THE EFFECT OF ENVIRONMENTAL CONDITIONS ON THE GERMINATION OF UREDOSPORES OF *PUCCINIA GRAMINIS-TRITICI* ERIKSS. AND E. HENN., RACE 56

By

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Abstract

Over a range of levels of certain environmental factors, the germination of uredospores of *Puccinia graminis-tritici*, Race 56, is characterized by qualitative as well as quantitative differences in response, as well as by pronounced variability between samples. Both of these features are probably dependent upon the level of a self-inhibitor evolved by these spores.

Evidence is presented for the existence of the following interactions:

a. In the presence of light, but not in its absence, the lowering of the carbon dioxide tension causes a reduction in percentage germination, and results in lysis of the germ tubes.

b. In terms of lower percentage germination, uredospores are more sensitive to the self-inhibitor at low carbon dioxide tension in the presence of light.

c. Uredospores are more sensitive to low oxygen tensions at low carbon dioxide tension, in the presence of light.

From the above considerations, it appeared likely that the effects of carbon dioxide, oxygen, and inhibitor concentrations were interrelated, and that the suppression of germination of submerged spores was probably associated with their sensitivity to the inhibitor.

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INTRODUCTION

At the present time, the major obstacle to the study of obligate parasitism in fungi, in this instance, Puccinia graminis-tritici Erikss. & E. Henn., Race 56, is that these organisms cannot be grown apart from their living hosts, their metabolic activities being intimately associated with, and hence obscured by, those of the host plant. There is, however, one relatively brief phase, the germination of fungal spores, which can proceed for a limited time independently of the host. For this reason, the physiology of spore germination has received considerable attention.

Apart from their value per se, studies on spore germination provide two means of revealing in greater detail the distinctive characteristics of obligately parasitic fungi: by comparing the germination behaviour of spores of the parasitic type with that of spores of normally saprophytic fungi, and by noting similarities and differences between the independent phase and the resulting parasitic one.

Unlike the majority of fungal spores, it was noted in this laboratory (Isaac), that Race 56 uredospores frequently did not germinate when covered with a thin layer of water.

Since this could conceivably be due to either an insufficient oxygen supply, or to an accumulation of an inhibitor known to be evolved by these spores, it was suggested that an enquiry into the relationships between the gaseous environment and germination, and also the role of the inhibitor, might be of some assistance in explaining this behaviour.

Before carrying out the main body of the investigation, certain inconsistencies in the germinability of the spore samples, involving storage conditions, required attention. These are dealt with in Part I. The effects of carbon dioxide concentration and oxygen supply, are treated in Parts II and III respectively. Finally, in Part IV, with an assessment of the information gathered from the previous sections, together with more direct evidence, an attempt is made to interpret the problem of why the germination of submerged spores is reduced.

METHODS AND MATERIALS

The Race 56 culture of uredospores of Puccinia graminis-tritici Erikss. & E. Henn. was originally obtained from a well-isolated pustule and grown continually on Little Club wheat (Triticum compactum Host.) under normal greenhouse conditions. The genetic stability of the rust collections was tested periodically on the standard set of twelve differential hosts. Both the rust material and the wheat seed were kindly provided by the Dominion Laboratory of Plant Pathology, Fort Garry, Manitoba.

Collection of the Spores.

The spores were collected daily with a cyclone harvester, which is shown diagramatically in Figure 1. It consisted of a 50 ml boiling-tube (A), a two-hole rubber stopper (B), and intake tube (C) with a 2 mm bore, and a 5 mm outlet tube (D), which was connected with rubber tubing to an aspirator pump. Tube (C) extended about halfway down the side of the boiling-tube, and was bent to an angle of about 45° (c_1), so that the spores were directed downwards in a circular direction as they were drawn into the boiling-tube.

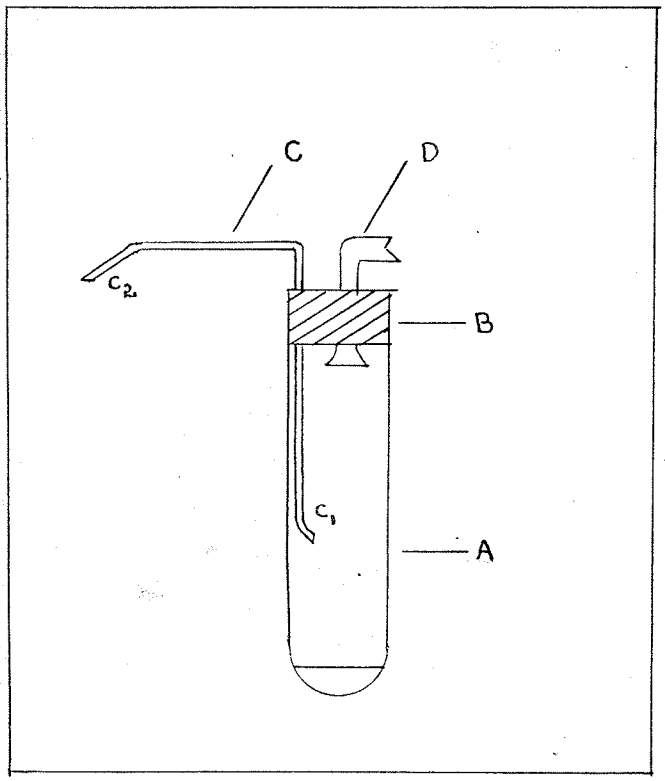


Fig 1. Diagram of apparatus used for the collection of uredospores. see text for details.

The tip (c_2) was slightly flattened horizontally. The outlet tube (D) did not extend as far into the boiling-tube as (C), and was flared at the inner end.

When the collector was attached to an aspirator pump, the velocity of the air passing through (C) was sufficient to carry the spores into the boiling-tube; here the drop in air velocity brought about through the increased cross-sectional area, caused the spores to be precipitated to the bottom of the tube.

Inoculation Technique.

The apparatus illustrated in Figure 2 assisted in dispersing the inoculum evenly over the surface of the medium, so that the spores were well separated from each other to facilitate counting. This was accomplished by vibrating the spores through a fine copper gauze screen and allowing the spores to settle onto a rotating petri dish.

A commercial vibrating tool (A) was fitted with a metal cylinder (B), to the bottom of which was soldered a circle of gauze screening. A turntable (C), such as that used for ringing microscope slides, was placed directly beneath the vibrator. When air currents caused the spores to drift, it

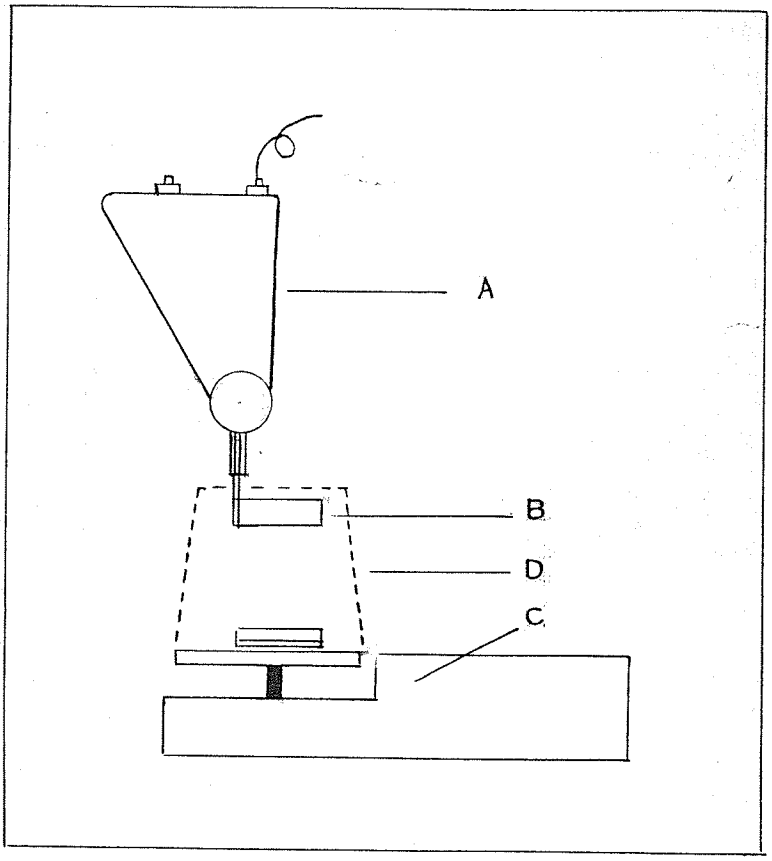


Fig 2. Diagram of apparatus used for the inoculation of uredospores. see text for details.

was sometimes found convenient to enclose the area above the petri dish with an inverted polythene container (D).

To inoculate the medium, the spores were placed in the container (B), the turntable and petri dish were set in motion, and the spores were subjected to vibration for five to fifteen seconds.

No significant difference in final percentage germination was found between spores "vibrated" in the above manner, and those dusted onto agar with a camel's hair brush.

Note.

Both pieces of apparatus described in this section were assembled in the Botany Department, U. of M.

Germination Conditions.

Many of the germination trials to be reported here were carried out in a growth-chamber which provided uniform conditions. These were:

Light ----- ca. 2400 foot-candles. When exclusion of light was desired, the germination vessels were wrapped in aluminum foil.

Temperature ----- 20° C $\pm$ 0.25

Humidity ----- 65% $\pm$ 2 Relative Humidity

Note.

By the following terms which appear throughout the work, the conditions implied are:

"Plain agar" --

Composition:

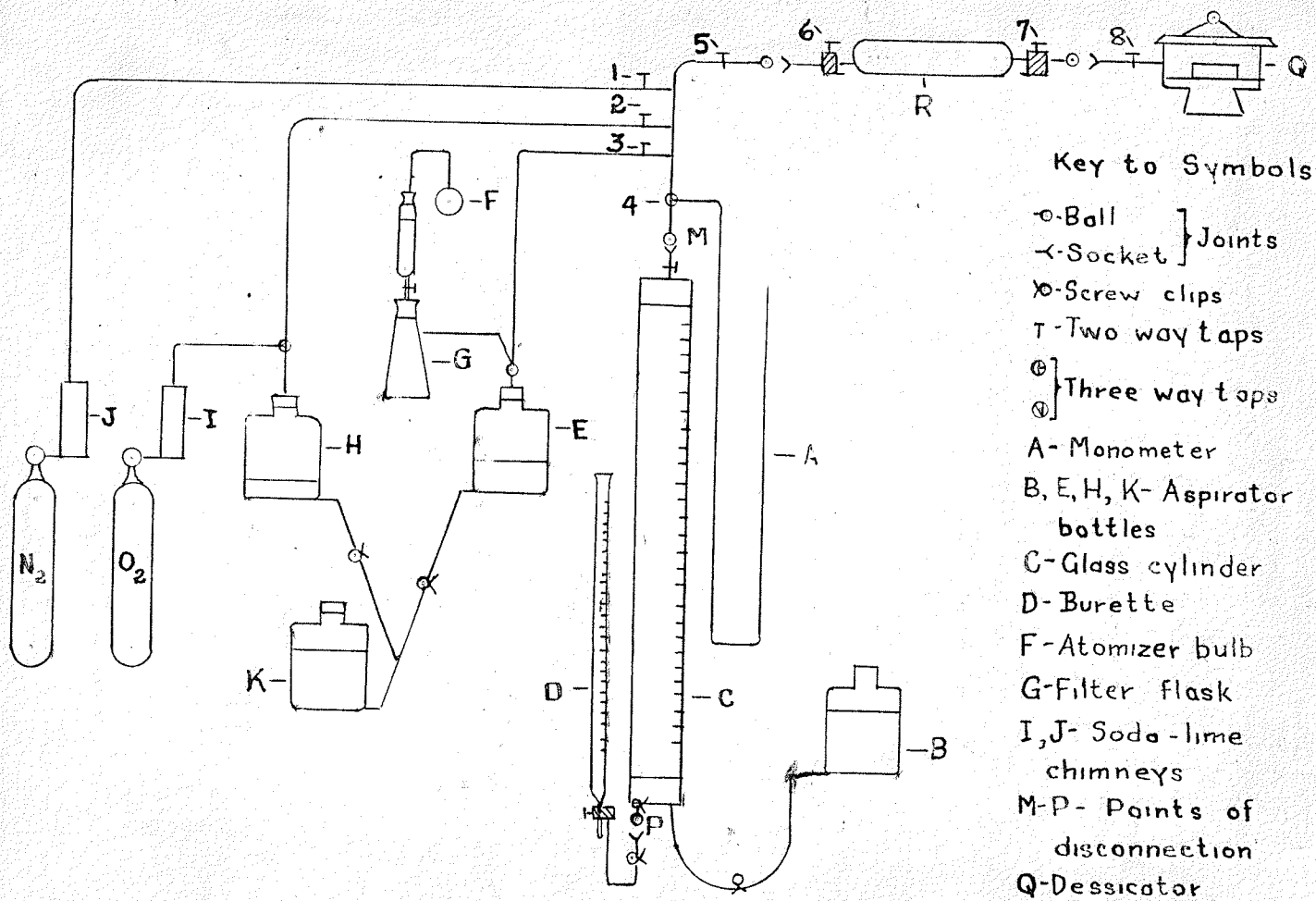
"Difco" agar	7.5 g
Distilled water	1000 ml

The agar was poured into 50 ml petri dishes, and used not more than four hours after preparation. In any experiment involving low carbon dioxide concentrations, the dishes were kept over 20% potassium hydroxide.

"fresh spores" -- spores that had been produced during the twenty-four hours previous to the experiment.

Apparatus for the Handling of Gas Mixtures.

