

THE INSTITUTE OF PAPER CHEMISTRY, APPLETON, WISCONSIN

ANTIMICROBIAL SUBSTANCES PRODUCED BY ISOLATED ASPEN
TISSUE GROWN IN VITRO

Project 2351

Report Two

A Progress Report

to

PIONEERING RESEARCH COMMITTEE

February 4, 1963

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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SUMMARY

Isolated aspen tissue grown in vitro, for three weeks on agar medium, produced antimicrobial materials which resulted in the production of inhibitory zones when the cultures were inoculated with various organisms. Growth of the following organisms was inhibited: Fusarium roseum, Saccharomyces cerevisiae, Bacillus subtilis, Penicillium roqueforti, Torula utilis, Sarcina lutea, Flavobacterium aquatile, Pullularia pullulans, and Staphylococcus aureus.

INTRODUCTION

The medicinal properties of various plant materials and extracts have been recognized since the beginning of the 5th century B.C. This recorded information along with the results of investigations performed in the late 19th and early 20th centuries and the advent of penicillin provided the impetus for the investigation of vast numbers of higher plants for antimicrobial substances. In 1943, Osborn (1) systematically tested 2,300 species of plants for activity against Staphylococcus aureus and Escherichia coli. Numerous investigations dealing with the screening of plants for active substances have been conducted as a result of increased interest in the search for antibiotic materials. The literature dealing with antibiotic surveys and individual materials is much too extensive to review in this report. Additional information containing detailed reference lists has been compiled by Nickell (2) and Skinner (3).

This report will be submitted for publication in Science, pending approval by the Pioneering Research Committee.

The occurrence of antibiotic materials in various members of the genus Populus have been reported by a number of workers. Grosjean (4) using boiling water extracts of the bark, reported fungistatic and fungicidal activity against Stereum purpureum. Populus candicans and P. trichocarpa produced extracts with strong activity while P. monilifera, P. Eugenii, P. robusta, P. fremontii, and P. Wislizenii produced extracts with very little activity. Activity was also found when extracts from P. regenerata, P. serotina erecta, and P. marilandica were used. A number of compounds with low fungistatic activity were extracted from the bark of P. candicans by Klöpping and van der Kerk (5). These materials included pyrocatechol, salicin, saligenin and salicylic acid derivatives. They also isolated two highly active substances which inhibited the growth of Botrytis cinerea, Penicillium italicum, Aspergillus niger, and Rhizopus nigricans. Bishop and MacDonald (6) obtained extracts from P. balsamifera and P. gileadensis which were active against Staphylococcus aureus and in some cases Escherichia coli. Extracts from P. alba did not inhibit the growth of these organisms. Azarowicz, Hughes, and Perkins (7) obtained extracts with antibiotic activity from P. fremontii. The antibacterial properties of the sesquiterpenes found in P. tacamahaca were investigated by Dull, Fairley, Gottshall, and Lucas (8) in 1957. Antibiotic activity has also been detected in extracts from P. alba (9), P. tacamahaca (10), and P. trichocarpa (11, 12).

Recently, Hubbes (13) extracted and isolated pyrocatechol from the bark of aspen (P. tremuloides). This material was found to inhibit the growth of Hypoxyylon pruinatum. The use of isolated tissues as a source of antifungal materials has been reported by Sussex, et al. (14). He concluded that antibiotic material was produced when lilac phloem explants were grown aseptically on agar medium. This material produced zones several mm. wide when phloem cultures were allowed to grow for a period of 3 to 15 days and then inoculated with Cytospora sp.

Much of the early work with antibiotic substances from higher plants centered around the use of extracts and juices from intact plants. The growth of isolated tissue from various plants may also be used in the detection of antimicrobial materials. Isolated tissue may be grown in large quantities (15) and may produce substances which differ from those found in the intact parent plant (16).

