

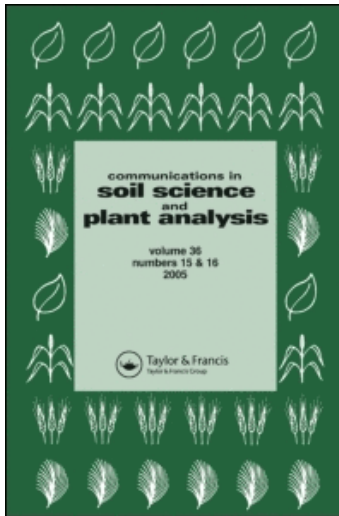
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Impact of Soil Nutrients and Environmental Factors on Podophyllotoxin Content among 28 *Podophyllum Hexandrum* Populations of Northwestern Himalayan Region Using Linear and Nonlinear Approaches

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Abstract: Podophyllotoxin is the active ingredient in the rhizome of an endangered Indian medicinal herb, *Podophyllum hexandrum*. Podophyllotoxin content in the *P. hexandrum* differs greatly in different natural habitats. The podophyllotoxin content reached more than 6.62% when soil pH value was about 4.82, soil organic carbon (C) was more than 3.23%, and nitrogen (N) content was more than 2.7% of soil dry weight. Available phosphorous (P) content of more than 0.419% and potassium (K) content of more than 1.56% resulted in low podophyllotoxin content. The linear relationship detected between podophyllotoxin and soil nutrients, environmental factors, and altitude suggested that further optimization of these factors are important in the conservation and exploitation of *P. hexandrum* in the northwestern Himalayan region, Himachal Pradesh, India. In this regard, like artificial neural network (ANN) and multiple linear regression (MLR), the prediction model used in this study to map the effect of these factors on podophyllotoxin yield will be helpful.

Keywords: Artificial neural network, environmental factors, multiple linear regressions, podophyllotoxin, *Podophyllum hexandrum*, soil nutrition

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INTRODUCTION

The Himalayan region is home to numerous highly valued medicinal herbs including *Podophyllum hexandrum* Royle (Berberidaceae), also known as Indian May Apple, which has an endangered status in India. It is distributed in very restricted pockets in the Himalayan zone at altitudes ranging from 1300 to 4300 m. It is recognized for its anticancer properties. The rhizomes and roots of *P. hexandrum* contain antitumor lignans such as podophyllotoxin, 4'-dimethyl podophyllotoxin, and podophyllotoxin 4-o-glucoside (Imbert 1998), which has long been used by the Himalayan natives and the American Indians as a life saving drug. Among the plethora of physiological activities and potential medicinal and agricultural applications, the antineoplastic and antiviral properties of podophyllotoxin congeners and their derivatives are arguably the most important from a pharmacological perspective. Semisynthetic derivatives of epipodophyllotoxin [e.g., etoposide (VP-16) (Allevi et al. 1993), etopophos (Schacter 1996), and teniposide (VM-26)] are effective agents in the treatment of lung cancer, a variety of leukemias, and other solid tumors (Van Uden et al. 1989). Growing demand in the world for anticancer drugs adds much to the importance of podophyllotoxin.

The total synthesis of podophyllotoxin is complicated because of the presence of four chiral centers, a rigid *trans*-lactone, and an axial 7-aryl substituent (Gordaliza et al. 2004). Hence, *P. hexandrum* (an Indian species) and *P. peltatum* (an American species) are presently the commercial sources of podophyllotoxin for the pharmaceutical industry. However, the yield of podophyllotoxin from *P. peltatum* is low (~0.25% based on dry weight) in comparison to *P. hexandrum*, which contain ~4% of podophyllotoxin by dry weight (Jackson and Dewick 1984). The demand for the compound continues to increase and thus encourages domestication and conservation of *P. hexandrum* in the Himalayan region. In an attempt at *ex situ* conservation, Sharma et al. (2000) collected root samples of *P. hexandrum* from Jalori Pass and Khajjiar (from high altitude) and grew them at Palampur (low altitude). This leads to reduction in podophyllotoxin content in the plant sample. Conservation strategies adopted at Y. S. Parmar University of Agricultural Science, Nauni, Himachal Pradesh, India, further resulted in reduction in podophyllotoxin content. Increased rate of reduction in podophyllotoxin content might be attributed to the difference in soil nutrients and climatic factors in the different habitat. This is obvious as the plant is very adaptable to a wide range of environmental conditions. It can survive under varying growing conditions and adapts well to the extreme low winter temperature of northern climates to the high summer temperatures and altitudes ranging from 1300 to 4300 m. Therefore, planting of *P. hexandrum* plants at lower altitude may not be useful

because of the small amount of podophyllotoxin. Further, we have been able to prove that the podophyllotoxin content is not related to genomic variation (data communicated for publication). Hence, it demands management of soil nutrients and optimization of climatic factors for the successful conservation of *P. hexandrum*. Statistical methods such as artificial neural network (ANN) and multiple linear regression (MLR) are very useful in this respect. The MLR method has been used to explain the spatial variations in soil nutrients and its impact on crop yield at field scale (Sudduth et al. 1996). However, MLR requires a normal distribution of the input variables, which is not always the case (Atkinson and Tatnall 1997). The nonlinear predictors such as ANNs were widely used to solve various problems in agriculture. For example, Sudduth et al. (1998) successfully predicted corn yield with back-propagation neural-network models based on soil texture, topography, pH, and some soil nutrient elements. This prediction model was superior to those of the nonparametric statistical benchmark methods. To the best of our knowledge, no work has been reported to map the yield of podophyllotoxin from *P. hexandrum* populations with respect to soil nutrients and climatic factors.

