IN VITRO CULTURE STUDIES OF *PUCCINIA RECONDITA* F. SP. *TRITICI*

By

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INTRODUCTION

When one organism can grow only by securing its food from another living organism, obligate parasitism is involved. In plant pathology, obligate parasites are generally considered to be the viruses, downy mildews (Peronosporales), powdery mildews (Erysiphales) and rusts (Uredinales) (18).

Puccinia recondita Rob. ex. Desm. f. sp. tritici, commonly called wheat leaf rust, is a heteroecious, long cycle rust that is an obligate parasite of Triticum spp. in the family Gramineae. The sporophytic generation can be classified into variants or races on the basis of virulence by the use of differential varieties (19).

In vitro culture of parasites currently considered to be obligate would be useful in physiological studies to better understand the intimate host-pathogen relationship exhibited between a host and an obligate parasite. Yarwood stated there were two different aspects to be considered when culturing obligate parasites: (a) successful culture on any non-living medium, and (b) analysis of nutrients necessary for growth (18).

This investigation began as a result of a report from Australia of successful vegetative growth of Puccinia graminis Pers. f. sp. tritici (Eriks. & E. Henn.) in vitro (16), and was designed to determine if wheat leaf rust could also be cultured on non-living media. The method might be a useful procedure for purification of cultures and for physiological studies.

LITERATURE REVIEW

Allen and Arthur reported that Ray in 1901 observed the saprophytic cultivation of rose rust and Euonymus rust fungi on a gelatin medium containing plant extracts and upon sterilized carrots resulting in the production of mycelium and teliospores (2,3). This report has not been generally accepted (2, 3, 11, 18).

Grecusnikov, in 1936, working in Russia with Puccinia spp., reported saprophytic growth of rust was obtained during a period of 12 days by removing ammonia and urea from the substrate by the addition of substances that absorbed them (9). Allen, in 1954, stated that ammonia and urea compounds have been found to inhibit development of rust spores, and susceptibility of a host was considered to be dependent upon the continued removal of these compounds by that host plant (2).

There have been many reports of successful tissue culture of a host, together with its pathogen, on artificial media.

Morel, in 1944, reported successful tissue culture of grapevine fragments together with the parasite Plasmopara viticola (12). The fungus developed normally, covering the surface of the grapevine fragments with typical conidiophores.

Hotson and Cutter, in 1951, working with callus tissue cultures of Juniperus tissue infected with Gymnosporangium juniperi-virginianae reported mycelial growth of a fungus from the callus tissue (11). This fungus growth was transferred to potato-dextrose agar where it continued to grow. With it they were also able to reinfect the alternate hosts

Crataegus spp.

Hotson, in 1953, reported the production of typical telial horns in callus tissue cultures of *Gymnosporangium juniperi-virginianae*. These horns would survive transplanting of the callus tissue (10).

Turel and Ledingham, in 1957, were unsuccessful in an attempt to repeat Hotson and Cutter's work, but they did report the production of aerial mycelium and urediospores by *Melampsora lini* on flax leaves in tissue culture (15). However, they stated that even with production of aerial mycelium the rust was still fully dependent upon the host, since any factor which affected the metabolism of the host also affected the production of aerial mycelium. This type of work was useful but still did not offer the opportunity for host and pathogen to be studied separately.

There also has been much work done on the infection structures formed by rusts on artificial media and under various environmental conditions. Dickinson, in 1949, working with the urediospore stage of several rusts concluded that the appressorium, substomatal vesicle, and infection hyphae can be induced by contact stimulus with artificial membranes (6). He later observed what he believed to be haustoria produced by *Puccinia triticina* and *Erysiphe graminis* when in contact with artificial membranes (7).

Emge, in 1958, observed that with *Puccinia graminis* f. sp. *tritici* infection structures occurred on artificial media under specific light and temperature conditions (8).

The observations of germinating urediospores on artificial media and studies of the various environmental factors that affected the nature of the structures formed was only partially successful. Studies

of this type were usually short, perhaps only 48 hours in length, and the formation of the infection structures seemed to be the termination point. Yarwood discussed this matter in 1956 and stated there was no good reason to believe that the formation of appressoria or substomatal vesicles was necessary for successful axenic culture of obligate parasites just because these organisms form such structures in nature (18).

In 1966, however, Williams, Scott, and Kuhl (16) reported from Australia in vitro vegetative growth of Puccinia graminis f. sp. tritici over an extended period and it might be now possible to study this, and perhaps other obligate parasites in the absence of the host. A later modification of their medium by the addition of 0.1% Evan's peptone resulted in more vigorous vegetative growth and in sporulation, both of urediospores and teliospores (17). Their work was substantiated, in 1968, by Bushnell in the United States who worked with the same Australian isolate (race 126-Anz-6,7) (5). However, he also stated in his paper that Williams, Scott, Kuhl, and Maclean had been able to culture 3 additional Australian races. He also tested several isolates of American stem rust races but had been unsuccessful with their in vitro culture. Thus, it seems evident that nutritional requirements of different uredial races vary, and the problem of culturing rust fungi in vitro is essentially nutritional as stated by Williams, Scott, and Kuhl (16).

MATERIALS AND METHODS

Preparation of Sterile Urediospores

Tests were made to determine the best method to obtain surface sterile urediospores. The technique finally adopted was a modification of that reported by Williams, Scott, and Kuhl in 1966 (16). Six day old plants of the wheat cultivar Cheyenne (C.I. 8885) were placed in a moist chamber and sprayed with a solution containing tap water and a surfactant (Tween-20, 4-5 drops per 1000 ml water). The plants were inoculated by brushing with the leaves of a plant infected with a specific race of *Puccinia recondita* f. sp. *tritici*, sprayed again, and left overnight. The next day the plants were placed in the greenhouse at 20 C.

In four to six days, when the leaves developed visible flecks, the most heavily infected leaves were harvested with scissors and placed in sterile Petri dishes with five leaves per dish.

