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Phytoalexin production by Aspen (Populus tremuloides Michx.) in response to infection by Hypoxylon mammatum (Wahl.) Mill and Alternaria spp.

By G. Flores and M. Hubbes

Receipt of Ms. 10. 8. 1978

Abstract

Freshly wounded stem sections of *P. tremuloides* Michx. produce phytoalexin when inoculated with mycelium of *Hypoxylon mammatum* (Wahl.) Mill., and *Alternaria sp.* These exudates are inhibitory against spore germination of these two fungi and, although inhibitory against mycelial growth of *Alternaria* sp., have no effect on the mycelial growth of *H. mammatum*. It was possible to correlate the amount of phytoalexin elicited by different strains with the inhibitory activity.

1 Introduction

In higher plants, attempts have been made to relate disease resistance to either the occurrence of structural or physical barriers, or to the presence of an inhibitory compound or compounds within the tissues of the host. The broad categories of structural and chemical defence mechanisms are often subdivided according to whether a particular mechanism is operative before infection (pre-infectional resistance) or develops as a direct consequence of the physiological interaction between the plant and the potential pathogen (post-infectional resistance), Ingham (1973).

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Until a few decades ago, little was known about the physiology of the host-parasite relationship of *Hypoxylon* canker caused by *Hypoxylon mammatum* (Wahl.) Mill. Subsequently, HUBBES (1962, 1962a, 1964) showed that aspen bark contained various compounds that were inhibitory to the growth of *H. mammatum*. These compounds could be classed as pre-infectional since they are found to be present in the host tissues and are operative before fungal attack.

The objective of this research was to investigate the role of post-infectional compounds as a direct result of the physiological interaction between the host and the pathogen.

2 Materials and methods

2.1 Isolation of H. mammatum

Diseased aspen (*Populus tremuloides*) material was collected from the Maple Woodlot, 25 miles north of Toronto, Ontario, Canada during the Fall of 1975. Small pieces of sapwood were cut and placed on 2 per cent malt agar. Isolations were made from various places above and below the cankers and the plates were incubated at 28 °C. Five isolates from the Maple Woodlot (MWL) vis: MWL I, MWL II, MWL III, MWL IV and MWL V, were used. One isolate from Quebec (Q) was provided by the Laurentian Forest Research Centre, Quebec City.

2.2 Production of exudates by Hypoxylon

Aspen stems about 9–10 years old were cut and stored at −35 °C. The frozen material was cut into sections 33 cm in length and split into quarters. The thin layer (phellem) and the green layer (cortex part of the secondary phloem) immediately beneath the outer layer, were removed at different intervals to form small debarked discs, approximately 1.5 cm in diameter and placed over petri dishes kept submerged in water in a polypropylene sterilizing pan 31.2×25.0×13.3 cm. The pan was covered with saran wrap to maintain high humidity. Agar plugs with actively growing *H. mammatum* were placed on the debarked discs and the pans were incubated at 28 °C. Exudates were collected by means of glass disposable Microsampling pipette and stored in the freezer at −35 °C.

2.3 Ascospore collection

H. mammatum ascospores were collected by placing the perithecia in 60×15 mm petri dish covers which in turn were kept in a 150×20 mm petri dish with water. A drop of sterile distilled water was put on top of perothecia and incubated for 24 hours at 28 °C. The ascospores which were released on the petri dish cover were washed with sterile distilled water and kept in suspension.

2.4 Hypoxylon ascospore germination test

The spore suspension was diluted to 100 000 spores/ml by using a Bright-Line Hemacytometer. By means of a disposable pipette two drops (0.02 ml) were transferred to each of three wax rings made on a glass slide with the help of a cork-borer No. 8. The glass slides were then placed over cotton rolls in a 150×20 mm sterile petri dish containing glass beads and water These were incubated at 35 °C until maximum germination of spores in the rings occurred. The germinated ascospores were then counted.

2.5 Isolation of Alternaria sp.

Leaves from *Populus deltoides* Bartr. with *Alternaria* leafspots were collected at Maple, Ontario. Isolation of the fungus from diseased leaf tissues, was carried out on 2 per cent malt agar and the plates incubated at 25 °C.

