

Allelopathic effects of *Viburnum nervosum* on seed germination and seedling growth of *Abies pindrow* Spach.

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(Received in revised form: May 3, 2013)

ABSTRACT

To understand the process of natural regeneration failure in *Abies pindrow* forests in western Himalayas, the allelopathic interactions of one under-storey shrub species (*Viburnum nervosum* D. Don) were studied with keystone species (*Abies pindrow* Spach). The effects of foliage leachates (0%, 5%, 10%, 15%, 20%) of *V. nervosum* were investigated on the seed germination, radicle, plumule and seedling growth of *A. pindrow*. The leachate concentrations inhibited the seed germination, radicle and plumule growth, seedling height and seedling basal diameter of the receptor species. Trans-5-p-coumaroylguinic acid, *p*-coumaric acid, *m*-coumaric acid and flavanol were identified as phenolic phytotoxins produced in *V. nervosum* leaves, which may be responsible for the inhibition of various physiological processes in receptor tree species. Allelopathic interactions of *V. nervosum* may explain the relative absence of seed germination, poor establishment and growth of seedlings of *A. pindrow* on the forest floor in these temperate forests. Knowing such tree/under-storey interactions can help the foresters in regenerating these forests by manipulating the silvicultural practices. However, further research is required to identify such other allelopathic interactions in natural habitat of forest tree species in the region.

Keywords: *Abies pindrow*, allelochemical, allelopathy, forest regeneration, phenolic compounds, seed germination, seedling growth, *Viburnum nervosum*.

INTRODUCTION

Allelopathy is the interference of one plant through substances produced and released by the plant into the environment. A rapidly growing body of data suggests that allelopathy is often important in the survival and growth of trees both in plantations and natural stands. An awareness of this phenomenon, and its potential effects on regeneration and site productivity, is essential in intensive silviculture (8). Allelopathic interactions may occur throughout the life of a stand, but most commonly observed during the reforestation or regeneration. The plants exhibit the allelopathic effects on seed germination, growth and development of other plants by releasing allelochemicals into the soil, either as exudates from living organisms or by decomposition of plant residues (21, 27).

The failure of forest tree regeneration is a major problem in natural conifer forests. Allelopathy, competition, soil nutrient imbalance and poor ectomycorrhization have been implicated in conifer regeneration failure in the presence of dense ericaceous under-story resulting from forest harvesting and fire in boreal forests and sub-alpine spruce

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forests (19). Pellisser and Souto (23) have reviewed the role of allelopathy in northern temperate and seminatural boreal forests. Allelopathic compounds may be produced in any part of the plant, but the highest concentrations appear to be in foliage, fruits and in roots and may be released to the environment by volatilization or by leaching and exudation (8). Different sensitivity of plant species to inhibitor substances depends on environmental conditions as well as physiological and biochemical characteristic of each specific species (2). The process of natural regeneration in *Abies pindrow* Spach (silver fir) forests is a big problem in western Himalayas. This problem was reported nearly a century ago and since then, numerous factors (unfavorable soil conditions, accumulation of dead needles in the presence of excess moisture in the soil, the forest duff provides some physical hindrance to seedling growth) were proposed to be responsible for it (10, 14, 33, 37). Studies on seed dispersal, germination and seedling establishment in natural forests of silver fir showed that of the many seeds dispersed on the forest floor (51.92-528.64/m²), only very few (0.63-6.93/m²) produces seedlings and many of them died before getting established. Despite good germination capacity of dispersed seeds of silver fir, poor germination in the field is due to factors other than germination capacity of seed. Seedling mortality (31.93 to 80.58 %) occurred in the first year in these forests with maximum mortality in the rainy season (28, 30, 31).

Few attempts have been made to correlate the flora of shrubs and herbs in the Himalayan coniferous forests with regeneration problem e.g. presence of *Strobilanthes artopurpureus* and balsams in forest are unfavorable for fir regeneration. Singh and Rattan (29) found inhibitory effects of leaf leachates of *Sarcococca saligna* (under-storey shrub) on seed germination, radicle, plumule and seedling growth of *A. pindrow* in western Himalayas. *Viburnum nervosum*, (Adoxaceae family), is common deciduous gregarious shrub in silver fir forests in this region as per the phyto-sociological data; *Polypodium negrocarpum* (fern), *V. nervosum* and *S. saligna* are the dominant shrubs in these conifer forests with Importance Value Index (IVI) of 130.96, 91.85 and 43.88, respectively (35). This study aimed to determine the allelopathic effects of *V. nervosum* leaves on the seed germination and early growth of *A. pindrow* and also to ascertain whether foliage leachates help in regeneration of the species.

