed Farms, Ames, Iowa. This seed had been treated with Arasan. Planting was done on May 22 at the Ash Avenue plots of Iowa State University. Hand planters were used and seed was planted one per hill with hill spacing to provide a population of 15,000 plants per acre. Four rows of each hybrid were planted in each of four replications.

On July 17, 27, and August 6 all plants were sprayed for corn borer control, as damage due to this insect was noted in the 1961 material. A 0.4% solution of DDT was sprayed liberally on leaves and stalks in each application. Effective control was achieved.

Collections of stalks were made on July 20, August 15, September 17, and October 26. Four stalks of each hybrid were collected from each of the four replicates on these dates. Stalk sections which included the first two nodes above the uppermost brace roots were brought into the laboratory. From each stalk the first node above the brace roots was excised in a three-inch length of stalk and immediately placed in the freezer. The internode immediately above was tested for strength and the

second node was split lengthwise into quarter sections and placed in moist chambers for detection of internally-borne organisms.

1963

Certified seed of each of the four hybrids was obtained from Clyde Black and Son Seed Farms. The seed had been treated with Arasan. Planting was done on May 17 at the Ash Avenue plots of Iowa State University. Hand planters were again used and seed was sown one per hill with hill spacing such as to provide a population of 15,000 plants per acre. Three rows of each hybrid were planted in each of five replications. The plants were treated with one application of DDT granules for corn borer control.

Collections of stalks were made on July 15, August 16, September 16, and October 16. Three stalks of each hybrid were collected from each of five replicates in every collection. Stalk sections including the first two nodes above the uppermost brace roots were brought into the laboratory and handled in a manner identical to that employed with the 1962 collections. Tests for detection of internally-borne organisms were performed, however, only with the July and August collections.

LABORATORY PROCEDURES

Stalk Strength Determinations

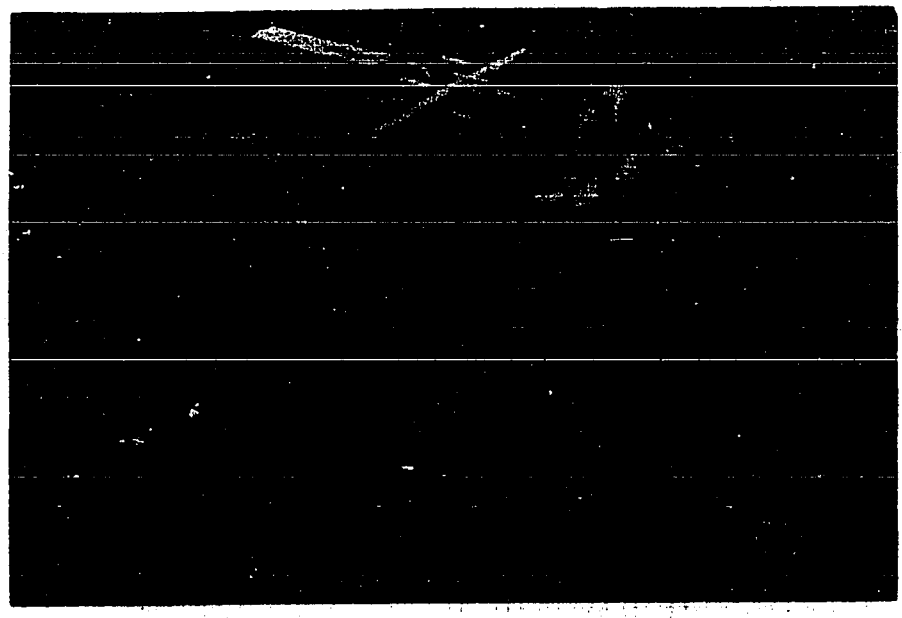
Stalk strength was determined by means of a specially constructed machine designed to apply gradually increasing force against the stalk until breakage of the stalk occurred (Figures 1 and 2). The stalk to be tested was inserted in a sliding two-piece carriage and subjected to gradually increasing lateral force. A spring scale simultaneously indicated the amount of force applied, and force required to break the stalk was read from a maximum value indicator on the scale. This force was designated as stalk strength.

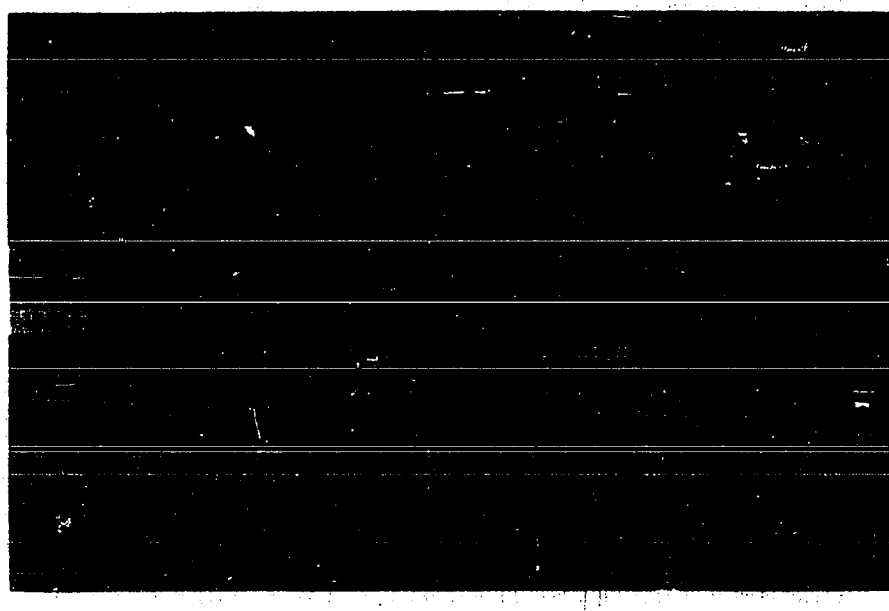
Detection of Internally-Borne Organisms

Individual moist chambers were prepared by placing strips of moistened filter paper in 210 x 27 mm. test tubes; the tubes were plugged with cotton and autoclaved. Stalk sections containing the second node above the brace roots were split lengthwise into quarters. One quarter was placed in a moist chamber after being surface sterilized by immersion for about ten seconds in 10% Clorox solution. The stalk sections prepared were incubated at room temperature to facilitate outgrowth of any organisms present within the tissue. Representative fungal colonies on stalk samples collected in July and August were transferred to slants of potato-dextrose agar (Difco) for identification.

Figure 1. Machine used in determining lateral force required to break corn stalks; power supplied by electric motor is transferred through a reducing gear system and chain to carriage into which stalk is placed for testing

Figure 2. Close-up view of sliding, two-piece carriage designed to apply lateral force to corn stalk; Points "A" and "B", connected to electric motor drive, travel to the right; force is transferred through the stalk to point "C", connected to the spring scale; maximum force applied to stalk prior to stalk breakage is read from maximum value indicator "D"





Extraction and Precipitation of Cellulase

Stalk samples collected in 1961 were used mainly in preliminary studies on techniques for extraction and assay of cellulase contained within the samples. Results of these tests influenced design of a standard technique for analyzing the 1962 and 1963 stalk samples.

Corn stalk nodal sections frozen after collection from the field were kept in the freezer until time of enzyme extraction procedures. Each stalk sample was processed separately for recovery of cellulase present. The section was first sliced into sections approximately three millimeters in thickness by using a powered rotary saw. In 1961 these slices were cut into cubes approximately five millimeters on a side for chopping in a Waring Blendor. Because small chunks of tissue remained intact after maceration in the Blendor, slices of 1962 and 1963 tissue were cut into thin strips to facilitate more complete tissue maceration.

A 10 gm. sample of the cut-up tissue was weighed out and added to 50 ml. of an extract solvent in a monel metal micro Waring Blendor cup. The solvent used for 1961 samples was deionized water. Variation in results and further study on cellulase activity later led to the decision to buffer the extraction and assay systems for the 1962 and 1963 samples with an acetate buffer as reported by Gascoigne and Gascoigne in their studies on Fusarium moniliforme (17). This buffer has

been used in other cellulase studies also (24). The pH selected was 5.0, a value close to that of the pKa of acetic acid (4.6) and generally found to be a value optimal for fungal cellulase activity (17, 24, 56). A 0.1 M solution of buffer (anhydrous sodium acetate and acetic acid in deionized water) was sufficient to prohibit changes in pH during the procedures to be described. A fresh supply of the buffer solution was prepared each week and stored under refrigeration.

In chopping the corn stalk tissue, one of the problems encountered, inherent in the use of a Waring Blendor, was that of heating of the cup during operation due to friction in the rotating shaft bearing. It was essential that all steps of the enzyme extraction process be carried out with the temperature as low as possible to avoid loss of enzyme through denaturation due to heat. Hence the Blendor was operated intermittently inside a $2^{o}C$ refrigerator directly in front of the cold air delivery stream. Intermittent operation was controlled by an automatic timing switch in such a manner that the Blendor operated for 30-second intervals with 90 seconds intervening between each running period. A total of ten cycles of the timing switch, or 300 running seconds, was used for each sample processed.

The resulting extract was filtered through a pre-chilled, coarse grade sintered glass filter funnel into a flask kept in an ice water bath, by means of suction provided by a water

aspirator. The solid residue was then returned to the Blendor cup, 25 ml. of water or buffer added, and the contents whipped for an additional 30-second period. This second extract was filtered through the same funnel into the same flask, and combined with that previously obtained. Suction was then closed off and the residue in the funnel washed with a 25 ml. portion of water or buffer solution; this washing was added to the contents of the filter flask.

The total 100 ml. volume of extract was poured into a 16 oz. prescription bottle. Precipitation of proteinaceous substances contained in the extract was effected by addition of four volumes (400 ml.) of C.P. grade acetone previously chilled to -20°C. The introduction of the acetone was done slowly, by means of a stopcock connected to the vessel containing the acetone, and was carried out in the 2°C refrigerator. A magnetic stirrer was used to agitate the contents of the bottle during this procedure.

Complete addition of the 400-ml. volume of acetone usually required approximately 20 minutes. Following this the prescription bottle was capped and placed in the freezer for at least four hours to allow completion of precipitation.

The resulting flocculent precipitate was recovered by decantation of excess supernatant liquid and centrifugation at 2,000 r.p.m. (810 r.c.f.) for 20 minutes. The supernatant remaining in the centrifuge bottle was poured off and the precipitate allowed to dry overnight in the open centrifuge bottle

at room temperature. The dried precipitate was light gray to dark "chocolate" brown in color. Normally this dried precipitate was prepared the following morning for cellulase assay tests; otherwise the centrifuge bottle was stoppered and placed in the refrigerator for storage. In most cases this storage did not exceed two days. Fungal cellulases are reported to be quite stable, capable of being stored in a dry state under refrigeration indefinitely (56).

Assay of Cellulase Activity

The method selected for assaying the amount of cellulase in the precipitated samples was that of measuring the breakdown of a soluble substrate, carboxymethylcellulose (CMC), through viscosimetric techniques. Of the methods commonly used for assaying cellulase, two were initially considered in view of practicality as related to materials and equipment available and to interfering substances possibly present in the prepared stalk enzyme sample. These were the CMC viscosimetric procedure, and the system whereby increase in reducing-sugars is measured after reaction of a cellulase sample with CMC has proceeded for a given length of time. According to Holden and Tracey (24) the former is the more sensitive technique. They report that if all chains in a CMC sample are split in half, the increase in the reducing-sugar value will be only in the neighborhood of 0.3%, while the viscosity increment is reduced considerably. Colorimetric determinations of reducing sugars

using dinitrosalicylic acid were tried in the present work but yielded results which were erratic, hence this method was not considered satisfactory for assay of processed cellulase samples.

A 1.0% solution, in deionized water, of Dow Methocel, MC Premium, 400 cps. was used for assaying the 1961 samples. Vigorous agitation, necessary for proper dissolving of the CMC powder, was provided by a Waring Blendor operated at low speed for ten minutes for all substrate preparations. Reese and Levinson (58) have reported that Methocel is resistant to hydrolysis by cellulolytic enzymes and that it is very effective in inhibiting cellulase. Due to this and to unexplained variability in results from using the Methocel, Hercules 7HSP CMC was used instead for the 1962 and 1963 assays. A 0.6% solution was prepared in 0.1 M acetate buffer; this concentration exhibited a reasonable efflux time (in the neighborhood of 200 seconds) in the viscometers utilized. The CMC solution was found to decrease slightly in viscosity with time following preparation, even when stored under refrigeration. An average decrease of 2% occurred over a period of one week. A new solution therefore was prepared each week. Preparations were made as identical as possible by weighing out the powder to the nearest 0.0001 gm. on a Mettler analytical balance and dissolving this in 500 ml. of buffer solution measured in one specific volumetric flask. In spite of this procedure a gradual decline

in original viscosity of the CMC solutions occurred in successive preparations of the solution in 1962, probably due to absorption of atmospheric moisture by the CMC powder. Before the assays of the 1963 samples were performed the CMC powder was dried in a 35°C. oven for 48 hours, after which it was kept in a tightly-sealed glass jar. This procedure resulted in uniform original viscosities of prepared solutions.

