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EFFECT OF PLANT AGE AND GEOGRAPHICAL LOCATION ON ACTIVE PAEONOL AND PAEONIFLORIN ACCUMULATION IN THE ROOTS OF *PAEONIA OSTII*

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Abstract

Paeonol and paeoniflorin, available in *Paeonia ostii* roots, are active compounds applicable for curing breast cancer and neuropathic pain. We investigated the impacts of growing locations and plant age on paeonol and paeoniflorin contents of the roots in *Paeonia ostii* from 3 to 6-year-old plants growing in the south, middle and north latitude of Anhui province, China. Root samples were analyzed by Ultra-Fast Liquid Chromatography (UFLC), and soil samples were analyzed by spectrophotometric methods. We found that both paeonol and paeoniflorin contents increased with plant age and also increased from south to north location in 6-years old plants but not in younger ones. We found that soil Mg^{2+}, Ca^{2+}, organic matter, and pH value had positive effects both on paeonol and paeoniflorin contents. Our study highlights the importance of plant age and geographical location on paeonol and paeoniflorin contents of *Paeonia ostii* roots.

Key words: Geographical locations, Plant age, *Paeonia ostii*, Paeonol, Paeoniflorin.

Introduction

Plants have been used in medicine for centuries in human history, and specific biochemicals derived from plants have remained a significant part of approved drugs worldwide (Oksman-Caldentey & Inze, 2004). Traditional herbal medicines play a vital role in the Chinese health system (Dong *et al*., 2011). *Paeonia ostii* is a hardy shrub in the family Paeoniaceae and is considered a primary source of cortex moutan (He *et al*., 2014). Cortex moutan contains a high level of active secondary plant metabolites such as paeonol and paeoniflorin (Ding *et al*., 2009; Long *et al*., 2012). The cortex moutan has been as a blood-cooling agent, heat-clearing dissipating blood stasis, promoting blood flow, and also as anti-inflammatory medicine in traditional Chinese herbal medicines (Huang 1999; Rho *et al*., 2005; Chun *et al*., 2007). It has been reported that cortex moutan can cure alcoholic steatohepatitis (Hu *et al*., 2010) and defend against severe hepatotoxicity (Park *et al*., 2011). Recent studies have reported that cortex moutan can prevent lipopolysaccharide-induced inflammation and Parkinson's disease, act as analgesic, and it is also useful for breast cancer treatment (Kim *et al*., 2014; Yin *et al*., 2016; Fu *et al*., 2012; Yun *et al*., 2013; Wu *et al*., 2016).

The medicinal plants had conventionally been used to assess their quality by ancient physicians in the past on the basis of morphological features. Now, the concept of geo-authentic herbal medicines has been established based on the association between the quality of medicinal plants and their growing locations. The most geo-authentic medicinal plants produced in their native geographical region have adequate effects for diseases. For example, *Picrorhiza scrophulariiflora* grown in Tibet and *Panax ginseng* in northeastern China legitimately recognised as Chinese medicines (Committee, 2010). Recently, utilisation of medicinal plants in some medical systems is being used based on the active secondary plant metabolites contained in extracts of these plants. Since extracts of medicinal plant develop their therapeutic properties from the active secondary plant metabolites, the driving forces involved in the accumulation and also in the production of such valuable metabolites in plants have great significance in standardising the quality of herbal medicines (Ncube *et al*., 2012).