EXPERIMENTAL

The experiments reported here were undertaken to determine a limited spectrum of activity for antimicrobial substances produced by isolated aspen tissue grown in vitro. Organisms were selected, on the basis of the results for use in future studies dealing with the characterization of these inhibitory materials.

Aspen tissue, originally isolated from the approximate cambial region of triploid stem sections on Dec. 26, 1961, was grown on an agar medium containing major nutrients (17), trace elements (18), 3 p.p.m. glycine, 0.1 p.p.m. thiamine, two per cent sucrose, ten per cent coconut milk, and 0.5 p.p.m. naphthaleneacetic acid. Occasionally, cultures became contaminated with various organisms. It was noticed that one of these contaminants (Bacillus sp.) did not grow in the region surrounding tissue of either diploid or triploid origin which had been growing for a period of 2 to 3 weeks. Measurements of the pH were made and it was concluded that a change in the acidity of the medium was not responsible for the production of the observed zones of inhibition. Experiments were designed to determine the sensitivity of a number of organisms to inhibitory materials produced by aspen tissue. In each experiment 3 pieces of tissue weighing approximately 6 milligrams each, were placed on the surface of the medium and allowed to grow for a period of 3 weeks in the dark at 27-29°C. After 3-weeks growth, the tissue was

removed and weighed and the surface of the agar was flooded with a suspension of the test organism in nutrient broth (Difco) or Staphylococcus broth (Difco). The excess inoculum was removed and the cultures were incubated until sufficient growth of the test organism was evident.

The results of these investigations (Table I) were based on the data obtained from cultures in which all 3 pieces of tissue gave consistent results. Occasionally, the degree of inhibition was difficult to determine because of poor growth of the test organism or the tissue and in some cases not all 3 pieces of tissue produced an inhibitory zone even though the test organism and the tissue grew well (see footnote to Table I). As a result of this variability, all experiments were performed at least 3 times. In most cases the inhibitory zones were quite extensive and clearly defined (Fig. 1). The diameter of the inhibitory zones was reported as an indication of the extent of inhibition. The amount of inhibition is not necessarily directly proportional to the amount of tissue growth. For example, an increase of 200 mg. in the fresh weight of the tissue, resulting in the production of inhibitory zones 20 mm. in diameter may not result in the production of zones 10 mm. in diameter when the growth of the tissue is 100 mg.

The test organisms selected for use in further investigation were Fusarium roseum, Bacillus subtilis, Sarcina lutea, and Pullularia pullulans. These organisms were selected because of uniform results and the production of clearly defined inhibitory zones. Additional studies will be directed toward the following objectives: (1) The characterization of inhibitory material or materials, (2) the determination of the occurrence of these materials in other members of the genus Populus.

TABLE I
THE INHIBITION OF VARIOUS MICRO-ORGANISMS BY ISOLATED
ASPEN TISSUE GROWN IN VITRO

| Organism | Tissue Growth, mg. fresh wt. | Diameter of Inhibitory Zone, mm. |
|---|---------------------------------|--|
| <u>Fusarium roseum</u> | 204 | 27 |
| <u>Saccharomyces cervisiae</u> ^a | 246 | 19 |
| <u>Aspergillus niger</u> | 91 | 0 |
| <u>Bacillus subtilis</u> | 77 | 23 |
| <u>Bacillus cereus</u> | 121 | 18 |
| <u>Proteus vulgaris</u> | 121 | 0 |
| <u>Penicillium expansum</u> ^a | 178 | 17 |
| <u>Penicillium roqueforti</u> | 155 | 20 |
| <u>Torula utilis</u> ^a | 107 | 15 |
| <u>Escherichia coli</u> | 257 | 0 |
| <u>Aerobacter aerogenes</u> | 91 | 0 |
| <u>Sarcina lutea</u> | 100 | 30 |
| <u>Serratia indica</u> | 98 | 0 |
| <u>Flavobacterium aquatile</u> | 110 | 18 |
| <u>Pseudomonas fluorescens</u> | 109 | 0 |
| <u>Pullularia pullulans</u> | 185 | 25 |
| <u>Staphylococcus aureus</u> ^a | 208 | 23 |
| <u>Chaetomium globosum</u> | 181 | 0 |
| <u>Salmonella gallinarum</u> | 220 | 0 |
| <u>Hypoxyylon pruinatum</u> | 171 | 0 |
| <u>Bacillus sp.</u> | 72 | 23 |

^aIndicates variable results.

