STUDIES ON THE BIOLOGY
OF
ECHINODONTIUM TINCTORIUM, E. AND E.

BY

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STUDIES ON THE BIOLOGY OF ECHINODONTIUM TINCTORIUM, E. AND E.

INTRODUCTION.

Echinodontium tinctorium E. and E. or the Indian Paint Fungus as it is commonly called, is responsible for considerable loss in the forests of the interior of the province of British Columbia. It is found only in the western part of North America and Japan. (9). Hubert (9) states that "the rot caused by this fungus is one of the most destructive of its kind. It is the principle rot of western hemlock, alpine fir and lowland fir, although species of other genera (such as Englemann spruce) are occasionally reported as hosts."

"It usually occurs as a trunk rot, uniformly circular when viewed at the ends of the logs. In older trees the rot occupies the entire heart-wood of the roots and larger branches. Very frequently the trunk is reduced to a mere shell of sap-wood."

Rot description. "A hidden stage of decay showing little or no discolouration is commonly found developing for some distance ahead of the early stage of decay. This hidden stage usually extends from one to five feet beyond the faintest discolouration. The first recognizable sign of early decay is a softening of the wood accompanied by a faint yellow discolouration which gradually deepens to a pale reddish brown. Fine reddish brown to rusty red lines forming irregular outlines are often distributed through the early decay."

Late stage. "In this stage the wood becomes soft, stringy, brownish, reddish brown or rusty red in colour, frequently showing rusty red streaks. Brownish to reddish zone lines are often present and at times the rot shows white patches in the brownish coloured rot. There is a frequent tendency to ring rot or a separation of the wood along the annual rings. In the very late stages of decay the butt section of the trunk may become hollow."

In addition to the above symptoms, areas having a water soaked appearance are common just outside the yellowish zone while a bluish area usually exists between the yellow and brown areas.

The causal organism is one of the bracket fungi belonging to the Basidiomycetes. It is of the order Hymeniales, family Hydnaceae and genus Echinodontium. This genus is characterized by the woody spines on the under surface of the sporophores. The scientific name is Echinodontium tinctorium, E. and E. the common name Indian Paint Fungus.

The pathology of Echinodontium tinctorium has been worked out very thoroughly by Weir and Hubert, (19) who demonstrated the constant association of the typical symptoms with the sporophores on western hemlock. Hubert, (9) reproduced the field symptoms by inoculating sterilized white fir blocks with the mycelium isolated from infected wood and from sporophores, leaving little doubt as to the identity of the causal organism.

A number of papers (2), (12), (15), (21), (22), have been published on the physiology and biology of many of the common wood-destroying fungi, dealing in detail with all phases of the development. Up to the present Echinodontium tinctorium has not been given a great deal of attention. Schmitz, (14) did some work in connection with the enzymes produced by this organism. It has been studied in culture along with other wood-rotting fungi by Fritz (5), and other workers, but a detailed study was not attempted. As it is the only hydnaceus fungus of its kind and the only member of its genus, (19) it was thought that a study of the biology of this fungus and a comparison with other wood-destroying fungi would provide an ample field for work.

The material for the study of this organism was obtained through the courtesy of the Research Division of the Forest Branch at Victoria, from their Forest Experimental Station at Aleza Lake, B. C. The material received consisted of sections from infected trees, showing all the characteristic symptoms, and sporophores found on these trees.

The fungus was readily isolated by aseptic methods. The infected wood was split and small sections removed with a sterile chisel from the freshly exposed surface. As Nutman, (13) Mounce, (12) and others have found that malt agar was the most suitable medium for the growth of wood-destroying fungi, these sections were planted on 'Difco' malt agar. The medium was prepared by using 33.36 gms. of the synthetic medium in 1000 ccs. of distilled water. The sporophores were also used for making isolations. They were split as in the case of the wood sections and the tissue from the freshly exposed surface used as inoculum. Isolations were also made from the spines. These were dipped for 10 seconds in 70% alcohol and then for 1 minute in an aqueous solution of mercuric chloride, (1:1000). The spines were then washed in sterile water, cut into small sections with a flamed scalpel and planted on the malt agar.

In making the original isolations, sections were taken from different zones of the rotted heartwood, represented by the different colours. The following were the sources:

1. Apparently normal wood outside the yellow zone and next to the sapwood.
2. Yellow area next to the normal wood.
3. Blue areas.
4. Water soaked areas in the normal wood.
5. Brown areas in the centre.
7. Sporophores, context and spines.

Echinodontium tinctorium, was obtained from all these sources. In the apparently normal wood however, only one piece produced mycelium. Growth was very slow in all cases and it was nearly a month before the mycelium had grown out on the agar sufficiently to be identified so that stock cultures could be made. The sporophores proved to be the best source, since not only was there less contamination but growth seemed to be more rapid in starting. From the punky rot in the centre no less than seven different organisms were found including E. tinctorium. A small amount of contamination occurred in cultures from the other zones but appeared to be less frequent when isolations were made from sections nearest to the normal wood.

In order to ascertain that the organism isolated was E. tinctorium, comparisons were made with authentic cultures obtained through the courtesy of Dr. Mounce.[1] Each organism was sown on 'Difco' potato dextrose agar, (40 gms. in 1000 ccs.) 'Difco' prune agar, (24 gms. in 1000 ccs.) and 'Difco' malt agar, (33.36 gms. in 1000 ccs.) In all cases the resulting medium was hard and firm. This type of medium is recommended by Etter, (4) as more favorable to the growth of wood-destroying fungi than a soft, watery medium.

The cultures from the wood and from the sporophore were practically identical with the authentic culture. Microscopic examination showed the same type of mycelium in all cases. The filaments are long, moderately branching and from 3.5 u to 5.6 u in width. Clamp connections appear at most of the septa which are fairly abundant. The aerial mycelium is generally hyaline and regular, while that from under the surface of the agar is dark, very irregular and usually bears chlamydospores.

In this test, prune agar proved to be by far the best medium. The mycelium had reached the edge of the petri dishes before it was more than half way across in the

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case of the malt agar and three quarters in the case of the potato dextrose agar.

In pure culture E. tinctorium, grows very slowly over the surface of the agar. A dark stain appears about the first week and diffuses through the agar ahead of the mycelium. There is a slight appearance of zonation especially when the fungus is observed through the bottom of the petri dish (Pl. 3). The mycelium is white at first turning dark yellow to light brown with age. There is prolific aerial growth with a tendency for massing in compact lumps particularly in the centre of the petri dish. Drops of a brownish watery liquid are frequently seen emerging from these masses. There is a very characteristic odour when the petri dish is opened. This is comparable to the odour emanating from the fresh white undersurface of the common Fomes pinicola Fr.

The Ph range of *Echinodontium tinctorium*, E. and E.

Zeller, (22) working on *Lenzites saepiaria*, *Fomes pinicola*, *Polystictus hirsutus*, *Polyporus lucidus* and others found that these fungi would not grow on alkaline media. Miss Mounce, (12) used media ranging from Ph 4.2 to Ph 6.2 in her study of *Fomes pinicola*, and refers to the work of Rumbold, who found that this organism would not grow under alkaline conditions. Wolpert, (18) found that the major portion of the growth curves of *Daedalea confragosa*, *Armillaria mellea*, *Pholiota adiposa*, *Polyporus adustus* and *Lenzites saepiaria*, is on the acid of neutrality and in the majority of cases wholly on the acid side. As it was thought desirable to compare the Ph range of *Echinodontium tinctorium*, with that of these other wood-destroying funge, a series of cultures was made up as follows:

Double strength potato dextrose agar was prepared according to the following formula:[1.]

1. This method of preparing a Ph series is taken from an unpublished manuscript by F. Dickson and J. W. Sinden.

Peeled potatoes-----400 gms.

Dextrose----------20 gms.

Sodium chloride-------5 gms.

Agar----------30 gms.

Distilled water----1000 ccs.

The potatoes were cut into cubes about 1 cm. in dimension and boiled until just soft. The extract was then decanted off. The agar was washed, soaked and added to the extract, which was then cooked until the agar had completely dissolved. The dextrose and sodium chloride were added and the solution made up to volume. It was then strained through cotton wool and cheesecloth. Erlenmeyer flasks of 250 ccs. capacity were used as containers, 80 ccs. of medium being added to each flask by means of a burette. The flasks were numbered from 1 to 15 and plugged with cotton wool.

Solutions (.1N) of hydrochloric acid and sodium hydroxide were next prepared and titrated against each other. They proved to be of exactly the same normality. A series of flasks were made up containing dilutions of these solutions as shown in table 1. These flasks were numbered in the order given in the table, plugged with cotton wool and sterilized for 20 minutes at 15 lbs. pressure together with the flasks containing the double strenght agar.

The flasks were removed from the autoclave, and the agar from each flask poured into the flask of acid or alkali bearing the corresponding number. The mixture was shaken thoroughly and immediately poured into petri dishes, about 20 ccs. to a petri dish. A sample was saved from each flask for a Ph determination which was made later by me means of a potentiometer. Care was taken to allow the flasks at the ends of the series to cool as much as possible before mixing. If these are mixed while too hot the agar at the acid end of the range will not solidify while at the alkaline end the sugar in the medium will be caramalized. Care must also be taken not to allow the agar to solidify before pouring as remelting will change the Ph. There is also a danger that the agar will not resolidify. In this experiment all plates solidified normally and none were caramalized

All Ph determinations were made by the 'Quinhydrone-calomel electrode method' A portion of the sample of agar saved for making the determination was macerated in a small vial with a little quinhydrone. The hard agar was used in order to avoid any possible change in Ph by melting. The gold leaf of the electrode was dipped in the macerated agar and the reading taken in millivolts. All determinations were made in triplicate. The average reading in millivolts was calculated and the Ph for this reading taken from the tables. After each reading the electrode

was thoroughly washed in distilled water before being used again. The Ph of the agar from each flask is given in table 1.

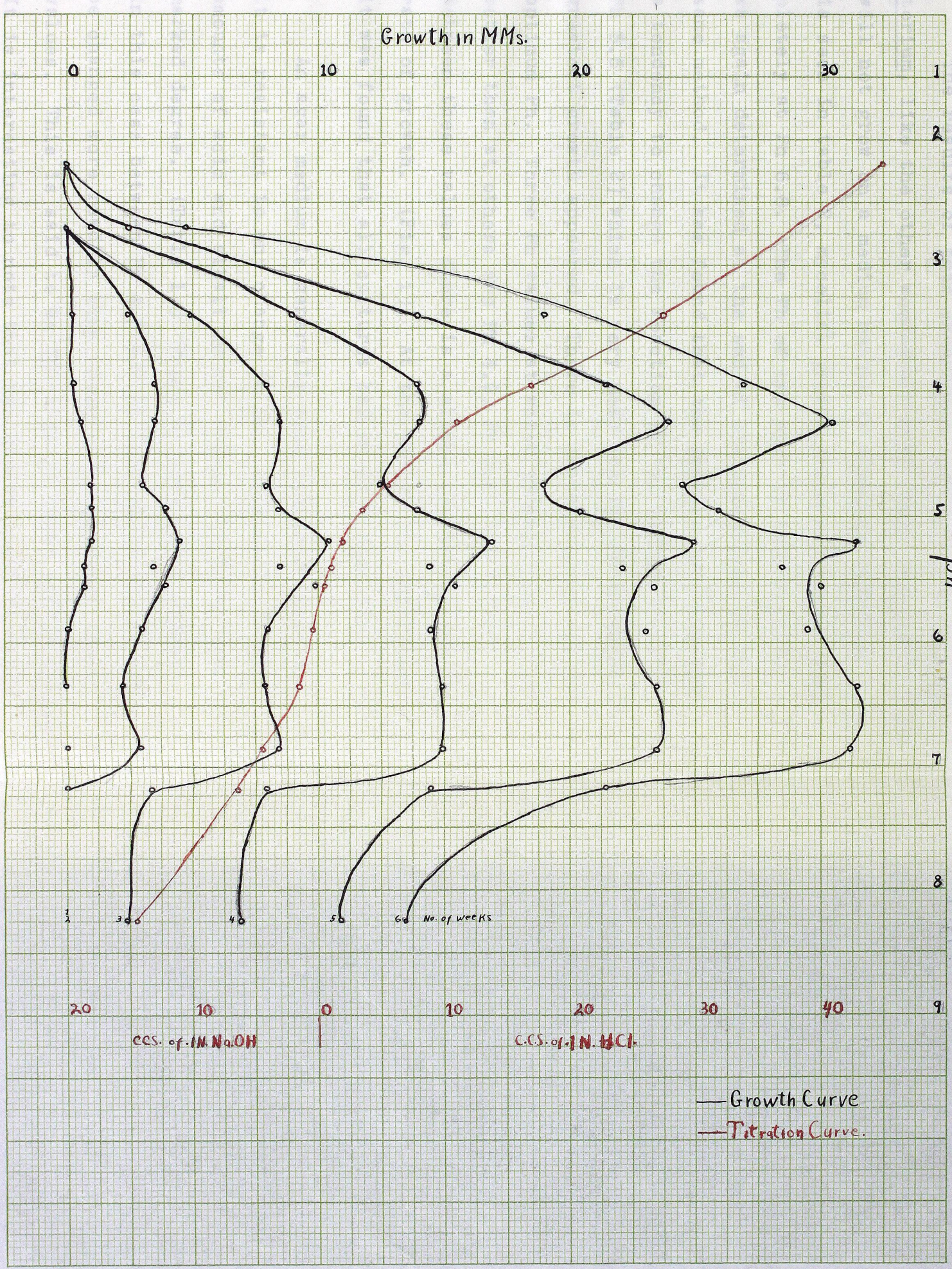
TABLE 1. Showing the amount of .1N. HCl, .1N. NaOH and water which were added to each 80 ccs. of the double strength agar and the resulting Ph values.

