PROTEIN BINDING PHENOLS AND THE
INHIBITION OF NITRIFICATION IN SUBALPINE BALSAM
FIR SOILS

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(Accepted 10 February 1983)

Summary Nitrification in a highly active A1 horizon of a balsam fir forest floor soil can be greatly inhibited by an aqueous methanol extract of the forest floor. This extract was fractionated in an attempt to identify the compounds responsible for the inhibition. Condensed tannins, smaller molecular weight phenolics, and their distribution on particulate matter in the extract were the most important inhibiting components of the extract. When all phenolic material was removed from the extract, the remaining solution stimulated nitrification.

INTRODUCTION

Olson and Reiners (1983) investigated possible factors inhibiting nitrification in the forest floor of a subalpine balsam fir forest. Through a series of incubation experiments, they eliminated denitrification, low pH, inadequate nitrifier populations, the lack of nutrients (including ammonium), and a lack of mineral surfaces as causes of the inhibition. Further analyses showed that lead and aluminium, the most abundant potentially toxic metals in the forest floor, were also unlikely inhibitors.

The total phenolic content of the forest floor was found to be three times greater than that of the A1 horizon, a zone of active nitrification underlying the forest floor. A phenolically active aqueous methanol extract of the forest floor inhibited nitrification in the A1. These results and the findings of others (Basaraba, 1964; Rice and Pancholy, 1973, 1974; Lodhi, 1977, 1978; Lodhi and Kilinglebeck, 1980; but see also Bohlool et al., 1977) suggest that polyphenols, especially tannins, could regulate nitrification in this system.

Tannins are large polyhydroxy compounds chemically categorized as hydrolyzable or condensed and functionally defined by their ability to bind proteins (Swain, 1979a). Tannins can be measured functionally by various protein binding techniques, or chemically by any one of numerous assays (Folin–Denis, cyanidin chloride, gallic acid, HHDA formation) which measure tannin functional groups or subunits. The measurements of protein binding assays, address the biological role of tannins (Rhodes and Cates, 1976) and recently, Schultz and Baldwin (1982) and Martin and Martin (1982) found the chemical and functional measurements not to be significantly correlated. Bate-Smith (1977) and Swain (1979b) addressed problems inherent in using chemical assays to evaluate tannin content.

We partitioned the aqueous methanol forest floor extract used by Olson and Reiners (1983) to determine whether polyphenolic compounds were responsible for the inhibition of nitrification in the incubation experiments and then to determine the relative contribution of tannins, \( \beta \)-tannins (see Swain, 1979a), and smaller non-binding phenolics.

MATERIALS AND METHODS

Preparation of the extract

It was experimentally determined (5 solvent to forest floor ratios, and 6 samples per ratio) that the minimum volume of aqueous methanol (1/1 v/v) (Swain, 1979a) required to quantitatively extract tannins and polyphenols in a single extraction step (Schultz et al., 1981) from the forest floor (FF) material was 5 ml solvent g\(^{-1}\) wet wt FF. After 3.30 kg of FF material was extracted in aqueous methanol under N\(_2\) (<1 \( \mu l \) O\(_2\) l\(^{-1}\)) at 77–88°C for 2 h, it was strained through cheesecloth. The resulting 17.5 l of extract were partitioned into seven fractions (Fig. 1) of equivalent weight (471 g) FF starting material.

Fractionation of the extract

Fraction 1 (Fig. 1: the whole slurry fraction) comprised 2.92 l of the FF extract rotary evaporated (63–70°C) to 200 ml. The remaining FF extract (14.58 l) was centrifuged (3600 rev min\(^{-1}\), 2 C) for 1 h, and the supernatant filtered through Whatman 1 filters under vacuum. Particulates were removed from filters by sonication, taken up in 750 ml H\(_2\)O, and rotary evaporated to 100 ml. 17 ml of this slurry were added to one sixth (3.92 g) of the N\(_2\) dried pellet of the centrifugation step. The combined particulates, taken up in 200 ml H\(_2\)O comprised Fraction 2 (pellet fraction). One fifth (2.92 l) of the filtered supernatant volume was rotary evaporated to 200 ml to comprise Fraction 3 (filtered slurry).