The dried precipitate samples were prepared for assay by re-dissolving in deionized water (1961 samples) or 0.1 M acetate buffer. A 2.0 ml. volume of solvent was added to the centrifuge bottle containing the dried precipitate; a rubber policeman was used to scrub the sides and bottom of the bottle and break the precipitate film into small fragments to speed dissolution. Due to higher cellulase activity found in the October samples, it was desired to obtain somewhat better separation of the samples with respect to enzyme activity. This was accomplished by re-dissolving the dried precipitate in 4.0 ml. of water or buffer instead of the usual 2.0 ml. Correction was made in treatment of the data as described later. The re-dissolving process was continued for at least two hours, with gentle agitation provided by a rotary shaker. The remaining residue was removed by centrifugation to prevent introduction of particles into the viscometers.

Assays were performed in Ostwald-Cannon-Fenske type viscometers, size 300, immersed in a circulating water bath main-

tained at $40^{\circ} \pm 0.2^{\circ}C$. The CMC solution was preheated to this temperature by immersing the container in the water bath. A 10 ml. volumetric pipette was used for introducing the CMC into each viscometer. An efflux time was determined for each viscometer, prior to each test run, with only the CMC; this time was termed the base rate and was used subsequently in calculations.

A 1.0 ml. volume of the re-dissolved cellulase sample was withdrawn and added to the viscometer. Mixing of the enzyme and substrate was first accomplished by removing the viscometer from the water bath immediately after addition of the enzyme and giving it five vigorous "snaps", after which it was returned to the bath. In assaying the 1962 and 1963 samples, more uniform agitation and less variance of the system due to thixotropy of the CMC were accomplished by gently blowing into the small tube of the viscometer as the enzyme was added and thereafter for ten seconds, thus bubbling the contents of the viscometer. Following initial agitation the liquid was drawn up in the viscometer and allowed to efflux once without timing. The viscometers were subjected to no further agitation.

The time at which the cellulase sample first entered the CMC solution was designated as zero incubation time. Efflux times of each sample were determined at designated time intervals following zero time, by means of electric timers graduated

to 0.1 second. For the 1962 and 1963 samples readings were taken at 5, 15, 30, 60, 90, and 120 minutes.

Treatment of Cellulase Activity Data

Inherent in viscosimetric assays are discrepancies in readings due to slight variations in dimensions of viscometers and slight differences in viscosities of CMC solutions prepared. In an attempt to standardize results, all viscometers were first tested with a common enzyme-substrate system. The viscometers were each supplied with 10.0 ml. of 0.6% Hercules 7HSP CMC. A commercially produced cellulase (no. 6225, Worthington Biochemical Corporation, Freehold, New Jersey) was prepared in a 0.001 mg./ml. solution, a 1.0 ml. sample of the solution added to each viscometer, and efflux times recorded. The per cent reduction (%R) in viscosity at given incubation times was computed for each viscometer system (Table 1) according to the relationship:

$$\%R = 100 - \left(\frac{\text{efflux time at given incubation time}}{\text{base rate}} \cdot 100\right).$$

It was assumed that the enzyme sample would catalyze the same amount of CMC breakdown in every viscometer in a given incubation time. The resulting figures for per cent reduction at 30 minutes were considered as being in such a range, experimental error recognized, as to validate the per cent reduction technique for reconciling differences in results between different viscometers.

Table 1. Per cent reduction values obtained with ten viscometers, each containing 10.0 ml. of 0.6% CMC and 1.0 ml. of 0.001 mg./ml. commercial cellulase; base rates ranged as follows: Test 1 - 179.0 to 231.0; Test 2 - 172.2 to 221.5; Test 3 - 187.2 to 238.5

Incubation time (min.)	Per cent reduction in viscosity in viscometer									
	D3	D6	D15	D25	D33	D39	D59	D61	D67	D97
					Test 1					
15	39.8	41.3	38.2	40.9	39.6	40.3	38.2	38.7	39.5	38.7
30	48.4	49.5	50.7	48.9	47.4	48.4	46.7	51.5	50.6	50.6
60	59.3	60.5	58.7	60.3	58.3	59.8	58.3	59.7	59.5	59.3
90	66.4	67.2	65.3	67.2	65.4	66.2	64.9	66.1	65.6	65.9
120	70.0	71.1	69.3	70.1	69.8	70.4	69.7	70.2	69.7	69.4
					Test 2					
15	37.8	38.2	39.0	38.8	38.8	39.2	38.1	38.6	39.8	38.7
30	46.5	46.2	46.2	46.8	46.9	49.0	44.6	46.8	50.1	48.3
60	57.5	56.3	57.1	58.1	57.9	57.8	55.1	57.6	57.9	56.2
90	64.2	63.2	63.3	64.2	64.1	63.2	61.9	64.5	64.5	63.1
120	68.5	67.6	67.7	68.5	68.7	67.9	67.4	69.0	68.9	67.5
					Test 3					
15	40.0	38.0	39.4	40.9	41.1	40.5	40.9	39.8	39.6	39.3
30	49.0	49.1	48.2	50.3	49.6	49.8	48.9	49.0	48.4	48.2
60	62.3	62.7	61.8	63.1	62.5	62.8	61.2	61.9	60.8	60.8
90	66.8	67.0	66.3	67.6	67.1	51.7	66.0	66.7	65.9	66.0
120	71.4	71.6	70.8	72.0	71.7	71.3	70.6	71.2	70.3	70.2

In an additional control experiment wherein selected concentrations of Hercules 7HSP CMC were tested with commercial cellulase, per cent reduction served in a satisfactory manner to standardize results under conditions of varying concentration or viscosity of CMC within the limits encountered in the experimental assaying (Tables 2 and 3). Efflux times for base rates during assays of 1962 and 1963 material varied over a range of 167.5 to 218.1 seconds.

The base rate was determined, as previously mentioned, with 10.0 ml. of CMC solution only, in the viscometer. Adding the 1.0 ml. sample of extracted enzyme diluted the CMC, reducing viscosity. Tests with an identical volume of buffer added as a blank indicated that this reduction was between 20% and 25%, hence this level was considered as indicating little or no enzyme activity in processed samples.

Per cent reduction in viscosity of the substrate, while providing an index of cellulase activity, did not however, present an accurate picture of enzyme activity per se. Dilution experiments with enzyme samples prepared from corn stalks by the methods outlined showed, for example, that a sample producing 80% reduction in a given time was not twice as concentrated as one producing 40% reduction.

The most active enzyme samples obtained could be diluted 1000 times and still exhibit activity. Activity was not observed with further dilutions of several samples tested, in

Table 2. Per cent reduction values obtained with varying concentrations of 7HSP CMC and 0.001 mg./ml. commercial cellulase; 10.0 ml. of CMC and 1.0 ml. of enzyme solution added to each viscometer

Incubation time (min.)	Per cent reduction in viscosity				
	Viscometer:	D6	D97	D3	D61	D59
	% CMC:	0.50	0.55	0.60	0.65	0.70
	Base rate:	122.5	157.5	200.3	253.9	328.3
5		24.4	26.0	27.6	29.7	31.2
15		30.4	32.1	33.8	35.7	36.8
30		37.4	39.4	40.4	42.2	43.1
60		46.9	48.6	50.1	51.4	52.1
90		53.1	55.2	56.4	57.4	59.0
120		57.7	59.6	61.0	62.1	63.6

Table 3. Per cent reduction values obtained with varying concentrations of 7HSP CMC and 0.01 mg./ml. commercial cellulase; 10.0 ml. of CMC and 1.0 ml. of enzyme solution added to each viscometer

Incubation time (min.)	Per cent reduction in viscosity				
	Viscometer:	D80	D25	D67	D33	D15
	%CMC:	0.50	0.55	0.60	0.65	0.70
	Base rate:	103.0	166.6	187.2	276.8	374.4
5		46.3	49.9	51.2	54.0	55.9
15		62.5	66.1	66.9	69.3	70.3
30		71.5	74.6	75.6	78.0	78.9
60		79.1	81.7	82.8	84.8	85.5
90		82.0	84.2	85.2	87.1	87.7
120		84.0	86.2	87.0	88.8	89.4

spite of attempts to obtain more active samples by combining several precipitated samples from rotted corn stalks.

A reference enzyme activity curve was consequently constructed from data of a carefully executed enzyme dilution

assay. A highly active sample was obtained by combining precipitates from four stalk samples. Dilutions of the sample were prepared with 0.1 M acetate buffer and assays of each dilution performed simultaneously, using the regular procedure. The concentrated sample was arbitrarily designated as having an activity of 1000, the 1:1000 dilution assigned an activity of 1, and appropriate values designated for other dilutions. A sigmoid curve resulted when the activity of each dilution was plotted against per cent reduction at a given incubation time (Figure 3).

All per cent reduction figures for the assayed samples were converted to enzyme activity values through the use of the reference curve. Greatest differences in activity between healthy and rotted corn stalk samples occurred at 30 minutes incubation time, hence the reference curve was constructed using data of 30 minutes and all conversions of assay data to enzyme activity values performed using 30-minute incubation time values.

The reference curve was constructed from data of a composite enzyme sample. Slight variation in dilution curves resulted with different enzyme samples, however the general sigmoid relationship still held. The reference curve merely served as a representative conversion relationship to which all experimental data were subjected.

As previously described, the assaying of the October samples of both years differed slightly from others in that the

Figure 3. Cellulase reference curve (per cent reduction in viscosity of 0.6% 7HSP CMC at 30 minutes incubation as related to enzyme activity)

PER-CENT REDUCTION IN VISCOSITY
0
20
40
60
80
100
CELLULASE ACTIVITY
1
10
100
1000

dried precipitate was re-dissolved in twice as much solvent as the earlier samples. To reconcile these October data with those for July, August, and September, the enzyme activity corresponding to the per cent reduction at 30 minutes was determined for each sample by means of the reference curve; this activity value was then multiplied by two to obtain the recorded enzyme activity value. The validity of this procedure was tested by processing eight stalk samples in the usual manner, re-dissolving the dried precipitate of each in 2.0 ml. of 0.1 M acetate buffer, and assaying the resulting solutions simultaneously with 50% dilutions of these solutions. The per cent reduction values at 30 minutes for the diluted samples were converted to enzyme activity figures using the reference curve and these activity figures multiplied by two. These values may be compared with those obtained directly from the concentrated samples (Table 4).

Table 4. Cellulase activity values obtained for full and 50% concentrations of re-dissolved precipitate samples; figures for 50% concentrations are doubled for comparison with those obtained for full concentrations

Stalk no.	Cellulase activity		
	50% conc.	50% conc. x 2	Full conc.
	September stalk samples		
A3-5	120	240	260
B3-5	500	1000	860
C2-5	21	42	42
D1-5	17	34	28
	October stalk samples		
A2-2	15	30	33
B3-2	9.0	18.0	17.6
C1-2	10.0	20	20
D2-2	14.3	28.6	27

Pith Deterioration Rating

At the time stalk sections were sliced for processing for enzyme recovery, visual estimates of the condition of the pith tissue were made from the cross-sections, approximately 3 cm. above the node. The arbitrary rating scale was established as follows:

0 = healthy stalk; yellow-green coloration throughout well-hydrated pith tissue; no white, dead (53) parenchyma cells in pith

1 = presence of white, dead parenchyma cells in pith but no cavity formation

2 = pith tissue containing smallest cavity - approximately 1/5 of cross-sectional area of pith

