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Some preliminary studies on the in vitro cultivation of Pseudotsuga menziesii callus and the dwarf mistletoe Arceuthobium douglasii

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SOME PRELIMINARY STUDIES ON THE IN VITRO CULTIVATION OF

PSEUDOTSUGA MENZIESII CALLUS AND THE DWARFMISTLETOE

ARCEUTHOBIUM DOUGLASII

by

LAWRENCE M. BLAKELY

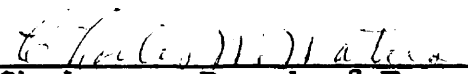B. A., Montana State University, 1956

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INTRODUCTION

Dwarfmistletoes: Historical

As a forest menace.

Most of the species of conifers of the forests of the western United States and Canada are susceptible to attack by one or more species of the genus *Arceuthobium*, the dwarf-mistletoes, a group of parasitic flowering plants belonging to the family Loranthaceae. Although early workers with the genus recognized their importance (Weir, 1916; Korstian and Long, 1922; Gill, 1935), it is only recently that their destructive potential is becoming generally acknowledged. Dwarfmistletoe damage may equal or surpass that caused by heart rot fungi, usually considered to be the most important of forest tree pathogens. Since dwarfmistletoes attack all age groups of trees, while heart rots are generally restricted to older trees, it is apparent that when the overmature, virgin stands have been cut over, *Arceuthobium* may become the most important forest pathogen. In some areas this has already become manifest. Kimmey (1957), reporting on the dwarfmistletoe problem in California, states that "it is now recognized that the reduction of damage caused by these parasites is the most important problem in applied forest pathology in the state".

Death, resulting solely from dwarfmistletoe infection,

occurs slowly (Gill, 1935). However, dwarfmistletoe infected
trees have a lower resistance to other pathogens, mainly
fungi and insects, and deaths are probably more commonly
due to the direct effect of these. In addition to death of
trees caused directly or indirectly by dwarfmistletoes,
more immediate effects become noticeable after infection.
These fall mainly into the categories of decreased growth
rate and deformities (Weir, 1916; Korstian and Long, 1922).
Korstian and Long (1922) present data on the growth rate of
infected as compared to healthy trees which show a direct
relation between decrease in growth rate and increase in
degree of infection. Witch's brooms (Fig. 1) and hyper-
trophies of stems usually occur in conjunction with infection.
Infected trees produce fewer seeds, of poorer viability,
than healthy trees (Weir, 1916; Korstian and Long, 1922).
These factors result in a poor timber crop and complicate
forest management.

Life history.

The life history of Arceuthobium is not only interest-
ing but a knowledge of it is necessary for physiological
considerations.

The seeds, to begin with, develop in an atypical manner.
No integuments are formed and a seed coat is lacking (Gill,
1935). The mature seed*, as it comes out of the berry,

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* In referring to Arceuthobium seeds, quotation marks are
often used (i.e., "seed") to indicate their anomalous nature.

Fig. 1. Witch's broom, caused by <u>Arceuthobium douglasii</u> Engelm., on Douglas fir in upper Pattee Canyon area.

consists of a simple cylindrical embryo embedded in the endosperm, both of which are rich in stored food. The endosperm is encased in the leathery endocarp and the latter is covered with mucilaginous cells of the mesocarp. (Figs. 2 and 3.)

Seeds are explosively dehiscent and may be shot to a distance of thirty feet or more. It is not necessary that seeds land on a host tree for germination to occur. Peirce (1905) found that seeds of A. campylopodum forma typicum (Engelm.) Gill (reported as A. occidentale Eng.) germinated, in nature, "anywhere where the air was moist and warm enough." Gill (1957) has had seeds of A. vaginatum (Willd.) Presl. and A. campylopodum Engelm. germinate on glass slides and the sides of tin cans. The writer has germinated seeds of A. douglasii Engelm. on agar and filter paper.

Germination consists of the elongation of the outward-directed end of the embryo (referred to as the radicle; see Kuijt, 1955). If germination occurs on a suitable host, the tip of the radicle comes in contact with the host's outer bark. The tip enlarges and becomes firmly attached to the cork. From this holdfast then emerge strands of cells which penetrate, intercellularly, into the host cortex (Peirce, 1905; Thoday and Johnson, 1930). By the time penetration has been effected the seed and radicle are shrivelled and may have been blown away (Peirce, 1905). The cortical strands branch freely in the cortex, and, by
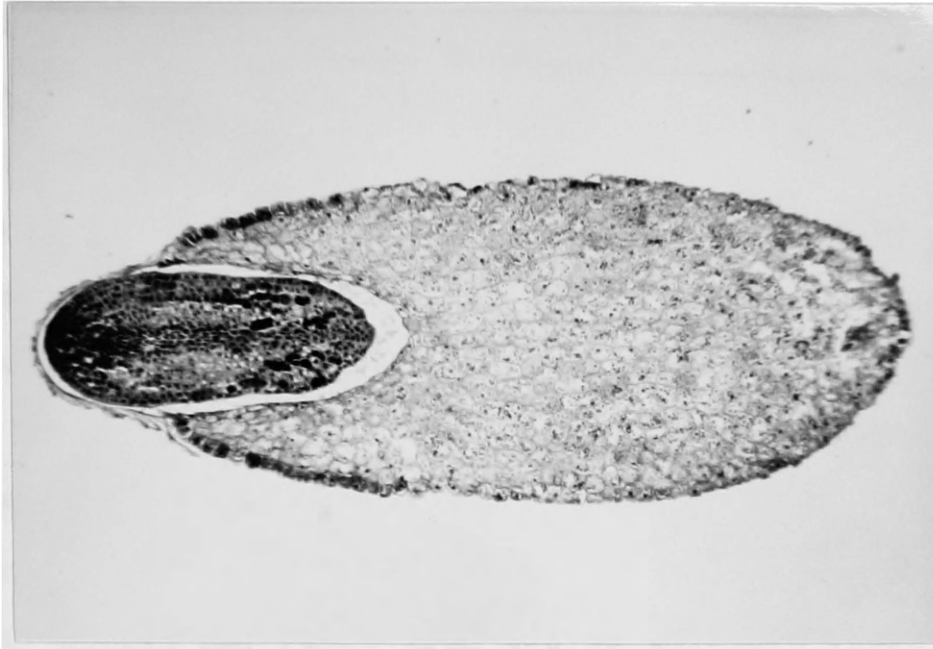
Fig. 2. Longisection of <u>Arceuthobium</u> <u>douglasii</u> embryo
(left center) enveloped by endosperm. Endocarp and viscin
cells removed. Note stored food particles in endosperm
cells. (x 40.)

Fig. 3. <u>A</u>. <u>douglasii</u> seed (at arrow) recently ejected
from berry to right of it. Aerial shoot below bears
turgid berries ready to dehisce. Scale divisions in mm.

sending "sinkers" through phloem rays, come in contact with the cambium; sinkers do not actually grow into the xylem but become embedded in it as new xylem is formed (Parke, 1951; Cohen, 1954). This system of cortical strands and sinkers comprises the "endophytic system" (Thoday and Johnson, 1930). (Fig. 4.)

Formation of aerial shoots does not occur until after the endophytic system has become well established. The interval between infection and appearance of the first aerial shoots may range from two to thirteen years in A. vaginatum forma cryptopodum (Engelm.) Gill (Gill and Hawksworth, 1954). Aerial shoots arise from buds, formed by the endophytic system, which grow outward, penetrating the host cork. In A. douglasii, shoots are generally less than two cm. in length(Fig. 5); in other species they may range from a few mm. to fifteen cm. (Kuijt, 1955). They are articulate, with greatly reduced, opposite, leaves. Flowers are imperfect and dioecious. Apparently all aerial shoots produce flowers, and are regarded as inflorescences by Gill (1935).

Physiology.

Little definite information on the physiology of the dwarfmistletoes is available, though there has been some speculation (see Kuijt, 1955). It is generally believed, although it has not been demonstrated, that the parasite absorbs from the host elaborated foods, in addition to
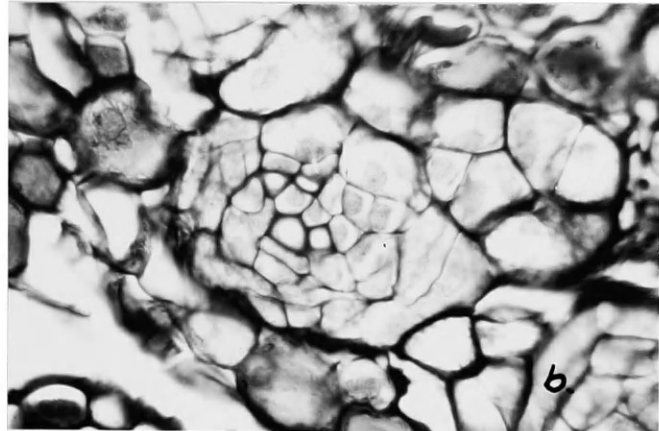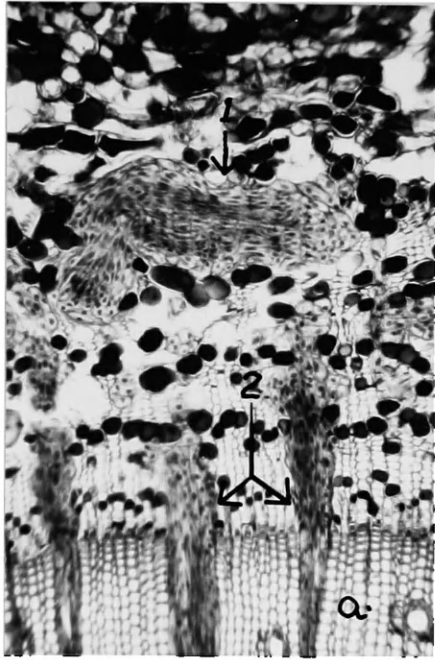
Fig. 4.   a. Transection of Douglas fir stem infected with
Arceuthobium douglasii, showing portion of endophytic
system.   Longitudinal view of a cortical strand at arrow 1;
sinkers at arrows 2.   (x 70.)   b. Young cortical strand in
transection with four tracheids distinguishable near
center.   (x 420.)

Fig. 5.   Fruit bearing aerial shoots of A. douglasii on
Douglas fir.   Photo taken September 24, 1957.   (slightly
enlarged.)

water and minerals (Gill, 1935). Phloem tissue has not
been found in the species studied (Kuijt, 1955), but
Thoday and Johnson (1930) working with A. pusillum Peck,
and Peirce (1905) with A. campylopodum forma typicum, found
cells of the endophytic system in contact with host phloem
cells. Although no sieve plates were located between host
and parasite cells, they believed that dissolved foods
could be absorbed by the parasite. Chlorophyll is present
in the aerial shoots of most or all species. Other pig-
ments may be present, masking the green color; this probably
accounts for reports of the absence of chlorophyll in some
species (Gill, 1935; Kuijt, 1955). Chlorophyll has been
found in the endophytic systems of some species but not in
others (Kuijt, 1955). However, it is felt that the endo-
phytic system would receive insufficient light to carry on
photosynthesis (Gill, 1935). Direct contact between host
tracheids and parasite tracheids (in the sinkers) has been
observed in some species but not in others (Kuijt, 1955).

During germination of the seed, the radicle is apparent-
ly negatively phototropic and negatively geotropic (Kuijt,
1955). As previously mentioned a "host stimulus" is not
required for the germination of the seeds of at least some
species.

Control.

The only known method of control is the pruning or
destroying of infected trees (Gill and Hawksworth, 1954;

Kimmey, 1957). Attempts to develop chemical control agents
for practical use have not yet met with success (Kimmey,
1957). It is interesting to note that there are occasionally
individuals of an Arceuthobium susceptible host species
that are resistant to dwarfmistletoe attack, indicating the
possibility of producing genetically resistant strains
(Kimmey, 1957).

Statement of Problem

One of the more pressing needs for a better under-
standing of the dwarfmistletoes is information on the
physiology of the parasite and the physiological inter-
actions between parasite and host. If Arceuthobium could
be cultured indefinitely apart from its host, its require-
ments could be studied in much more detail and with more
certainty than if this were studied while in the host. Loo
(1946) succeeded in culturing, in vitro, isolated stem tips
of dodder (Cuscuta campestris Yuncker) over a period of five
months (when his paper was written they were still growing)
on a simple, chemically defined, culture medium. It might
be inferred from the intimate relationship between Arceuthobium
and its host that a more complex nutrient medium would be
required for the indefinite culture of dwarfmistletoe
separate from its host. In addition to the likely necessity
for a complex medium, there are other possible complications.
Since the external shoots appear to be expressly modified

for the production of flowers, it may be impossible to culture them indefinitely in a vegetative state. Culturing isolated cortical strands would be difficult owing to their small size. The indefinite culture of the radicle may prove possible, given the right nutrient medium, but the growth rate of dwarfmistletoe radicles is very slow. Another possibility might be the induction and maintenance of callus tissue. In some of his earlier experiments the writer attempted to induce callus formation on aerial shoot segments of A. campylopodum forma laricis (Piper) Gill and A. douglasii, using simple culture media, containing auxin, but was unsuccessful.

An alternative, though less satisfactory, approach to the study of Arceuthobium physiology, but a more direct approach to the study of host-parasite interrelationships, might be the culture of isolated host tissues infected with the parasite. Using tissue culture technique, such cultures, if it proved possible to establish them, could be studied under rigorously controlled conditions, and a large number of samples could be handled in a small space. Comparisons of the responses of healthy versus infected tissues to various agents, might be studied. It is interesting to note that a similar approach has been used by Hotson (1953) with some success. He was able to culture callus of Juniperus virginiana L. infected with the rust Gymnosporangium juniperi-virginianae Schw.