The apparatus shown schematically in Figure 3 was designed to prepare mixtures of carbon dioxide, oxygen, and nitrogen of any desired combination. Essentially this is achieved by withdrawing a known volume of liquid from a graduated cylinder, creating a partial vacuum, then bringing the pressure back to atmospheric by adding the required gas to the cylinder.



Key to Symbols

- Ball, Socket } Joints
- Screw clips
- Two way taps
- Three way taps
- A- Monometer
- B, E, H, K- Aspirator bottles
- C- Glass cylinder
- D- Burette
- F- Atomizer bulb
- G- Filter flask
- I, J- Soda-lime chimneys
- M-P- Points of disconnection
- Q- Dessicator
- R- Gas sampling tube

Fig 3 - Diagram of apparatus for the preparing and handling of gas mixtures

The vertical glass cylinder (C), with a capacity of two liters, and graduated in 10 ml intervals, is connected at the base with a four-liter aspirator bottle (B), containing 5% sulphuric acid. A "fine adjustment" in the form of a 50 ml graduated burette (D), which was also attached to the main cylinder (C), provided a means of withdrawing measured volumes of acid of less than 10 ml.

Carbon dioxide is generated by forcing sulphuric acid into the filter flask (G), containing sodium carbonate, by means of an atomizer bulb (F) with a one-way valve. As the carbon dioxide is evolved, it is drawn into one of the two eight-liter aspirator bottles (E) over 5% sulphuric acid.

Cylinders of compressed nitrogen and oxygen, as well as the carbon dioxide supply are connected to the main cylinder (C) through the taps (1), (2), and (3) respectively. The manometer (A), containing acidified Brodie's fluid, opens to the manifold through a three-way tap (4).

For prepared oxygen and nitrogen mixtures, an extra storage bottle (H) was found convenient, and the soda-lime chimneys (I & J) were added to the oxygen and nitrogen lines as a precaution against carbon dioxide contamination. Glass tubing or thick-walled rubber tubing was used throughout.

Since a positive pressure was maintained in the gas lines during the preparation of the gas mixtures, contamination by atmospheric carbon dioxide was probably a negligible factor.

The germination vessels (Q) consisted of 600 ml desiccators, each of which was bored to admit a rubber stopper and a Pyrex tap.

Those parts of the apparatus that were frequently disconnected (e.g., desiccators and sampling-tube) were fitted with ball and socket joints.

For the analysis of the gas mixtures, a Haldane-type of apparatus, the PRECISION model of the Fischer Scientific Company, was employed. Its sensitivity was found to be approximately 0.1 per cent.

To prepare the gas mixture, the manometer fluid was drawn up to tap (4), and re-levelled by admitting nitrogen. This tap was then closed. The gas lines were flushed out with their respective gases and taps (1), (2), and (3) were turned off. Finally, the main cylinder (C) and the manifold were filled with acid and tap (5), at the outlet of the manifold, was shut off.

The gases were drawn into the main cylinder one at a time by lowering the aspirator bottle (B) and allowing the

acid to flow out of the cylinder, meanwhile introducing small amounts of the gas, so that a reduced pressure was maintained in the cylinder. The liquid flow was stopped at the appropriate level (i.e., at the nearest 10 ml mark on the cylinder plus the amount removed through the burette (D)). Tap (4) was opened at this point, and the pressure in the cylinder was brought to atmospheric by introducing small additional amounts of gas. The process was repeated for each gas required.

To mix the gases, all taps were closed and the cylinder was detached from the rest of the apparatus at (M) and (P). It was inverted a few times, and re-connected to the manifold.

The gas mixture was now ready for use. One or two inoculated petri dishes were placed in each desiccator, and a desiccator was attached to a gas-sampling tube (R). Both were evacuated for one minute* and connected to the gas mixing apparatus.

* No significant difference in final percentage germination could be attributed to this treatment.

The manifold was flushed out by turning tap (6) of the gas sampling-tube so that some of the gas mixture was forced through the manifold to the external air. The appropriate taps were then turned to establish connection between the desiccator and the main cylinder.

Acid from the aspirator bottle (B) was admitted to the cylinder, and when the pressure in the system was approximately atmospheric, the manometer tap (4) was opened, and a final adjustment was made. Taps (6) and (7) of the sampling-tube and (8) of the desiccator were closed, and the two were disconnected.

After each analysis, the sampling-tube was flushed with air; the desiccators were washed thoroughly after each germination trial.

PART I

THE PROBLEM OF VARIABILITY. A. THE EFFECT OF STORAGE CONDITIONS

At the outset of investigations of germination under various conditions, we were confronted by variations in the spore samples, such that the percentage germination varied from fifty to ninety-five. Unaccountable variations of a similar nature have also been encountered by other workers (2, 3, 19, 22, 26, 37).

Alternatively, in some experiments the controls agreed within around one per cent of each other.

It was felt necessary to devote some time to trying to trace this source of variability, because if there is a marked difference between the "control" results in essentially similar experimental conditions, it is invalid to attribute any result obtained to the factors being tested and no comparison can be made between experiments.

A number of factors were studied with a view to reducing the large error variance; such likely ones as age of pustules, time of day that the spores were collected, position of the pustule on the leaf, differences between plants,

differences between leaves on the same plant, greenhouse temperature[1] and humidity[2] during spore formation, were all found to be without significant effect under the conditions of this series of experiments.

The following experiments were carried out in an attempt to determine the particular conditions of storage and germination that caused this variation.

Experiment I. The Effect of Storage Conditions And Mixing on Percentage Germination

Fresh spores from four large plants were bulked and divided into two lots, A and B. Lot A was mixed thoroughly with a camel's hair brush, while Lot B was disturbed as little as possible.

Samples from A and B were shaken directly onto plain agar and kept at 20° C.

[1] Temperatures tested--85° F and 55° F

[2] Relative humidities--50% and 75%

The remaining portions of A and B were again subdivided into Lots C, D, E and F, and kept at 5° C for two days. At the end of this time, C and E (from A and B respectively) were mixed thoroughly, while D and F (from A and B) were left unmixed. A_1 and A_2, both taken from A, and serving as controls on the treatments at 5° C, were left standing in the laboratory at approximately 20° C for two days. Samples from all treatments were then shaken onto agar and germinated at 20° C.

There were four replicates, that is, four petri dishes, for each treatment.

The results are summarized diagramatically in Figure 4.

Fresh spores, apparently, were highly viable and uniform in their behaviour, whereas those that had been stored in the refrigerator exhibited a significantly lower percentage germination. This decrease, furthermore, did not occur uniformly throughout the sample, since the replicates from unmixed samples possessed a higher standard error.

Heterogeneity in this case could not be attributed to growing conditions, since there was no significant difference between A and B, but must have occurred after removal from the plant.

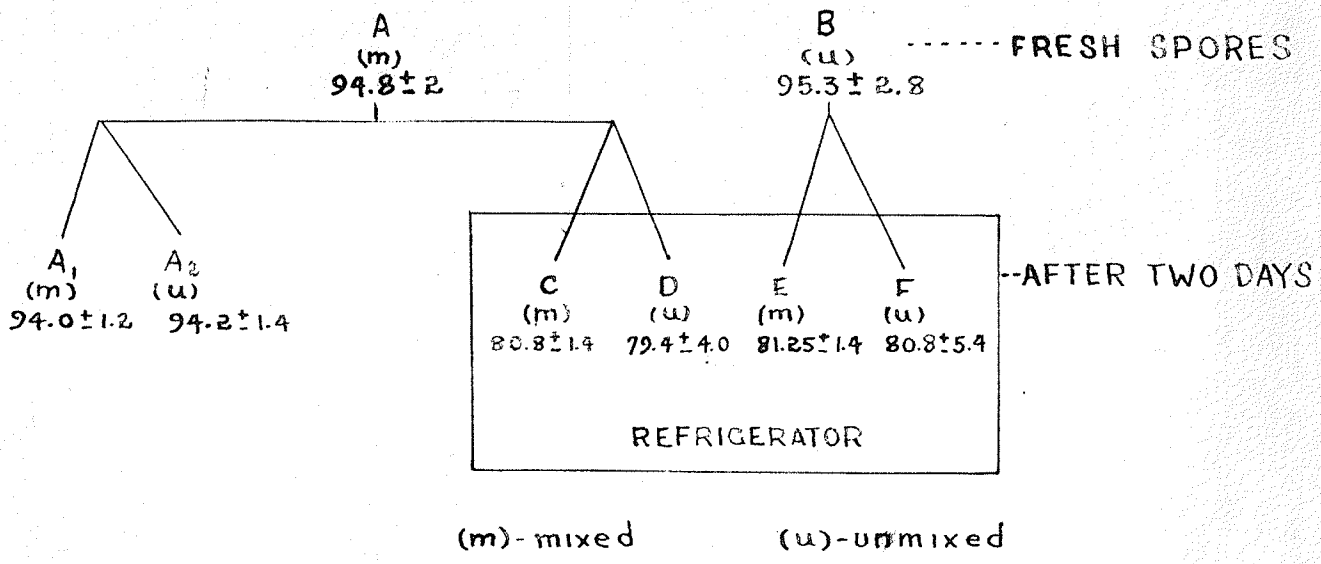


Fig.4. Diagram of the effect of mixing and of storage conditions on percentage germination.

There was no significant difference between C, D, E and F, although spores that had not been mixed gave more variable results. C, D, E and F, however, were all noticeably lower than A_1 and A_2.

Since there was no significant reduction in percentage germination after two days' storage under room conditions, it was concluded that a large part of the variation occurred after collection, and was probably associated with conditions in the refrigerator.

Experiment II. The Effect of Storage in The Refrigerator

Allen (2), working with Race 56, found that prolonged storage tended to decrease the rate of germination, and to increase the time required for germination to commence. Similar effects have been noted in this laboratory, but from preliminary experiments, it was found that there was no direct correlation between age of spores and decrease in viability, up to around six months. (Figure 5.) In some instances, there was a sharp reduction in percentage germination after as little as two hours in the refrigerator; on other occasions, little change occurred over a period of ten days. When successive

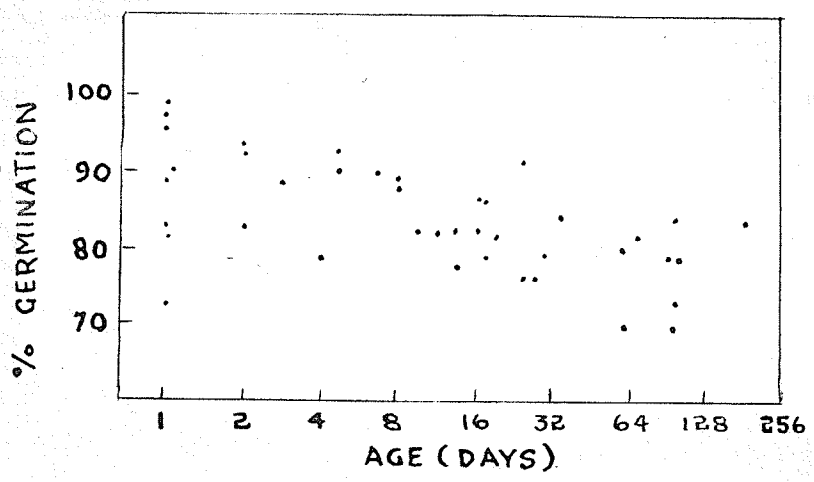


Fig.5. Relationship between age of spores and percentage germination. Bulked data from all experiments

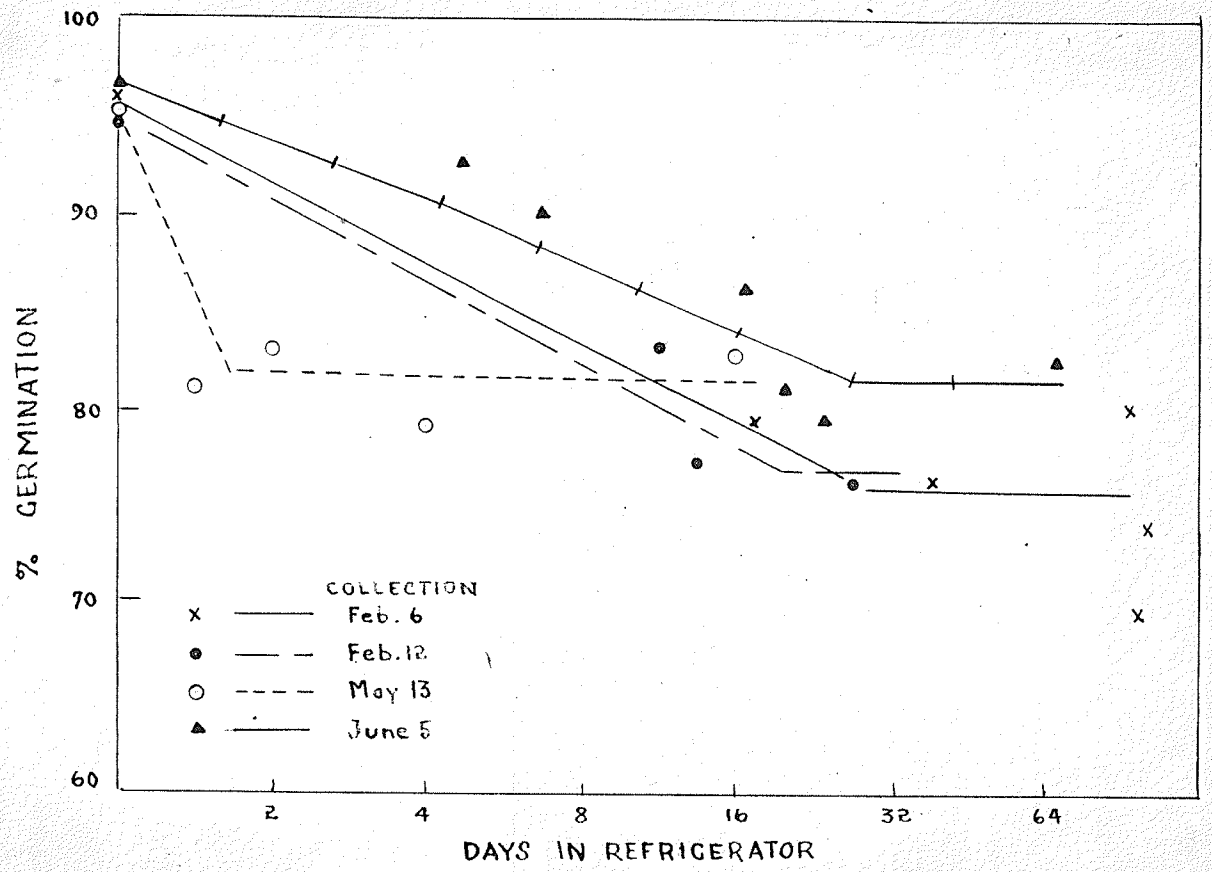


Fig. 6. Percentage germination of individual spore collections with storage time in the refrigerator.

samples were withdrawn from the same spore collection (Figure 6), it was found that after an initial rapid reduction, the rate of which seemed to vary considerably between collections, a more or less constant level was reached and maintained over a period of months.