Flask No.	Ccs. of .1N. solutions.		Ccs. of water.	Resulting. Ph of agar.
	HCl.	NaOH.		
1.	44.5	----	35.5	2.20
2.	27.5	----	52.5	2.68
3.	17.2	----	62.8	3.40
4.	11.2	----	68.8	3.95
5.	8.0	----	72.0	4.25
6.	5.2	----	74.8	4.77
7.	3.3	----	76.7	4.94
8.	2.0	----	78.0	5.20
9.	1.0	----	79.0	5.42
10.	0.4	----	79.6	5.55
11.	-----	0.4	79.6	5.91
12.	----	1.7	78.3	6.35
13.	----	3.7	76.3	6.85
14.	----	6.4	73.6	7.20
15.	----	14.5	65.5	8.30

The plates were inoculated in duplicate with uniform pieces of mycelium taken from the edge of an actively growing culture of E. tinctorium. At the end of each week the edge of the growth was marked on the bottom of the petri dish with India ink. After six weeks the mycelium of the most actively growing cultures had reached the edge of the petri dishes. The growth at the end of each week was then measured by taking the distance between each ring and the centre of the inoculum.
This distance was measured in several directions, the results averaged and plotted. These are shown in table 2 and are given in graph form in fig. 1.

TABLE 2. SHOWING THE GROWTH OF ECHINODONTIUM TINCTORIUM, AT THE END OF EACH WEEK IN CULTURES OF DIFFERENT Ph VALUES AND THE Ph VALUES OF THESE CULTURES AT THE CONCLUSION OF THE EXPERIMENT.

No.	Orig. Ph.	Final Ph.	Average Radial Growth in mms.					
			1st. week	2nd. week	3rd. week	4th. week	5th. week	6th. week
1.	2.20	2.50	Nil.	Nil.	Nil.	Nil.	Nil.	Nil.
2.	2.68	2.93	Nil.	Nil.	----	2.5	3.5	4.5
3.	3.40	3.62	----	2.5	5.0	9.0	14.0	19.0
4.	3.95	4.33	----	3.5	8.0	14.0	21.5	27.0
5.	4.25	4.72	----	3.5	8.5	14.0	24.0	30.5
6.	4.77	4.98	1.0	3.0	8.0	12.5	19.0	24.5
7.	4.94	5.18	1.0	4.0	8.5	14.0	20.5	26.0
8.	5.20	5.39	1.0	4.5	10.5	17.0	25.0	31.5
9.	5.42	5.46	----	3.5	8.5	14.5	22.2	28.5
10.	5.55	5.53	----	4.0	10.0	15.5	23.7	30.0
11.	5.91	5.60	----	3.0	8.5	14.5	22.8	29.5
12.	6.35	5.68	Nil.	2.5	8.0	15.0	23.5	31.5
13.	6.85	5.93	Nil.	3.0	8.5	15.0	23.5	30.0
14.	7.20	6.40	Nil.	----	3.5	8.0	14.7	21.5
15.	8.30	6.72	Nil.	----	2.5	7.0	11.0	16.0


Growth in MMs.
0
10
20
30
1
2
3
4
5
6
7
8
9
Ph
½
3
4
5
6
No. of weeks
20
10
0
10
20
30
40
c.cs. of .1N. NaOH
c.cs. of .1N. HCl
Growth Curve
Titration Curve.

The results of this experiment indicate that E. tinctorium, like the other wood-destroying fungi mentioned above will not grow on a medium having an alkaline reaction. As is seen in table 2, mycelial growth falls off almost completely at Ph 6.8. The Ph of the agar in each culture wa again determined at the end of the experiment by the same method. It was found that the growing mycelium had a tendency to change the reaction of the medium towards Ph 5.5 (Table 2) which is very near the the Ph at which greatest growth appeared. There is apparently no single optimum Ph. Three Ph values at which growth seems to be better than at others are Ph 4.2, Ph 5.2, and Ph 6.2. Between these values there is a slight falling off in the rate of growth. (Fig. 1). At the end of the experiment it was found that all cultures had an acid reaction.

An agar medium is generally well buffered, that is, it is resistant to any change in Ph. Relatively large amounts of acid and alkali do not change the Ph to any marked degree. (Table 1.) There is however, a region, in this case between Ph 4.0 and Ph 7.0, where the Ph can be changed more readily than above or below these these values. This is said to be the area of least buffer action. It is interesting to note that the growth curve flattens out in this area, but falls of steeply at each side to correspond with the titration curve. (Fig. 1). The

explanation of this is probably that the growing mycelium has a tendency to adjust the Ph of the medium towards the optimum. (Table 2). In the area of least buffer action this change will be more readily effected so that although the original Ph values had a comparatively wide range, after growth had once started they would be almost the same. This is seen to be the actual case. (Table 2.) Outside of this zone of least buffer action the mycelium cannot change the reaction of the medium so readily and so the growth curve falls off rapidly.

Heterothallism in *Echinodontium tinctorium*, E. and E.

In 1918 Mlle. Bensaude, (12) working with Coprinus lagopus, proved that heterthallec species exist among the Basidiomycetes. Miss Newton, (12) found heterothallism in another member of the genus, namely Coprinus rostrupianus. Miss Mounce, (12) was the first to demonstrate the presence of sexuality in a large wood-destroying polypore in her study of *Fomes pinicola* Fr. "Both Bensaude and Kneip have shown that in a mycelium bearing clamp connections the nuclei occur in pairs. That is the mycelium is in the diploid condition. Thus the absence of clamp connections on compound mycelia resulting from the union of two monosporous mycelia of opposite sex provide a reliable criterion for the determination of heterothallism. (12) " In order to determine whether *Echinodontium tinctorium*, was also heterothallic a number of single spores were isolated. The presence of clamp connections in paired - and - monosporous cultures and their absence in single monosporous cultures was used as evidence of heterothallism.

The isolation of single spores proved to be rather a difficult task. Spore suspensions were readily obtained

by mascerating the spines in a petri dish containing sterile water. Two types of spores were present in this suspension. The majority were smooth elipsoid, hyaline spores measuring about 4 u by 6 u. The others were the same except that instead of being hyaline they were distinctly brown. To isolate these spores a suspension was put in an atomizer and sprayed on a glass plate. The drops were examined microscopically and the suspension adjusted so that on the average one spore was found in each drop. This suspension was sprayed on a thin film of agar on a petri dish. The drops were examined through the bottom of the dish with the low power of the microscope. About 50 drops containing single spores of both types were ringed. Not only did these spores fail to germinate but no growth appeared on any of the plates from the unringed spores. Drops of the spore suspension were mounted in van-Tieghem cells and carefully watched but no sign of germination was observed. Slides placed under sporophores in a moist chamber also failed to yield germinating spores.

It is interesting to note at this point that White, (21) experienced great difficulty in obtaining germinating spores of Fomes applanatus.(Pers.) Wallr. In his germination studies he used the hanging drop method in van-Tieghem cells. Solutions of the different sugars, of acid and alkali, of

peptone and of wood decotions were tried with indifferent success. He was unable to distinguish any difference between viable and unviable spores but did notice that some sporophores produced more viable spores than others. He estimates that only a small fraction of 1% of the spores germinate.

In a final effort to obtain germinating spores of Echinodontium fresh sporophores were obtained. Suspensions of the spores from these were made in dilute sugar solutions, in distilled water and in extracts from amabalis fir. Drops of these suspensions were mounted in van-Tieghem cells and watched carefully for several days but no germinating spores were observed. Finally a thin film of agar was poured into a number of petri dishes and the most likely looking spines attached to the lid by means of a drop of balsam so that they would shed their spores on the surface of the agar. These could be seen through the bottom of the petri dish with the low power of the microscope. When a sufficient number had been shed in one spot they were ringed and the lid turned so that the next spores would be shed in a different spot. These spores were examined daily for about 10 days but no germination was evident. A final examination before discarding the plates resulted in the finding of one or two germinated spores, and at the end of three weeks about

25 germinated spores had been isolated. The germ tube grows very slowly and spores germinated three or four days previously were at the right stage for isolation.

Isolations were very difficult to make as the spores are shed very profusely and only a very small percentage germinate. In order to insure that only one spore was being taken the following technique was adopted. A capillary point was made by flaming a glass tube and drawing it out to the desired shape. This was mounted in a cork which was then screwed into the microscope in place of the oil immersion lens. This was adjusted so that a spore could be picked out with the high power, centred in the field and then the nose piece revolved so that by screwing down carefully the spore could be ringed by the capillary tube.[1] The ringed section of agar was then picked out under the binocular and planted on an agar slant. This proved to be the most satisfactory method although many spores were lost.

It was observed that only spores from certain spines germinated. These spines were traced back to a single sporophore. The spines of this sporophore were covered with the

mycelium of Echinodontium tinctorium. Whether some of the spores had germinated in situ—probably due to the moist conditions under which sporophores were packed--or whether the trama of the spines had started to grow is not known. It was from those spines covered with mycelium that practically all the germinating spores were obtained.

The mycelium originating from these spores was of several different types. These types ranged from normal, to a very dark, slow growing, closely appressed growth with irregular mycelium and numerous chlamydospores. Other cultures ranged between these extremes in appearance. Miss Mounce, (12) found that the mycelium from monosporous cultures of Fomes pinicola Fr. had a wide range of variation. She attributed this to individual and racial variation. These cultures, (Fomes pinicola) when grown side by side in a petri dish, except in two cases formed a definite line of aversion even when of opposite sex. It was only when a mycelium was paired with itself that no line of aversion was formed.

As the appearance of the monosporous cultures of E. tinctorium suggested that there was distinct racial variation, they were planted in pairs on plates of 'Difco' potato dextrose agar. This agar was made up with the extract from

the heartwood of amabalis fir (The extract from 200 gms. of wood in 1 litre of water) instead of distilled water. In pairing the mycelia, a small piece of agar bearing the actively growing mycelium from a monosporous culture was planted near the centre of the petri dish. Another piece of agar bearing the mycelium from another monosporous culture was planted about an inch and a half from the first piece. The monosporous cultures were numbered and all possible combinations were used. In two cases the mycelium was paired with itself. A distinct line of aversion appeared in all but these two cases (Pl. 3). In these the two mycelia blended as one. These results suggest that Echinodontium tinctorium, like Fomes pinicola, gives rise to races with a natural aversion to one another. No reason can be suggested for this.

Except in one or two cases the mycelia from the monosporous cultures had no clamp connections. In these cases we had reason to believe that the cultures were not from a single spore. When the cultures were paired as described above in several cases clamp connections were found in the paired cultures and were particularly abundant where the cultures came in contact. Camera lucida drawings of the growing mycelia (Plate 2) shows mycelia from the edge of the culture away from the line of aversion, (Fig 1.) the

mycelia at the line of aversion (Fig. 2) and mycelia growing out from the meeting point of the cultures, (Fig.3) when the single spore isolations, were being made, in several cases a number of spores were taken together. Clamp connections were found in several of these cultures. In the cultures which we were reasonably sure were monosporous no clamp connections were found. These observations though not definite or conclusive leave little doubt in the writer's mind that Echinodontium tinctorium is a heterothallic species.

THE PRODUCTION OF SPOROPHORES IN CULTURE

Wood-rotting fungi as a general rule do not produce fruiting bodies in ordinary culture. Typical sporophores can not be developed inside containers but seem to need air, and therefore a porous medium is necessary (3). Miss Mounce, (12) found that blocks of wood about 2' by 2" by 4" placed obliquely in 2 quart jars on layers of saturated cotton were the most satisfactory for producing sporophores of Fomes pinicola. White, (21) working on Fomes applanatus found that this fungus would not fruit on an agar medium but only on sterilized wood in large containers.

In an endeavour to induce Echinodontium tinctorium to produce fruiting bodies in culture a number of sections of balsam fir about 1" by 1" by 5" were selected and placed in quart sealers. About 2" of cotton wool was placed in the bottom of each jar. Sufficient half-strength 'Difco' prune agar, (12 gms. in 1000 ccs.) to saturate the cotton was added. The object of this was to provide the fungus with plenty of food material and moisture. The jars were plugged with cotton wool and then autoclaved for 30 minutes at 15 lbs. pressure. Both eastern balsam (A. balsamea) and

western balsam (A. amabalis) were used in this experiment.

