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STUDIES ON THE
NATURE OF RESISTANCE IN WHEAT
TO STEM RUST.

by

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S T U D I E S
O N T H E N A T U R E O F R E S I S T A N C E
I N W H E A T T O S T E M R U S T

by

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A T H E S I S

Submitted to the University of Alberta in
Partial Fulfilment of the Requirements for
the Degree of Master of Science

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I

INTRODUCTION

The study of disease resistance in plants, on account of the great economic importance of the problem, has occasioned many contributions from various workers. The resistance of plants to various diseases may present a variety of conditions, some relatively simple, others complex, but from the work which has been done in numerous investigations of this plant character the evidence is in favour of the situation in most cases being very complex. The ramifications of the problem are many, but they may be roughly grouped under the two headings, mechanical and chemical resistance.

When resistance is attributable to the physiology of the plant, the lack of exact knowledge of the chemical reactions of the plant cell forms a very real difficulty. We know, however, that in certain plants there are known or unknown characters present which enable them to resist the attacks of various diseases.

The investigation which is reported in subsequent sections of this thesis was concerned with two possibilities; first, that the physico-chemical properties of the plant tissue fluids might be related to resistance to Puccinia graminis tritici, and second, that the inhibitory or accessory substances present in the plant fluids might effect resistance or susceptibility of wheat varieties to the stem rust fungus. Before dealing with such problems, it will be advisable to review some of the more general aspects of disease resistance.

II

GENERAL REVIEW OF THE LITERATURE

MECHANICAL RESISTANCE

In the successful avoidance of diseases mechanical resistance has played an important part; that is, some structural feature which a plant possesses may serve as an effective barrier to the entry of the disease organism. While this might perhaps be termed avoidance of disease, rather than resistance, it is of sufficient interest to warrant inclusion in any discussion of resistance; more particularly so when such structural details may result in complete immunity.

External Structures

An example of such structural immunity is found in the case of young beet leaves. The stomatal openings are not of sufficient size to permit the entry of the fungus Arcospora beticola, and the leaves are therefore immune to the disease caused by this organism.

The thickness of the cuticle is another feature which may have a pronounced influence on disease resistance in those cases where infection is not accomplished through the stomata. Willamen, Pervier and Triebold, (57) in an investigation of brown rot of plums, measured the relative toughness of the cuticle by determining the pressure required to rupture the skin. They found a definite relationship existing between the cuticle strength and resistance to the rot-causing fungus. Another example of a similar condition is reported by Hurd (27) in the ability of seed to withstand infection by Penicilliums and Rhizopus nigricans only as long as the testa remained intact.

Melander and Craigie (43) investigated cuticle thickness as related to resistance of Berberis to the aecial stage of Puccinia graminis. The thickness of the cuticle of several species of barberry was measured by puncture tests similar to those of the work cited above. Cuticle thickness was found to vary with different species, and in all cases where a thick cuticle was found there was a corresponding resistance to Puccinia graminis. No evidence of entry was found in immune species and all species resistant to puncture were found resistant to the rust fungus. The problem, however, was found to include physiological resistance since some resistant species possessed a thin, readily punctured cuticle, and these workers concluded that in the case of Berberis resistance to rust was both mechanical and physiological.

In 1924 Hursh (31) carried out an investigation of the resistance of wheat to stem rust. Leaves of Kota, Kanred, Khapli, Marquis and Little Club wheats were examined for the relative number of hairs. The resistant wheats were found to have more hairs, and in microscopic examination of inoculated leaves germination tubes of the spores could, in several cases, be seen wrapped around leaf hairs. In cases where the amount of inoculum is small the relative hairiness of the leaf might influence the severity of the infection in the manner indicated. The number of spores present on any leaf is far more likely to be large enough so that a few entangled germination tubes would make very little difference. On hairy leaves, of course, the thin film of water necessary for germination of the spores, may be prevented from coming into close contact with the epidermis. This is the only way in which hairs could be regarded as serious obstacles to the entry of the rust fungus.

The size of the stomatal openings does not influence the susceptibility of wheat to Puccinia graminis tritici. Kanred wheat is less susceptible to stem rust than many other varieties, and Allen (1) suggested that the smaller stomata of this variety might have some bearing upon this feature. Unfortunately, subsequent work by Newton (44) has shown that the stomata of Kanred, though of lesser diameter than more susceptible varieties, are more than sufficient for the entry of rust infection hyphae.

The numbers of stomata on resistant varieties of wheat Hursh found to be slightly smaller than on susceptible ones. He pointed out in this respect that even the smaller numbers (41 per microscopic field) were amply sufficient to allow copious infection. There remains only the possibility that the stomatal mechanism may be of importance. If the guard cells were influenced by the appressorium of a germinating spore, and the closing of the stoma resulted, it is obvious that the entry of the parasite in question might be prevented. No evidence has been adduced to show that the guard cell is influenced in this way, and the whole subject of exterior structures and their influence upon infection by Puccinia graminis may be dismissed with the remark that since even in resistant varieties entry is always accomplished, such structures are unimportant. With respect to other diseases, of course they may have a much greater significance.

Internal Structures.

With respect to the internal anatomy of the plant and its influence on infection by disease organisms, there is also but little to say. Hursh (31) pointed out that large amounts of sclerenchymatous tissue had the effect of limiting the growth of the rust fungus to a certain extent. The parasite is naturally confined to the collenchymatous regions of the plant, and when, as in Kota, these tissues are separated by hard, thick walled, sclerenchymatous cells, the fungus' growth is somewhat restricted. In a discussion of Webster wheat (51) it was suggested that this variety is less damaged by stem rust in the field because of the large amounts of sclerenchyma which surrounded the collenchymatous tissue.

The only other example of such resistance which will be mentioned, is that found in the resistance of the potato to Pythium debaryanum (24). It was determined in this investigation that the entry of the fungus was by mechanical means. A rupture of the epidermis first occurred, followed by subsequent rupture of the underlying cells. Measurements of the relative toughness of the internal tissues were made and definite proof was obtained that the resistance of potato varieties to the fungus was dependent upon the toughness of the cell walls.

The importance of mechanical resistance as outlined cannot be overlooked. When immunity to disease results from structural features, it would appear to be a comparatively straightforward matter to select the desirable varieties. Yet it is really in only a few cases that morphological features are responsible for disease resistance. Even then there is no real security. The structure of the host may be so modified by growing conditions that the once adequate defence becomes depreciated. The problem may be further complicated by its dependence upon a combination of structural and physiological factors.

CHEMICAL RESISTANCE

In the case of a great number of parasitic fungi, including stem rusts, the true basis of resistance is found in the individual plant cell. This physiological resistance may be regarded as chemical in nature. In this paper it will not be attempted to cover all the phases of chemical resistance, but the principal forms such resistance may take will be classified and the main features of each class will be mentioned.

There are several theories for the inability of a fungus or bacterial disease to establish itself within the host plant. It is common knowledge that many specific fungal parasites enter organisms other than their natural hosts and are unable to develop further. One explanation of this fact is that there may be present in the plant cell some substance which is toxic, or at least detrimental, to the parasite. The possible nature of this substance will be discussed later. Another explanation is that in such cells there may be a lack of some substance essential to the invading organism's growth. The only other alternative seems to be that either there are mechanical barriers, or that physical conditions such as the acidity of the host sap, are not suited to parasites' requirements. Only in some such way could we explain why, for example, wheat rusts cannot infect a variety of other plants.

Inhibitory Substances.

The theory involving the presence of some toxic substance in the host is supported by a fair amount of evidence, and some indication has been found as to the nature of such substances.

Pigments.

Walker (52,53) noted that white onions were more susceptible to attacks by onion smudge than were colored bulbs, and that when the dry colored scales were removed the onion lost its resistant qualities. The fungus readily penetrated the dry colorless scales, so resistance evidently was not mechanical. Germination tests of the spores of Colletotrichum showed that while a pronounced inhibitory effect resulted on the introduction of colored portions of scales, the uncolored portions of the same scales produced no such reaction. Several other workers are cited by Walker as having found a similar relationship between pigments and plant diseases. Sorauer noted that red potatoes were more resistant than white. Voges is also mentioned in connection with his unsupported statement that the red color of apples constitutes a defence against scab.

Tannins.

Tannins are found very frequently in a large number of plant species, and the significance of such substances is still largely a matter of conjecture. Investigators have endeavoured to explain their presence by many different hypotheses, and they are mentioned in connection with a number of plant problems. No very conclusive work has resulted. There is, however, a possibility that these substances may have some relation to the inability of disease organisms successfully to attack resistant or immune species. Cook and Wilson (8), working with several members of the genus Endothia found in most cases that this fungus growth was retarded by percentages of 0.8% of tannin added to the cultural media. In the case of Endothia parasitica, the organism responsible for chestnut blight, it was found that not only was the presence

of tannin not detrimental, but that this species could utilize such substances for food. In general they claimed that tannins and related substances exerted a certain inhibitory effect. Tannins are closely related chemically to pigments, and the findings of Cook and Wilson thus strengthen Walker's theory that the pigments in onion scales are responsible for the resistance of colored onions to Colletotrichum. Experiments have also been carried out (33) on the effect of tannic acid on various fungi growing in nutrient media. Twenty-three different fungi were examined, and of these only two remained alive when 10% of tannic acid was added to the cultures. These two fungi utilized the tannic acid for food by secreting tannase and so obtaining a supply of gallic acid.

Unknown substances influencing infection.