Plant age is a significant factor that impacts on phytochemicals concentrations of medicinal plants. As reported by Achakzai *et al*., (2009) that young plant of *Rhododendron* species had the highest level of flavonoid content and total phenolic compound in its stem and leaves while the *Nerium oleander'*s leaves contained a minimal amount of phenolic compounds. It has been discovered that phenolic compounds concentration and antioxidants activity rise in a mature plant that increases its abilities to provide energy for secondary metabolism. In contrast, some plants need to utilize this energy during their early age primary metabolic processes that are necessary for their growth (Fritz *et al*., 2001; Paśko *et al*., 2009). Thus, the concentration of phytochemicals decrease or increase with the age of medicinal plants. Therefore, to avoid any change in optimum therapeutic potency of medicinal plants, it is recommended that medicinal plants should be harvested at a specific age for manufacturing medicine (Anon., 2002; Mutalib, 2015). Peony roots are generally taken from at least two years (preferably four years to five years) of age from cultivated plants (Chillemi & Michael, 2013). From previous studies, we have vague results about the production of paeoniflorin in roots of *Paeonia lactiflora* (another species of Paeoniaceae). According to the study of Jian *et al*., (2010) contents of paeoniflorin upsurge with the older age of the plant. But contradictory results were described by Zha *et al*., (2012) that the contents of paeoniflorin in the *P. lactiflora* roots gradually dropped with increasing plant age. And no data is available about the production of paeonol according to age. To clarify the ambiguous and missing research, study of paeonol and paeoniflorin production according to different ages of plants is crucial.

Secondary compounds symbolise a chemical interface between the growing environment and the plant, and their biosynthesis is often affected by environmental conditions (Kutchan, 2001). Previous studies have revealed that medicinal plants grown-up in different locations produces dissimilar contents of secondary compounds, resulting in alterations in their therapeutic qualities (Dong *et al*., 2011). Various environmental factors such as latitude, altitude, rainfall, temperature and soil factors including soil organic matter, soil pH value and available nutrients can affect the growth and development of plants which can bring changes in the quality of active secondary metabolites present in a specific species even if it is produced from the same country (Kokate *et al*., 2004; Liu *et al*., 2015).

Hence, plant age and growing locations are key factors that influence the quality of herbal medicine due to the variations in its active secondary metabolites (Achakzai *et al*., 2009; Liu *et al*., 2015; Mutalib, 2015; Liu *et al*., 2016). Due to the numerous benefits of *Paeonia ostii* root's active compounds specially paeonol and paeoniflorin (Hu *et al*., 2010; Sevim *et al*., 2013; Yin *et al*., 2016), it is important to conduct further studies to find out the impact of plant age and growing locations on the production of these active compounds. We hypothesized that contents of paeonol and paeoniflorin may change in *Paeonia ostii* roots with the plant growing locations and plant age as well. To validate our hypothesis we visited north, middle and south latitude of Anhui province (a core production area for *Paeonia ostii* in China) at the start of October 2015 to collect required samples.

Materials and Methods

Study area: *Paeonia ostii* is native to Anhui Province, China, and is widely cultivated to obtain the bark of its roots (Sevim *et al*., 2013, Zhou *et al*., 2014). Anhui Province is located in eastern region of China. Its average annual temperature ranges from 15.4-17.0°C, average annual precipitation is 718-1470 mm, annual average relative humidity is 63-74% and annual sunshine duration time approximately 1792–2018 h (Table 1).

Sampling design

(a) Root collection: Normally a four to five-year-old peony plant is used for taking roots in Chinese medicine (He and Dai, 2011). We selected 3, 4, 5 and 6-year-old plants (propagated during 2009, 2010, 2011, 2012 respectively, according to grower's information). In each location, three replicates of 3, 4, 5 and 6-year-old plants were collected randomly. The distance between the adjacent replicates was kept at least 50–100 m to see variations within each population. Roots of randomly selected plants were dug by spade. After digging roots, taproot and secondary roots from each plant were selected, cleaned, packed and labeled.

(b) Soil sample collection: For soil analysis, 0.5 kg from 0–20 cm deep of topsoil was taken from every root digging location, mixed well, packed and labeled.

(c) Climate data: Related data of climate factors including January average temperature, July mean temperature, mean annual temperature, mean annual precipitation, annual sunshine duration and relative humidity of the recent 6 years (from seed sowing August 2009 to root harvesting period October 2015) were obtained from Anhui meteorological bureau (station) for the three study sites (Table 1).