The wood was inoculated by placing pieces of agar bearing mycelium from an actively growing culture at the base of the blocks. The fungus grew rapidly over the surface of the agar and then onto the blocks until the whole surface of the exposed heart and sap wood was covered. The fungus did not grow over the bark, which was present on some of the blocks, to any appreciable extent. On one of the blocks of A. amabalis however the mycelium began to mass on the bark and after 12 months had formed a mass about 1 cm. wide by 1 cm. high by 2½ cm. long. This was thought to be a young sporophore. It was dirty white in colour, becoming darker towards the base, while the context was a reddish brown.

THE GROWTH OF ECHINODONTIUM TINCTORIUM, E. AND E. ON WATER SOLUBLE EXTRACTS OF DIFFERENT WOODS.

In the above experiment there was no growth in the cultures containing the blocks of Abies balsamea. Growth was completely inhibited (except in one case where there was a very slight growth on the agar) even on the surface of the agar. This suggested that some water soluble substance, toxic to Echinodontium tinctorium, was present in Abies balsamea, It was thought that it would be worth while to run a toxicity test on the extracts of the different woods, in particular of Abies balsamea, to see if a diffusable toxic substance were present and if possible to identify it.

The chemical products of a tree include such substances as tannins, resins, dyes, alkaloids, gums and oils. These substances are considered to be an integral part of the cell. They usually occur in the xylem and they are not consistent but vary with the species of wood. (1). Many of these substances are soluble ar partly soluble in water. It has been shown that woods naturally resistant to decay contain water soluble extractives in large amounts,

whereas, woods susceptible to decay are very deficient in these extractives. (7). The relative durability of heartwood and sapwood of various species has not been thoroughly worked out (1). Such workers as Hawley, (7) have found that heartwood extracts are more toxic than the corresponding sapwood extracts and it is generally conceded that heartwood is less susceptible to decay than sapwood. In as Much as Abies balsamea, appeared to contain some diffusable substance definitely toxic to E. tinctorium, it was decided to set up a series of cultures on media containing the water soluble extracts of the different woods.

The woods selected for this experiment were Abies grandis, (grand fir) Abies amabalis, (amabalis fir) and Thuja heterophylla, (western hemlock) which are listed as common hosts; Pseudothuja taxifolia, which is a rare host; Pices sitchensis, (sitka spruce) and Thuja occidentalis, which are not naturally susceptible and Abies balsamea, which is not listed as a host. The heartwood and the sapwood (bearing bark) of these trees were extracted separately. In order to obtain the extracts 40 gms. of the oven dried wood were cut in shavings and put in large Florence flasks. To each flask was added 150 ccs. of distilled water. The flasks were then placed in the autoclave for 30 minutes at 20 lbs. pressure. The solutions were allowed

to stand for 24 hours and again autoclaved at the same pressure and for the same time as before. The extracts were filtered and made up to 100 ccs. To 60 ccs. of these extracts were added 2.4 gms. of 'Difco' potato dextrose agar. The flasks containing the extracts plus the agar were then autoclaved for 15 minutes at 15 lbs. pressure. They were then poured into petri dishes, about 20 ccs. to a petri dish. A small sample from each flask was saved for a Ph determination which was made later by the 'Quinhydrone, Calomel electrode' method. The plates were inoculated with uniform pieces of agar containing the actively growing mycelium from the edge of a petri dish culture. The margin of the mycelial growth was marked in India ink on the bottom of the petri dish at the end of each week, as in the Ph determination experiment. After six weeks the mycelium in the most actively growing cultures had reached the edge of the plates. The radial growth at the end of each week was then measured as before and the results tabulated as in table 3.

TABLE 3. SHOWING THE AVERAGE RADIAL GROWTH IN MMS. OF ECHINODONTIUM TINCTORIUM, AT THE END OF EACH WEEK ON 'DIFCO' POTATO DEXTROSE AGAR - WATER SOLUBLE EXTRACTS OF DIFFERENT WOODS.

Culture No.	Extract from.	Average Radial Growth in mms.						
		Ph of Ext.	1st. week	2nd. week	3rd. week	4th. week	5th. week	6th. week
1h.[1]	Thuja occidentalis	4.75	----	Nil.	Nil.	Nil.	Nil.	Nil.
1s.	"	4.67	6.0	14.0	23.0	31.5	44.0	54.0
2h.	Abies amabalis	4.92	5.0	11.0	21.5	31.5	41.0	51.5
2s.	"	4.75	4.0	10.0	20.0	33.5	45.0	56.0
3h.	Abies grandis	4.90	8.0	16.5	25.0	33.5	47.0	58.5
3s.	"	4.67	3.0	6.5	17.5	28.5	40 0	48.0
4h.	Abies balsamea	4.62	6.0	18.0	24.0	42.5	45.0	56.0
4s.	"	4.53	----	2.0	6.0	9.5	13.0	17.0
5h.	Thuja heterophylla	4.90	5.0	9.5	23.0	32.5	43.0	52.0
5s.	"	4.75	7.5	----	22.0	30.0	42.5	52.0
6h.	Pseudothuja taxifolia	4.70	6.0	14.0	24.0	33.5	44.5	53.0
6s.		4.50	----	8.0	19.5	28.0	38.5	45.0
7h.	Picea sitchensis	4.60	----	Nil.	Nil.	Nil.	Nil.	Nil.
7s.	"	4.52	----	----	----	5.0	----	7.0
8.	Distilled water	5.05	2.0	6.0	22.0	24.0	31.5	38.5

1. h--Extract from hearwood
s--Extract from sapwood (and bark).

From these results the following observations were made: 1. Echinodontium tinctorium appears to grow equally well on the extracts from the heartwood and from the sapwood (plus bark) of the susceptible trees namely, amabalis fir, grand fir, western hemlock and Douglas fir. 2. The fungus grow better when the extracts from these woods are added to the medium. 3. The heartwood of eastern balsam contains no water soluble toxic substance as normal growth occurred on this extract. There does however, appear to be a definite toxic substance in the sapwood and bark which inhibits the growth of the fungus to a marked degree. 4. The heartwood of cedar contains a water soluble toxic substance which is not present in the sapwood and bark. 5. Sitka spruce contains a water soluble substance both in the heartwood and sapwood which is inhibitory to the growth of E. tinctorium at this concentration.

Anderson, (7) found a definite difference in the toxicity of water soluble extracts from kiln dried and air dried yellow pine to Lenzites saepiaria. He suggests that the toxic substances are volatile. As in our experiment the blocks had been dried in the oven it was thought advisable to repeat it using extracts from air dried wood, and also more concentrated extracts.

In this second experiment 100 gms. instead of 40 gms. of wood were used so that 1 cc. of extract came from 1 gm. of wood. The results were the same as before with the exception that the growth was definitely better on the extracts from the sapwood and bark than on those from the heartwood. This is rather strange in view of the fact that we are dealing with an organism causing a heart rot. It does however corresponde with the results obtained by Anderson,(1) who found that although there was no pronounced difference in the relative toxicity of the two types of extract, in general the extract from the heartwood was slightly more toxic than the corresponding extract from the sapwood.

As a check on the apparent toxicity of the extracts from Abies balsomea, (eastern balsam) the following experiment was performed using only Abies balsomea and Abies amabalis (western balsam). About 30 gms. of the extracted shavings of each of these woods were further extracted by boiling in a litre of water for 30 minutes. This water was decanted off and the operation repeated twice. The shavings were then thoroughly washed and placed in small Erlenmeyer flasks. The each flask was added 10 ccs. of distilled water and 10 ccs. of Czapek's synthetic medium[1]. Shavings

1.Czapek's Synthetic Agar.
0.5 g. magnesium sulphate. (Cont. on page 29.)

of the normal wood of these two species were also placed in flasks only in this case 25 ccs. of water were added instead of 10 ccs. This was to allow for the absorption of water by the wood. All flasks were plugged with cotton wool and autoclaved for 15 minutes at 15 lbs. pressure. They were inoculated with actively growing mycelium from an agar culture and placed in an incubator at 22°C. After four months the flasks were examined and the growth in each recorded. The following observations were made.

1. Eastern balsam,--extracted heartwood----no growth.
2. " " -- " sapwood------ " "
3. " " --normal heartwood---- " "
4. " " -- " sapwood------ " "
5. Western balsam,--extracted heartwood----fair growth.
6. " " -- " sapwood------abundant growth.
7. " " --normal heartwood----moderate growth.
8. " " -- " sapwood------abundant growth.

This experiment was set up in triplicate and the results were uniform throughout. From these observations there appears to be little doubt that eastern balsam contains some substance definitely toxic to Echinodontium tinctorium. Relatively large pieces of agar were used to inoculate all flasks. There were placed with the mycelial

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(Cont. from page 28.)

1.0 g. mono-potassium phosphate.
0.5 g. potassium chloride.
0.01 g. ferrous sulphate.
2.0 g sodium nitrate.
30.0 g. dextrose.
25.0 g. agar.
1 litre distilled water.

covered surface downwards. In the case of the western balsam the mycelium grew first on the upper surface of the inoculum. In that of eastern balsam there was no growth even on the inoculum. It appears that the toxic substance is not completely soluble in water as there was no growth even on the extracted wood. The abundant growth on the sapwood of western balsam is what we would expect in view of the results of the previous experiment.

The fact that Echinodontium tinctorium grew on the extract from that wood to some extent, and not on the wood itself lead us to the conclusion that the toxic substance is only slightly soluble and unless comparatively large quantities of wood are used as was the case in the cultures made up for the purpose of sporophore production is not concentrated enough to inhibit growth.

THE RELATION OF HEARTWOOD AND SAPWOOD AND SATURATED AND UNSATURATED WOOD OF DIFFERENT SPECIES TO DECAY BY E. TINCTORIUM.

The method adopted by Zeller, (22) Schmitz, (15) Snell (16) and others to determine the wood destroying proclivities of a fungus is as follows: The wood is cut in blocks and dried to a constant weight. It is then placed on a layer of saturated cotton or sawdust in large containers, sterilized and inoculated with the mycelium of the fungus. After a certain period the blocks are again dried to a constant weight. The loss in weight is the weight of the wood which has been destroyed and is a criterion of the wood destroying proclivity of the fungus.

There are many factors which influence the amount of decay brought about by wood rotting fungi. Temperature, water content of the tracheids, (in that it has a direct bearing on the air content) texture of the wood, resins, etc. all affect the growth of the fungus and hence the rate of decay. Westerdijk, (20) says in regard to wood destroying fungi in the tropics: "The heavy reanfalls, combined with the abundant transpiration--owing to the intense heat, must cause a high water-content and a small air content of the wood-vessels of the trees, thereby making a substratum poor

in air. This fact, combined with the high temperature, would explain the rare occurrence of Hymenomycetae and other wood-destroying fungi in the tropics." Zeller, (22) found that although Lenzites saepiaria grew abundantly over the surface of saturated blocks the mycelium did not penetrate more than two or three tracheids until the blocks had started to dry. Snell, (16) found that saturated wood was not so susceptible to decay as unsaturated wood. Schmitz, (15) working with Fomes pinicola, found that the sapwood of Pinus ponderosa, was more readily decayed than the corresponding heartwood. Hubert, (10) says that in general heartwood is more durable than sapwood. In connection with the previous experiment it was thought desirable to run a series of cultures to get some idea of the rate of decay induced by Echinodontium tinctorium, on different species of wood. In conjunction with this it was decided to compare the rate of decay in heartwood and sapwood, and in saturated and unsaturated blocks of these woods.

Accordingly twelve blocks aproximately 1" by 1" by 1" were cut from each wood chosen. These were cut from both the heartwood and sapwood. They were numbered by burning with a hot iron and dried at 80°C. to a constant weight. Six blocks (3 of heartwood and 3 of sapwood) of each wood were then boiled until saturated. These were placed in

pint sealers the bottoms of which were covered with cotton wool. 20 ccs. of distilled water and 10 ccs. of standard 'Difco' potato dextrose agar were added to each sealer. This was just enough to saturate the cotton. The blocks sere placed in the jars so that each block was in direct contact with the saturated cotton.

The remaining six blocks were not boiled but placed in sealers as above, 40 ccs. of water being added instead of 20ccs. This was to allow the wood to absorb a certain amount and still leave the cotton saturated. All jars were then plugged and sterilized for 20 minutes at 15 lbs. pressure. Each jar was inoculated in two places with a large piece of agar containing the actively growing mycelium. To further insure equal inoculation 5ccs. of a suspension of mycelium obtained from actively growing cultures was poured over the surface of the blocks by means of a sterile pipette.

After five and one half months the blocks were taken out. The mycelium was rubbed off as much as possible and the blocks again dried at 80°C. to a constant weight. The loss in weight was taken as a criterion of the destructive power of E.tinctorium on the different woods.

The mycelium grew rapidly over the saturated blocks

and had a tendency to mass in definite areas. The context of these masses was orange in colour. On the unsaturated wood the surface growth was not nearly so luxurient, but the blocks themselves seemed to be more decayed. This was found to be the case when the final weights had been taken. On two of the blocks of Abies amabalis the typical red zone lines found in natural infactions were observed.