Parallelling the work on tannins is an investigation by Hawley, Fleck and Richards (25), who found that water extracts of various resistant woods caused inhibition of the growth of wood-rotting fungi. Extracts of susceptible sap wood had no effect upon the parasitic organisms. An indication is given in this manner that resistance to wood rots may result from the presence of substances in the wood, toxic to the rot-causing fungi.

Lee (35) found that in actively growing sugar cane cuttings, where the developing aerial shoot draws its nutriment through the old cutting, fungi and other micro-organisms did not enter the cutting. Once the shoot established its own roots, the entry of the fungi was very rapid. Lee forwarded the theory that the turgidity of the cells might be decreased when the aerial shoot became independent; or that some inhibitory substance was secreted by the shoot, and as long as it remained in contact with the cutting, this substance prevented the growth of the fungi in the plant tissues.

There is apparently no limit to the number of substances which may occur in plants, and which may influence the resistance or susceptibility of that plant. The deleterious effect of straw on young seedlings is explained by Collison (7) on the basis of the presence in the straw of vanillin, cumarin, tyrosine and dihydroxi-stearic acid. In hay, which also inhibits seedling growth, were found vanillin and salicylic acid. These substances proved remarkably detrimental to plant growth, and even in dilute solutions were usually toxic. The relation such substances may have in disease resistance is at least worthy of thought. When toxic^{to}/seedlings there is every reason to suppose that they will exert a similar effect on disease organisms. With these or similar substances present in the host cell, and invading parasite, if at all susceptible to such compounds, would experience considerable difficulty in successfully attacking the plant.

Resistance as outlined in the preceding paragraphs constitutes one of the most interesting and at the same time one of the least understood problems connected with plant investigations. The determination of the specific substance responsible for resistance or immunity to any disease is beset by so many difficulties as to appear an almost impossible task. The intricate relationship which exists between host and parasite may be very easily upset by even minute amounts of the substance in question, and there is always the possibility that the attempt to determine these substances results in a change in the physiology of the host or the parasite. In the case of culturable parasites, the problem, though delicate, is not so intricate as that of the obligate parasite which can only be examined upon the host plant. Our knowledge of such conditions is limited to the supposition that there are present in some plants substances which may inhibit the growth of parasitic invading organisms.

Lack of Some Accessory Substance.

Vitamines.

Resistance resulting from the lack of some substance essential to the disease organism has already been mentioned as a possibility. Willaman (56) investigated "the bare essentials of life" for *Sclerotinia cinerea*, and found it totally unable to grow on artificial media until a little plant juice was added. Further investigation revealed the fact that a vitamine preparation obtained from yeast could be substituted for the plant juice.

With the need in one case demonstrated, it seems possible that many other fungi may require a vitamine. If the vitamine differs for different organisms or groups of organisms, we would have a possible explanation of the inability of certain fungi to infect plant varieties susceptible to other fungi. The presence of at least three different vitamins in plants has been established, and if it be granted that fungi may in this respect have specific requirements, the absence of the essential vitamine in a given host provides a possible basis of immunity.

Intermediate products of metabolism.

Resistance to disease resulting from the lack of some essential substance need not be limited to the absence of vitamins. Plant products are infinite in variety, as are also the intermediate products of metabolism, and any one of such products might be specifically required by the parasite. There are over forty physiological forms of stem rust, each differing from the other in ability to infect certain varieties of wheat. It has been as yet impossible to culture such rust on artificial media, and this fact suggests that the ability of infection depends upon the presence in the host of certain intermediate products.

Bacterial diseases of plants may result in slightly different plant reactions. There is the possibility that associated with such diseases, there may be a transmissible lytic principle similar to d'Herelle's bacteriophage. Hallman and Hemstreet (40) prepared a filtrate from rotting cabbage and demonstrated that such filtrate was capable of strongly inhibiting the growth of the rot-producing bacteria. Solutions of 1 to 1×10^{17} still retained inhibitory powers and combined with the fact that the inhibitory effect was lost at 63°C. , this occasioned the theory that the effect noticed was due to some organism rather than any chemical substance. Only from diseased cabbage was this lytic filtrate procurable..

With fungal diseases of plants the possibility of bacteriophage influencing infection does not apply directly, but with bacterial diseases it may constitute an important factor in the relative resistance or susceptibility of a species to the disease.

SUMMARY OF THE LITERATURE REVIEWED

Concluding this general review of the literature, the points may be best summarized by the construction of an imaginary case. Suppose a plant "A" is resistant to a disease parasite "B". Such resistance may be the result of morphological (mechanical) protection; the presence of some inhibitory chemical substance; the lack of some substance in the host essential to the invading parasite; or to an intricate combination of all these factors. It is apparent at this point that in the last case a very slight change in one factor might upset the whole balance, especially if the host "A" is not strongly resistant. Now in the disease parasite let us introduce a suppositional bacterial disease which so weakens the fungal organism that it barely

is able to maintain its hold in "A". The last point we may imagine is the presence of a bacteriophage in the parasite "B" which attacks the disease bacteria. With the resistance of the host remaining unchanged, it is this bacteriophage which will determine whether or not infection of "A" by the parasite will be severe.

No evidence of the occurrence of a case such as the above has been obtained, yet in view of the complexity of interrelationships which may exist in parasitic growths, the example, though highly imaginative, remains a possibility, and serves to demonstrate the multiplicity of the factors which may enter into the problem.

THE SIGNIFICANCE OF THE PHYSICO-CHEMICAL PROPERTIES OF
EXPRESSED TISSUE FLUIDS.

During the summer of 1926 an investigation was carried out to determine whether any relationship existed between certain physico-chemical constants and the wheat plant's ability to withstand attacks of Puccinia graminis tritici. Physico-chemical determinations have been frequently used in recent investigations of plant problems and before presenting the results of the investigations it will be advisable to review briefly the literature on the subject.

CORRELATION OF PHYSICO-CHEMICAL CONSTANTS
WITH VARIOUS FACTORS

The significance of such constants has been demonstrated so frequently that it would be impossible in this review to deal with all the problems to which the determinations may be applied. This discussion will be limited to those fields which best indicate the significance of physico-chemical determinations, and only a few outstanding examples will be mentioned in each case.

Relation to Environment.

In 1907 Drabble and Drabble (13) demonstrated that plants from a physiologically dry habitat had higher osmotic pressures than those where the available water supply was abundant. This work was done using De Vries plasmolytic method, which is open to criticism on several points. Only the mesophyll tissue was examined and the results obtained can only be regarded as an indication of the relationships which we now know to exist.

The work of the Drabbles has been supported by a number of subsequent investigators. Korstian (34) examined the expressed leaf fluids of forest trees and found consistently higher osmotic pressures in trees with restricted water supplies. He also demonstrated that any factor which influenced the plant's physiology exerted a certain influence upon the osmotic pressure,

In a detailed investigation by Harris, Gortner, and their co-workers (17) a definite relationship was established between osmotic pressure and the water supply of indicator plants from six major divisions. The specific electrical conductivity of the tissue fluids was shown to be slightly influenced by the same environmental factor.

In concordance with Korstian's work, Lewis and Tuttle (35) found a seasonal variation occurring in conifers. The highest concentrations were obtained in the spring before the water supply was abundant. In Populus and Pyrola a variation occurred and the maximum concentrations were found during December. In Pyrola a steady decrease in concentration was evident from December to June. The reason for this apparent abnormality is difficult to determine, but it was suggested that the change from starch to sugars which occurs in different plants at different times may have been partly responsible.

The effect of local environmental conditions has been investigated by Harris, Lawrence and Gortner (22) who examined the tissue fluids of plants in different desert environments. The regions were classed as follows: (1) the foothills canyons; (2) cliffs, ledges, and the steeper rocky slopes; (3) bajada slopes; (4) arroyos; and (5) salt spots. In each case decreasing water supply resulted in increased osmotic pressure.

The work of McCool and Miller (42) is also of interest at this point. Plants grown in soils of different moisture contents showed demonstrable differences in osmotic pressure, the greater values being found in the plants from the less humid soils. A daily variation of from one to one and one-half atmospheres was found and in explanation of such fluctuations it was suggested that the increasing intensity of light during the early morning accelerated photosynthesis, resulting in increased osmotic pressures.

Martin (41) demonstrated that higher osmotic pressures were usually found in plants with the ability to withstand periods of drought. The work was primarily concerned with the bound-water values of drought resistant species and it was pointed out that while greater osmotic pressures were found in drought resistant plants, the values for this property were not sufficiently regular to provide a reliable indication of the plants' relative ability to withstand periods of water scarcity.

The work reviewed shows that osmotic pressure, at least, is influenced strongly by the environmental conditions to which the plant is subject. The abnormally high values of 125 to 150 atmospheres obtained in desert plants by Fitting (14) and Harris et al (18), serve to emphasize this point. In the case of electrical conductivity there is an indication that a similar relationship exists.

Relation to Tissue Position and the Age of Tissue.

The physico-chemical constants of the tissue fluids may be affected also by the position of the tissue on the plant. Dixon (12) concluded that differences in the constants did exist, lower leaves having in general a lower osmotic pressure than more elevated leaves. Indications were found also that such pressure increased with the age of the leaf. Iljen (32) corroborated Dixon's findings. Lower osmotic pressures were found in

roots than in stems, and higher pressures in the upper leaves than in the lower ones. McCool's work (42) is confirmatory also. Lower pressures again were found in roots than in stems; roughly two to four and one-half atmospheres difference was demonstrated.