2.6 Spore germination test with Alternaria sp.

Spore suspensions of *Alternaria* sp. were prepared by washing the spores in 20 ml of distilled water from actively growing cultures on malt agar plates. The suspension containing the spores and mycelium was centrifuged at 3600 r.p.m. and filtered through cheese-cloth to remove the mycelium. The spore suspension was diluted so that each ml of suspension contained approximately 100.000 spores. Spore germination was tested in the same manner as described in section 2.4. However, the spores were incubated at 25 °C for 5 hours.

2.7 Action of exudates against Hypoxylon ascospore germination

Ascospore germinations were tested in the same manner as described in section 2.4, except in the treatments 20 microlitres of crude exudate were incorporated to the spore suspension (0.02 ml) with the help of glass disposable Micro-sampling pipette. The controls were treated with equal amounts of sterile distilled water.

2.8 Action of exudates against Alternaria spore germination

Procedures followed for this part of the study were the same as described in sections 2.6 and 2.7.

2.9 Action of exudates against Alternaria mycelial growth

20 ml of the spore suspension, adjusted to 100000 spores/ml, was mixed thoroughly with 250 ml of 2 per cent malt agar just before it started to solidify. The agar was poured into 100×25 mm glass petri dishes and allowed to solidify. Sterile filter paper discs were then soaked in respective exudates and placed on the seeded agar plates. Sterile filter paper discs soaked in sterile distilled water were used as controls and placed in the center of the plates. The plates were incubated at 25 °C for 2 days.

2.10 Host-wound-parasite relation

The methods followed for this experiment were the same as described under section 2.2 except that one set of strips was not debarked and was inoculated with actively growing *Hypoxylon* mycelial plugs. The other set of debarked strips was inoculated with actively growing *Hypoxylon* mycelial plugs. The third set of strips was debarked and inoculated with just sterile agar plugs. This was done so that conditions with wounded bark plus fungus, wounded bark and no fungus, and unwounded bark plus fungus were acquired.

2.11 Production of exudates by Alternaria sp.

Aspen bolts were cut into strips and debarked. *Alternaria* mycelial plugs were placed over the debarked areas. The pans were incubated at 25 °C.

2.12 Determination of the dry weights of the exudates

The dry weights of the crude exudates were determined by taking 1 ml of the exudates in pre-weighted test-tubes and evaporated to dryness in a Vortex Evaporator (Searle) at 50°C until constant weight was achieved.

3 Results

3.1 Isolation of H. mammatum

It was not possible to isolate *Hypoxylum mammatum* from diseased material collected during fall and winter seasons. However, the fungus was easily isolated during the months of March and April. Best isolations were achieved during the flushing of aspen leaves.

3.2 Effect of the exudates on the germination of Hypoxylon ascospores

Having isolated the pathogen, experiments could be initiated to investigate the role of phytoalexins during the process of infection. Therefore, freshly wounded aspen bolts were inoculated with mycelial plugs from different strains of *H. mammatum*. After four days of inoculations, honey coloured to dark brown exudates were collected and tested against the germination of *Hypoxylon* ascospores to determine whether these exudates had any inhibitory activity against ascospore germination (Table 1).

Table 1

Inhibition of Hypoxylon mammatum ascospore germination by the exudates produced by different strains on inoculated aspen bolts

Strains	Average germination percentage		Inhibition %
	Treatment	Control	
MWL I	10.0	50.0	80.0
MWL II	2.4	49.2	95.12
MWL III	6.0	50.8	88.19
MWL IV	13.2	51.2	74.22
MWL V	3.4	49.8	93.17
Q	13.0	50.0	74.0

The data are the average of five readings. Each reading was the result of counting 100 spores. The inhibitory power varied from 95.12 per cent to 74.0 per cent depending on the fungal strain.

3.3 Action of the exudates against Alternaria sp. spore germination

To determine whether these exudates could fulfill MÜLLER's postulate regarding the non-specific toxic nature of the aspen phytoalexins towards fungi, these exudates were tested against the *Alternaria* sp. which was isolated from leaves of hybrid poplars (Table 2).