The fungus grew well on the blocks of grand fir, (A. amabalis) amabalis fir, (A. amabalis) Douglas fir, (Pseudotsuga taxifolia) and western hemlock (T. Heterophylla). The results of this experiment are tabulated on table 4.

TABLE 4. SHOWING THE RELATIVE LOSS IN WEIGHT OF HEARTWOOD AND SAPWOOD, OF SATURATED AND UNSATURATED WOOD AND OF VARIETIES, FIVE AND ONE HALF MONTHS AFTER INOCULATION WITH ECHINODONTIUM TINCTORIUM.

				Significance of Results			
Species.	Saturated or unsaturated	Av. % loss in wt. in gms. with probable error. Heartwood	Sapwood	Heartwood vs sapwood	Saturated vs unsaturated Heartwood	Saturated vs unsaturated Sapwood	Order of susceptibility based on significance of differences between specie (Unsat. wood).
Abies amabalis	saturated	4.13 ± .14 [1.]	7.12 ± .28	+	-----	----	1.
	unsaturated	discarded [2.]	discarded	-------	-----	----	
Abies grandis	saturated	0.96 ± .03	4.24 ± .17	+	+	+	2.
	unsaturated	2.41 ± .10	5.24 ± .21	+			
Thuja heterophylla	saturated	1.05 ± .03	2.65 ± .10	+	+	+	3.
	unsaturated	2.33 ± .09	3.32 ± .13	+			
Pseudothuja taxifolia	saturated	0.77 ± .03	0.52 ± --- [3.]	-------	+	+	4.
		1.56 ± .06	3.45 ± .14	+			
Abies balsamea	saturated	negligible	negligible [4.]	-----	---	---	5.
	unsaturated	"	"	-------	---	---	

1. Foot notes on page 36.

In table 4. the varieties were compared be the 'deviation of the mean method'. The saturated and the unsaturated and the heartwood and sapwood were compared by 'Students method' (8). In each case three replications were used.

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1. A difference of three times the probable error is considered to be a significant difference.

2. The figures for the unsaturated wood of Abies amabalis, were discarded as for some reason the growth on the blocks was comparatively meagre and the loss in weight comparatively large. These blocks had been placed on the bottom of the drying oven and had a scorched appearance. All the other blocks had been placed on the shelf and were not scorched. By comparison with the results obtained for the other woods it is evident that these figures wood be even higher than those for the saturated wood.

3. In one replication the loss was negligible.

4. In Abies balsamea, the loss was .03%.

In the above experiment the heartwood and sapwood were in the same container which makes these results even more significant.

THE ENZYMES SECRETED BY ECHINODONTIUM TINCTORIUM.

Comparatively speaking, work on the enzymes of the wood-destroying fungi is very meagre, and relatively few papers have been published dealing with this subject. "In 1895 Bourquelot and Herissey investigated the enzymes from the juice of Polyporus sulphureus. Czapek, in 1899 found in natural infections of Merulius lacrymans, an active principle capable of liberating from lignin the substance which gives the lignin reactions in alcoholic extracts. This substance he called hadromal". (22). In 1906 Buller, (2) worked on the enzymes secreted by the sporophores of Polyporus squamosus, and discovered the presence of eight distinct enzymes. Zeller's, (22) work in 1916 in connection with the enzymes secreted by Lenzites saepiaria, is perhaps one of the most outstanding papers on this subject. In 1925, Schmitz, (14) published a paper on the enzymes secreted by Echinodontium tinctorium, in which he demonstrates the positive presence of twelve enzymes namely; esterase, maltase, lactase, sucrase, raffinase, diastase, inulase, cellulase, hemicellulase, urease, rennet, and catalase. It was thought however that it would be worth while to

repeat this work and if possible add to the above list.

The mycelium for making enzyme dispersions was obtained from the petri dish cultures from previous experiments. These experiments had all run for about eight weeks and the agar in the petri dishes was practically dry. The lids of these petri dishes were removed and the agar allowed to dry thoroughly. No contamination occurred on the rapidly drying agar. The mycelial mats were then readily scraped off with a scalpel. These mats were ground to powder in a meat chopper. 5 gms. of the powder was then extracted with 100 ccs. of distilled water for twenty four hours. The extract was then filtered, about 5ccs. of toluol added as an antiseptic and placed in the frigidaire at 0°C. until needed. An extract from 5 gms. of the powdered sporophore was made in a similar manner and used in all experiments to compare with the mycelial extract.

ENZYMES HYDROLYZING POLYSACCHARIDES, DISACCHARIDES AND MONOSACCHARIDES

DIASTASE. In testing for this enzyme 1 cc. of the enzyme dispersions was added to 20 ccs. of a five percent solution of soluble starch in small erlenmeyer flasks. A few drops of toluol were added as an antiseptic. As a control a

similar solution was made up only in this case the dispersion was first boiled. All flasks were then placed in the incubator at 28°C. After 12 hrs. 5 ccs. of each solution was added to 5ccs. of Fehling's solution and heated. The following results were obtained.

	12 hrs.	24 hrs.
Mycelial extract.	Heavy precipitate.	Very Heavy precip.
Sporophore extract.	Slight "	Heavy precip.
Control.	Very slight Precip.	Very sl. precip.

The slight precipitate of cuprous oxide in the control was due to reducing sugars present in the starch as impurities. The same amount of precipitate was obtained with the freshly made up starch solution. The complete hydrolysis of the starch in the mycelial extract culture was confirmed by the gradual disappearance of the blue reaction on testing with iodine. From the above results it was ~~concluded~~ that diastase is present in the mycelium of E. tinctorium and to a less extent in the sporophores.

INVERTASE. To 20 ccs. of a five percent sol. of sucrose in small Erlenmeyer flasks were added 2 ccs. of the enzyme dispersions. A control was made by adding the boiled extract. A few drops of toluol were added to each flask as an antiseptic. The flasks were placed in the incubator at 28°C. After 12 and 24 hrs. the solutions in each flask were tested for reducing sugars with Fehlin's solution

5 ccs. of the solution were added to 5 ccs. of Fehling's solution and the mixture heated. A marked precipitate of cuprous oxide was obtained in the case of the mycelial extract after 24 hrs. There was a slight precipitate with the extract from the sporophore while the control remained perfectly clear. These results indicated that invertase is present in the mycelium and sporophores of E. tinctorium but to a much less extent in the sporophore.

LACTASE. A 2% solution of lactose was made up and 10 ccs. were placed in each of three test tubes. 1cc. of the different extracts were added to different tubes. For a control the boiled extract was used. Toluol was added as an antiseptic as before and the tubes placed in the incubator at 28°C. After 48 hrs. 5ccs. of Barfoed's solution[1] was added to 5ccs. of the different solutions and the mixture heated. A slight precipitate of cuprous oxide was formed in the tube containing the mycelial extract. A very slight precipitate was present in the case of the sporophore extract while the control remained clear. The results indicate the lactase is present in the sporophores and mycelium of E. tinctorium but only to a slight extent.

MALTASE. A 2% solution of maltose was made up and treated in exactly the same manner as was the lactose. After 48 hrs. tests were made with Barfoed's solution for reducing sugars. A slight precipitate was obtained in the case of the mycelial extract. The control and the solution containing the extract from the sporophore remained quite clear. This would suggest that maltose is present in the mycelium but not to any appreciable extent in the sporophores of E. tinctorium.

ZYMASE. To test for this enzyme with breaks down dextrose into carbon dioxide and alcohol 1% solutions of dextrose and galactose were made up. Both these sugars reduce Barfoed's solution but it was thought that if this enzyme were present there would only be a slight precipitate if any after incubation with the fungus extract. "2 ccs. of the extracts were added to 10 ccs. of the sugar solutions in different test tubes. A few drops of toluol were added as an antiseptic as before. A control was made by using the boiled extract. After 48 hrs. incubation at 28°C. tests were made by adding 5 ccs. of Barfoed's solution to 5 ccs. of the solutions from the different tubes. The following results were obtained.

Dextrose.

Mycelial dispersion-----small precipitate of Cuprous oxide

Sporophore " -----Moderate precipitate.

Control----------Normal precipitate.

Galactose.

Same as dextrose.

As a check on these results 5 ccs. of each of the solutions of dextrose (and enzyme) were taken and diluted to about 20ccs. in small Erlenmeyer flasks. These solutions were brought to a boil and Fehlings solution added drop by drop by means of a burette. The flasks were not shaken and as the Fehling's solution was added the cuprous oxide settled to the bottom. When there appeared to be no further precipitate the solutions were filtered. The flasks and filter were then thoroughly washed with distilled water which was added to the filtrate, and this was then made up to 100 ccs. This was brought to a boil and Fehling's solution again added. A very slight precipitate occurred in each case but the filtrate soon started to turn blue. A reading was taken at the first trace of blue color against a white background. The following results were obtained.

	Ccs. of Fehling's reduced by 5 ccs. of sol.
Mycelial extract	16.1 ccs.
Sporophore extract	19.2 ccs.
Control.	21.2 ccs.

From these readings it was concluded that the zymase in the mycelial extract had broken down the dextrose which would have reduced 5.6 ccs. of Fehlings solution. It is also evident that the enzyme is not present in the sporophore to as great a degree as in the mycelium.

COAGULATING ENZYMES.

RENNIN. To each of 3 test tubes each containing 10 ccs. of fresh milk were added 1 cc. of the extracts from the mycelium from the sporophore and the boiled extract respectively. A drop of toluol was added to each tube as an antiseptic. The tubes were then placed in the incubator at 28°C. and examined every few minutes. Ager 40 minutes the milk containing the extract from the mycelium had coagulated. After 90 minutes the milk with the extract from the sporophore had also coagulated. The control was uncoagulated after 24 hrs. From these results it was concluded that rennin was present in the mycelium and in the sporophores of E. tinctorium

FAT HYDROLYZING ENZYMES.

LIPASE. The qualitative determination of the presence of lipase is based on the fact that the action of the enzyme upon a neutral fat produces an increase in the content of

the fatty acids. In our experiment butter fat was used as the neutral fat. 10 ccs. of well shaken cream was pipetted into each of four test tubes. To the first was added 2 ccs. of the mycelial extract to the second 2 ccs. of the sporophore extract, to the third 2ccs. of the boiled extract, and to the fourth 2 ccs. of distilled water. A few drops of toluol were added to each tube as an antiseptic. The tubes were then placed in the incubator for 12 hrs. at 28°C. The contents of these were then well shaken and 10 ccs. from each titrated against a .1N. solution of potassium hydroxide. Phenolphthalein was used as an indicator and the first trace of pink taken as the end point. The readings were recorded as follows:

10ccs. of cream plus 2 ccs. extract	Ccs. of .1N. KOH to neutralize.
1. From mycelium	4.7
2. From sporophore	3.4
3. Boiled	3.2
4. Distilled Water	3.1

In view of the fact that it took 1.6 ccs. more .1N. KOH to neutralize 10 ccs. of cream plus the mycelial extract than the cream plus the distilled water it is concluded that lipase is present in the mycelium of E. tinctorium. There is also evidence that it occurs in the sporophores but the difference in readings is not great enough to be significant.

PROTEIN HYDROLYZING ENZYMES.

A preliminary experiment to find out if proteolytic enzymes were present was performed as follows: A 7% solution of unneutralized gelatin was pipetted into tubes, 10 ccs. being put in each tube. The tubes were then divided into two series A and B. Series A was cooled to 40°C. and then 2 ccs. of the different extracts were added to different tubes. Series B was allowed to solidify before adding the extracts. A drop of toluol was added to each tube as an antiseptic. The tubes were kept at room temperature and examined after 12 and 24 hours. The following observations were made.

Series A.

Gelatin plus	12 hours.	24 hours.
1. Mycelial extract	liquid	liquid
2. Sporophore "	solid	liquid
3. Boiled "	solid	solid

Series B.

	Depth of liquifaction.	
1. Mycelial extract	2 mm.	5 mm.
2. Sporophore "	none	1 mm.
3. Boiled "	none	none

These results led to the conclusion that proteolytic enzymes are present in E. tinctorium. The common proteolytic enzymes are pepsin, trypsin and erepsin. It was thought that by comparing the mycelial and sporophore extracts with known solutions of these enzymes, circumstantial evidence could be

obtained as to which were present. Unfortunately we were not able to obtain a dispersion of erepsin, but the other two were used. The following facts were considered in comparing the different extracts.

1. Pepsin has an optimum Ph of 2.0 and will not act in alkaline solution.
2. Trysin and Erepsin have an optimum Ph of 8.5 and will not act in an acid medium.
3. Pepsin hydrolyses proteins to albuminoses and peptones.
4. Trypsin and erepsin hydrolyse proteins to amino acids and polypeptides.

Gelatin and peptone were used as substrates in comparative studies. A 7% solution of gelatin was made up as before and again divided into two series A and B. A .1N. solution of KOH was added to A until a Ph of 8.5 had been reached. Series B was acidified with .1N. HCl to Ph 2.0. The solutions were tubed as before, 10 ccs. to a tube, cooled to 40°C. and the various extracts and dispersions added to different tubes. 2 ccs. of extract were added to each tube and a drop of toluol for an antiseptic. After 24 hours at room temperature all the tubes were liquid, including those to which the acid or alkali had been added. All tubes were then placed in the frigidaire until the controls had thoroughly solidified. They were then taken out and examined. The following observations were made.