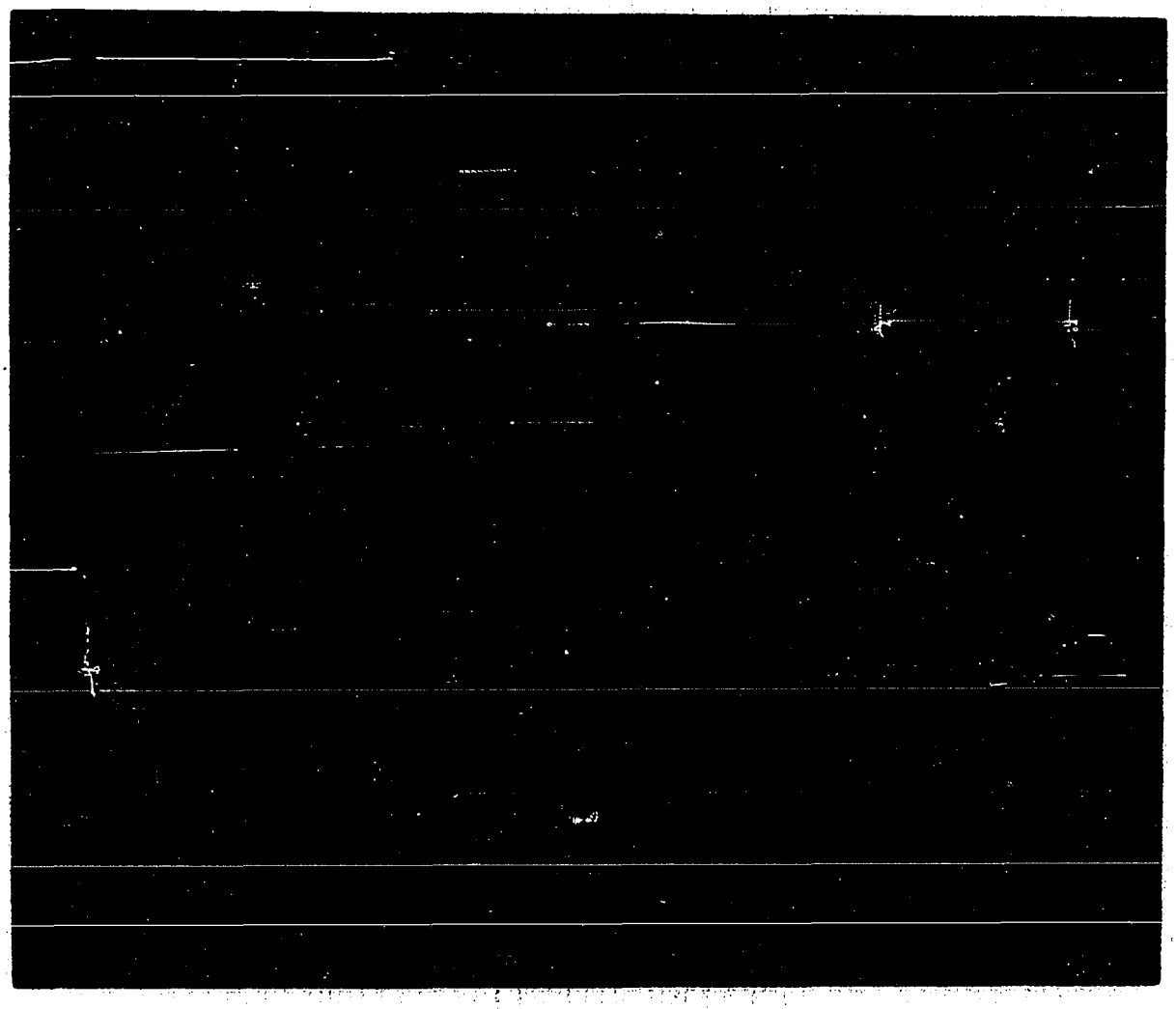
3 = pith tissue containing moderate cavity - approximately 1/2 pith area

4 = pith tissue containing large cavity - approximately 3/4 pith area

5 = little pith tissue remaining; all stalk tissue very dry; little more than hollow cylinder of rind.

Representative sections illustrating these ratings are shown in Figure 4. These pith deterioration ratings were used as the basis for evaluations of stalk rot severity throughout the present work.

Figure 4. Typical pith tissue condition exhibited in healthy and rotted stalks; numbers indicate representative pith deterioration ratings



RESULTS

Corn stalk rot varied in severity from year to year; in 1962 there was more severe rotting than in 1963. Gross differences in extent of rotting between the resistant and susceptible hybrids were usually first noticed in the September collections. In general, stalks of the susceptible hybrid, Os420 x 187-2, were characterized by being light in weight and containing a marked amount of dead, white, fluffy parenchyma tissue. Stalks of B14 x C103, on the other hand, were markedly solid with well hydrated, seemingly healthy tissue. Stalks of WF9 x W22 and Hy x 38-11 were intermediate between these extremes in the characteristics mentioned. In October, these differences were more pronounced; many stalks of Os420 x 187-2 were little more than hollow cylinders of rind tissue, much of the parenchyma tissue being disintegrated, while the same solidity noted in the B14 x C103 stalks in September prevailed in October.

Stalk Strength

Individual stalk strength determinations were averaged for hybrids, times of sampling, and years (Figures 5 and 8). In both 1962 and 1963, stalk strength for all hybrids was greater in mid-August than in mid-July. This increase in strength occurred during the period just prior to, during, and following elongation and flowering. After the mid-August sampling, stalk

strength decreased during 1962. A possible exception was Hy x 38-11, stalk strength of which remained essentially constant after mid-August.

In 1963, with less stalk rot in general than in 1962, hybrids B14 x C103 and WF9 x W22 gained in stalk strength until the September 16 sampling date; there were noticeable drops in stalk strength for both hybrids between September 16 and October 16. Hy x 38-11 and Os420 x 187-2 gained in stalk strength from mid-July to mid-August but showed subsequent continuous declines.

Pith Deterioration

Examination of cross-sections of corn stalks at intervals during the season revealed, usually beginning in August, successive stages of breakdown or disintegration of the parenchymatous pith tissue. Unaffected pith tissue was well-hydrated and of a yellow-green color. There appeared as the first evidence of breakdown a white, fluffy, dry tissue, composed of dead parenchyma cells. This transformation commenced at the center of the stalk and gradually progressed toward the periphery. Following the development of such tissue a cavity sometimes formed in cross-section; this cavity appeared to start as a small crack near the center of the stalk and progress in a somewhat radial pattern. Generally the cavity did not assume a circular form but progressed as a series of cracks or splits, be-

coming more or less "star-shaped". Eventually the disintegrated portion enlarged outwardly to the rind, the cavity gradually taking on a circular outline. In severely rotted stalks a hollow cylinder of rind tissue was all that remained. Steps in this progress of deterioration were rated as outlined under "Laboratory Procedures".

Individual pith deterioration ratings were averaged according to corn hybrid and time of sampling, and so depicted in Figures 6 and 9. Progressive disintegration of pith tissue was more pronounced in all hybrids in 1962 than in 1963. The resistant hybrid, B14 x C103, showed the least disintegration in both years while susceptible Os420 x 187-2 was given the highest average deterioration ratings. B14 x C103 and Hy x 38-11 showed little or no disintegration until October with no cavities in the majority of stalks even in October; some amount of dry pith tissue was found, however, in most samples of Hy x 38-11 after July of 1962. In contrast was the pronounced deterioration in Os420 x 187-2 in October of 1962; WF9 x W22 approached this condition also.

Cellulase Activity

Individual cellulase activity values were averaged with respect to hybrid and collection date for the two years (Figures 7 and 10). Generally, activity values were considerably higher for 1962 than for 1963, the only exception being stalks of the

Figure 5. Average stalk strength values for four hybrids in 1962, as determined from second internode above uppermost brace roots

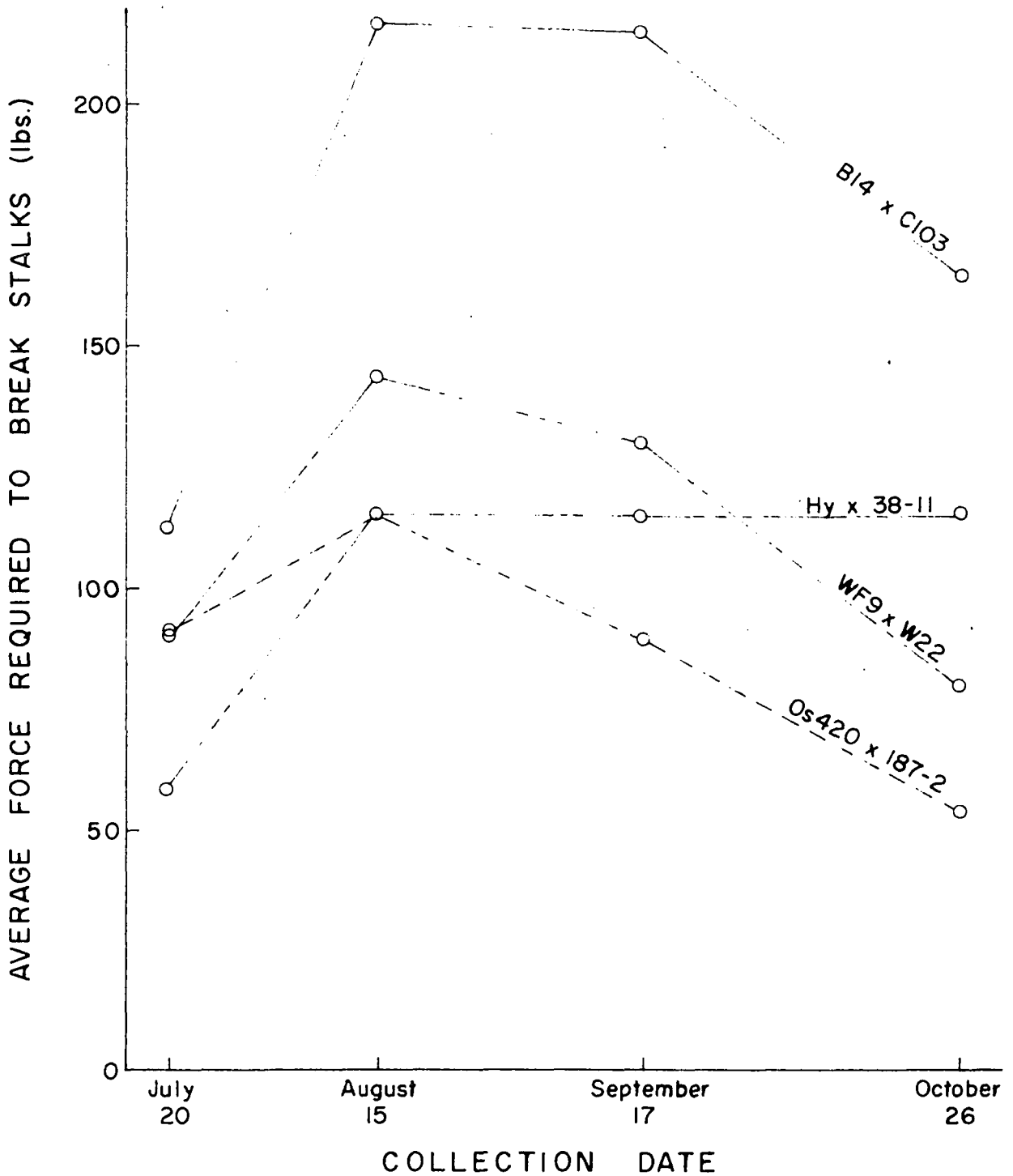

AVERAGE FORCE REQUIRED TO BREAK STALKS (lbs.)
200
150
100
50
0
B14 x C103
Hy x 38-11
WF9 x W22
Os420 x 187-2
July 20
August 15
September 17
October 26
COLLECTION DATE

Figure 6. Average pith deterioration ratings for four hybrids in 1962, as determined from cross-section of stalk approximately 3 cm. above first node above uppermost brace roots

