INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
13–1410/2014/16–4–819–824
http://www.fspublishers.org

Full Length Article

Changes in Leaf Phenolics Concentrations Determine the Survival of Evening Primrose (*Oenothera biensis*) in Various Seasons

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Abstract

Plants are important source of medicines and major resources of healthcare since ancient times. Of these evening primrose (*Oenothera biensis* L.) is used in the treatment of a number of diseases due to its distinct antioxidative and medicinal properties. The studies pertaining to seasonal patterns of some secondary metabolites accumulation in leaves of different ages are not reported thus far. In this research, evening primrose was investigated for changes in the levels of secondary products such as alkaloids, saponins, tannins, phenolics and flavonoids. Results revealed that secondary metabolites accumulation in evening primrose substantially increased in summer season as compared to other seasons. All metabolites i.e., alkaloids, saponins, tannins, phenolics, flavonoids increased in summer season except anthocyanin, which decreased. A comparison of leaves of various ages indicated that the accumulation of secondary metabolites was more prominent in penultimate and middle leaves as compared to old bottom leaves. Results showed that for the leaves of three ages, seasonal changes were the most important to change evening primrose metabolism These changes switch the plant metabolic phenomena in a way that survival of young growing parts is ensured under harsh environmental conditions. Of the studied metabolites, contribution of phenolics was more explicit than the alkaloids and saponins. © 2014 Friends Science Publishers

Keywords: Phenolics; Summer season; Survival; Evening primrose; Metabolites adjustments

Introduction

Plants synthesize many aromatic and non-aromatic substances, referred to as secondary metabolites, which are used as defense arsenal in plants against predation by insects, microorganisms and herbivores (Hartmann, 2004). According to an estimate, there are 2619 different phytochemicals isolated from medicinal plants (Kennedy and Wightman, 2011). These are also involved in plant odor, flavor and pigmentation such as terpenoids (odor), capsscin (flavor) and tannins (pigmentation). The most important use of these compounds is that they exhibit different medicinal properties (Cowan, 1999). More important phytochemicals are alkaloids, tannins, glycosides, flavonoids, saponins, terpenes and other polysaccharides (Okwu, 2004). These phytochemicals are taken as fresh plant products such as fruits, vegetables or in the form of extract, powder or pills. These phytochemicals are also used in the preparations of different drugs (Sofowora, 1993).

Seasonal variation is a changing pattern around a trend line during the specific time period such as in one year or less. The important environmental factors are light, temperature and water that change with season. They produce various kinds of damages in plant, which range from structural to metabolic adjustments, and ultimately affect the plant performance. Pakistan has distinct four seasons i.e., winter (Nov–Jan), spring (Feb–Apr), summer (May–Jul) and autumn (Aug–Oct). The land plants growing solitary in nature display tolerance to ambient conditions by means of various metabolic adjustments. These metabolic adjustments may be due to the synthesis of primary or secondary metabolites. Furthermore, the responses of leaves of various ages may also be varying.

Secondary metabolites are not essential for growth but are beneficial for defense against biotic and abiotic stresses in plants. These are produced from the intermediates of primary carbon metabolism such as shikmate, malonate or mevalonte pathways (Taiz and Zeiger, 2010). They play protective role under abiotic and biotic stresses (Wahid and Ghazanfar, 2006; Wahid, 2007). Evening primrose (*Oenothera biensis* L.) is an industrially and medicinally important plant. It survives throughout the year, but the basis of the success of its survival especially during extreme climatic conditions has not been reported yet. It is hypothesized that metabolites adjustment in leaves of various ages is a possible mechanism whereby the evening primrose can survive under adverse seasonal conditions. The objectives of this research were to investigate the pattern of some secondary metabolites synthesis in the leaves of three ages and to determine their possible relationship with the changing seasonal conditions over monthly intervals.

