Inhibitory activity of pine needle tannin extracts on some agriculturally resourceful microbes

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Abstract Crude extracts of water and solvent extractable tannin fractions from pine needles were found to contain tannin concentrations of 10.15% and 13.15% tannic acid equivalents respectively. Thin Layer Chromatography revealed the presence of four distinct phenolic compounds, amongst which two were tannic acid like compounds. Both the extracts were found to be inhibitory to several microbes of agricultural importance. Amongst the bacterial strains studied, *Azotobacter* sp (VL-A2) was able to tolerate upto 1000 ppm of crude tannin concentration without any growth inhibition. While growth of *Rhizobium* (VL-R1) and *Bacillus halodurans* (MTCC 7181) was inhibited by crude tannin concentrations of 50 and 100 ppm respectively of both water and solvent extracted tannins. Among the fungal genera, *Pleurotus djamor* was found to tolerate up to 10000 ppm of crude tannins, while *Trichoderma virescens* (MTCC 6321) and *T. reesii* could tolerate up to 3000 ppm of both water extractable and acetone extractable crude tannins without any growth inhibition.

Keywords Pine needle · Tannin · Inhibitory activity · Agriculture · Microbial inoculants.

Introduction

Pine (*Pinus roxburghii*) is a coniferous tree that grows well in the mid-altitudes of the Indian Himalayas. Besides timber and resin, pine forests contribute to the agro-ecosystem in the form of plant nutrients, which are made available by the decomposition of pine needle litter. Being a coniferous litter rich in silica and phenolic compounds, it is highly resistant to decomposition. Among the various phenolic compounds present in pine needles, tannins are most important. Tannins are commonly defined as water-soluble polyphenolic compounds ranging in molecular weight from 500 to 3000 Daltons that have the ability to precipitate proteins¹. Tannins are of two types viz., hydrolysable and condensed. The hydrolysable tannins are polyphenols with a polyol central core, while condensed tannins are polymers of flavon-3-ol, which are linked by carbon – carbon bonds. Gymnosperms like pine are known to produce only condensed tannins while dicots produce both hydrolysable and condensed tannins. The tannins that enter the soil affect soil nutrient dynamics by delaying organic matter decomposition and mineralization². Pine needles are a rich source of condensed tannins, which inhibit the decomposition process to a great extent³. The molecular weight of pine needle tannins seems to be an important factor that influences the inhibition of soil microbial processes. While the light fractions strongly enhanced respiration and decreased net N mineralization, indicating higher immobilization of N in the microbial biomass, the heavy fractions reduced respiration and slightly increased net N mineralization, suggesting toxic or protein-precipitating effects⁴. This study aims at studying the effect of two different pine needle crude tannin extracts on the

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growth and development some agriculturally resourceful microbes.

Materials and Methods

Fresh pine needles collected from the pine forests adjoining Almora (1650 m amsl) were used to prepare tannin extracts. Crude aqueous tannin extracts was obtained using double distilled water, while acetone (Merck®, India) was used for the solvent extraction according to the method of Broadhorst and Jones (1978). The tannin content in both the extracts was estimated by the Folin Denis method⁶. Thin layer chromatography was performed on silica gel coated plates according to Harbourne (1989), using tannic acid as the reference standard. The solvent system used was n-butanol, acetic acid and water in the ratio of 4:1:5.

The phosphate solubilizing bacterial strain *Bacillus halodurans* (MTCC 7181) and *Rhizobium* sp (VL –R1) were isolated from the rhizosphere and nodules of rajmash and pea respectively. *Azotobacter* (VL-A2) was isolated from cultivated soils. The litter decomposing fungal strains *Trichoderma virescens* (MTCC 6321) and *Trichoderma reesii* (T 21) were isolated from naturally decomposing pine needle litter while *Pleurotus djamor* was obtained from the mushroom unit of Tamil Nadu Agricultural University, Coimbatore.