The objective of the present study is to understand the soil management and optimization of environmental and geographical factors for the domestication, conservation, and sustainable utilization of *P. hexandrum* at a commercial scale from the Himalayan region. The effects of soil nutrient and environmental factors on the podophyllotoxin production in the root of *P. hexandrum* were investigated for the first time in northwestern Himalayan region, Himachal Pradesh, India.

MATERIALS AND METHODS

Sample Stations and Plant Materials

Populations of *Podophyllum hexandrum* were sampled from 28 sites covering 11 forest divisions at different altitudes (1300–4300 m) from the northwestern Himalayan region, Himachal Pradesh, India, in July to August 2006. From each site, representative plant samples were collected in replicates of three. The interval between replicates was 2–5 m. Each patch was considered one accession and coded according to the site of collection. A total of 8–10 plants were collected from each sampling site. The pairwise distance between populations within a forest division was 0.5–32 km, whereas the pairwise distance between forest divisions was 10–400 km (Figure 1). The fresh roots of *P. hexandrum* were trimmed from uprooted plants and washed with running tap water to remove the soil particles, followed by washing with doubly distilled water. The washed roots were

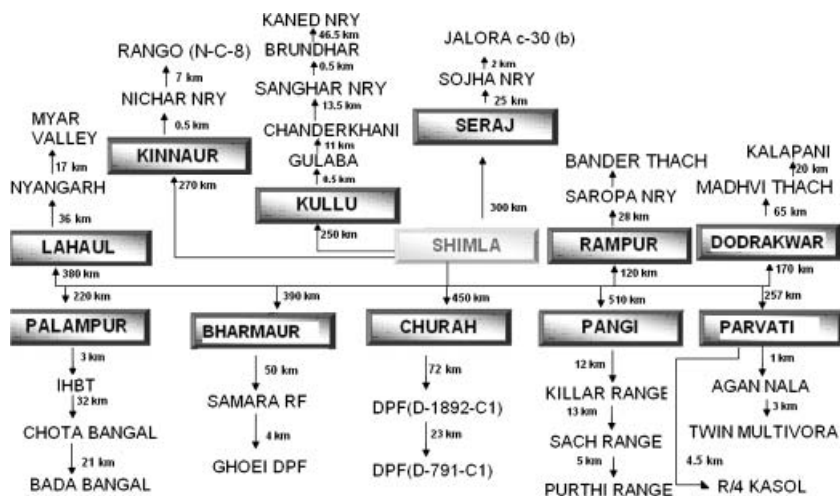


Figure 1. Difference in distance (Km) between sampling sites of respective forest divisions measured from a center point in Shimla.

then dried separately at 60 °C for 24 h in an oven and used for podophyllotoxin estimations. To diminish the effects of the age differences on podophyllotoxin content, only 3-year-old plants were selected.

Soil samples (root layer) from each sampling site were collected in replicates of three after uprooting the plants and brought to the laboratory for analysis of soil nutrients. The soils in the Himalayan region originated from weathering of sedimentary rocks and are technically known as podzol. The soil is mostly rock mixed with humus. However, the texture of the soil may be sandy loam, mica shift, loamy, or rock clay. Because the sampling was done from the wild, there was no human intervention. The forest types were generally subalpine to alpine, temperate conifers and temperate broadleaf at altitudes between 1300 m and 3000 m. However, the forest types were subalpine to alpine pasture and temperate conifers above 3000 m altitude, with wide variation in topography and geographical variation in the Himalayan region. From each sampling site, the data on environmental factors such as temperature, humidity, and rainfall was taken as monthly averages from January to December 2006 and properly documented.

Extraction of Podophyllotoxin and Quantification

Dried roots were ground to a powder in a pestle and mortar. Podophyllotoxin was extracted following the procedure of Broomhead

and Dewick (1990). Fifty mg of root powder was suspended in 20 mL of ethanol and continuously stirred at 60 °C for 20 min using a magnetic stirrer. The extract was filtered through Whatman filter paper no. 1. Second, third, and fourth extractions of the same samples were done with 10 mL ethanol for 10 min under the conditions mentioned. All the extracts were pooled, and ethanol was evaporated to dryness in a water bath shaker at 60 °C. The resultant residue was dissolved in 10 mL acetonitrile (high-performance liquid chromatography, HPLC, grade) and filtered with 0.22- μm durapore membrane filter (Millipore) for HPLC analysis.

The HPLC analysis was carried out using Nova Pack C18 cartridge column (250 \times 4.6 mm) in HPLC system (Water). Acetonitrile/water/methanol (37:58:5) was used as a mobile phase with a flow rate of 1.0 mL min⁻¹. Crude extract (20 μL) was used for injection into the HPLC system. Podophyllotoxin was detected at 230 nm (490 E Multiwavelength Detector, Waters). Podophyllotoxin (0.1 g L⁻¹, Sigma, P-4405) was used as a standard for calculating podophyllotoxin content in the samples on the basis of peak heights. All the experiments on extraction of podophyllotoxin and HPLC analysis were repeated three times.