These observations seemed to indicate that some casual factor, or factors, apart from aging processes alone, was responsible for the reduction in germination, while the data from Experiment I would suggest that this source of variability could be connected with storage in the refrigerator.

The following experiment was designed to compare specifically the effect of storage under room conditions and in the refrigerator, as well as combinations of the two.

Fresh spores were mixed thoroughly, and approximately equal quantities were dusted evenly over the bottoms of 50 ml glass containers, which were covered with watch-glasses.

The following treatments, with five replicates for each, were carried out:

A. stored in the refrigerator for 2 days.

B. stored in the refrigerator for 1 day, then exposed to room temperature for 1 day.

C. stored at room temperature for 2 days.

D. stored at room temperature for 1 day, then placed in the refrigerator for 1 day

Those kept under room conditions were stored in a dark cupboard, with a temperature of approximately 23° C.

TABLE I

THE EFFECT OF STORAGE CONDITIONS ON PERCENTAGE GERMINATION

Replicate	Treatment			
	A	B	C	D
1	87	88	90	93
2	84	83	90	86
3	90	86	85	91
4	94	90	93	89
5	90	91	91	84
Mean Percentage	89.0 ± 3.3	87.6 ± 2.8	89.8 ± 2.6	88.6 ± 3.2
No. spores counted	754	728	737	735

The percentage germination of the spores after collection, and before storage, was 96.7 ± 2.2.

Contrary to expectation, there did not appear to be any significant difference in percentage germination between any of the four treatments. All four treatments, however, gave significantly lower results than did freshly collected spores.

In Experiment I, it was found that a reduction in germination resulted with spores kept in the refrigerator; in

this experiment, there appeared to be no difference between room and refrigerator storage, although both yielded a lower percentage germination than that of fresh spores.

It was concluded that some factor (or factors), which was associated with storage in the refrigerator most of the time, but only casually connected with room conditions, was tending to reduce the germinability of the spores.

Experiment III. The Effect of Light, During Storage, Upon Subsequent Germination

In examining the previous two experiments for any discrepancies in conditions that might account for the reduction in percentage germination with room storage in Experiment II, but not in Experiment I, it will be noted that only in the former were the containers kept in the dark.

The effect of light on dry spores is generally considered to be injurious, the effect apparently being a function of the light intensity and wave-length, duration of exposure, type of spore pigmentation, and of the species under study (7, 8, 12, 19, 20, 38, 54).

The action of light, however, does not always appear to follow a simple relationship.

Bolley (7) found that uredospores exposed in test-tubes to direct sunlight for one month showed only fifty per cent germination, while if the test-tubes were kept in the shade for the same length of time, they retained their viability. In a later paper, however, he reported that spores exposed to direct sunlight for twelve days gave eighty to one hundred per cent germination (8).

Cochrane (12), working with Phragmidium mucronatum, obtained a marked reduction in germination after only three hours of light exposure (ca. 1000 f.c.), but no significant reduction in germination after short exposure periods when the spores were kept in thin-walled glass phials.

Hwang (38) noted that uredospores appeared to be more resistant to light after storage (although the results were not consistent), and that samples covered with cellophane were more resistant than uncovered samples to the direct action of light. A similar observation has been made in this laboratory; it was found that when fresh spores were exposed to fluoures-cent light (ca. 1000 f.c.) in open containers for two days, the subsequent percentage germination was only about ten per cent; on the other hand, when the spores were kept in covered glass containers, no significant reduction in germination, as

compared with that of fresh spores, was apparent. The above results would suggest that the physiological condition of the spores, and perhaps also, the ultra-violet light intensity governs the reaction of the spores to light.

The ensuing experiment was carried out to determine whether the exclusion of light, which was the case with refrigerator storage and under the room conditions in Experiment II, may also be responsible for a reduction in germination.

Similar samples of fresh spores were dusted evenly over the bottoms of ten 50 mm petri dishes. The lids were replaced, and five petri dishes were wrapped in aluminum foil, and five were left unwrapped.

The dishes were placed on a laboratory bench, under a bank of fluourescent lights (ca. 1000 f.c.), for two days. At the end of this time, the contents of the dishes were mixed with a camel's hair brush, and samples were germinated on plain agar at room temperature (22.5° C).

TABLE II

Replicate No.	Dark (wrapped)	Light (unwrapped)
1	93	95
2	86	97
3	95	96
4	85	94
5	90	92
Mean Percentage	89.8	94.8
Total No. Spores Counted	1036	995

Using the "t" test for the comparison of unpaired differences (Fisher 1938), a "t" value of 2.33 was obtained, which with eight degrees of freedom, was just significant at the five per cent level. It was concluded from this that the reduction in germination often observed during storage was probably associated, directly or indirectly, with absence of light.

The apparent discrepancies in the literature concerning the effect of light may perhaps be accounted for by attendant variations in other factors, such as the amount of air circulation about the sample, and by the fact that the effect of darkness, as well as the effect of light, must be considered.

THE PROBLEM OF VARIABILITY. B. THE EFFECT OF GERMINATION CONDITIONS

In the previous section, the suggestion was made that a large part of the experimental error occurred during the storage of the spores, when standardized conditions were employed for germination.

The remaining portion of Part I deals with what was subsequently found to be the most important source of variability in the controls when standardized germination conditions were not employed.

Experiment IV. The Effect of The Initial Agar Temperature on Percentage Germination

The optimum temperature for the germination of uredospores of most races of wheat stemrust generally lies within the range from ten to twenty-five degrees Centigrade (40, 55). It has been noted here that Race 56 uredospores will not germinate at 29° C., although they can withstand this treatment for at least eight hours, with a high subsequent percentage germination at lower temperatures.

Instances have been recorded where brief exposures to extra-optimal temperatures, followed by more normal conditions,

exert a decided effect upon the course of germination.

Duggar (22) and Eriksson and Henning (25), working with aecidiospores of Puccinia graminis, found that the highest percentage germination was obtained by placing the spores for a time on melting ice, then sowing them on water. The next best results were obtained when the spores were soaked for three hours at 3° C. The poorest results were secured when the spores were sown directly at room temperature. Similar results were found with uredospores. In view of the results obtained by Allen (2), it would appear likely that the temperature effect was confounded with that of "presoaking", which has been shown to largely overcome a source of inhibition.

Dickinson (16) subjected germinating uredospores and Erysiphe graminis conidia to rising and falling temperatures; no significant difference in percentage germination was observed when the spores were placed in a refrigerator, the mechanism of which was then turned off, and the temperature was allowed to rise from 6° C to room temperature overnight. When the reverse process was executed (i.e., from room temperature to 6° C), the spores failed to germinate.

Raeder and Bever (49) found that by transferring uredospores of Puccinia glumarum, P. graminis, and P. triticina after all germination had ceased at 29° C. to 9 or 10° C. for forty-eight hours resulted in renewed germination. Similarly, the transferring of spores from freezing-point to room temperature resulted in renewed germination.

Dodge (21) and Shear and Dodge (53) were successful in breaking the dormancy of Ascobolus and Monilia spores by subjecting the inoculated media to temperatures of over 75° C. for brief periods.

Goddard (31) was able to stimulate germination of ascospores of Neurospora sitophila by a similar method; this worker found, moreover, that the effect of heat activation could be reversed by placing the spores under anaerobic conditions or in the presence of cyanide, and that reactivation occurred after the spores were exposed once more to high temperatures.

Curran and Evans (13) found that a temperature of 95° C. for ten minutes resulted in the activation of the spores of a number of thermo-tolerant bacteria.

Cochrane (11) obtained an increase in germination for uredospores of Phragmidium mucronatum when the inoculated

material was kept at 27° C. for ten minutes. The maximum increase occurred within eight hours of sowing; after twenty-four hours, the temperature treatment failed to stimulate the process.

Agar plates were left for three hours after preparation at the following temperatures: 5°, 20°, and 29° C. Each plate was then inoculated quickly with three and one-quarter month old spores, and placed at 20° to germinate. Old spores were used so that any increase in percentage would be recorded; with fresh spores, the percentage of germinated spores already approaches its top limit.

TABLE III

THE EFFECT OF INITIAL AGAR TEMPERATURE ON FINAL PERCENTAGE GERMINATION

Replicate No.	Initial Temperature		
	5°	20°	29°
1	52	73	86
2	49	68	89
3	67	68	88
4	42	70	82
Mean %	52.5 ± 9.1	69.7 ± 2.0	86.25 ± 2.6
Total No. Spores Counted	587	604	702

To summarize, evidence was presented for the following:

1. Placing uredospores on chilled agar (5° C.), and allowing the temperature to rise to 20° C., resulted in a significant decrease in percentage germination, as compared to those maintained in 20° throughout the experiment.

2. Placing uredospores on warm agar (29° C.) and allowing the temperature to drop to 20°, produced a significant increase in percentage germination.

The above results appear to conflict with the findings of Eriksson and Henning (25) and Duggar (22), who found that pre-chilling increased germination. The greater variability associated with the pre-chilling treatment would suggest that other uncontrolled factors exert a more critical influence at this temperature. The adverse effect of chilling, incidentally, has been obtained with spores germinated on distilled water as well as on agar, and with spores subjected to different treatments.

A first approximation of the time required for the temperature of the contents of the petri dish to come to equilibrium with the temperature of the growth-chamber (20° C.) would be around twenty minutes, suggesting that the residual

effect of the initial temperature involved either a physical phenomenom, or a process of an enzymatic nature. In either event, the initial treatment had important consequences on the subsequent behaviour of the spore, and constituted an easily overlooked source of error.

DISCUSSION AND CONCLUSIONS

The activity of massive samples of spores during storage can perhaps be divided into two phases: an initial rapid reduction in germinability, usually occurring within a few days, and a more or less constant level, lasting for periods of up to at least six months, in which seventy or eighty per cent of the spores retain the ability to germinate under standard conditions.

The lack of correlation between length of storage time and percentage germination recorded in Figure 5 was probably due to the marked differences between individual collections, and to the sensitivity of the spores, before germination, to environmental disturbances occasioned by removing samples, exposure to room conditions for varying lengths of time, etc. When the collections were kept in the refrigerator continually, and the contents disturbed as little as possible, a more consistent trend was seen (Fig. 6).

While factors such as the depth of the inoculum and the amount of air circulation about the sample probably accounts for the observed differences in reduction in germination of various collections stored in the refrigerator, there was no direct evidence for these assumptions. There was evidence, however, that storing samples in closed containers in the absence of light resulted in decreased germinability, as compared with those exposed to a light intensity of around 1000 foot-candles.

It will be observed that the spores are not necessarily killed by storage treatment, but only inhibited from germinating, since this effect can be largely overcome by a brief exposure to temperatures of around 29° C.

Allen (2) noted that old spore collections germinated with difficulty, and with variable results, but that if measures were taken to remove the inhibitor (e.g., by "pre-floating"), a high percentage germination was reached. It seems likely that at least some of the phenomena noted above can also be attributed to the activity of a self-inhibitor or its precursors.

The immediate aim of obtaining reasonably uniform spore samples was achieved by using spores not more than one week old, which had been left in containers covered with a watch-glass at room temperatures, in the presence of light. The theoretical aspects of the problem, however, remain obscure.

PART II

THE EFFECT OF CARBON DIOXIDE CONCENTRATION
A. LOW CONCENTRATIONS

An external supply of carbon dioxide appears to be required by a number of microorganisms. Probably the first demonstration of the stimulatory action of carbon dioxide on the growth of heterotrophic organisms was that of Bang (4) and Nowak (46) for Brucella abortus. By 1927 it was known that the spore germination of Basisporium gallarum (24), Saccharomyces (51), Aspergillus (50), and the growth of a large number of bacteria (51, 59), were stimulated by the presence of carbon dioxide.

Rockwell and Highberger (51) found that the inhibition of growth brought about through the lowering of the carbon dioxide tension was greater in acid media, and that with increased amounts of inoculum, low carbon dioxide tensions were not as effective in reducing growth.

Golding (34), for Penicillium roqueforti, observed that the stimulation in growth brought about by the addition of small amounts of carbon dioxide took place sooner at the lower temperatures.