A necessary prerequisite to the latter approach is the ability to culture healthy host tissue indefinitely. The major portion of the present work is concerned with the problem of inducing and maintaining callus tissue derived from host stems, using tissue culture technique. It was thought that it might be possible to infect healthy callus cultures, in vitro, with Arceuthobium seeds. Or, as another possible method of obtaining infected callus, it was thought that callus formed by infected host tissues might become invaded by the parasite. Pertinent to the latter method is the following observation of Parke (1951): "Most of the parenchyma cells of the strand retain meristematic properties. The peripheral parenchyma [of cortical strands] are capable of producing new strands, thereby providing a means of perennating the endophytic system in subsequently formed host tissue."

The host chosen was Douglas fir (Pseudotsuga menziesii [Mirb.] Franco) which is attacked by Arceuthobium douglasii. Some observations on the germination of seeds of A. douglasii were also made.

The Culture of Coniferous Callus Tissue
in vitro: Historical

There are only a few reported cases of the in vitro cultivation of callus tissues derived from coniferous species, in comparison to the large number of cases of the culture of callus of species belonging to other taxa. Con-

iferous callus cultures may be divided into two groups:
Those which are adversely affected by the presence of the
enzyme phenol oxidase and those which are not.

Callus cultures not adversely affected by phenol oxidase.

Callus cultures of Sequoia sempervirens (D. Don) Endl.
have been established by Ball (1950). The callus was stim-
ulated to form on the cut ends of one centimeter segments
from green shoots, which had formed on a burl. The nutrient
medium contained only mineral salts, sucrose, and an auxin
(indoleacetic acid at one mg. per liter).

Straus (1958) is growing callus cultures derived from
staminate cone axes of Libocedrus Endl. and Cupressus L.
Callus was also derived from the stems of Libocedrus.
Libocedrus callus is grown on White's basic medium (see p.
25) plus 20% coconut milk and 2,4-dichlorophenoxyacetic acid
at 2 mg/l. For Cupressus the same medium is used except
that the auxin (2,4-D) is omitted.

The culture of callus formed by Pinus banksiana Lamb.
and P. strobus L. seedlings (two weeks old) was reported by
Loewenberg and Skoog (1952). In addition to mineral salts
and some known organic supplements, including an auxin
(naphthaleneacetic acid at 1 mg/l), it was necessary to
supply the seedlings, and the subsequent callus cultures
derived from them, with an extract of malted barley seeds.
It was necessary to sterilize this extract by passing it
through a bacterial filter (Seitz) rather than by autoclaving.

In the remaining instances of conifer callus culture
it appeared that the activity of the enzyme phenol oxidase
was responsible for the poor results initially obtained.
Special treatment was necessary to overcome this. Since
it appears that the action of this enzyme may also be
hampering the culture of Douglas fir callus, an account of
the nature of this enzyme will be given before considering
these cases.

The nature of phenol oxidase and its role in the problem of culturing pine and spruce callus.

Phenol oxidase* is widely distributed throughout the
plant kingdom where one of its more commonly observed man-
ifestations is the production of brown or black pigments.
This may occur under normal conditions, as in the browning
of seed coats and bark, or it may be the result of wounding,
as in the case of the browning of the flesh of apples,
potatos, and numerous other plant tissues after injury
(Mason, 1955). It may also play a role in lignin formation
in conifers (Manskaya, 1948). The proposition that it may
function as a terminal oxidase has been challenged by recent
workers. Bonner (1957) feels that there is insufficient
evidence to support this hypothesis, but suggests that it
may function in an anaerobic step in electron transfer.

Phenol oxidase is a copper containing enzyme. It is

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* Two common synonyms of phenol oxidase are tyrosinase
and polyphenol oxidase (Bonner, 1957).

assumed that a change in valence of the copper accompanies
substrate oxidation, but this has not been demonstrated
(Bonner, 1957). Its substrates are compounds with a mono-
or ortho-dihydroxy phenolic configuration:
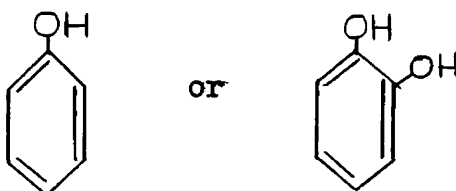
In the presence of oxygen, monohydroxy phenols are oxidized
to o-dihydroxy phenols. The latter are oxidized to the
corresponding quinones:
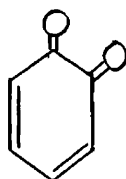
In summary:
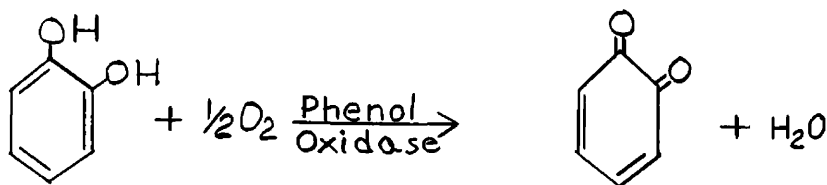
(Dawson and Tarpley, 1951).

The quinones formed undergo polymerization and further
oxidation without the participation of enzymes. This
process leads to the formation of the dark pigments. The
potential substrates for this reaction are numerous, includ-
ing tannins and lignin precursors. However, it appears that
injury to cells is generally necessary to make substrates
available to the enzyme (Mason, 1955).

It is possible to prevent browning when conditions
are otherwise favorable. One method is to use substances
which combine with copper, such as Na-diethyldithiocarbamate,
K-ethylxanthate, cysteine and glutathione. Another method
is the use of strong reducing agents, such as ascorbic acid,
to reduce quinones back to phenols as soon as they are form-
ed (Dawson and Tarpley, 1951).

The following generalized scheme illustrates the reac-
tions that have been discussed:
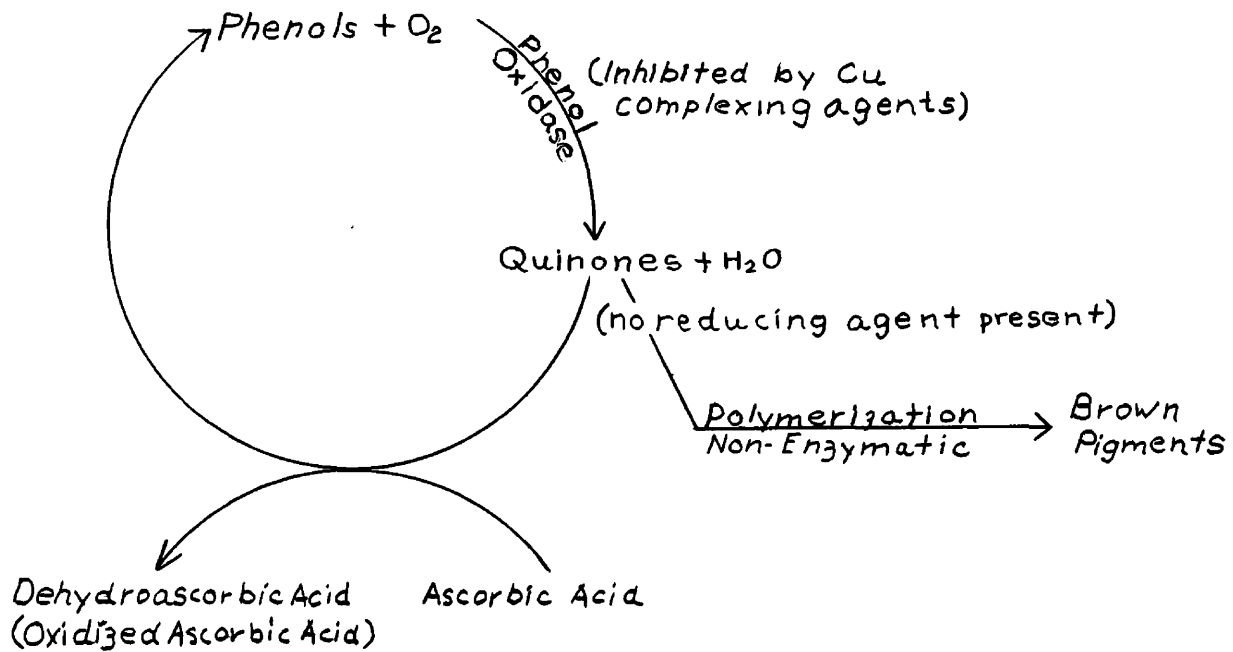
One further consideration is the inactivation of
auxin by phenol oxidase. Briggs and Ray (1956), using
a commercial phenol oxidase preparation and a phenol
oxidase containing extract of Osmunda cinnamomea L.,
demonstrated the inactivation of indoleacetic acid by
reactions involving this enzyme. It was found that in-

activation occurred when catechol or pyrogallol were used
as substrates, but not with tyrosine or dihydroxyphenyl-
alanine (phenolic amino acids). All four of these were
oxidized by the enzyme, as was evidenced by the formation
of pigments. The final oxidation products alone were not
effective in inactivating the auxin. It was therefore
thought that some intermediate products were responsible.

It should also be mentioned that other enzymes are
capable of inactivating auxin, or at least indoleacetic
acid (IAA). Bonner (1957) notes: "It is apparent from
consideration of the evidence relating to IAA oxidation
that there is no one specific IAA oxidase; rather, we are
presented with a variety of IAA oxidizing systems that have
been produced as a result of cell rupture."

Wetmore and Morel (unpublished, see Loewenberg and
Skoog, 1952; Reinert and White, 1956) have established
callus cultures derived from the cambium of branches of
Pinus strobus. Their work, however, was apparently hamper-
ed by the activity of phenol oxidase (Wetmore, 1957). They
believed that the destruction of auxin by this enzyme was
responsible for the poor growth which was initially observ-
ed. This difficulty was obviated by the use of ascorbic
acid, according to the principle given above; i.e., the
reduction of quinones back to phenols as soon as they are
formed. They found that after three to five days on a medium

containing ascorbic acid at a concentration of 176 mg/l
(10^{-3} molar -- see Wetmore and Morel, 1949), in addition to
other nutrients, the cut surfaces had become covered with
newly proliferated cells, "thereby closing the enzyme with-
in intact cell membranes again, with the result that the
rapid oxidative action, with the availability of free
oxygen, is no longer carried on." (Wetmore, 1957). The
cultures were then transferred to media without ascorbic
acid. This latter step was performed because ascorbic acid
may act as an auxin antagonist (see Tonzig and Trezzi, 1950).
Every time their cultures were cut up into smaller pieces
for subculturing, it was necessary to employ the ascorbic
acid treatment.

A medium which gave consistently good results con-
tained (with or without ascorbic acid) the mineral nutrients
as given by Nitsch (1951), 2% sucrose, 15% coconut milk
(sterilized by autoclaving), and naphthaleneacetic acid at
0.05 mg/l(Wetmore, 1957). According to Reinert and White
(1956) and Loewenberg and Skoog (1952), they also grew the
callus on a chemically defined medium, the coconut milk
being supplanted by a mixture of B vitamins.

Reinert (1955, 1956) and Reinert and White (1956)
report the successful culture of _Picea glauca_ (Moench) Voss
cambium derived callus. They also encountered difficulties
which they ascribed to phenol oxidase. When small blocks
containing cambium tissue, excised from mature trees, were

planted on nutrient medium, callus formation occurred and
growth progressed for two to three weeks. The callus soon
began turning brown, however, and growth ceased. If the
callus was cut away from the original tissue before brown-
ing set in, and transferred to fresh medium, it began turn-
ing brown within three to four hours. Pretreatment with
ascorbic acid was without effect as a means of preventing
browning. When ascorbic acid or the phenol oxidase inhibi-
tors Na-diethyldithiocarbamate, Na-ethylxanthate, or gluta-
thione were used as permanent components of the medium,
the calli grew for a longer time. Results were best with
glutathione or ascorbic acid at concentrations of 10 or
100 mg/l. However, on these media, the cultures died after
two or three subcultures. They also tried tyrosine (a
phenol oxidase substrate) at 40 mg/l together with copper
sulfate at 0.01 mg/l. With these compounds growth was better
at first, but the cultures died on subculturing. When
tyrosine alone was used, at the same concentration, the
calli continued to grow on subculturing, and through all
subsequent subcultures as well. At the time of publica-
tion (Reinert and White, 1956), cultures on media contain-
ing tyrosine had gone through seventeen subcultures and were
one and a half years old.

The writers pointed out that the manner in which phenol
oxidase brought about growth restriction of spruce callus
was not elucidated in their studies. It was not known

whether growth restriction was due to tyrosine destruction
or to toxic intermediate products of phenol oxidase activity.
The auxin generally used was 2,4-D at 0.05 mg/l. If indole-
acetic acid was used instead of 2,4-D, much higher concen-
trations were necessary to stimulate callus formation.
Whether phenol oxidase was responsible for the apparent
destruction of IAA, or whether other IAA oxidases were
involved, was not clear.

The medium which they used contained (in addition to
White's basic nutrients, tyrosine, and 2,4-D) numerous
organic compounds, principally vitamins and amino acids.
Though the medium was a complex one, it was chemically
defined. They found that malt extract was growth promoting,
but it was not necessary to add it to the medium.

As mentioned earlier, Hotson (1953) cultured callus of
Juniperus virginiana. Uninfected callus was not adversely
affected by phenol oxidase. However, the culture of infect-
ed callus derived from galls of infected eastern redcedar
was hampered by the formation of dark pigments in the callus.
Hotson used ascorbic acid at 150 mg/l, present as a permanent
constituent of the medium, to overcome this.

Another work which may be pertinent to this study,
although with non-coniferous tissues, is that of Jacquiot
(1947). He found that oak and chestnut cambium fragments
would not proliferate in vitro. On the assumption that

tannins, or their oxidation products, after diffusing
from the fragments into the medium and being reabsorbed,
were inhibiting callus formation, he (1) subcultured the
fragments to fresh media a few days after explantation,
thus removing the fragments from the influence of tannins
which had diffused into the original medium, or (2)
cultured the fragments for a few days on medium containing
caffeine, followed by subculturing on medium without
caffeine. (Caffeine precipitates tannins and is of
relatively low toxicity.) The second method gave the best
results.