Quantitative Analysis of Soil Nutrition

The soil samples were collected in replicates of three from each site. About 200 g of soil were collected from the root level, air dried to a constant weight, and then sieved through a 2-mm mesh. The fine soil (particles <2 mm) was used for nutrient analysis. Soil water pH was determined by dissolving 5 g of air-dried soil sample into 5 mL of SMP buffer [12.9 mM paranitrophenol; 15.4 mM of potassium chromate, K₂CrO₄; 0.361 M of calcium chloride, CaCl₂·2H₂O; 12.6 mM of calcium acetate, Ca(OAc)₂; and adjusting to pH 7.50 with 15% sodium hydroxide, NaOH] and measuring pH value with the pH meter (Mehlich 1976). The soil organic matter was determined by measuring organic carbon (C) content according to the wet-oxidation procedure described by Mebius (1960). Total nitrogen (N) was determined by Kjeldahl digestion, steam distillation of the resulting ammonium into boric acid, and titration with 0.10 N or 0.02 N hydrochloric acid (HCL) to pH 4.6 using automatic titrator. This method measures both organic and inorganic forms of N, which were reported as dry-weight percentage (Rump and Krist 1992; Kimble, Knox, and Holzhey 1993). Total phosphorus (P, organic and inorganic) was determined by perchloric acid digestion (Olsen and Sommers 1982). Total potassium (K) was analyzed by sodium hydroxide digestion and estimated by atomic absorption spectrometry.

Statistical Analysis

The correlation and regression analysis between the podophyllotoxin content and soil nutrients, environmental factors, and altitude were examined using MINITAB statistical package.

Neural-Network Data-Mapping Model Development

Neural networks are useful tools for pattern recognition, identification, and classification. A neural-network model can determine the input-output relationship for a complicated system based on the strength of their interconnections presented in a set of sample data (Howard and Mark 2000). Such a model can provide data-approximation and signal-filtering functions beyond optimal linear techniques (Clifford and Lau 1992). Therefore, neural-network models provide more robust results for complicated system analysis than conventional mathematical models. In this study, a back-propagation (BP) neural-network model was created using Stuttgart Neural Network Simulator package [SNNS version 4.2; Institute for Parallel and Distributed High Performance Systems (IPVR) at the University of Stuttgart, Germany] and trained using the environmental factors and soil nutrition parameters as the inputs and the measured corresponding podophyllotoxin reading as the output. The topological structure of this neural-network model consisted of 13 input neurons in the input layer and one output neuron in the output layer to match the 13:1 input-output pattern of the training data set. One hidden layer with eight neurons was the optimal topology for the neural-network model determined by a trial-and-error method (Figure 2). The evaluation criterion for determining the optimal topology was the best correlation value of the training set. The neural-network model was trained in an iterative training process using the obtained training set as follows:

$$D_T, i = \{0.090 \ 0.086 \ 0.255 \ 0.702 \ 0.190 \ 0.786 \ 0.739 \ 0.403 \ 0.235 \ 0.299 \\ 0.688 \ 0.805 \ 0.266\}$$

The first value referred to altitude, the next six numbers are the climatic factors, the next five values belong to soil nutrition parameters, and the last number is the corresponding average podophyllotoxin content measured from the roots collected from the corresponding site. To avoid possible bias, the order of input-output data pair in a training data set was randomized before the training process. During the training process, the BP training algorithm compares the estimated output value with the target value (namely the measured value), then tunes weighting values, connecting all the neurons to minimize the difference between the estimated and the target values until the error is smaller than a predefined level or until the number

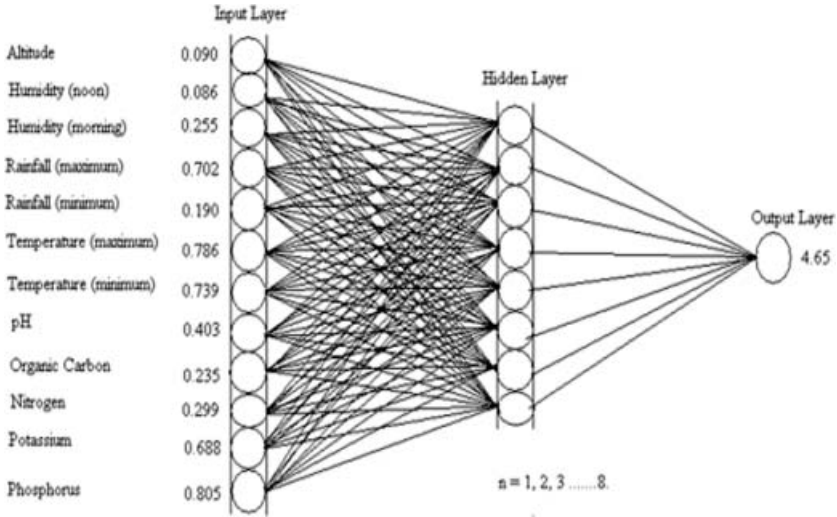


Figure 2. Layers and connection of a feed-forward back-propagating ANN.

of the iteration reached a preset maximum number. The constructed model was trained with the input data for an epoch of 10,000 with 0.1 learning rate. After completing the training process, all weighting indices describing the interconnection strengths between neighboring neurons are fixed, and the neural-network model will then be capable of mapping input variables to an estimated output promptly and accurately.

The neural-network model developed here applied a sigmoid transfer function to compute the strength of interconnection between each pair of neurons. The input variables in this model were normalized based on their possible ranges to avoid data saturation using the following equation:

$$x_{norm} = \frac{x - x_{min}}{x_{max} - x_{min}}$$

where x , x_{min} , x_{max} , and x_{norm} are the real-valued input variable, the minimum and maximum possible values of the input variable, and its normalized value, respectively. The output from this neural-network model is an indexed value that corresponds to the input variable. To get the real-valued output, the indexed output value needs to be denormalized according to the following equation:

$$y = y_{norm}(y_{max} - y_{min}) + y_{min}$$

where y , y_{min} , y_{max} , and y_{norm} are the real-valued output variable, the minimum and maximum possible values of the real-valued output, and the indexed output value from the neural-network model, respectively.