Two-fifths (5.84 l) of the filtered extract were rotary evaporated to 2196 ml, shaken for 40 min with 120 g dry wt pre-swollen technical grade casein (Eastman) in a mechanical shaker and filtered through Whatman 1. The filtrate was rotary evaporated to 715 ml, and the casein step was repeated with 40 g dry wt pre-swollen casein. One half of the final filtrate (153 ml) made up Fraction 4. If \( \beta \)-tannins were
present, it is likely that they would not have been removed by the casein shake and show preferential solubility in ether (Rhoades, 1977). Hence to separate casein shake, and solvent extraction for phenolic constituents of the methanol extract, the remaining half of the filtrate was shaken with eight 50 ml volumes of petroleum (pet) ether (J. T. Baker Resi-Grade). The inulin of /j-tannins from the remaining contents was collected every 5 ml and analyzed for total tannin fractions were combined and dialyzed against H₂O which was tested for phenolics and changed every 2 h. The dialysed condensed tannin fraction (Fraction 9) was taken up in 200 ml H₂O. All fractions were kept at 5 C under N₂ until the later.

**RESULTS**

All treatments established characteristic NO₃⁻ production rates early in the experiment (by weeks 2–4) and retained these characteristics for the 10-week duration (Fig. 2): NO₃⁻ production was relatively constant with few lags or accumulations. The FF extract contained compounds that both inhibited and stimulated nitrification. The treatments can be separated into five statistically significant subgroups (P < 0.01): a stimulatory group (Fraction 6), a neutral group (Fraction 4, casein and water controls) and three inhibiting groups in increasing order of inhibitory effects—Fraction 9 (condensed tannins), Fraction 3 (filtered slurry) and both pellet containing fractions 1 and 2.

Chemical measurements

A negative KIO₃ test (Bate-Smith, 1981) indicated that hydrolysable tannins were absent from the forest floor extract. Total phenolic content was estimated by the Folin–Denis procedure of Swain and Hillis (1959) and expressed as tannic acid equivalents (FD-TAE). Condensed tannins were measured as proanthocyanidins (Bate-Smith, 1975) between 547–556 nm and expressed as purified red oak tannin equivalents (PA⁻⁷⁻ROT) g⁻¹ dry wt soil (Schultz and Baldwin, 1982). Tannin capacity was measured as relative astringency using a hemoglobin binding technique (Schultz et al., 1981) and also expressed as tannic acid equivalents (Heme−⁻⁻TAE). NH₄⁺ and NO₃⁻ were determined with a Technicon Auto-analyzer II. NH₄⁺ analyses were done by an alkaline phenol method (Technicon Industrial Method No 98-7000) which can overestimate NH₄⁺ when particular free amino acids are in sample (White and Gosz, 1981). Carbon was measured with a Coleman carbon–hydrogen analyzer and methanol was determined by capillary gas chromatography on a bonded phase fused silica column in a modified Perkin–Elmer 3920 GC with a flame ionization detector.

Nitrification potential experiments

The effect of each FF extract fraction on nitrification in samples from the A1 soil horizon of the balsam fir forest was used as a measure of nitrification inhibition potential. Each fraction was partitioned into 10 replicates, each replicate (with the exception of the condensed tannin fraction) representing the extract of a dry weight of forest floor equal to the dry weight of each A1 sample. Each fraction replicate was mixed with an A1 soil sample and placed in a plastic funnel (Olson and Reiners, 1983). The samples were placed on filters of glass wool and acid washed sand in the funnel stems. The funnels were lightly covered by foil caps. At the beginning of the experiment and then every week, 100 ml deionized water were added to each funnel and then drawn through by vacuum after 15 min. In addition to the seven extract fractions, casein, petroleum ether, and water controls were also tested.

NO₃⁻ output was determined for 10 weeks after which all funnels were extracted with 2 N KCl and analysed for NH₄⁺. Since pet ether may be toxic to nitrifiers, the two experimental subgroups which were not aqueous solutions (Fraction 5 and the pet ether blank) were reincubated with nitrifiers from the same A1 soil after the initial extract addition.

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**Fig. 1.** Partitioning of the methanolic forest floor extract. See text for details.
output was not significantly different from the blank pet ether fraction.