MATERIALS AND METHODS

Preparation of plant material

The fresh leaves of *V. nervosum* were collected from ten randomly selected plants in the natural *A. pindrow* forests in Deovan forest in Chakrata forest division, Uttarakhand, India (Altitude - 2,500 to 2,800m, Latitude - 20° 26' and 31° 2' North and Longitude - 77° 38' and 78° 4' East) during July 2008 and brought to the laboratory for leachate preparations. The leachate extracts of species were prepared as per method of Djurdjevic *et al.* (7). Three hundred g foliage was soaked in 600 ml distilled water for 24 h in plastic tub at room temperature, and then shifted to sieved plastic cylinder to collect foliage leachate. The leachate was filtered through Whatman #1 filter paper. It was considered as 100 % concentration leachate and stored at 4°C in Refrigerator for further experiments. For bioassay, it was further diluted to 0, 5, 10, 15, 20% concentrations with distilled water.

The seeds of *A. pindrow* and leaves of *V. nervosum* were collected from evenly aged trees of natural forests of Chakrata during October 2008, the seeds had no dormancy.

Laboratory bioassay

The seeds were soaked in distilled water for 24 h before germination tests, and then seeds were sterilized with 0.05% mercuric chloride solution for 5 min. The seeds were washed thrice with distilled water to remove traces of mercuric chloride before germination test. Fifty seeds of *A. pindrow* were evenly placed on Whatman #1 filter paper in sterile Petri dishes (10 cm dia). Five ml leachate solutions was added to each Petri dish as per treatment and distilled water was used as control. Each treatment was replicated four times and the Petri dishes were placed in the germination chamber (light intensity :750 lux 16 h light and 30°C temperature). Germination count was recorded daily and the germination test was run for 28 days. Seeds were considered germinate when the 1 cm long radicle emerged. The data of seed germination were recorded and quantified according to ISTA (12) rules. At the end of experiment i.e. after 28 days, the radicle and plumule length of germinated seeds was measured using a centimeter scale.

Nursery experiments

To study the effect of foliage leachates of *V. nervosum* on the growth of *A. pindrow* seedlings, nursery experiments were conducted at Deovan forest nursery in Chakrata Forest Division, Uttarakhand. One year old seedlings, grown in polythene bags (9 × 6 cm) containing soil + FYM + sand in equal proportion, were used in the trial.. There were 12 seedlings per treatment in 4 replications in Randomized Block Design and there was only one seedling per polythene bag. The leachates at 100 ml/ seedling were applied every month as per treatments from March 2009 to February 2010. The initial and final height and stem basal diameter of the seedlings was measured after one year application of leachates.

Identification of phenolic compounds

Phenolic compounds in leachates of *V. nervosum* were identified using HPLC as per method of Batish *et al.* (3). The specification of HPLC system used were what man ODS-2, gradient pump, photo diode array detector (PDA), interfaced with an auto sampler using ODS-2 column. The mobile phase consisted of methanol and water in the ratio of 20: 80. Leachates were injected in one volume of 10 µl, calibrated at 256.8 nm. The system was run for 0 - 50 min. The flow rate was kept constant throughout the analysis at 1 ml/min. Compounds were identified by tracing the retention time according to external standards.

Statistical calculations

The observations were computed for analysis of variance (ANOVA) as per Sukhatme and Amble (32). The critical difference (CD) was used to determine significant difference between treatments and control. Analysis of covariance (ANCOVA) was also used to observe variations in different treatments for seedlings height and collar diameter. The data was statistically analyzed using SPSS version 16.

Response Index (RI) was calculated using the formula of Richardson and Williamson (26) to observe the magnitude of inhibition versus stimulation by various leachates on seed germination, radicle, plumule and seedling growth.

RESULTS AND DISCUSSION

Seed germination

All concentrations of foliage leachates *V. nervosum* inhibited the seed germination of *A. pindrow* (Table 1). The maximum (33.68%) and minimum germination (8.65%) was recorded in control and 5% leachates, respectively. The seed germination at 5% and 10% leachates concentrations differed significantly ($p > 0.01$) from control, with no significant difference at higher leachate concentrations. The response index of germination with different leachate concentrations also revealed the inhibition of -73.75%, -69.17%, -26.92%, -17.38% in germination at 5%, 10%, 15%, 20% concentrations, respectively (Fig. 1). Thus foliage leachates of *V. nervosum* inhibited the seed germination of *A. pindrow* in all applied concentrations. However inhibition was more at lower concentrations with decrease at higher concentrations and such inconsistent pattern in germination was observed in both years.