To cite this paper: Fardus, S., A. Wahid, F. Javed and B. Sadia. 2014. Changes in leaf phenolics concentrations determine the survival of evening primrose (*Oenothera biensis*) in various seasons. *Int. J. Agric. Biol.,* 16: 819–824

Materials and Methods

A selected local population of evening primrose (*Oenothera biensis* L.) maintained for the last eight years was used to find out the changes in primary and secondary metabolites in leaves of three ages (penultimate, middle and bottom) consecutively for two years (2010 and 2011). For analysis, leaves of three ages/positions; penultimate (second fully expanded second leaf from the top), middle (leaf from middle) and bottom (green leaf from the bottom) were sampled in triplicate. At each sampling time, half samples were deep frozen at -30ºC for fresh analysis, while the other half were oven dried.

For the determination of soluble phenolics, anthocyanins, flavonoids, the frozen fresh material was used. For the estimation of alkaloids, saponins and tannins, powdered dry material was defatted following the method of AOAC (1990). Plant sample (3 g) was mixed with 30 mL hexane. This mixture was left on a shaker at 100 rpm for 24 h. Then this mixture was centrifuged at 3500×*g* for 18 min. The residue was again mixed with hexane followed by centrifugation and allowed to stand for 24 h; centrifuged and separated the supernatant and residue. This residue was oven dried and used as fat free sample for further analysis.

Alkaloids were determined by using the method of Harborne (1973). Fat free sample (0.5 g) was added to 20 mL of 10% acetic acid (in ethanol; v/v) in 250 mL beaker. The test tubes were covered and allowed to stand for 4 h. The mixture was filtered and filtrate was heated on a water bath at 90^{o}C till one quarter of the original volume of extract was obtained. After heating, concentrated NH_4OH was added drop-wise until the formation of precipitates, which were collected, washed with NH_4OH and re-filtered. The residue was oven dried and weighed. The total saponin contents were evaluated according to the method of Chapagain and Wiesman (2005). Fat free sample (0.1 g) was taken after drying for 24 h and added 30 mL of methanol. The mixture was shaken on a shaker at 100 rpm for two days and centrifuged followed by three consecutive extractions using methanol. The solvent was evaporated and a yellowish crystalline residue was obtained, which was carefully weighed and saponins were estimated.

The extraction of tannins was carried out by following the procedure of Van-Burden and Robinson (1981) with some modifications. Fat free sample was mixed with 50 mL distilled water. This mixture was shaken for 1 h at 100 rpm, filtered and volume made up to 50 mL. Filtrate (5 mL) was taken and mixed with 0.1 M $FeCl_3$ (in 0.1 N HCl) and 0.008 M potassium ferrocyanide. Absorbance of the mixture was taken at 605 nm. For construction of standard curve, 10, 20, 30, 40 and 50 µg/mL concentrations of tannic acid were used and run along with the unknown samples. Total soluble phenolics were evaluated by using the method of Julkenen-Titto (1985). Fresh plant material (0.5 g) was extracted in 80% acetone; extract centrifuged at 12,000 and supernatant collected. A 100 µL of the extract was mixed with 0.5 mL of Folin-Ciocalteu's phenol reagent and 2.5 mL of 20% Na_2CO_3. The volume of mixture was made up to 5 mL and vortexed. Absorbance of the reaction mixture was noted at 750 nm.

Flavonoids contents were determined by the method of Zhishen *et al.* (1999). Plant material (0.1 g) was extracted in 80% acetone and 1 mL of extract was added in a 10 mL volumetric flask containing 4 mL of distilled water. The reaction mixture was added with 0.6 mL of 5% $NaNO_2$, 0.5 mL of 10% $AlCl_3$ after 5 min, and 2 mL of 1 M NaOH after 1 min. The reaction mixture was diluted with 2.4 mL of distilled water and mixed. The absorbance was taken at 510 nm. The quercetin was used as a standard for the calibration curve. Anthocyanins were determined with the method of Stark and Wray (1989). For this, fresh leaf material (0.1 g) was extracted in 1 mL acidified methanol (1% HCl; v/v). The mixture was heated at 50^{o}C for 1 h and extract was filtered. The absorbance of the filtrate was measured at 535 nm by using a spectrophotometer, and changes in the anthocyanins were expressed as λ_{535} of the extract.

Weather data was obtained from the Agro-Meteorological Observatory, Department of Crop Physiology, University of Agriculture Faisalabad, Pakistan. The design of the experiments was factorial randomized complete block, with three replications for all parameters. Computer software Statistix (v. 8.1) was used to perform analysis of variance (ANOVA) and ascertain the differences between different factors and their interactions. The means were separated by putting letter on the data points. The correlations of different metabolites and environmental variables were drawn using the above software.