RESULTS

Podophyllotoxin Content in the Root of *P. hexandrum*

Podophyllotoxin content was extracted and analyzed in triplicate from 28 populations of *P. hexandrum*. This investigation will guide selection of soil type, altitude height, and environmental factors for the cultivation of *P. hexandrum* to improve the podophyllotoxin content. It was found that the podophyllotoxin content in the root of plants obtained from Lahaul forest division was comparatively more (8.857 to 9.533% on dry-weight basis) than that in the root samples collected from other forest divisions with a minimum from Parvati, 3.020 to 4.753% on a dry-weight basis (Table 1). The variation in podophyllotoxin content was significant among the populations ($F = 17.22$, $P < 0.001$) as well as between the forest divisions ($F = 3.70$, $P < 0.009$).

Effect of Altitude

All the 28 sites chosen for sampling of *P. hexandrum* populations were at different geographical locations with altitude ranging from a minimum of 1300 m (Parvati forest division) to a maximum of 4300 m (Lahaul forest division) (Table 1). The podophyllotoxin content in the root sampled from these sites increased progressively from low altitude to high altitude. Figure 3 shows the linear regression analysis between the altitude and podophyllotoxin content, including all the sampling sites. The respective correlation coefficient (r) was 0.928 and reached statistical significance level ($P < 0.001$), which is a symbol of accelerated correlation and indicates that podophyllotoxin production was significantly favored at increased altitude.

Effect of Environmental Factors on Podophyllotoxin Content

The environmental factors recorded during the course of the experiment are given in Figure 4. It revealed wide range in climatic factors like temperature (minimum and maximum), rainfall (minimum and maximum), and relative humidity (before noon and after noon) among the different sites from where the samples were collected. At these sites, the minimum temperature ranges from 2 to -10 °C, maximum temperature ranges from 12 to 35 °C, rainfall varies from a minimum of 0.00 cm to a maximum of 400 cm, and relative humidity varies from 30% to 90% at forenoon and 60% to 20% at afternoon. The variation in podophyllotoxin content in the root of podophyllum is highly dependent on these

Table 1. Twenty-eight populations of *Podophyllum hexandrum* collected from different sites at different altitudes covering 11 forest divisions and their podophyllotoxin content

Forest division	Sampling site	Altitude (m)	Podophyllotoxin ^a (% dry weight) (mean \pm sd)	Predicted value using ANN ^b	Predicted value using MLR ^c
Parvati	Twin Multivora	1300.00	3.567 \pm 0.747	4.65	4.286062
	R/4, Kasol(C-II-a-Nry)	1570.00	4.753 \pm 0.796	3.595	3.495285
Kullu	Anganoala (R/9) Rajgiri	1300.00	3.020 \pm 0.524	3.054	3.547704
	Brundhar	1916.00	4.077 \pm 0.270	6.692	5.786373
	Gulaba	2895.00	5.943 \pm 0.591	8.348	8.343126
	Chander Khani	3352.80	8.033 \pm 0.454	5.713	6.209076
	Kaned Nry	2150.00	4.657 \pm 0.850	8.913	9.133802
	Sanghar Nry	2100.00	4.173 \pm 0.276	9.511	9.427568
Dodrakwar	Madhvi Thach	3048.00	6.207 \pm 0.743	4.315	4.209545
	Kala Pani	2743.20	5.800 \pm 0.212	5.864	5.5872
Seraj	Jalora Pass (Sojha Nry)	2667.00	6.607 \pm 0.348	7.975	7.944759
	Jalora c-30(b)	2473.20	6.790 \pm 0.855	6.395	6.802259
Churah	DPF (D-1892-C1) (Chaoundi)	3750.00	8.487 \pm 0.565	4.03	4.357002
	DPF (D-791-C1)	2700.00	5.753 \pm 0.411	4.643	4.569688
Lahaul	Myar Valley	4300.00	9.533 \pm 0.484	5.625	5.860158
	Nayan Ghar	4300.00	8.857 \pm 0.427	6.615	6.559633
Palampur	Bada Bangal	2895.00	7.097 \pm 0.797	5.055	5.930285
	Chota Bangal	2700.00	6.573 \pm 0.827	7.115	6.655357
	IHBT	2800.00	5.183 \pm 0.780	6.627	6.541235

Table 1. Continued

Forest division	Sampling site	Altitude (m)	Podophyllotoxin ^a (% dry weight) (mean \pm sd)	Predicted value using ANN ^b	Predicted value using MLR ^c
Rampur	Bander Thach	2895.00	6.773 \pm 0.640	6.348	5.853001
	Saropa Nry	2499.40	6.097 \pm 0.942	5.864	5.89382
Kinnaur	Nichar Nry	2190.00	4.760 \pm 0.291	4.568	4.897322
	Rango (N-C-8)	2710.00	5.797 \pm 0.552	5.648	5.842633
Pangi	Sach Range	2712.70	6.133 \pm 0.216	6.307	5.967629
	Killer Range	2850.00	5.967 \pm 0.692	5.769	6.221935
	Purthi Range	2900.00	6.233 \pm 0.790	6.314	6.47006
Bharmaur	Ghoei DPF	2080.00	5.700 \pm 0.692	5.686	6.040152
	Samara RF	2590.80	6.030 \pm 0.825	6.243	6.133434

^aPodophyllotoxin content varied significantly among 28 populations ($F = 17.22$, $P < 0.001$) as well as between 11 forest divisions ($F = 3.70$, $P < 0.009$).

^bANN, artificial neural network.

^cMLR, multiple linear regression.