Table 1. Effects of foliage leachates of *V. nervosum* on seed germination, radicle and plumule length of *A. pindrow* in petri plates

Leachates conc (%)	Germination (%)	Radicle length (cm)	Plumule length (cm)
0 (Control)	33.68	2.52	1.46
5	8.65	2.47	1.44
10	10.25	2.39	1.45
15	24.50	2.22	1.45
20	27.75	1.95	1.56
CD (0.01)	9.89	0.61	0.33

CD = Critical difference

Less inhibition in seed germination at higher concentrations or beyond threshold concentrations of leachates may be explained with the redundant entry of leachates due to restricted osmotic pressure, hereby, causing less penetration of allelochemicals in the seeds. Li *et al.* (25) reported that the aqueous extracts of walnut leaves had inhibitory effects on germination of four medicinal plants (*Isatis tinctoria*, *Atractyloides macrocephal*, *Polygala tenuifolia* and *Salvia miltiorrhiza*) at 5% concentrations. Jaderlund *et al.* (13) also found that water leachate of green leaves of bilberry was more inhibitory to germination and root growth of Norway spruce than brown leaves.

Seedling growth

Radicle length: The foliage leachates of *V. nervosum*, also inhibited the radicle growth, however, treatments did not differ significantly ($p > 0.01$) in both years. There was inhibition of -1.98%, -5.27%, -12.14%, -22.57% in radicle length at 5%, 10%, 15%, 20%

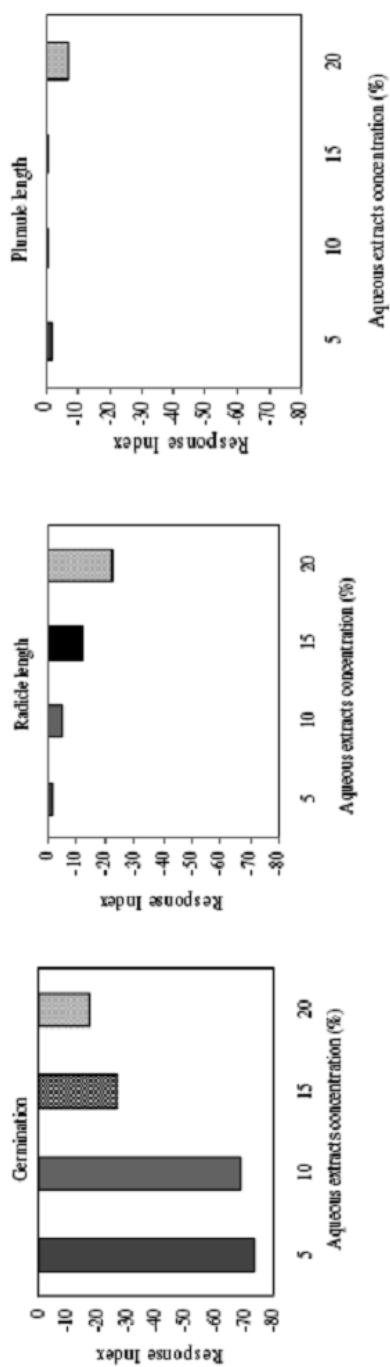


Figure 1. Response Index (RI) of foliar leachates of *V. nervosum* on germination and seedling growth of *A. pindrow* in petriplate bioassay

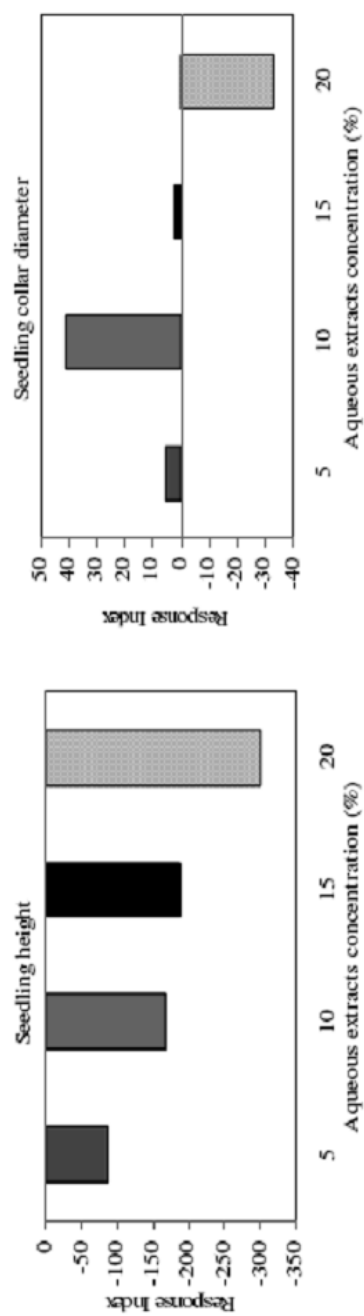


Figure 2. Response Index (RI) of foliar leachates of *V. nervosum* on seedling height and seedling collar diameter of *A. pindrow* in pot culture.

concentrations during both the years (Table 1, Fig. 1). The rate of inhibition increased with the increase in concentrations showing toxic effects of leachates on radicle growth of *A. pindrow*.