MATERIALS AND METHODS

<u>Arceuthobium</u> <u>douglasii</u> seeds were collected on
September 23, 1957*, along the east side of Flathead Lake
in the vicinity of the Montana State University Biological
Station (located in Lake County, Montana). Both <u>A</u>.
<u>douglasii</u> and <u>A</u>. <u>campylopodum</u> f. <u>laricis</u> are very abundant
in this area. Uninfected <u>Pseudotsuga</u> material was taken
from an approximately 40 year old tree located about fifty
yards west of the main entrance to the Natural Science
building at Montana State University. Dwarfmistletoe
infected material was collected in the vicinity of the
Pattee Canyon Recreation Area, immediately southeast of
Missoula, Montana, and also in the same area in which seeds
were collected.

Experimental Douglas fir material consisted of stem
disks approximately two to four mm. in diameter and two
mm. thick. These were cut from three to five year old
stems (from which the needles had been removed) by making
transverse cuts with a sharp razor blade at two mm. intervals.

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* The date of collection was two or three days after
peak seed dissemination. They were collected, as Gill
(1957) suggests, by placing fruit bearing aerial shoots
in paper bags, which are then closed and shaken occasional-
ly over a period of 3 to 4 days. At the end of this time
most of the seeds have been ejected from the berries and
are clinging to the paper, from which they are removed
with tweezers.

Unless otherwise indicated the material used in the experiments was non-mistletoe infected. Material of this size was chosen because infected stems of this size regularly contain actively growing mistletoe. Work, then, could be shifted to comparable material when it became desirable to use infected stems. Stems were generally selected for a high bark-wood ratio. In some cases the cork was cut away prior to slicing; in others it was left intact. The disks, then, consisted of pith, xylem, cambium, phloem, cortex, and cork; or all of these except the cork (and a portion of the cortex cut off along with the cork).

Stem segments were surface sterilized, before being cut into disks or removing the cork, by either (1) soaking in a 0.1% mercuric chloride solution for 10 minutes, followed by rinsing in sterile distilled water, or (2), as White (1954) recommends, rinsing in 95% ethyl alcohol and burning off the alcohol, followed by the mercuric chloride treatment and rinsing as in (1). The latter method gave better results.

The stem disks were planted on agar-solidified nutrient medium in 18 X 150 mm. test tubes. The agar was allowed to solidify so that the surface was sharply slanted. Two disks were placed in each tube and the tubes were stoppered with cotton plugs. A typical set-up is shown in Fig. 6. Experiments were begun with twenty cultures (ten tubes) for each different medium employed, unless otherwise noted.

Fig. 6.   Typical set up of Douglas fir stem disk cultures.
Callus formation on these, which are 25 days old, has
obscured the original tissues.   (x 1/3.)

Generally, only the best ten cultures were used for sub-culturing purposes, and only one subculture per tube. Many experiments were repeated, with or without some modification. Stem disks were distributed to the various components of an experiment in such a manner as to insure comparable material on different media. The cultures were observed with the aid of a binocular dissecting microscope, and notes taken, every two or three days.

Arceuthobium seeds were freed of endocarp and viscin cells before use, because of the difficulty of working with seeds with the mucilaginous viscin cells intact, and to eliminate possible restriction of radicle elongation by the endocarp. Seeds were sterilized by placing in Chlorox (a commercial bleach containing 5.25% by weight of sodium hypochlorite) for 10 minutes, followed by rinsing in sterile distilled water. This method of sterilization gave good results at first, but as the age of the seeds increased they seemed to become more susceptible to damage caused by the Chlorox. Diluting the Chlorox with an equal volume of water was of no benefit. No further attempts were made to improve the sterilizing technique. Seeds (intact) were stored in a celluloid covered box placed near a window in the laboratory. Perhaps a cooler or moister location would have been better. Some seeds were kept in the culture room, but they became mold covered.

Manipulations to be carried out aseptically were per-

formed in a wooden and glass transfer chamber, fitted with cloth sleeves, and equipped with a small ultraviolet lamp. Prior to use the floor of the chamber was washed with an antiseptic solution; the ultraviolet lamp was then turned on and left on for at least an hour before work commenced. Care was taken to protect living material from the ultraviolet radiation. They were kept in glass containers when the lamp was on; the lamp was shut off once work commenced. Contaminations which could be traced to operations carried out in this chamber were exceedingly rare.

The basic nutrient medium used was that of White (given in Reinert and White, 1956) with two minor changes. Instead of ferric sulfate, ferrous sulfate was used; instead of 65 mg/l of potassium chloride, 80 mg/l was used. Each liter of nutrient contained:

Substance	Concentration (mg/l)	Substance	Concentration (mg/l)
$MgSO_4$	360	$ZnSO_4$	1.5
$Ca(NO_3)_2$	200	H_3BO_3	1.5
Na_2SO_4	200	KI	0.75
KNO_3	80	glycine	3.0
KCl	80	nicotinic acid	0.5
NaH_2PO_4	16.5	pyridoxine	0.1
$FeSO_4$	2.5	thiamin	0.1
$MnSO_4$	4.5	sucrose	20,000

The sucrose and salts were of reagent grade. Organic compounds other than sucrose were obtained from Nutritional Biochemicals Corporation. The tyrosine and cysteine used in some experiments were both levo isomers. The malt

extract which was used in some experiments, and the agar ("Bacto") were products of Difco. The stock salt solution, less the ferrous sulfate and ten times the concentration used in media, was stored in a brown bottle in a dark cabinet. The glycine and vitamins listed above were made up in a stock solution 100 times the concentration used and stored in a deep freezer at $-15^\circ C$. The stock solutions were diluted, and other components were added, when media were prepared. The other components, such as ferrous sulfate, were generally freshly dissolved at the time of making media. Occasionally, stock solutions of auxin, or other organic compounds used at low concnetrations, were kept in a refrigerator for as long as a month for use in making media. Double distilled water was used in all media. It was prepared by redistilling, with glass apparatus, distilled water obtained from the Chemistry Department, and was stored in Pyrex flasks. The pH was not adjusted or determined for any of the media used. White's basic nutrient medium has a pH of about 5.5 (White, 1954). Unless otherwise noted, media were made up complete, transferred to tubes (10 ml. per tube) and autoclaved at 15 pounds pressure for 20 minutes.

Culture tubes were cleaned by soaking overnight or longer in trisodium phosphate solution, followed by rinsing several times in warm tap water. They were then filled with sulfuric acid potassium dichromate cleaning solution and

allowed to stand overnight again, followed by eight or more rinsings in warm tap water and three rinsings in distilled water. They were drain-dried overnight in a closed cabinet and then stored in large, covered, clean glass dishes. The minimum cleaning treatment given other glassware, such as that used in making media, was four hours in sulfuric acid potassium dichromate solution followed by rinsing as above.

Cultures were kept in an environment-controlled room in which the temperature was maintained at $20^{\circ}\pm 2^{\circ}C.$, and the relative humidity at $75\%\pm 5\%$*. Light was supplied by two daylight type 40 watt fluorescent lamps over one bench. The light intensity at the level of the bench top was 70 foot candles. All _Arceuthobium_ seeds were placed here. Douglas fir cultures, unless otherwise noted, were placed on a different bench, where the light intensity was less than 5 foot candles.

Material to be sectioned for microscopic examination was fixed in formalin-acetic-alcohol (FAA; Johansen, 1940), dehydrated in n-butyl alcohol, and embedded in $54-56^{\circ}$

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* Temperature and humidity were automatically maintained at the indicated values. A thermometer and a hygrograph were present in the culture room at all times and these were inspected whenever the culture room was entered, but a continuous record of temperature and relative humidity was not kept during the entire course of this study. During the summer months the relative humidity was subject to somewhat more fluctuation than indicated, apparently caused by the cooling system. However, only one experiment here recorded, the first one with Douglas fir mentioned under the section on the Effect of caffeine, was performed during this period.

paraffin. Soft material was sectioned at 10μ. Longi-
sections of wood containing material were cut at $10-15\mu$.
Transections of woody material were cut at $15-20\mu$. Woody
materials were sometimes softened by hydrofluoric acid
treatment. All sections were stained with safranin and
fast green.

Photographs were taken with either Kodak plus-x or
panatomic-x 35 mm. film. Photomicrographs were taken with
an AO Spencer 35 mm. Photomicrographic Camera attached to
an AO Spencer Microstar trinocular microscope illuminated
by a Silge & Kuhne Ortho-Illuminator B. Close-up pictures
were taken with a single lens reflex camera either attached
to a binocular dissecting microscope or fitted with extension
tubes.

RESULTS

Arceuthobium douglasii Seed Germination

One of the original intentions of initiating studies
on A. douglasii seed germination was to investigate the
possibility of inducing callus proliferation on the develop-
ing embryos. This method has proved fruitful for other
species when embryos or young seedlings were placed on
media containing auxin (for instance, Loewenberg and Skoog,
1951).

Some seeds were placed on moist filter paper in Petri
dishes a few days after collection on September 23, 1957.
Within a few days definite radicle elongation had occurred
in most of them, though the amount was small, being general-
ly one mm. or less. The radicles of some were curved away
from the window next to which they had been placed. These
and other similar observations suggested that the seeds
were not dormant and that germination would proceed without
special treatment. An experiment was then set up to test
the effect of several growth substances on radicle develop-
ment. The media employed were:

1. White's basic nutrients, solidified with 1% agar.
   (White's)
2. White's + yeast extract at 1 g/l. (White's + yeast)
3. White's + yeast + naphthaleneacetic acid at 1 mg/l.
4. White's + yeast + gibberellic acid* at 1 mg/l.

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* Kindly supplied by Dr. M. Chessin, who in turn obtained
it from Dr. P. W. Brian of Akers Research Laboratories,
Welwyn, England.

5. White's + yeast + coconut milk* at 5%.
6. White's + yeast + coconut milk* at 15%.

The media were distributed to 125 ml. Erlenmeyer flasks,
50 ml. per flask. Three flasks each of media 1. through 4.,
one flask of medium 5. and two flasks of medium 6. were
used. Five seeds were planted in each flask, and the
flasks were placed under the lamps in the culture room.
The experiment was begun one month after the seeds were
collected and was maintained for three months. No sub-
cultures were made. Photographs of some of the seeds
after three months are given in Fig. 7. Some were lost
due to contamination.

The fraction germinating on the several media, in
general, decreased with increasing number in the list given
above; in order they were: 12/15, 9/15, 6/15, 5/15, 3/5,
and 2/10. Due to the small number of seeds used, the abso-
lute significance of these figures is doubtful, but they do
seem to show that elongation may be affected by growth
substances.

Elongation of most radicles, especially on medium 2.,
did not exceed one mm., this length being attained within
two weeks. Others continued to grow uninterruptedly over
the period, reaching as much as six mm. The ratio of the
two types of elongation was about 1:1 on most media, with a

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* Liquid endosperm of mature coconuts purchased at a
local market. In these media only, the coconut milk was
sterilized by cold, Seitz, filtration.
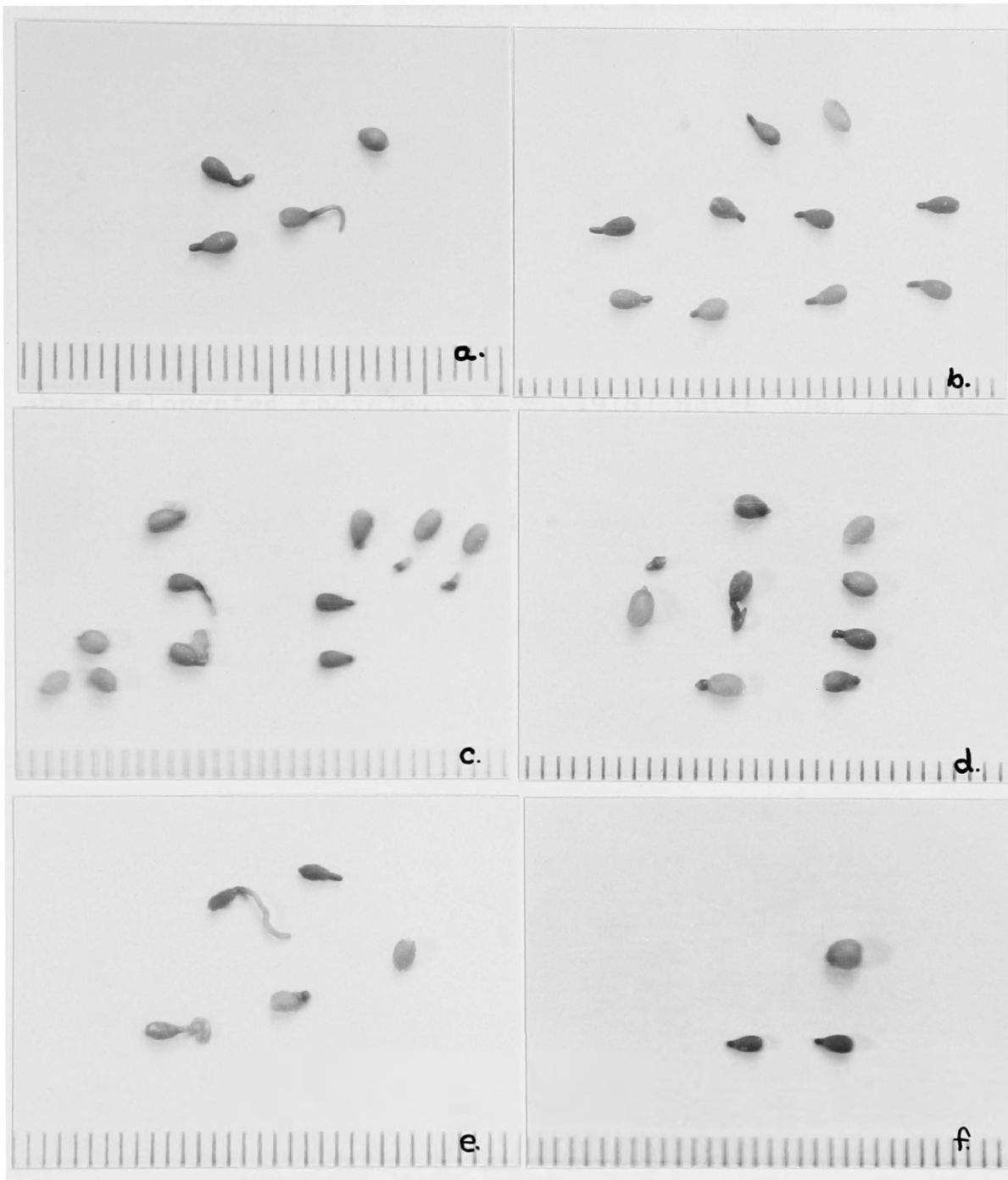
Fig. 7. A. douglasii seeds after three months on the
media listed on pp. 29 and 30. Those in a. were on medium
1.; those in b. on medium 2.; c. on medium 3.; d. on medium
4.; e. on medium 5.; and f. on medium 6. Scale divisions
in mm. Note short-elongation (all of those in b.) and
short, stubby, radicles (two lowermost ones and one to
left in d.). The radicles became detached from the endo-
sperm of two in c. and one in d. on handling.

notable exception on medium 2., on which none elongated
over one mm. These two types of elongation have also been
observed in seeds germinated on filter paper and on agar
without added substances. It appears that the shorter
elongation can be largely accounted for by simple cell
elongation in the radicle (see Fig. 8). However, a mitotic
figure was found near the growing point in a section of a
short-elongated radicle. Weir (1918) noted that low temp-
eratures seemed to be beneficial to Arceuthobium seeds;
perhaps low temperatures would induce a higher percentage
of radicles to elongate over one mm.