The antibacterial activity of the extracts was determined by the standard agar well diffusion technique⁸ on seeded media in Petri dishes (110 mm). Rehydrated culture media (Hi-Media) viz., yeast extract mannitol agar, Ashby's mannitol agar and nutrient agar were used for *Rhizobium*, *Azotobacter* and *Bacillus halodurans* (MTCC 7181) respectively. Wells (6 mm dia) drilled in the media, were filled with 20 µl of filter-sterilized extracts of the desired concentrations viz., (10, 50, 100, 500, 1000, 2000, 3000 and 4000 ppm), while the respective extractant served as control. The plates were incubated at 30°C for 48–72 h and the diameter of the inhibition zone was measured. Each test was replicated thrice. The anti fungal nature was determined by the poisoned food technique⁹ using Czapeks Dox agar (Hi Media) in Petri dishes (110 mm). Mycelial discs (0.5 cm dia) were used for inoculation, and plates were incubated at 27°C for period of 48–72 h. The percent inhibition (T) was calculated using the formula,

$$T = (D_R - D_O) / D_R \times 100$$

Where D_R = Diameter of mycelia in the control plates

D_O = Diameter of mycelia in the tannin incorporated plates

Results and Discussion

It was observed that the extraction efficiency of tannins varied with the extractant used. When 70% acetone was used as an extractant, the tannin content in terms of tannic acid equivalent was higher (13.15%), than water as an extractant (10.15%). Though both the fractions were inhibitory, the water extractable fraction had higher inhibitory activity. Thin layer chromatography revealed the presence of four distinct compounds in both the extracts (Table 1). Compounds 1 and 2 with R_f values of 0.24 and 0.59 are predicted to be phenolic compounds with less complicated structure than tannic acid, since both the compounds have a higher degree of polarity. Compounds 3 and 4 with R_f values of 0.80 and 0.88, respectively are predicted to be very similar to tannic acid which had a R_f value of 0.83.

Among the bacterial strains studied it was observed that *Azotobacter* sp (VL-A2) was the most resistant to crude extracts of pine needle tannin extracts, when compared to *Rhizobium* (VL –R1) and *Bacillus halodurans* (Table 2). *Azotobacter* sp (VL-A2) could withstand upto 1000 ppm of both water extractable and acetone extractable tannins, with out any inhibition, while concentrations higher than 1000 ppm were found to be inhibitory. *Rhizobium* (VL–R1) and *Bacillus halodurans* (MTCC 7181) were inhibited by crude tannin concentrations of 50 and 100 ppm respectively of both water and solvent extracted tannins. The extent of inhibition was more in the case of *Rhizobium* (VL –R1) than *Bacillus halodurans* (MTCC 7181).

The fungal genera used in this study were more resistant to pine needle tannins than the bacterial genera (Table 3). Among the fungal strains studied *Pleurotus djamor* was able to tolerate crude tannin concentrations up to 10000 ppm (1%) without any growth inhibition. Further higher concentrations of crude tannins were found to be slightly inhibitory to *Pleurotus djamor* (Data not shown). *Trichoderma virescens* (MTCC 6321) and *T. reesii* could tolerate up to 3000 ppm of both water and acetone extractable crude tannins without any growth inhibition. Progressive growth inhibition of *T. virescens* (MTCC 6321) and *T. reesii* (VL -T 21) was observed with increasing crude tannin concentrations.

Table 1 Relative mobility of major fractions of pine needle tannin extracts.

Compound	R_f (Resolution factor)
1	0.24
2	0.59
3	0.80
4	0.88
Tannic acid (Standard)	0.83

Table 2 Effect of different crude extracts of pine needle tannins on *Rhizobium*, *Azotobacter* and *Bacillus halodurans* (MTCC 7181).

Tannin Concentration *	<i>Rhizobium</i> sp (VL –R1)		<i>Azotobacter</i> sp (VL-A2)		<i>Bacillus halodurans</i> (MTCC 7181)	
	Diameter of the zone of inhibition (mm)					
	Water Extract	Acetone Extract	Water Extract	Acetone Extract	Water Extract	Acetone Extract
10 ppm	NI	NI	NI	NI	NI	NI
50 ppm	6.0	5.0	NI	NI	NI	NI
100 ppm	9.0	7.0	NI	NI	11.0	10.0
500 ppm	13.0	10.0	NI	NI	12.0	11.0
1000 ppm	15.0	12.0	NI	NI	13.0	12.0
2000 ppm	18.0	16.0	9.0	5.0	16.0	14.0
3000 ppm	21.0	20.0	10.0	7.0	18.0	15.0
4000 ppm	23.0	21.0	13.0	9.0	20.0	18.0

* - Tannic Acid Equivalent; NI – No inhibition.

Table 3 Effect of different crude extracts of pine needle tannins on *Trichoderma* spp and *Pleurotus djamor*.