(d) UFLC method of paeonol and paeoniflorin determination: For paeonol and paeoniflorin determination, root extract for UFLC was prepared by previously used method (Guan *et al*., 2013) with little modifications. In detail, the root samples were cleaned, washed and the cortex of the root was peeled. The root's cortex was dried at room temperature, milled and passed through 80 mesh sieve. For UFLC analysis 500 mg fine powder was mixed with 1ml HPLC grade methanol in a microfuge tube, incubated in an ultrasonic water bath (JK-300DB) for an hour at 30°C, and centrifuged by SIGMA laboratory centrifuges 3K15 at a speed of 10000rpm for 10 minutes. The 500µl supernatant was taken in 8-425 thread screw neck vials (32x11.6mm) for UFLC analysis. And the same process was done with 5 mg of each standard compounds powder. Paeoniflorin (110736-201539) and paeonol (110708-201407) UFLC standards were bought from National Institutes for Food and Drug Control (NIFDC). UFLC was performed using a SHIMADZU UFLC (Ultra-Fast Liquid Chromatograph). The chromatographic separations of samples (10µL, 5 mg/mL) were carried out on Atlantis® T3 column (30×150mm, 3um) at 30°C, eluted with water (W) and acetonitrile (A) mixture. The linear gradient program was set at low-pressure mode. W-A (v/v) from 95:05, 70:30, 0:100 and 95:05 in 0 to 10, 11 to 30, 31 to 50 and 51 to 60 minutes respectively with a flow rate of 0.4 mL/min. The maximum temperature of the oven was kept at 80°C. Absorbance was monitored at 254nm. A standard sample of paeoniflorin was detected after 18.044 minutes of retention time, and paeonol was detected at 27.12 minutes. With the comparison of these reference samples retention time, the curve at 18.044 ± 0.05 minutes was considered as the paeoniflorin curve and curve at 27.12 ± 0.05 minutes was considered as the paeonol curve (Fig. 1).

(e) Soil analysis: Soil samples were dried at room temperature. Macro and micronutrients were measured with iCAP 6000 Series ICP Spectrometer following laboratory protocol. In detail: dried samples were sieved, and 0.5 g of soil were dissolved in 10 ml mixture (9:1) of absolute nitric acid and absolute perchloric acid. This mixture was then baked at 230°C until the resultant compound became white and filtered through Whatman filter paper into a sterilised 50 ml volumetric flask and filled it with distilled water up to mark. 20 ml aliquot was taken as a final sample to analyse the primary macronutrients: Nitrogen (N), Phosphorus (P), Potassium (K) and secondary macronutrients Magnesium (Mg^{2+}), Calcium (Ca^{2+}) and Sulfur (S). Micronutrients like Manganese (Mn), Zinc (Zn), Boron (B), Copper (Cu), Chlorine (Cl) and Iron (Fe) by iCAP 6000 Series ICP Spectrometer. Soil organic matter was determined by near-infrared spectroscopy as described by (Fidencio *et al*., 2002). Soil pH value was measured by mixing soil and 0.01M calcium dichloride in 1:5 ratio respectively (NF ISO 10390) with METTLER TOLEDO pH meter. All measured variables of the soil of all locations are recorded in Table 2.

Table 1. Climatic conditions.

Variable	South	Middle	North
Latitude (° N)	30.00 ± 0.00	31.00 ± 0.00	33.00 ± 0.00
Longitude (° E)	117.00 ± 0.00	116.00 ± 0.00	115.00 ± 0.00
Elevation (m a.s.l.)	45.00 ± 0.00	37.00 ± 0.00	32.00 ± 0.00
MAT (°C)	17.00 ± 0.02	16.10 ± 0.03	15.44 ± 0.03
MAP (mm)	1470.27 ± 9.64	1045.13 ± 7.56	718.74 ± 5.09
Humidity (%)	72.84 ± 0.45	74.57 ± 0.08	63.80 ± 0.17
SSD (hours)	1792.33 ± 10.86	1736.01 ± 6.69	2018.23 ± 11.52
HT (°C)	38.44 ± 0.04	38.22 ± 0.05	39.26 ± 0.06
LT (°C)	-4.64 ± 0.05	-9.02 ± 0.10	-9.10 ± 0.08
JNT (°C)	3.50 ± 0.13	2.18 ± 0.15	1.10 ± 0.10
JLT (°C)	29.07 ± 0.08	28.43 ± 0.08	28.22 ± 0.06