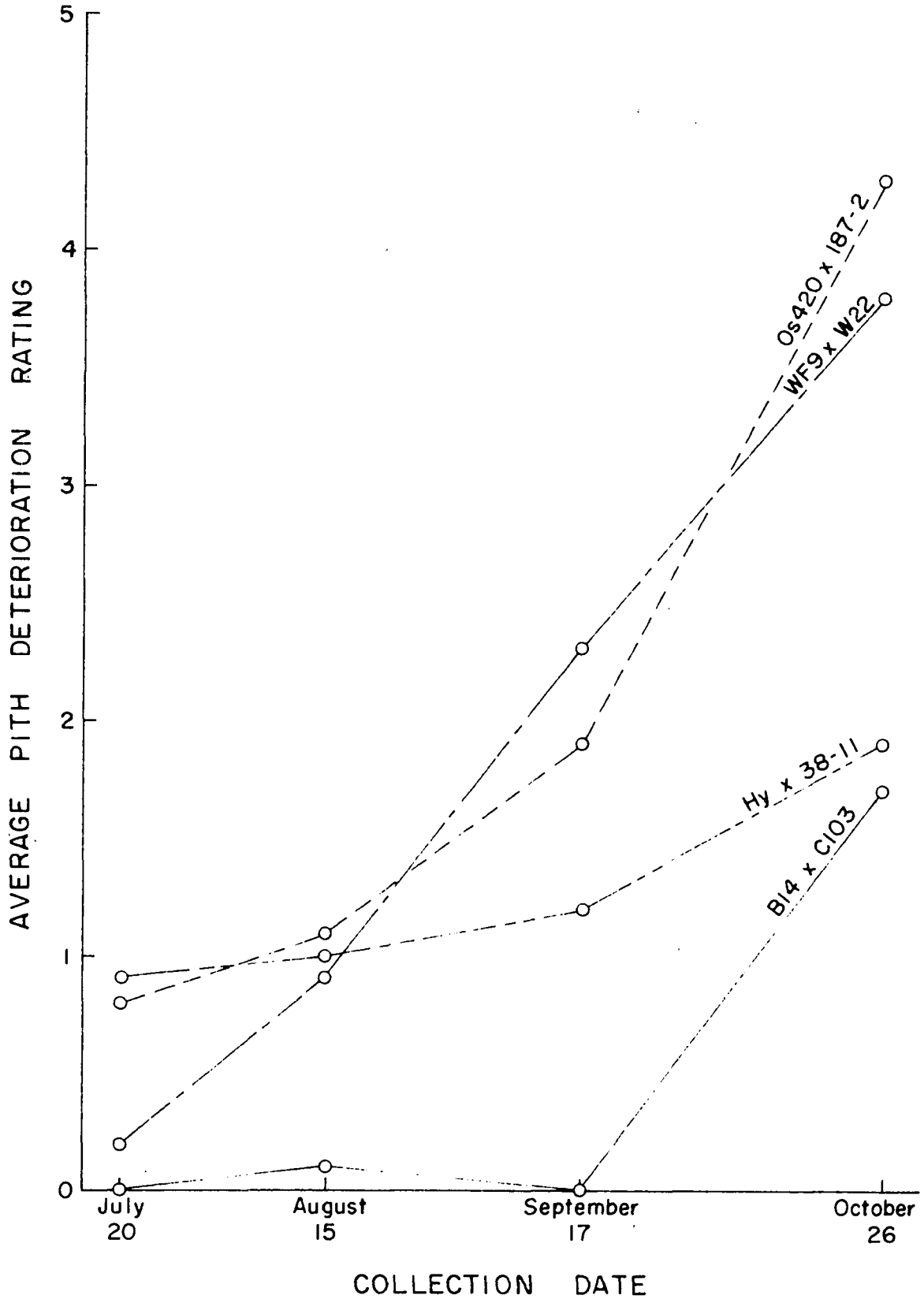

AVERAGE PITH DETERIORATION RATING
5
4
3
2
1
0
Os420 x 187-2
WF9 x W22
Hy x 38-11
B14 x C103
July 20
August 15
September 17
October 26
COLLECTION DATE

Figure 7. Average cellulase activity values for four hybrids in 1962, as determined from first node above uppermost brace roots

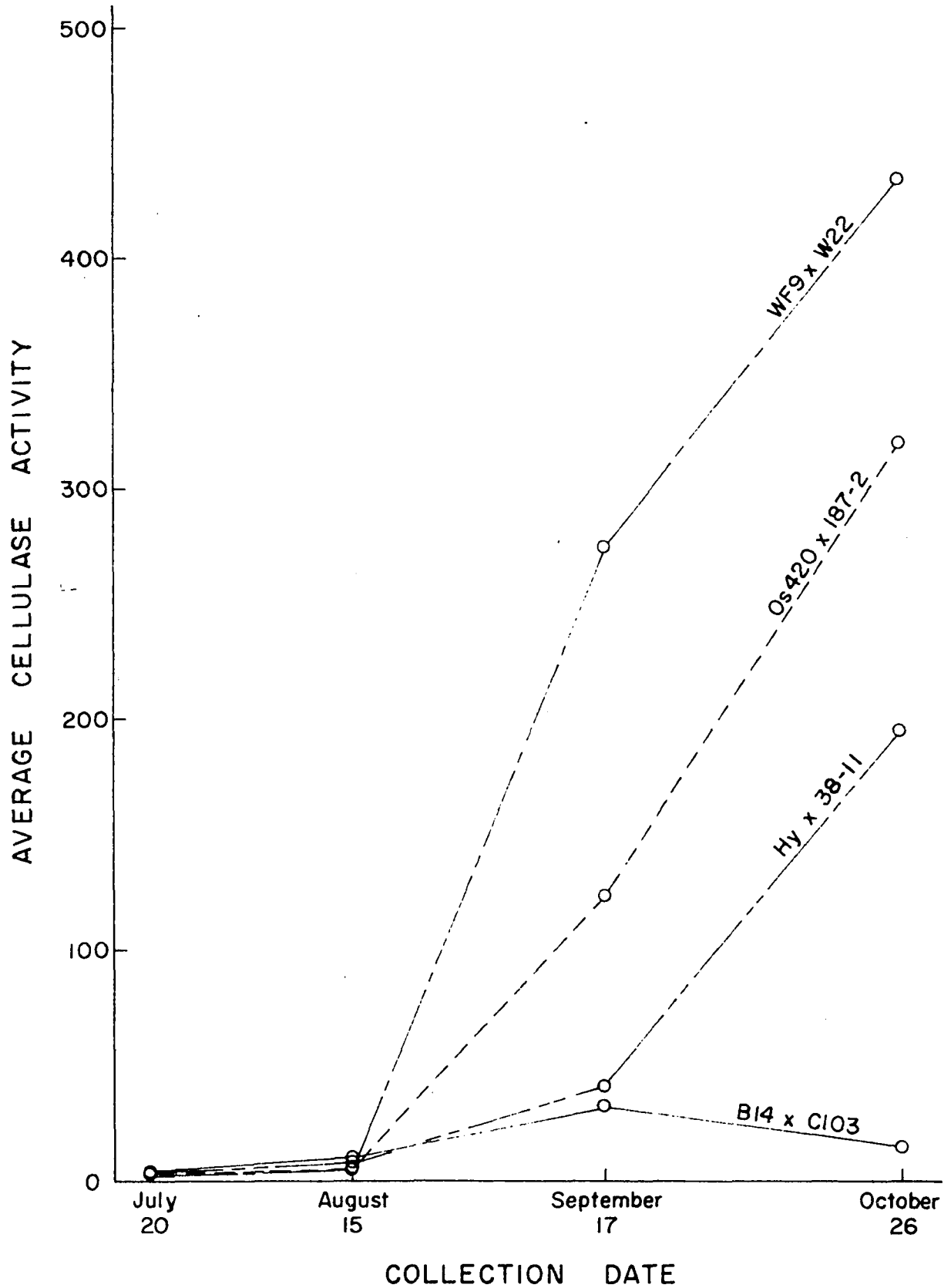

500
400
300
200
100
0
AVERAGE CELLULASE ACTIVITY
WF9 x W22
Os420 x 187-2
Hy x 38-11
B14 x C103
July 20
August 15
September 17
October 26
COLLECTION DATE

Figure 8. Average stalk strength values for four hybrids in 1963, as determined from second internode above uppermost brace roots

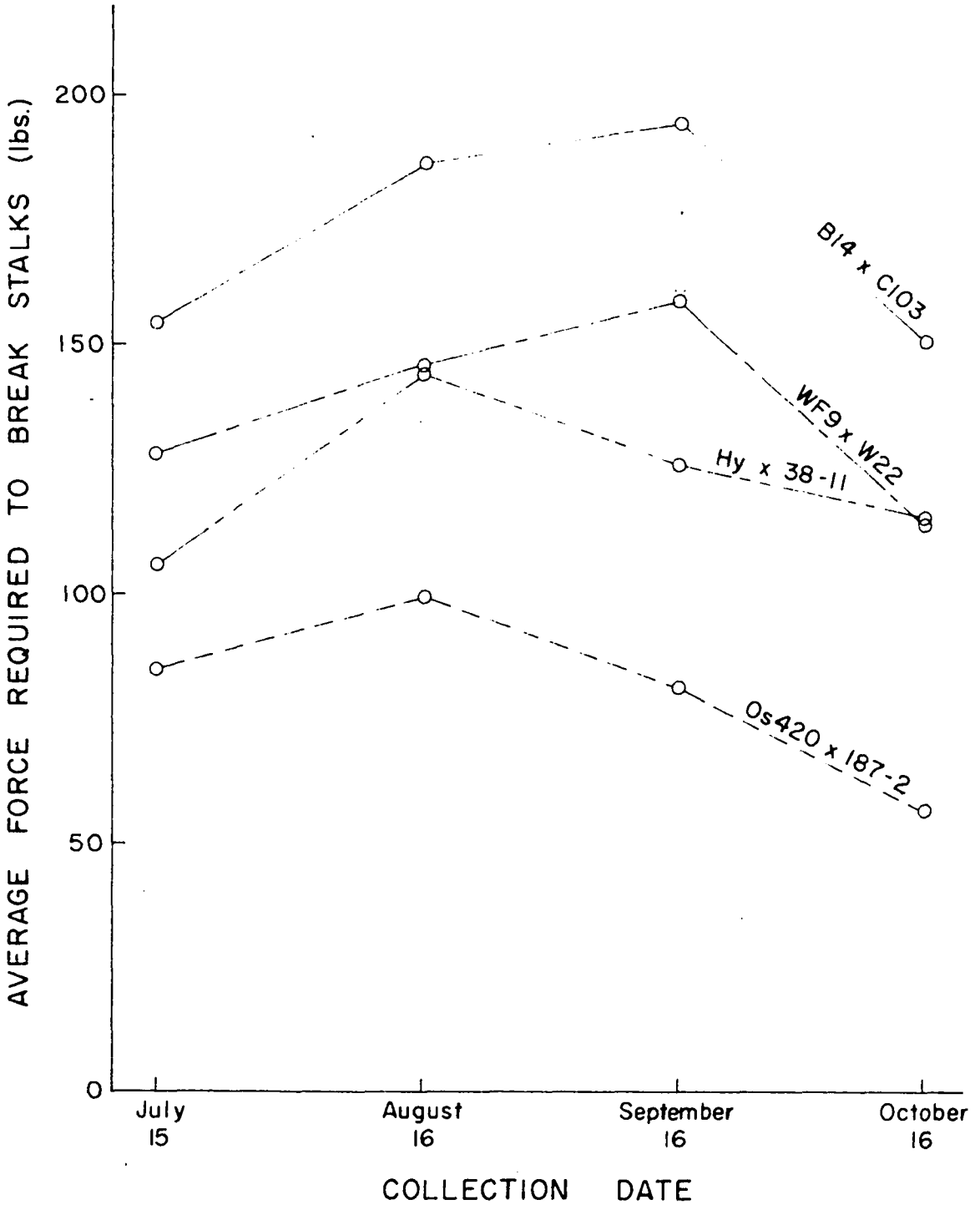

AVERAGE FORCE REQUIRED TO BREAK STALKS (lbs.)
200
150
100
50
0
B14 x C103
WF9 x W22
Hy x 38-11
Os420 x 187-2
July 15
August 16
September 16
October 16
COLLECTION DATE

Figure 9. Average pith deterioration ratings for four hybrids in 1963, as determined from cross-section of stalk approximately 3 cm above first node above uppermost brace roots