Each leaf was surface sterilized in one of several fresh dilutions of sodium hypochlorite (5.2%, 3.9%, 2.6% and 0.52%) and a surfactant (Tween-20, 5 drops per 500 ml sodium hypochlorite). These dilutions of sodium hypochlorite were used to determine which dilution was the most effective for a given length of time. The leaf was removed with forceps sterilized in the same solution. The leaf was rinsed twice in sterile distilled water, and placed on water agar containing 20-25 ppm of benzimidazole. Fresh solutions were used for each leaf. The excised end of the leaf was placed in the agar with the adaxial side up. The

top and bottom of each Petri dish were taped together with masking tape but not sealed. This permitted extensive handling of the plates without the usual degree of contamination. The plates were placed in a desiccator over calcium chloride. Maximum sporulation usually occurred nine or ten days after inoculation.

The urediospores were transferred with sterile cotton swabs to plates containing the medium on which growth was to be tested. The cotton swabs were prepared by autoclaving toothpicks for two one-hour periods in tap water at 15 psi, to free the toothpicks of dormant spores of bacteria. They were then cooled, air dried, and stored. Later the toothpicks were tipped with cotton and placed in individual test tubes plugged with cotton. The cotton swabs were then steam sterilized (15 lb. pressure for 20 minutes) and stored at least overnight before use.

The benzimidazole agar was prepared by mixing 40 ml of 500 ppm benzimidazole stock solution, 960 ml distilled water, and 20 gm of agar. This mixture was autoclaved at 15 pounds pressure for 20 minutes, and poured into sterile Petri dishes at the rate of 20-25 ml per plate. These plates were stored in sealed plastic bags at 17 C.

Preparation of Media

Basically, the medium used here was the same as that used by Williams and his co-workers (16, 17) and by Bushnell (5). Some modifications were made later (Appendix Table I).

The general procedure for preparation of media was as follows. The Czapek Dox Broth, peptone, and yeast extract were added to distilled water and mixed thoroughly with a magnetic stirrer, after which agar was added. The pH was then adjusted to the desired level with 2M hydrochlo-

ric acid or 0.1 N sodium hydroxide while the mixture was continuously stirred. The medium was autoclaved 20 to 30 minutes at 15 pounds pressure, aseptically poured into sterile glass or plastic Petri dishes, and stored at 17 C.

Inoculation Procedure and Incubation of Plates

Cotton swabs were used to pick up sterile spores from detached leaves and transfer them to the desired medium. The cotton swab was touched to the agar surface in three to four places depositing the spores, or by lightly brushing the agar surface. Inoculated plates were sealed with masking tape after a technique used by Bushnell (personal communication). The inoculated plates were incubated without light at 17 ± 1 C in an enclosed glass cylinder suspended over a solution of copper sulfate and distilled water (2g copper sulfate per 1000 ml distilled water).

Method used for Taking Pictures

Pictures were taken with a Leica M2 35 mm camera coupled to a Leitz Ortholuz microscope. Most sequential pictures were taken through the bottom of Petri dishes. For this purpose plastic Petri dishes were better than glass Petri dishes, because they were more uniform in thickness and free from defects.

Measurements of Growth

In an attempt to give a numerical value to the growth that was observed, counts were made of the number of secondary branches or branch buds extending from the primary germ tube. The total number of second-

ary branches was divided by the number of germ tubes counted to give an average number of branches per germ tube. Branches were counted only if the germ tube could be followed back and identified as coming from a specific urediospore. As with the photographs, counts were made by observing the cultures through the bottom of a Petri dish.

Procedure for Subepidermal Inoculation of Wheat Leaves

The technique used here was a modification of one used by Sharp and Emge in 1958 (13). Seven to eight day-old wheat seedlings (cv Cheyenne C.I. 8885), were used for this purpose. The abaxial epidermis was cut with a sharp razor blade and stripped away from the mesophyll cells 5 to 10 cm with forceps. A small amount of rust mycelium and agar was placed on mesophyll cells at the base of the stripped epidermis. The epidermis was laid back in place and that portion of the leaf was covered with scotch tape to prevent the transplant from drying out too rapidly. The tape also helped support the leaf. Inoculated plants were maintained in a saturated atmosphere overnight. They were then incubated in a clear plastic cage in a growth chamber at a temperature of 20-23 C, with 900-1100 footcandles of light in 12 hour cycles.

RESULTS

Preparation of Surface Sterile Urediospores

Production of urediospores free from surface contaminants was essential. The concentration of sodium hypochlorite, the presence of a surfactant, and time, were found to be important factors associated with the procedure of producing surface sterile urediospores.

Various dilutions of sodium hypochlorite and distilled water were tried (0.525%, 2.6%, 3.9% and 5.25%). The results indicated that a 5.2% solution of sodium hypochlorite was the most effective.

It was found that a surfactant (Tween-20, 5 drops per 500 ml) was necessary in order that the complete surface of the leaf would come in contact with the sterilizing agent.

The length of the surface sterilization period was also important. If a time period less than three minutes was used, surface sterilization was usually not sufficient. If the leaves were surface sterilized for four minutes or more, certain areas of the leaf, particularly the margins became bleached or yellow. Leaves damaged in this manner would produce few, if any spores. Leaves that were surface sterilized for three minutes in a solution of 5.25% sodium hypochlorite and Tween-20 (5 drops per 500 ml sodium hypochlorite) produced a quantity of surface sterile urediospores, but even with this procedure about 10% of the leaves became contaminated.

After 5 to 6 days on benzimidazole agar the unsterile leaves could

be detected by a water soaked appearance and sparse sporulation. Such leaves were discarded. If contaminated leaves were not detected, and urediospores from these leaves were used to inoculate the growth medium, colonies of what appeared to be bacteria developed in 2-3 days on the inoculated plates.

Completely sealing the 9 cm Petri plate top and bottom together proved to be a very important step. Complete sealing with masking tape permitted handling of the plates without too much danger of outside contamination. However, even with this precaution, 1 to 5% of the plates did become contaminated. The contaminates were either fungal or bacterial and appeared first around the outer edge of the plates.

It was also found that plates had to be inverted to prevent condensation on top of the plates which would flood the agar surface if disturbed.

Transferring surface sterile spores with cotton swabs was more effective than using glass rods or dusting spores on by shaking infected leaves above the agar. A glass rod was not very effective in picking up a quantity of spores. Shaking leaves over agar did not equally distribute spores over the area inoculated and increased the danger of contamination. Spores were easily picked up with cotton swabs and placement of the spores on the plates could be controlled.