Steinberg (54) revealed an interaction between certain trace elements and the carbon dioxide supply on the growth of Aspergillus niger; he found that the effect of removal of carbon dioxide was greater, in terms of decreased yields, when the following elements were lacking in the nutrient media: gallium, zinc, copper, molybdenum and iron.

For a review of some of the known paths of carbon dioxide fixation, see Krebs (42) and the S.E.B. Number V (56).

Allen (2), working with Race 56 uredospores of wheat stem-rust, found a marked increase in percentage germination in the presence of inhibitor when carbon dioxide was added to the atmosphere; however, at very low inhibitor concentrations, the rate of germination appeared to be stimulated by the removal of carbon dioxide from the air-stream.

Experiment V. The Effect of Low Carbon Dioxide Levels on Percentage Germination

Early experiments on Race 56 uredospores, with the object of testing low oxygen concentrations, tended to be very erratic when the gas mixture was made up with tank oxygen and nitrogen and nitrogen, but less so when the same concentrations were obtained by diluting laboratory air with nitrogen. It was

decided that this effect might be due to a difference in carbon dioxide levels.

A mixture of nine- to seventeen-day old spores were mixed thoroughly, shaken onto plain agar, and exposed to different carbon dioxide concentrations, as described in METHODS AND MATERIALS.

The desiccators were flushed with tank nitrogen for five minutes, evacuated and filled with the gas mixture twice, then placed in the growth chamber. The oxygen concentration for all treatments was 20%. The "0" per cent carbon dioxide treatment refers to the desiccators containing 20 ml of 10% potassium hydroxide.

TABLE IV

THE EFFECT OF LOW CARBON DIOXIDE CONCENTRATIONS ON PERCENTAGE GERMINATION

% CO_2	% Germination	
	Trial I	Trial II
"0"	61	74
0.003	70	79
0.006	77	81
0.012	91	85
0.025	89	82
0.25	89	80
1.00	87	84

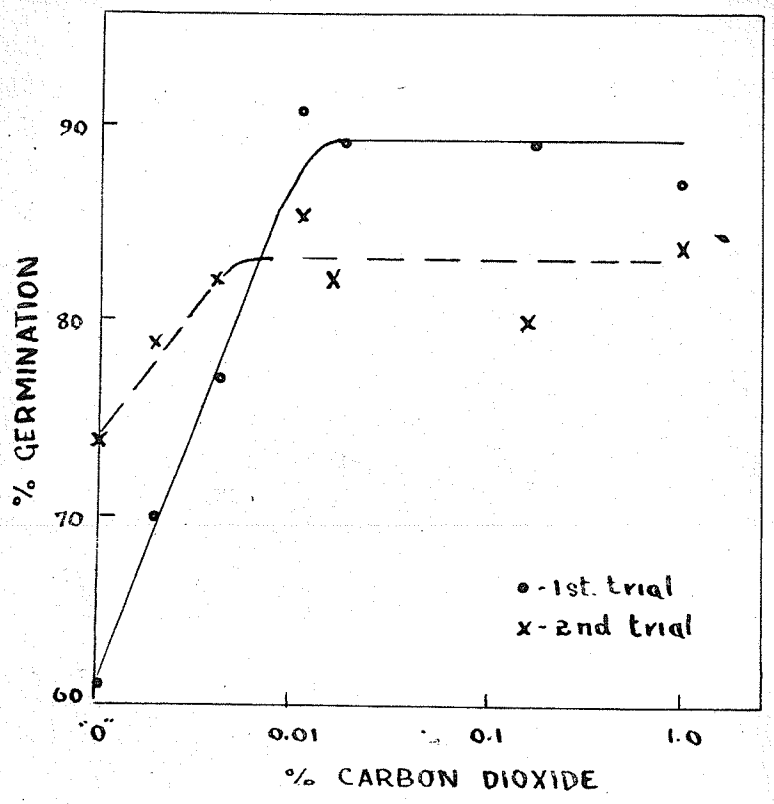


Fig.7. The relationship between percentage germination and low CO_2 concentrations

The presence of burst tips* was much more noticeable at "0" and 0.003% carbon dioxide than at the higher levels. The following are rough estimates of the proportion of spores with "bursts":

"0"% CO_2	50%+
0.003	15%
0.025	less than 1%
↓	
1.00	" " 1%

From an inspection of Table IV and Figure 7, there appeared to be an approximately linear relationship with percentage germination from "0" to 0.012% carbon dioxide and a levelling-off in percentage from 0.012 to 1.00% carbon dioxide.

Experiment VI. The Effect of Density of Inoculum On Percentage Germination at Low Carbon Dioxide Concentrations

Uredospores evolve carbon dioxide during normal germination procedures; hence, as shown by Rockwell and Highberger (51), a possible source of variation when carbon dioxide becomes

* For an example of this effect, see Plate III, Fig. 2.

limiting for normal germination could be due to the effect of spore density.

Varying amounts of fresh spores were shaken onto agar, and placed in individual 600 ml desiccators, over 20% potassium hydroxide. The desiccators were put under a bank of fluoures-cent lights (ca. 1000 foot-candles) at a temperature of approxi-mately 22° C. As controls, the spores were treated in similar fashion, except that they were not placed in desiccators.

TABLE V

THE RELATIONSHIP BETWEEN PERCENTAGE GERMINATION AND SPORE DENSITY OVER KOH

Spore Density	% Germination	
	Over KOH	Controls
2.4	36	
3.4	56	
3.7		94
4.0	54	
5.0	67	
6.0		96
7.0	72	95
8.0	79	
8.4	82	
9.5		92
11.5	93	
13.7	91	
18.2		93

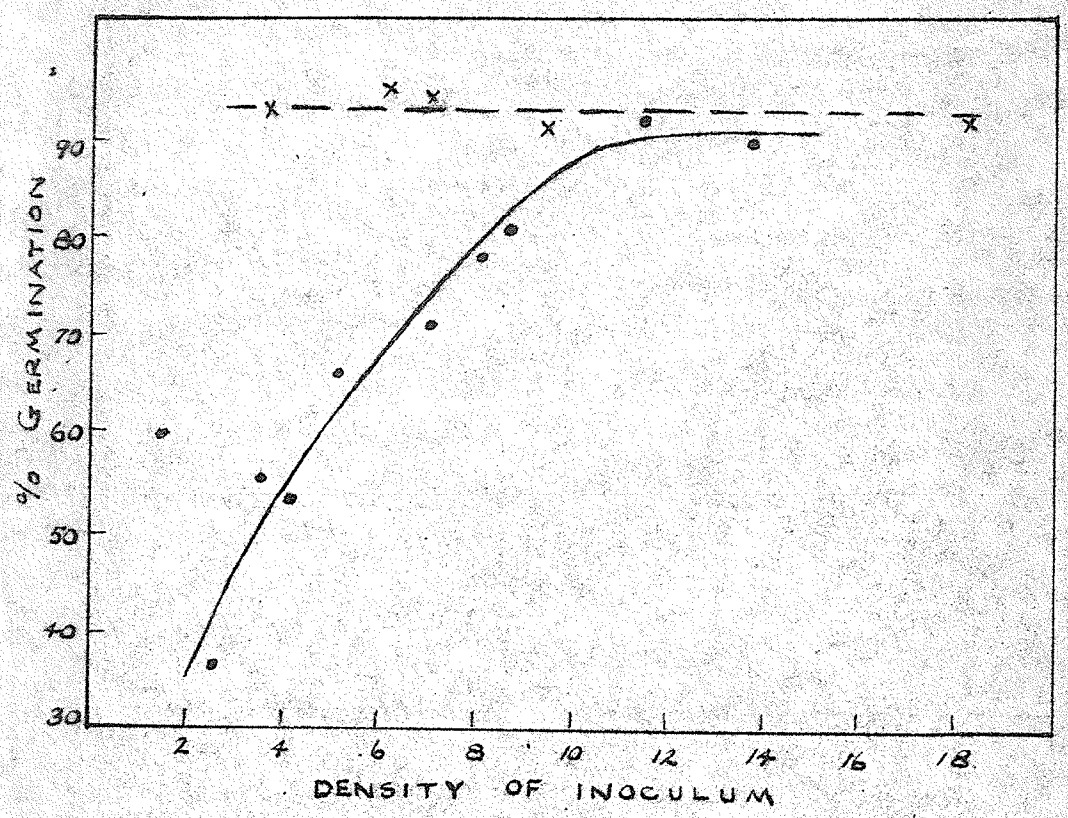


Fig. 8. The relationship between spore density and germination over KOH.

In appearance, the spores exhibited a graded series from extreme effects at low spore-densities to no observable differences from the controls at the higher densities. At a density of 2.4,* the tubes were short and distorted, with frequent swellings and burst tips, as in Plate III, Figs. 1 and 2. The criterion used to distinguish "germinated" from "ungerminated" spores was that the length of the germ tube had to be greater than the longer dimension of the spores; since at the lower densities, many of the germ tubes were extremely short, classification was often difficult.

It was concluded that carbon dioxide is probably evolved by the germinating spores,** even when the atmospheric supply is low, and that in any assessment of the effects of low carbon dioxide concentration, the factor of spore density must be taken into consideration.

* The figures represent the average number of spores per microscope field (approximately 1 sq. mm).

** Compare Fig. 8 and the response to low carbon dioxide concentrations, Fig. 7.

Experiment VII. The Effect of Light on The Reaction Of Spores to Low Carbon Dioxide Concentrations

Although a large part of the experimental variation could be attributed to the amount of inoculum per petri dish, large errors in percentage germination over potassium hydroxide also appeared when the spores were germinated under room conditions, as compared to germination in the growth-chamber.

Since experiments had been performed at different times of the day, it appeared probable that light intensity was another factor that had to be considered.

Germinating spores are more susceptible to the action of light than dry spores, according to Dillon Weston (19).

Stock (55) reported no effect of strong light on the germination of uredospores of Puccinia triticina, P. dispersa, and P. coronata, except a slight delay in the last two species in the time of movement of the protoplast into the germ tube. In the case of Puccinia graminis, germination was somewhat delayed by strong light, but it eventually attained as high a level in the light as in the dark. Hart (37) noted no difference between the germination of uredospores of Melampsora lini in the light and in the dark.

The experimental design consisted of a 2 x 2 factorial, with one large desiccator for each treatment combination:

a. exposed to light (L), at atmospheric CO_2 (+)

b. " " " (L), over KOH (-)

c. kept in the dark (D) at atmospheric CO_2 (+)

d. " " " " (D) over KOH (-)

The desiccators for the dark treatments were wrapped in aluminum foil, and for the low carbon dioxide treatments, a shallow layer of 10% KOH was kept in the bottom of the desiccators.

TABLE VI

THE EFFECT OF LIGHT AND DARKNESS ON PERCENTAGE GERMINATION AT TWO LEVELS OF CARBON DIOXIDE

Replicate No.	D		L	
	+	-	+	-
1	93	90	94	82
2	94	96	97	88
3	97	93	93	86
4	95	94	95	74
5	94	91	93	98
6	94	95	96	88
7	97	91	96	84
8	97	93	94	91
Mean %	95.1 ± 1.5	92.9 ± 1.9	94.75 ± 1.4	86.4 ± 6.5

The effect of a low carbon dioxide tension on the growth habit in the presence of light was much more marked than it would appear from an inspection of Table VI. Even the replicates with about ninety per cent germination exhibited stunted and distorted germ tubes with abundant swellings and burst tips.

The remaining three combinations, + and - carbon dioxide in the dark, and + carbon dioxide in light were morphologically indistinguishable from each other; they all exhibited long germ tubes of an even diameter, with branched and unbranched forms, and some that appeared stretched across the surface of the agar.

From the above information, it would appear that an external source of carbon dioxide is required in the presence of light for normal germination, but not in the absence of light.

In the dark, lowering of the carbon dioxide tension by the presence of 10% KOH seemed to be of little consequence--morphologically, or on a percentage basis.

In the presence of carbon dioxide, there was no significant difference in percentage germination between spores germinated in the light, and in the dark.

As far as can be ascertained from the published information on other heterotrophic organisms, uredospores are unique with respect to this light intensity:low carbon dioxide interaction.

Experiment VIII. The Effect of "Inhibitor" on Percentage Germination at Two Levels of Carbon Dioxide

Dickson (18) found that a number of phenomena associated with the germination of Puccinia graminis-tritici uredospores could be explained on the basis of the evolution of a natural inhibitor.

Forsyth (28) observed that the uredospores of Race 15B and unidentified field collections of P. graminis-tritici, as well as those of P. coronata, germinated poorly in closed Warburg flasks, as compared with germination in open containers. This inhibition could be overcome by increasing the amount of water in the flasks, and by the addition of the following substances to the centre-well: 20% $AgNO_3$, 1.4% acetone, or 0.06M ammonia.

Since the absorption spectrum of the absorbed inhibitor closely approximated that of trimethylethylene, and this substance possessed physiological properties similar to those of

the natural inhibitor, Forsyth concluded that the two were probably identical. The effect of the inhibitor appears to be that of preventing germination.

Allen (2), working with the same race of rust as used in this laboratory, found that the effect of the inhibitor could be largely overcome by the addition of carbon dioxide to the germination vessels; the effective concentration varied with the spore collection.

To determine whether the same effect could be obtained with a different culture of the same race of rust, an experiment similar to Allen's was carried out.

One-tenth of a gram of "old" spores were dusted onto shallow petri dishes containing 25 ml of distilled water, and placed in each of the 600 ml desiccators labelled "I", for the production of inhibitor. The spores were left for four hours in a dark cupboard, after which time, the petri dishes were quickly removed, so that the atmosphere within the desiccator was disturbed as little as possible. Fresh spores were used each day of the actual experiment, and germination was carried out at a temperature of 24-25° C., under a bank of fluourescent lights.