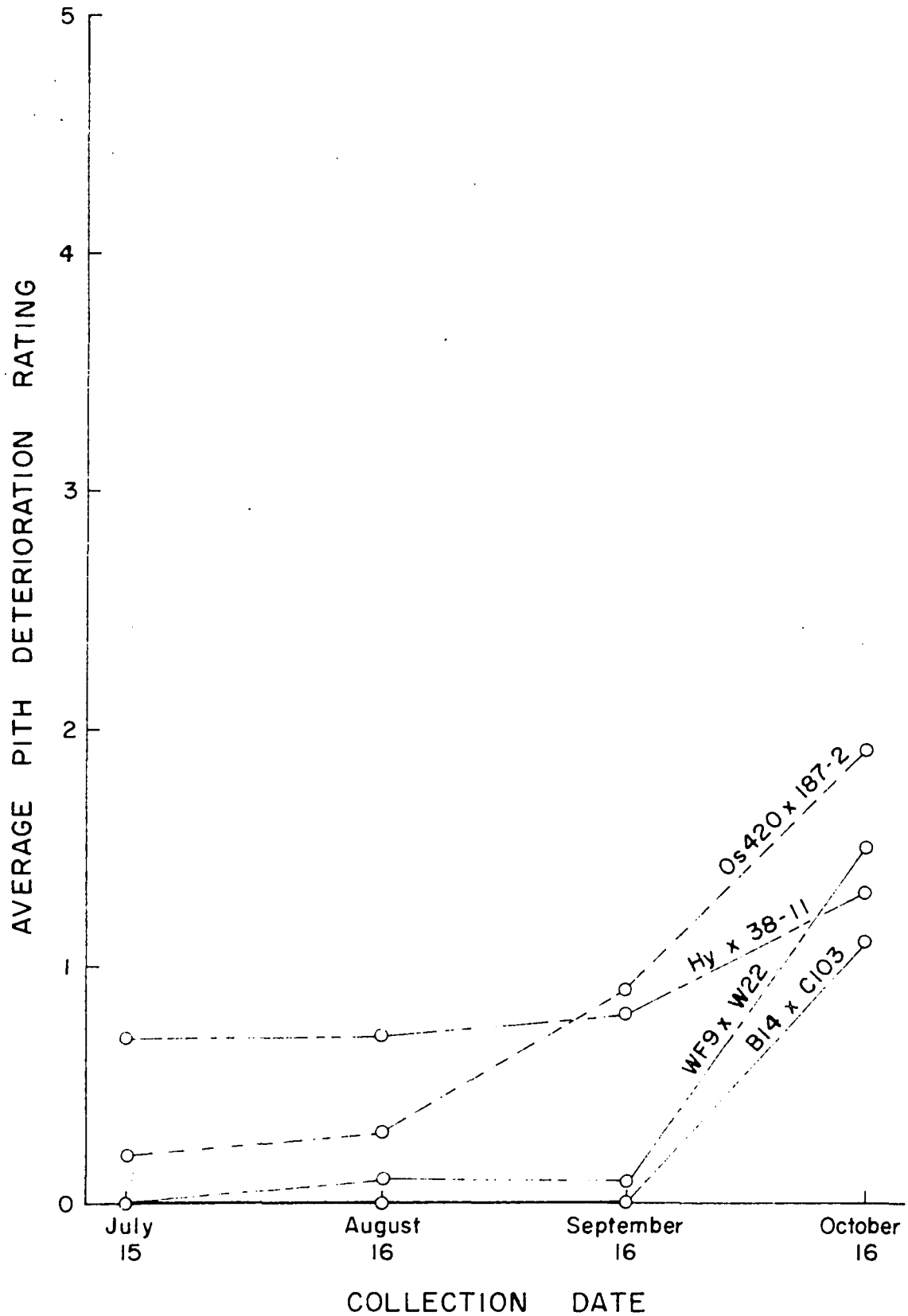
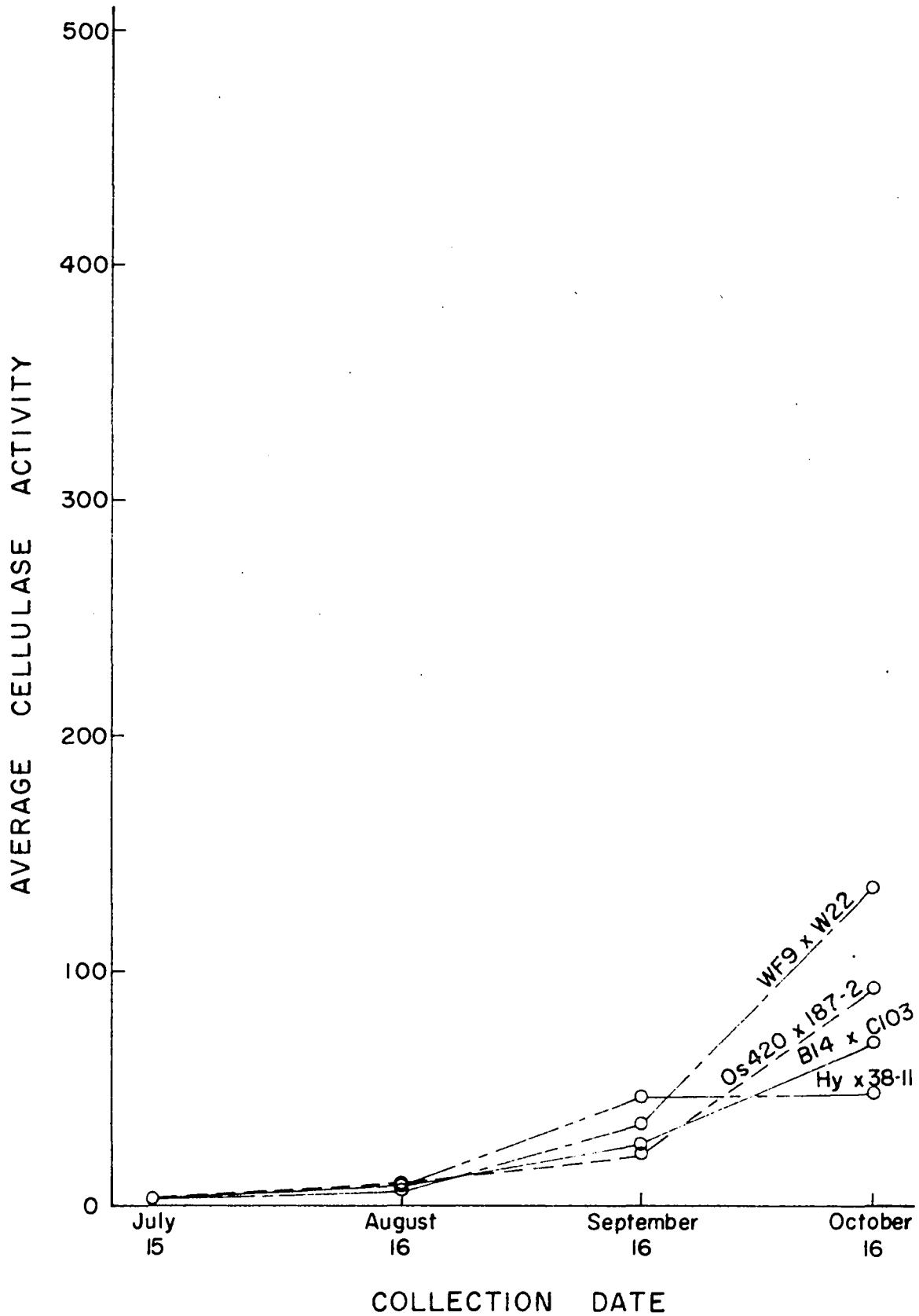

AVERAGE PITH DETERIORATION RATING
5
4
3
2
1
0
July 15
August 16
September 16
October 16
COLLECTION DATE
Os420 x 187-2
Hy x 38-11
WF9 x W22
B14 x C103

Figure 10. Average cellulase activity values for four hybrids in 1963, as determined from first node above uppermost brace roots


500
400
300
200
100
0
AVERAGE CELLULASE ACTIVITY
WF9 x W22
Os420 x 187-2
B14 x C103
Hy x 38-11
July 15
August 16
September 16
October 16
COLLECTION DATE

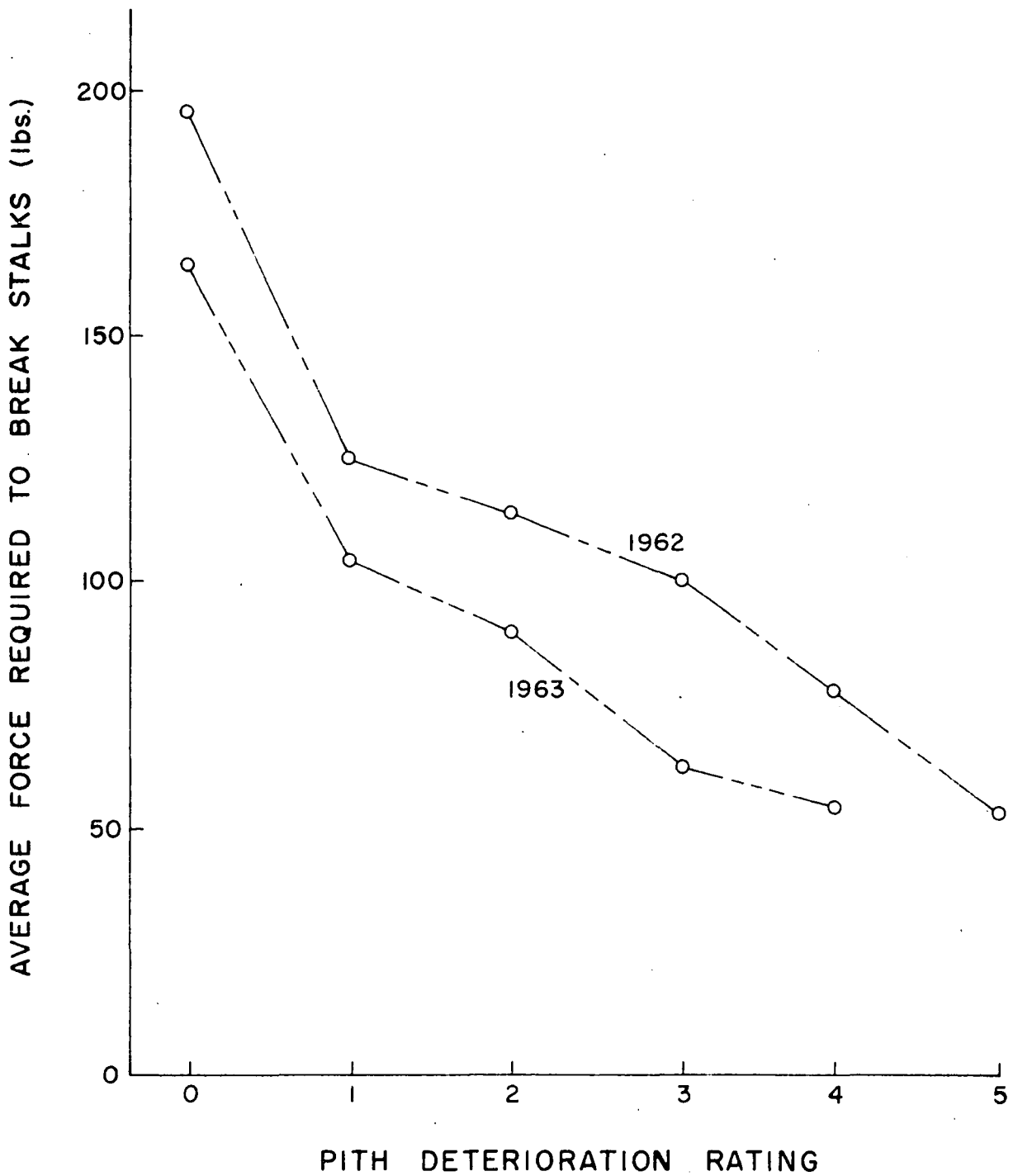
resistant hybrid B14 x C103. In 1962 enzyme activity values increased to a noticeable extent after the August 15 collection. The same was true in 1963, however increases were of lesser magnitude. Notably high in enzyme activity values, especially in 1962, were Os420 x 187-2 and WF9 x W22, the latter being the highest in both years. Generally it was evident that cellulase activity in the stalks tested was higher as the season progressed, with low but detectable levels of activity in July and August.

Figures 5 through 10 have been arranged so as to facilitate comparison, with respect to time of sampling season, of values for stalk strength, pith deterioration, and cellulase activity within each year. During progression of the sampling season stalk strength was inversely related to both pith deterioration and magnitude of cellulase activities.

Stalk Strength as Related to Pith Deterioration

For each pith deterioration rating, individual stalk strength figures were averaged to include all four hybrids for 1962 and 1963 (Figure 11). Computations of these averages were made, however, excluding data of July samplings. It was evident that there was progressive anatomical stalk development until mid-August in most cases, hence low stalk strength figures for July would markedly influence average strength figures in a manner misleading in interpretations of effects of rotting.

Figure 11. Average stalk strength values by pith deterioration ratings for 1962 and 1963; inclusive of data of all hybrids for August, September, and October collection dates


AVERAGE FORCE REQUIRED TO BREAK STALKS (lbs.)
200
150
100
50
0
1962
1963
0
1
2
3
4
5
PITH DETERIORATION RATING

While over-all stalk strength was somewhat less in 1963 than in 1962, strength decline was definitely associated with increasing pith decay. Pith deterioration was not as pronounced in 1962; no representative stalk samples were encountered with a rating of 5.

Pith Deterioration as Related to Cellulase Activity

As a means of categorizing data, the full range of cellulase activity values, 1 to 1000, was divided into classes. Reference to Figure 3 will show that the logarithmic scale may be divided into six sections of similar length at 3.0, 10.0, 30, 100, and 300. Pith deterioration ratings were averaged with respect to enzyme activity class, including all hybrids for each year (Figure 12). As seen previously, average pith deterioration was more pronounced in 1962 than in 1963. In both years, however, there was a marked trend toward higher ratings, i.e. more pith decay, with increasing enzyme activity.