7% Gelatin	Reaction of Medium	
	Acid	Alkaline.
1. Mycelial extract	liquid	liquid
2. Sporophore "	liquid	liquid
3. Pepsin	liquid	solid
4. Trypsin	solid	liquid
5. Boiled extract	solid	solid

These results led us to believe that both trypsin (or erepsin) and pepsin are present. In order to confirm this and also to determine whether the enzymes present would hydrolyse peptone, 1% solution of peptone was made up. This was divided in two parts, one part was acidified with 1N. HCL to Ph 2.0 and to the other was added 1N. KOH until a Ph of 8.5 was obtained. 2 ccs. of the dispersions were used to inoculate the tubes. A few drops of toluol were added to each tube as an antiseptic. The tubes were then placed in the incubator for 48 hours at 28°C. They were then taken out and the Burette test applied. This consists of adding a solution of copper sulphate to an alkaline solution of the substance. If protein is present a dark purple colour results. This colour changes from red to a light pink as the protein is hydrolyzed to peptone. After the peptone stage there is no colour except the blue of the copper sulphate.

5 ccs. of the solutions to be tested were placed in tubes. A drop of saturated copper sulphate was added to each tube. Then in the case of the acid solutions

1N. KOH was added drop by drop until a reaction was obtained. The following results were recorded.

1% Peptone	Reaction of medium	
2 ccs. of extract from	Acid	alkaline
1. Mycelial	Deep blue heavy ppt.	Deep blue heavy ppt.
2. Sporophore	Bluish pink clear	Bluish pink clear
3. Pepsin	Bluish pink clear	Bluish pink clear
4. Trypsin	Deep blue heavy ppt.	Bluish pink clear
5. Boiled	Pink clear	Bluish pink clear
6. Original solution	Pink clear	Bluish pink clear

The pepsin and trypsin give the results which we would expect. As pepsin does not act on peptones we would expect no hydrolysis, and therefore the test for peptone. As trypsin only acts in an alkaline medium we would expect no hydrolysis of the peptone in an acid medium. It is evident from the results that an enzyme capable of hydrolyzing peptone both in an acid and an alkaline medium is present in the mycelium of E. tinctorium. From this and the preceding experiment we concluded that both pepsin and trypsin (or erepsin) are present and possibly other proteolytic enzymes. It is also evident that they are not present to any appreciable extent in the sporophores but mainly in the mycelium.

OXIDIZING ENZYMES.

LACCASE. To determine the presence of laccase 5 ccs. of the different extracts were added to 10 ccs. portions of a 5% solution of hydroquinone. One control was made by using the boiled extract. Another control was a straight 5% solution of the substrate. The flasks were plugged with cotton wool and kept at room remperature. After 30 minutes the solution to which the mycelial extract had been added began to turn pink. This deepened in colour until after 24 hours it was a rich golden brown. The solution containing the sporophore extract was much lighter in colour while the two controls remained a very light pink and were identical. The colour in all flasks darkened until at the end of a week the first two were quite black while the controls were a light brown. This was considered as positive evidence as to the presence of laccase. As in the case with the other enzymes laccase is more abundant in the mycelium than in the sporophores.

CATALASE. This enzyme was tested for by adding the mycelial extract to a 3% solution of hydrogen peroxide. This was added drop by drop and in small amounts. No trace of effervescence was observed, and this enzyme had to be listed as not present. Schmitz,(14) citation however includes catalase in his list of enzymes found in E. tinctorium.

ENZYMES HYDROLYSING MORE COMPLEX POLYSACCHARIDES AND GLUCOCIDES.

TANNASE. A 1% solution of tannic acid was made up. This solution gave a distinct rose color on the addition of a drop of a saturated solution of ammonium molybdate plus an equal volume of saturated ammonium chloride. This reagent gives a copius yellow precipitate with many tannins and according to Haas and Hill (6) Gardiner, considers that this affords a means of distinguishing glucocide tannin from tannic acid. A change in colour would indicate the decomposition of the tannic acid. 2ccs. of the extract from the mycelium from the sporophore the boiled extract and 2 ccs. of distilled water were added to 10 ccs. portions of the 1% tannic acid. After 48 hours these solutions were tested as described above for tannic acid. All solutions gave the same typical rose colour. The tests were the same after 15 days so it was concluded that tannase was not present.

PECTINASE. The substrate for testing for this enzyme was obtained by grinding up an apple and expressing the juice. This was filtered through several layers of cheesecloth and put in a series of tubes, 5ccs. to a tube. To the first series was added 1 cc. of the different extracts and to the second series 2 ccs. A drop of toluol

was added to each tube as an antiseptic. After 12 hours the juice to which the extracts from the mycelium and sporophore had been added was quite thick and somewhat cloudy. The controls remained normal. Although no further change took place the evidence of coagulation indicated that pectinase was present.

Additional evidence as to the presence of pectinase was obtained histologically. Transverse sections of the infected wood showed quite clearly the pulling apart of the cell walls. caused apparently by the disolution of the middle lamella, which is composed of calcium pectate. The fact that the mycelium sends off short lateral branches which appear to penetrate the cell wall as the haustoria in other fungi penetrate the cells and that these short branches appear to stop at the middle lamella and is additional evidence that the fungus feeds on the pectin contained in this part of the wall.

CELLULASE. This is said to be an endoenzyme insoluble in water. Histological evidence only was obtained in regard to its presence. Sections of the rotted wood showed quite clearly that the cell walls become thinner and thinner until only a mere thread is left and this finally disappears, leaving pocket-like hollows throughout the section. This

entire disappearance of the cell wall leaves little doubt as to the presence of cellulase especially in view of the fact that cellulose has been demonstrated to be present during certain stages of the decay.

HADROMASE. This is the enzyme which splits ligno-cellulose into its component parts, namely lignin and cellulose. Michro-chemical tests leave little doubt as to its presence. With the calcium chloride iodine solution the normal cells have yellowish brown walls of a very uniform colour. In the rotted sections a very definite rose colour which is given by cellulose was observed. As the cell wall is composed primarily of ligno-cellulose and normal cells give no test for cellulose it is fairly definite evidence that hadromase is present.

HISTOLOGICAL STUDIES.

As stated in the introduction there are several stages in the decay induced by Echinodontium tinctorium. These stages are represented by the different zones of colour. It was thought that a histological examination of these zones would be well worth while.

Small blocks were cut from each of the areas from which the original isolations had been made. These areas were as follows; brown area in centre, blue area, yellow ares, water-soaked area and apparently normal wood. Blocks were also cut in which the red zonation lines were present. These blocks were boiled for an hour in a 4% solution of glycerine and then sectioned. The sections were stained by a method devised by Hubert, (11). The sections were first flooded with a 2% solution of Bismark brown in 70% alcohol for 1 min. after which they were washed in distilled water. They were then immersed in a dilute solution of methyl green (4 parts of a saturated aqueous solution of methyl green in 12 parts of water) for 2 minutes and again washed with distilled water. They were then examined for depth of staining. The time in each stain varies slightly with the sections but after one or two attempts it can be correctly adjusted. The sections were then allowed to dry slowly on a warming plate. A cover glass should be used to keep the sections flat. When

dry they were dipped in xylol and mounted in balsam. This method stains the fungus hyphae a deep brown and the cell walls a light green.

BROWN AREAS NEXT TO ACTUAL DECAY.

It is in these sections that the medullary rays are seen to play such an important part in the spread of the mycelium. Mycelium could be found in the medullary ray cells when it was not apparent in any of the adjacent tracheids. Delignification is also first apparent in the medullary rays as shown by later microchemical tests. Breaking down of the cells can be seen in all medulary rays entering the zone of actual decay. (Pl. XIV) This breaking down of the cells in many cases may be traced across several annual rings, where it is not apparent in the ordinary tracheids beyond the two or three lying adjacent to the edge of the section, except in those in close proximity to the medullary ray. There the progress of decay may be observed very well.

The medullary ray cells in the summer wood break down first until only a fine line is left where the cell wall once was. (Pl. VI) The tracheids next to this area are the next to break down. This leaves a pocket-like area od decay where the medullary ray passes through the spring

wood. These pockets may be found in the spring wood of several adjacent annual rings, while the summer wood remains normal. (Pl. XIV.)

Mycelium is very abundant in these sections and is of two types. The first is coarse, indistinctly septate and sparsely branched. (Pl. VII and VIII,) Filaments of this can be traced across 20 or 30 tracheids, and seem to pass equally well through summer and spring wood. Bordered pits are not used except when directly in the path of the mycelium. This mycelium appears in the transverse sections as commonly as in the radial. It does however use the medullary rays to a very marked degree. When the mycelium penetrates a cell wall it first flattens out in the form of an appressorium-like structure and then sends a narrow penetration tube through the wall resuming its natural size on the other side. (Pl. VIII) The penetration seems to be purely a mechanical process as no trace of dissolution of the wall can be found around the points of penetration and microchemical tests failed to show any trace of delignification. Many of the appresoria-like structures continue to grow along the wall and send penetration tubes through at intervals. (Pl. VIII) These are the only points at which the mycelium seems to branch. This mycelium ranges from 4.2--6.3 microns in width with an average of about 5.6 microns.

The other type of mycelium is much finer, ranging from 2.1--3.5 μ with an average of 2.8 μ in width. (Pl. IX) As far as could be determined it is identical with mycelium from authentic cultures. Branching is common and wherever septa could be found clamp connections were usually seen. This mycelium also penetrates the walls directly but seems to make greater use of the bordered pits. It is also more closely associated with delignification and appears wherever the cell walls are breaking down. (Pl. X.) Many bordered pits however were observed in these sections, where delignification seemed to be taking place without the close proximity of any mycelium. (Pl. VII).

SECTIONS CONTAINING RED BANDS.

The red lines which appear in the decaying wood are seen under the microscope to be caused by densely packed mycelium in the cells (Pl. XIII). Usually four or five adjacent tracheids are completely plugged with a reddish mycelium. The red colour is not confined to the cells containing the mycelium but diffuses into the adjoining tracheids causing a definite red band. (Pl. XIII). This mycelium is mostly of the Echinodontium type. It is however interwoven with very fine filaments and dense knobby outgrowths. These different types of mycelium were also observed in the irregular mycelium of some of the monosporous cultures.

Filaments of mycelium were frequently observed in these bands with short lateral branches which appeared to enter the cell wall only as far as the middle lamella, (Pl. XI and XV.) and then to form an appressorium-like structure against it. This was found commonly in sections from wood inoculated with E. tinctorium. It is rather significant in view of the fact that Echinodontium causes a stringy rot due to the fact that it dissolves out the middle lamella, thus causing the tracheids to fall apart.

The remarkable thing about these bands is that the mycelium is usually confined to the tracheid in which it is growing and does not often pass laterally through the wall. (Pl. IX). These choked cells are always the cells of the spring wood and may appear in several adjacent annual rings. The narrow tracheids of the summer wood are never plugged.

Both the coarse and normal mycelium were found in all the sections from the brown to the normal wood. The amount of mycelium however decreased from the brown to the blue areas and again in the yellow. In the normal wood both types were found but were very scarce. In the irregular mycelia of some of the monosporous cultures all types of mycelium found in the wood were observed. Hubert (9) says that the water soaked areas are the entrance points of the mycelium in the normal wood from the yellow zones.

It is evident from these studies that ; (1). The medullary rays are the greatest avenues for the spread of the

mycelium. (2). The red bands are caused by the actual plugging of the cells by mycelium and by the diffusion of colouring matter. Probably from some substance freed by the dissolution of the cell wall, by enzymes secreted by the fungus. The only noticeable differency in the histological sections from the different zones of colour was in the amount of mycelium present. The amount of mycelium appeared to decrease from the brown areas to the blue, from the blue to the yellow and so on to the normal wood.

MICROCHEMICAL TESTS.

In order to get some idea of the progress of delignification in the heartwood microchemical tests were made on sections from the different zones of decay. The first method tried was the iodine, sulphuric acid test for cellulose and lignin. The sections were dipped in a dilute solution of iodine for two or three minutes until they had taken the colour of the iodine. They were then dipped in a strong solution of sulphuric acid. This caused the cellulose to swell considerably and to turn blue.

The acid was first used at full commercial strength. Under the microscope the cell walls could be seen to swell and rapidly turn black. The acid was gradually diluted until a concentration was reached at which the cells retained their normal shape but the delignified parts were a bright blue. In these sections the differentiation was exceptionally good. The middle lamella was a dark brown, the normal cell walls were a clear yellow while the delignified portions were distinctly blue.

The difficulty with this method is to obtain the correct concentration of sulphuric acid. This does not seem to be constant but varies with the sections. The acid also seems to become weaker so that when a series of sections are being made the last sections will not stain.