Benzimidazole agar was used in place of the nutrient medium suggested by Williams et al. (16) for incubating surface sterile wheat leaves. This medium was stored (17 C) in sealed plastic bags for weeks at a time without affecting its usefulness.

Growth In Vitro

The first three growth media (I, II, III) (Appendix Table I) used were modifications of the media used by Williams et al. (16, 17), and Bushnell (5). In these media 0.1% Difco Bacto-peptone was substituted for 0.1% Evan's Bacteriological Peptone. Each of the three media and plain water agar were inoculated with Puccinia recondita f. sp. tritici race UN1-NA65-1 (19) and incubated at 17 C and 100% relative humidity without light. Eleven days after inoculation, it was apparent that medium I stimulated greater differentiation of the germ tubes of leaf rust than any of the other media used (Figures 1, 2, 3, 4). Medium I contained yeast extract, while the other media did not. These plates were held for 122 days after inoculation (Figures 5, 6, 7), but it was evident that on these media very little vegetative growth or differentiation occurred after eleven days.

It was evident that if the leaf rust fungus were culturable, as was the Australian stem rust fungus, then the peptone source was probably very important.

A quantity of Evan's Bacteriological Peptone was secured and a medium was prepared which was identical to medium IA except for the peptone source. This became medium IV (Appendix Table I). Both medium IA and medium IV were adjusted to pH 6.4. Two physiological races of Puccinia recondita f. sp. tritici, UN1-NA65-1 and UN1-NA65-9, were inoculated into both of these media. It was clearly evident that there was more vegetative growth by both races on the medium containing 0.1% Evan's Bacteriological Peptone than on the medium containing 0.1% Bacto-peptone prepared by Difco. The most vigorous vegetative growth was produced by UN1-NA65-9.

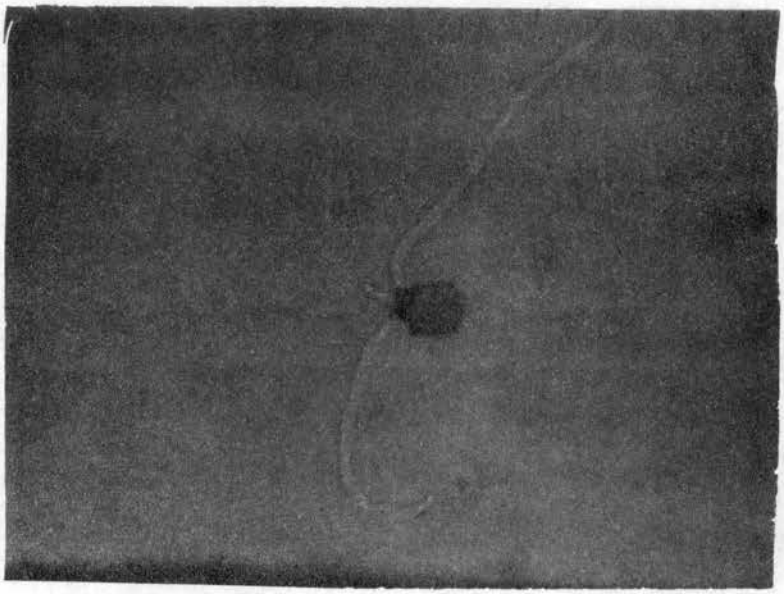

Figure 1. Puccinia recondita tritici race UN1-NA65-1 on plain water agar medium at pH 6.4, 11 days after inoculation. 275X

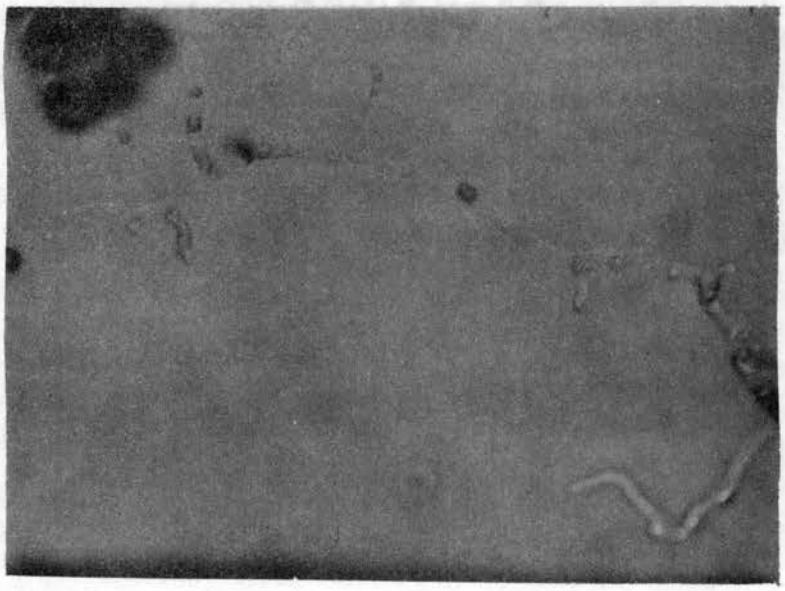

Figure 2. Puccinia recondita tritici race UN1-NA65-1 on medium I at pH 6.4, 11 days after inoculation. 275X