Cellulase Activity as Related to Stalk Strength

Average stalk strengths were computed for each class of enzyme activity, including all hybrids for each year (Figure 13). July data were again excluded in computing the averages for reason previously mentioned. Average stalk strength was similar in 1962 and 1963 with respect to enzyme activities. In both years decrease in stalk strength was generally associated

Figure 12. Average pith deterioration ratings by cellulase activity classes for 1962 and 1963; inclusive of data of all hybrids for all collection dates

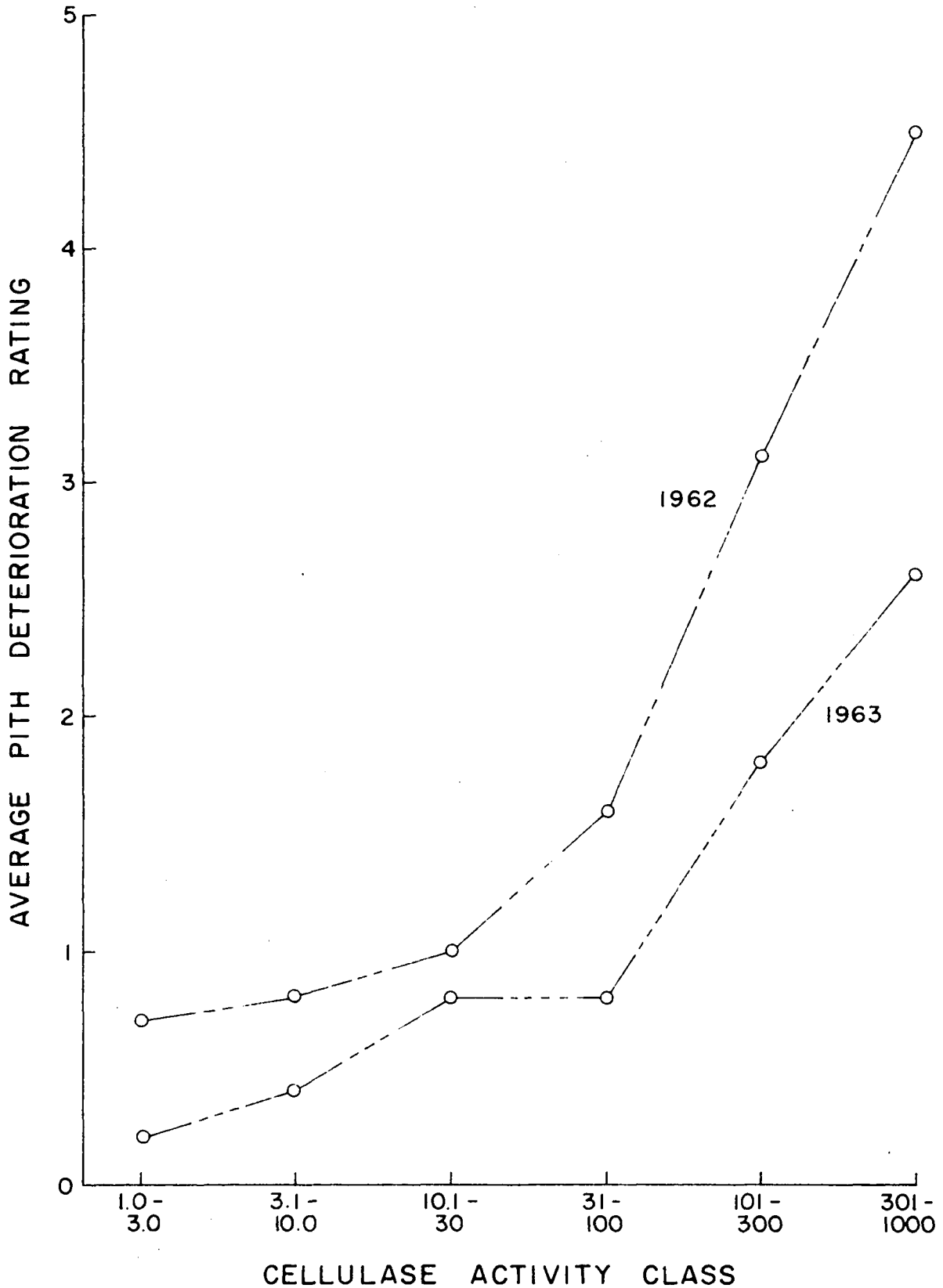
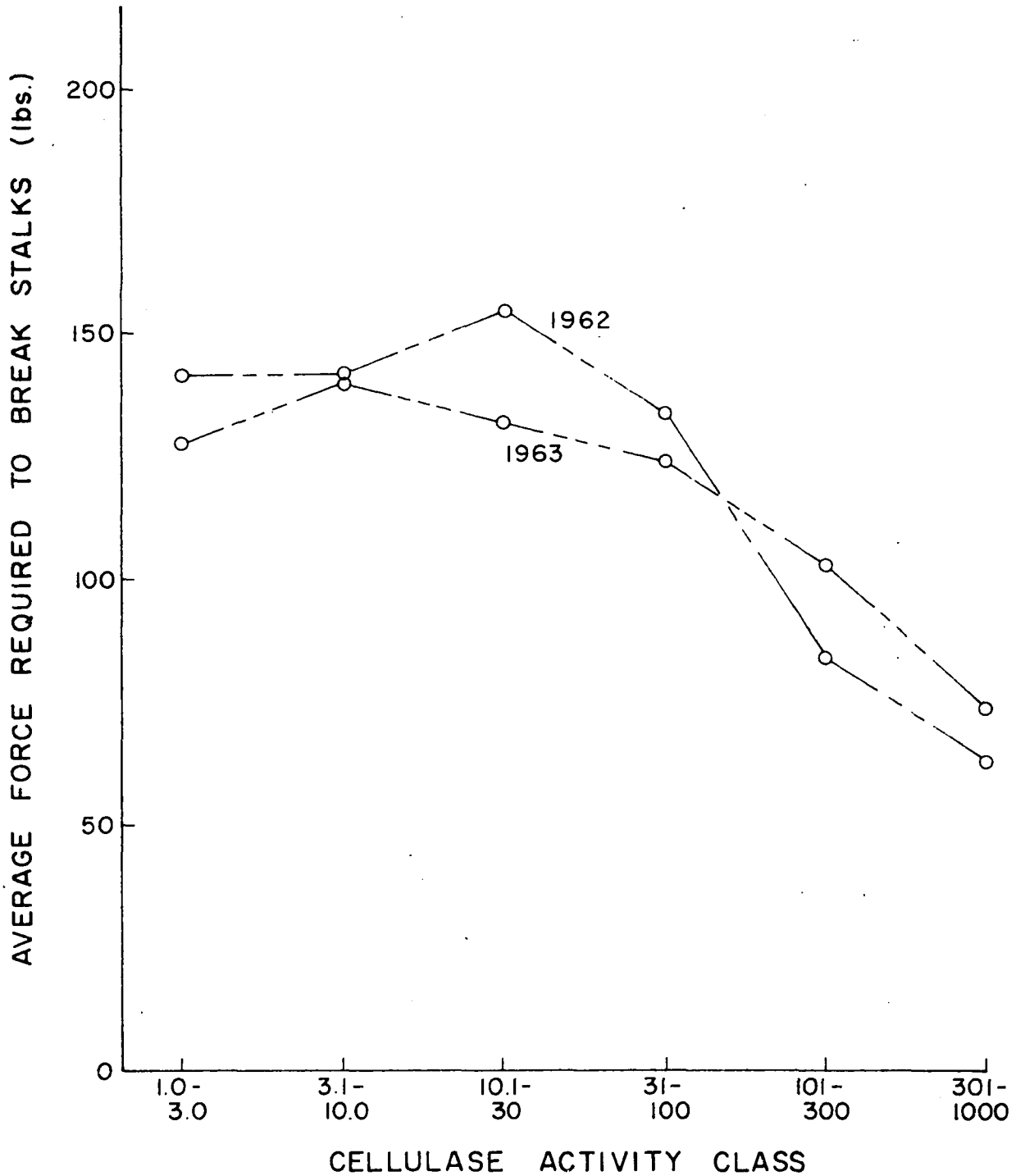

AVERAGE PITH DETERIORATION RATING
0
1
2
3
4
5
1962
1963
1.0 - 3.0
3.1 - 10.0
10.1 - 30
31 - 100
101 - 300
301 - 1000
CELLULASE ACTIVITY CLASS

Figure 13. Average stalk strength values by cellulase activity classes for 1962 and 1963; inclusive of data of all hybrids for August, September and October collection dates


AVERAGE FORCE REQUIRED TO BREAK STALKS (lbs.)
200
150
100
50
0
1962
1963
1.0-
3.0
3.1-
10.0
10.1-
30
31-
100
101-
300
301-
1000
CELLULASE ACTIVITY CLASS

with increase in enzyme activity.

General Relationships between Stalk Strength, Pith Deterioration, and Cellulase Activity

Rotting of pith tissue in 1962 was pronounced in WF9 x W22 and Os420 x 187-2; deterioration progressed rapidly in these hybrids throughout the period of sampling, reaching in October a point of severity highest among all samples tested in both years. Strength of deteriorating stalks of these hybrids decreased substantially following the August sampling. B14 x C103 did not show severe rotting; markedly healthy pith tissue was noted in all months, with deterioration evident in some stalks only in October. Concurrently there was little loss in stalk strength in this hybrid until October. Pith deterioration also was consistently low in Hy x 38-11, and stalk strength remained rather constant throughout August, September, and October.

1963 samples were characterized by milder rotting than observed in 1962, and this was accompanied by less stalk weakening. Both WF9 x W22 and B14 x C103 displayed an increase in strength up through September, instead of weakening after the August sampling as in 1962. Pith deterioration was nil in these varieties until October. Evidently anatomical developmental processes progressed in the absence of noticeable deterioration through September, considerably past the pollination period. Os420 x 187-2 and Hy x 38-11 exhibited progressive loss of stalk strength in September and October, with some pith deterioration

evident in July and August and more pronounced rotting in the latter months.

In both years it was generally observed that stalk strength decreased with the first appearance of white, dead parenchyma cells in the pith and continued to decrease as parenchyma cell deterioration became more pronounced. Furthermore, in the absence of noticeable deterioration, stalks continued to increase in strength even beyond silking dates.

Both pith decay and cellulase activity were more pronounced in 1962 than in 1963. In both years severity of rotting increased with increasing amounts of cellulase activity, especially within the activity range 31 to 1000. Cellulase activity increased during the growing season to a maximum in October in all hybrids, except B14 x C103 in 1962.

As stalk strength decreased with increasing magnitudes of pith deterioration, and as severity of pith deterioration was directly associated with increasing cellulase activity, so stalk strength was found to decrease with increasing levels of enzyme activity in the stalk. These relationships were evident not only in progression through the sampling season but also in direct comparisons of data for the three aspects.

Detection of *Fusarium moniliforme* in Corn Stalks

Upon incubation in moist chambers, corn stalks usually yielded growth of *Fusarium moniliforme* in 80% to 100% of cases

tested. This organism typically exhibited a characteristic habit of growth upon the stalk tissue under such circumstances, i.e. a slightly pinkish mat of mycelium in close proximity to the surface of the stalk section. Aerial mycelium occurred only after several weeks, if at all. Once a positive identification of the organism was made with a representative number of stalks, characteristic appearance of macroscopic growth was used as the identifying criterion for presence of F. moniliforme on the incubated stalk sections.

Pure colonies of F. moniliforme usually occurred on stalk samples collected in July and August. Several stalk samples from September and October collections yielded colonies of other fungi which often overwhelmed any growth similar to that identifiable as F. moniliforme. In such cases positive identification of F. moniliforme could not be made; however, failure to identify F. moniliforme was not considered to preclude its presence in the stalk tissue.

Percentages of detection of F. moniliforme in incubated stalks were lower in July of 1963; however, August figures for both years indicated 100% of stalks infected (Table 5).

Table 5. Incidence of detection of *Fusarium moniliforme* from corn stalk sections (second node above uppermost brace roots) incubated in moist chambers at room temperature

Hybrid	July		August	
	Total no. of stalks tested	Percent stalk sections yielding *F. monil.*	Total no. of stalks tested	Percent stalk sections yielding *F. monil.*
		1962		
WF9 x W22	14	100	15	100
Os420 x 187-2	16	94	16	100
B14 x C103	16	100	16	100
Hy x 38-11	16	88	14	100
		1963		
WF9 x W22	15	47	15	100
Os420 x 187-2	15	73	14	100
B14 x C103	15	93	15	100
Hy x 38-11	15	80	15	100

DISCUSSION AND CONCLUSIONS

Strength of a corn stalk internode is dependent, to a degree, upon certain structural characteristics, such as degree of lignification or amount of sclerenchyma tissue in the rind and around peripheral vascular bundles (8, 26, 29, 39, 71). With corn stalks tested in 1962 and 1963 there was increase in stalk strength after the first sampling date, in mid-July, even until the third sampling date, in mid-September, in 1963, in spite of some cellulase activity and pith deterioration. Discoloration or decomposition of parenchymatous pith tissue within the stalk does not appear to provide quite the same evaluation of stalk strength as actual field "standability" (25, 39, 67, 71).