All of the radicles, even the short ones, curved
downward toward the medium, with which most made contact.
However, none ever penetrated the medium. Radicles, once
they reached the surface of the medium, usually followed an
eratic path on further elongation, as shown in some of the
photographs of Fig. 7. Some seeds on media 3. and 4.,
whose radicles did not appear until about two months after
the beginning of the experiment, had short, stubby, radicles
develop at this time. The tips were swollen and green, not
the characteristic red color of other radicles. There was
little noticeable increase in these radicles during the
remaining month. It appears possible that they represented
a chemically induced abnormal growth.

For short term experiments, as the one described,
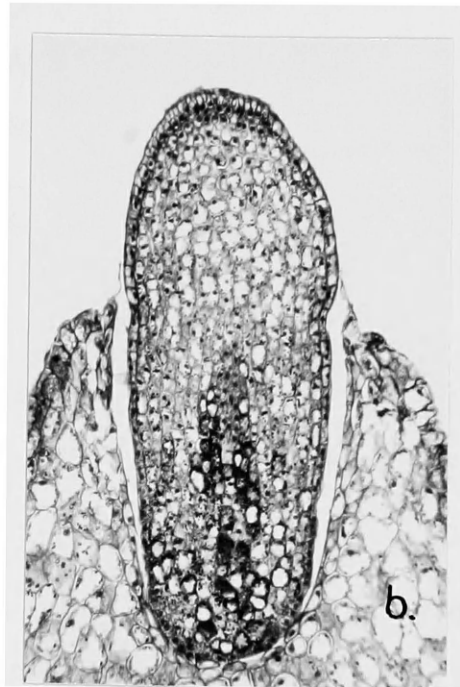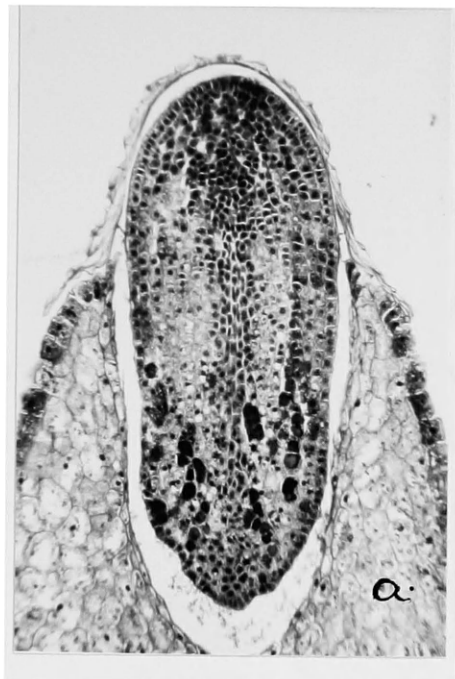the addition of salts and sucrose is probably unnecessary

Fig. 8. a. Longisection of embryo of ungerminated A. douglasii seed. b. Same of "short-elongated" radicle after 25 days; note larger cells. c. Portion of "long-elongated" radicle after 29 days. Xylem elements present in row of elongated cells at center. (all x 70.)

since the endosperm and embryo contain abundant stored food. Seeds have been grown on agar alone, with the resulting growth comparable to that on medium 1.

Pseudotsuga Callus Culture

Callus growth is easily stimulated on Douglas fir stem disks. It has not been possible, however, to maintain this callus. Callus growth generally proceeds well for three to four weeks but generally during the fourth week the growth rate seems to decline. After five weeks growth appears to cease, whether or not the tissues are subcultured to fresh media. Within six to nine weeks the callus regularly turns a dark brown color.

This is the sequence of events that takes place on control medium and on several modifications of the control medium. The sequence of events on control medium will be covered in more detail below. This will be followed by a discussion of growth on other media. Certain other possible pertinent findings will also be introduced.

The control medium, which was used in most experiments, consisted of White's basic nutrients (p. 25) solidified with 0.8% agar to which was added naphthaleneacetic acid (NAA) at a concentration of 0.5 mg/l. A higher NAA concentration (5.0 mg/l) was less effective in promoting callus formation. On White's with no auxin added, or on agar alone, Douglas fir stem disks formed little or no callus.

Consistently good growth occurred on White's plus NAA at
0.5 mg/l (this medium will hereafter be referred to as
White's-NAA) taking into account variability of the plant
material. (See Figs. 9 and 10.)

Growth on White's-NAA.

Within a few hours after cutting disks and placing
them on media, a slight amount of browning occurs on the
exposed cut surfaces. Greener disks tend to show less
initial browning of this sort. Otherwise little or no
visible change occurs on the surface for three or four
days. By about the fifth day healing commences, and a
day or two later a prominent ridge generally forms over the
cambial region. Within another day or two proliferation
over the cortical-phloem region equals that over the cambium
and soon surpasses it. Growth proceeds rapidly for the
next two or three weeks, a doughnut shaped callus dome
being formed over the original disk. Callus spills over the
xylem*, and proliferation within the cortex and phloem
occurs, causing distortion and lateral expansion. Prolifer-
ation occasionally occurs over the pith also. By three to
four weeks the cultures may be two to four times the origi-
nal size and appear as in Figs. 9c, 12, and 15a,b. In

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* A simple method of estimating the extent of callus
formation consisted of noting the diameter of the opening
over the xylem-pith (larger when callus was less extensive).
Shallower callus over the cortex-phloem and localized rather
than overall callus formation were other characteristics of
poor callus production.
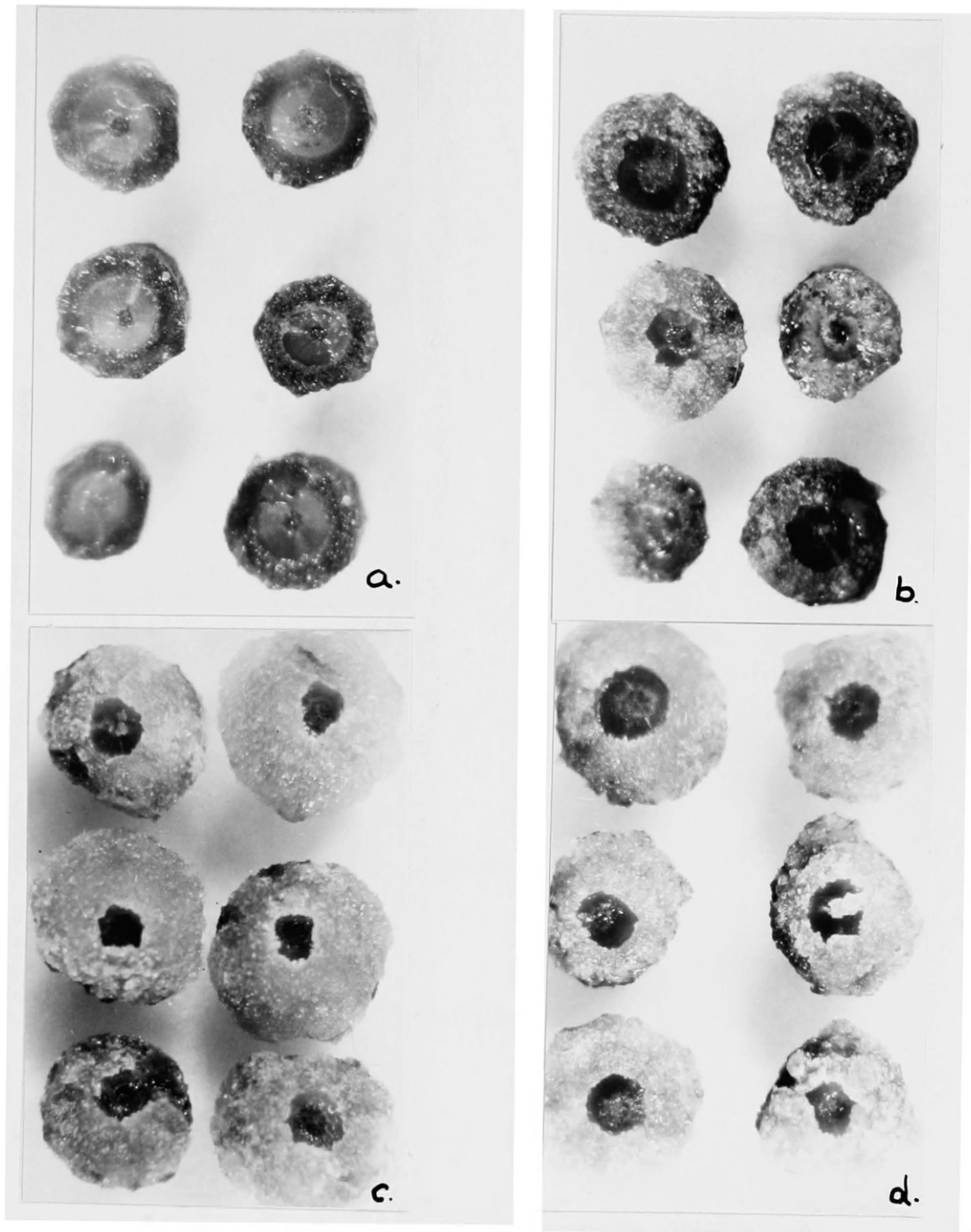
Fig. 9. Each photograph pictures six representative cultures from sets of 16 to 18 grown on the following media for 27 days (cork removed in all): a. on agar alone; b. on White's; c. on White's-NAA (0.5 mg/l); d. on White's-NAA (5.0 mg/l). (all x 5.) Quantitative data taken from cultures represented here are given in Fig. 10.
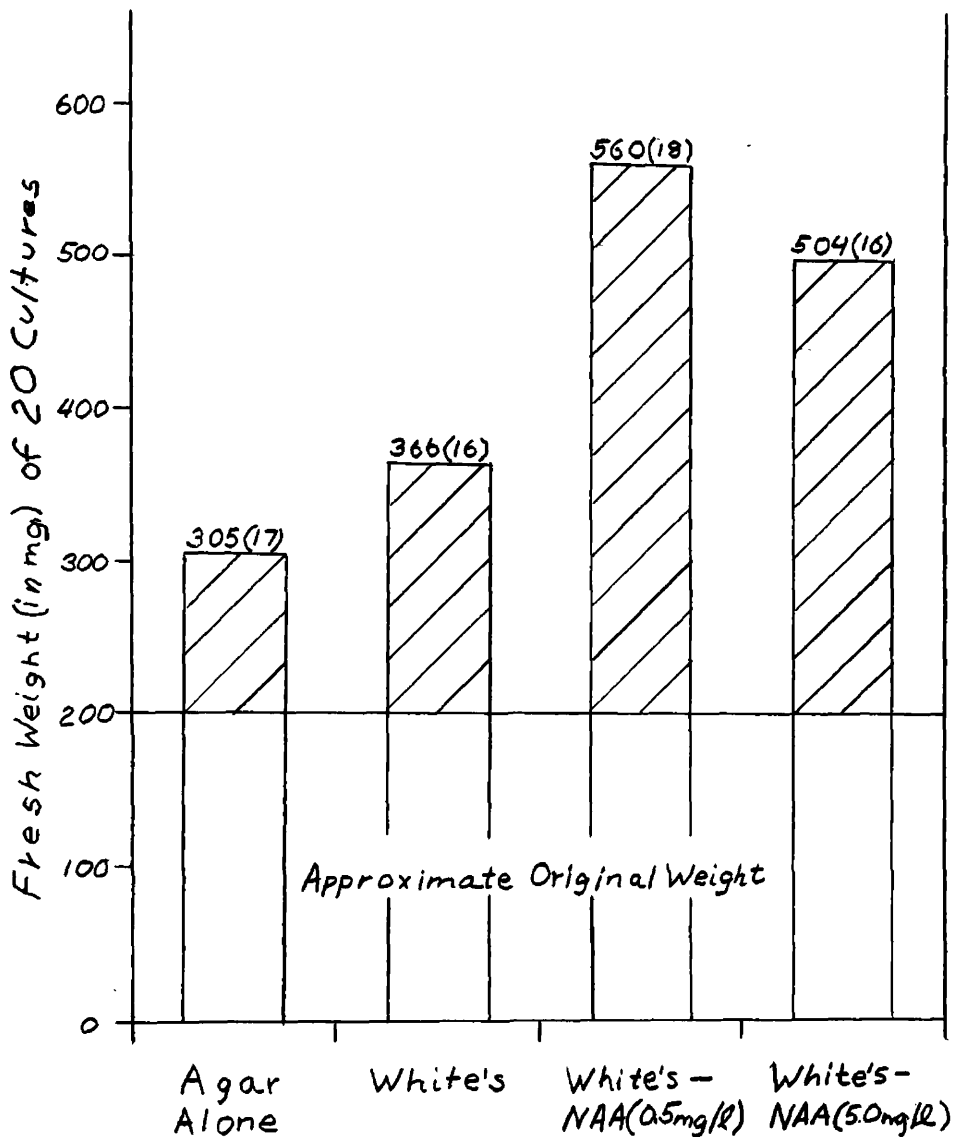
Fig. 10. Total fresh weights of 20 cultures grown on the indicated media for 27 days. The total weight of 20 cultures is given at the top of each bar, as well as, in parentheses, the actual number of cultures from which this figure was determined. The approximate original weight (200 mg/20 disks) was determined from a set of 20 disks similar to those used in experiments.

median longisection they appear as in Fig. 11b. After
three to four weeks, growth slows and apparently soon
ceases.