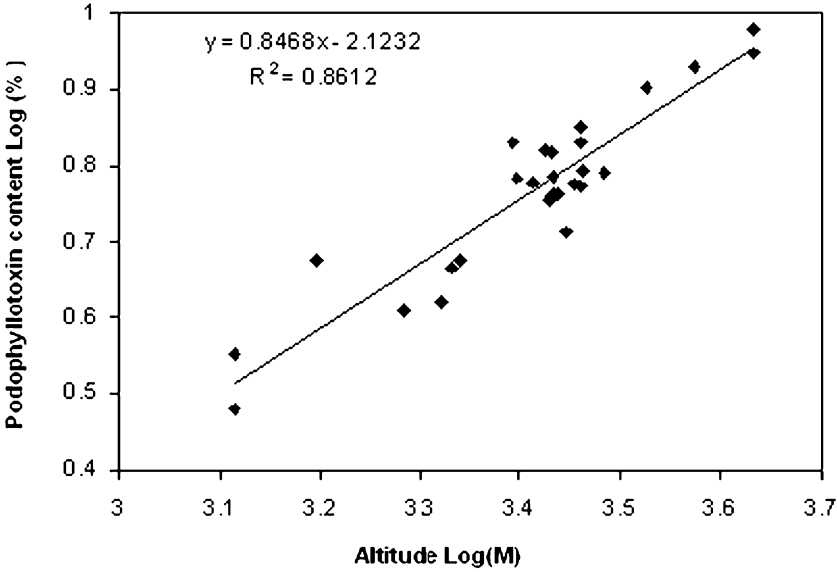


Figure 3. Regression analysis based on \log_{10} podophyllotoxin content and \log_{10} M (altitude) between 28 populations of *P. hexandrum*.

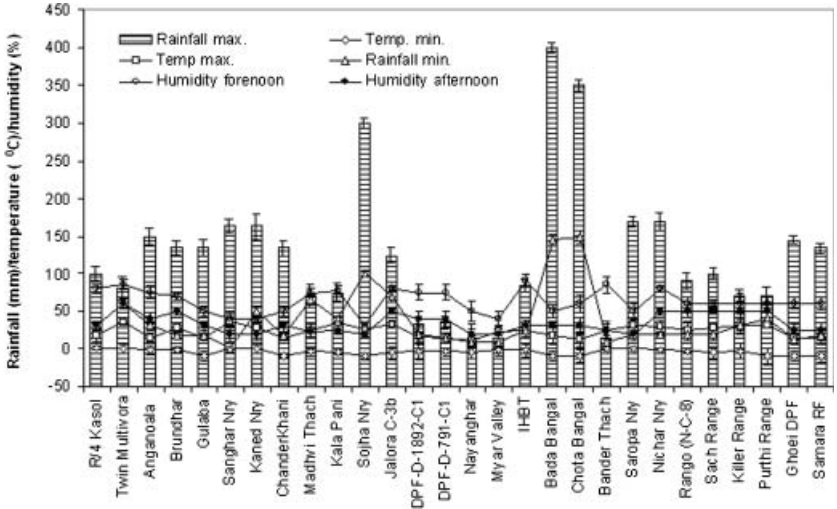


Figure 4. Meteorological observations made during the course of the experiment with respect to the site of collection of *P. hexandrum* populations. The values are the average of data collected in each site from January to December 2006. The vertical bars represented standard error of mean (SEM).

climatic factors. The variation in podophyllotoxin content was related positively with humidity; $r = 0.825$ (afternoon) and $r = 0.844$ (forenoon) and reached statistical significance level $P < 0.001$ (Figure 5a and 5b). The correlation coefficient between podophyllotoxin content was -0.595 (significant at $P < 0.01$) with maximum rainfall and 0.717 (significant at $P < 0.001$) with minimum rainfall (Figures 5c and 5d). The linear correlation coefficient (r) was -0.720 for maximum temperature (significant at $P < 0.001$) and -0.635 ($P < 0.001$) for minimum

