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FUNGISTATIC ACTIVITY OF PINE (*PINUS KESIYA* ROYLE) NEEDLE EXTRACTS

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Effect of pine (*Pinus kesiya* Royle) needle extracts on the growth and spore germination of three phylloplane fungi viz., *Alternaria alternata*, *Bipolaris monoceras* and *Cladosporium herbarum* has been investigated. The fungistatic property of the extracts varied with the age of needles, solvents used and doses of the extract. The results are discussed in the light of available literature.

INTRODUCTION

Last (1955) first time suggested that leaf surface has a characteristic environment and it supports a microbial community. Leaf surface microbes are always in a dynamic equilibrium with the changing microhabitat of the leaves. Colonization of leaf surface started virtually from zero to a maximum at the senescence and death (Dickinson, 1967). Quite a good amount of literature has been reviewed by Preece and Dickinson (1971) and Dickinson and Preece (1976).

Coniferous leaves and needles are comparatively resistant to the fungal colonization. Very narrow spectrum of fungi can colonize the pine needle surface. So far little attention has been paid towards the fungal inhibitory substances in pine needles. Recently, Barlocher *et al.* (1978) has worked out this factor with relation to aquatic hyphomycetes.

Barlocher and Oertli (1978) proved that there is no correlation between the phenolic compounds concentration and the inhibition of fungal growth. They have noted two substances inhibitory to fungal growth of which one was phenolic and the other non-phenolic. While the effect of leaf extracts on the growth and germination of soil and litter fungi has received considerable attention, the effect of these extracts on the phylloplane fungi has received very little attention. In the present study it has been tried to work out the behaviour of these two sets of substances on the growth and spore germination of pine phylloplane fungi.

MATERIALS AND METHODS

One month old (young), one year old (mature) and attached dead needles from five years old pine (*Pinus kesiya* Royle) plantation were collected from approximately same position of the trees. The leaves were sorted out for any visible sign of fungal or insect damage. Apparently healthy needles were mixed thoroughly and air dried in sun. These were then powdered in a grinder and sieved through a 250 μm mesh.

Three dematiaceous fungi *Cladosporium herbarum*, *Alternaria alternata* and *Bipolaris monoceras* isolated from the phylloplane of pine needles were used as test fungi. 25 gm of air dried powder was put in a 1000 ml conical flask which was then filled with 500 ml desired solvent, i.e. water and ether. The flasks were wrist shaken two hourly for first 12 hrs and incubated for 24 hrs at 10°C. After 24 hrs the mixture was shaked thoroughly by hand and then filtered through Whatman No. 1 filter paper followed by 0·2 μ millipore filters. The volume of each extract was made 200 ml with the respective solvent by vaccum evaporator at 20°C. For water extracts, sterile double glass distilled water was used and always aseptic precautions were taken. 1, 2 and 5 ml of the extracts were poured in the petri dishes which were then filled with 20 ml of 0·25% malt extract agar at 50°C (0·25% malt extract, 1·5% bacteriological agar, BDH) and the suspension was stirred by gentle shaking and rotating the petri dishes to ensure proper mixing of the extracts to the medium. The medium in each petri dish was then inoculated with single agar disc (4 mm diameter) at the centre overgrown with a two week old colony from the periphery of one of the three fungi viz., *A. alternata*, *B. monoceras* and *C. herbarum*.

Control sets were maintained separately for each set of treatment and organism. Three replicates were maintained in each case. The plates were then incubated at 20°C and the diameter measured at every 24 hrs for ten days.

Spore germination and germ tube length were estimated in a cavity slide. 0·02 ml of the extracts was put on the sterilized cover glass and spores from two weeks old cultures were transferred to this droplet with the help of a sterile inoculation needle and it was ensured that the spores are mixed properly in the droplet. The drop was then inverted in the cavity which was provided with sterile water to maintain humidity in the cavity. The cavity was sealed with sealing wax. These slides were then kept in petri dishes on glass rod containing water to maintain humidity. For each treatment and control three replicates were maintained. The germination and germ tube length were noted at 24 hrs intervals in 10 non-overlapping fields under high power microscope.

RESULTS

Results are depicted in Tables 1 and 2.

Bipolaris monoceras

Table 1 shows that the water extracts of needles of all the three ages were inhibitory. In the case of young needle extract the inhibitory property decreased with the increase in quantity of extract while reverse was true with extracts of mature and dead needle. The ether extracts of young and mature needles while

TABLE 1: Mean colony diameter after 8 days of incubation at 20°C (cm)

Organisms	Control	Solvent	Young 1 ml	Young 2 ml	Young 5 ml	Mature 1 ml	Mature 2 ml	Mature 5 ml	Dead 1 ml	Dead 2 ml	Dead 5 ml
B. monoceras	6·20	Water	1·20	1·45	2·50	5·76	5·3	5·11	4·75	4·59	3·60
		Ether	6·66	6·33	5·57	7·2	5·36	4·8	5·85	5·03	4·43
A. alternata	6·65	Water	2·40	0·65	1·70	6·00	4·00	1·85	5·66	4·60	2·80
		Ether	7·43	7·15	6·16	6·56	6·45	4·36	5·93	5·80	5·[illegible]6
C. herbarum	2·43	Water	0·70	0·78	0·78	2·20	1·75	0·78	1·25	1·30	1·60
		Ether	2·73	2·83	2·83	2·16	2·73	2·00	2·03	2·03	2·53

TABLE 2. Percentage germination, mean tube length, and germ tube elongation rate (after 24 hours)