In early stages of growth the callus surface generally
appears rough, with individual cells clearly distinguish-
able under magnification. In later stages individual cells
become less noticeable and the surface takes on a mushy
appearance. Some cultures retain the rough, granular,
appearance on a portion of the surface for longer periods
of time. The color of the callus generally changes from
white or light green, through shades of cream or tan, to,
eventually, darker shades of brown.

Browning of intact cultures, subcultured or not,
begins after about five or six weeks, and cultures are
dark brown and appear dead usually within two more weeks.
The callus dome, when cut away from the original material
and subcultured, turns brown sooner. Within two or three
days after transferring callus subcultures (i.e., callus
domes cut away and subcultured), they begin browning around
the edges, and are usually completely brown within two
weeks. The rate of browning of callus subcultures varies
with their size. Smaller, thinner, ones turn brown faster
than larger, thicker, ones*. Larger callus subcultures

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* Callus subcultures from disks grown on growth restrict-
ing media regularly turn brown sooner than callus sub-
cultures from disks grown on the control medium. In most
cases this seems to be due to the smaller size of the
former since cultures left intact on growth restricting
media (when not highly toxic) turn brown at about the
same time as intact controls.

Fig. 11. a. Radial section of stem disk similar to those used in experiments. b. Radial section of a 29 day old culture (with cork removed) showing callus formed by proliferation of cells of cortex, phloem, and cambium. A lateral meristem is indicated by arrow 1, the outer limit of the expanded phloem by arrow 2, and proliferation within the cambium at arrow 3. Xy., xylem; cam., cambium; phlo., phloem; cort., cortex. (both x 40.)
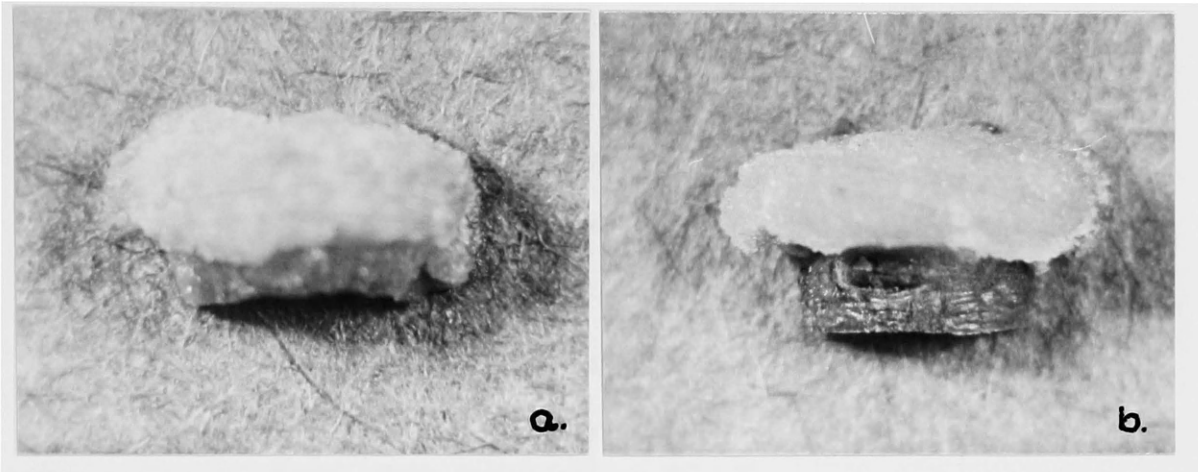
Fig. 12. Edge views of 30 day old cultures grown on White's-NAA. a. Cork removed. b. Cork intact. Lateral expansion restricted in b. (both x 10.)
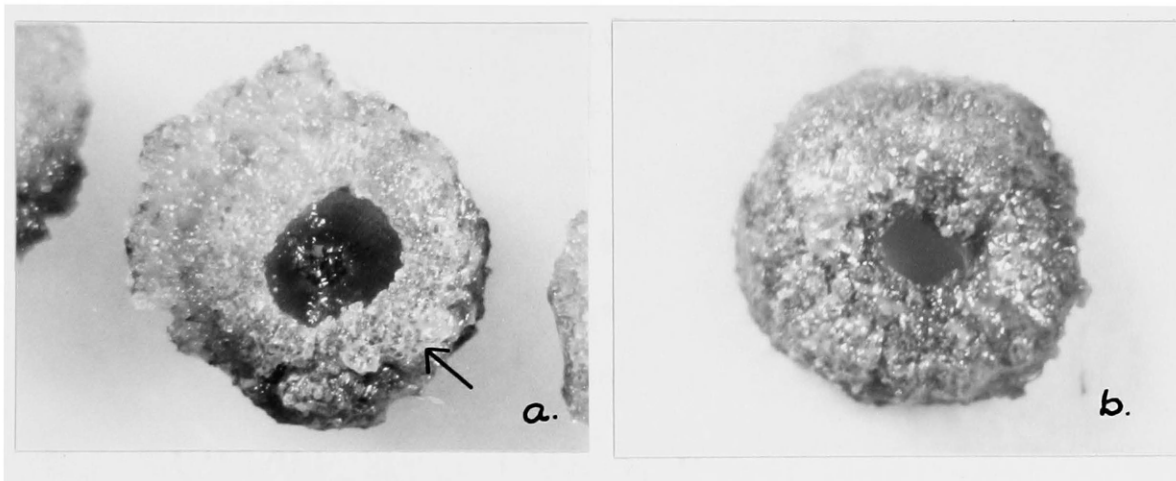
Fig. 13. Extracellular deposit on callus surfaces. a. Extracellular deposit (most evident at arrow) on 27 day old culture grown on White's-NAA (5.0 mg/l). It is light colored, and little browning of the callus has occurred. b. Brown deposit on a 61 day old culture. This is callus only, which was cut away from the original stem disk and subcultured after 35 days on White's-NAA. It was trans- ferred to White's-NAA plus ascorbic acid (100 mg/l) and after 5 days transferred again to White's-NAA without ascorbic acid. This treatment failed to prevent browning and extensive formation of the extracellular deposit. (both x 10.)

may remain light tan for three or four weeks after sub-
culturing. In one case, five larger (ca. 2 mm. thick)
callus subcultures of this type were subcultured again
after 28 days in the first subculture, but after three
days in the second subculture they had turned dark brown.

In association with growth decline, there generally
appears on the callus surface (extracellularly) a cream
colored substance. This generally appears somewhat before
browning in intact cultures, but, when callus is subcultur-
ed before this substance appears, it is formed coincident
with browning. Smaller callus subcultures may not form
this substance. Following its appearance this extracell-
ular deposit becomes evident over more of the surface,
thickens, and becomes darker in color as the outer cells
themselves darken (Figs. 13 and 14b). It is chemically
rather inert and appears to be of the same nature as the
brown pigments within the tissues. It is not attacked
by concentrated acid (HNO_3), ethyl alcohol, or ether, is
bleached by hydrogen peroxide, and is either slowly or not
at all attacked by concentrated alkali (NaOH)*. These
reactions suggest a relationship to pigments produced by
phenol oxidase (see Thomas, 1955). The brown pigments
within the tissues exhibit similar reactions.

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* When brown cultures are placed in concentrated NaOH, a
reddish-brown material slowly diffuses into the solution,
but the extracellular deposit does not diminish perceptibly
after a period of one hour.

A slight darkening of the medium immediately below three weeks or older cultures is commonly observed. Perhaps there is a transfer of pigment precursors to the medium where they are further oxidized.

Peripheral cells of cultures regularly contain more granular material than interior cells, the amount increasing with age and growth decline. The granular material consists in part of starch grains and what appear to be oil droplets. (See Fig. 14c.)

The death of cells progresses centripetally. Older cultures that are dark brown in color and appear dead exhibit on microexamination outer layers of dead cells while inner cells still appear to be alive (Fig. 14b). A few old and apparently dead cultures, which had not been discarded, were noted to produce small localized amounts of new callus. However, the new callus soon turned brown.

Effect of leaving the cork intact.

In earlier experiments the cork was removed from stem disks to decrease contamination. In one early experiment extensive Protococcus Agardh contamination occurred when the cork was left intact. Later it was found that soaking stem segments in 95% ethyl alcohol followed by a short burning (see White, 1954) previous to mercuric chloride treatment, effectively eliminated contam-
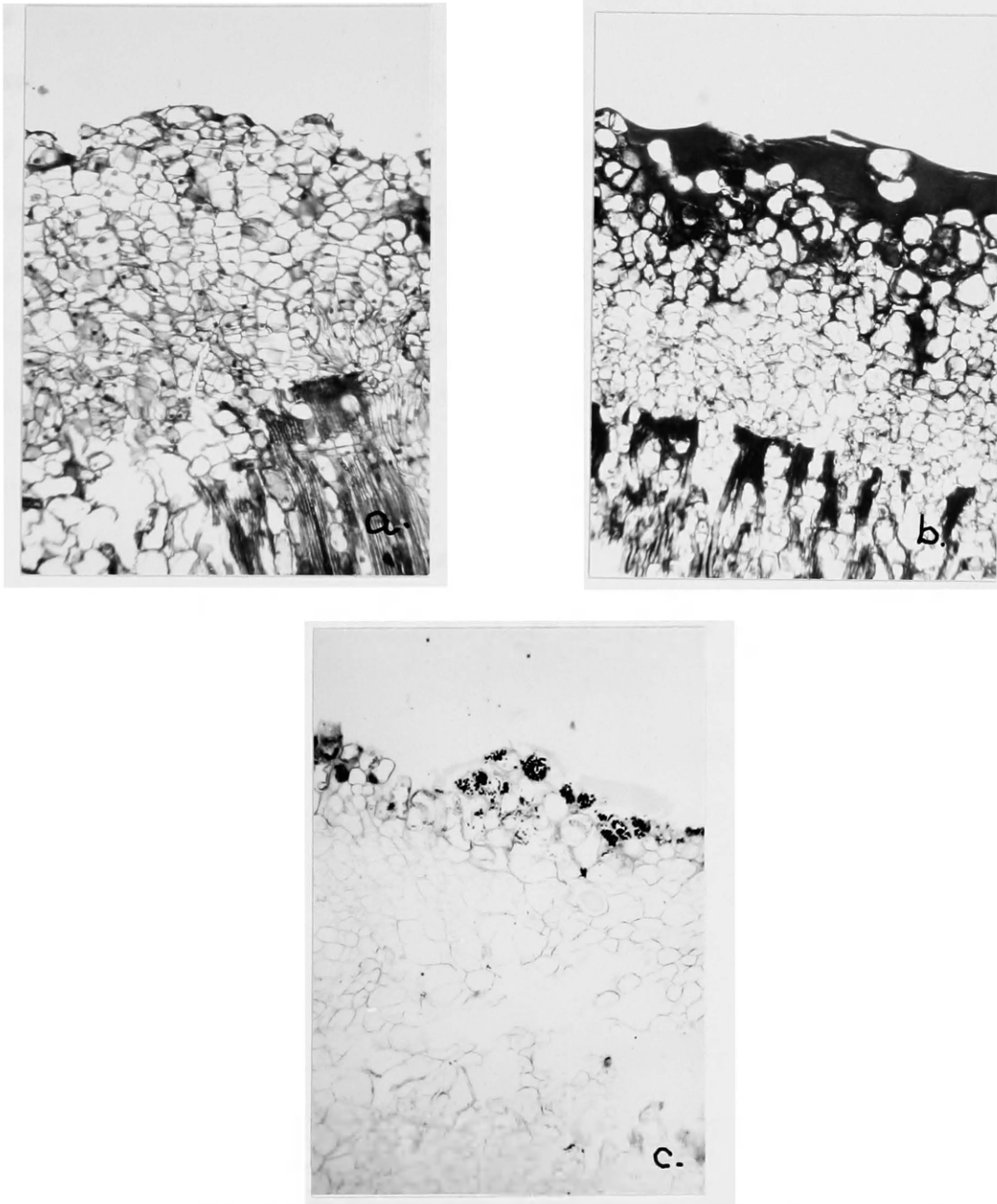
Fig. 14. a. Section of callus on a 29 day old culture. b. Section of callus on a 67 day old culture showing heavy pigment deposit on and among peripheral cells. Though this culture appeared dead when intact, as shown in this section there are still nucleated and apparently living cells below the dead outer cells. c. Section of callus on a 67 day old culture stained with I_2-KI. Note concentration of starch grains in peripheral cells. All cultured on White's-NAA. (all x 80.)

ination from the cork surface. Increase in fresh weight
is less when the cork is left intact but this is apparently
due mainly to the restriction of lateral expansion. Equal-
ly high callus domes occur over disks with or without cork.
The surface of callus formed on stem disks with cork
intact seems to retain a healthier appearance for a some-
what longer time. In later experiments the cork was
regularly left intact. (Compare Figs. 12a,b; 15a,b; 16.)

Effect of light.