Plumule length: The foliage leachates of *V. nervosum*, also inhibited the plumule length, however, treatments did not differ significantly. The response index showed inhibition of -1.67%, -0.66%, -0.66% and stimulation of +6.83% plumule length at 5%, 10%, 15% and 20% concentrations, respectively (Fig. 1). However more inhibitory effect was observed at lower concentrations with decreasing trend at higher concentrations and slight stimulation at 20% concentration in the study.

Comparatively more inhibition of radicle growth than plumule growth of receptor plant by the leachates in this study is supported by earlier studies where water extract of allelopathic plants had more pronounced effects on radicle growth than on hypocotyls or shoot growth (5). Such an outcome might be expected, because the roots are the first to absorb the allelochemicals or autotoxic compounds from the environment (34). Li *et al.* (25) also observed that root length of medicinal plants was relatively more sensitive to aqueous leachate of walnut leaves than shoot length. Colvin and Gliessman (6) found that increasing the concentrations of fennel leaf leachate inhibited the root growth of 11 introduced test weed species except one forb species, whose growth was promoted as the leachate concentration increased. They also reported that the shoot growth of all test species was inhibited except in two forb and one grass species, where increasing concentrations stimulated shoot growth.

Seedling height: All applied concentrations of foliage leachates of *V. nervosum* inhibited the seedling height of *A. pindrow* in nursery during one year of study. However, the seedling height was not differed significantly in treatments. The height growth of 0.83, 0.11, -0.57, -0.73 and -1.65 cm was observed in control, 5%, 10%, 15% and 20% leachate concentrations, respectively. The rate of inhibition increased with the increase in concentrations with more pronounced effect at 20% concentration of leachates. The response index showed growth inhibition of -86.75%, -168.67%, -187.95%, -298.80 in 5%, 10%, 15%, 20% leachate concentrations, respectively (Table 2, Fig. 2). These results showed that the foliage leachates of *V. nervosum* inhibited the seedling height of recipient species.

Table 2. Effect of foliage leachates of *V. nervosum* on seedling growth of *Abies pindrow* in pot culture

Leachates conc. (%)	Seedling height (cm)			Seedling collar diameter (mm)		
	Initial	Final	Growth	Initial	Final	Growth
0 (Control)	7.07	7.90	0.83	2.07	3.02	0.95
5	6.75	6.86	0.11	1.78	2.78	1.00
10	6.90	6.33	-0.57	1.80	3.14	1.34
15	6.85	6.12	-0.73	1.92	2.89	0.97
20	7.20	5.55	-1.65	2.14	2.78	0.64
CD (0.01)	..	3.23	NS	..
F- Value (ANCOVA)	..	5.22	0.31	..

CD = Critical difference

Seedling collar diameter: The concentrations of foliage leachates of *V. nervosum* also inhibited the seedling collar diameter of *A. pindrow* in nursery but only at higher concentrations. The seedling collar diameter did not differ significantly in various treatments. The response index showed +5.26%, +41.05%, +2.11% stimulation in 5%, 10% & 10% concentrations, respectively, but inhibition of -32.63% at 20% concentration of leachates (Table 2, Fig. 2). This might be ascribed to different allelochemicals in donor plant.

Such growth inhibition, as observed here in silver fir seedlings by the leachates of under-storey plant, was also reported by Krause (12) in jack pine (*Pinus banksiana* Lamb.) in the presence of *Kalmia angustifolia* in New Brunswick, Canada. The other ericaceous shrubs such as salal (*Gaultheria shallon* Pursh.) in coastal oceanic temperate rainforests of west coast of Canada and several *Vaccinium* species (e.g. *Vaccinium alaskaense* L.) in high elevation forests of Pacific Northwest of USA have also been reported to cause growth stagnation of conifers such as western red cedar (*Tsuga plicata* Donn), western hemlock (*Thuja heterophylla* (Raf. Sarge)), Sitka spruce (*Picea sitchensis*) and Amabilis fir (*Abies amabilis* Dougl.) (4, 9, 20, 24).