All values are given in mean ± SEM. MAT Mean annual temperature, MAP Mean annual precipitation, RH Relative humidity, SSD Sunshine duration, HT Annual high temperature, LT Annual low temperature, JNT January average temperature, JLT July average temperature and m a.s.l. meters above sea level

Table 2. Soil characters analyzed.

Variable	South	Middle	North
OM (mg/kg)	206.04 ± 10.66	75.37 ± 3.50	259.77 ± 9.61
pH value	4.55 ± 0.08	4.72 ± 0.08	7.67 ± 0.08
N (mg/kg)	1200 ± 0.00	900 ± 0.00	1000 ± 0.00
P (mg/kg)	1.37 ± 0.10	0.85 ± 0.04	1.80 ± 0.04
K (mg/kg)	89.52 ± 1.97	60.02 ± 5.54	63.35 ± 2.55
S (mg/kg)	676.56 ± 6.22	439.08 ± 32.61	349.20 ± 12.45
B (mg/kg)	231.02 ± 3.51	140.29 ± 9.00	140.59 ± 3.88
Ca (mg/kg)	21.97 ± 0.70	30.23 ± 1.59	277.35 ± 5.98
Cu (mg/kg)	0.41 ± 0.00	0.24 ± 0.01	0.26 ± 0.01
Fe (mg/kg)	401.48 ± 1.75	265.35 ± 13.64	247.61 ± 6.60
Mg (mg/kg)	46.43 ± 0.32	43.72 ± 3.20	57.14 ± 1.66
Mn (mg/kg)	14.27 ± 0.07	5.90 ± 0.35	6.14 ± 0.20
Cl (mg/kg)	3.82 ± 0.16	4.07 ± 0.18	4.10 ± 0.12
Zn (mg/kg)	1.43 ± 0.02	0.69 ± 0.04	0.83 ± 0.04

All values are given in mean ± SEM. OM total organic matter contents of soil, pH value Soil pH value. The data were collected from south, middle and north latitude of Anhui province, China.

Data analysis: UFLC data was analysed by Shimadzu's LC Solution software. To examine the effects of plant age and growing location on paeonol and paeoniflorin concentrations, we used linear models with plant age, location and their interaction as predictors. We used Bartlett's test and Shapiro–Wilk's test for the assumptions of homogeneity and normality, and we found that both assumptions were violated at $\alpha = 0.05$. To mitigate the violations to these assumptions and to improve coefficient estimates for small sample size ($n = 4$ for each age class and location), we bootstrapped the fitted coefficients of all models by 1000 iterations using the *boot* package (Canty & Ripley, 2016). To examine the extent of correlation among paeonol and paeoniflorin concentrations and measured site climate and soil properties, we used the *corrgram* package to visualize the results data. All data were analyzed with R 3.3.1.

Results

Effect of plant age and growing locations: Both paeonol and paeoniflorin concentrations in *Paeonia ostii* roots differed significantly with plant age and locations (Fig. 2). Paeonol concentration increased with plant age in all study locations, and on average increased from south to north locations (Fig. 2). From north locations Paeonol concentration was on average 0.34±0.04, 0.63±0.02, 0.83±0.05 and 1.32± 0.1 mg/g, from middle location was 0.57±0.3, 0.68±0.03, 0.75±0.04 and 1.00± 0.1 mg/g from south locations was 0.27±0.02, 0.36±0.02, 0.47± 0.06 and 0.63± 0.0 5mg/g of root sample in 3, 4, 5 and 6 year-old plants, respectively (Fig. 2). Paeoniflorin concentration was also increased with plant age, and the difference among locations was most apparent in 6-year-old plants, with higher concentrations in the north rather than south locations. Paeoniflorin concentration in *Paeonia ostii* roots from north locations was 0.18±0.09, 0.11±0.03, 0.21± 0.07 and 0.92± 0.1 mg/g, from middle location was 0.12±0.06, 0.21±0.05, 0.35± 0.03 and 0.66± 0.09 mg/g, from south location was 0.17±0.08, 0.31±0.03, 0.35± 0.06 and 0.40± 0.01 mg/g of root sample from 3, 4, 5 and 6 year old plants, respectively (Fig. 2).