The calcium chloride, iodine test was next tried and this proved to be very satisfactory. The solution is made up as follows. A saturated solution of calcium chloride is made up and for each 10 ccs. of this solution there is added .5 gms. of potassium iodide and .1 gms. of iodine. The mixture is then heated and filtered through glass wool.

The wood sections were immersed in the hot solution and left for several minutes until they had become a distinct rose colour. They were then taken out and destained in water until the required depth of staining was obtained. They were then mounted in a cold dilute Calcium chloride iodine solution. With this solution the lignin stains from clear yellow to brown while the cellulose stains a rose red.

OBSERVATIONS. The most noticeable characteristic is that delignification occurs in pits or pockets which gradually enlarge and fuse until a definite band is formed.(Pl.XII) These pits only are observed in the tangential sections and in every case occur around a medullary ray. Marked delignification occurs only next to the advanced stage of the rot, and in the sections showing red bands. Sections cut as close to the actual rot as possible show that all the decayed tissue has been delignified and the whole edge of the section gives the rose colour which is the test for cellulose. This marked colour does not extend across more than two or three tracheids. In the radial sections however the delignification

can be traced along the medullary rays, commonly across several annual rings.

In the transverse sections it can be seen that delignification first occurs nearest to the centre of the cell and works in towards the middle lamella. In these sections many cells were observed where the part next to the middle lamella. In these sections many cells were observed where the part next to the middle lammella was the normal yellow colour while a distinct rose colour was observed around the inside of the cell wall. There was no appreciable difference in the amount of delignification in the summer and spring wood. Those cells which lay along the medullary rays however were markedly more delignified than those which lay farther away.

No trace of delignification could be found where the mycelium was observed to penetrate the cell wall. This indicates that penetration is purely mechanical. Histological examinations indicated also that penetration is mechanical as the mycelium appears to force its way through and no sign of dissolution of the wall can be seen around the points of penetration.

SUMMARY.

1. Echinodontium tinctorium has a Ph range of from 2.9 Ph to 6.8 Ph, but there is apparently no single optimum.

2. There is evidence of heterothallism and also of individual or racial variation in E. tinctorium.

3. E. tinctorium does not produce fruiting bodies readily in culture.

4. There appears to be some water soluble substance in Abies balsamea which is definitely toxic to E. tinctorium.

5. There is a direct relationship between water content of the wood and decay be E. tinctorium.

6. Sapwood is more susceptible than heartwood to decay by E. tinctorium.

7. Abies amabalis appears to be more susceptible to decay by E. tinctorium than the other species. The order of susceptibility is (1) A. Amabalis (2) A. grandis (3) T. heterophylla (4) P. taxifolia.

8. Enzymes secreted by E. tinctorium include, diastase, invertase, maltase, lactase, zymase, rennin, lipase, pepsin trypsin, laccase, pectinase, cellulase, and hadromase.

9. Histological studies indicate that there are several types of mycelium in E. tinctorium. Penetration appears to be purely mechanical as there is no evidence of delignification, where the hyphae penetrate the cell wall. The medullary rays play an important part in the spread of the

hyphae through the wood.

10. Microchemical studies show that cellulose is not liberated from the cell walls until the decay is well advanced. Delignification starts in pockets around the medullary rays, gradually extending to form a band.

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PLATE I.

Showing spores of Echinodontium tinctorium, E. and E. X400 and stages in the germination.

PLATE I

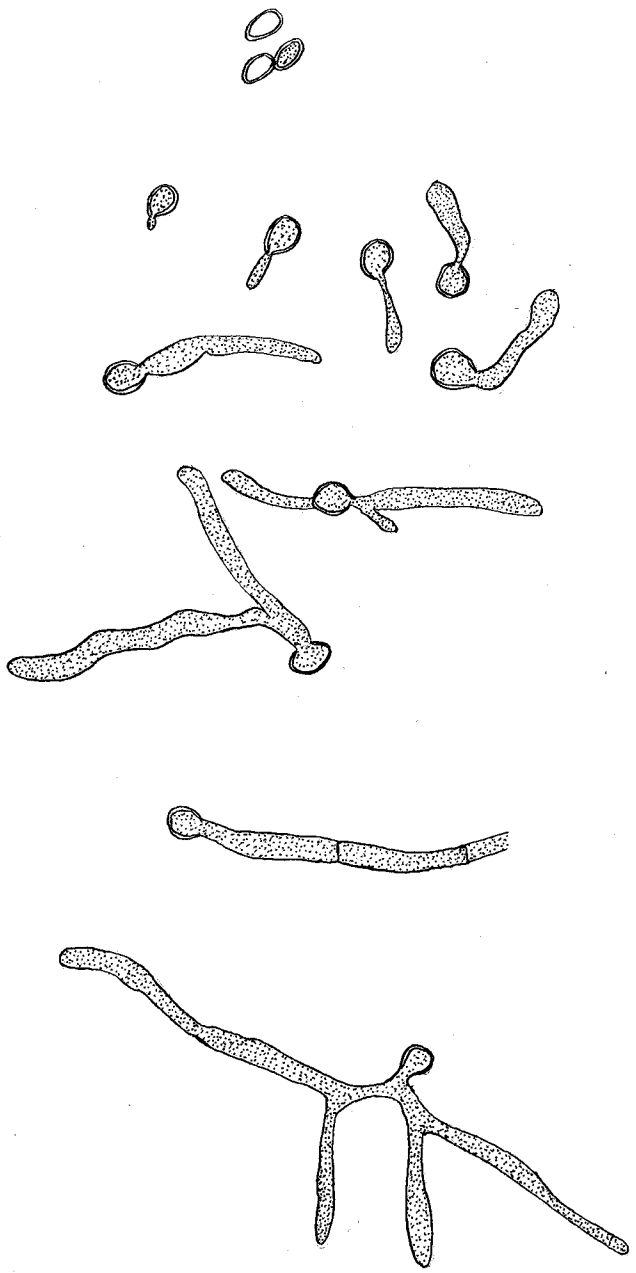


PLATE II.

Fig. 1. Mycelium of monosporous cultures of *Echinodontium tinctorium.* X400.

Fig. 2. Mycelium at meeting place of two monosporous cultures of *Echinodontium tinctorium.* X400.

Fig. 3. Mycelium of *Echinodontium tinctorium* growing out from meeting point of the same monosporous cultures. X400.

PLATE II

Fig. 1.

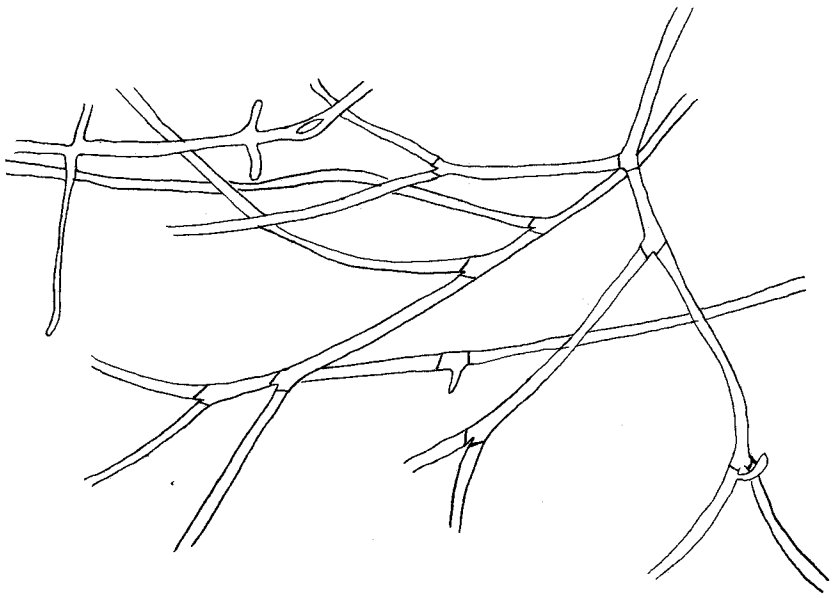


Fig. 2.

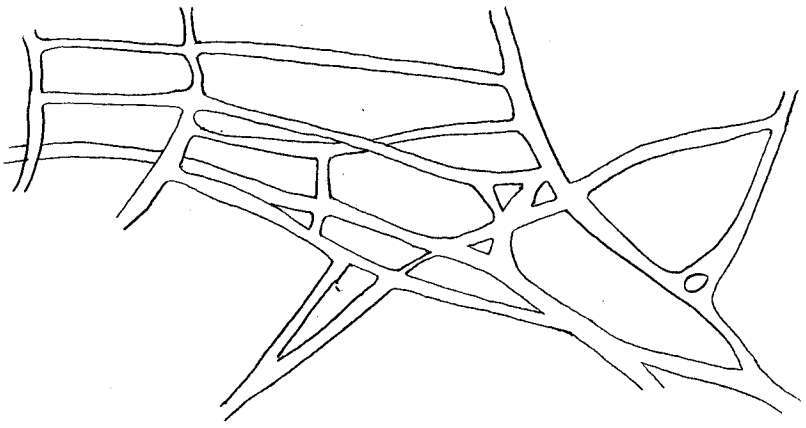


Fig. 3.

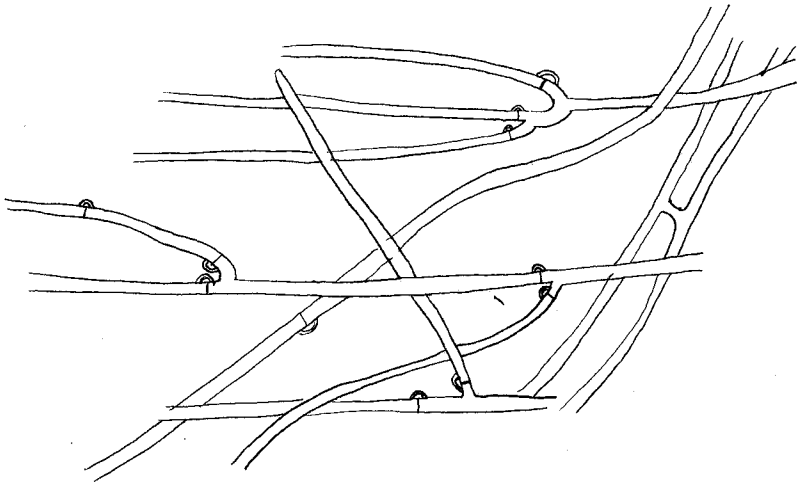
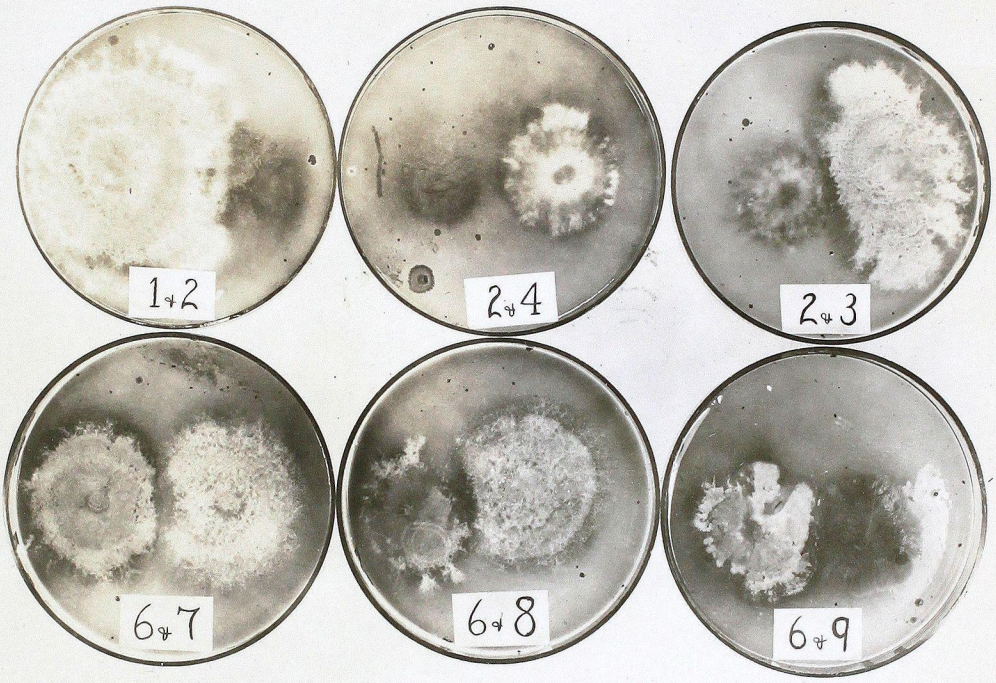


PLATE III.



Showing the pairing of monosporous cultures of Echinodontium tinctorium, Note the lines of aversion in cultures 2 & 3, 6 & 7, 6 & 8 and 6 & 9. Note also the individual variation in the cultures.

PLATE IV.

Showing the beginning of sporophore developement of *Echinodontium tinctorium* in culture.

PLATE V.

Showing the growth of Echinodontium tinctorium, on water soluble extracts of : 1. Abies balsamea. 2. Abies amabalis. 3. Abies grandis. The cultures on the left are those made up with the extracts from the sapwood (and bark) those on the right from the heartwood. Note the appearance of zonation in 2h.