Results

Averaged meteorological data for the years 2010 and 2011 showed that respective average minimum and maximum temperatures were 4 and 16^{o}C in January, which increased to a minimum and maximum of 26 and 41^{o}C in May and June (Fig. 1). These temperature ranges decline from July onwards and in December, the minimum and maximum temperatures decreased to 4 and 21^{o}C, respectively. In both the years, a highest average relative humidity was recorded in the rainy months of August and September (~75%) and in cool and humid months of January and February (73%) while it was the lowest in driest month of the year i.e., May (43%). The precipitation (rainfall) pattern was very erratic, being the maximum in September, July and August (155, 118 and 93 mm, respectively) of both the years but with great variations (as evident from the error bars). On the other hand, months of January, November and December in both the years did not experience any rain. Changes in the patterns of evapotranspiration were closely related to seasonal changes in minimum and maximum temperatures. This variable was higher (~6 mm) in hot and dry months (May and June) while lower (1.2 mm) in cool and humid months (January and February).

Table 1: Analyses of variance (mean squares) for seasonal variation on secondary metabolites synthesis in evening primrose leaves

SOV	df	Alkaloids	Saponins	Tannins	Anthocyanin	Phenolics	Flavonoids
Block (B)	2	26.40	0.24	6.54	0.02	8.71	0.09
Leaf (L)	2	7135.24**	327.04**	2489.94**	6.10**	2016.98**	85.35**
Month (M)	11	675.53**	15.98**	70.81**	0.40**	98.46**	8.92**
L × M	22	368.86**	3.08**	8.79ns	0.26**	15.25**	1.90**
Error	70	14.12	0.39	5.56	0.01	8.31	0.47

Significant at: ns, non-significant; * and **, significant at P<0.05 and P<0.01 levels

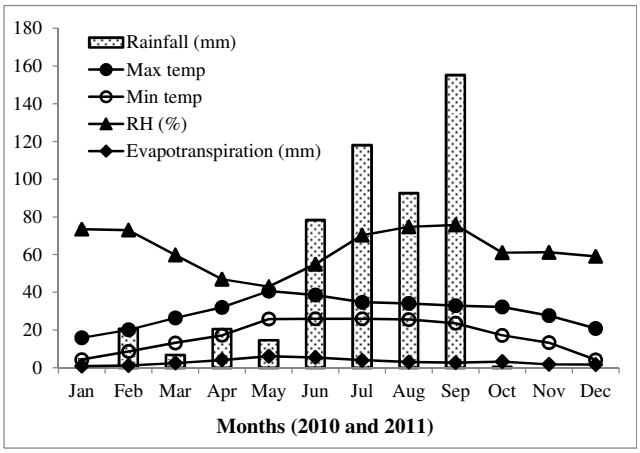

Fig. 1: Average monthly data of meteorological conditions during the year 2010 and 2011

Statistical analysis of data revealed that there were significant differences in the leaves of different ages and sampling months for various secondary metabolites. Moreover, there were significant interactions of leaves and months except a non-significant interaction in case of tannins (Table 1).

For all secondary metabolites the responses of different leaves were different. Middle leaf showed the highest alkaloids production followed by penultimate leaf. A higher alkaloids synthesis was observed during August–September (autumn season) months while their content during other months was low and not much different from each other in penultimate leaf. Middle leaf indicated most of alkaloids accumulation in July and August followed by June and January. Bottom leaf showed a lowest contents but accumulation was greater during summer months (Fig. 2). The trend of saponins synthesis in leaves of different age/position was different. Penultimate and middle leaves indicated increased sponins during December–January (winter months), declined gradually up to May and then increased during June–August and declined thereafter, although there were differences in their contents in the leaves. Bottom leaf showed the greater changes in the saponins synthesis although their pattern of accumulation remained essentially similar as noted for penultimate and middle leaves (Fig. 3).