During the course of his extensive work in the Wassatch mountains, Korstian (34) demonstrated differences in the osmotic pressures of forest leaves at the base, and at the top of the tree. Korstian suggested that such differences might be attributable to the increased formation of soluble carbohydrates which occurred by reason of the more favorable conditions for such activities at the top of the tree. Roots were shown to be four atmospheres less in osmotic pressure than even low leaves.

With reference to the electrical conductivity in various plant tissues, the work of Heald (26) is of interest. Stems, roots and leaves were examined, and while the differences between the values for stems and leaves were not always significant, the roots showed consistently lower values for this constant. In the case of Nuphar progressively increasing values were obtained for root, rhizome, petiole and leaf.

The last example which will be mentioned in this connection is the work of Harris, Gortner and Lawrence. The expressed juice of a proliferation of the carpels in the fruit of Passiflora gracilis was examined. Lower values for osmotic pressure and electrical conductivity were obtained, which adds weight to the evidence for significant differences in the physico-chemical constants in differing tissues.

The conclusions to be drawn from the examples which have been mentioned can be definite on only one point. The osmotic pressure of

the plant tissues at various heights is not constant. According to Dixon's theory for the ascent of sap, the root must have the lowest osmotic concentrations, while stem and leaves have correspondingly greater pressures. Dixon's own work and the findings of other experimenters confirm this theory. There also seems to be some evidence that the electrical conductivity value increases progressively from root to leaf.

Relation to Growth form.

In the previously mentioned investigation by Harris, Lawrence and Gortner of the flora of the Arizona desert (22), the plants examined were grouped into ligneous and herbaceous forms. In each of the five areas examined the osmotic pressures were greatest for trees, shrubs and other ligneous species. Differences of 20 to 40 per cent were evident in the depression of the freezing points for ligneous and herbaceous plants.

In subsequent work (19,20) Gortner and his associates conducted experiments on mesophytic plants. Different ligneous and herbaceous species were examined from the Jamaican montane forests and the Long Island forests, and the results obtained were in complete concordance with the previous desert investigations. The results of the Tocoale valley project (17) also demonstrated a pronounced tendency for ligneous forms to develop higher osmotic pressures than the herbaceous species. Korstian (34) found the same relationship between the growth form and osmotic pressure was present in the plants of the Wassatah mountain regions.

Relation to Parasitism.

The relationship between host and parasite has been shown to be very delicate, and by investigations of the physico-chemical constants as related to such conditions several workers have attempted to gain some insight of the balance which exists. Hurd (28,29,30) investigated the possibility that the acidity of the cell fluids of certain wheats might prove detrimental to the parasitic stem rust fungus. The pH values for resistant and susceptible hosts were determined, but no correlation was found between rust invasions and the acidity of the plant fluids. The effect of parasitic infection was also investigated and slight increases in acidity were demonstrated, but since uninfected plants in poor condition showed correspondingly high acidities the increase could not be attributed to the rust infection.

Experiments on the course of acidity changes during the growing season also yielded negative results with respect to rust infections of cereal crops. Acidities were low in seedling stages, remained low during the growing season and increased with ripening. The wheat plant however, undergoes no corresponding variations in susceptibility, and such differences as were obtained could not be considered as significant.

Hursh (31) investigated osmotic pressures and hydrogen-ion concentrations in an attempt to explain the resistance of wheats to rust, but was unable to discover any significant relationship between rust infection and the physico-chemical constants in question. Other workers (50, 52, 55) also have found that no relationships exist between physico-chemical constants and resistance to pathogenic fungi.

Of more encouraging nature is the work of Harris and his associates on the mistletoe (16,21,23). Parasitic Loranthaceae in the Jamaican rain forests and the Arizona desert were examined. In most cases a higher osmotic pressure was found in the parasite than in the host tissues, although in a few mistletoe lower osmotic pressures predominated. Electrical conductivities were determined only upon desert species, where higher values were obtained showing that the parasite accumulated and retained in solution larger quantities of dissociated salts or organic acids than did the host.

The advantage resulting in the mistletoe from the higher osmotic pressure is not as great as it might seem. The parasite and the host both draw their water supply from the trachea of the host, and as long as the osmotic pressure of the parasitic haustoria is greater than that of the tracheal solutions, and as long as there is no water scarcity, the parasite will continue to thrive. The situation is much the same as that noted by Dixon, where young leaves of low osmotic pressures compete successfully with more mature leaves with greater pressures. Only when the supply of soil water is inadequate for both host and parasite will any beneficial effects from higher osmotic concentrations be evidenced.

The main points of this discussion of physico-chemical constants may be summarized as follows:

- (1) A distinct relationship exists between those factors, such as temperature, water supply and light intensity, which may be considered as comprising the plant's environment, and the physico-chemical constants of expressed tissue fluids.

- (2) Such constants are also influenced by the tissue positions and the age of the tissue or tissues.
- (3) Variations in these constants can be correlated with the habit or growth form of the plant or plants from which the sap is extracted.
- (4) The parasitic habit of organisms may influence certain physico-chemical constants with respect to the differences in such constants demonstrable between host and parasite.

EXAMINATION OF THE PRESS JUICE OF A SERIES OF WHEAT VARIETIES DIFFERING WIDELY IN RUST RESISTANCE

From the immediately preceding summary it is evident that for any physico-chemical investigation to be trustworthy, certain precautions must be taken in selecting and collecting the material used. In the light of the numerous correlations possible the work will not be of greatest value if confined to one group of determinations. Differences in the time of day at which the collection of tissue is made, in the part of the plant selected, and in the age of the plant, will all influence the results. It is evident then that determinations made at one season on one variety cannot be compared with similar determinations upon another variety at a different season. In the investigation which follows, all collections were made at the same time of day and at regular intervals during the growing season. Eight wheat varieties were examined; namely, Little Club, Marquis, Mindun, Kubanka, Kota, Kanred, "Common" Emmer and Khapli, and the order in which they have been named corresponds to the order of increasing resistance to stem rust. Little Club is very susceptible and Khapli almost immune.

During the season in which the wheat was in the green stage, four series of collections were made and the values for total solids, bound water, osmotic pressure, specific electrical conductivity and hydrogen-ion concentration of the expressed juices were determined for each of the eight wheats.

Method.

It is of interest to discuss the methods which have been developed for the determination of these physico-chemical constants.

Method of expressing the tissue fluids.

Cryoscopy necessitates the expression of the plant tissue fluids which may be accomplished in several ways. For most studies the tissue has been frozen, either by liquid air as Dixon (12) outlined or in an ice salt mixture after the method of Gortner and his associates. (15, 19, 20). The material is then thawed and subjected to pressure. The preliminary freezing seemed necessary in the light of investigations on frozen and unfrozen tissue, which showed that unequal pressures on unfrozen samples resulted in unrepresentative juice. In some examinations, however, the freezing of the tissue was not advisable, in view of the changes resulting in the colloidal state of the juices. For the bound water determination, the initial freezing of the plant tissues would tend to invalidate the results. In such investigations the expression of the sap was accomplished by the method described by Newton (45, 46, 49), where the tissue is passed through a meat chopper, subjected to constant pressure and finally is centrifuged at a slow speed to remove the extraneous material. The press juice so obtained has proved satisfactory in every way, and this method is the one used in the present investigation.

Time factor.

In the work carried out by Martin (41) it was shown that the expressed juice undergoes appreciable changes in thirty minutes even, when stored in an ice bath. It was not always feasible to complete the determinations in this time, but they were carried out with as much promptness as possible.

The determination of the osmotic pressure.

Osmotic pressures were determined in the usual way, by the measurement of the freezing point of the press juice as compared to the freezing point of distilled water. For this measurement a Beckman thermometer graduated to one one-hundredth of a degree proved eminently satisfactory. The alternative in this respect is a delicate thermocouple. The principal advantage of the latter method is the small amount of juice required, but in this work an abundance of juice was available.

Methods for total solids, electrical conductivity, and pH.

The total solid content was determined by the refractometric method proposed by Gortner and Hoffman (14b). A correction in the value read was made from tables prepared by Martin (41). Conductivity values and hydrogen-ion concentrations were determined in the usual manner, the former on a Wheatstone bridge and the latter by means of a Leeds-Northrup potentiometer.

Methods for the bound water determination.

Bound water serves as a reliable index of the colloidal nature of the cell fluids. It may be described as the water held by the colloidal particles with such tenacity as to render it unavailable for the solution of sucrose, and has been found by Newton (46, 47), and Martin (41) to be closely related to the problems of frost and drought

resistance. The method for its determination is not described in detail, since the literature cited contains lucid descriptions. The bound water value is calculated from the amount by which the freezing point depression of press-juice, to which a molar concentration of sugar has been added, is in excess of the sum of that of the pure juice and that of a molar solution of sugar in water.

Collection of leaf material.

All collections were made at eight o'clock in the morning. Blades were cut or plucked from the plant and packed in jars surrounded by ice. Expression of the tissue fluids and subsequent determination were carried out in the laboratory as described.

Results.

The values obtained for the physico-chemical constants under investigation are presented in Table 1. Four series of determinations are reported, together with the averages for each wheat variety and the date of the separate determinations. The varieties are listed in the probable order of increasing resistance to stem rust.

TABLE I. Physico-chemical constants of the press-juice of a series of wheats arranged in the order of increasing resistance to stem rust.