Phenolic compounds

Four phenolic compounds namely, *p*-coumaric acid, *m*-coumaric acid, trans-5-*p*-coumaroylguinic acid and flavanol were identified from foliage leachates of *V. nervosum* on the basis of eight peaks presented in the HPLC analyzed chromatogram (Fig. 3). The trans-5-*p*-coumaroylguinic acid has the maximum area of 57.20%, while *m*-coumaric acid has the minimum area of 0.49%.

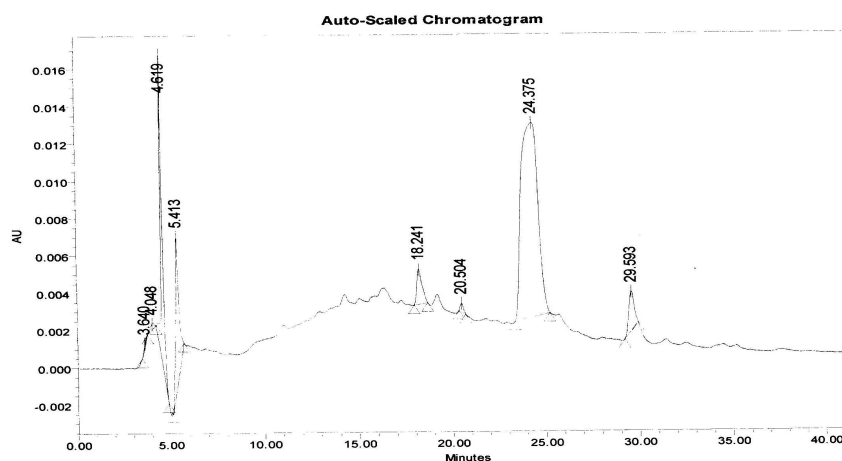


Figure 3. Chromatogram of foliage leachates of *Viburnum nervosum*

The presence of four allelochemicals in foliage leachates of *V. nervosum* may be responsible for inhibition of various processes (germination, radicle, plumule and seedling

growth) of *A. pindrow*. These findings are supported by the study where ericaceous litter, having an array of phenolic compounds, inhibited the conifer seed germination, primary root growth and ectomycorrhizal growth (19). Many of these phenolic compounds can create soil nutrients imbalance by reducing the available N (by forming protein-phenol complex) and increasing the amounts of Fe, Zn, K, Ca, Mg and Mn leading to long - term site degradation (11). Wollenweber and Kohorst (36) have extracted epicuticular leaf flavonoids from kalmia and salal and speculated that these compounds may have growth inhibitory effects on conifers. Andre (1) suggested that high phenolic contents of a *Vaccinium*-dominated humus layer on the forest floor restrict the development of mycorrhizae of Norway spruce seedlings in sub-alpine forests.

Allelopathic interactions may occur throughout the life of a forest stand but are most commonly observed during reforestation or regeneration and some tree species cannot regenerate due to these interactions (8). The inhibitory effects of *V. nervosum* on seed germination and seedling growth of *A. pindrow* may explain regeneration failure in the west Himalayan forests. Similarly natural regeneration failure of Norway spruce in the presence of bilberry (*Vaccinium myrtillus*) is mainly due to the germination inhibition of the spruce under a *Vaccinium* canopy (18,22), whereas black spruce regeneration in the presence of *Kalmia angustifolia* is caused not so much by germination inhibition but by root growth inhibition, competition, and nutrients deficiency (16,17).

CONCLUSIONS

The foliage leachates of *V. nervosum* inhibited the seed germination, radicle, plumule and seedling growth of *A. pindrow* in all applied concentrations in the study. Trans-5-p-coumaroylguinic acid, *p*-coumaric acid, *m*-coumaric acid and flavanol were identified as phenolic phytotoxins produced in *V. nervosum* leaves, which may be responsible for inhibition of various processes of receptor tree species. Allelopathic interactions of *V. nervosum* may explain the relative absence of seed germination, poor establishment and growth of seedlings of *A. pindrow* on forest floor in temperate forests of western Himalayas. Knowing such tree/under-storey interactions can ease the foresters in regenerating the forests by avoiding such situations in site preparations and eradication of ericaceous shrubs. However, further research is required to identify such other allelopathic interactions in natural habitat of the species in the region.

ACKNOWLEDGEMENTS

Authors greatly acknowledge the support of laboratory staff of Forest Tree Seed Laboratory, Dehradun and field staff of State Forest Department, Chakarata, Uttarakhand. The study was funded by Indian Council of Forestry and Education, Dehradun.

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