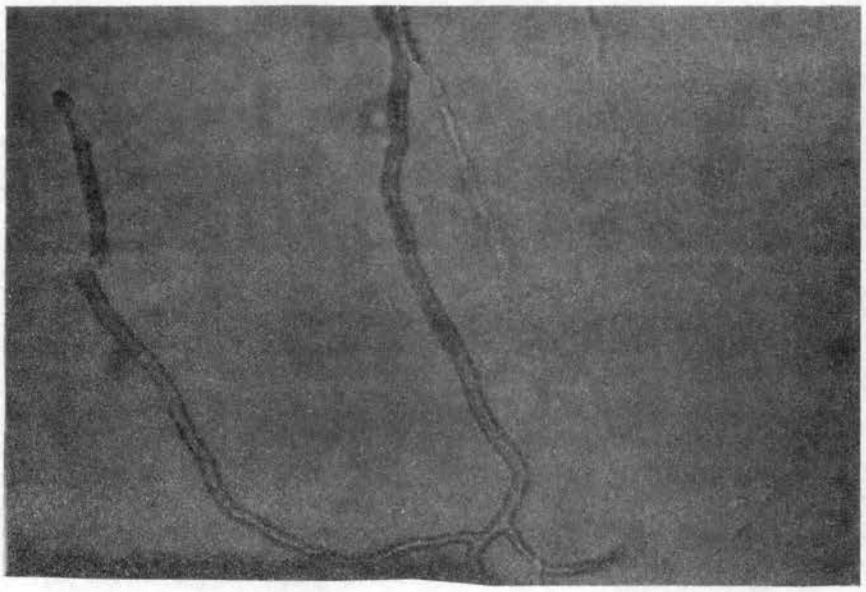

Figure 3. Puccinia recondita tritici race UN1-NA65-1 on medium II at pH 6.4, 11 days after inoculation. 275X

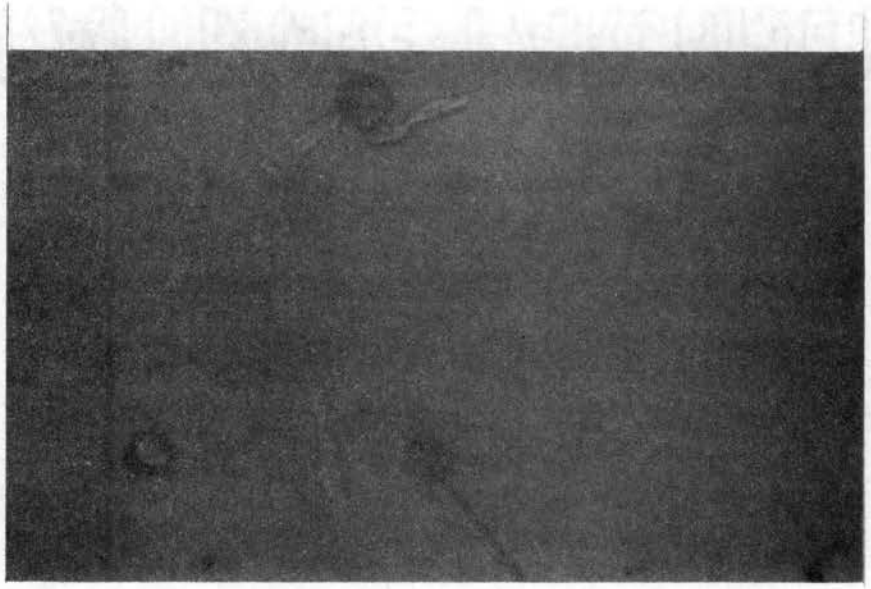

Figure 4. Puccinia recondita tritici race UN1-NA65-1 on medium III at pH 6.4, 11 days after inoculation. 275X

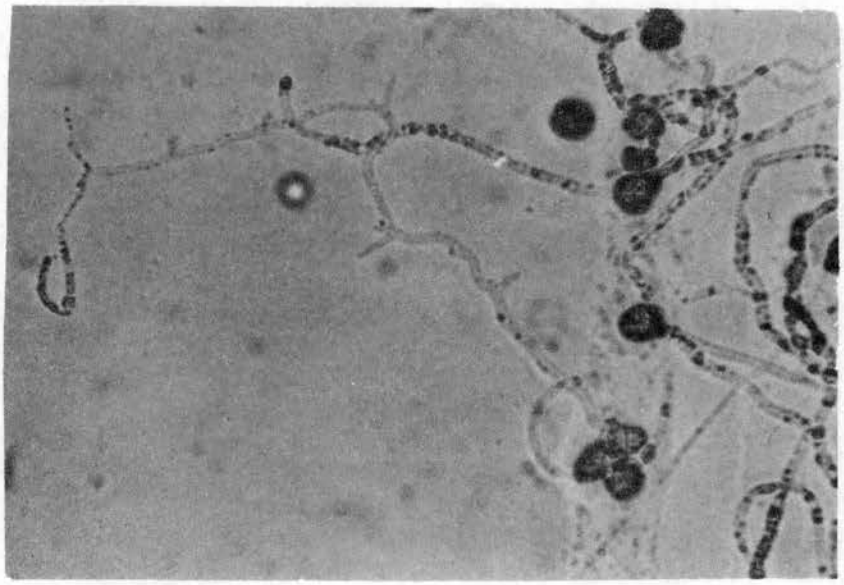

Figure 5. Puccinia recondita tritici race UN1-NA65-1 on medium I at pH 6.4, 122 days after inoculation. 140X

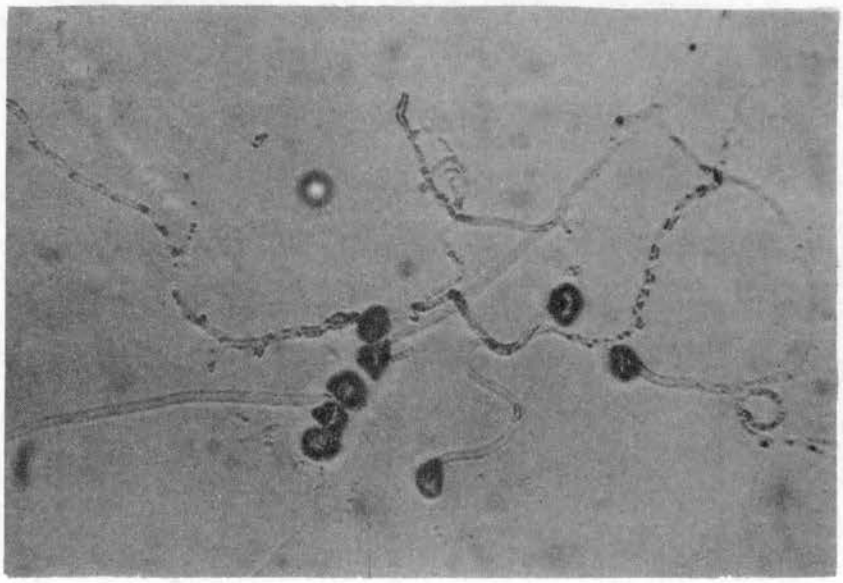

Figure 6. Puccinia recondita tritici race UN1-NA65-1 on medium II at pH 6.4, 122 days after inoculation. 140X