Effect of soil factors: There were various extents of correlations among paeonol and paeoniflorin concentrations and measured soil attributes (Fig. 3). Across all plant ages and locations, paeonol concentrations were significantly positively correlated to soil pH, OM, Ca^{2+}, and Mg^{2+}. Paeonol concentration was significantly negatively correlated with soil pH, N, K, S, B, Cu, Fe, Mn, and Zn. While paeoniflorin concentration had significant and weak positive correlation with OM, pH, and N. Paeoniflorin concentrations showed a significant negative correlation to chlorine in the soil.

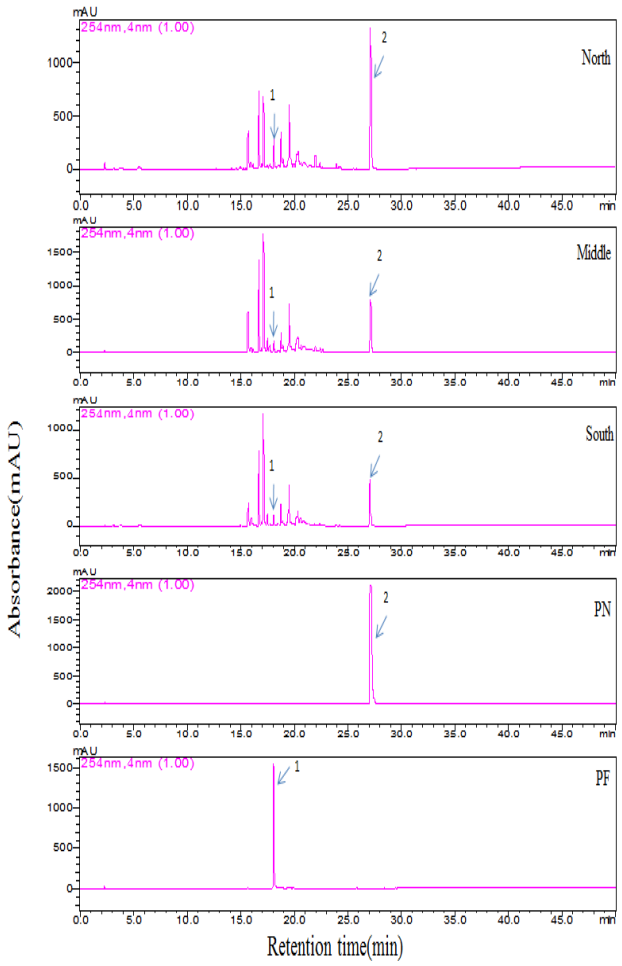

Fig. 1. UFLC chromatogram of root sample of different locations. In this figure PF is chromatogram of paeoniflorin standard sample, PN shows chromatogram of paeonol standard sample, peak 1 is for paeoniflorin, and peak two (2) is for paeonol.