There were two levels of carbon dioxide---atmospheric (+), and over 20% KOH (-), and two levels of inhibitor---I and I_o;

I_o signifies that no inhibitor was present, except that produced by the fresh spores themselves.

TABLE VII

THE EFFECT OF "INHIBITOR" AT TWO LEVELS OF CARBON DIOXIDE ON PERCENTAGE GERMINATION

Series	I		I_o	
	+	-	+	-
1st Trial	92 93	11 31	97 94	72 80
2nd Trial	92 89	5 5	91 95	59 64
Mean %	91.5 ± 1.5	13.0 ± 10.7	94.25 ± 2.1	68.7 ± 7.9

The symptoms of carbon dioxide deficiency were typical in the case of the spores germinated over KOH, in the absence of added inhibitor. When inhibitor was added, and the carbon dioxide tension lowered, the effects were even more drastic; spores with no visible germ tubes had burst, releasing their contents to the surrounding medium, while the germ tubes that had developed were exceedingly short and distorted.

When atmospheric carbon dioxide was available, there were no visually discernible differences between the treatments with or without added inhibitor.

Evidence was presented for the following:

a. Desiccators in which large quantities of old spores had been germinated exerted a residual effect on subsequent spore germination, tending to reduce the percentage germination.

b. The residual effect was greater when the carbon dioxide tension was lowered by the presence of KOH.

It appeared likely that the residual effects noted above were due to the activity of a volatile product of spore germination, a self-inhibitor, as reported by Allen (2).

THE EFFECT OF CARBON DIOXIDE
B. HIGH CONCENTRATIONS

The retarding effect of high carbon dioxide concentrations varies markedly with the species and the experimental conditions.

Fränkel (29), as quoted by Brown (9), found that certain species of the true beer yeasts could grow as well in an atmosphere of one-hundred per cent carbon dioxide as in air, although the majority of fungi were inhibited by such treatment.

Adams and Miller (1), on the other hand, noted that carbon dioxide concentrations greater than four per cent depressed ascospore formation in Saccharomyces cerevisiae.

Golding (34, 35) obtained growth in cultures of Penicillium roqueforti, Aspergillus niger, P. expansum, A. flavus, and Oospora lactis upon exposure to seventy per cent carbon dioxide, although the amount of growth was significantly reduced. The inhibiting effect on growth appeared to be proportional to the solubility of the carbon dioxide in the mycelium or medium. In support of this is the observation that carbon dioxide becomes more effective in retarding growth with decrease in temperature.

Lopriore (43) noted a decrease in the rate of germination of Mucor mucedo spores at ten per cent carbon dioxide.

Brown (9), on studying the effect of carbon dioxide on germination in a number of fruit-rot organisms,* found that spores sown in water were inhibited at concentrations of twenty to thirty per cent, although the concentration producing the same effect was increased two- or threefold when the spores were sown in nutrient media. Penicillium glaucum and Fusarium sp. were particularly resistant to carbon dioxide.

* Aspergillus repens, Mucor sp. Botrytis cinerea, Monilia cinerea, Phoma roseola, Alternaria grossulariae, Rhizopus nigricans, B. parasitica, Sphaeropsis malorum, Fusarium sp. P. glaucum.

Gassner and Straib (30) noted no difference in percentage germination of cereal rust uredospores in atmospheres containing up to 7.5% carbon dioxide; Stock (55) obtained full germination in the presence of 20% carbon dioxide, although the rate of germination, compared with that in air, was reduced with each increase in carbon dioxide concentration.

When germination and growth are inhibited by carbon dioxide, however, the material is not necessarily killed by this treatment; see Brown (9), Golding (34), and Lopriore (43). In this laboratory it was observed that uredospores sown on 0.2M sodium bicarbonate did not germinate within twenty-four hours, although a high percentage germination was attained when they were subsequently transferred to a large volume of distilled water.

In the opinion of Thornton (57), as exemplified by experiments on Sclerotinia fructicola, the inhibiting effect of carbon dioxide is due to an increase in the alkalinity of the living fungal hyphae, perhaps due to the formation of bicarbonates.

Experiment IX. The Effect of High Carbon Dioxide Concentrations on Percentage Germination

The gas mixtures were prepared as described in METHODS AND MATERIALS. Fresh spores, which had been stored in the refrigerator for ten hours, were used. The desiccators, each containing an inoculated petri dish, were evacuated once, filled with the gas mixture, and placed on the laboratory bench at approximately 22° C. The oxygen level in all cases was twenty per cent.

TABLE VIII

THE EFFECT OF HIGH CARBON DIOXIDE CONCENTRATIONS ON PERCENTAGE GERMINATION

% CO_2	% Germination 1	% Germination 2	Appearance[2]
.03[1]	96	92	stretching
	93	87	"
5.5	87	92	"
15.0	80	---	gnarling and staghorns
17.0	78	---	gnarling
20.0	67	79	"
21.0	73	77	germ tubes short and gnarled
25.5	52	48	germ tubes short
27.7	---	0	
30.0	0		

1/ controls--evacuated once, and released to air.
2/ terminology is primarily that of Dickinson (17).

The drop in percentage germination with higher carbon dioxide concentrations was quite pronounced, producing complete inhibition at 28 per cent carbon dioxide.

The branching habit also seemed to be influenced by the carbon dioxide level; the appearance of "stretched" germ tubes (see Plate 1, fig. 2) was observed only in the controls and at 5.5% carbon dioxide---at higher concentrations, the branches were further divided, but shorter in length, giving the "staghorn" appearance (15% CO_2), and finally, only rudimentary knobs were observed ("gnarling").

NOTE: While germination was inhibited at carbon dioxide concentrations higher than 27.5%, the spores were not necessarily killed by this treatment. It was subsequently found that while ten-day old spores did not germinate at 28% carbon dioxide, they could be exposed to this concentration for at least three days and still germinate after air was flushed through the desiccators. The final percentage germination was about eighty per cent, and the germ tubes were long and abundantly branched.

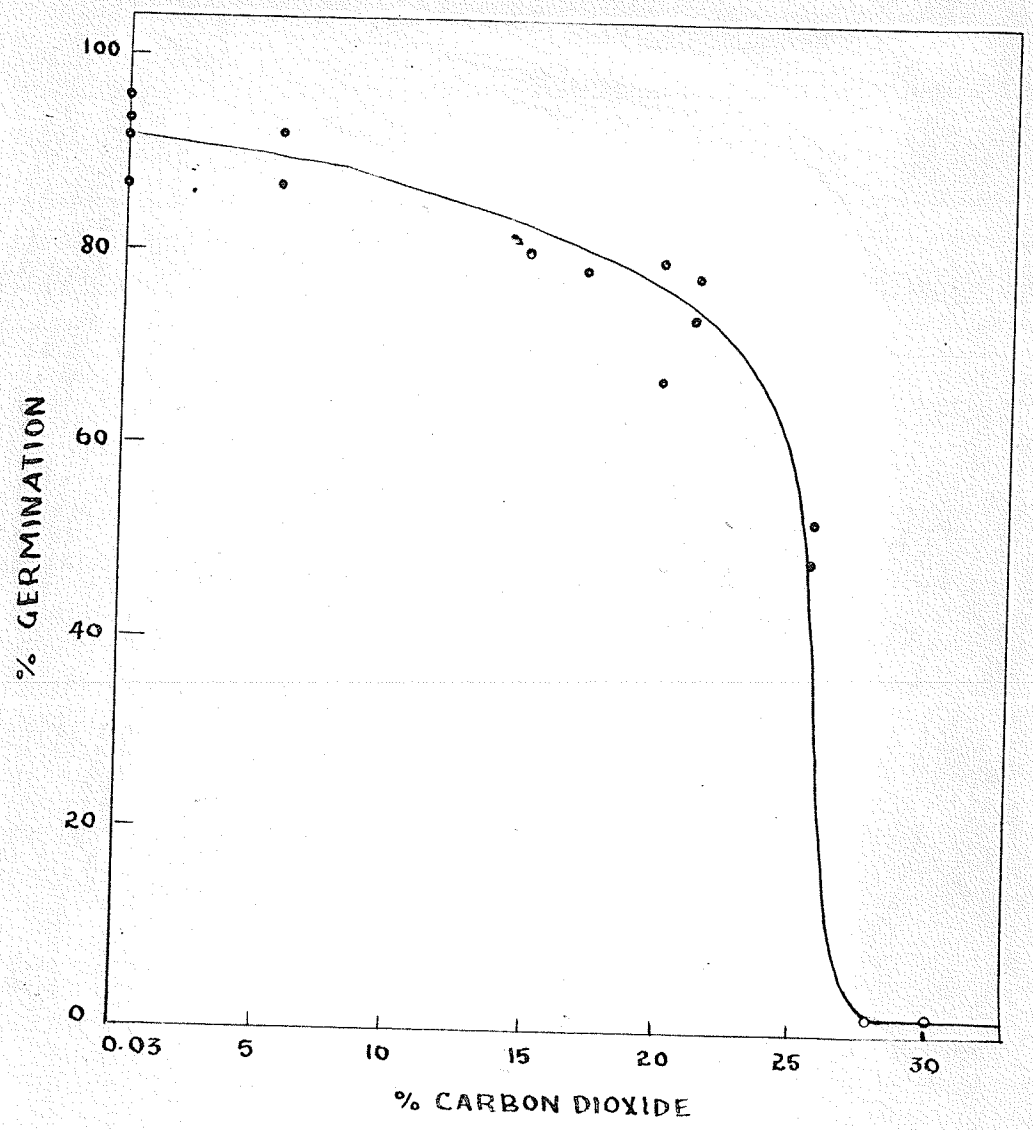


Fig. 9. The effect of high carbon dioxide concentrations on percentage germination.

PART III

THE EFFECT OF OXYGEN

The majority of fungi appear to be insensitive to variations in oxygen pressure, within wide limits.

Porodko (48), in an extensive survey of the oxygen requirements of a number of fungi, such as Phycomyces nitens, Aspergillus niger, Penicillium glaucum, and Mucor stolonifer (as well as a number of bacteria), found that the minimum oxygen concentration required was often below one per cent.

Similarly, Brown (9) found that a suspension of Botrytis spores was not inhibited from germinating until the oxygen concentration of the surrounding atmosphere was reduced to about one per cent.

Denny (15), investigating the oxygen requirements of Neurospora sitophila, obtained good mycelial growth at 0.3% oxygen, although only a trace of growth occurred at 0.01%; the formation of perithecia, however, was inhibited below 0.5% oxygen.

Stock (55) obtained equally good germination in "pure" oxygen, nitrogen plus 2% oxygen, and air, for the uredospores of several cereal rusts. In nitrogen with only a trace of

oxygen, germination in Puccinia triticina was reduced from 25% (in air) to 8%.

The last observation was repeated in this laboratory, with Puccinia graminis-tritici; it was found that when flushed periodically with tank nitrogen (ca. 0.25% O_2), uredospores commenced to germinate after approximately forty-eight hours, attaining a high final percentage.

Allen (2) recorded a sharp increase in rate of germination from "0" to approximately 5% oxygen, for pre-floated uredospores, with a levelling-off in rate of germination from 5% to 20% oxygen. For untreated uredospores, there appeared to be a well-defined optimum at from 4% to 8% oxygen.

Scheffer and Livingston (52) found that the minimum oxygen pressure for the growth of Polystictus versicolor was approximately 1.5 mm; they found, moreover, that this level was not influenced to any great extent from 17.5° to 33.5° C. The general rule, however, appears to be that the minimum required oxygen concentration rises with increase in temperature (see, for example, Golding (32, 34)).

Many workers found no increase in germination at oxygen concentrations exceeding that of air (52, 55), while in some cases, high oxygen concentrations were inhibitory (9, 41).

It is believed, however, that none of the filamentous fungi can exist anaerobically.

Experiment X. The Effect of High Oxygen Concentration On Percentage Germination at Two Levels of Carbon Dioxide

Samples of a five- to seven-day old mixture of spores from the refrigerator were exposed to two levels of oxygen (95% and 20%), with and without added carbon dioxide (3% and "0"). The desiccators with no added carbon dioxide contained 15 ml of 20% KOH in the bottom well; those with added carbon dioxide contained an equal amount of 20% $CuSO_4$ to compensate for humidity changes brought about by the KOH*. A total of eight desiccators, with two petri dishes in each, were employed, providing two desiccators for each treatment combination. Germination was carried out in the growth-chamber.

* A later experiment revealed no significant difference in percentage germination due to variations in relative humidity from 30% to 99% (approx.).

TABLE IX

PERCENTAGE GERMINATION AT NINETY-FIVE PER CENT OXYGEN, AND THE EFFECT OF CARBON DIOXIDE

	20% O_2		95% O_2	
	3% CO_2	"0"	3% CO_2	"0"
	91) 83)	48) 61)	87) 94)	57) 55)
	86) 76)	53) 51)	88) 80)	55) 53)
Mean	84.0 ± 5.4	53.25 ± 4.8	87.25 ± 4.9	55.0 ± 1.4

The figures enclosed in brackets represent the two petri dishes from the same desiccator.

As in the previous experiments, germinating spores over potassium hydroxide resulted in deleterious effects---morphologically, and on a percentage basis.

The effect of low carbon dioxide tensions did not appear to be modified by increasing the oxygen concentration from twenty per cent to ninety-five per cent.

It was concluded that oxygen concentrations above that of air had little effect on percentage germination, and hence, that high oxygen concentrations could be neglected in further studies.

Experiment XI. The Effect of Four Levels of Oxygen, In Combination with Four Levels of Carbon Dioxide, on Percentage Germination

While information concerning the effects of oxygen and carbon dioxide on spore germination is fairly well represented in the literature, there are no indications of the interrelationship of these two factors. The following experiment was carried out in the hope that this short-coming would be at least partly met, for Puccinia graminis-tritici.