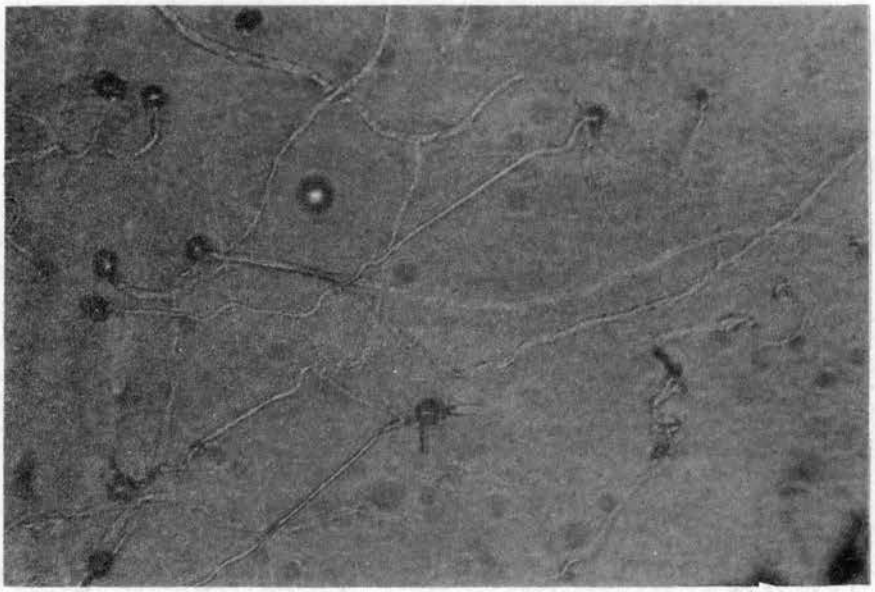

Figure 7. *Puccinia recondita tritici* race UN1-NA65-1 on medium III at pH 6.4, 122 days after inoculation. 140X

pH Study

A study was then initiated to determine if pH of the medium had any influence on vegetative development or sporulation of the leaf rust fungus on Evan's peptone medium. Race UN1-NA65-9 on medium IV was used throughout this study. The pH of the medium was adjusted to the desired level with 2M hydrochloric acid. The levels of pH used were 6.0, 6.2, 6.4, and 6.6.

It was difficult to find a means to measure the amount of growth that took place. Two methods were used: (1) one involved counting the number of urediospore germ tubes that produced a proliferated branching (Figure 8) and (2) the other involved determining the average number of secondary branches per ger tube. In this study the percent of 500 urediospore germ tubes that produced a proliferated branching after 93 days was determined. These data are given in Table I. It was obvious that as the pH increased from 6.0 to 6.6 the number of proliferated germ tubes decreased from 32.4% to 8.6%. The amount of germ tube proliferation at pH 6.0 is illustrated in Figure 9.

In view of the fact that much better growth occurred at pH 6.0, it was decided to make another comparison of the different peptone sources particularly since the first test was made at pH 6.4. Water agar, also adjusted to pH 6.0, was used as a check. Only race UN1-NA65-9 was used. In this case the results were based upon the average number of secondary branches per germ tube. At first, 100 germ tubes were counted, but later it was found that data from only 20 germ tubes were equally significant and therefore only 20 germ tubes were counted. The data are illustrated graphically in Figure 10. Growth of this fungus on the medium containing Evan's peptone was dramatically greater than on the

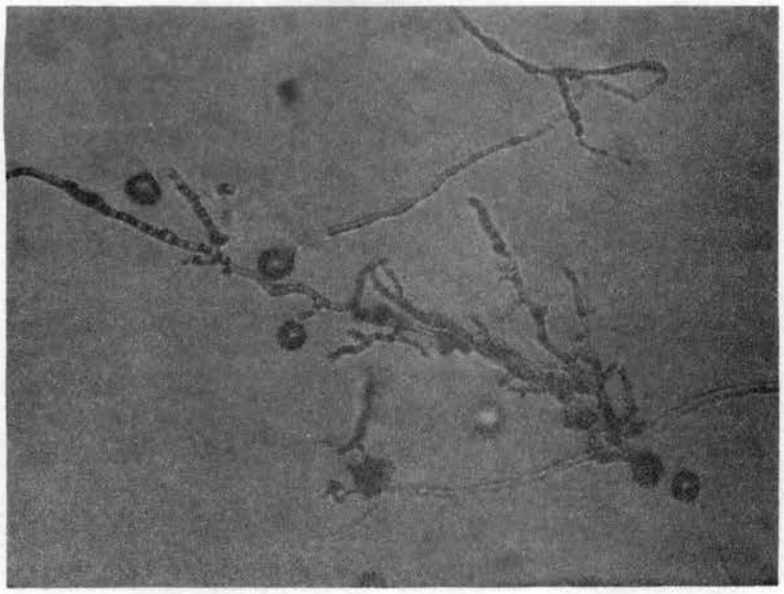

Figure 8. A proliferated germ tube of Puccinia recondita tritici race UN1-NA65-9 on medium IV at pH 6.2, 53 days after inoculation. 140X

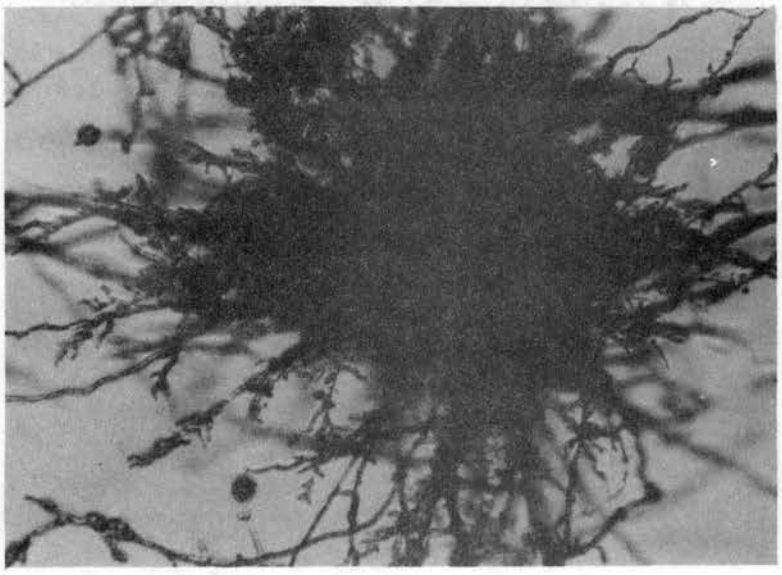

Figure 9. Puccinia recondita tritici race UN1-NA65-9 on medium IV at pH 6.0, 53 days after inoculation. 82X