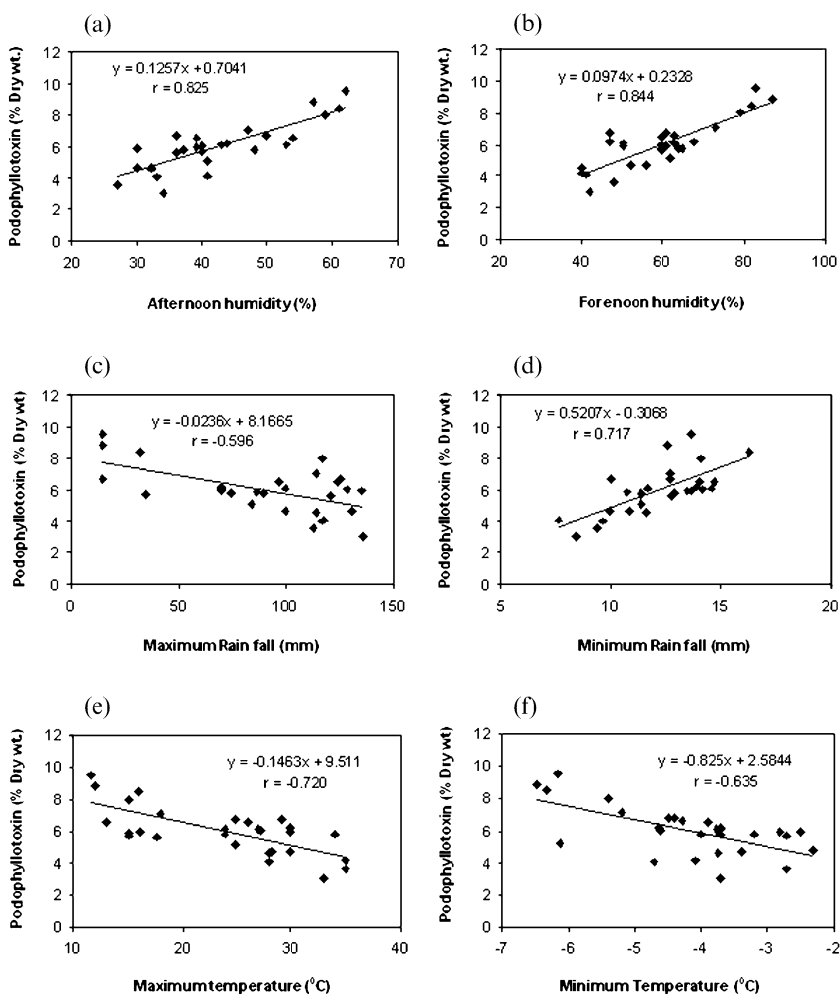


Figure 5. Relationship between environmental variables humidity (% , afternoon and forenoon), rainfall (maximum and minimum), temperature ($^{\circ}\text{C}$, maximum and minimum), and podophyllotoxin contents in the root of *P. hexandrum* at different altitudes in the northwestern Himalayan region.

temperature and are negatively correlated with podophyllotoxin content (Figure 5e and 5f).

Effect of Soil Organic Carbon (C)

According to our investigation, soil organic C content of respective sites mainly ranged between 2.26 (Anganoala) and 8.07% (Myar Valley). Figures 6a and 6b shows the statistical analysis results of the linear regression between soil organic C contents and podophyllotoxin contents in the root of *P. hexandrum* of all populations between the altitudes of 1300 m

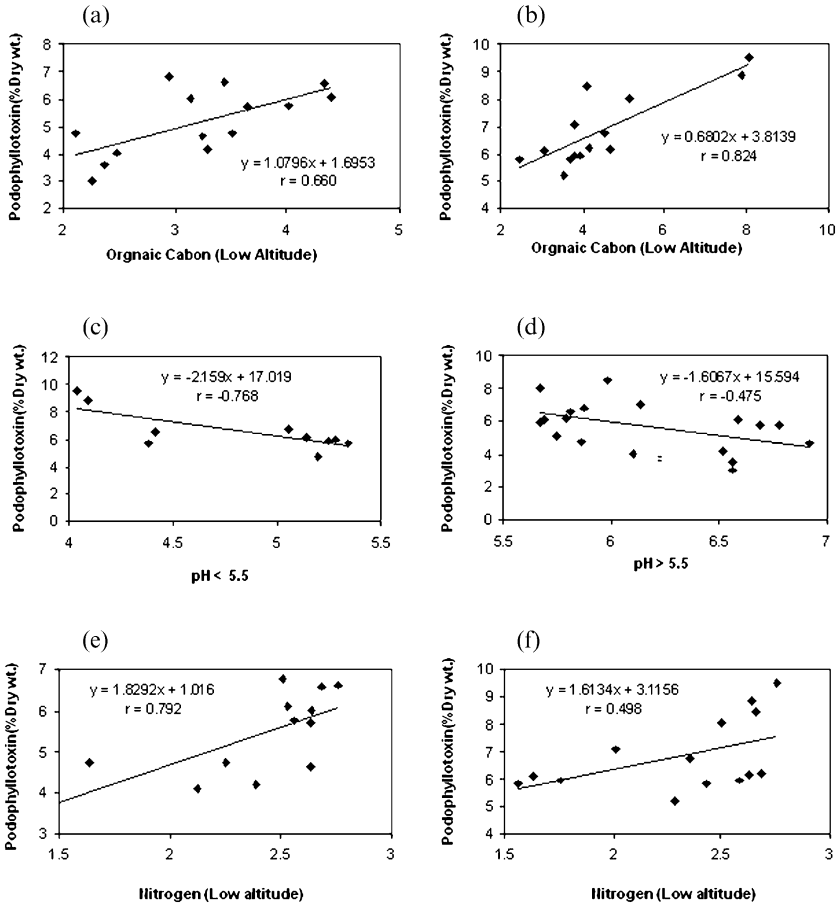


Figure 6. Relationship of soil organic C, pH, N, and podophyllotoxin contents in the root of *P. hexandrum* at different altitudes in the northwestern Himalayan region. Each pattern was classified according to low and high altitude.

to 2700 m (low altitude) and 2710 m to 4300 m (high altitude). In both groups, the correlation coefficients (r) were larger than 0.660 and are statistically significant at $P < 0.001$ (Figures 6a and 6b). The podophyllotoxin content in the root reached 5.182% (on average) in the soil organic C content of 3.23% (on average). However, increase in the organic C with respect to altitude of more than 3.23% revealed an increase in podophyllotoxin content up to 6.86% (on average). The results demonstrated that high soil organic C significantly favored podophyllotoxin production in the root of *P. hexandrum* at the altitude higher than 2700 m in Himachal Pradesh.

Effect of Soil pH

Figures 6c and 6d show that the soil water pH value at the altitudes above 1300 m in Himachal Pradesh ranges from 4.04 to 6.92. Therefore, *P. hexandrum* grew well in acidic soil conditions. The statistical analysis demonstrated that podophyllotoxin contents in the root of *P. hexandrum* grown at varying soil pH ($pH < 5.5$ and $pH > 5.5$) values had significantly negative linear correlation in both the population groups. The correlation coefficient (r) in both the groups was larger than -0.475 and reached statistical significance levels ($P < 0.05$). Podophyllotoxin contents reached about 6.62% (on average) when the soil pH value was 4.82 (on average), whereas the podophyllotoxin content decreased to 0.93% when the soil pH value was more than 5.5. This revealed that the acidic pH in the soil favored the podophyllotoxin accumulation in the roots of *P. hexandrum*.