Date	Total solids (%)			Bound water (%)			Osmotic pressure (atm.)			Electrical conductivity ($\times 10^3$)			Hydrogen-ion concentration (pH)																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					
	June 9-12	June 21-24	July 5-8	June 9-12	June 21-24	July 5-8	 

Feb. 11

Discussion.

The results show upon even a cursory examination that there is no definite relationship between these constants and the varietal susceptibility to Puccinia graminis tritici. In each case there are differences apparent, but these differences are not related to the degree of susceptibility. In the bound water values, for example, the high percentages obtained for Emmer and Khapli on July 22 cannot be considered as significant. Khapli was ripening much more rapidly at this date than were any of the others, which would tend to result in higher values. Martin's work (41) brought out the fact that Emmer was the most drought resistant of any of the varieties studied, and the value in this case is probably related to the drought resistant properties rather than to rust resistance.

With respect to osmotic pressures, a similar condition arises. A seasonal increase is demonstrated, but neither the rate of increase nor the averages for the eight varieties show any indication of a relationship with the problem. The values for Marquis and Mindum, two readily susceptible varieties, are about the same as those for Khapli and Emmer, which are much more resistant.

The conductivity and pH values also show no sign of any relationship existing with stem rust resistance.

Perhaps the most definite proof for the absence of any possible relationship is that no evidence has been adduced to show that resistance to rust varies with the increasing age of the plant. Since seasonal variations of the physico-chemical constants do occur, the very fact of their occurrence seems proof that no relationship can exist. Varietal differences in the wheats examined are probably related to frost and drought resistance.

IV.

THE INVESTIGATION OF INHIBITORY AND ACCESSORY
SUBSTANCES

In section II of this thesis the presence of inhibitory or accessory substances in the tissue fluids was postulated. Applied to stem rust resistance, the hypothesis would be that in a resistant variety, such as Emmer of Khapli, there might be present a substance of a toxic or inhibitory nature, thus preventing the growth of the parasitic organism in tissues so protected. Or, conversely, that in resistant varieties some substance essential to the fungus might be lacking, thus preventing the growth of the parasite in such deficient tissues. A third hypothesis is possible. Allen (1, 2, 3) demonstrated that when an haustorium enters a resistant host, the cell activities are at first stimulated. Newton (44) points out that in reality the immune varieties may be merely excessively susceptible ones. The invaded cell of a susceptible host lives for some time after an haustorium has penetrated; in the immune host, however, the invaded cell dies almost immediately and chlorotic flecks result. It might be possible, therefore, that a resistant host owes its freedom from infection to the fact that the cell fluids are hypersensitive to the parasite. In this case the death of the host cell would follow hard upon invasion by the parasite, and the starvation of the latter cause a failure of the infection.

To investigate these hypotheses it was first necessary to devise methods for feeding plant juices to the desired wheat variety.

If, for example, the juice of a resistant host were fed to a susceptible one and after or during such treatment the susceptibility of the plant was found to be decreased, it would demonstrate, assuming adequate controls, that in the resistant tissue fluids was present some substance inhibitory to the rust parasite.

GENERAL METHODS

Maintenance of Rust Cultures

In all the experiments reported physiological Form 21 of Puccinia graminis tritici was used. This culture was obtained from the rust laboratory at Winnipeg. Stock pustules were maintained on Marquis and Little Club hosts, inoculations being made in the following manner. Young seedlings in the pots were thoroughly sprayed with distilled water and the leaves drawn gently through the fingers to ensure a film of water in close contact with the leaf surface. Spores were carried from a stock pustule to the moist leaf by means of a scalpel or spear-headed needle, and the plant was placed in a moist chamber for thirty-six hours. During the winter 1925-26 it was exceedingly difficult to obtain infections. In 1926, however, the greenhouse was fitted with powerful artificial lights and while pustules during the winter tended to be smaller and less frequent than in spring and summer, it was possible to carry the culture through the period of short days successfully.

Definition of Terms.

For the sake of convenience several terms have been applied to the plant-tissue fluids. In the work which follows "press juice" refers to the untreated fluid obtained by pressing out the pulp

obtained by grinding fresh green leaves and centrifuging; "filtrate" is used to describe juice which has been sterilized by filtration; and "extract" refers to the supernatant fluid of juice sterilized and precipitated by boiling. The term "juice" or "plant juice" is reserved as a non-specific term applicable to any of the other three. Thus Marquis juice might be press juice, Marquis filtrate or Marquis extract.

Methods of Preparing Plant Juices.

Two methods of sterilizing press juice were devised. One consisted of boiling gently for ten minutes under a reflux condenser, decanting through a filter paper and receiving the liquid passing the filter in a sterile container. This vessel was then plugged with cotton wool and the liquid heated again just to the boiling point to ensure sterility. This liquid was termed "extract."

The second method of sterilization consisted of filtration. Press juice was passed through a #12 folded filter paper. The first few cc. passing through the filter were very little changed by this process, but in a few minutes the paper became impregnated with colloidal material and a clear amber colored filtrate was obtained. With some wheat varieties the juice refused to pass the impregnated filter after a short time, and in such cases it was necessary to renew the filter paper. Filtration was slow and for that reason it was carried out in a refrigerator at 0° to 3° centigrade. After the clear filtrate was obtained this liquid was filtered through a Pasteur-Chamberland filter for sterilization. This operation also was carried out in the refrigerator to retard as much as possible the fermentations which occur in standing juice. It is to this sterilized plant juice

that the term "filtrate" is applied. The juice filtrate was received in sterile containers plugged with sterile cotton wool.

The sterility of this juice filtrate was tested quite simply by pouring nutrient agar plates and inoculating with a few c.c.'s of filtrate. In four dishes so poured no bacterial or fungal growth occurred over a period of 14 days.

In connection with experiment No. 4 of the Petri dish cultures which will be mentioned later the sterility of the Khapli filtrate was tested again. The juice in this instance was added to sterile sucrose solutions by exactly similar methods to those used in the experiment. No growth of any kind appeared.

PETRI DISH CULTURES

Historical

One of the methods developed for studying the effect of the plant juice upon the resistance of wheat varieties was that of placing cut leaves or portions of cut leaves in Petri dishes and adding the cultural solutions. In 1902 Ward (54) described a method for growing sterile seedlings in closed containers. Mains (39) included in the work he reported in 1917 a study of leaves floated on nutrient solutions. Blackman and Welsford (4) also made use of the Petri dish method in examining the infection of Botrytis cinerea. Their method is described as follows. "Before infection the leaves are washed with a gentle stream of sterile water to remove as far as possible extraneous spores and dust. They are then placed on damp filter paper in a sterile Petri dish and drops of the prepared solution containing spores placed on their upper surfaces."

In 1924 Clinton and McCormack (6) described in considerable detail a method for the infection of leaves in Petri dishes and compared it with the pot method for the production of the spores of several rusts. They found that the Petri dish method was satisfactory for all hosts with a hardy leaf. Grasses they found to be less adapted to this cultural method. Leaves were washed in a stream of tap water and placed in sterile Petri dishes in which enough water had been placed to insure a humid atmosphere during the course of the experiment. The leaves were placed either on glass rods or on rubber bands stretched across the lower dish. In no cases were the leaves floated on solutions.

Preliminary Experiments

The first attempts to obtain rust on leaves in Petri dishes were unsuccessful. Experiments were usually terminated in a very short time by the prolific growth of foreign fungi on the cultural solutions. It is obviously impossible to sterilize the leaves by any such methods as can be applied to apparatus, yet the necessity for retarding the growth of extraneous fungi was evident. Partial sterilization with a 1 per cent solution of bichloride of mercury proved valueless, as a slight increase in the number of foreign molds resulted. Other disinfectants gave similar results and it was finally decided that only very careful washing and rapid handling of the leaves subsequently could be used to avoid the inclusion of foreign spores with the inoculated leaves.

On May 1, 1926, the first successful attempt to grow rust in Petri dishes was set up. All leaves were from Marquis seedlings two weeks old; three leaves were placed in each dish. The leaves were

cut under water, washed in a gentle stream of tap water, inoculated with a flamed needle and placed in sterile Petri dishes, arranged as follows:

1. Leaves supported on glass rods, their cut ends dipping into 6% sucrose solution.
2. Leaves arranged similarly over Knop's full nutrient solution.
3. Leaves supported on rods over distilled water.
4. Leaves floated on a 6% sucrose solution.
5. Leaves floated on Knop's solution.
6. Leaves floated on distilled water.
- 7 and 8. Leaves with cut ends submerged in a 6% sucrose agar.

The dishes were placed in a dark cupboard for 36 hours subsequent to inoculation, and then placed on a laboratory bench where no direct sunlight could reach them. The following symbols were used to denote the degree of bleaching which occurred.

B0 No bleaching. Leaves fresh, normal, green.

B1 Very slightly bleached. Paling of green color just apparent.

B2 Slightly bleached. Definite difference in color in part of the leaf.

B3 Bleached. Part of the leaf white, yellow, or brown; the leaf often presents a mottled appearance

B4 Much bleached. At least half leaf white, yellow, or brown.

B5 Very much bleached. Only pale green color left in basal quarter.

B6 Bleached to dead yellow. No color left in any but the base.

B7 Dead.