The data are the average of six readings. Each reading was the result of counting 100 spores. It was observed that the germinating spores produced "Halos" around the emerging germ tubes.

Table 2

Inhibition of the Alternaria sp. spore germination by the exudates from inoculated aspen bolts

Strains	Average germination percentage		Inhibition %
	Treatment	Control	
MWL I	6.8	83.0	91.8
MWL III	7.5	83.4	91.01
MWL V	25.1	83.8	70.05

3.4 The effect of exudates on mycelial growth of Alternaria sp. and Hypoxylon

The exudates were tested against the mycelial growth of *Alternaria* sp. on seeded plates. After 48 hours inhibition zones appeared around the filter paper discs impregnated to saturation with the water extract. The colony density of *Alternaria* sp. decreased with four days of incubation (Table 3). Impregnated sterile filter paper discs were placed near the mycelial fangs of *H. mammatum* to see whether these exudates could inhibit the mycelial growth of *Hypoxylon*. The exudates were not capable of inhibiting mycelial growth of *Hypoxylon*, indicating the differential action of the exudates against fungi. *Hypoxylon* continued its normal growth over, as well as under, the sterile filter paper discs.

Table 3

Inhibition of mycelial growth of Alternaria sp.

Inhibition zones in cms	Average inhibition zone in cms
3.5 3.0 2.7 2.5	2.9

Table 4

Dry weight of the exudates

Strains	Dry weight of exudates
MWL I	0.0012 g
MWL II	0.0020 g
MWL III	0.0038 g
MWL IV	0.0038 g
MWL V	0.0021 g
Q	0.0016 g

3.5 Dry weight of the exudates

From the foregoing results it appears as if there is a difference in the inhibitory power of exudates according to the fungal strains (Table 1). However, this difference could be a quantitative or a qualitative one. To test the first assumption, the dry weight of the exudates was determined (Table 4).

Based on the dry weights, there is a difference in the ability of different fungal strains to trigger the exudate formation by the host. Therefore, based on the dry weights and also on the inhibitory capacity, the strains selected for further work were MWL I, MWL III and MWL V.

3.6 Production of exudates by the fungus

Experiments were carried out to see whether the exudates were produced either by fungus alone, by the host, or whether they were produced due to the effect of wounding without any fungal implication. Only wounded wood inoculated with the fungus produced exudates, whereas wounded wood inoculated with sterile agar plugs did not produce exudates. Similarly, non-wounded wood inoculated with the fungus failed to produce any exudates.

There is, therefore, an interaction between the host and the parasite, in the presence of a wound, for the production of exudates.

3.7 The possibility of Hypoxylon ascospores producing exudates

Tests were done to determine if the ascospores of *Hypoxylon* could induce the host to produce exudates during the process of germination. For these tests, aspen strips were prepared as described in section 3.2. Instead of mycelial plugs, the debarked areas were inoculated with a spore suspension at a concentration of 100000 spores/ml.

After four days of incubation no exudates were produced.

3.8 To test whether an increase in mycelial inoculum would increase the exudate production

Since the production of exudates, following the method using circular mycelial plugs on debarked rings was small, it was decided to see if an increase in mycelial inoculum would induce the host to produce larger amounts of exudates. For this purpose aspen strips were debarked lengthwise to the length of the strip instead of debarked rings as mentioned in section 3.2. Aspen strips were inoculated with agar strips containing actively growing fungus.

The host failed to produce any exudates after the required time of incubation. Interestingly, the fungus colonized the whole surface area of the host after about 72 hours.

3.9 Test to show that the exudates are produced by the host and not by the fungus

The problem of the biological origin of toxic compounds that are formed in host-parasite interactions has, in many cases, been in doubt. Clearly, the above compounds designated as phytoalexins are of host origin and they are not formed as specific responses to specific fungi (CRUICKSHANK 1963). To confirm this conclusion, aspen strips were inoculated with *Alternaria* sp. mycelial plugs and incubated at 28°C. At the end of the incubation period, the host produced a considerable amount of exudates. Thus, aspen could be induced to produce exudates not only by *H. mammatum* but also by another fungus which is a leaf parasite on poplars but not a trunk parasite on trembling aspen. This indicated that the exudates were not produced by the fungus but by the host itself.