Cultures were regularly grown in dim light (less than
5 f.c.). Though young calli were often of a greenish cast,
this was principally due to green tissues in the original
stem disk. When greenish appearing callus was cut away
from disks for subculturing, it was found to be actually
white or cream. In an experiment to be described later
(in which germinated Arceuthobium seeds were placed in
contact with thirteen day old dim light grown cultures)
two tubes were placed in the light (70 f.c.). The remainder
were placed in dim light as usual. Within fifteen days
under the light, both cultures of one tube had become
bright green (the others, both poor callus producers, had
become brown). They remained green for about two months,
but appeared dead within another two weeks. The dim light
cultures appeared to dead about a month earlier. However,
the green ones formed no more callus than those grown in
dim light.

Soon after this greening was noticed ten cultures (five tubes) on White's-NAA were started in the light. By fifteen days some of them were definitely greener than those in dim light set up at the same time. Curiously, only one of the cultures in each tube turned green. In each case it was the one toward the stopper end of the tube that had turned green. The one toward the bottom end of each tube was of the same appearance as the dim light grown controls. Since the tubes were slanted, while the agar surfaces were parallel to the bench top and fluorescent lamps, the only apparently important difference in position between the two cultures in each tube was their relative proximity to the upper glass wall of the tube. Perhaps the amount of light was near the critical point for chlorophyll formation, and the glass walls of the tubes acted as lenses, concentrating the light more in the vicinity of the cultures located toward the stopper ends. In each case the green culture formed less callus than the non green culture. This type of greening response was also noted in two other tubes of a different experiment.

When the callus domes on the ten light grown cultures were cut away and subcultured after 23 days, they turned brown as did the similarly subcultured dim light controls.

Effect of malt extract.

When malt extract at a concentration of 300 mg/l was added to White's-NAA it was somewhat stimulatory.

(Compare Figs. 15a,d; 16.)

Effect of tyrosine.

Douglas fir callus production on White's-NAA containing tyrosine (40 mg/l) was decidedly inhibited, and the callus turned brown when subcultured. A total of 80 cultures were grown on tyrosine containing media. In one experiment 20 stem disks were cultured on each of the following media:

1. White's-NAA.
2. White's-NAA + tyrosine (40 mg/l).
3. White's-NAA + tyrosine (40 mg/l) + cysteine (20 mg/l, autoclaved; see Effect of cysteine).

After 25 days the callus which had formed on the disks was cut away and subcultured on the same media.

Growth on medium 1. was as usual. There was little noticeable difference between growth on media 2. and 3., in both cases being poorer than on medium 1.

The results of another experiment involving tyrosine (40 mg/l) are given in Figs. 15 and 16.

Effect of ascorbic acid and coconut milk.

The coconut milk containing medium which Wetmore and Morel used (as described on p. 17, differing as used here in that the NAA was used at 0.5 mg/l) was not effective either in stimulating or maintaining callus growth, whether or not ascorbic acid (at 176 or 1000 mg/l) pretreatment was employed. A total of 100 cultures were grown on this medium; 60 received ascorbic pretreatment
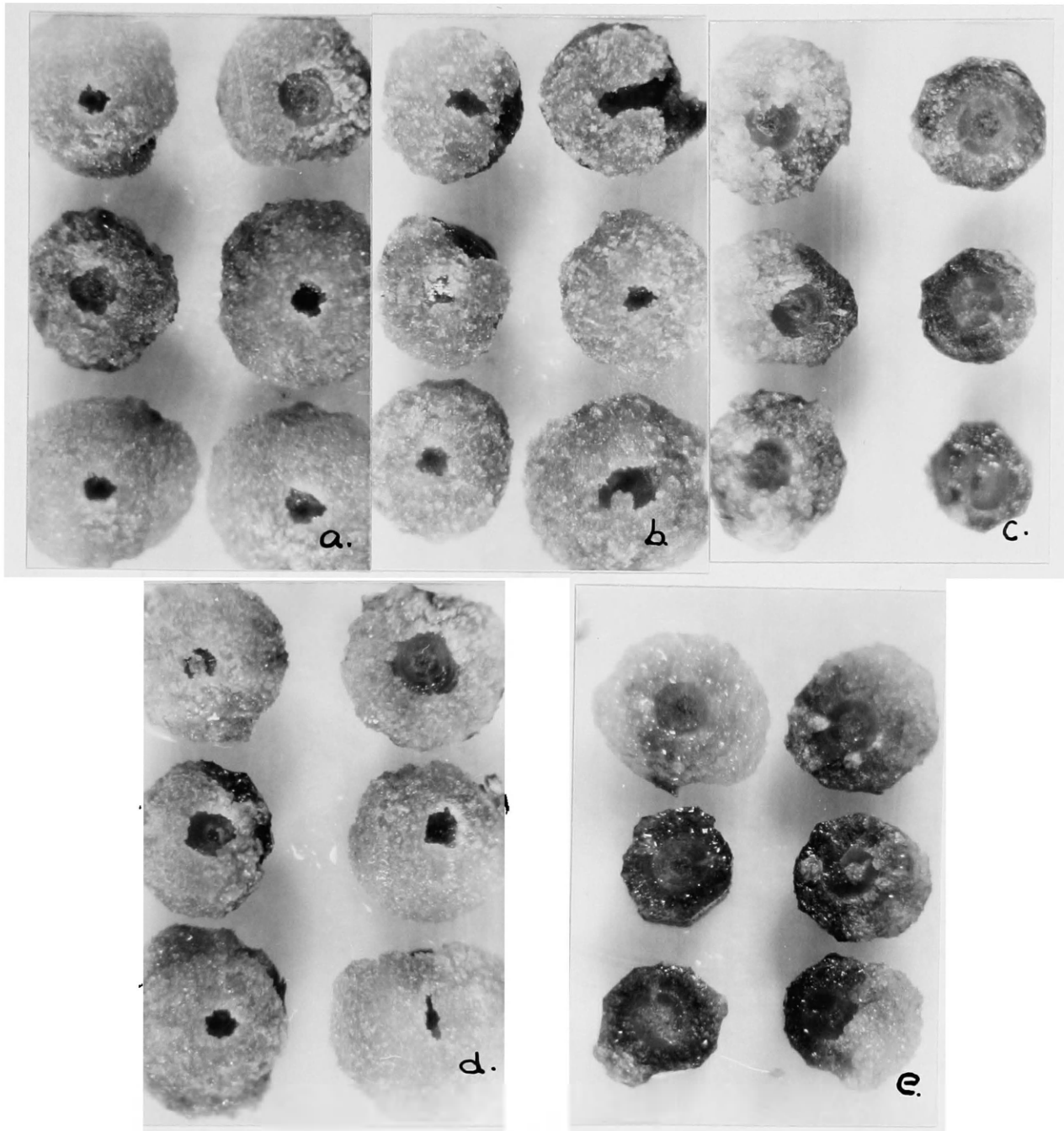
Fig. 15. Each photograph pictures six representative cultures from sets of 18 to 20 grown on the following media for 30 days (cork removed except in b.): a. on White's-NAA; b. on White's-NAA with cork intact; c. on White's-NAA plus tyrosine (40 mg/l); d. on White's-NAA plus malt extract (300 mg/l); e. on White's-NAA plus tyrosine (40 mg/l) plus malt extract (300 mg/l). (all x 5.) Quantitative data taken from cultures represented here are given in Fig. 16.
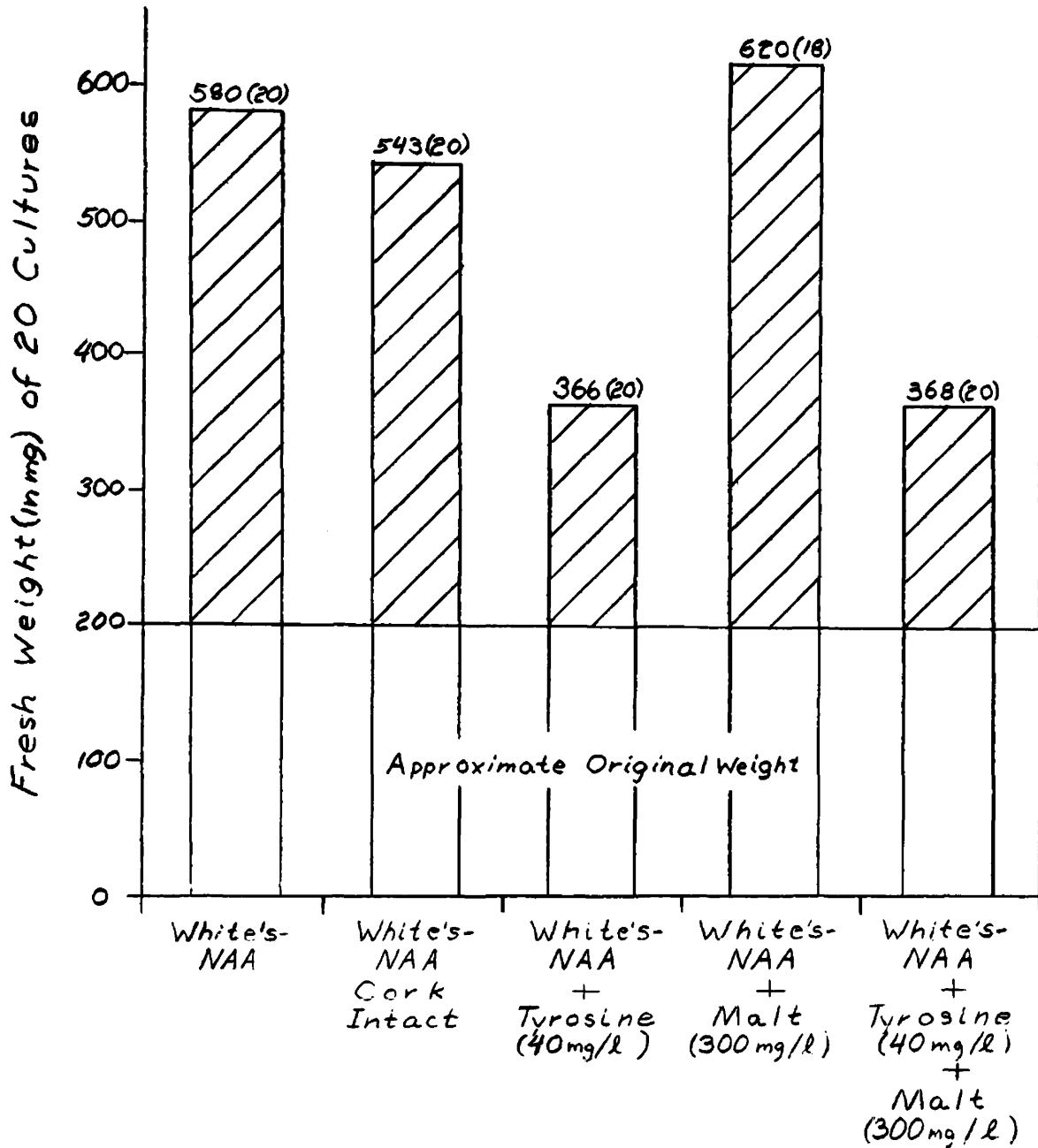
Fig. 16. Total fresh weights of 20 cultures grown on the indicated media for 30 days. (Malt = malt extract.) The total weight of 20 cultures is given at the top of each bar, as well as, in parentheses, the actual number of cultures from which this figure was determined. The approximate original weight (200 mg/20 disks) was determined from a set of 20 stem disks similar to those used in experiments.

and 40 did not. In all cases callus formation was slight and generally only observable under magnification. The extracellular deposit regularly formed on the callus within two weeks. Forty cultures with and 20 cultures without ascorbic acid treatment were set up at the same time, with similar Douglas fir material, as cultures grown on White's-NAA. Growth on White's-NAA was as has been described earlier.

One set of 20 disks grown on the medium described on p. 17 (with 0.5 mg/l NAA) without coconut milk, produced, comparatively, considerably more callus. The coconut milk used in these experiments was removed from mature nuts, which had been obtained from a local store. Often the milk in these nuts was putrid, but that used in media appeared not to be contaminated, having a fresh taste and odor. It was filtered twice through Whatman #1 filter paper before being used. In two experiments the coconut milk was used after it had been stored frozen for some time. In another experiment the milk was removed from nuts obtained on the same day the medium was prepared. Results were the same in either case. Apparently either the quality of the coconut milk was poor, or coconut milk at 15%, under the conditions of these experiments, exerts a toxic effect on Douglas fir tissues.

In another experiment involving ascorbic acid, 30 cultures were grown on White's-NAA for 35 days. The 20

best cultures were divided into two similar sets of ten.
The callus from one set was cut away and subcultured on
White's-NAA. Callus from the other set was subcultured on
White's-NAA plus ascorbic acid at 100 mg/l. After six
days, nine of the latter calli were transferred to White's-
NAA without ascorbic acid. One was left on the ascorbic
acid medium.

Without ascorbic acid treatment browning commenced
within two or three days after subculturing and proceeded
as usual. Those transferred to ascorbic acid remained
fresh appearing while on the ascorbic acid medium, but
began turning tan two or three days after transfer to
medium without ascorbic acid. The one left on ascorbic
acid medium remained fresh appearing for a day or two
longer. Cultures with ascorbic acid treatment differed
from the controls only in the slightly longer period of
time required for browning to take place.

In another experiment 20 cultures were started on
each of the following media:

1. White's-NAA.
2. White's-NAA + ascorbic acid (10 mg/l).
3. White's-NAA + ascorbic acid (100 mg/l).

The amount of callus formation was similar on all three
media. Formation of the extracellular deposit was delayed
a few days on the ascorbic acid media. It was delayed
longer on medium 2. About half of the cultures on each
medium were turning brown by 36 days.

Effect of cysteine.

The use of cysteine, which can inhibit phenol oxidase activity by combining with the enzyme's copper (Dawson and Tarply, 1951), has not proved effective in preventing browning of Pseudotsuga callus. One experiment employing cysteine has been mentioned under the Effect of tyrosine. The cysteine in that case was added to the medium prior to autoclaving. In two other experiments cysteine was added to the medium after the latter had been autoclaved and cooled to 45°C., since Nitsch (1957) reports that auto- claving causes the breakdown of the sulfhydryl group of cysteine*. It was added as a solution which had been sterilized by passage through a Seitz filter.