Two days after this experiment was started the leaves in all but the sucrose solutions were bleached to an appreciable extent. On the third day the sucrose solution leaves were at the B1 stage, the others were B3. After five days bleaching was more advanced and the presence of foreign fungi was noticed at the cut ends of the leaves. The growth of foreign fungi was very rapid and in three or four days almost completely concealed the leaves in the dishes other than those containing sucrose solution. At the end of 8 days pustules were forming on leaves in dish No. 1. Bleaching had advanced to the B4 stage in both sucrose dishes and in No. 4 the foreign fungi partially concealed the leaves. Surrounding each pustule could be seen a green "island" of unbleached leaf tissue. On May 11 the experiment was terminated. Pustules were present only on the leaves in dish No. 1, which were by this time bleached to the B5 stage, except for the islands of green mentioned previously. In dish No. 4 bleaching was classed as B5 and the growth of extraneous fungi was equal to that of dish No. 1. All the other leaves had been discarded at the B6-7 stage on the tenth day of the experiment. No evidence of rust infection of these leaves was found. These results are summarized in Table 2.

Table 2--Effect of various nutrient solutions on rust-inoculated

Dish No.	Description	Nutrient	cut leaves in Petri dishes Duration of Experiment			
			2 days	5 days	8 days	11 days
1	Leaves on rods	6% sucrose	B0 no f.f.	B2-3 no.f.f.	B4 4pustules	B6 4 pustules
2	"	Knop's dist.	B1 no.f.f.	B6 f.f.pres.	B6--no pustules	Discarded
3	"	water	B1 no f.f.	B6--f.f. present	B6--no pustules	"
4	leaves floated	6% sucrose	B0 no f.f.	B2--f.f. present	B4--no pustules	B5--no pustules
5	leaves floated	Knop's dist.	B1 no f.f.	B5-6 f.f. present	B7--no pustules	Discarded
6	leaves floated	water	B1 no f.f.	B6--f.f. present	B7--no pustules	"
7 & 8	leaf ends submerged in agar		B2 no f.f.	B6-7.f.f. present	B7--no pustules	"

From this experiment it was evident that leaves supported on glass rods were more likely to give satisfactory results than those floating on the solutions. Further experiments showed that such was indeed the case. Sucrose solution was easily the most satisfactory nutrient.

A variation of the Petri dish method was also tested. Leaves washed in tap water, followed by rinsing in sterile distilled water, were placed upright in large sterile test tubes to which 20 c.c.'s of sucrose had been added. Spore development was very scanty, only one of the fifteen leaves tested showing any sign of infection. Moreover, the cotton plug used to stopper the tube permitted considerable evaporation, and the increasing concentration of the sucrose solution seemed to accelerate the bleaching of the leaf.

A second variation was tried in which leaves with stems attached were sealed in the stoppers of test tubes with grafting wax, the cut stem projecting below into the nutrient solutions. Six control leaves in sucrose and six test leaves in sucrose plus press juice were set up. The solutions became infected after a day or so, and fermented rapidly. On the test leaves a few very small pustules developed. The test leaves died before the end of the infection period. When the stoppers were replaced with cotton wool plugs the sterility of the solutions remained but evaporation was such that the cut end of the stem was exposed and the leaf died. Neither method gave encouraging results.

The rapid bleaching noted in the first experiment was thought to be due to two causes: first the lack of adequate illumination, and second the possibility that the leaf might not transpire in the moist atmosphere of the Petri dish. These points were examined in the order mentioned. Leaves were arranged on glass rods in a dish containing

a sucrose nutrient. Four dishes were placed in direct light in the greenhouse, four in diffused light under a white cloth screen, and four in the laboratory where the light was poorer than for either of the greenhouse series. Contrary to expectation, the leaves under the cloth screen did not prove satisfactory. Both greenhouse series bleached more rapidly than the laboratory set up. Bleaching was most rapid in direct sunlight where large scalded areas developed on the leaves, intermediate on those in diffused light and slowest in those leaves in the laboratory dishes. Pustules were produced only after the tenth day and appeared only on the leaves in the laboratory. The greenhouse leaves died in four and seven days respectively. No foreign fungi appeared in any dishes.

The effect of a dryer atmosphere was investigated quite simply by raising the cover of the dishes slightly by means of rubber bands. No beneficial effects resulted and there was naturally an increase in the number of extraneous fungi. The best results were invariably obtained in dishes of which the covers were in place and in which the leaves were supported on glass rods.

Method Adopted.

Bearing in mind the points brought out by these preliminary experiments the following method was adopted for Petri dish cultures. The dishes were sterilized in a hot air oven at 110° C. for several days. Sucrose solutions of 6 per cent concentration were placed in 10 cc. portions in test tubes plugged with cotton wool and sterilized in an autoclave at 20 pounds pressure for thirty minutes. The sterilization of the sucrose solutions was carried out in this way, since it was found to require less time to pour the solutions from

test tubes than to pipette the required amount from a sterilized stock solution. In view of the possibility of the conducting elements of the leaf becoming plugged with air and the danger of introducing foreign spores during the manipulation of the dishes the most rapid introduction of the nutrients into the dishes was deemed a necessity. The plant juices, of course, could not be sterilized in this manner, and for their introduction sterile pipettes were used, a separate pipette for each liquid. In each dish was placed 10 cc. of sucrose solution plus 10 cc. of plant juice.

The leaves used in these experiments were all from seedlings two to three weeks old. The terminal leaf was found most satisfactory, cut with a portion of the stem remaining. The best results were obtained with leaves of a size to permit their being placed in the dish whole. While this was not always feasible it was carried out whenever possible. The cut end of the stem was observed to be the centre of infection for the foreign fungi which could never be totally excluded and when two cut surfaces were present the growth of such organisms tended to be more severe.

The treatment of the leaves was as follows:

1. Seedlings bearing the desired leaves were cut at ground level and immediately placed in water.
2. The suitable leaf was severed under water.
3. The leaves were then thoroughly washed in a gentle stream of tap water by drawing them gently through the fingers. This was done both to insure a film of water in close contact with the leaf in which the rust spores could germinate and to remove as many foreign spores as possible.
4. Leaves were then rinsed in a stream of sterile distilled water and placed on a glass plate which previously had been scrupulously washed and rinsed with sterile water.

5. Leaves were inoculated at a point about midway between the base and the tip with a flamed needle. Spores were placed on both dorsal and ventral surfaces.
6. The last operation was the transference of the leaves to the dishes and their arrangement therein by means of sterile forceps. Leaves were arranged so that the cut end was between the rods on which the leaves rested, where it would be well covered by the nutrient solutions.

These operations were carried out as rapidly as possible and after three leaves had been placed in a dish the cultural solutions were added. The glass plate used for inoculation was washed with sterile water after the leaves for any one dish had been in contact with it. Each dish was placed in a dark cupboard for 36 hours and then removed to the laboratory shelf.

It was found to be almost impossible to remove all the foreign spores from the preparations. Two sources of contamination were present. One of these was the adherence of such spores to the leaf surface with such tenacity as to resist the efforts to remove them by washing. The other was the inoculation which might have been made inadvertently with the spores of the rust fungus. Mains (39) obtained sterile seedlings and sterile rust spores by following the method outlined by Ward (54). In the present investigation this was deemed unnecessary, since by following the method outlined very few fungi appeared in the sugar solutions used in preliminary experiments. Unfortunately, with solutions to which plant juices were added there was

an increase in the growth of such organisms, sometimes to such an extent that experiments were discarded before any growth of rust could be expected. It was possible, however, to keep leaves for the required two-week period with only a slight and generally localized growth of foreign organisms. It is difficult to estimate the influence which these extraneous growths may have had upon the rust pustules and the cultural work in Petri dishes is open to criticism on this point.

Experiments with Plant Tissues

The effect of various solutions fed to the leaves was investigated at different times following the adoption of the method described. The numbers of the experiments are not the original numbers, but they are used as a simple method of referring to each experiment.

In these and in subsequent experiments four wheat varieties were used; Marquis and Little Club, both very susceptible to Form 21 of Puccinia graminis; and Vernal Emmer and Khapli, two varieties very resistant and immune respectively.

In the first experiment with Marquis leaves in the Khapli juice the results were somewhat indefinite. Three control dishes were used with leaves in 6% sucrose solution, 3 dishes containing leaves over equal parts of Khapli press juice and sucrose solution, and three dishes containing only Khapli press juice. As was to be expected, the leaves over Khapli juice alone died very quickly. With even greater rapidity did the foreign fungi develop in both sets of dishes containing the press juice. During the seven days the leaves in the fungus infested dishes were visible there was a definite difference noticeable in the size of the pustules on the control and experimental leaves but whether this was attributable

to the prolific growth of foreign fungi or to the influence of the Khapli juice was uncertain. The control leaves were completely free from foreign fungi and developed pustules in four to five days. The infection was excellent, and 8 pustules were found on the nine control leaves. Pustules on the test leaves in juice plus sucrose developed two days later, were less numerous and smaller. The leaves in pure press juice were discarded at the end of five days because of the intense growth of foreign fungi.

In the second experiment Khapli leaves were supported in Marquis press juice, and Marquis extract in sugar solutions, and in sterile water. In each case three dishes were used for each solution. No pustules were obtained in any dishes. Foreign fungi were most numerous in press juice sucrose dishes, intermediate in press juice water dishes and sparse in the dishes containing juice extract and in the controls. It was after this experiment that the method of sterilizing the juice by filtration was developed.

In experiment number three Khapli leaves were used. Press juice was obviously unsuited for these cultural investigations and was not included in the ones which follow. It was also evident that since foreign fungi might appreciably influence the results a second control was necessary. This second control consisted of dishes in which leaves were supported in sucrose solutions plus equal parts of the juice from the same variety of wheat from which the leaves had been removed. The set-up for experiment number three is given.