TABLE I

THE PERCENT OF UREDIOSPORE GERM TUBES OF *PUCCINIA RECONDITA TRITICI* RACE UN1-NA65-9 WITH SECONDARY BRANCHES AFTER 93 DAYS ON MEDIUM IV WITH EVAN'S PEPTONE SOURCE AT DIFFERENT pH LEVELS

	pH Level			
	6.0	6.2	6.4	6.6
Percent of germ tubes with secondary branches	32.4	29.4	18.0	8.6

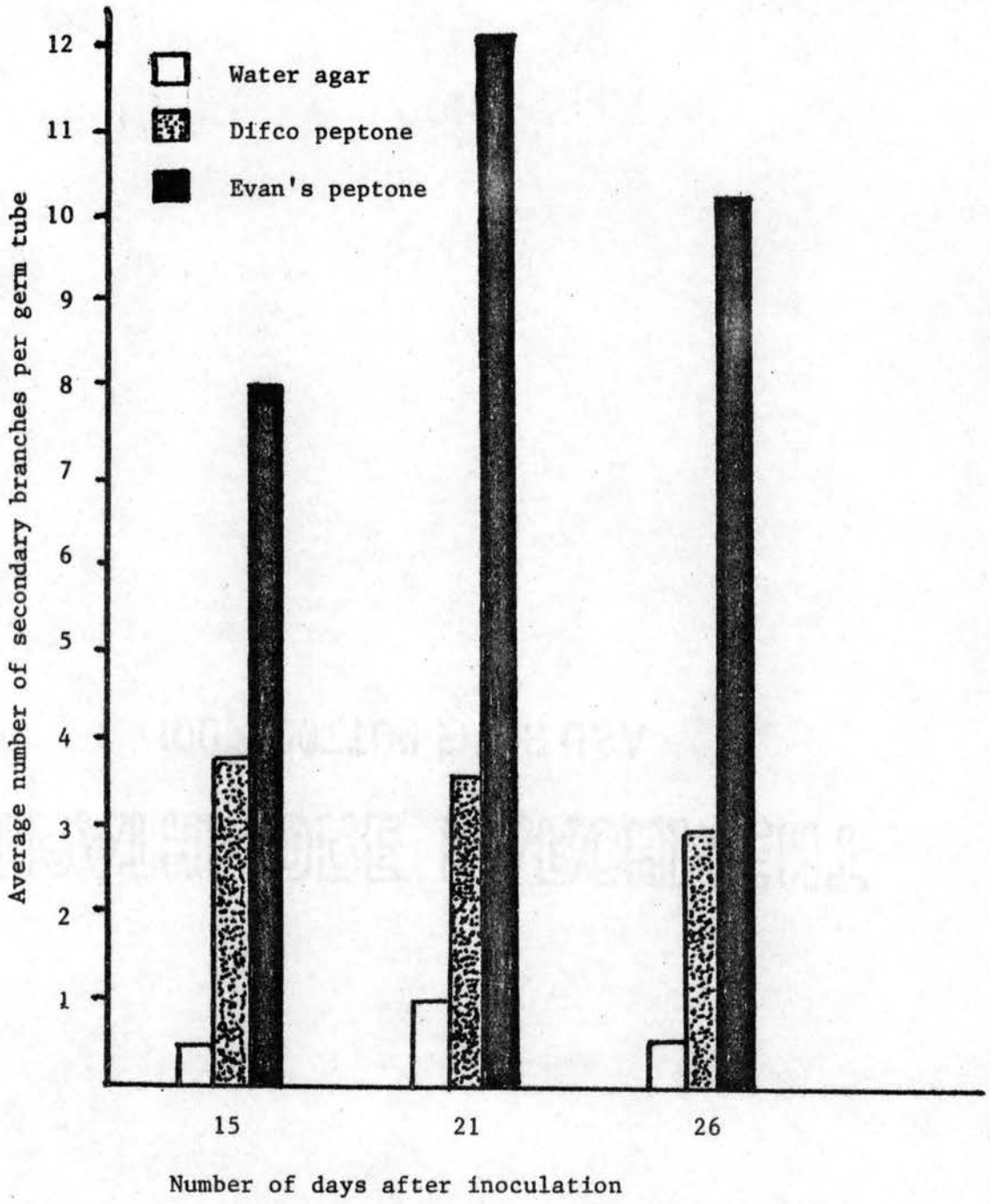

Figure 10. A comparison of the average number of secondary branches produced by Puccinia recondita tritici race UN1-NA65-9 on water agar, medium IV with 0.1% Evan's Bacteriological peptone, and medium IA with 0.1% Difco Bacto-peptone at pH 6.0.

other two media, just as it had been in the test made at pH 6.4. A t-test (14) was applied to the data given in Figure 10 and differences were statistically significant. Differences in growth on the two sources of peptone are also illustrated in Figure 11.

Subepidermal Transfer to Leaves of Wheat Seedlings

Throughout all tests with all media at all levels of pH even after 93 days of growth, no spores, either urediospores or teliospores, were produced. Consequently, attempts were made to transfer branched germ tubes to wheat by placing them under the epidermis of a seedling wheat leaf as previously described. Repeated attempts were made, but failed to produce evidence of infection on the inoculated leaf. It was noted during the course of these tests, however, that the portion of the epidermis which had been peeled back for inoculation dried out quite rapidly (within a matter of hours) and may have led to desiccation of inoculum before it could become established in the host.