The tannins synthesis was the lowest in penultimate leaf followed by middle leaf but was the highest in bottom leaf. As regards their accumulation patterns in different seasons, penultimate leaf exhibited some tannins accumulation in the months of May and June, which declined afterwards. Middle leaf showed some accumulation in winter season (January) but much more in summer (May–July), which decreased in the following seasons. Bottom leaf showed much higher tannins synthesis, which was the highest in summer season (May–July) followed by winter season but was the lowest in autumn season (Fig. 4). Penultimate and middle leaves showed greater synthesis of anthocyanins while the bottom leaf indicated the lowest synthesis. As far as seasons were concerned, the anthocyanins accumulation was the lowest in winter and summer months while spring and autumn seasons indicated their highest accumulation in penultimate as well as middle leaves. However, bottom leaf showed the least synthesis and variation in anthocyanins accumulation in all the seasons (Fig. 5).

A comparison of leaves showed that trend of soluble phenolics accumulation was similar in penultimate and middle leaves across the seasons, while their accumulation in bottom (aged) leaf was higher and variable across the seasons. A comparison of months indicated that soluble phenolics accumulation was generally lower in spring and autumn months while it was greater in winter and summer months (Fig. 6). The flavonoids accumulation was the highest in penultimate leaf followed by bottom leaf across the seasons. As regards sampling months (seasons), the flavonoids accumulation was higher in summer months (June–July) than winter months, while spring and autumn seasons indicated their lowered accumulation (Fig. 7).

Discussion

Secondary metabolites are not essential to the plant growth but are beneficial for plants. Plant produces these substances to protect itself from different diseases, as well as some of these compounds help in the pollination (Harborne, 2001). Determined here were metabolites from two classes; nitrogen containing (alkaloids, and saponins) and non-nitrogen containing (tannins, phenolics, flavonoids and anthocyanins) secondary products (Hussain *et al.,* 2012). The changes in the levels of these metabolites revealed that anthocyanins showed accumulation in spring and autumn seasons, while rest displayed accumulation mainly in the

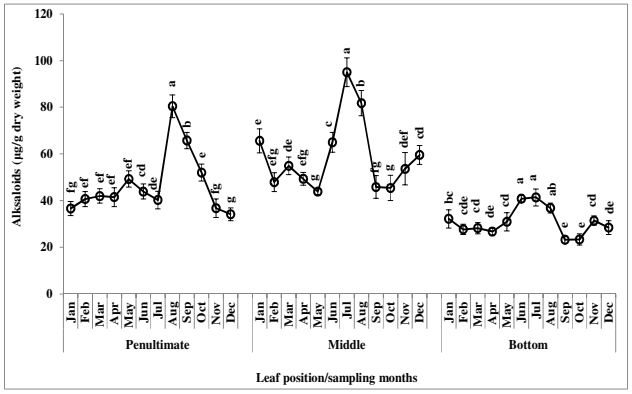

Fig. 2: Influence of seasonal condition on alkaloids contents in the penultimate, middle and bottom leaves of evening primrose. The vertical bars are standard deviations of three replicates.

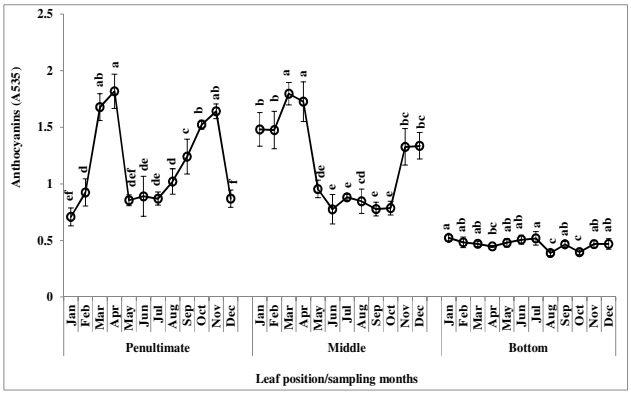

Fig. 5: Influence of seasonal condition on anthocyanins contents in the penultimate, middle and bottom leaves of evening primrose. The vertical bars are standard deviations of three replicates.