Dish No. Cultural Solution

1-3 (control)	20 cc.'s 6% sucrose solution
4-6 "	10 " Khapli filtrate plus 10 cc.'s sucrose solution
7-9	10 cc.'s Marquis filtrate plus 10 cc.'s sucrose solution
10-12	10 cc.'s Marquis extract plus 10 cc.'s sucrose solution

The foreign fungi dominated by Rhizopus nigricans increased with great rapidity in the Khapli filtrate, much more so than in the other dishes. No evidence of infection by Puccinia Graminis was observed in any of the dishes.

Experiment number four was with Marquis leaves. Three sets of controls were used, two as already described with pure sucrose and Marquis filtrate plus sucrose; the third with similar proportions of Marquis extract and sucrose solutions. The experimental dishes contained the usual amounts of sucrose solution in addition to Khapli filtrate, Vernal Emmer filtrate and Vernal Emmer extract. The washing of the leaves was very thorough, but in spite of the utmost precautions the growth of foreign fungi was very rapid in the Khapli filtrate. Two dishes were completely filled with Rhizopus nigricans and the other was partially covered by the sporangiophores of this fungus. It is interesting to note that it was in one of the densely filled dishes that the one pustule found in these three dishes developed. The results of this experiment are presented in Table 3. The abbreviations used are self evident except for "f.f." which represents foreign fungi or bacteria" and the initials "M" for Marquis, "K" for Khapli, and "V.E." for Vernal Emmer.

TABLE 3. Effect of plant juices on infection of Maquis leaves by stem rust in Petri dish cultures.

Dish Nos.	Cultural solution	Condition of leaf	Relative amounts of f.f.	Evidence of infection	Remarks
1 to 3	Sucrose solution	B 4	None	Pustules in five days	Excellent infection
4 to 7	Sucrose plus M. filtrate	B 5	Sparse growth of f.f.	Pustules in five days smaller than #1-3	Fair infection
8 to 10	Sucrose plus M. extract	B 5-6	No f.f. Great bacterial action	Pustules in 5 days equal to #4-7	Bacterial action reduced. Number of f.f. present. Fair infection
11 to 13	Sucrose plus K. extract	B 6	Large amounts of f.f. filled 2 dishes. #12 less infected	One very small pustule in eight days	F f. may have decreased infection by rust fungus
14 to 16	Sucrose plus V.E. filtrate	B 5-6	F f. prevalent, less so than #11-13, greater than #4-7	No pustules in 9 days	Influence of f.f. doubtful less severe than #11-13
17 to 19	Sucrose plus V.E. extract	B 5	None. Great bact. action	Pustules in five days equal to control	Evidence that extract ineffective in limiting infection

The greater development of foreign fungi in Khapli filtrate was found in a number of different experiments and its significance is not evident. The leaves used were the same for each series and the treatment was identical. Why, then, does an increased growth of such organisms (mainly Rhizopus nigricans) occur in such solutions? Since the number of foreign spores is probably similar in all the dishes and since, as mentioned under the heading "Methods" the Khapli filtrate was found to be sterile, it seems logical to conclude that Khapli juice is much more adapted to the growth of Rhizopus than are the other plant juices examined. Khapli is probably the most resistant to stem rust of any wheat variety and the coincidence of the increased growth of other fungi in such juice is interesting. It is not suggested, however, that such a condition is directly associated with rust resistance.

The last experiment which will be mentioned in connection with the Petri dish cultures is No. 5, in which Little Club leaves were supported in various solutions. Three controls were again set up as in experiment No. 4, and test leaves were treated with Khapli filtrate, Vernal Emmer filtrate, Khapli extract and Vernal Emmer extract. Four dishes, each containing three Little Club leaves, were used for each solution. Foreign fungi were again most prevalent in Khapli filtrate. Three of the four dishes were densely packed with Rhizopus nigricans at the end of five days. Control dishes and the extract dishes were almost free from foreign organisms, while Vernal Emmer filtrate showed a much less severe infection by these fungi than did the Khapli filtrate solutions. Pustules appeared on the sucrose controls in five days; on the juice controls and the leaves in Vernal Emmer and Khapli extract in seven days; and no pustules were found on leaves treated with Khapli

and Vernal Emmer filtrate. Again these results were rendered less trustworthy by the amounts of foreign fungi in the test dishes. The results of experiment No. 5 are shown in Table 4.

In Table 5 are presented the data from all the experiments reported and it will be seen from this table that the experiments with Petri dish cultures have not been productive of conclusive results. The effect of the foreign fungi is possibly great enough to make any conclusions unreliable, yet in the experiments which have been carried out there seems to be an indication that inhibitory effects may be exerted on the rust parasite by the resistant plant juices of Khapli and Vernal Emmer. Only the juice filtrates have exhibited this effect. With juice extracts of resistant varieties no increase in the resistance of Marquis or Little Club has been demonstrated. The hypothesis for the presence of accessory substances received no support at all. In no case examined was it possible to increase the infection of resistant Khapli leaves by supplying to these leaves the juices of susceptible varieties such as Marquis and Little Club.

The influence which the foreign fungi may have had was perhaps not as great as might be imagined. The fungi were all saprophytic and in cases where the growth was greatest it was evident that the leaves were not attacked. In such cases, however, the diminution of the light resulting from the enveloping of the leaves by the fungal mycelium might effectually inhibit the production of pustules. Little more can be said of the Petri dish cultures than that doubtful indications were found that the resistant juices might inhibit infection of susceptible leaves.

TABLE 4. Effect of plant juices on infection of Little Club leaves by stem rust in Petri dish cultures.

Dish no.	Cultural solution	Condition of leaf	Relative amounts of f.f.	Evidence of infection	Remarks
1-4	sucrose solution	B 4	none	25 pustules in 5 days	excellent infection
5-8	sucrose solution plus L.C. filt.	B 5-6	sparse growth of <i>R. nigricans</i> in 2 dishes	pustules less numerous and slightly smaller than on sucrose control	good infection
9-12	sucrose solution plus L.C. ext.	B 6	no f.f. bacterial growth pronounced	pustules fewer in number than #5-8	fair infection
13-16	sucrose solution plus K. filt.	B 4-5	dishes filled in 5 days by <i>R. nigricans</i> .	no infection on any of 12 leaves	lack of infection, possibly due to f.f. dishes discarded after 6 days
17-20	sucrose solution plus V.E. filt.	B 6	vigorous growth of f.f. but less than in #13-16	No infection in 8 days	f.f. may have had inhibitory effect on rust fungus
21-24	sucrose solution plus K. ext.	B 6	no f.f. bacterial growth pronounced	feeble pustules on 4 leaves	fair infection
25-28	sucrose solution plus V.E. ext.	B 6	no f.f. bacterial growth pronounced	feeble pustules on 5 leaves	fair infection

TABLE 5. Summary of results of Petri dish cultures.

Ex. No.	Variety of leaf	Cultural solution	R e s u l t s			Test leaves	Remarks
			Control #1	Control #2			
1	Marquis	pure K. p.j.	pustules in 5 days	-		discarded in 4 days	discarded before end of infection period great amounts of f.f. makes result doubtful
		K.p.j. + sucrose	pustules in 5 days	-		very small pustules in 7 days	
		M.p.j. + sucrose					
		M.ext. + water					
2	Knapl1	M.p.j. + water	no pustules	-		no pustules in any of 12 dishes	f.f. most numerous in p.j. + sucrose, fewer in p.j. + water, bact. action pronounced in ext. + sucrose or water
		M.ext. + water					
3	Knapl1	M.filt. + sucrose	no pustules	no pustules		no pustules	f.f. most prevalent in K. filt control
		M.ext. + sucrose					
4	Marquis	K. filt. + sucrose	excellent infection in 5 days	good infection in 5 days		1 very small pustule	large amounts of f.f.
		V.E.filt. + sucrose	do	do		no pustules	large amounts of f.f. but less than K. filt.
		V.E.ext. + sucrose	do	do		good infection in 5 days	pronounced bact. activity
		K.filt. + sucrose	excellent infection	fair infection		no pustules	large amounts f.f. tend to invalidate result
5	Little Club	V.E.filt. + sucrose	do	do		no pustules	large amounts f.f. but less than K. filt.
		K.ext. + sucrose	do	do		fair infection	pronounced bact. activity
		V.E.ext. + sucrose	do	do		fair infection	pronounced bact. activity

WATER CULTURES

Outline of Method

Water cultures have frequently been made use of in the investigations of plant requirements and in the studies on the secretions of plants. In connection with cereal rusts, however, it has not been used so extensively. Ward (54) used it for the development of sterile seedlings and Mains (39) adapted Ward's method for studying the effects of solutions lacking certain salts on rust development. This method of growing seedlings is very simple, but for the purposes of this investigation proved rather unsatisfactory.

The method of growing seedlings in water cultures is well illustrated in Plate 1. Wide mouthed four ounce pintment jars of amber glass were used in these experiments, supplemented when necessary by somewhat smaller crystallizing dishes. In the photograph there is an unfortunate omission; the crystallizing dishes of clear glass were in all cases but the one shown, wrapped with heavy brown paper to exclude the light. Circular wax covers were cut from commercial "Parowax" to fit the top of the jars and small holes were bored through the wax to accommodate the seeds, which were held in place by cotton wool plugs moistened to promote germination. Germination was not always regular, but in most cases at least five of the eight seeds produced healthy plants. Knop's full nutrient solution was used throughout, to which was added the desired plant juice.