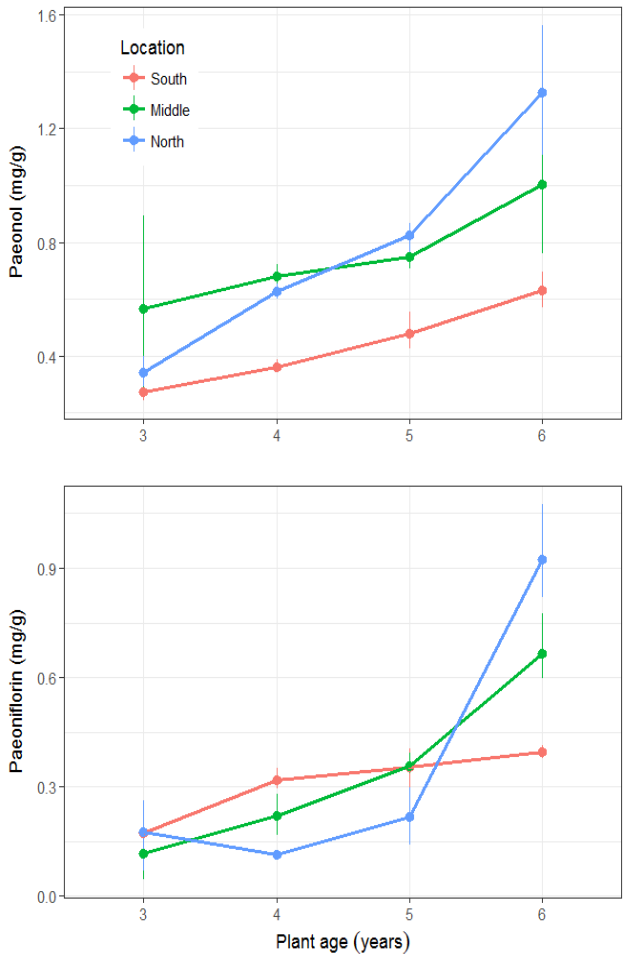

Fig. 2. Paeonol and paeoniflorin concentrations of *P. ostii* roots in relation to plant age and growing locations. South, Middle and North; production areas at south, middle and north latitude of Anhui province, China. All values are shown in mean ± SEM and were compared at $p<0.01$ sigificant level.

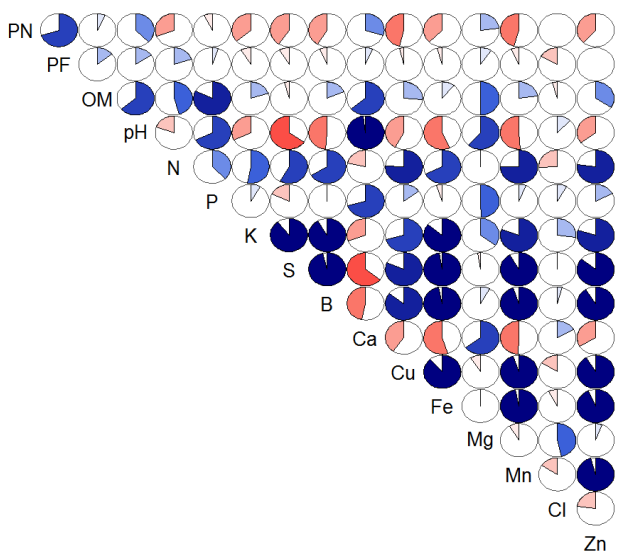

Fig. 3. Correlation between soil Factors, paeonol and paeoniflorin. Blue and red color show the positive and negative correlation respectively. The strength of each color represents the strength of correlation ($p<0.05$). In this figure PN stands for paeonol, PF stands for paeoniflorin, OM is total organic matter contents of soil, pH value represents soil pH value.

Discussion

Various plant species have adjusted to the distinct environments by adopting different strategies, with the production of surplus secondary metabolites being one of the best survival strategies. These secondary plant metabolites are a defensive system of plants which often use these secondary metabolites against environmental stresses (Seigler, 1998). Secondary metabolites concentrations strongly rely on the plants growing conditions and affect the metabolic pathways of related secondary metabolites. Hence, under different growing conditions, accumulation level of active compounds in medicinal plants is also different (Liu *et al*., 2015; Li *et al., 2018*). In this study, concentrations of paeonol and paeoniflorin showed substantial effects of growing locations (Fig. 2). Generally, as moving from south to north latitude, contents of both secondary metabolites increase. Our results are in consistent with Lätti *et al.*, (2008), where they found that bilberry populations growing on northern latitude had high anthocyanin level are compared to the bilberry of southern latitude.