With the exception of Fraction 4, which stimulated mineralization, all treatment fractions showed the inverse relationship between NO$_3^-$ and exchangeable NH$_4^+$ (Table 1) which characterizes inhibited nitrification (Viro, 1963; Rice and Pancholy, 1973, 1974; Lodhi, 1978). The phenolic data of Table 2 are expressed either as red oak tannin or tannic acid equivalents g$^{-1}$ dry weight soil. Fraction 9 is an attempt to account for all the original extract's tanning activity in terms of

### Table 1. Cumulative NO$_3^-$, NH$_4^+$ at the tenth week, and total measured nitrogen from all nitrification potential experiments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>NH$_4^+$ (µg g$^{-1}$)</th>
<th>Total NO$_3^-$ (µg g$^{-1}$)</th>
<th>Total measured nitrogen (µg g$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-pellet fraction</td>
<td>598.7 (128)</td>
<td>419.7 (220)</td>
<td>553.4</td>
</tr>
<tr>
<td>1-whole slurry</td>
<td>508.1 (832)</td>
<td>502.8 (292)</td>
<td>508.7</td>
</tr>
<tr>
<td>3-filtered slurry</td>
<td>470.7 (48)</td>
<td>727.3 (393)</td>
<td>530.3</td>
</tr>
<tr>
<td>9-condensed tannins</td>
<td>305.3 (422)</td>
<td>1342 (314)</td>
<td>540.7</td>
</tr>
<tr>
<td>4-post casein shake</td>
<td>665.3 (94)</td>
<td>1820.3 (240)</td>
<td>928.5</td>
</tr>
<tr>
<td>8-water control</td>
<td>238.3 (281)</td>
<td>1982.1 (153)</td>
<td>576.9</td>
</tr>
<tr>
<td>10-casein control</td>
<td>194.8 (11)</td>
<td>2001.3 (173)</td>
<td>603.5</td>
</tr>
<tr>
<td>6-aqueous fraction</td>
<td>167.5 (5)</td>
<td>2357.8 (79)</td>
<td>654.9</td>
</tr>
<tr>
<td>5-pet ether fraction</td>
<td>485.5 (653)</td>
<td>1559.3 (833)</td>
<td>729.7</td>
</tr>
<tr>
<td>7-pet ether control</td>
<td>470.5 (453)</td>
<td>1561.2 (1483)</td>
<td>718.5</td>
</tr>
</tbody>
</table>

Values are the mean of 10 (SD) expressed on a g$^{-1}$ dry wt, soil basis.

### Table 2. Phenolic and carbon data from aqueous fractions of the forest floor extract

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Carbon (mg C$^{-1}$ dry wt soil)</th>
<th>Total phenolics (FD$^{-1}$ TAE)</th>
<th>Condensed tannins (%PA$^{-1}$ROU)</th>
<th>Tanning capacity (Henr$^{-1}$ TAE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-whole slurry</td>
<td>42.3</td>
<td>ND</td>
<td>3.701</td>
<td>ND</td>
</tr>
<tr>
<td>2-pellet fraction</td>
<td>11.3</td>
<td>ND</td>
<td>2.130</td>
<td>ND</td>
</tr>
<tr>
<td>9-condensed tannins</td>
<td>7.4</td>
<td>8.07</td>
<td>2.757</td>
<td>20.20</td>
</tr>
<tr>
<td>3-filtered slurry</td>
<td>7.5</td>
<td>7.65</td>
<td>1.570</td>
<td>27.51</td>
</tr>
<tr>
<td>4-post casein shake fraction</td>
<td>1.9</td>
<td>1.68</td>
<td>0.022</td>
<td>0.00</td>
</tr>
<tr>
<td>6-aqueous fraction</td>
<td>1.9</td>
<td>0.91</td>
<td>0.022</td>
<td>0.00</td>
</tr>
<tr>
<td>10-casein control</td>
<td>0.08</td>
<td>0.00</td>
<td>0.000</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Values are the means of three measurements. See text for an explanation of phenolic data. ND means that phenolic measurements were not possible due to interfering particulate matter.
condensed tannins; however, only 73% of the tanning activity of Fraction 3 was restored with 1.75 times the original extract's concentration of condensed tannins, and 1.05 times the total phenolics. Hence the extent to which one can attribute nitrification inhibition to tannin content depends on how the tannin content is measured. From the cumulative NO$_3^-$ values (Table 1) Fraction 9 had 51.4% of the inhibitory potential of Fraction 3, the filtered slurry fraction. Adjusting this value for the concentration of tannins in Fraction 3 (Table 2), tannins account for 70.0% of the inhibitory potential using the functional measurement-% tanning activity—but only 29.3% if the chemical measurement-% condensed tannins—is used.