Plate 1. Water cultures showing method used for growing seedlings
in nutrient solutions.

Experiments with Plant Juices

The suitable concentration of the juice to be added was first determined and was found to be about 10 per cent. In even 5 per cent solutions of press juice the growth of the plant was materially affected. Press juice was found on the whole to be a most unsatisfactory cultural solution; oxidation and enzymatic actions resulted in a precipitation of the chlorophyll and very rapid putrefaction. In preliminary experiments with Marquis seedlings Emmer press juice was added when roots were only an inch or so long. No further root development resulted. In six days the roots were brown and covered by molds, and the leaves were drooping badly in all but concentrations under 5 per cent. In twelve days the food reserves of the seed had been exhausted and the seedlings of the higher concentrations were dead. Rust pustules developed prolifically on all shoots in concentrations less than 5 per cent juice. The higher concentrations wilted before the end of the infection period.

In similar experiments the juice was changed at intervals of a week, five days and three days. Little difference was noted. Concentrations of over 10 per cent juice strongly inhibited the growth of the roots, though with changes every three days the seedlings just survived in this concentration. No modification of infection resulted, but since the Emmer plants from which the juice was obtained proved later to be susceptible to Form 21 of Puccinia graminis tritici this was to be expected.

With Emmer seedlings in Marquis juice the susceptibility of this variety was discovered and the other experiments which had been set up with this variety were discarded. With the resumption of the

water-culture experiments after the work on the physico-chemical constants had been completed, Vernal Hammer seeds were available and plants of this variety were grown in 5, 10, and 15 per cent solutions of Marquis juice. Three jars were used for each concentration and a series of five experiments was carried out with the juices added at various times after germination. Twelve jars were used as controls with the seedlings growing in pure Knop's solution. Changes of these solutions were made every two days except when in very warm weather coagulation and fermentation occurred, necessitating daily changes. With shoots in 15 per cent juice, growth was very poor and seedlings died after the food reserves of the seeds had been depleted. In 10 per cent concentrations good seedlings were obtained except in those jars where juice was added before the roots were an inch long. In 5 per cent juice solutions the growth was normal whether the juice was added on the day of the set up, at the germination of the seedlings, three days after germination, a week after germination, or nine days after germination. In all, 45 jars were under examination exclusive of the twelve control jars, with an average of five plants per jar. Of the 225 leaves examined only 4 showed evidence of infections and all the pustules came within the range of Vernal Hammer's resistance.

The next three experiments were carried out similarly. Marquis, Little Club, and Khapli seedlings were grown in Knop's solutions for two weeks. Four control jars of pure Knop's solution were used and in addition four jars as secondary controls in which 7 per cent of the juice from the same variety as the seedlings, was added. The Little Club and Marquis seedlings were

Ex. No.	Varieties used	Cultural solution	Reaction of test plants	Reaction of control	Remarks
1	Marquis	"Common" Emmer. p.j. at various concs.	15% and over died 10% small pustules; poor plants 5% no pustules; good plants 3% normal pustules; good plants.	Normal infection	"Common" Emmer found to be susceptible to Form 21
2	"Common" Emmer	M. p.j. at various concs.	15% and over died. Lower concs. all showed heavy inf.	Heavy infection	"Common" Emmer found susceptible
3	"Common" Emmer & Marquis	M. and E. p.j. M in M p.j. & E in E p.j.	Normal infection	Normal infection	Juice changed every 2 days
4	V. Emmer; juice added at set up	M. p.j. at 5, 10, 15% concs.	No infection	No infection	Juice changed every 2 days
5	V.E.; juice added at germination	M. p.j. as in #4	Very small pustule within the range of V.E. reaction	No infection	Juice changed every 2 days
6	V.E.; juice added 3 days after germination	M.p.j. as in #4	Very small pust. as in #5	No infection	Juice changed every 2 days
7	V.E.; juice added 6 days after germination	M.p.j. as in #4	No pustules	No infection	Juice changed every 2 days
8	V.E.; juice added 9 days after germination	M. p.j. as in #4	Very small pustule as in Nos. 4 & 5	No infection	Juice changed every 2 days
9	Vernal Emmer	M. filt.	No infection	Very small pustules on 1 leaf	Juice changed every 2 days. Erysiphe graminis prevalent
10	Marquis	K. filt. and V.E. filt	Died	Sparse infection	Erysiphe graminis prevalent
11	Little Club	K. filt and V.E. filt	Died	Sparse infection	Erysiphe graminis prevalent
12	Vernal Emmer	M. filt and L.O. filt	Died	No infection	Erysiphe graminis prevalent

treated with Khapli filtrate and Vernal Emmer filtrate, while the Khapli seedlings were treated with Marquis and Little Club filtrates. For each of the three wheat varieties, therefore, there were 16 jars, 8 controls, and 4 each of different juice filtrates. Rustules were obtained only on the Marquis and Little Club seedlings in pure Knop's solution after eleven days. The secondary controls and the test seedlings were all in very poor condition at this time and the experiment was discontinued.

In Table 6 a summary of the water culture experiments is given. No definite results were obtained with any of the water culture experiments and it was decided that this method was unsatisfactory for supplying the cultural solutions of plant juices to the wheat host plant.

INJECTION EXPERIMENTS

Historical

The third method developed for the examination of plant juices for possible inhibitory effects was that of injecting these fluids into the leaf itself while still attached to the plant. It has not proved possible to obtain any definite references to such operations. Rumboldt (49) reviewed the literature on injections in 1920 and showed that various chemicals had been successfully introduced into trees. The opinions of the workers she cited expressed the view that injections are either detrimental or non-effective. The method she used was not described in detail, but from her remarks it was evident that it involved boring a hole in the tree trunk and sealing a glass tube in the aperture so formed.

Lipman and Gordon (38) developed a similar method for injecting trees. A glass tube connected to a raised reservoir was sealed in a hole bored in the trunk and the liquid was supplied to the conducting elements by the force of gravity. By this treatment it proved possible to correct the chlorosis of leaves of lemon trees and it was suggested that further applications of the method could be made in the freeing of plants from insect pests and fungal parasites and in protecting it from frost damage by the injection of electrolytes.

As far as it has been possible to ascertain, very little work has been done on the injection of liquids into herbaceous plants. A French worker, Lumiere, has conducted experiments along this line in an attempt to determine whether anaphylactic reactions could be demonstrated, but the reference to this work was not available.

Development of Method

The injection of the leaves of wheat seedlings proved to be a more delicate undertaking than at first imagined. An ordinary glass surgical hypodermic syringe was used, fitted with the finest needle procurable. Even this needle, number "0.0", was much coarser than would have been desired. With this apparatus a considerable time was spent practising on wheat leaves of various ages. At first it was impossible to insert the needle in any but maturing wheat leaves but after several weeks experimenting the injection of young seedling leaves was effected successfully. The leaf to be injected was held in the left hand, stretched flat over the index finger. The hypodermic was then placed with the slanted edge of the needle flat against the leaf, where it was supported by the index finger.

The needle was then tipped down very slightly and pushed gently forward just far enough for the tip to puncture the epidermis. At the same time a gentle pressure was applied to the plunger. Successful injections were evidenced by a darkening of the leaf color in the regions where the liquid penetrated. With leaf tissues of the right age the whole leaf could be flooded by three injections at different points. The greatest care was necessary in making the injections, first to avoid tearing the tissue unduly and second to prevent the rupture of the cells from too vigorous pressure on the plunger. Only very slight pressures were required where the needle was neatly inserted. Too great pressure resulted invariably in large dead patches surrounding the point of injection. In plate 2, Marquis leaf number 2 and 5 can be seen an example of a case where such dead patches were occasioned. In a number of cases creasing of the leaf followed at the point where dead areas appeared. When more than one set of injections were to be made on the same leaf even greater care was necessary in carrying out the first ones, since even a very small dead area effectively barred the path of the liquid on subsequent injections. This necessitated more numerous needle insertions and a material increase in the areas of damaged tissue.

Experiments with Plant Juices

The first experiment with injected juice was carried out with Khapli seedlings. This variety is immune to Form 21 of Puccinia graminis and is well suited to injections in possessing

broad thick leaves. The injected substance was Marquis extract freshly prepared. Leaves were injected at four points immediately to one side of the mid rib at intervals of about one inch. In pot No. 1 the leaves were inoculated after the second set of injections and died on the third day. In pots No. 2 and 3 the leaves wilted severely at the first injection, but recovered before the next day when the third set of injections was made and the fourth set proved fatal. In Pot No. 4 with inoculations made two days before injection the plants lived for six days with daily injections. On the seventh day there was no sign of infection by P. graminis and the leaves were so tattered that further injections were impossible.

Whether the death of the leaves was due to the injected substance or to the mechanical injury resulting from the numerous needle insertions is an open question. At the present time no more than two sets of injections have been made without seriously damaging the leaf, but the injury inflicted in nearly every case was directly attributable to needle tears or to too vigorous pressure on the plunger of the syringe. The hypodermic is at best ~~a~~ rather unwieldy instrument for the injection of wheat leaves, and when the work is continued it will be advisable to obtain some more delicate injection apparatus. The interest of the investigation would be greatly increased when details such as the possible occurrence of anaphylactic reactions could be observed uninfluenced by the mechanical damage the leaf had sustained. Chambers (5) described a micro-injection apparatus which could be used for very delicate injections. With the ability to inject even single cells of the host plant it should

be possible to increase the scope of the experiments tremendously. It was possible, however, to obtain two good sets of injections with a hypodermic needle.