3.10 Can Populus deltoides produce exudates when inoculated with Hypoxylon?

Populus deltoides is resistant to *H. mammatum* (BERBEE and ROGERS 1964). This resistance could be due to the ability of the host to produce phytoalexins or due to some other factors. If all the answers pointed towards the ability of phytoalexin production then was this ability a quantitative or qualitative one?

To elucidate these topics, experiments were carried out to find if *P. deltoides* could produce exudates and, if so, to compare them with those produced by *P. tremuloides* for their inhibitory power against the germination of spores. *P. deltoides* strips were inoculated with *Hypoxylon* and after the required incubation period exudates were produced in large quantities, as compared to those produced by *P. tremuloides*. Spore germination tests with *Alternaria* sp. showed that the exudates produced from *P. deltoides* had a low inhibitory power as compared to those produced by *P. tremuloides* (Table 5).

Although *P. deltoides* produced larger amounts of exudates, these did not have the same inhibitory power as those produced by *P. tremuloides*.

Table 5

Inhibition of Alternaria sp. spore germination by exudates from P. deltoides and P. tremuloides

Exudates	Average germination percentage	Inhibition percentage
P. deltoides	86.83	8.26
P. tremuloides	3.70	96.1
Control	94.65	0

4 Discussion

During the present study, the isolation of *H. mammatum* was not successful during Fall or Winter months. Possible reasons for the success in isolating the fungus in the Spring, could be that fungal activity is probably greater in Spring because of the low inhibitory power of the bark (HUBBES 1964). Also, there could be a close relationship between the increase in the sap flow and availability of particular nutrients during the growing season influencing fungal activity.

Freshly wounded aspen stems could be induced to produce phytoalexins by a fungus (*Alternaria* sp.) other than *H. mammatum*. This indicates that these compounds are of host origin and are not formed as a specific response to specific fungi which is similar to the results obtained by CRUICKSHANK (1963).

Phytoalexins were only produced when smaller areas of the stem were wounded. When larger areas were wounded, no phytoalexins were formed. MÜLLER (1958) and UEHARA (1959) have shown a correlation between inoculum concentration and fungitoxic activity of the substance by the host. Similar studies showed a direct relationship between concentration of inoculum and concentration of pisatin formed (CRUICKSHANK and PERRIN 1963). The failure of the host to produce phytoalexin due to the increase of inoculum on wounded bark strips indicates that the increase in inoculum will not induce the host to produce larger amounts of exudates. Therefore, this failure could be attributed to the host being overcome by the parasite before it could react, or due to the rapid death of the underlying tissues caused by excessive wounding of the host.

When the aspen phytoalexins were tested against the germination of *Hypoxylon* ascospores, they were inhibitory in nature and the inhibitory capacity depended on the strains (FLORES 1977). In addition, they showed a high inhibition against the germination of *Alternaria* sp. spores, indicating that these phytoalexins are not specific in nature against particular fungi. This agrees with the phytoalexin theory by MÜLLER and BORGER (1940).

When these phytoalexins were tested against mycelial growth of *H. mammatum*, they had no inhibitory effect. However, they were inhibitory against mycelial growth of *Alternaria* sp. The conflicting reports regarding infection of aspens by ascospores of *H. mammatum* could be due to phytoalexins which are inhibitory against ascospore germination. BERBEE and ROGERS (1964) and SHEA (1963) found that artificial inoculations were successful only when mycelial plugs were used. Therefore, aspen phytoalexin probably plays an important role during the time of infection due to its inhibitory power against ascospore germination and its non-toxic effect against mycelium.

A great number of plants can be induced to produce phytoalexins when inoculated with the spore suspension of pathogenic or even non-pathogenic fungi (CRUICKSHANK 1963; MÜLLER 1958; UEHARA 1959). However, *H. mammatum* ascospores did not induce aspen stem to produce any phytoalexin. The failure of the spore suspension to induce the host to produce phytoalexin could be attributed to the low germination percentage and relatively long periods of incubation (HUBBES 1966) of *Hypoxylon* ascospores under normal conditions.