One-week old spores, which had been kept at room temperature, were exposed to various concentrations of oxygen and carbon dioxide, in every combination of the two:

% carbon dioxide---

C_1	C_2	C_3	C_4
"0"	0.03	5.0	10.0

% oxygen---

O_1	O_2	O_3	O_4
0.5	1.0	5.0	20.0

There were four replicates for every combination, giving $4 \times 4 \times 4 = 64$ single determinations (one petri dish per desiccator).

The desiccators were evacuated and filled with the prepared gas mixture twice, and germination was carried out at

room temperature (21-23° C.) under a bank of fluourescent lights for a period of eight hours (± 5 mins.).

At the end of the germination period, the spores were "fixed" for approximately 15 mins. by placing the petri dishes in a container over glacial acetic acid.

The mean percentage germination for each treatment combination is recorded in Table X. For the statistical treatment, see Appendix II.

TABLE X

MEAN PERCENTAGE GERMINATION, AFTER EIGHT HOURS, AT FOUR LEVELS OF CARBON DIOXIDE AND FOUR LEVELS OF OXYGEN

O/C	"0"	0.03	5.0	10.0	Mean
0.5	52.5	84.5	84.5	85.0	76.6
1.0	80.2	89.5	91.0	90.7	87.9
5.0	90.5	94.5	93.5	90.0	92.1
20.0	89.0	96.7	95.0	91.0	92.9
Mean	78.1	91.3	91.0	89.2	

Minimum significant difference for treatment means = 5.45.

Minimum significant difference for column and row means = 2.66.

TABLE XI

THE EFFECT OF CARBON DIOXIDE AND OXYGEN COMBINATIONS ON THE APPEARANCE OF THE GERM TUBES

O^{C}	"0"	0.03	5.0	10.0
0.5	Spherical swellings Swollen tips Tubes short Bursts	Spherical swellings Swelled tips Bursts Gnarling	Gnarling	Spherical swellings along tube Gnarling and branching
1.0	Tubes fairly long; some bursts Gnarling	Gnarling and branching Some bursts	Gnarling and stretching	Staghorns
5.0	Some bursts Gnarling	Gnarling and branching	Free branching Staghorns Some with secondary tubes	Staghorns and gnarling
20.0	Gnarling and staghorns Some bursts	Excessively branched Some with short secondary tubes	Staghorns Gnarling Some with secondary tubes	Staghorns Gnarling

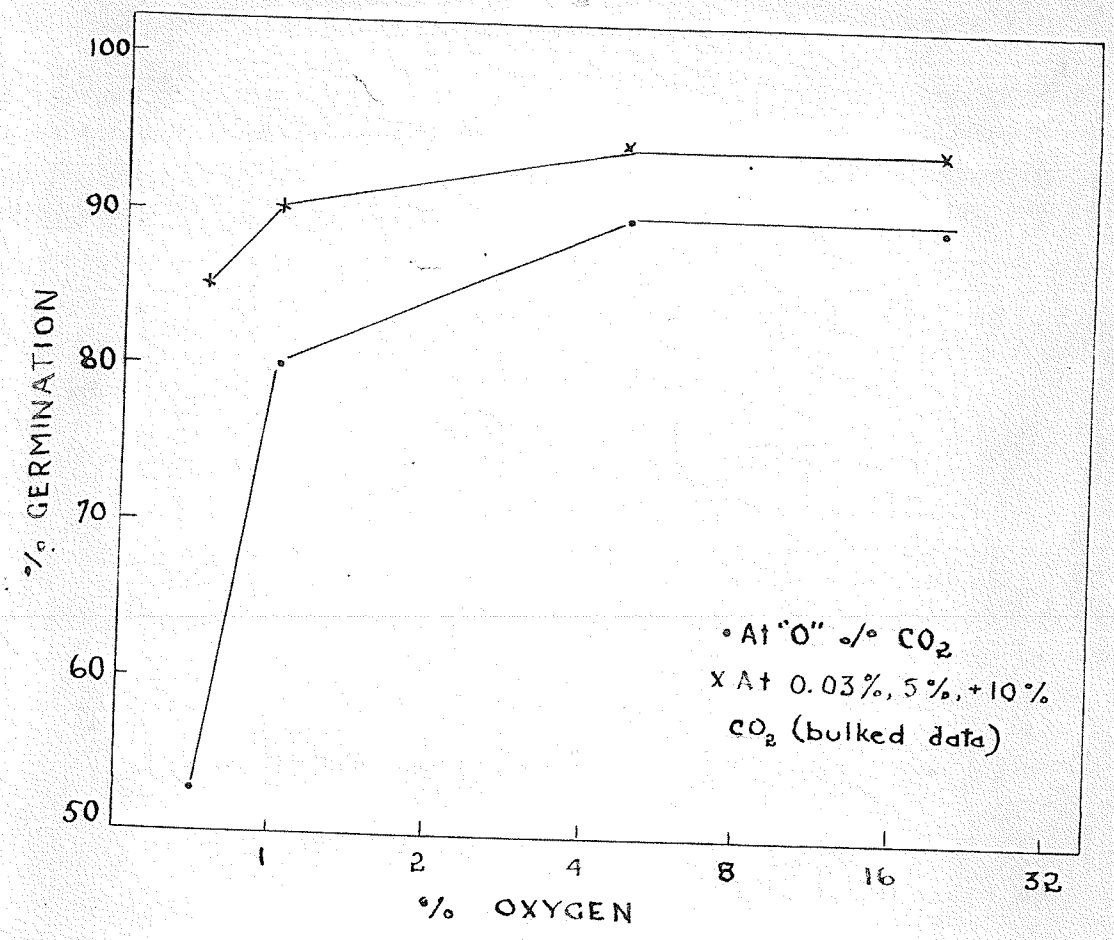


Fig. 10. The effect of oxygen concentrations on percentage germination at four levels of CO_2.

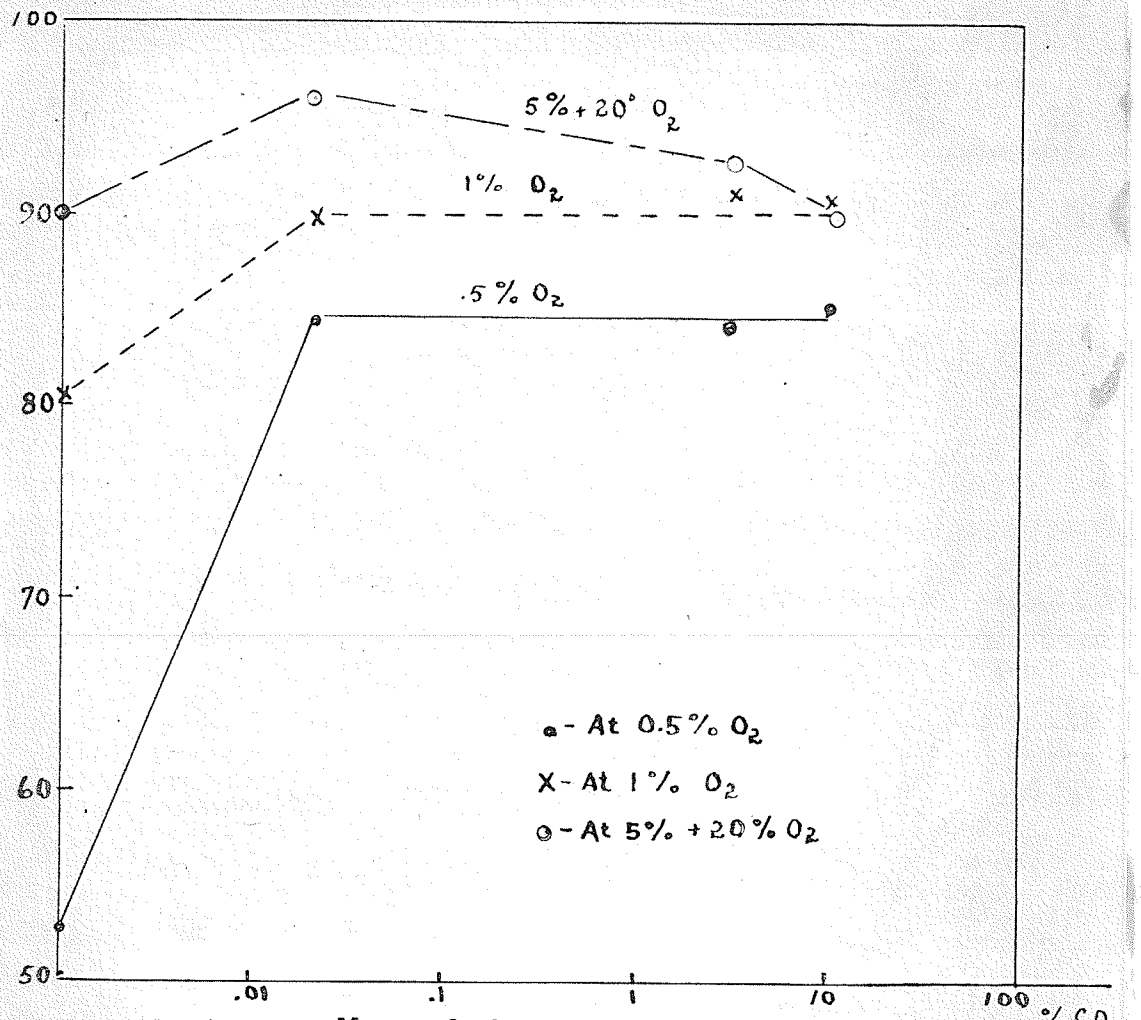


Fig.11. The effect of CO_2 concentration on percentage germination at four levels of O_2

b. At the low oxygen levels, no significant difference in percentage germination was recorded from 0.03% to 10% carbon dioxide; at the high oxygen levels, on the other hand, a decrease in percentage germination was noted when the carbon dioxide concentration was increased from 0.03 to 10%.

The differences recorded above, while significant, are not large; they may represent effects upon the rate of germination, rather than upon the final percentage germination, since the trial was only conducted over a period of eight hours.

In any case, the low concentrations of oxygen and carbon dioxide found in distilled water[1] would not account for the complete inhibition often found for submerged spores. From calculations,[2] the concentration of oxygen and of carbon dioxide in distilled water (v/v), in equilibrium with that of the atmosphere was estimated to be approximately 0.68% and 0.026% respectively. In the present experiment, at comparable concentrations (0.5% and 0.003%), the percentage germination was eighty-five.

[1]The pH of a suspension of spores over a period of 24 hours was not found to be less than 6.8.

[2]Calculated from gas solubility tables (Handbook of Chemistry and Physics. 25th ed. Cleveland. 1941) at 20° C., assuming that Henry's Law holds over the entire range.

PART IV

THE GERMINATION OF SUBMERGED SPORES

The spores of a number of fungi, such as Urocystis tritici (45), Aspergillus (22), and the fruit-rot organisms studied by Brown (9), germinate well in aqueous suspension, or under a layer of water. Brown observed, however, that for Botrytis cinerea, the percentage germination and the average length of the germ tube were markedly diminished with increase in spore density.

There have been indications throughout the literature, particularly with reference to the obligate parasites, that the spores of certain species are adversely affected by submersion.

As early as 1855, Tulasne (58) pointed out that when the spores of Aecidium ranunculacearum were submerged in water, they did not germinate as readily as when placed in a damp atmosphere. DeBary (14) also experienced difficulty in germinating spores of Puccinia graminis under water. The general belief was that lack of oxygen was the causative factor.

Instances of a similar nature have also been recorded for Puccinia triticina (44) and Erysiphe graminis (10).

Blackman (6) found that teleutospores of Phragmidium rubi did not form sporidia under water.

Durell (23), in his suggestive study of the factors affecting uredospore germination in Puccinia coronata, found that spores in suspension failed to germinate, or did so very slightly, but that when the drop cultures were in contact with paraffin oil or vaseline, the percentage germination was materially increased.

Allen (2), who observed that inhibitor-depleted (i.e., "pre-floated") uredospores germinated readily when submerged or floating, whereas untreated spores did not germinate when submerged, concluded that the reduction was probably not due to lack of oxygen, but to the presence of inhibitor.

In the present study, germination under water was characterized by extreme variability, reminiscent of the results in Part I.

There were three general observations, however, which served to suggest an outline for further work:

a. submerged spores, while inhibited from germinating, remained highly viable for at least three days.

b. a high percentage germination was obtained in depths of up to at least 20 cms of water when the spores were spread

evenly over the surface of a glass stirring-rod, but not when the spores were clumped together.

c. germination was not stimulated by reducing the temperature from 20° C., which would be expected if availability of oxygen was the limiting factor.

Experiment XII. Germination at Different Depths of Agar

If oxygen is the main limiting factor for the germination of submerged spores, germination should depend upon the diffusion gradient of the gas, and hence upon the depth of the spores beneath the surface. For convenience, 0.75% agar was used in place of water in testing this assumption.

Freshly-prepared agar was poured into 50 mm petri dishes, and allowed to cool for two hours. Three day old spores from the refrigerator were shaken onto these plates, and warm agar (40° C.) was poured over the spores to varying depths. After germination in the growth chamber, the depth of the spores was measured by means of the graduated fine adjustment of a microscope.

TABLE XII

THE EFFECT OF AGAR DEPTH ON PERCENTAGE GERMINATION

Mean Depth (mm)	% Germination
0	90, 86
0.6	85
0.9	84
1.1	88
1.6	89
2.1	78
2.4	91, 89
4.0	86
4.1	89
4.6	79
5.1	75

In all cases, the germ tubes were long and unbranched.