For the second experiment Khapli leaves were used with Little Club filtrate as the injected substance. Three pots of ten seedlings each were used with a fourth pot for control. The tissue was aged two weeks, three weeks, and one month. Injections were made at two day intervals with inoculations at the time of the first injection.

Pot 1.

The first injection resulted in very little apparent damage and the leaves showed only grey flecks where the needle had been inserted. Recovery was complete. The second injection was less successful--more insertions were required and the area of the flecks mentioned was therefore increased. The third injection was valueless and resulted in rather tattered leaves. No evidence of altered resistance of the host was obtained.

Pot 3, seedlings 3 weeks.

Conformed closely to pot 2. The first two injections were successful, the third less so and no evidence of any alteration in the degree of the host's resistance was obtained.

Pot 4, seedlings 4 weeks.

The first injection was difficult, since the liquid refused to flow evenly from the point of injection. Subsequent injections were increasingly unsatisfactory. The leaves were so tattered that they were discarded after the second injection.

It was evident from these results that the injections should be made in young seedlings, and that the number of injections should be limited to two sets. The third and fourth experiments with Marquis and Little Club extracts injected into Khapli seedlings emphasized this point, through unproductive of any change in the host's resistance. The maximum number of injections made on any one leaf was eight sets, but since each set required approximately twice the number of insertions made in the previous one, the rapidity with which the leaf became tattered can well be imagined.

In the last experiments Marquis and Little Club seedlings were used. With these two susceptible varieties more encouraging results were obtained. The seedlings were all 14 days old at the time of the first injection. In this work the Marquis plants were injected with Khapli filtrate and the Little Club with Vernal Emmer filtrate. Injections were made on the first leaf in every case. Only two sets of injections were made, with an interval of six days. In each pot eight seedlings were injected. The set up for both these experiments is here indicated:

- Pot 1. Control, no injections
- " 2. Injected with resistant filtrate two days prior to inoculation
- " 3. Injected with Marquis (or Little Club) filtrate two days prior to inoculation. Secondary control for pot 2.
- " 4. Injected with resistant filtrate on day of inoculation.
- " 5. Secondary control for pot 4.
- " 6. Injected with resistant filtrate two days subsequent to inoculation.
- " 7. Secondary control for pot 6.

The results of these experiments are given in Tables 7 and 8 and in Plates 2 and 3. Representative leaves were selected for these plates seven days after the appearance of the pustules. In preparation for photographing some of the spores were lost and for this reason Marquis No. 3 and Little Club No. 3 and 5 do not contrast with their respective test leaves as strongly as they should. In Plate 3 this is particularly noticeable. The white patches on the leaves are due in most cases to injury caused during injections and to attacks of Erysiphe graminis. The Little Club leaves were relatively free from this mildew and the needle marks are plainly evident.

It is evident from the preceding data that differences in the infection of injected leaves have occurred. The number of leaves examined was small, but the delay in infection and the decrease in the number and size of the pustules on the leaves injected with resistant juice were too regular to be explained satisfactorily on the basis of chance.

The size of the pustules may have been influenced to a certain extent by the condition of the leaf, but as shown in Tables 7 and 8, leaf damage was usually equal, in the test pots and their controls; in some cases (Marquis No. 5) the controls, with larger pustules, were more damaged.

The effect of severe attacks by Erysiphe graminis may have influenced the results slightly. Damage by this fungus was fairly uniform, however, Marquis seedlings were more heavily infected than Little Club but each variety was uniformly infected except in Pot 1

TABLE 7. Effect of injected Khapli filtrate on the susceptibility of Marquis seedlings to *Puccinia graminis*.

Pot No.	Injected substance	Infection period	No. of pustules	Relative size of pustules	Condition of leaves
1	None	13 days	27 on 8 leaves	normal infection	damaged by attacks of <i>Erysiphe graminis</i>
2	K. filt.	17 days	10 on 7 leaves	slightly smaller than #3	damaged by E. gram.
3	M. filt.	13 days	19 on 8 leaves	normal as on #1	slightly more damaged by needle insertions and mildew.
4	K. filt.	17 days	6 on 3 leaves	small, not convergent	considerably damaged by inject. and mildew
5	M. filt.	17 days	18 on 5 leaves	large, as on #1 and #3	damage about same as #4, slightly more dead tissue
6	K. filt.	18 days	1	very small pustule	poorer condition than control #7
7	M. filt.	13 to 14 days	5 on 4 leaves	large, well formed	slightly less damaged than #6

TABLE 8. Effect of injected Vernal Emmer filtrate on the susceptibility of Little Club seedlings to *Puccinia graminis*.

Pot No.	Injected substance	Infection period	No. of pustules	Relative size of pustules	Condition of leaves.
1	None	12 days	8 on 5 leaves	large, convergent	little damage from injections, heavily infected by E. gram.
2	V.E. filt.	16 days	3 on 3 leaves	minute, smaller than control #3	damaged slightly, but comparable to controls
3	L. C. filt.	13 days	6 on 4 leaves	somewhat smaller than #1. but considerably larger than #2	little damaged
4	V. E. filt.	-	none	-	4 leaves with dead areas from faulty infections
5	L. C. filt.	12 days	4 on 2 leaves	comparable with #3	considerably damaged by injections, 5 leaves with large dead areas
6	V. E. filt.	16 days	1	minute	3 leaves withered, remainder damaged slightly
7	L. C. filt.	12 days	5 on 3 leaves	comparable with #3 & #5	damaged more than #6

Plate 2. The effect of injected resistant filtrate on the infection of Marquis and Little Club leaves by Puccinia graminis. Arrows indicate pustules.
 2, 4, 6, Test leaves
 3, 5, 7, Secondary controls
 1, Uninjected control.

Plate 3. The effect of injections with Vernal Enmer filtrate on the infection of Little Club leaves by Puccinia graminis.

1, Uninjected. 2, Injected with V. E. filtrate.
3, Injected with L. G. filtrate.

of Little Club, the uninfected control, which was considerably more damaged than the others. The mildew, however, may have been responsible for the excessively long infection period of 13 days.

The injection method has proved the most satisfactory one for supplying plant solutions to wheat leaves. Fairly definite evidence was obtained that the resistant juices exerted an inhibitory effect on the growth of Puccinia graminis, and although the condition of the leaves and attacks by other parasites may have influenced the results to a certain extent, such influence does not seem to have been great enough to account for the differences noted. It seems a more logical conclusion that in the resistant juice some inhibitory substance was present.

DISCUSSION

While definite conclusions from any individual experiments would be unwarranted, when examined collectively the experiments suggest that the resistance of wheat to Puccinia graminis may result from the presence of inhibitory substances in the plant juice. One difficulty of explaining resistance by an inhibitory substance is found in the fact that wheat varieties behave differently to the various physiological forms of the rust parasite. If the substance occurred in a definitely toxic form it would seem that the variety in which it occurred should be equally resistant to all the forms of stem rust. To explain the condition which exists, it is suggested that the inhibitory substance may occur as some chemical compound or in a colloidal state, which is not effective until acted upon by the fungal secretions. With different secretions the reaction may result in either a toxic or an inactive substance. The physiology of the parasite is probably quite as important as that of the host in determining whether resistance or susceptibility

will be exhibited by the host variety. Such an explanation seems adequate for the different degrees of resistance which are found in plant reactions to Puccinia graminis.

The nature of this inhibitory substance has not been determined. In no cases were inhibitory effects obtained with juice extract, and the substance responsible for the inhibitory effect seems to have been either precipitates with the coaguable proteins, or so altered that it was ineffective. There is a possibility, also, that a very volatile substance might be lost in the preparation of the extract in spite of the reflux condenser used in all such preparations.

On resistant hosts a characteristic feature is the presence of a sharply defined chlorotic area surrounding the stem rust pustule and it might be expected that susceptible leaves injected with resistant juice might also show this chlorotic area. On some of the leaves of the last injection experiment lighter green areas could be seen surrounding the pustules. (See Plate 2). Marquis leaves, however, show this area frequently on normal leaves.

In the Little Club leaves no such area was found on the uninjected leaves of the experiment, on the leaves of stock hosts or on control leaves. In leaves No. 2 and ⁶ 7, Plate 2, the chlorotic area surrounding the very small pustule is quite definite and it approaches the condition found in Vernal Hammer seedlings infected with rust. Only slight significance can be attached to the presence of this area, since Marquis, only slightly more resistant to stem rust, shows a similar area when not infected.

V

SUMMARY

The nature of resistance of wheat varieties to stem rust has been investigated in two ways. An examination of the physico-chemical properties of the plant juices has been made and an investigation of these juices for the presence of inhibitory and accessory substances has been carried out.

No indications were obtained that the physico-chemical properties of the plant juice were in any way connected with the problem.

Three methods of determining the effect of plant juices on the susceptibility of wheat varieties were developed:

(1) by Petri dish cultures; (2) by water cultures; and (3) by injections. Of these the water cultures were the least satisfactory, and the injection method the most encouraging.

A filtration method was developed for the sterilization of the expressed tissue fluids of wheat plants, without heat.

By the injection of sterile filtrate prepared from resistant leaves into the leaves/^{of}susceptible varieties, an indication was obtained that resistant plant juices exerted an inhibitory influence on the growth of the rust parasite.

No evidence was obtained that boiled resistant juice exerted any inhibitory influence.

No evidence of decreased resistance was obtained with susceptible juice introduced into resistant leaves.

VI.

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