Effect of Soil Nitrogen (N)

In Himachal Pradesh from the altitude of 1300 m to 4300 m, the total soil N contents ranged between 1.25% and 4.16%. At the altitude above 1300 m, the accumulation of podophyllotoxin in the root increased with the increasing total N content in the soil. The linear correlation coefficients (r) were 0.792 (among populations at altitude 1300 m to 2700 m) and 0.498 (among populations at 2710 m to 4300 m), and both reached the statistical significance levels ($P < 0.001$ and $P < 0.05$) respectively (Figures 6e and 6f). The podophyllotoxin content reached 6.86% (on average) when the soil N content was more than 2.27%.

Effect of Phosphorus (P)

Among 28 sampling sites, total P content in the soil ranged from 0.106% to 0.24%. Figures 7a and 7b showed the statistical results of the

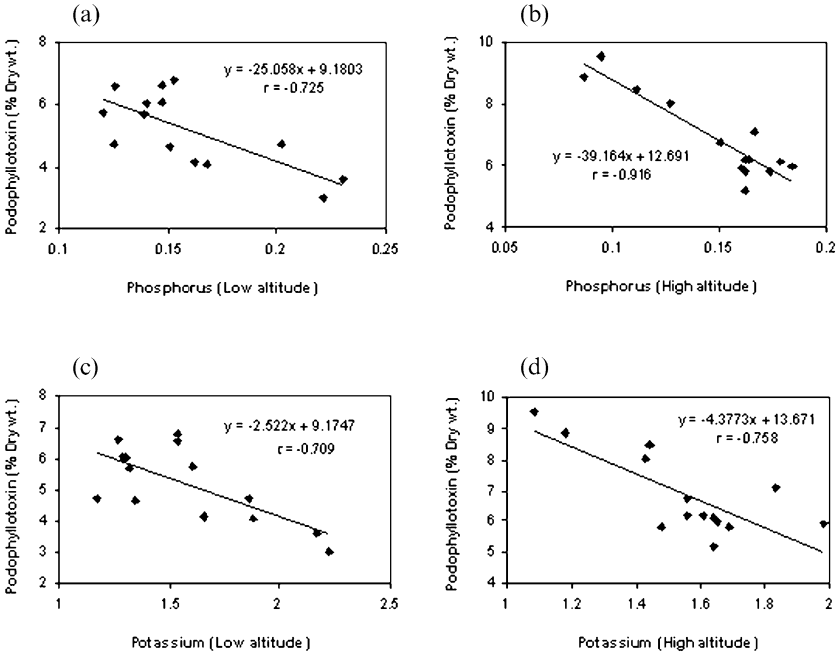


Figure 7. Relationship of P, K, and podophyllotoxin contents in the root of *P. hexandrum* at different altitudes in the northwestern Himalayan region. Each pattern was classified according to low and high altitude.

relationships between soil P contents and podophyllotoxin contents in roots of *P. hexandrum*. The linear correlation coefficients (r) were -0.725 and -0.916 between both the groups of populations at altitudes of 1300–2700 m and 2710–4300 m and reached significant levels of the negative linear relationship ($P < 0.001$). The results demonstrated that the increase of soil P content above 0.149% inhibited podophyllotoxin accumulation in the root of *P. hexandrum*.

Effects of Soil Potassium (K)

Total soil K contents ranged between 1.08% to a maximum of 2.22% (Figures 7c and 7d). The correlation coefficients (r) of the negative linear relationship between podophyllotoxin content and K content were -0.709 and -0.758 at the altitudes of 1300 to 2700 m and 2710 to 4300 m, respectively, and reached a significant level ($P < 0.001$). This indicates that soil total K content also had a significant relationship with the podophyllotoxin production in the root of *P. hexandrum*.

Performance Measure of ANN and MLR Model

The prediction results of the ANN model determinate from the prediction phase are presented in Table 1. It revealed better prediction of yield ($r^2 = 0.9905$) in comparison to the experimental measurement, and the root mean square error (RMSE) was also very low (0.0399). To assess the performance of the neural-network model more thoroughly, a comparison study between the neural-network model and a baseline regression model was made. The baseline model was a best-fit regression model obtained from similar parameters that has been used for ANN prediction. Regression analysis of the best-fit model estimated values resulted in r^2 of 0.9302. The r^2 values from estimated podophyllotoxin values using both the neural-network model and the best-fit regression model including the exponential model were compared, and the neural network model shows stronger correlation than the other prediction models (Figure 8). Similarly, the RMSE was 0.2939 from the best-fit model in comparison with 0.0399 from the neural-network model. By comparing results (the r^2 value and RMSE), we verified that the neural-network model can provide more accurate estimations of podophyllotoxin values than a best-fit regression model could. More importantly, a well trained neural-network model can be implemented in real time to

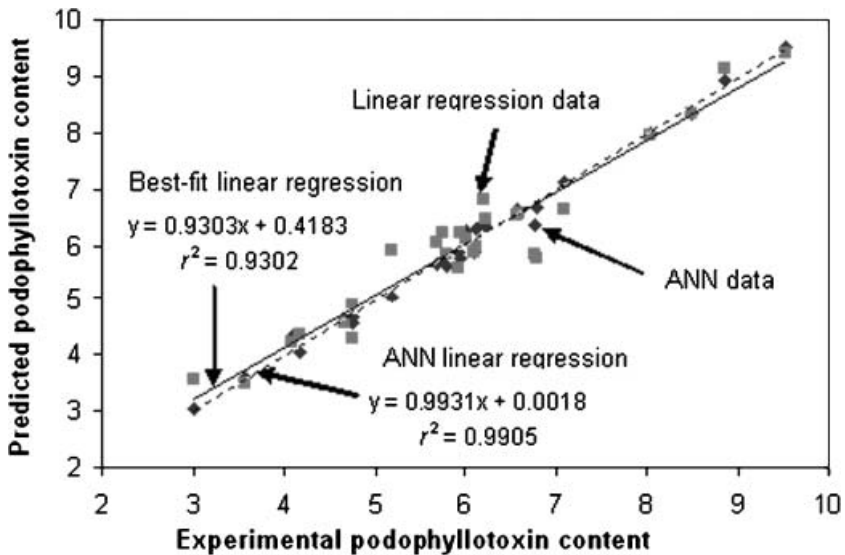


Figure 8. Comparison of estimated podophyllotoxin content using an ANN model (---) and a best-fit regression model (—, MLR). Here r^2 represents coefficient of determination.

estimate the required podophyllotoxin content in terms of changed environmental and soil factors with a minimal computational load.