There did not appear to be any correlation between the depth of agar in which the spores were submerged, and the percentage germination, at least to depths of around five millimeters.

A second series was run, using similar methods, except that 300 ml beakers were used, and warm agar was poured to depths of up to eight centimeters. As in the first series, no correlation was found, and the average percentage germination was approximately eighty-five per cent. In this series, there was a delay of about twenty-four hours before germination commenced, however, suggesting that while the rates of diffusion were not

limiting during germination, a certain minimum level of oxygen and/or carbon dioxide had to be attained before commencement of germination.

Experiment XIII. The Effect of Dilution on The Germination of Submerged Spores

If a product of spore germination is responsible for the behaviour of submerged spores, the inhibition effect would depend upon the density of the spore suspension.

A suspension of one-month old spores was prepared by shaking up 0.05 g of dry spores with 30 ml of distilled water. A known volume of this suspension was pipetted into 50 mm petri dishes, and distilled water was added to make the final volume five milliliters in each case. The petri dishes were placed on the laboratory and germinated in diffuse light.

TABLE XIII

THE EFFECT OF SPORE DENSITY ON GERMINATION UNDER WATER

ml spore Suspension	% Germination			Mean %
	1	2	3	
0.1	85	82	84	83.6
1.0	70	80	75	75.0
2.0	49	62	64	58.3
3.0	42	40	46	42.6
4.0	40	31	36	35.6

A change in the appearance of the spores was noted with increase in spore density; at the lower densities, the germ tubes were of even diameter throughout their length, while in the petri dishes containing three and four milliliters of spore suspension, the germ tubes exhibited spherical or irregular swellings. (See Plate II, fig. 2.)

It will be seen from Table XIII that there was a definite relationship between spore density and percentage germination for submerged spores, with a reduction in germination of about forty per cent with increase in spore density from 0.03 to 1.28 mg/ml.

Under the conditions of this experiment it was not likely that such marked reductions in germination with increase in spore density were due to competition for oxygen; this is indicated by the following observations:

a. no increase in the germination of submerged spores was obtained at lower temperatures, which would be expected if oxygen solubility was the limiting factor.

b. a high percentage germination was apparent at oxygen and carbon dioxide concentrations roughly equivalent to those of distilled water (Exp. XI).

The observation by Allen (2), that "pre-floating" the

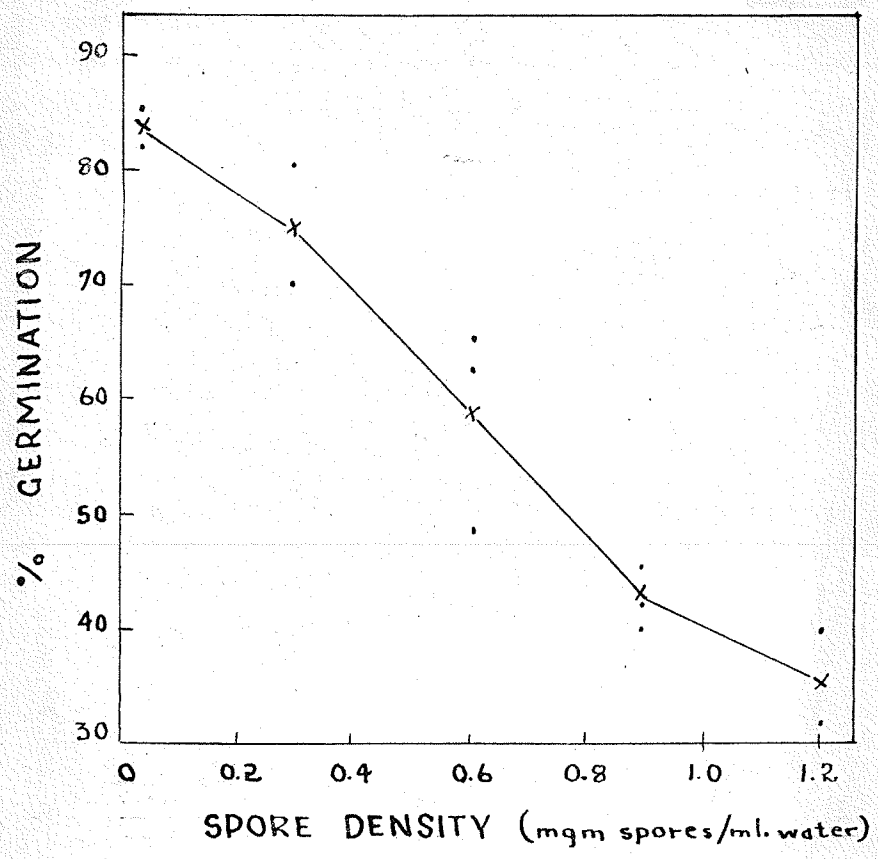


Fig 12. The effect of spore density on germination under water

spores increased their subsequent germination under water would lend weight to the belief that the reduction in germination with increase in spore density, as noted above, was due to the accumulation of a substance (s) given off by the spores.

DISCUSSION AND CONCLUSIONS

The emphasis, in Part I, on the questionability of making comparisons between experiments containing a large degree of variability appeared justified; it was seen that the reaction to any given factor depended in part upon the previous history of the spore sample, as well as upon the level of other experimental factors.

Because of this interdependence, simple effects were often difficult to assess; overlooked differences in technique and cultural conditions may alter markedly the reactions to the main experimental factors.

The following outline is given in the hope that it will at least assist in stating the problem in more concrete terms.

There are factors associated with storage which tend to reduce the subsequent germination. The rate of reduction varies with the particular spore collection, and is probably brought about by such factors as the absence of light, the rate of air diffusion through the sample, and perhaps the amount of spore material present. After the initial reduction, the spores exhibit a much slower decrease in germinability, with seventy to eighty per cent of the spores still functional after as long

as one-hundred and ninety days. The spores do not appear to be killed to any great extent during storage, however, since the percentage can be increased by exposing them, on agar, to a temperature of 29° C. for several minutes. The behaviour of dry spores, as outlined above, was tentatively ascribed to the activity of a self-inhibitor.

In the presence of light, uredospores appear to require an external source of carbon dioxide for normal germination, since germination is reduced over potassium hydroxide, and it can be increased by the addition of carbon dioxide to the germination vessels. At low carbon dioxide concentrations, the length of the germ tube appears to be inversely related to the light intensity (Appendix I). In the dark, the presence or absence of carbon dioxide made little difference.

When the oxygen concentration was reduced below five per cent, the percentage germination was also reduced, particularly in the absence of carbon dioxide.

It was concluded, in agreement with the findings of Allen (2), that the low and highly variable rates of germination accompanying the submersion of uredospores was more probably due to the accumulation of a natural inhibitor than to low oxygen tensions, but inasmuch as interactions exist

between inhibitor "sensitivity" and carbon dioxide concentration, and between carbon dioxide and oxygen concentrations, there may also be an indirect interaction between oxygen concentration and inhibitor "sensitivity"; hence, at low oxygen tensions, such as occur under water, uredospores could be more 'sensitive' to the inhibitor.

It was not found possible, with the information at hand, to elucidate the apparently diverse effects of light on germination, viz., that for 'dry' spores, the absence of light resulted in a decrease in germinability, whereas for spores germinating under low carbon dioxide tensions, the presence of light was injurious. Probably, with the commencement of germination, abrupt changes in metabolism take place, with attendant alterations in the response to the environment. It may be that the reaction of germinating spores to light intensity, carbon dioxide tensions, and inhibitor "sensitivity" is a function of the preceding dark-storage period.

Undoubtedly the most characteristic feature of work in this field is the critical importance of interactions between environmental and physiological factors; moreover, these interactions must be taken into account in providing an accurate description of the behaviour of uredospores.

Acknowledgments

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APPENDIX I

FURTHER OBSERVATIONS ON THE EFFECT OF LIGHT AT LOW CARBON DIOXIDE TENSIONS

Another observation which tended to support the view that light intensity plays a decisive role at low carbon dioxide tensions is given below. The effect produced was an accidental result of the experimental design, but might be of interest at this point.

Fresh spores were stored in the dark, in stoppered phials, for approximately four hours. Each replicate was taken from a separate phial, and the treatments were carried out in random order. Two large desiccators were used, one containing 10% sulphuric acid ($+CO_2$), the other, 10% KOH ($-CO_2$). The inoculated petri dishes were placed in both desiccators, but due to restrictions in space, they had to be arranged in two layers (Top and Bottom). The desiccators were then placed in the growth-chamber.

TABLE XIV

THE EFFECT OF LIGHT INTENSITY ON PERCENTAGE GERMINATION OVER KOH

+ CO_2		- CO_2	
Top	Bottom	Top	Bottom
87	94	45	74
84	86	64	73
90	89	70	66
93	90	45	79
86	90	52	76
84	93	55	83
88	85	63	64
91	91	30	73
90	91	53	82
83	90	50	82
Mean = 87.6 ± 3.2	89.9 ± 2.6	52.7 ± 10.8	75.2 ± 6.2

The characteristic appearance of distorted germ tubes, with swollen and burst tips, was seen in the plates from the top layer of the desiccator containing KOH. The spores from the bottom layer of this desiccator* were not quite as extremely modified in appearance, but represented an intermediate stage between the markedly affected top layer, and the normal-appearing spores germinated over sulphuric acid.

* See Plate IV, fig. 1.

The average length of the germ tubes of the spores over KOH was also noticeably shorter in the top layer, compared with those from the bottom layer.

Before reaching the bottom layer of petri dishes, the light had to pass through the first layer, which consisted of an extra thickness of glass, and a plaque of 0.75% agar, about 7 mm thick. It was deemed more probable that the change in the intensity, rather than the quality of the light, was responsible for the significant difference between the two layers.

While there may also have existed a temperature gradient between the two layers, judging from the previous experiments, any such difference in temperature would not be sufficiently large to account for such marked changes.

APPENDIX II

STATISTICAL TREATMENT FOR EXPERIMENT XI

Two sets of eight desiccators were employed per day, for four successive days, with the arrangement of treatment combinations shown in Table XV. The enclosed figures, which represent the percentage germination obtained in each case, are based upon an average count of approximately one-hundred and sixty spores.

TABLE XV

	O_1	O_2	O_3	O_4
C_1	44, 61	73, 87	87, 93	93, 85
C_2	81, 87	91, 92	94, 89	97, 95

1st and 3rd days

	O_1	O_2	O_3	O_4
C_3	90, 83	97, 90	90, 94	95, 92
C_4	87, 84	90, 90	87, 92	91, 86

1st and 3rd days

	O_1	O_2		O_3	O_4
C_1	57	78	C_1	94	87
	48	83		88	91
C_2	85	86	C_2	97	98
	85	89		98	97
C_3	79	85	C_3	93	96
	86	92		97	97
C_4	83	91	C_4	90	93
	86	92		91	94
	2nd & 4th day			2nd & 4th day	

TABLE XVI

ANALYSIS OF VARIANCE FOR EXPERIMENT XI

	Variation due to:	D.F.	Sum of Squares	Mean Squares	Variance Ratio
Treatments	(CO_2 conc.	3	1898.4	632.8	42.8*
	(O_2 conc.	3	2709.0	903.0	61.0*
	(CO_2 x O_2	9	1732.6	192.5	13.0*
	Total	15	6340.0		
	Error	48	709.2	14.77	
	Total	63	7049.2		

Standard error of single determination = $\sqrt{14.77}$ = 3.823

* Significant at the one per cent level.

Mean difference between 0.03% and 10% CO_2 levels:

a. at low O_2 concentrations---

$$\frac{(84.5 + 89.5) - (85.0 + 90.7)}{2} = -0.85$$

b. at high O_2 concentrations---

$$\frac{(94.5 + 96.7) - (90.0 + 91.0)}{2} = 5.10$$

Minimum significant difference ($P = .05$) =

$$2.01 \sqrt{\frac{2 \times 14.77}{8}} = \underline{\underline{3.82}}.$$

APPENDIX III

THE EFFECT OF TOLUENE

Isaac (38) found that Race 56 uredospores could germinate in toluene-saturated water. The following two experiments indicate some additional properties of toluene with respect to uredospore germination.

EXPERIMENT A

Three-day old spores from the refrigerator were shaken onto a solution of toluene (3 parts of water saturated with toluene:7 parts of distilled water), or onto an equivalent amount of water. One-half of the 20 ml glass vessels used for the experiment were placed in a large desiccator containing 20% KOH, and the remainder were left on the laboratory bench, covered with watch-glasses. There were three replicates for each treatment, and all were continuously exposed to fluoures-cent light. The following results represent only approximations of the percentage germination.

a. Spores germinated on distilled water, at reduced CO_2 tension (over KOH)---40% germination; tubes short and unbranched.

b. Spores germinated on toluene solution, over KOH---75%

germination; tubes long, with long straight branches.

c. Spores germinated on distilled water, exposed to atmospheric CO_2---over 80% germination; tubes long, mostly unbranched.

d. Spores germinated on toluene solution, in the presence of atmospheric CO_2---similar to c.

Under room conditions, germinating uredospores appeared to be able to withstand a concentration of at least $0.3 \times .047^{16}$ gm toluene per 100 ml water. At this concentration, furthermore, the usual reduction in germination by low carbon dioxide tensions was apparently overcome to some extent.

EXPERIMENT B

The Effect of Toluene on the Germination of Submerged Spores

A suspension of one-month old spores was prepared from 0.2 g of dry spores in 60 ml of distilled water. Seven milliliter-aliquots were pipetted into eight petri dishes; three milliliters of a toluene-saturated water extract were added to four of these, and three milliliters of distilled water to the other four.