The two pellet containing Fractions (1 and 2) were the most inhibitory fractions, but due to the large mineral component (15%, dry wt) only condensed tannin measurements of the pellet-containing fractions were possible. The pellet contained 1.4 times the amount of condensed tannins in solution (Fraction 3) but only reduced the 10 week cumulative NO$_3^-$ output by 310 µg g$^{-1}$ dry weight soil compared to Fraction 3. Fraction 1, which had a condensed tannin content equal to the combined tannin contents of Fraction 2 and 3, could not be distinguished statistically from Fraction 2 alone.

Shaking the filtered extract with the insoluble protein casein removed all the tanning (Table 2) and inhibitory characteristics (Table 1) of the extract. This fraction still had 22% of the total phenolic activity of the filtered slurry. Removing an additional 10% of the total phenolic activity with the pet ether wash produced a fraction (6) which stimulated nitrification. However, as indicated by the results of Fractions 5 and 7, the phenolics removed in the pet ether wash did not affect nitrification rates of soil reinoculated with nitrifiers.

Aliquots removed before and after each rotary evaporation showed that the evaporation conditions did not influence the extract's tanning and total phenolic activity. Methanol concentrations were below the detection limit (1 ng g$^{-1}$) in all fractions.

**DISCUSSION**

Removing protein binding phenolics from the filtered FF extract eliminated the extract's capacity to inhibit nitrification in the experimental microcosms. Similarly, the addition of purified condensed tannins from the same FF extract inhibited nitrification. Condensed tannins and other protein binding phenolics are clearly responsible for the inhibitory characteristics of the FF extract, but the tanning activity of the condensed tannin fraction alone cannot account for all the observed inhibition.

The casein shake removed 98.6% of the condensed tannin component of the filtered extract while also eliminating the filtered extract's tanning and inhibitory activity. This implies that condensed tannins play an inhibitory role, although the relationship may be exaggerated by the techniques used. Shaking a solution of phenolics with an insoluble protein, such as casein, may be a more efficient method of removing phenolic compounds than shaking the same solution with a soluble protein, such as hemoglobin, which was our method of determining tanning activity. Hence phenolics too small to precipitate protein, but still inhibitory to nitrification (Rice and Panholly, 1974) may have been removed in the casein shaking step. The inhibitory potential of Fraction 4, which contained compounds not removed in the casein shake, may have been greater had a soluble protein been used as the binding substrate. The role of smaller phenolics in the inhibition process is also suggested by the inability of the purified condensed tannins to account for all the inhibitory activity of the filtered extract.

The importance of utilizing the functional definition of tannins is underscored in the calculations of tannin concentrations in the purified condensed tannin fraction. The two procedures employed in the preparation of this fraction, dialysis and gel permeation, while efficiently separating the condensed tannin chemical subunit, dramatically reduced the tanning activity of the condensed tannin fraction. While nothing is known of the structural reasons for this loss of activity, dialysis substantially reduced the tannin's water solubility, possibly indicating the dependence of larger molecular weight moieties on smaller phenolics for their water solubility and tanning activity.

It should be emphasized that the observed inhibition of nitrification is not primarily the result of the immobilization of nitrogen due to carbon addition (Basaraba, 1964). In our soil microcosm, tannins and phenolics specifically inhibited nitrification and had relatively minor influence on net ammonification. A weak trend ($r^2 = 0.2$) exists between our measurement of total nitrogen lost (Table 1) from the microcosms and the amount of carbon added (Table 2) in the treatments. The validity of the trend is, however, dependent on the assumption of no NH$_4^+$ mobility in the incubation funnels, for our only measurement of NH$_4^+$ is of exchangeable ammonium at the end of the experiment. Hence, the observed trend could have resulted from an increase in NH$_4^+$ mobility due to carbon addition rather than the immobilization of nitrogen.

The total tannin content of the forest floor may indicate the degree to which nitrification is regulated. However, other factors such as forest floor leaching rates and microbial manipulations of these compounds may be as important in determining a system's nitrification rate. Moreover, balsam fir foliage may influence soil nitrification before it becomes an integral part of the forest floor. Recently, Thibault et al. (1982) found foliar leachates and bud extracts of balsam fir to dramatically inhibit nitrification in a laboratory percolation device.

**Acknowledgements**—We thank P. Vitousek for reading and improving the manuscript, J. C. Schultz and P. J. Nothnagle for suggesting the role of phenolics, and M. J. Richards for drawing the figures.

**REFERENCES**