The differentiation and development of globose cells by germ tubes was observed on medium IV at pH 6.0 within 16-21 days after inoculation (Figure 12). These structures were frequently observed in cultures of race UN1-NA65-9 (32.4% of the germ tubes) on medium IV but not on other media. They were also observed in cultures of races UN1-NA65-1 and UN6-NA65-19 on the same medium but the frequency of occurrence was lower. Only 9.0 percent of the germ tubes of race UN1-NA65-1 and 3.2 percent of the germ tubes of race UN6-NA65-19 developed globose cells based on observation of 500 spores of each culture.

Most observations and pictures reported here were made of germ tubes lying on the agar surface or growing below the surface. However,

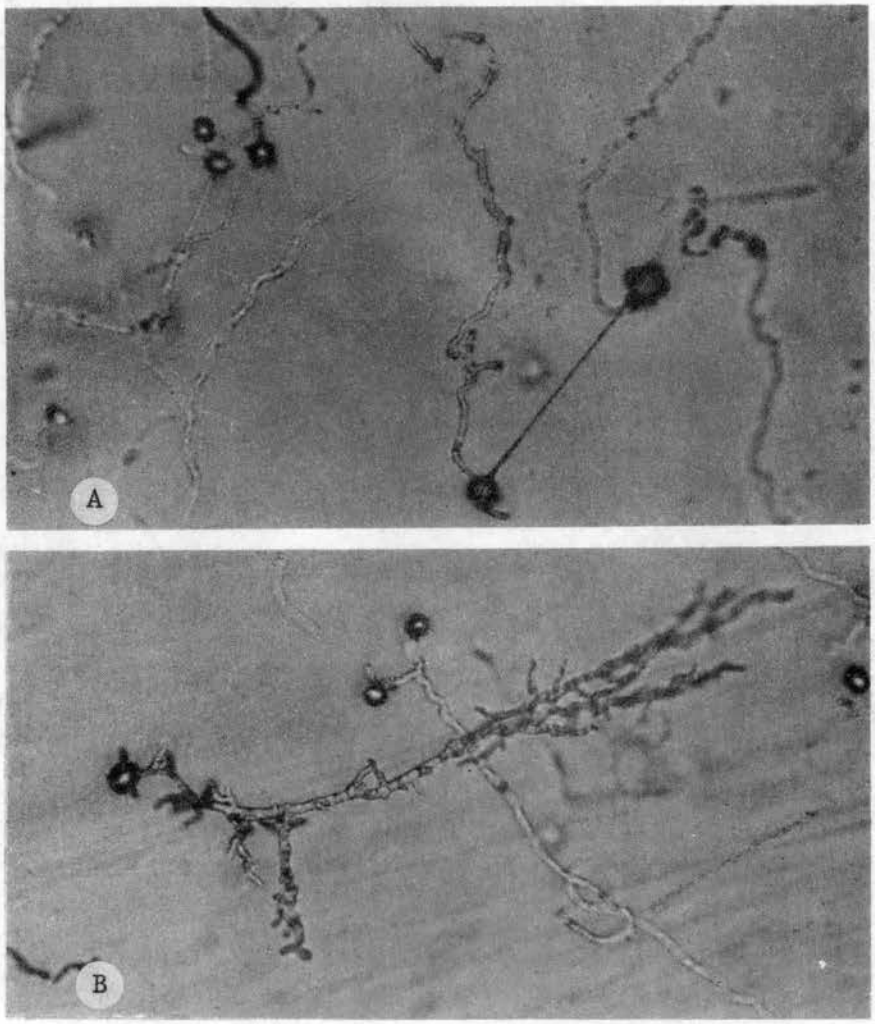

Figure 11. A comparison of the branching of uredio-spore germ tubes of Puccinia recondita tritici race UN1-NA65-9 at pH 6.0 16 days after inoculation, on media (A) containing Difco Bacto-peptone and (B) containing Evan's Bacteriological Pep-tone.

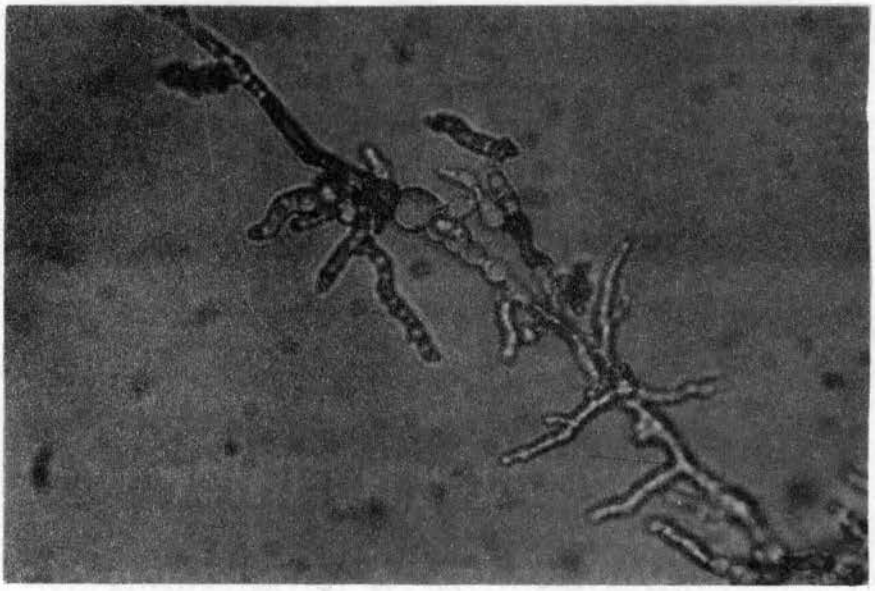

Figure 12. Globose cells produced on germ tubes of Puccinia recondita tritici race UN1-NA65-9 on medium IV at pH 6.0, 21 days after inoculation. 275X

rather extensive growth of aerial mycelium has often been observed in cultures of race UN1-NA65-9 on medium IV at pH 6.0. This mycelial growth is hyaline to white at first, turning darker with age. Observations indicated that production of urediospores did not occur in 4-5 weeks on Evan's peptone medium, although stem rust isolates were reported to sporulate within that period of time (5, 17).