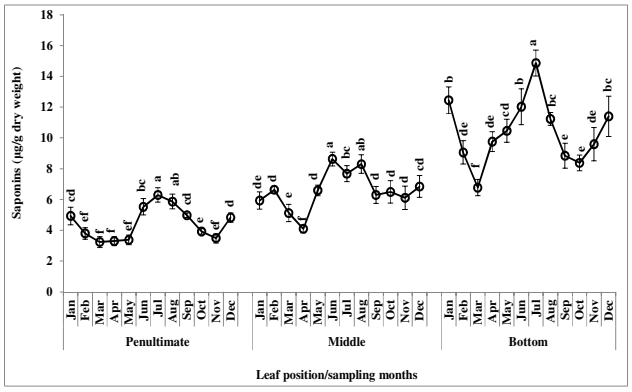

Fig. 3: Influence of seasonal condition on saponin contents in the penultimate, middle and bottom leaves of evening primrose. The vertical bars are standard deviations of three replicates.

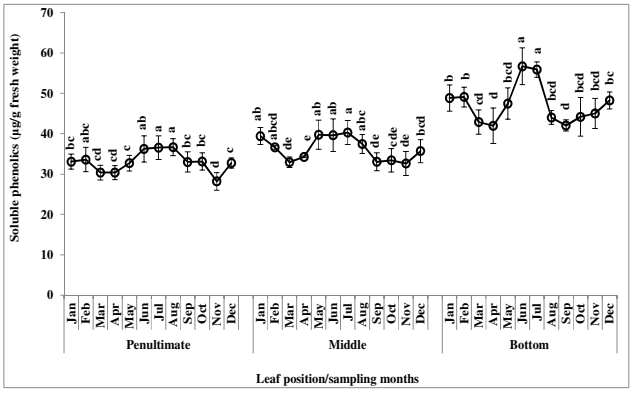

Fig. 6: Influence of seasonal condition on soluble phenolics in the penultimate, middle and bottom leaves of evening primrose. The vertical bars are standard deviations of three replicates

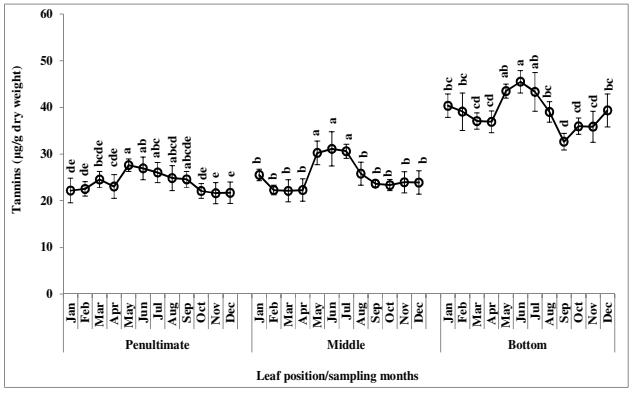

Fig. 4: Influence of seasonal condition on tannin contents in the penultimate, middle and bottom leaves of evening primrose. The vertical bars are standard deviations of three replicates

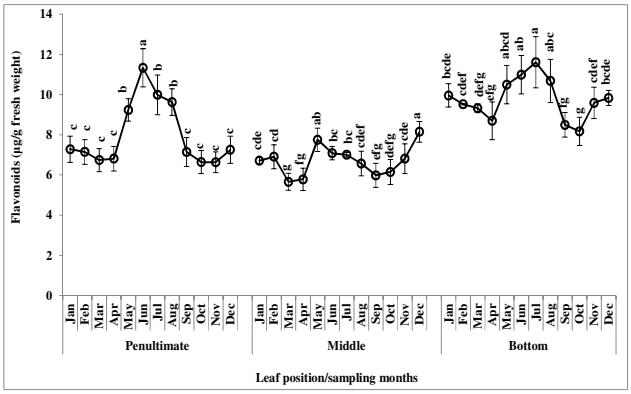

Fig. 7: Influence of seasonal condition on flavonoids contents in the penultimate, middle and bottom leaves of evening primrose. The vertical bars are standard deviations of three replicates

summer season in all leaves. However, differences were notable in their levels in leaves of various ages/position; bottom leaf indicated their higher accumulation except alkaloids and anthocyanins. Although a number of studies show a positive relationship of anthocyanins synthesis with abiotic stress tolerance in plants (Chalker-Scott, 1999; Wahid and Ghazanfar, 2006; Wahid *et al*., 2007), we speculate that such a response in evening primrose appears

to be due to sensitivity of naringenin oxidase enzyme to convert naringenin into anthocyanidins (Buchanan *et al.*, 2000), which is further related to age and stage of leaf growth.