PLATE VI.

Fig. 1. Showing the coarse mycelium in the medullary ray. Note the fine mycelium in the tracheids below.

Fig. 2. Showing delignification and breaking down of the medullary ray cells. Note the reduction of the thichness of the wall in the tracheids.

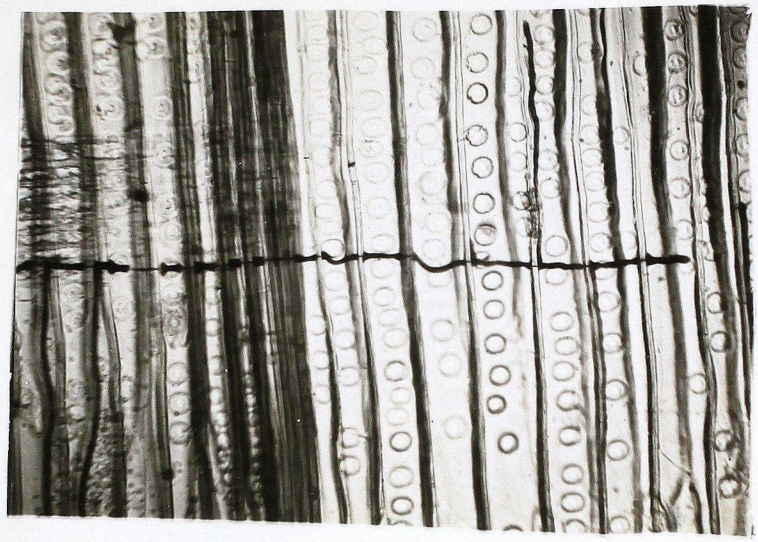


Fig. 1. Radial section of Abies amabalis, showing the coarse mycelium penetrating the walls of the spring and summer wood. Note the delignification in the bordered pits in the 4th. tracheid from the summer wood and also the branching of the mycelium at the point of penetration and the appressorium like structures from which the penetration tubes are sent out.

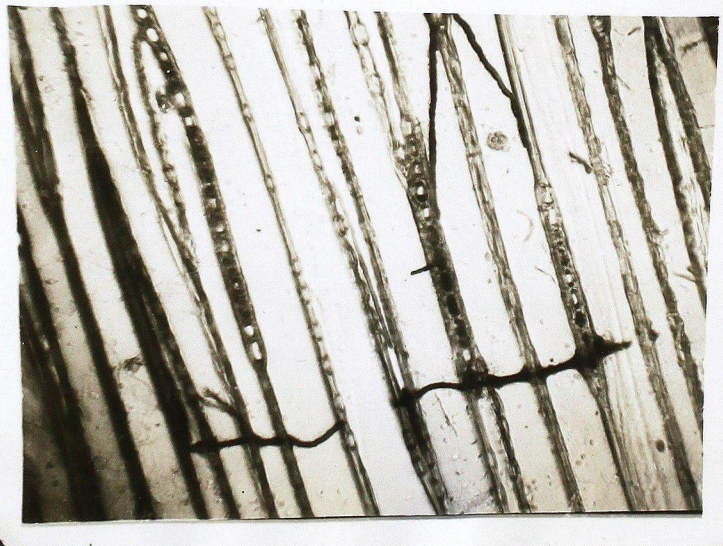


Fig. 2. Tangential section of Abies amabalis, showing the coarse mycelium of Echinodontium tinctorium.

PLATE VIII.

Showing the coarse type of mycelium found in sections of *Abies amabalis*, naturally infected with *Echinodontium tinctorium*. Note the method of penetration. X600

PLATE XV.

Fig. 1.

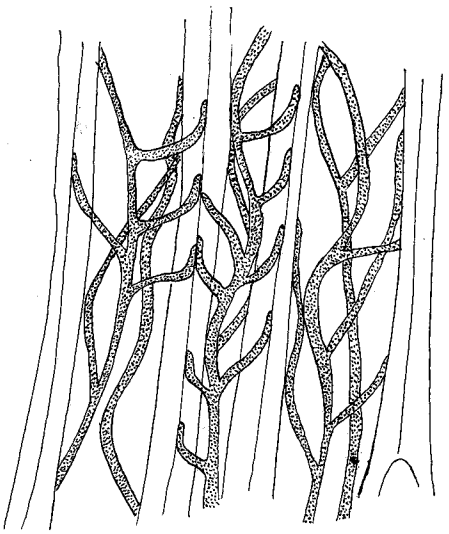


Fig. 2.

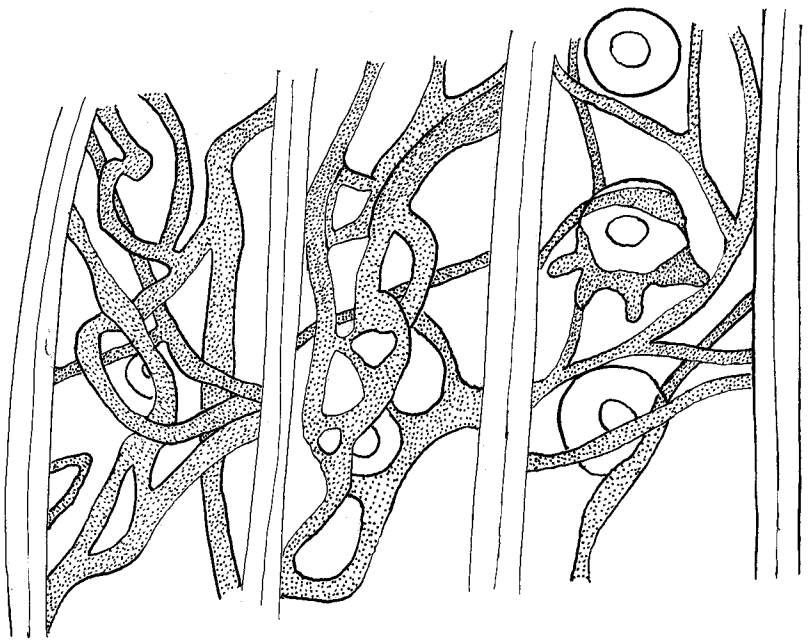


PLATE XV

Fig. 1. Showing mycelium of Echinodontium tinctorium, in tracheids of Abies amabalis. Note the short lateral branches and the affinity of the mycelium to the middle lamella. ×400.

Fig. 2. Mycelium of Echinodontium tinctorium, in tracheids of Abies amabalis, which had been inoculated with the fungus. ×400

PLATE VIII

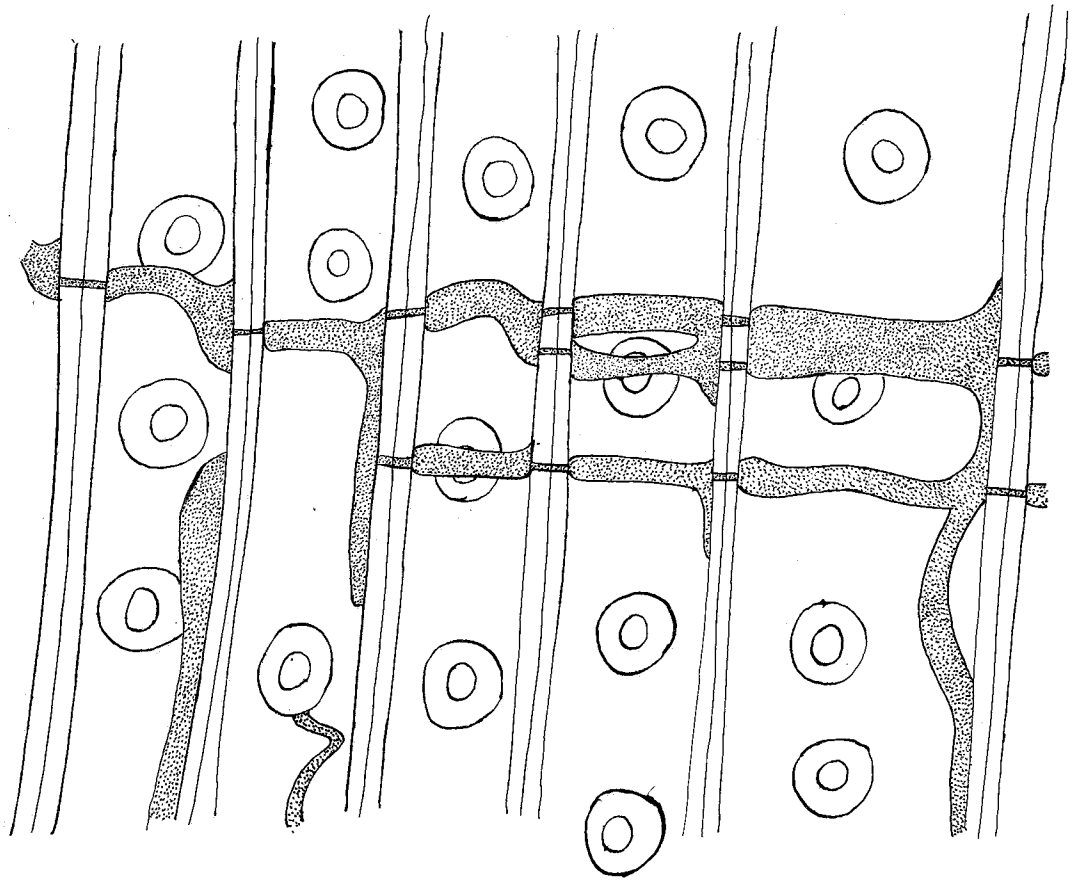


PLATE IX.

Showing types of mycelium found in naturally infected wood of Abies amabalis. ×400.

PLATE IX

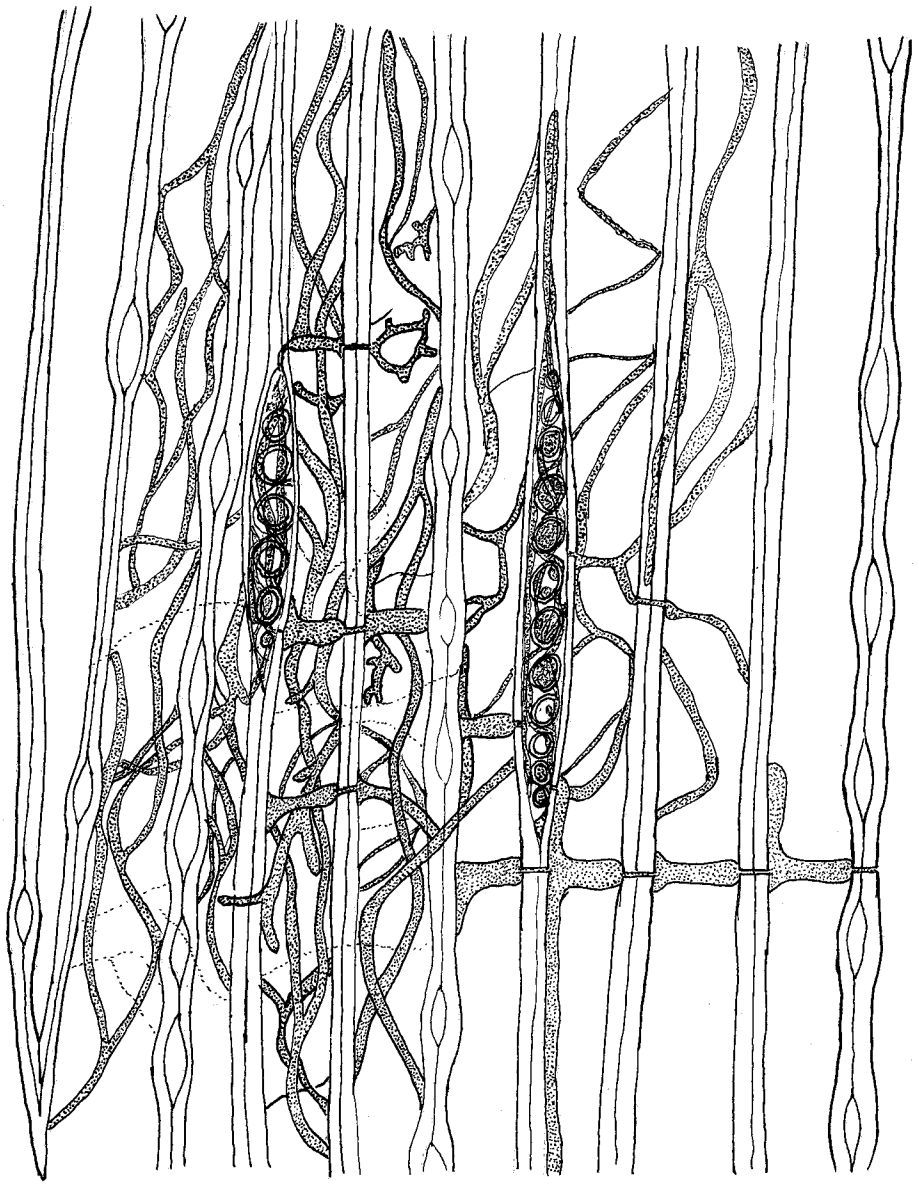


PLATE X.