After twenty-four hours in the growth-chamber, the spores to which the toluene had been added showed about fifty

per cent germination, while those receiving only distilled water possessed less than one per cent of germinated spores.

While too much emphasis can be placed upon two such isolated observations, they may provide a means of correlating the two phenomena of the inhibitor:carbon dioxide interaction (Exp. VIII) and the reduction in germination of submerged spores.

The suggestion was put forth by Allen (2) that carbon dioxide assumed a role in counteracting the effects of the self-inhibitor; there is also evidence that an inhibitor is involved in the case of spores covered with a thin layer of water. If the inhibitor were rendered more soluble by the presence of non-toxic amounts of toluene, it could perhaps be more readily removed from the site of production, thus reducing the injurious effects of lowering the carbon dioxide tension in the presence of light, and overcoming the inhibition associated with immersion.

Forsyth (28), for example, found that the inhibitor given off by germinating spores in Warburg flasks could be absorbed in solutions of acetone, ammonium hydroxide, and silver nitrate, thus stimulating germination.

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EXPLANATION OF PLATES I - IV
spores photographed *in situ*

PLATE I

fig. 1. "Normal" germination, showing branched and unbranched germ tubes.

fig. 2. Germ tubes showing "stretched" appearance.

PLATE II

fig. 1. "Gnarling" and "staghorns". See Dickinson (17).

fig. 2. Germination under water. Note pigmented bands along the germ tube, and spherical swellings.

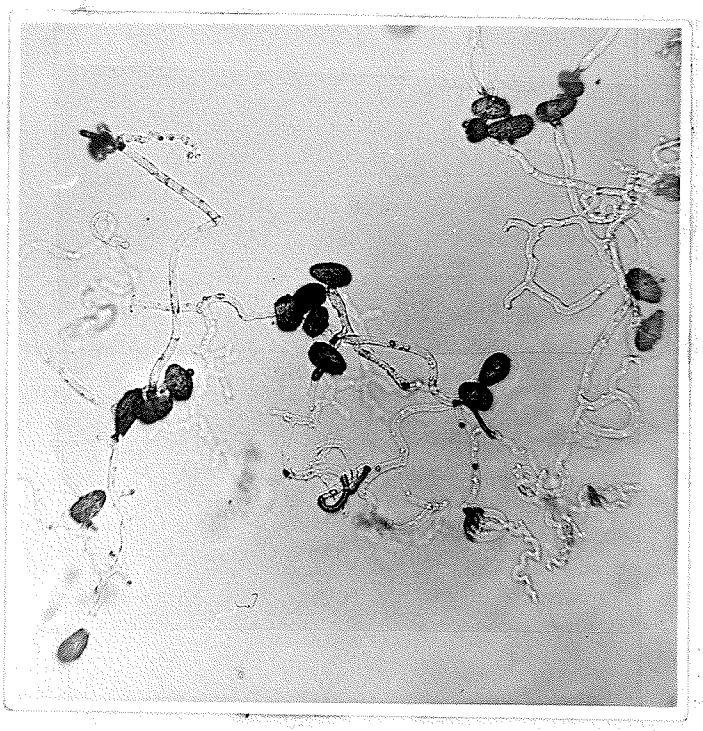
PLATE III
germination at low carbon dioxide tensions

fig. 1. Note swollen tips and short length of germ tubes.

fig. 2. Note burst tips, and irregular appearance of germ tubes.

PLATE IV

fig. 1. Germination under conditions comparable to those in fig. 1 of Plate III, but under lower light intensity (bottom layer of desiccator in Appendix I). Some damage evident, but the proportion of "bursts" not as high, and the average length of the germ tubes not as short as in fig. 1 of Plate III.



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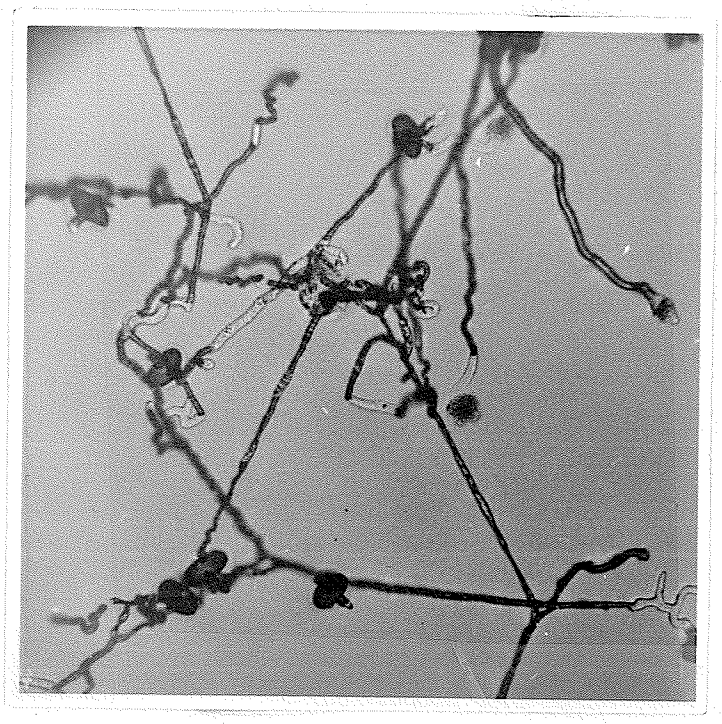

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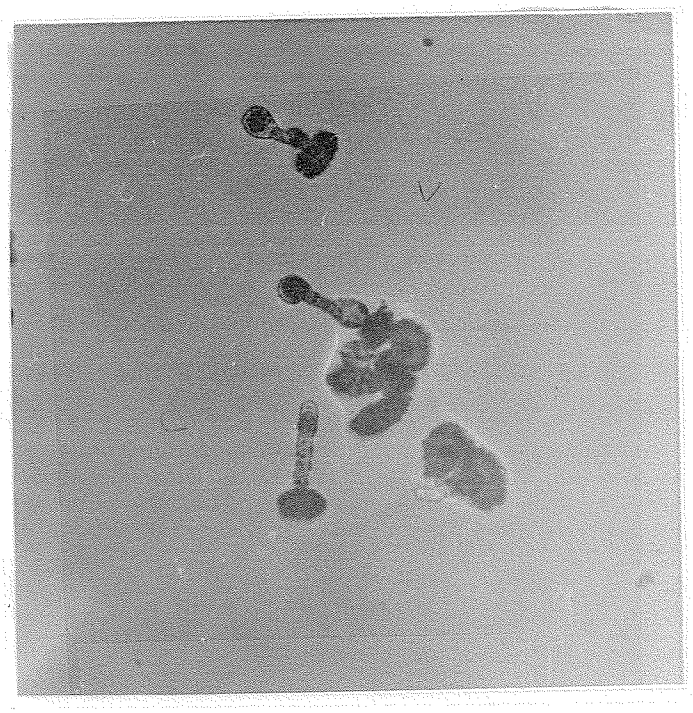

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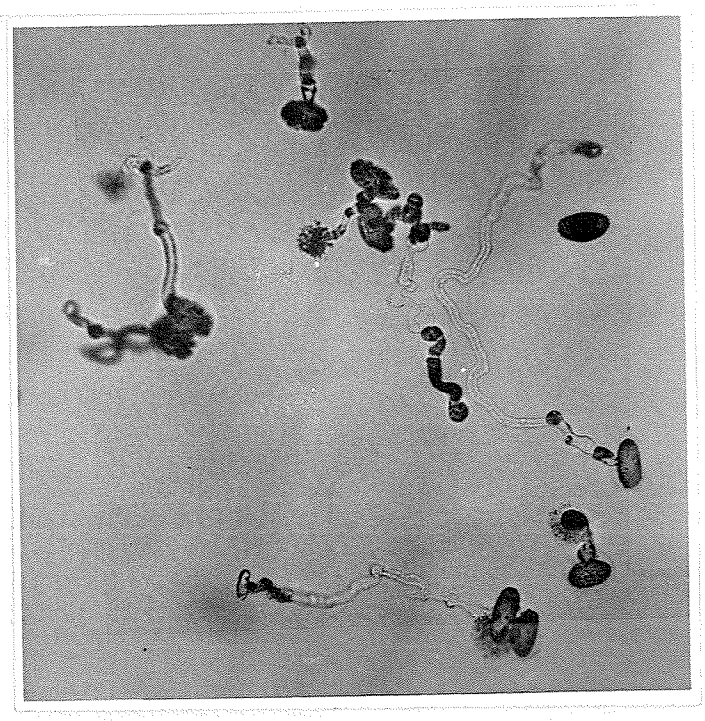

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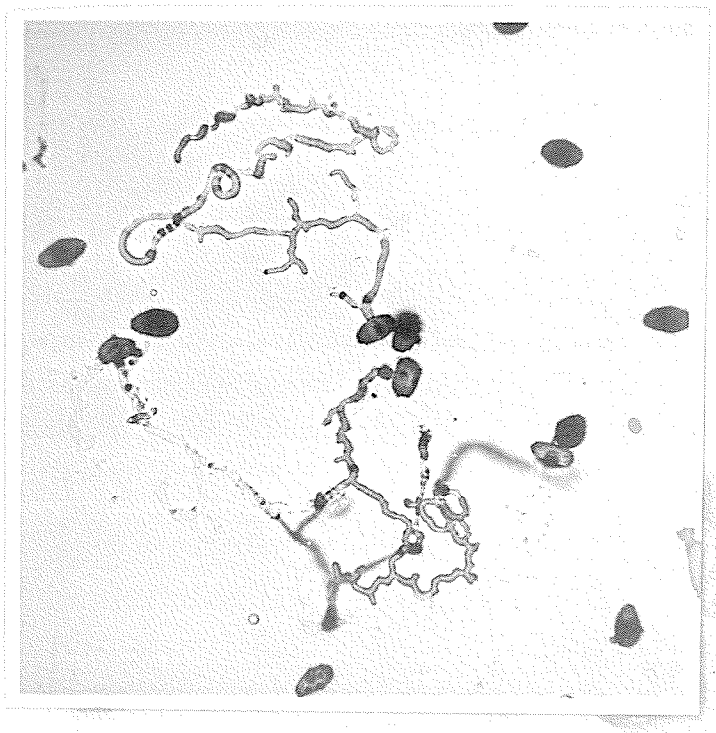

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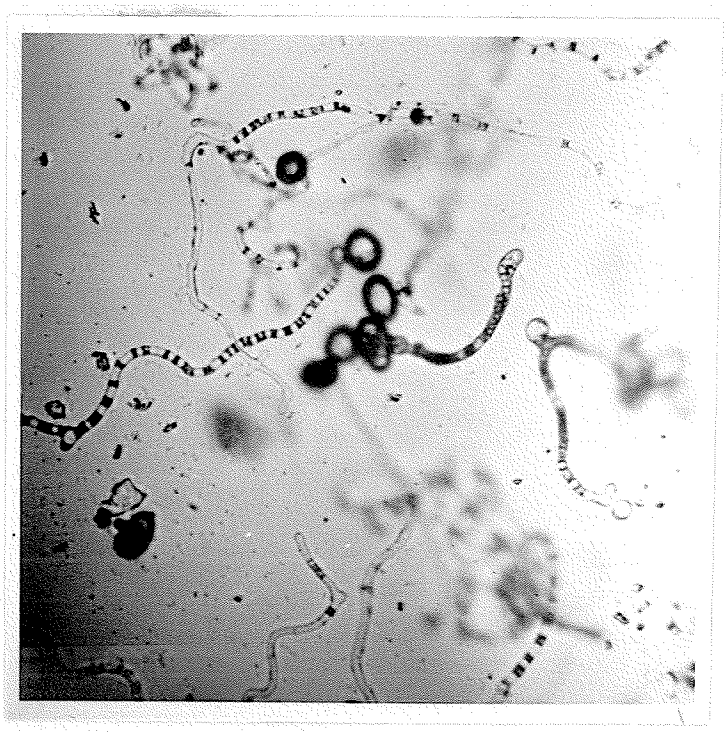

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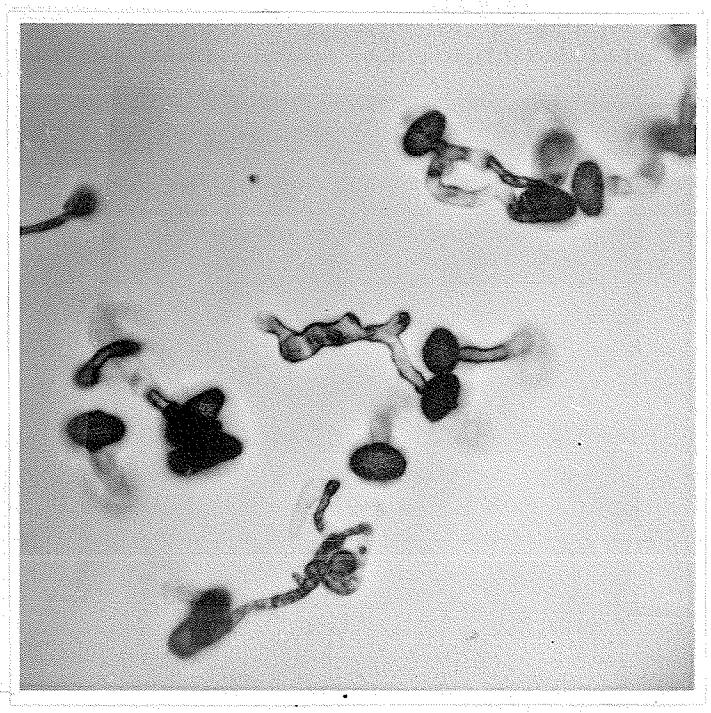

PL. IV.