Among the classes of compounds non-nitrogen containing secondary metabolites appeared to have a major role in the plant survival than the nitrogen containing metabolites. This is plausible because phenolics have aromatic ring, which is important in the dissipation of excess of solar energy falling on the plant surface. Secondly, the soluble phenolics, flavonoids, and anthocyanins are water soluble and provide tolerance against abiotic stresses (Rivero *et al.,* 2004; Wahid and Tariq, 2008; Fini *et al.,* 2011). However, tannins being more complex molecules provide structural strength to the plant thereby playing indirect role in tolerance to sub- and supra-optimal conditions (Escaray *et al.,* 2007). The synthesis of most of these compounds was greater in the bottom leaf, which indicated that they were synthesized more in the aged tissues plausibly for storage purpose. The alkaloid and anthocyanins production was the lowest in the bottom leaf, which indicated that they both might have more physiological roles in the actively metabolizing tissues. To explore the possible interactive roles of these metabolites, their interrelationships were established (Table 2).

The changing environmental conditions are pivotal in determining the physiological phenomena in the plant survival. The correlation of environmental data with the secondary metabolites revealed that tannins was correlated with maximum temperature, minimum temperature and ET in penultimate (0.780**, 0.819**, 0.821** respectively) and middle leaves (0.597*, 0.620*, 0.686* respectively) while flavonoids was correlated with maximum temperature (0.615*), minimum temperature (0.686*) and ET (0.659*) in penultimate leaf. Alkaloids and saponins and phenolics was correlated with RH (0.586*, 0.695*, 0.596*) in penultimate leaf. These results suggested that secondary metabolites of phenolic in nature were more responsive to the changes in the seasonal conditions in penultimate leaf, while these tendencies were not well marked in middle leaf and not seen in the aged (bottom) leaf. This further substantiated that since most of the phenolics studies here are water-soluble, so they are likely to have antioxidative roles in tolerance to adverse conditions such as temperature extremes and excessive water loss in summer season.

Table 2: Mutual correlations coefficients (*r*) of secondary metabolites with the changing seasonal conditions (n = 12)

X Variable	Y variable	Penultimate	Middle	Bottom
Alkaloids	Saponins	0.328ns	0.565ns	0.780**
	Tannins	0.320ns	0.502ns	0.791**
	Phenolics	0.425ns	0.552ns	0.772**
	Flavonoids	0.249ns	0.171ns	0.942**
	Anthocyanins	-0.008ns	-0.247ns	0.398ns
Saponins	Tannins	0.304ns	0.673*	0.686*
	Phenolics	0.840**	0.713*	0.754**
	Flavonoids	0.662*	0.506ns	0.790**
	Anthocyanins	-0.618*	-0.686*	0.477ns
Tannins	Phenolics	0.517ns	0.819**	0.850**
	Flavonoids	0.804**	0.518ns	0.852**
	Anthocyanins	-0.324ns	-0.618*	0.510ns
Phenolics	Flavonoids	0.783**	0.627*	0.778**
	Anthocyanins	-0.697*	-0.413ns	0.665*
Flavonoids	Anthocyanins	-0.576*	-0.264ns	0.462ns

Significant at: ns, non-significant; * and **, significant at P<0.05 and P<0.01 levels

In conclusion, the pattern of secondary metabolites synthesis were well evident with the changing seasons. The alkaloids and saponins appear to have no significant role in the tolerance of evening primrose leaves to seasonal changes. However, most of the soluble phenolic compounds were effective in the adjustment of evening primrose leaves to prevailing climatic conditions which appears to be due to their established role in averting the oxidative damage on cells and tissues.

Acknowledgements

The authors pay tribute to Higher Education Commission of Pakistan, Islamabad for the awards of scholarship to first authors under 5000 Indigenous Ph.D. scheme, and Chairman, Department of Crop Physiology for provision of weather data.

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(Received 06 December 2013; Accepted 15 January 2014)