Fig. 1. Showing the mycelium of Echinodontium tinctorium, in tracheids of Abies amabalis, inoculated with the fungus. Note the pulling away of the secondary wall from the middle lamella. ×400.

Fig. 2. Showing the breaking down of the secondary wall in wood naturally infected with Echinodontium tinctorium. ×400.

PLATE X

Fig. 1

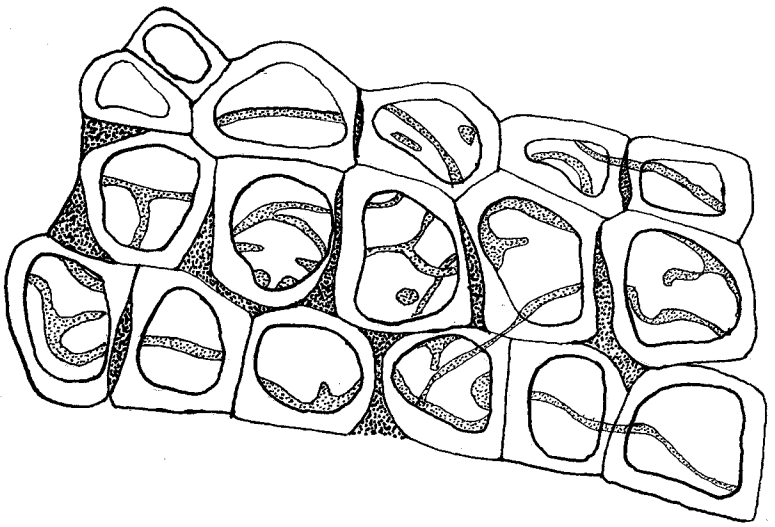


Fig. 2.

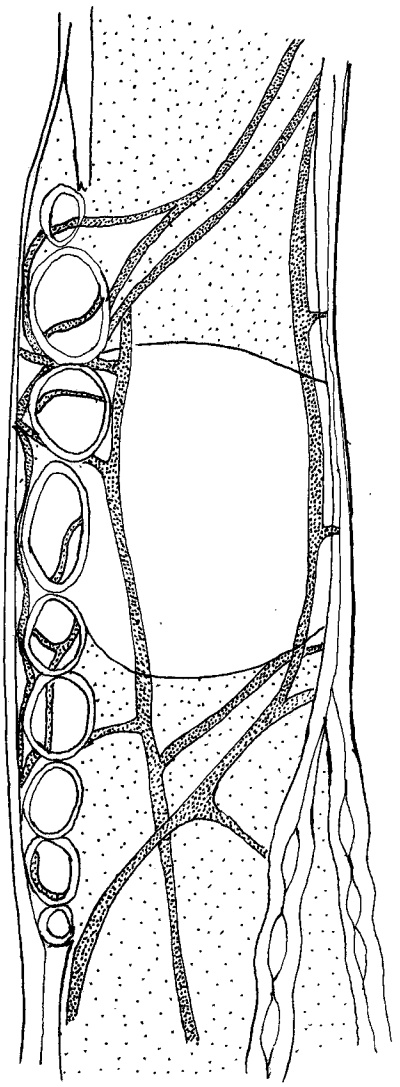


Fig. 1. Mycelium of Echinodontium tinctorium, in tracheids of wood inoculated with this fungus. Note the short lateral branches which seem to penetrate the cell wall only as far as the middle lamella.

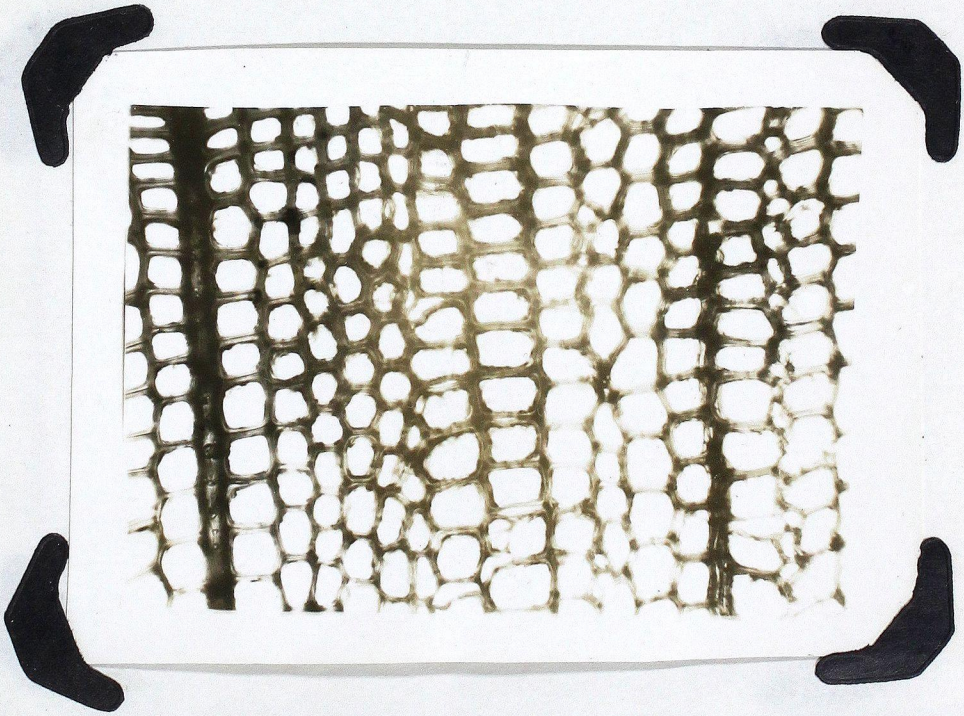


Fig. 2. Showing the pulling away of the secondary wall from the middle lamella. This section was taken from wood inoculated with Echinodontium tinctorium.

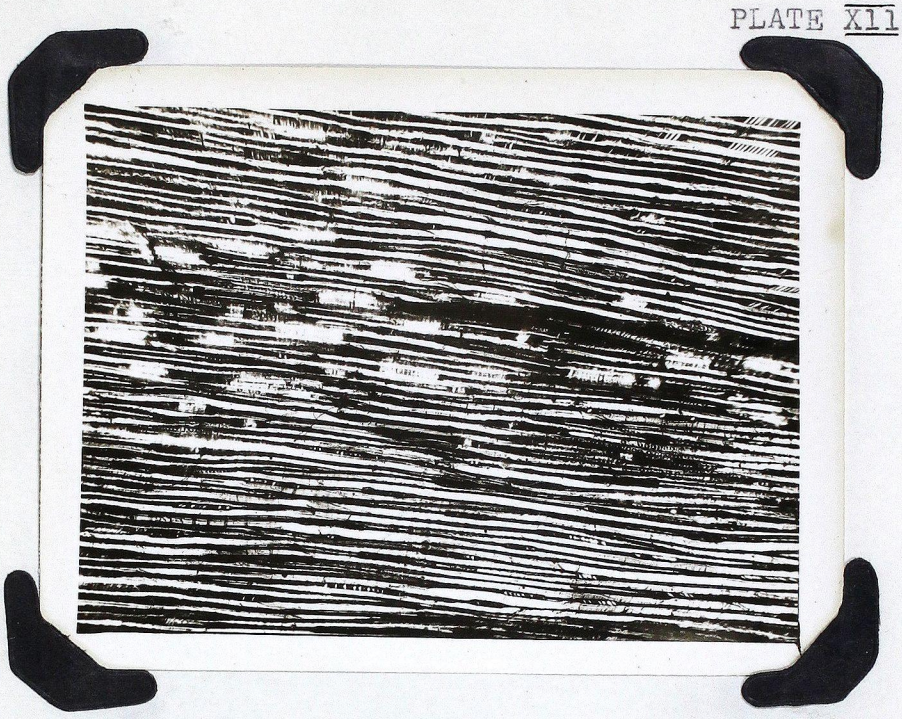


Fig. Showing the delignification in pockets around the medullary rays in wood infected with Echinodontium tinctorium, The white areas are the areas which gave the test for cellulose.

Fig. 2. Showing the localized areas of delignification. The dark areas gave the cellulose reaction. (Colour screen). Note the faint lines of delignification across the 3rd. and 4th. tracheid from the bottom.

PLATE XIII.

Fig. 1. Showing the plugging of a tracheid in Abies amabalis infected with Echinodontium tinctorium, Note that the adjoining cells are comparatively free of myceliim This section is from the area showing the red zonation lines.

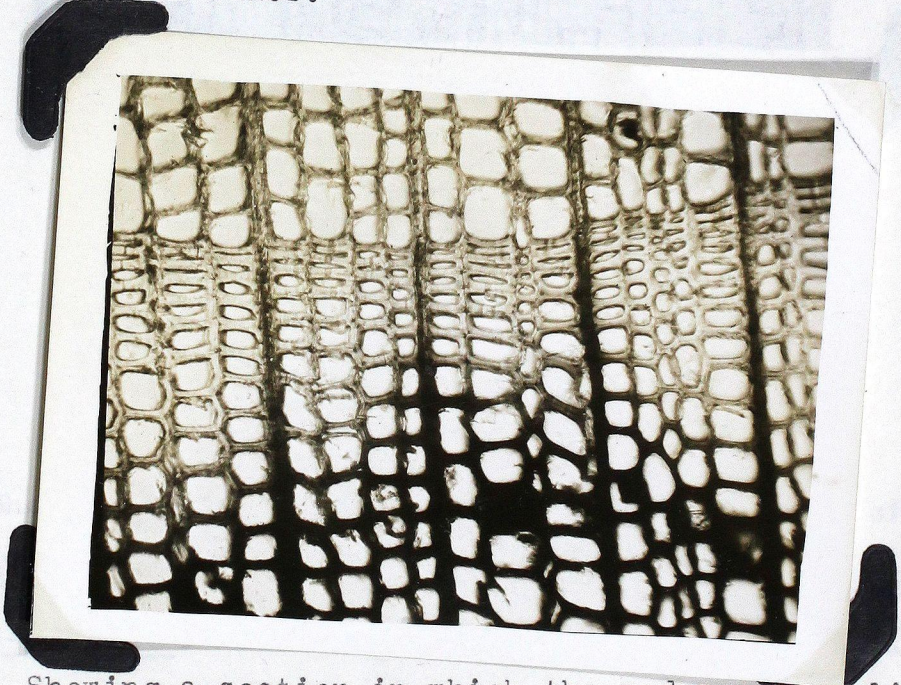
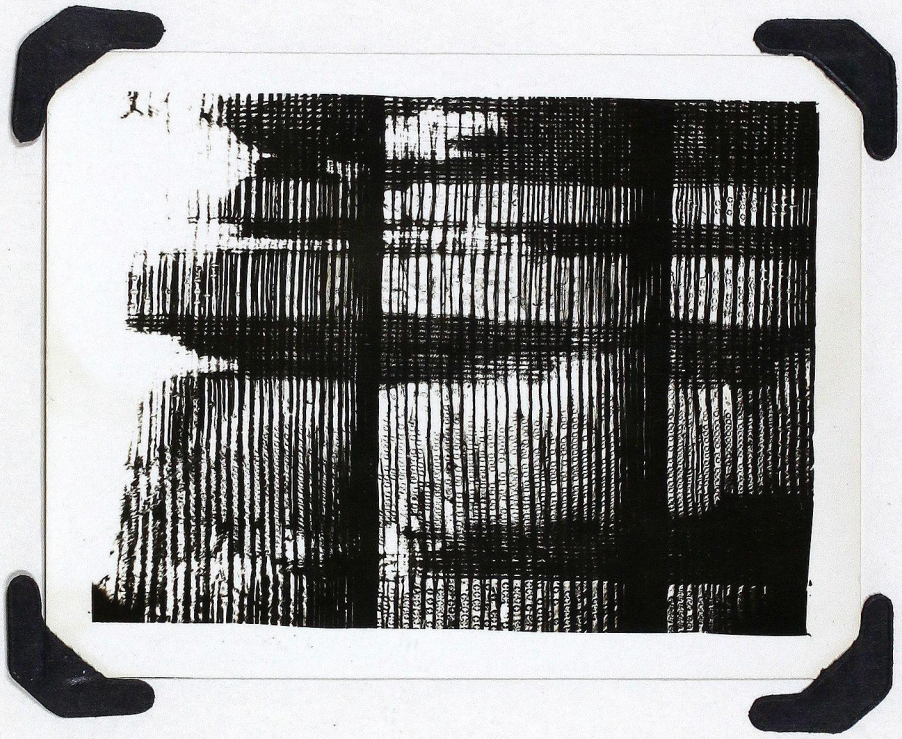


Fig. 2. Showing a section in which the red zonation line is represented. Note the plugging of certain cells and the diffusion of colour particularly along the medullary rays.

PLATE XlV.



Showing delignification in the medullary rays of Abies amabalis naturally infected with Echinodontium tinctorium. Note that the cells in the spring wood are the first to break down. The white areas in the medullary ray gave the cellulose reaction in the microchemical tests.