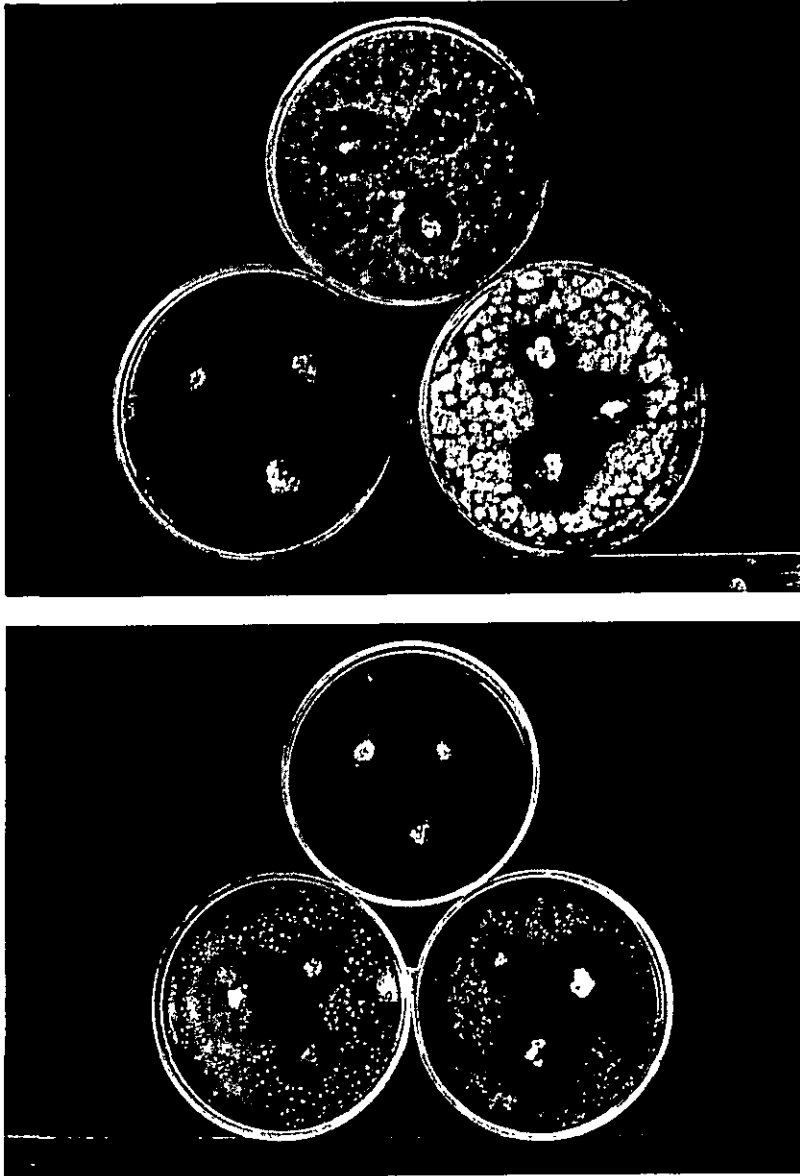


Figure 1. The Production of Inhibitory Materials by Isolated Aspen Tissue Grown* In Vitro

Inhibitory zones produced after inoculation with:


- A. (above) Penicillium expansum
(below)-left Pullularia pullulans
right Fusarium roseum
- B. (above) Flavobacterium aquatile
(below)-left Bacillus subtilis
right Sarcina lutea

*Grown on agar medium for a period of 3 weeks in the dark at 27-29°C.

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Martin C. Mathes, Research Aide