Various methods have been used in laboratory testing of stalk strength, including magnitude of force required to crush a stalk section longitudinally (71), and force required to break a stalk upon lateral application (8). The laterally-applied force technique reported here, while not in itself providing actual field "standability" data, does simulate field conditions conducive to stalk breakage or lodging. It seems reasonable to postulate that a stalk containing healthy parenchyma tissue would not be expected to collapse and/or break under a lateral force (e.g. wind) as readily as one containing deteriorated parenchyma tissue.

From data presented it is evident that pith condition is

not solely responsible for stalk strength. Minimum and maximum strength values varied among hybrids tested, even where pith deterioration was similar, especially in 1963. More severe pith deterioration and higher cellulase activities were recorded in 1962 than in 1963; over-all stalk strength for each hybrid, however, was similar in the two years. Such observations indicate the role of inherent structure in stalk strength. Nevertheless, the data show that stalk strength definitely decreased with progressive pith deterioration, particularly in September and October. Such was evident not only as a progressive phenomenon through the sampling season, but also in direct comparisons of stalk strength and pith deterioration data.

With all hybrids general increases in cellulase activity were coincident with increases in pith deterioration and decreases in stalk strength. Again, these relationships were demonstrated with respect to season (time of sampling) and in direct comparisons of respective data. Utilization of cellulose by Diplodia zeae has been reported (7), and cellulolytic enzymes extracted from corn stalk tissue and from cultures of Fusarium moniliforme have been shown to disintegrate cell walls of corn stalk parenchyma tissue and soften rind tissue sections[1] (13, 14). It is therefore concluded that cellulolytic enzymes in the corn stalk act to soften and disintegrate cell wall structure

[1]Foley, D. C. Assoc. Prof. of Botany and Plant Pathology, Iowa State University, Ames, Iowa. Presence of cellulase in corn stalks. Private communication. 1963.

and thus bring about weakening of the stalk. Since initiation of stalk weakening occurred not at the same level of enzyme activity for all four hybrids, no single threshold of enzyme activity appears to be related to onset of stalk weakening. This fact may also be related to the anatomical features of the respective hybrids.

Whitaker (68) believes cellulase to be a single component enzyme; much evidence, however, has been accumulated (30, 56) in support of the multiple component concept developed by Reese and his co-workers (56, 57, 58). If this concept is valid, the methods used in the present study may have served to assay for the Cx component, or that which is capable of hydrolyzing modified cellulose only. Nonetheless, the presence of such a component in the stalks would very likely be an index of presence of a C_1 component, supposedly necessary for "native" cellulose breakdown, in the stalk tissue. The Cx component thus served as a convenient assay index for the action of a C_1 fraction evidently present in stalks which were rotted.

The C_1 fraction has rarely been observed in extract preparations, either from plant tissue or fungal cultures. In the majority of cases the actual presence of a fungal cellulase-producing organism has been necessary for attack on "native" cellulose substrates (30, 61). Because of this, results obtained

by Foley[1] and Fagle (13) are of special interest; various extracts prepared possessed capabilities of disintegrating corn stalk parenchyma cell walls and weakening rind sections. Evidently the extraction procedures used, quite similar to those involved here, recovered a C_1 fraction from the stalk tissue and from fungal cultures, or possibly the thin, rather delicate cell walls of the pith parenchyma tissue are not resistant to attack by the Cx component, thus resembling the nature of CMC (a modified cellulose) rather than that usually attributed to "native" cellulose.

The presence of cellulase in the corn stalk necessarily raises the question as to its origin within the tissue. Very low activity levels of cellulase have been found in seeds and meristematic regions of vascular plants. It has been suggested that this cellulase is involved in breakdown of cell wall cellulose, deemed necessary for cell enlargement, and hence is produced endogenously by the plant (50, 63). While low levels of cellulase activity found in the stalks in July and August could, as a remote possibility, be due to endogenously-produced enzyme, levels found in the latter months of sampling are substantially higher than reported for "naturally-occurring" cellulases.

D. zeae, a common stalk rotting fungus, is capable of

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[1]Foley, D. C. Assoc. Prof. of Botany and Plant Pathology, Iowa State University, Ames, Iowa. Presence of cellulase in corn stalks. Private communication. 1963.

utilizing cellulose (8, 61). While the author was unable to locate publications citing Gibberella zeae as a producer of cellulase, this organism would most likely also be capable of production of this enzyme in view of its role in stalk rotting. The presence of these organisms in stalks undoubtedly provides a source of cellulolytic enzymes. The ability of F. moniliforme to produce cellulolytic enzymes, however, has been well demonstrated (13, 14, 17, 61). The present study and others (e.g. 9, 11, 14, 16, 27, 31, 55, 60) have demonstrated the very frequent presence of this organism in corn seed and stalks and leave little doubt that stalks are commonly infected with this fungus, even as early as July. Infection by this fungus is far more common than that by other stalk rotting organisms. Koehler (31) has reported that near the middle of September, when sampling in the reported work was done, approximately 25% of corn plants exhibited lesions characteristic of D. zeae infection, about 45% displayed G. zeae lesions, while nearly 90% of stalks showed lesions caused by F. moniliforme. In these experiments 100% of stalks tested were infected with F. moniliforme as early as mid-August. Coincident with such high incidence of infection, no stalk samples of nearly 500 tested during the two-year period, representative of July through October, were without some amount of cellulase, the enzyme levels in September being substantially high.

Foley found that activity of cellulolytic agents was higher

in nodes than in inter-nodes of the corn stalk (14), that the first visible evidence of cell wall deterioration occurred in nodal tissue (15), and that breakage due to rot occurred at nodes in more than 90% of broken stalks (15). Coincident with these findings was the more frequent isolation of F. moniliforme from the nodes than from internodes (16). Observations reported here strongly substantiate the suggestion by Foley (16) that F. moniliforme is the agent responsible for at least early stalk deterioration resulting in weakening of the stalk. It is concluded, therefore, that F. moniliforme is an active stalk rotting pathogen which incites stalk tissue decay earlier in the growing season than other stalk rotting organisms and, because of its widespread occurrence and cellulolytic activity, continues to act as a destructive pathogen throughout the growing season. It is further suggested that early attack on corn stalk tissue by this organism, producing dead and weakened stalk tissue, may even predispose plants to entry and activity of additional stalk rotting pathogens later in the season.

There have been various studies of mechanisms of stalk rot resistance (28, 54, 71). It has been shown that celluloses vary in susceptibility to attack by cellulolytic enzymes according to molecular structure and configuration (19, 20, 30, 37, 59). While resistance to stalk rotting could possibly be attributed to certain cellulosic structure in pith tissue, the results reported here lead to the conclusion that resistance is afforded

by some mechanism which determines levels of cellulase in the stalk tissues. As stalks are commonly infected with F. moniliforme even in July, various physiological changes occurring in the stalk during pollination time probably incite initiation of more abundant cellulase production by the fungus.

SUMMARY

Quantitative determinations of cellulolytic enzymes in corn stalk tissue were made by assaying enzyme samples precipitated from aqueous stalk extracts. Tests of stalk strength against laterally-applied force and visual estimates of pith tissue deterioration were also made with the same stalks. Four single-cross hybrids were used: B14 x C103, resistant to stalk rot; Os420 x 187-2, susceptible; and two intermediate in resistance, WF9 x W22 and Hy x 38-11.

There were more marked pith deteriorations, higher enzyme activity values, and greater declines in stalk strength in 1962 than in 1963. However, in general with all four hybrids, magnitude of enzyme activity values was directly related to extent of pith deterioration and inversely related to stalk strength. The hybrids exhibiting more and faster pith decay were characteristically higher in enzyme activity. There was seasonal progression of pith deterioration and increase in enzyme activity values, with highest levels of both in the October samplings. In varieties which underwent stalk strength decline, the decline was usually evident in the September and October samplings.

Considering individual hybrids: The resistant hybrid B14 x C103 was characterized by very little pith deterioration until after September samplings, low enzyme activity even at the September and October sampling dates, and consistently high stalk

strength with slight decline evident after September samplings. The susceptible hybrid Os420 x 187-2 exhibited progressive marked pith deterioration throughout the sampling period, a similar progressive increase in enzyme activity values, which increase was marked in the September and October samplings, and a progressive decline in stalk strength following the August samplings. Hybrid WF9 x W22, intermediate in resistance, was similar to the susceptible Os420 x 187-2 except that pith deterioration and decline in stalk strength occurred later in 1963. Intermediate hybrid Hy x 38-11 was intermediate between the resistant and susceptible hybrids in magnitude of seasonal pith deterioration, enzyme activity values, and stalk strength, and all these characters were more constant during the season than for any of the other three hybrids.

It is concluded that within varieties there was association between progressive seasonal increase in enzyme activity values, increase in pith deterioration and decline in stalk strength. It is further concluded that cellulolytic enzymes in the corn stalk soften and disintegrate stalk tissue thus causing weakening of the stalk.

While hybrid evaluations of stalk strength may have been complicated by inherent anatomical structure and development of the respective hybrids, comparisons of the four on the basis of enzyme activity, pith deterioration ratings, and stalk strength determinations would leave them ranked essentially: resistant,

B14 x C103; susceptible, Os420 x 187-2; intermediate, Hy x 38-11 and WF9 x W22.

Fusarium moniliforme was isolated from a high percentage of stalks tested; 100% recovery was effected in September of 1962 and 1963. Due to such infection, as compared with prevalence of other major stalk rotting organisms, it is concluded that this organism is a destructive pathogen responsible for production of cellulolytic enzymes found in the stalks.

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ACKNOWLEDGMENTS

The author wishes to express sincere appreciation to Dr. D. C. Foley for his suggestion of the topic of study and for helpful guidance and criticism during the development of the investigation and preparation of the dissertation. His originality of thought, especially concerning the research project, has served as a distinct challenge to the author in discovering and developing concepts in scientific research.

Sincere gratitude is also expressed to Dr. W. F. Buchholtz for his professional and personal abilities in assuming the duties of the author's major professor following Dr. Foley's transfer to the Uruguay AID project. Dr. Buchholtz' capacity to quickly grasp the concepts embodied in the research project and to render guidance in preparation of the manuscript is both appreciated and admired.

The author is also indebted to many other staff members of the Department of Botany and Plant Pathology for their willingness to help in various aspects of the study.

Throughout the entire term of graduate study - in course work, research, and preparation of the dissertation - the author has been continually aware of unequaled guidance and encouragement in "the depth of the riches and wisdom and knowledge of God"[1] as experienced through His Son Jesus Christ. The writer's gratitude for these benefits goes far beyond that which could be expressed in written acknowledgment.

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[1]Romans 11:33 (R.S.V.)

APPENDIX

Table 6. Stalk strength in pounds, cellulase activity at 30 minutes incubation, pith deterioration ratings, and detection of *Fusarium moniliforme* (July and August only), for corn stalk samples of four hybrids tested in 1962 and 1963

Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]	Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]
				July 1962					
	WF9 x W22					Os420 x 187-2			
1	lost				1	88	2.7	0	+
2	134	4.9	0	+	2	85	1.5	0	+
3	130	1.0	0	+	3	108	2.4	1	+
4	115	1.3	0	+	4	98	1.6	1	+
5	90	3.2	0	+	5	85	7.9	0	+
6	112	1.0	0	+	6	85	1.0	1	+
7	75	1.4	0	+	7	80	1.7	1	+
8	92	1.0	0	+	8	104	2.7	1	+
9	50	3.4	0	+	9	110	14.2	0	+
10	120	1.5	0	+	10	80	1.6	1	-
11	120	1.0	1	+	11	75	1.4	1	+
12	75	5.1	1	+	12	95	1.6	1	+
13	135	1.3	0	+	13	125	1.2	1	+
14	55	2.5	0	+	14	58	1.9	1	+
15	lost				15	92	4.8	1	+
16	98	1.3	1	+	16	95	1.4	1	+
	B14 x C103					Hy x 38-11			
1	92	1.9	0	+	1	78	1.6	1	+
2	40	2.3	0	+	2	37	3.8	0	+
3	80	1.7	0	+	3	40	3.2	1	+
4	112	2.1	0	+	4	62	2.5	1	+
5	142	2.5	0	+	5	74	1.9	1	+
6	145	1.8	0	+	6	52	1.0	1	+
7	134	3.7	0	+	7	72	1.7	1	+
8	156	3.3	0	+	8	85	2.0	1	+