In the first experiment with non-autoclaved cysteine, 50 cultures were started on White's-NAA. After 23 days the callus was removed from the best 30 cultures; ten were transferred to each of the following media:

1. White's-NAA.
2. White's-NAA + malt extract (300 mg/l).
3. White's-NAA + malt extract (300 mg/l) + cysteine
      (20 mg/l).

After five days they had all turned light brown, the amount of browning increasing as usual with age.

In another experiment cultures were both started on and, after 20 days, transferred to the same three media given above. The results were essentially the same.

* Apparently, it is the sulfhydryl group of cysteine which combines with copper -- see Dawson and Tarply, 1951.

Effect of caffeine.

In an early experiment, which was not repeated, a method similar to those used by Jacquiot (1947) with oak and chestnut was performed with Douglas fir stem disks. Disks were placed initially in solutions of 1% sucrose plus caffeine at 0.1%, 0.2%, and 0.5%, and allowed to remain in each of these solutions for one, two, and three days. There were 20 disks for each combination of caffeine concentration and length of exposure to caffeine. After caffeine treatment they were transferred to White's-NAA, as were 20 untreated disks. After one month the cultures were transferred intact to fresh White's-NAA. They all turned brown within two or three weeks after subculturing.

Later, another experiment involving caffeine was set up. This time caffeine was used as a permanent constituent of the medium at 100 mg/l (added to White's-NAA). By 18 days cultures on this medium had turned a greyish color, as constrasted to the greenish color of the controls, and most of them had noticeable amounts of the extracellular deposit as contrasted to the controls which had none. By 27 days they had produced about 1/2 as much callus as the controls, and some were turning brown. They were all brown by 36 days and had heavy extracellular deposits.

Differentiation in the callus.

Sections for microscopic examination were prepared from about 30 cultures. Complete or partially complete

serial sections were prepared from several of these. Only one clear case of the formation of other than parenchyma cells in the callus was observed. In a culture grown on White's-NAA a column of elongated spiral tracheids, in various stages of development, and with a sheath of elongated thin walled cells, was formed in proliferated cells within and above the cortex. It terminated, in the callus above the cortex, in a group of deeply staining tracheids. Other than this, the only other sort of differentiation which has been noted has been in the manner of arrangement and size of callus cells. What were apparently localized meristematic areas were common in the callus. Lateral meristems, appearing like rib meristems in longisection, were often observed in the outer layers of the original cortex (Fig. 11b).

Some attempts at _in vitro_ infection of _Pseudotsuga_ cultures with germinated _Arceuthobium_ seeds.

Eighty days after the beginning of the experiment with _A. douglasii_ seeds mentioned earlier, one of the flasks (of medium 1., p. 29) became contaminated with a fungus. One of the seeds in this flask, not near the fungus, and which had elongated about 3 mm., was removed and placed against a 30 day old Douglas fir culture on White's-NAA, from which the callus had been removed. In about a month the tip of the radicle was noted to have swollen slightly. It continued to enlarge slowly and in

another month had developed into a structure resembling a
holdfast. Fig. 17a pictures this radicle after 94 days,
with the senescent Douglas fir culture. There was apparent-
ly no connection between the radicle tip and the culture.

Soon after this radicle tip was observed to be swell-
ing, seven seeds with elongated radicles, which had been
grown on plain agar for 88 days, were placed against 13
day old cultures growing on White's-NAA. Two of these
were placed in the light and the remainder in dim light.
The tubes placed in the light each contained two Douglas
fir cultures, though only one of each was supplied with a
seed. Two of the Douglas fir cultures placed in the light
turned green, as noted under the Effect of light. The
radicle tip on the green Douglas fir culture bent downward,
and, apparently due to growth at the tip, the radicle became
arched over the callus, with only the tip making contact
with it. No swelling occurred after 66 days, however, and
the bending was probably only a phototropic response. The
other radicle in the light was not observed to undergo
any change.

Of those in dim light, one apparently died and another
became contaminated. The radicle tip of a third, after a
month, began to swell slightly, but increased in size only
slightly thereafter. The tips of two others split open
with the formation of callus like cells; one after two
weeks and the other after about a month and a half. In

both cases the proliferation was slight. In the first one the exposed cells were mushy appearing and green. The second one produced healthier looking callus type cells, with individual cells clearly distinguishable under magnification, and which were white. A photograph is given in Fig. 17b. It was transferred to a fresher Douglas fir callus after 64 days, but, during the process, the tip came in contact with the medium and water was absorbed by the tip. Afew days later, the tip had turned grey in color and soon died.

In all of these cases in which radicle tips were observed to undergo growth transformations when placed against Douglas fir cultures, it cannot be stated that they would not have done so had they not been brought in contact with host material. Further experiments of this type should probably be postponed until the host tissue can be cultured indefinitely. Since the radicles grew very slowly, the Douglas fir cultures used in these few experiments were generally brown before transformations of the radicle tips began.

Callus production on dwarfmistletoe infected stem disks.

Callus formation on A. douglasii infected Douglas fir stem disks proceeds similarly to callus formation on uninfected stem disks. In the first experiment with infected material, 20 disks, collected from two young, stunted, trees in the Pattee Canyon area, were cultured on White's-
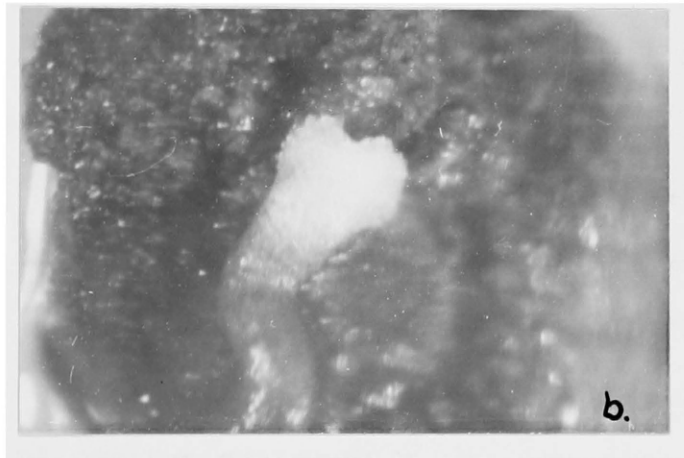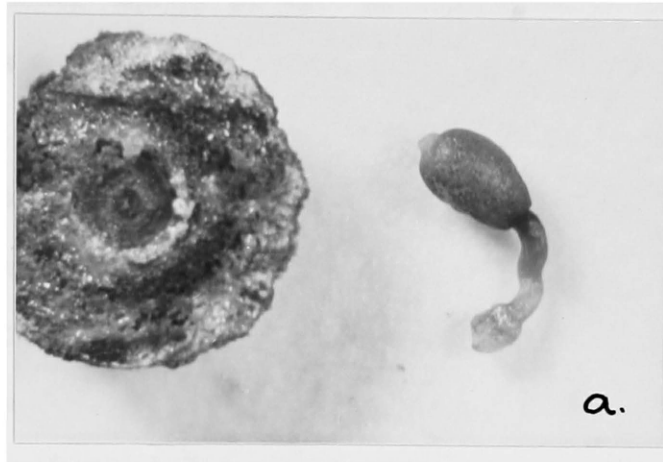
Fig. 17. a. A 174 day old <u>A</u>. <u>douglasii</u> seed, the tip of
the radicle of which had rested on the Douglas fir culture
at left for 94 days. (x 6.) b. Callus tipped radicle
of a 152 day old <u>A</u>. <u>douglasii</u> seed which had rested against
the Douglas fir culture beneath it for 64 days. (x 20.)

NAA in dim light. Disks from one of these trees proliferated only slightly, while from the other, proliferation of some equaled that which could be expected from the uninfected material used. At 29 days the extracellular deposit was beginning to form on the surfaces. Sections for microscopic examination prepared from the two best ones failed to reveal any clear indications of mistletoe penetration of the callus.

Forty stem disks collected from a young, vigorous appearing, dwarfmistletoe infected tree growing in the Flathead Lake area, produced callus more uniformly than the Pattee Canyon material. Callus development progressed similarly to that on a set of uninfected stem disks started three days after the infected material, with two exceptions. (1) On most of them small but quite noticeable amounts of callus formed on the disk face embedded in the agar. On uninfected material there have only occasionally been very slight amounts of callus formed in this area. (2) Though the callus was cut away from a few infected cultures and subcultured, after 26 days, and these turned brown and apparently died as with uninfected material, those left intact on the original medium continued growing after the unsubcultured uninfected material had apparently ceased growing and was turning brown. The infected cultures apparently continued growing through the sixth week. Though most of them began turning a muddy-

cream color by 33 days, browning progressed very slowly and extracellular deposit formation was slight. At 49 days, when they were more noticeably brown, they were subcultured intact to White's-NAA plus malt extract at 100 mg/l. By 60 days they had become a uniform brown color.

Before growth ceased several of the infected cultures reached 8 to 9 mm. in diameter, which is a larger size than any other cultures have achieved. This size seems to have been achieved more as a result of a longer period of growth than of a faster growth rate, since it appeared that they grew no faster, or even slower, than the uninfected material started at about the same time, until the latter ceased growing.

It is not possible at present to say whether this greater growth on infected stem disks was the result of the association with dwarfmistletoe, or the result of using material which was no doubt genetically and physiologically (exclusive of the influence of dwarfmistletoe) different from that used in the other experiments, or both.

Four of these infected cultures were placed in the light (70 f.c.). They grew similarly to those mentioned under the Effect of light. The callus was cut away from two of them and subcultured after 26 days. By 28 days the intact light grown cultures had begun to form the extracellular deposit, and the subcultures had begun turning brown. All four of them were killed and fixed at

this date. Some of the dim light cultures were also killed and fixed at this time and some at later dates. Sections were prepared from nine of these.

On examination of these sections it was found that dwarfmistletoe had definitely penetrated the callus of at least three cultures. Coincidentally or not these were all light grown cultures. Both subcultured calli and one intact culture had strands in the callus similar to young cortical strands. In three others (one light grown and two dim light grown) dwarfmistletoe of the cortex had apparently been "buoyed up" into the callus by proliferating cells. Two of these (one light grown and one dim light grown) were only partially sectioned. In three others (all dim light grown, aged 28, 34, and 37 days old -- the 28 day old one only partially sectioned) dwarf-mistletoe was not noticeable in the callus.

<u>A</u>. <u>douglasii</u> present in the callus was readily re-cognizeable. Living cells of the parasite are generally smaller, and, especially those in contact with host cells, take the fast green more strongly, than host cells. Tracheids, when present, take the safranin less strongly than do host tracheids; they are easily distinguished from host tracheids by their shorter length, scalariform thick-ening, and the absence of bordered pits (see Parke, 1951). Parasite nuclei are smaller and denser than host nuclei. There seems to be little possibility of confusing dwarf-

mistletoe strands with differentiation of the host callus.
Tips of the expanded phloem at the boundary between
original tissues and proliferated cells above, due to their
staining darkly with fast green, could, on cursory exam-
ination, be mistaken for thin dwarfmistletoe strands.

Penetration of a dwarfmistletoe strand into the callus
of a 28 day old light grown (but non-green) culture, the
callus of which had been cut away and subcultured after 26
days, was easily followed through 62 serial longitudinal
10 μ sections. One of these sections is shown in Fig. 18
while a diagrammatic reconstruction of the strand is given
in Fig. 19. The lower left portion of the reconstruction
(up to 50 μ in height and to 150 μ in length on the scale)
represents principally what was apparently a portion of a
strand present in the original tissues. A branch, formed
on the upper right of this, grew up into the callus. It
narrowed to a single cell in width at 300 μ, widened again,
and, after forming a short branch at 500 μ, terminated
among the outermost layer of callus cells. There was only
one dwarfmistletoe cell in the last 10 μ section, and only
three in the 3 preceding sections.
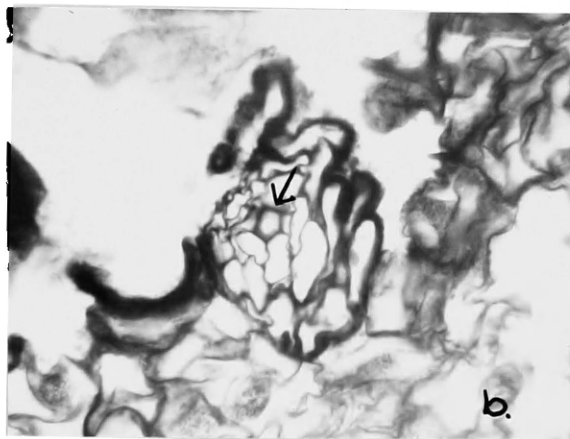
8 day old callus, cut away
disk at 26 days. Cortical
igh power view of cortical
w. Compare Fig. 4b. (x 400.)