In the case of stem rust, urediospores were usually produced at the end of 4 to 5 weeks on a medium containing Evan's peptone (5, 17). However, this has not been the case with the 3 isolates of leaf rust tested so far. No spores of any kind have been observed to form with these cultures of leaf rust even after as long as 93 days.

DISCUSSION

It was very important to obtain urediospores free from surface contaminates. There were many experiments in this study which had to be terminated because the urediospores used were not free from surface contaminates. Much of the initial work has devoted to developing the aseptic technique described here.

A suggestion from Bushnell (personal communication) to completely seal the inoculated Petri plates with masking tape was also very helpful. It would be virtually impossible to maintain growth in Petri dishes free from outside contaminates without sealing the plates in such a manner. Also, the sealed plates could be handled freely during the process of making observations and photographs without the problem of contamination.

Benzimidazole agar was used instead of a nutrient medium mainly because of the simplicity of preparation. Water solutions of benzimidazole had been used previously for essentially the same purpose (4). The preparation of benzimidazole agar was simply a modification of that technique. The benzimidazole agar apparently accomplished the same purpose as the nutrient medium (16). It is doubtful that rust urediospores produced on wheat leaves on benzimidazole agar would greatly differ physiologically from those produced on wheat leaves on nutrient medium, since the end results were apparently the same.

The growth media used in this study were modifications of the media described by Williams *et al.* (16, 17) and Bushnell (5). One exception

was noted. Medium III, described by Bushnell (5), had an initial pH of over 6.4 and therefore, he used dilute hydrochloric acid to adjust to pH 6.4. When this medium was prepared for studies reported here, the initial pH was less than pH 6.4 and 0.1N sodium hydroxide was used to adjust to 6.4. All ingredients were prepared shelf chemicals except the Czapek minerals. The latter were prepared according to Ainsworth and Bisby (1). If changes in the ingredients were involved, they probably involved the Czapek minerals. In any case, the duplication of medium III was not obtained.

At the beginning of the study, it was postulated that the more avirulent a physiological race, the better possibility that it would grow on an artificial medium. For this reason races UN1-NA65-1 and UN1-NA65-9 were chosen for these studies. These races were the least virulent on the differentials used (19). Although no data presented in this study would give definite proof that avirulence was related to growth in vitro, still race UN6-NA65-19 which is more virulent on the differentials did not appear to grow as well as either race UN1-NA65-1 or race UN1-NA65-9.

The results of the various experiments reported here clearly point out that the pH of the medium, peptone source, and the physiological race of the pathogen were very important factors in development of growth in vitro. The stimulation of vegetative growth was attributed to the proper combination of these three variables. The results definitely indicate that Evan's peptone source was superior to the Difco peptone source in development of vegetative growth, at least of physiological race UN1-NA65-9 of Puccinia recondita tritici. Similarly, a pH of 6.0 was far superior to pH 6.4 for in vitro culture of this organism. How-

ever, a pH of 6.0 cannot be considered the optimum since no pH levels lower than 6.0 were investigated. Lastly, it also was clearly evident that certain races performed better in culture than others. However, the interaction between race, pH, and peptone source was not investigated further.

Evaluation of what has been accomplished shows there is still much to be done. Williams et al. in 1967 pointed out three conditions that must be fulfilled before successful culture of an obligate parasite on artificial media can be accepted (17). This study did not completely fulfill any of the conditions. (1) The pathogen was cultivated apart from the host but did not produce any characteristic fruiting bodies, and could not definitely be identified. (2) Reinfection of the host with material produced in artificial culture was attempted but was not successfully accomplished. This, however, may be just a matter of dexterity or technique rather than complete failure of infection. Nevertheless, without this step (3) the pathogen could not be reisolated from the host and studied on artificial media.

Regardless of all of the above facts, a greater degree of differentiation occurred on the specialized media than occurred on water agar alone. Presumably, then some additional growth occurred as a result of the aquisition of food for energy from sources other than reserves in the urediospore itself. Formation of the globose type of cells was also considered to be evidence that the fungus was carrying on metabolic processes and was growing. It is possible these structures were the beginning of a spore form. Certainly a greater degree of in vitro vegetative growth of Puccinia recondita tritici occurred in these studies than

reported before, although additional work is needed to attain fructification.

SUMMARY

1. A technique was devised to surface sterilize wheat leaves in order to obtain uncontaminated urediospores of *Puccinia recondita* f. sp. *tritici*.
2. Benzimidazole agar was found satisfactory for supporting growth of detached wheat leaves.
3. The in vitro vegetative development of *Puccinia recondita* f. sp. *tritici* was influenced by:
   (a) the race used
   (b) pH of the growth medium
   (c) the peptone source
4. Greater vegetative differentiation and development was obtained than previously reported for *Puccinia recondita tritici* in vitro.
5. The development of globose cells in the developing germ tube in vitro was observed.

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A P P E N D I X

APPENDIX

Table I. Composition and preparation of media used for in vitro culture of races of *Puccinia recondita tritici*.

Medium I

35g Czapek Dox Broth, Difco
1g Bacto-peptone, Difco
1g Yeast extract, Difco
20g Bacto-Agar, Difco
1000ml Distilled water
Autoclaved 20 min at 121 C.

Medium IA

Same as Medium I, but autoclaved for 30 min at 121 C.

Medium II

35g Czapek Dox Broth, Difco
1g Bacto-peptone, Difco
20g Bacto-agar, Difco
1000ml Distilled water
Autoclaved 20 min at 121 C.

Medium III

1g Bacto-peptone, Difco
30g Glucose, Nutritional Biochemical Co.
4g Czapek minerals (prepared according to Ains. & Bisby).
20g Bacto-Agar, Difco.
1000ml Distilled water.
Autoclaved 30 min at 121 C.

Medium IV

1g Bacteriological Peptone, Evan's
35g Czapek Dox Broth, Difco
1g Yeast extract, Difco
20g Bacto-Agar, Difco
1000ml Distilled water
Autoclaved for 30 min at 121 C.

Water Agar

20g Agar

1000ml Distilled water
Autoclaved for 30 min at 121 C.

VITA

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