[a]Growth of *F. moniliforme* indicated as +; no positive growth as -

Table 6. Continued

Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil[a]	Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil[a]
				July 1962					
		B14 x C103					Hy x 38-11		
9	132	28	0	+	9	60	2.0	1	+
10	110	1.7	0	+	10	55	4.1	1	+
11	122	3.6	0	+	11	24	1.9	1	+
12	150	2.3	0	+	12	52	1.6	1	+
13	142	2.4	0	+	13	30	1.3	1	-
14	84	1.6	0	+	14	55	3.5	1	+
15	55	2.1	0	+	15	94	1.6	1	-
16	90	7.2	0	+	16	56	5.8	1	+
				August 1962					
		WF9 x W22					Os420 x 187-2		
1	lost				1	95	1.0	1	+
2	145	1.3	1	+	2	100	1.4	1	+
3	110	1.0	1	+	3	100	1.1	2	+
4	154	1.0	1	+	4	132	1.1	1	+
5	130	1.0	1	+	5	108	1.7	1	+
6	115	1.4	1	+	6	118	6.6	1	+
7	222	6.5	0	+	7	102	2.7	1	+
8	112	1.0	1	+	8	122	3.9	1	+
9	112	2.7	1	+	9	156	2.5	2	+
10	170	3.1	1	+	10	141	3.1	1	+
11	108	2.6	1	+	11	106	6.6	1	+
12	143	2.6	1	+	12	122	2.7	1	+
13	132	7.8	0	+	13	103	5.0	1	+
14	134	2.1	1	+	14	138	4.3	1	+
15	174	2.8	1	+	15	100	3.5	1	+
16	153	25	1	+	16	82	2.6	1	+
		B14 x C103					Hy x 38-11		
1	230	1.4	0	+	1	110	30	1	+
2	250	14.2	0	+	2	lost			
3	250	2.1	0	+	3	132	2.1	1	+
4	250	1.2	0	+	4	lost			

Table 6. Continued

Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]	Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]
					August 1962				
	B14 x C103						Hy x 38-11		
5	250	9.1	0	+	5	75	1.5	1	+
6	210	4.0	0	+	6	100	4.6	1	+
7	250	13.7	0	+	7	120	8.3	2	+
8	176	28	1	+	8	120	1.4	1	+
9	250	2.6	0	+	9	128	2.5	1	+
10	202	31	0	+	10	110	2.1	1	+
11	184	19.5	0	+	11	148	3.2	1	+
12	250	2.7	0	+	12	102	2.5	1	+
13	152	7.0	0	+	13	115	3.1	1	+
14	228	2.6	0	+	14	96	2.8	0	+
15	169	4.7	0	+	15	119	3.6	1	+
16	163	2.7	0	+	16	111	4.6	1	+
					September 1962				
	WF9 x W22						Os420 x 187-2		
1	131	1000	5		1	25	1000	5	
2	94	880	3		2	78	74	2	
3	178	61	1		3	155	50	0	
4	126	42	2		4	94	25	2	
5	93	90	3		5	128	64	1	
6	95	51	3		6	86	53	2	
7	103	19.0	3		7	56	59	2	
8	159	110	0		8	102	21	2	
9	190	9.6	0		9	92	73	2	
10	103	1000	3		10	85	110	2	
11	124	1000	4		11	94	36	2	
12	97	53	3		12	49	260	3	
13	244	40	2		13	95	34	2	
14	92	23	2		14	94	10.5	0	
15	114	22	3		15	102	29	2	
16	134	15.0	0		16	88	76	2	
	B14 x C103						Hy x 38-11		
1	207	15.0	0		1	92	210	2	
2	248	100	0		2	146	120	1	
3	238	11.8	0		3	132	130	1	
4	210	130	0		4	136	6.9	1	

Table 6. Continued

Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of *F. monil*[a]	Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of *F. monil*[a]
					September 1962				
		B14 x C103					Hy x 38-11		
5	250	17.3	0		5	126	29	0	
6	219	35	0		6	112	13.0	1	
7	240	14.3	0		7	85	27	2	
8	250	24	0		8	112	7.8	1	
9	240	30	0		9	126	18.5	1	
10	144	11.8	0		10	99	26	0	
11	181	31	0		11	95	18.4	1	
12	230	25	0		12	144	18.0	2	
13	137	9.2	0		13	138	6.0	2	
14	250	55	0		14	90	6.1	2	
15	162	14.0	0		15	114	17.5	1	
16	232	5.6	0		16	64	8.0	1	
					October 1962				
		WF9 x W22					Os420 x 187-2		
1	50	1000	5		1	46	1000	5	
2	122	1000	3		2	54	580	4	
3	66	168	2		3	lost			
4	59	1000	5		4	42	1000	5	
5	66	190	4		5	32	240	5	
6	52	380	5		6	86	26	2	
7	80	380	4		7	44	600	4	
8	65	720	5		8	30	180	5	
9	115	7.8	0		9	49	164	5	
10	52	1000	5		10	64	118	3	
11	40	1000	5		11	51	230	5	
12	111	4.8	3		12	65	260	5	
13	79	540	5		13	41	170	5	
14	101	62	4		14	772	4.8	2	
15	160	6.6	1		15	84	102	5	
16	42	440	5		16	53	380	5	
		B14 x C103					Hy x 38-11		
1	196	5.4	0		1	196	6.2	1	
2	220	18.4	0		2	155	64	1	
3	80	78	3		3	32	810	4	
4	221	50	2		4	121	44	0	

Table 6. Continued

Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]	Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]
				October 1962					
	B14 x C103						Hy x 38-11		
5	142	10.8	3		5	135	24	0	
6	242	10.4	0		6	111	4.4	1	
7	96	11.0	2		7	lost			
8	162	6.0	0		8	206	6.6	1	
9	126	3.2	1		9	32	1000	5	
10	212	13.8	2		10	115	50	1	
11	116	8.8	3		11	145	68	1	
12	250	4.0	0		12	135	2.8	1	
13	126	10.2	2		13	62	390	5	
14	122	33.2	4		14	36	420	5	
15	168	3.2	2		15	110	4.4	1	
16	128	10.2	3		16	99	10.2	2	
				July 1963					
	WF9 x W22						Os420 x 187-2		
1	110	2.2	0	-	1	64	2.7	2	-
2	123	2.7	0	+	2	92	2.7	0	-
3	128	2.7	0	-	3	132	3.0	0	+
4	115	2.6	0	-	4	74	3.4	0	+
5	114	2.5	0	-	5	94	3.5	0	+
6	129	2.2	0	+	6	87	2.3	0	+
7	147	7.8	0	-	7	53	3.2	1	+
8	102	3.3	0	+	8	115	4.0	0	+
9	128	3.0	0	+	9	54	2.9	0	-
10	110	2.9	0	-	10	96	2.2	0	+
11	109	2.0	0	+	11	110	2.8	0	+
12	178	2.3	0	-	12	101	2.2	0	+
13	104	2.7	0	-	13	62	2.8	0	+
14	178	2.2	0	+	14	48	2.9	0	+
15	150	2.6	0	+	15	85	2.9	0	-
				July 1963					
	B14 x C103						Hy x 38-11		
1	136	1.9	0	-	1	56	3.4	1	+
2	64	2.7	0	+	2	118	2.3	1	+
3	172	3.1	0	+	3	144	2.7	0	+

Table 6. Continued

Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]	Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]
					July 1963				
		B14 x C103					Hy x 38-11		
4	105	3.1	0	+	4	75	2.7	1	+
5	151	2.8	0	+	5	70	3.1	1	-
6	166	2.7	0	+	6	181	2.8	0	-
7	162	2.9	0	+	7	87	3.3	0	+
8	169	2.9	0	+	8	82	3.6	1	+
9	210	1.9	0	+	9	72	3.9	1	+
10	140	2.8	0	+	10	76	3.1	1	+
11	145	3.5	0	+	11	65	4.4	1	+
12	192	2.4	0	+	12	221	2.9	0	-
13	124	3.1	0	+	13	100	2.8	1	+
14	177	2.8	0	+	14	70	2.2	1	+
15	179	3.5	0	+	15	140	2.2	0	+
					August 1963				
		WF9 x W22					Os420 x 187-2		
1	160	6.0	0	+	1	98	6.5	1	+
2	130	5.0	0	+	2	120	4.4	0	+
3	110	2.6	0	+	3	85	4.9	0	+
4	146	6.0	0	+	4	138	4.5	0	+
5	162	3.3	0	+	5	lost			
6	171	5.7	0	+	6	100	6.0	0	+
7	180	7.7	0	+	7	85	3.9	0	+
8	156	5.8	0	+	8	80	4.3	1	+
9	157	9.7	0	+	9	86	7.7	1	+
10	216	8.5	0	+	10	82	6.8	0	+
11	77	3.9	0	+	11	105	42	0	+
12	106	3.0	1	+	12	98	9.7	0	+
13	148	4.8	0	+	13	77	4.7	1	+
14	147	4.7	0	+	14	91	3.6	0	+
15	120	14.0	0	+	15	115	25	0	+
		B14 x C103					Hy x 38-11		
1	238	19.0	0	+	1	135	3.2	1	+
2	172	7.0	0	+	2	155	6.5	0	+
3	191	4.3	0	+	3	152	10.0	1	+

Table 6. Continued

Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]	Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]
				August 1963					
	B14 x C103						Hy x 38-11		
4	250	8.2	0	+	4	132	20	1	+
5	193	6.5	0	+	5	250	4.3	0	+
6	162	13.5	0	+	6	177	3.4	0	+
7	140	7.3	0	+	7	112	2.2	1	+
8	246	6.1	0	+	8	92	4.2	1	+
9	167	3.7	0	+	9	116	11.8	11	+
10	172	4.8	0	+	10	110	8.0	1	+
11	170	3.6	0	+	11	130	3.3	1	+
12	179	17.0	0	+	12	124	3.3	1	+
13	179	5.9	0	+	13	190	23	0	+
14	187	6.2	0	+	14	89	3.4	1	+
15	143	6.2	0	+	15	191	11.6	0	+
				September 1963					
	WF9 x W22						Os420 x 187-2		
1	192	10.8	0		1	99	33	1	
2	96	54	0		2	25	34	1	
3	215	24	0		3	62	20	1	
4	129	52	0		4	86	33	1	
5	198	27	0		5	75	22	0	
6	218	42	0		6	93	8.2	1	
7	203	65	0		7	114	37	1	
8	155	13.3	0		8	81	35	1	
9	110	18.0	0		9	62	16.5	1	
10	115	39	1		10	lost			
11	117	40	1		11	68	13.7	1	
12	188	30	0		12	78	23	1	
13	135	9.0	0		13	85	17.2	1	
14	132	48	0		14	55	10.7	1	
15	175	33	0		15	79	9.4	1	
	B14 x C103						Hy x 38-11		
1	250	19.0	0		1	116	60	1	
2	212	105	0		2	109	78	1	
3	208	41	0		3	126	110	1	

Table 6. Continued

Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]	Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of F. monil.[a]
					September 1963				
	B14 x C103					Hy x 38-11			
4	176	13.0	0		4	120	10.5	1	
5	200	16.0	0		5	151	73	0	
6	201	22	0		6	181	14.6	0	
7	184	8.1	1		7	101	44	1	
8	172	19.5	0		8	138	24	1	
9	208	8.8	0		9	131	30	1	
10	195	26	0		10	87	11.0	1	
11	197	33	0		11	151	60	1	
12	193	10.8	0		12	116	42	1	
13	140	55	0		13	122	13.3	1	
14	200	9.8	0		14	106	46	1	
15	171	24	0		15	133	95	0	
					October 1963				
	WF9 x W22					Os420 x 187-2			
1	115	18.0	1		1	48	10.0	2	
2	151	13.4	2		2	82	142	3	
3	54	1000	4		3	47	100	3	
4	64	22	2		4	43	166	3	
5	166	34	0		5	55	16.0	1	
6	98	510	3		6	54	12.8	2	
7	143	14.8	0		7	56	11.2	1	
8	73	18.0	2		8	51	5.4	2	
9	175	13.8	0		9	64	15.6	1	
10	115	8.8	0		10	52	180	2	
11	114	360	1		11	70	29	2	
12	124	9.4	2		12	68	12.6	2	
13	93	13.6	2		13	63	31	1	
14	97	11.0	2		14	80	36	1	
15	97	12.8	2		15	26	32	2	
	B14 x C103					Hy x 38-11			
1	206	27	0		1	146	17.2	1	
2	158	86	1		2	136	67	1	
3	198	70	1		3	72	38	1	
4	144	15.6	2		4	102	14.8	2	
5	100	10.6	2		5	60	320	2	
6	180	16.0	0		6	137	47	2	

Table 6. Continued

Stalk no.	Stalk str.	Enz. act. value	Pith deter. rating	Detec. of *F. monil.*[a]	Stalk no.	Stalk str.	Enz. act. Value	Pith deter. rating	Detec. of *F. monil.*[a]
					October 1963				
	B14 x C103							Hy x 38-11	
7	188	22	2		7	110	14.4	1	
8	151	28	1		8	84	25	1	
9	170	25	0		9	76	19.0	1	
10	214	10.4	0		10	90	32	2	
11	230	38	0		11	100	18.2	1	
12	75	13.8	2		12	110	14.8	1	
13	78	58	2		13	129	64	1	
14	104	14.8	0		14	230	14.0	0	
15	45	600	3		15	122	9.6	2	