Organisms	Control % germination	Control Mean tube length (µm)	Control Germ tube elongation rate (µm/h)		Young % germination	Young Mean tube length (µm)	Young Germ tube elongation rate (µm/h)	Mature % germination	Mature Mean tube length (µm)	Mature Germ tube elongation rate (µm/l.)	Dead % germination	Dead Mean tube length (µm)	Dead Germ tube elongation (µm/h)
B. monoceras	100	362·5	15·10	Water	9[illegible]·33	1006·25	41·92	98·28	41·5	1·72	98·50	25·92	1·08
				Ether	100	742·17	30·92	100	55·30	2·30	100	1328·12	55·33
A. alternata	87·50	847·5	35·31	Water	85·00	516·5	21·52	64·33	43·65	1·81	30	26·5	1·10
				Ether	100	1031·25	42·96	87·5	790·82	32·95	93·33	666·65	27·77
C. herbarum	57·60	19·5	2·37	Water	2[illegible]·50	20·75	2·91	55	12·9	2·08	5·5	12·62	2·08
				Ether	85	22·75	0·95	60	41·65	1·73	10	17·93	0·75

stimulated the growth in small doses proved inhibitory at the higher doses. The extract of dead needle had an inhibitory property which increased with increase in dose.

A perusal of Table 2 shows that the water extract of young needles showed a little decrease in the percentage germination but the mean tube length and germ tube elongation rate was significantly high. While the percentage germination was not affected much in the extracts of mature and dead needle, the germ tube growth has been significantly reduced. The ether extract of mature needles had an inhibitory effect on germ tube growth, young and dead needles on the other hand showed a stimulatory effect.

Alternaria alternata

Water extract of needles of all ages proved inhibitory which increased with the increase in doses except in the case of the young needles where the middle does was found to be most inhibitory. In low doses the ether extract stimulated in the young needle extract but the high doses were found inhibitory. The inhibition was dose dependent (Table 1).

The percentage spore germination, mean tube length and germ tube elongation rate were inhibited with the water extracts of needles of all the ages. This inhibition increased with the increase in age of the needles. The ether extract was stimulatory in case of young needles but there was a tendency of increase in inhibition as the needles age (Table 2).

Cladosporium herbarum

While water extract of young needles was considerably inhibitory the ether extract was found to be stimulatory. Water extract of mature needle showed a dose dependent inhibition. Middle concentration of mature needles ether extract was stimulatory while low and high doses were inhibitory. In the case of dead needles, the water extract was dose dependent inhibitory while the ether extract was inhibitory in lower doses but stimulatory in highest dose (Table 1).

The young needle extract was inhibitory for spore germination but the germ tube growth was a little stimulated. Very little inhibition in percentage spore germination in mature needles extract was noted, while it was significantly inhibited in the case of dead needle extracts. The germ tube growth was inhibited to almost the same level in both the extracts. The ether extract stimulated the spore germination percentage in the case of young and mature needles but inhibited in the case of dead needle extracts showing an increased tendency of inhibition with the needle age. The germ tube growth showed a mixed behaviour, not showing any definite relationship witn the age of the needle (Table 2).

DISCUSSION

The results depicted in Tables 1 and 2 demonstrate that both inhibition and stimulation of growth, spore germination and germ tube growth could be observed with the extracts of the pine needles. The pronounced inhibition of most of the growth parameters by the water extract was found. There is a clear indication that most of the inhibitory substances are water soluble. As evidenced from the studies (Schmidt, 1955 ; Seikel, 1964 ; Barlocher and Oertli, 1978) such inhibitory substances may be phenolic compounds. General tendency of dose dependent decrease in growth (Table 1) shows that the inhibition is directly related with the quantity of the leaf extract. These results are against the finding to Barlocher and Oertli (1978) who have noted no correlation between colorimetrically determined phenol content of the extract and its antifungal activity. The young needle extract in the case of *B. monoceras* showed a reduction in the inhibitory activity as the dose increases which may probably be due to the nutrients of the extracts (Table 1).

The ether extract in low doses was always found to be stimulatory for the fungal growth. In higher doses the effect was inhibitory (Table 1). This shows that the ether extracts do have certain inhibitory substance (s) which is effective only in comparatively higher concentration. The results further prove that the water soluble phenolic compounds are the most important inhibitory substances in the pine needles.

Water extract of young needles was always most inhibitory in comparison to the other two for the growth of the fungi (Table 1). Soft, thin-walled tissue of the young needles probably favoured the leaching of the toxic substances even if they are in lower amount. This may probably be a reason why the younger leaves harbour less number of micro-organisms as reported by Dickinson (1967).

Comparing the results of the percentage spore germination, and germ tube growth with the colony growth it is evident that no single study can give a complete and true picture regarding the response of the fungus to any particular treatment. From a perusal of tables 1 and 2 it is clear that while the highest colony diameter inhibition was noted with the young needle water extracts, the germination of the fungal spores was inhibited in the extracts of dead needle. The increased inhibition in spore germination and hyphal growth with the increase in needle age was probably due to the presence of more inhibitory compounds and for less nutrients present in the aged needles (Table 2).

The results of this study are very useful in understanding the behaviour of these fungi on the phylloplane. These three fungi which are common phylloplane organisms (Sharma and Mukerji, 1976) are found to be dominant colonizer of pine needle phylloplane (Das, unpublished). These fungi live together probably because of their different adaptations. *B. monoceras* has hundred percent germination and moderate hyphal growth rate, *A. alternata* has a high percentage spore germination and a very high hyphal growth rate whereas *C. herbarum* has a delayed and less percentage spore germination and slow hyphal growth rate. These are the probable ecological adaptations which make them dominant colonizers of leaf surfaces.

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