Tannin Concentration *	<i>Trichoderma virescens</i> (MTCC 6321)		<i>Trichoderma reesii</i> (VL – T21)		<i>Pleurotus djamor</i>	
	% Inhibition					
	Water Extract	Acetone Extract	Water Extract	Acetone Extract	Water Extract	Acetone Extract
3000 ppm	NI	NI	NI	NI	NI	NI
4000 ppm	32.9	30.0	30.0	25.0	NI	NI
5000 ppm	37.0	33.5	32.6	30.5	NI	NI
10000 ppm	64.5	59.2	60.0	58.7	NI	NI

* - Tannic Acid Equivalent; NI – No inhibition.

Tannins enter the agricultural ecosystem by leaching and litter inputs both above and below ground. A large fraction of tannins and simpler phenolics appear to enter the soil as foliar leachate¹⁰. These tannins are known to affect several microbial processes such as decomposition and nitrogen fixation¹¹. Acetone or methanol is usually used to extract tannins from plant material, but a significant fraction of the tannins (5–50%) are non-extractable¹². In the present study the higher extraction efficiency of 70% acetone could be attributed to the lower polarity of acetone when compared to water. Though both the fractions produced similar patterns on the Thin Layer Chromatogram, the water extracted crude tannin fraction was found to possess higher inhibitory activity than the solvent extractable fraction. This could be attributed either to the differences in the structural composition of the extracts or due to the presence of other inhibitory compounds that were not detected on the chromatogram. The presence of two simple phenolic compounds and two compounds with R_f values close to tannic acid in the both the extracts is a clear indication of the presence of inhibitory compounds in both the extracts. Fredaliana (2005), made a similar observation that aqueous and acetone extracts of tannins from galls of *Quercus infectoria* possessed antimicrobial activities and that the

inhibitory activity of the aqueous extract was higher than the acetone extract.

The inhibitory range of pine needle tannins observed in this study is in accordance with the previous reports, wherein concentrations of 3000 ppm – 12000 ppm were found inhibitory to fungi, while concentrations of 100 ppm – 20000 ppm were inhibitory to bacteria¹⁴. The mechanisms by which bacteria can overcome inhibition include tannin modification/degradation, dissociation of tannin–substrate complexes, tannin inactivation by high-affinity binders, and membrane modification/repair and metal ion sequestration¹⁵, while fungi overcome tannin inhibition mainly by the production of tannases¹⁶. Though there is less evidence regarding the bacterial utilization of tannins, few studies conducted with *Azotobacter* revealed that this genus could utilize tannins as a sole C source and it was more resistant to tannins¹⁷. Though it was not possible to demonstrate the utilization of tannins by the *Azotobacter* strain used in this study, the higher resistance level of *Azotobacter* to tannins could be attributed to its ability to produce higher levels of exopolysaccharides, which complexes with the tannins thus reducing their efficiency¹⁸. *Bacillus subtilis* and *Bacillus stearothermophilus* are known to be affected by tannins¹⁴, but the effect of tannins on *Bacillus halodurans* is not

clearly known so far. The results of this study clearly point out to the inhibition of *Bacillus halodurans* by pine needle tannins. *Bradyrhizobium japonicum* has been known to utilize catechin a condensed tannin, and the tannin utilizing enzymes are known to be inducible¹⁹. But the rhizobial strain used in this study was the most sensitive to pine needle tannins; this may be attributed to the chemical complexity of the pine needle tannins.

In the present investigation *Pleurotus djamor* was found to be the most resistant in tolerating higher levels (1%) of pine needle crude tannins with out any growth inhibition when compared to *Trichoderma virescens* and *T. reesii*. Both the *Trichoderma* species tested were able to tolerate upto 3000 ppm of pine needle crude tannins without any growth inhibition, while growth was severely inhibited by increasing the concentrations beyond 3000 ppm. The ability of white rot fungi in particular to withstand and degrade high levels of tannins is well known²⁰, besides several ericoid and ectomycorrhizal fungi have been found to degrade tannins²¹. Recent studies have used *Rhizopus oryzae* and *Aspergillus foetidus* for the biotransformation of tannin rich substrates²². The results of the present study are in accordance with the earlier observations²³ that fungi are more resistant to inhibition by pine needle tannins. This study reinforces the need for selection of tannin resistant microbial inoculants, for use in tannin rich agro-ecosystems.

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