Scales in Microns
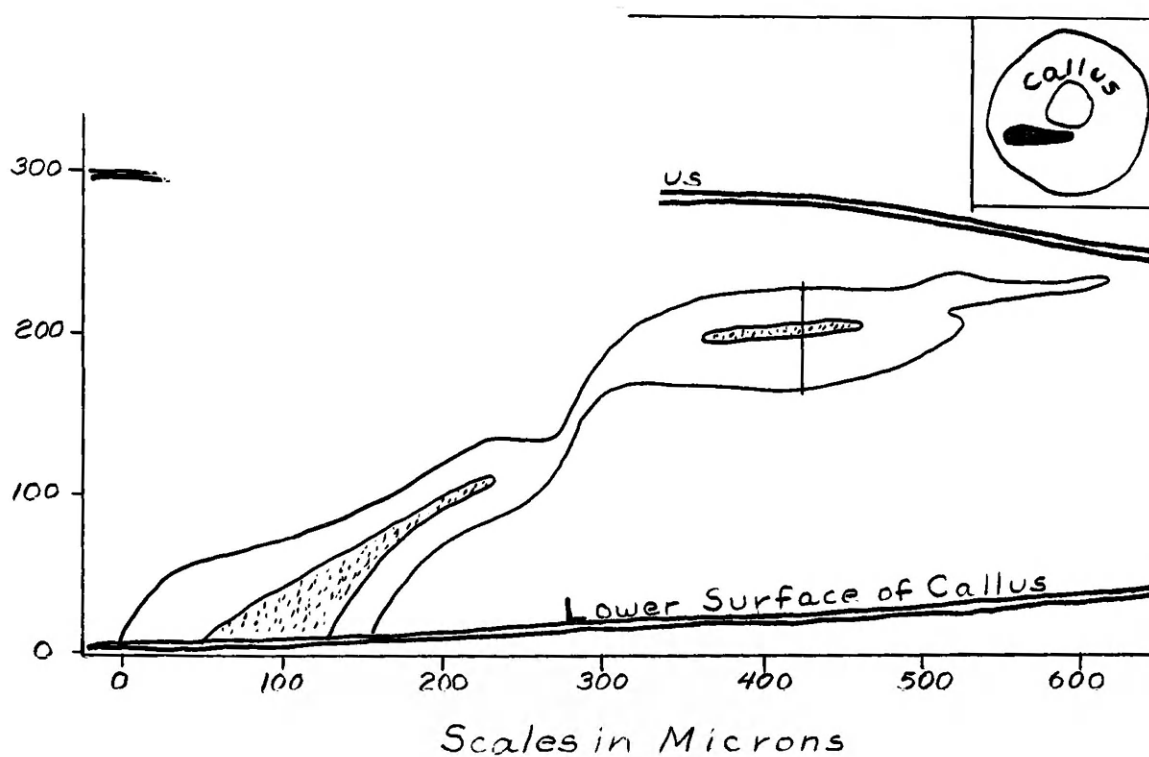
Fig. 19. Diagrammatic reconstruction, prepared from 62
serial 10 μ sections, of an <u>A. douglasii</u> cortical strand
penetrating callus formed on an infected stem disk.
Stippled areas indicate location of tracheids. Vertical
line at 420 μ indicates location of section pictured in
Fig. 18. Inset at top right shows the approximate location
of the strand (solid black) in top view.

DISCUSSION

The maintenance of Douglas fir callus has been hampered by the early cessation of growth and eventual death of callus cells, though fairly abundant callus may be formed before death commences. Methods similar to those used by others (Reinert and White, 1956; Wetmore, 1957) to inhibit or nullify the action of phenol oxidase, have failed to prevent browning and death of Pseudotsuga callus. It nevertheless seems likely that it is the activity of this enzyme which is mainly or wholly responsible for the growth decline and death of the cultures used in the experiments here recorded, and that the failure to maintain Douglas fir callus is principally due to insufficiently extensive utilization of phenol oxidase activity restricting methods. However, it has not been demonstrated that phenol oxidase is active in the browning of Douglas fir cultures nor that it is actually present. It could be that the browning of cultures is a result of the entirely non-enzymatic oxidation of pigment precursors. Also, it can not be stated with any certainty that the lack of substances essential for growth, not supplied by the media, does not play a role in growth cessation and death.

If the browning of Douglas fir cultures is enzymatic,

as has been assumed in this study, then the enzyme
responsible for browning is most likely phenol oxidase
(or a very similar, possibly identical [Bonner, 1957] enzyme
-- laccase); Mason (1957) states that " . . . the contri-
bution of other oxidizing systems [other than phenol
oxidase or laccase] is questionable." Tannins, being
phenolic substances, may serve as substrates for phenol
oxidase (Mason, 1955); their presence in fresh stem disks
and in both young and old (brown) cultures can be readily
demonstrated by the ferric chloride test (Johansen, 1940).
Freehand sections of fresh stem disks treated with ferric
chloride solution develop the characteristic blue color,
indicating tannins, in the tissues between cork and xylem
(and also in the pith). Sections of intact callus
cultures, when similarly treated, develop the blue color in
the callus cells also, with the strongest color in the
callus being developed in the peripheral layers. That
they are more concentrated in the peripheral layers is
interesting in that certain other substances also seem to
be more concentrated in this area, as indicated by the
accumulation of stored food particles as mentioned earlier.
It may be that there is a general "flow" of dissolved
substances to the surface cell layers, which occurs to a
greater extent than would be accounted for by a concentration
gradient. In the case of tannins, this appears to be
particularly favorable to the activity of phenol oxidase,

which, when acting at the surface, is in the best location
for utilizing the oxygen of the atmosphere. Further
evidence for the tannin "flow" viewpoint has been gained
from examination of prepared slides of stem disks and
cultures. Numerous tannin cells are located in the cortex
of sections of stem disks, as shown in Fig. 11a., while the
abundance of such cells in this area is regularly less in
sections of cultures (Fig. 11b). In the latter sections,
tannin cells can generally be distinguished at and near the
upper surface, in proliferated cells. If phenol oxidase
is present, and it utilizes these tannins, it has an
abundant reservoir of substrate material. Other, less
obvious phenol oxidase substrates, may also be present.

As has been mentioned, the formation of an extra-
cellular deposit on the callus surface precedes or
accompanies browning. When it first appears it is light
colored but becomes darker as the outer callus cells
darken. It probably represents excreted quinones under-
going further non-enzymatic oxidation and polymerization.

Since intact cultures regularly turned brown in the
course of a few weeks, callus was cut away from the original
tissues in many of the experiments in order to remove the
callus from possible deleterious influences of the starting
material. But it appears that by the time sufficient
callus has been formed, this influence, which appears to
be the transport of pigment precursors to the callus, has

already been effected.

Callus subcultures always turned dark brown sooner than intact cultures. In general, the only factor which affected the rate of browning of callus subcultures was their size. Thicker callus subcultures remained light brown for some time, not turning dark brown in some cases until soon before intact cultures of the same experiment turned dark brown. This correlation between size and browning rate of callus subcultures is probably due to the lower accessability of oxygen to more deeply seated cells. However, intact cultures did not begin turning brown until a week or more after callus subcultures had started turning brown. Injury to cells, which stimulates phenol oxidase activity (Mason, 1955), probably accounts for the earlier browning of callus subcultures. Perhaps intact cultures turn brown only when a higher concentration of pigment precursors are present in the peripheral cells.

Wetmore and Morel (Wetmore, 1957) thought that auxin destruction by phenol oxidase was responsible for the poor growth of pine callus which occurred when ascorbic acid was not used. Reinert and White (1956) found that "fair initial growth" occurred on White's plus NAA and certain other organic supplements, but that the cultures blackened and ceased growing within a few weeks. Ascorbic acid pre-treatment did not prevent browning. Though they thought that phenol oxidase was mainly responsible for the termin-

ation of growth of spruce callus, they were not certain

as to the manner of its action in causing growth termina-

tion. In the case of Douglas fir this is likewise not

certain, but it appears that death, and perhaps the growth

cessation also, might be accounted for by the toxic influence

of high concentrations of quinones, since death occurs in

those areas where the quinone concentration is presumably

highest. It has yet to be determined, however, whether it

is the accumulation of quinones that causes growth decline

or whether it is growth decline that results in a "piling

up" of quinones, followed by death.

Some considerations for further study of this problem

based on the results given here will now be presented.

It will be assumed that growth cessation and death of

Douglas fir callus is caused primarily by reactions or

reactants involved in browning. It will also be assumed

that phenol oxidase is present in the tissues.

Since initial callus growth is apparently not greatly

affected by phenol oxidase, a pretreatment of the starting

material with reducing substances such as ascorbic acid

does not appear to be necessary. However, the use of brief

treatments with reducing substances may prove to be an

effective method for the successful subculturing of callus.

Though this method was not effective here (see p. 50),

this may have been due solely to the inability of the

subcultures to heal over the cut surface due to the lack

of substances necessary for growth. A more growth stimulating medium, in addition to ascorbic acid treatment whenever cultures are cut up for subculturing, may be all that is necessary. Also, it may have been that autoclaving destroyed most of the ascorbic acid.

As an alternative to the use of occasional treatments to restrict the browning reaction, methods designed to be active continually during the growth of cultures might be used. Reinert and White found that tyrosine present as a permanent constituent of the medium limited browning and allowed for the continuous culture of Picea callus. It is difficult to assess the reason for the ineffectiveness and even inhibiting influence of tyrosine with Pseudotsuga. Reinert and White used tissue fragments from the trunks of older trees. The difference in age of tissues used, in addition to genetic differences, may be the reason. Another possibility may be the method of sterilization. Reinert and White do not specify the method they used to sterilize tyrosine. They mention only that "relatively heat-labile substances" were sterilized by passage through a bacterial filter. Perhaps tyrosine is sufficiently altered on autoclaving to destroy its effectiveness and to cause it to be toxic. However, tyrosine is stable to 344°C. (Meister, 1957).

Methods similar to those used by Jacquiot (1947) may prove successful with Douglas fir, but there are some

apparently basic differences between the culture of Douglas fir stem disks, and the material he used, to be taken into account. His cultures would not proliferate unless treatments to remove the influence of tannins were used. His use of caffeine to precipate tannins was based on the argument that tannins, or their oxidation products, diffused into the medium, inhibiting growth on being reabsorbed by the tissues. Tannins were precipitated while in the medium. Transferring tissues to fresh media at several early stages, removing them from tannins diffused into the media, also gave partially successful results. In the case of Douglas fir, proliferation is not apparently greatly inhibited. Also, it has been observed from examining sections of the cultures, that the number of tannin cells does not decrease as much from the lower half of the original material as it does from the upper half, indicating that there is not so much diffusion into the medium (though slight browning of the medium immediately under cultures occurs), as diffusion from the original material into the callus above. Something which will enter the original material and cause tannins (or other phenol oxidase substrates) to be unavailable to phenol oxidase, without being toxic, is necessary. Caffeine used properly may accomplish this. The observation that cultures supplied with a continuous source of caffeine (100mg/1), formed phenol oxidase products sooner than the controls, and formed

less callus, indicates that caffeine at this concentration
is toxic to <u>Pseudotsuga</u> and that perhaps it was degraded
before it could act on the tannins. It should also be
mentioned that Reinert and White did not find caffeine
effective with <u>Picea glauca</u> (White, 1957).

A possibly less fruitful method of overcoming phenol
oxidase activity may be the use of substances which inhibit
this enzyme directly, such as glutathione, cysteine, or
Na-ethylxanthate. At least, the use of such substances
was not effective with the cultures of Wetmore and Morel
(Wetmore, 1957) or Reinert and White (1956). Cysteine,
used at a concentration approximately equimolar to the
glutathione concentration used by Reinert and White, seemed
to be somewhat inhibitory to the growth of Douglas fir
callus.

Though Reinert and White grew their cultures in the
dark (it is not known if Wetmore and Morel did so also),
perhaps more extensive studies of the effect of light
should be made, especially if it becomes evident that
dwarfmistletoe penetration of callus is more consistent
in light grown cultures. The results with light (70 f.c.)
grown cultures given here indicate that a modification of
the medium may be necessary for light grown cultures,
since those grown in the light, most noticeably those
which turned green, produced less callus than those grown
in dim light.

Callus derived from Douglas fir material other than young stem disks may possibly be more easily maintained. (Compare the work of Loewenberg and Skoog with Pinus strobus and the work of Wetmore and Morel with the same species.) However, the use of such material as a substrate for dwarf-mistletoe may be more restricted unless it could be infected in vitro by Arceuthobium seeds.

Experiments with Arceuthobium seeds seem to indicate that radicle elongation can be inhibited by chemicals. Perhaps, for preventative purposes in dwarfmistletoe threatened areas, chemical sprays could be developed which would block infection by preventing seed germination.

It has been found that A. douglasii can penetrate callus formed on infected Douglas fir stem disks. It would obviously be very interesting from a purely botanical standpoint to be able to culture, indefinitely, dwarf-mistletoe infected host tissues. If this were possible, certain physiological studies, such as on the nutritional requirements of infected as compared to uninfected callus, could be made. A study of Arceuthobium morphology and histology while in the callus, and under a variety of conditions, might be rewarding. If it should prove possible to culture infected Pseudotsuga callus indefinitely, one possible practical use of such material may be in the study of Arceuthobium control, keeping in mind that in vitro

results may not be directly applicable to intact plants.
A search could be made for substances that might destroy
dwarfmistletoe while not adversely affecting the host.
This could be done in much smaller space, in less time,
and under more uniform environmental conditions, than would
be possible if such experiments were carried out in the
field. Field studies could be carried out with the more
promising ones.

SUMMARY

The dwarfmistletoes (genus *Arceuthobium*) are a group
of flowering plants which are parasitic on many species
of conifers. Little is known of the physiology of the
parasite or of the physiology of host-parasite inter-
relationships. As a possible approach to these problems,
work has been initiated on a study of the *in vitro* culture
of Douglas fir (*Pseudotsuga menziesii*) callus with the
intention of using such material as a substrate for *A.
douglasii* (which is parasitic on Douglas fir). It has
been determined that the callus which develops on *A. douglasii*
infected *Pseudotsuga* stem material may be invaded by the
dwarfmistletoe, suggesting that it might be possible to
establish cultures of Douglas fir callus infected with
the parasite once it proves possible to culture the callus
indefinitely.

The present work has been primarily concerned with
the problem of establishing cultures of uninfected Douglas
fir callus. Though callus can be easily stimulated to
form on Douglas fir stem material, the callus soon ceases
growing and turns brown. Attempts to overcome growth
cessation and browning have not been successful. However,
it appears that it will prove possible to culture
Pseudotsuga callus indefinitely, since callus cultures

derived from other conifer species have been established
by other workers, even though similar problems were en-
countered. Some considerations for further work are
given based on the results presented here.

Results of a few experiments on A. douglasii seeds
are also given, which indicate (1) that A. douglasii
seed germination can be influenced by chemical agents,
and (2) that the tips of radicles of seeds grown in
vitro may undergo changes in morphology.

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