Only recently wounded aspen stems inoculated with *Hypoxylon* mycelium, produced phytoalexin, whereas wounded aspen strips inoculated with sterile agar plugs did not. Also non-wounded aspen strips inoculated with *Hypoxylon* failed to produce phytoalexin. These results give a clear indication for the host-parasite interaction in the presence of a wound. For the production of phytoalexin in aspen, one would have to consider three equally important factors: host, parasite, and wound.

Experiments carried out to determine whether *Populus deltoides* could produce phytoalexin, indicate that the amount of phytoalexin produced by *P. deltoides* was greater than that produced by *P. tremuloides*. However, when tested at original concentration, against the germination of *Alternaria* sp. spores, phytoalexin from *P. deltoides* showed a lower inhibitory power compared to the one produced by *P. tremuloides*. This could be due to a higher amount of phytoalexin produced by *P. deltoides* but having a lower concentration as that of *P. tremuloides*. Other possible reasons for *P. deltoides* being resistant to *H. mammatum* could be due to its ability to produce callus faster than *P. tremuloides*, to avoid infection by *H. mammatum*. The possibility of other chemical factors being present in *P. deltoides* should not be ruled out.

No experiments were performed to test if there is a difference in phytoalexin production between the different stem sections, or a variation with seasons or sites. Since these experiments were all performed *in vitro* on stem sections it would be interesting to investigate the possibility of phytoalexin production in nature.

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Summary

Freshly wounded stems of *Populus tremuloides* produced phytoalexin when inoculated with mycelial plugs of *Hypoxylon mammatum* and *Alternaria* sp. which was inhibitory against spore germination of *H. mammatum* and *Alternaria* sp. However, *H. mammatum* ascospores failed to elicit the host to produce phytoalexin. The phytoalexin inhibited the growth of *Alternaria* sp. but failed to inhibit mycelial growth of *H. mammatum*. Increase in size of wound and inoculum did not produce any phytoalexin. The production of phytoalexin was the result of host-parasite interaction.

Résumé

La production de phytoalexin par Populus tremuloides Michx. comme résultat de l'infection avec Hypoxylon mammatum (Wahl.) Mill. et Alternaria spp.

Des sections fraîches du tronc du *Populus tremuloides* (Michx.) produisent des phytoalexins après inoculation de l'écorce blessé avec mycelium du *Hypoxylon mammatum* (Wahl) Mill. et d'*Alternaria* sp. Ces phytoalexins inhibent la croissance du mycélium d'*Alternaria* sp., la germination des conidies d'*Alternaria* sp., la germination des ascospores du *Hypoxylon mammatum* mass mais ne pas la croissance de sa mycélium.

Zusammenfassung

Die Bildung von Phytoalexin durch Populus tremuloides Michx. als Folge der Infektion mit Hypoxylon mammatum (Wahl.) Mill. und Alternaria spp.

Frisch geschnittene Stammabschnitte von *Populus tremuloides* (Michx.) erzeugen Phytoalexine nach Beimpfung der verwundeten Rinde mit Myzel von *Hypoxylon mammatum* und *Alternaria* sp. Die Hemmwirkung dieser Phytoalexine erstreckt sich auf die Ascosporenkeimung von *H. mammatum*, die Konidienkeimung von *Alternaria* sp., das Myzelwachstum von *Alternaria* sp., nicht aber auf das Myzelwachstum von *H. mammatum*.

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The influence of chemical factors on the selectivity of Pinus resinosa stumps for Fomes annosus

I. In vitro studies on chemical changes in Pinus resinosa as a response to wounding

By J.-Y. C. Lin and M. Hubbes

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Abstract

Fresh pine stump surfaces act selectively for primary invaders such as *Fomes annosus* that competes successfully against other microorganisms. However, a series of chemical reactions on the stump surface due to aeriation and the hosts response to wounding may be implicated in the selectivity of the host for certain microorganisms. The theory of stump selectivity of coniferous trees for *F. annosus* as well as the theory of wound reaction and protection wood are discussed. The sequence of events after wounding is complicated and the present investigation concentrates on the processes associated with the host response to wounding.

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