The production of secondary metabolites is often low (not more than 1% of plant dry weight) and depend

greatly on the age of the plant (Rao & Ravishankar, 2002). Phenolic compounds and antioxidants activity rise in mature plants that enhance its abilities to provide energy for secondary metabolism and to strengthen its defensive system. In this study, paeonol contents increased with increasing age of plants on every location (Fig. 2). In north location, from 3rd to 4th year of age paeoniflorin contents of *Paeonia ostii* were decreased (Zha *et al*., 2012), during the fifth year it began to increase again and in the sixth year there was massive increase in its concentration but in southern and middle location paeoniflorin contents of *Paeonia ostii* increased gradually with the increasing age (Fig. 2). Previous studies also revealed that plant age might affect the concentrations of secondary metabolites (Jian *et al*., 2010). For example, the structural outcome of glucosinolate activation is regulated by plant age and other factors in *Arabidopsis thaliana* (Wentzell & Kliebenstein 2008). Achakzai *et al*., (2009) reported that levels of alkaloids, saponins, tannins and total phenolic contents were changed with the age of different medicinal plants. Achakzai *et al.,* (2017) also showed that cumulatively there was an increased level of 29.25% of polyphenols (*viz.,* flavonoids, phenolics, and tannins) in reproductive as compared to the respective foliar stage of eight common plant species of Asteraceae. In our study, collectively, the contents of paeonol and paeoniflorin were increased with increasing plant age (Fig. 2).

Soil fertility effects active ingredient contents of the plants. Excessive soil salts result in cellular dehydration that leads to water removal from the plant cells resulting in a reduction of the vacuolar volumes and cytosol. This condition creates both osmotic as well as ionic stress in plants and results in increase or decrease of their secondary metabolites (Mahajan & Tuteja, 2005). For example, in *Panax quinquefolius*, saponin contents were enhanced by the repetitive use of organic fertilizer (Liu, 1995). In our study paeoniflorin, contents and soil organic matter show a positive correlation (p<0.05, Fig. 3) which is in agreement with a study conducted by Liu *et al*., (2015). They demonstrated that organic matter content of soil correlated positively with the secondary metabolites of *Sinopodophyllum hexandrum*. The polyphenol contents in leaves of young tomato plants growing in greenhouse increased significantly in response to less availability of Nitrogen (Dumas *et al*., 1993) as did the paeonol in this study (Fig. 3). The inorganic elements including K, S, B, Cu, Ca^{2+}, Mg^{2+} Fe, Mn, Cl and Zn of the soil affect secondary metabolites of *Paeonia ostii.* These results are also in line with the results obtained by Guo *et al*., (2013), whereby they proved that inorganic soil elements were significantly correlated to secondary metabolites of *S. baicalensis.* Soil pH value was positively correlated to paeonol, and paeoniflorin contents agreed with the research of Liu *et al*., (2015).

Our study further reveals that many soil factors weakly correlate to active secondary metabolites contents (Fig. 3). We found that in three to six-years old plant the concentration of paeonol and paeoniflorin was still increasing. Hence further research is needed to find out the ideal age of plants containing ultimate levels of paeonol and paeoniflorin concentration. Roots of *Paeonia ostii* taken from north location contained the highest amount of paeonol and paeoniflorin content as compared to middle and south location respectively. Hence, a north location is favourable for the production of good quality *Paeonia ostii* cortex. Moreover, though this study relates to *Paeonia ostii* only, this procedure can be applied broadly to quality assessment and production of good quality medicinal and edible plants.

Conclusions

The geographical locations significantly affected the paeonol and paeoniflorin production in the roots of *Paeonia ostii*. Soil factors like pH value, Ca^{2+} and Mg^{2+} may enhance paeonol and paeoniflorin biosynthesis in *Paeonia ostii* roots. Integrating the results of production locations and plant ages, it was concluded that paeonol and paeoniflorin production increased from south to north growing locations and 6-year-old plants have the highest concentration of paeonol and paeoniflorin in the roots as compared to the younger plants.

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