DISCUSSION

The existing variations in podophyllotoxin content were proven to be coupled with altitude, environmental variables, and soil nutritional factors. This result is very much supported by the studies done by Sharma et al. (2000). They reported considerable reduction in the podophyllotoxin content in the roots of plants collected from higher altitude and after growing at lower altitude. The altitude ranges from 1300 m (Parvati forest division) to a maximum of 4300 m (Lahaul forest division). As a result, there is wide variation in climatic factors among the sites of collection of samples and thus there is variation in podophyllotoxin content among the populations used in the study.

This is the first report demonstrating that high podophyllotoxin production in the root of *P. hexandrum* has close relationship to the high soil C level, low soil pH value, high soil N level, as well as low concentration of soil P and K and environmental variables. Among these soil factors, soil pH, soil organic matter, and soil N are most significantly correlated to the podophyllotoxin production. Our work demonstrated that the soil in northwestern Himalayan region, Himchal Pradesh, is acidic and that *P. hexandrum* has not only adapted well it to but also produces more podophyllotoxin in the acidic soil. Other research also reported that pH value influenced secondary metabolites production. Cell culture of *Lupinus polyphyllus* increased alkaloid production when pH value decreased from 5.5 to 3.5 in the culture medium (Endress 1994). Hydrogen ions in soil change the membrane permeability of the root cell so that soil pH affects the growth of plants directly. It also affects the uptake of the soil nutrients by plants indirectly (Endress 1994). Therefore, the high podophyllotoxin yield in acidic soil may be caused by the influence of the acidic soil on the availabilities and uptakes of soil elements such as N, P, and K (Bhojwani and Razdan 1996).

Soil organic matter provides plants with N, P, and K and essential metal cofactors for metabolism. High soil organic-matter content can uniformly supply the nutrition to plants, guarantee the plants a good growth and metabolic status, and enhance the resistance of the plants to stresses. All these are the basis of secondary metabolism. Our work demonstrated that high soil organic matter favors the high podophyllotoxin yield of *P. hexandrum*. Another soil factor significantly affecting the podophyllotoxin production is N. Soil N has a close positive relationship with plant growth and metabolism because the N is a structural component of amino acids from which proteins are synthesized and

wide metabolisms take place. Nitrogen is needed in the production of phenylalanine, which is the starting point of the general phenylpropanoid pathway leading to podophyllotoxin production. Nitrogen forms, such as organic N nutrients, might be more important to high podophyllotoxin yield than the inorganic ones. Therefore, examining how N nutrients work and which N forms take part in the biosynthesis process of podophyllotoxin is essential for improving podophyllotoxin yield in the future study.

High P and K contents in soil inhibited the podophyllotoxin yield. The reduced phosphate content in the culture medium was also reported to increase secondary metabolite accumulation in other plant species (Knobloch and Berlin 1983; Endress 1994). The level of exchangeable K in the forest conditions is rarely a problem for plant growth (Li and Huang 1989; Xi 1994), but certain species are sensitive to it (Bhojwani and Razdan 1996). The mechanism of P and K affecting the podophyllotoxin yield in the cellular bioprocess remains unclear. It might be because both elements regulate the activity of certain enzymes involved in podophyllotoxin biosynthesis, in N metabolism, or in the cell's energy level, such as ATP, during these processes (Endress 1994).

Many Indian medicinal herbs have greater medicinal productivity in their original habitat than in cultivated lands. Soil nutrient characteristics and environmental factors similar to original habitats must be most suitable for the active compound production. According to the results in this research, podophyllotoxin production of *P. hexandrum* or other bioactive compound production from herbs cultivated in farms can be improved through the soil management to mimic the soil conditions similar to the original ones. The first approach is to measure the key soil nutrient factors such as pH value, organic-matter content, and N content, as well as P and K contents, when choosing a farm for the cultivation of *P. hexandrum*. The second approach is balanced fertilization. It is important and necessary to increase and maintain the soil organic matter and N contents at high levels during the period of the plant growth and harvesting season. Organic N fertilizer is suggested for the cultivation of *P. hexandrum*.

The soil matrix is a complex organic ecosystem with its own constructors and interaction between nutrition and microorganisms. Furthermore, plant secondary metabolism itself is also a complex physiological process. The secondary metabolite production is influenced by the plant's own physiological age, status, and other environmental factors. Therefore, the effects of soil on podophyllotoxin production of *P. hexandrum* are far more complicated than those mentioned. The impact of these topological and climatic factors on podophyllotoxin content is unstudied so far. However, it is assumed that the variation in podophyllotoxin content is dependent on these factors, which needs

further research. The prediction models like ANN and MLR developed in this study to map the effect of these factors on podophyllotoxin yield will be helpful to a certain extent for conservation aspects of the plant to get maximum yield. The results showed that using a combination of topographic, soil, and environmental data, we were able to successfully predict podophyllotoxin yield with ANN and MLR. Both the models could provide useful information regarding selection of sites and optimization of soil and environmental factors to increase the yield of podophyllotoxin and thus are very important for planning any conservation aspects.

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