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Research Article

CYANOGENESIS AND β- GLUCOSIDASE ACTIVITY IN *MADHUCA LONGIFOLIA, EMBLICA OFFICINALIS* AND *DIOSPYROS MELANOXYLON*

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ABSTRACT
Dependence on natural plant species for dietary supplement has greatly increased in tropical regions. To describe the safety of plant products, present study was conducted to analyze the level of cyanogenesis (HCN production) and its regulation in *Madhuca longifolia*, *Emblica officinalis* and *Diospyros melanoxylan* commonly utilized as both food and folk medicines. Cyanogenic activity was significantly high in young stages of plants and plant parts whereas, at maturity cyanogenic activity recedes considerably. *D. melanoxylan* contained highest amount of cyanogenic compounds (62.35 mg HCN Kg^{-1} f.w.) among the highest ever reported for mature leaves. Significant variation in cyanogens content was also observed in different parts of the same plant. β-glucosidase that releases corresponding cyanohydrins exhibit its higher activity in young developing fruits in comparison to matured fruits and highest activity was observed in young fruits of *E. officinalis* (78.46mg-p-nitrophenol $hr^{-1}g^{-1}$ f.w.). Production of cyanogenic compounds depends on cellular pool of free amino acids and was positively correlated with the production of toxic amount of hydrocyanic acid (HCN) in the above plant species. Present reports show the higher amount of cyanogens (HCN) in *M. longifolia*, *E. officinalis* and *D. melanoxylan* at younger stages of plant and plant parts. Level of cyanogens may be reduced at maturity by the developmental losses or by the unavailability of free amino acids for the synthesis of cyanogenic glycosides. Evaluation of cyanogenic level in natural edible plants is necessary before it consumption as food, fodder and medicine.

***Keywords*:** *Amino Acids Content, β- Glucosidase, Cyanogenesis, Cyanogenic Glycosides, Ethnic People, Natural Plant Communities*

INTRODUCTION
Despite the progress in the agricultural sector in tropical countries, majority of tribes continue to procure their sustenance on the forest and its minor products. They evolved a way of life, which could be understood in context with nature-man-spirit complex (Vidyarthi, 1996). Since, the plants are everything to them in their day to day struggles, presence of biologically active nutrients, cyanogenic compounds and other secondary metabolites in natural resources are of great importance. (Miller *et al.*, 2006; Singh, *et al.*, 2007) The administration of various naturally occurring biological compounds has been reported to respond differently to human physiology and metabolism (Dhar *et al.*, 1968; Herberman, 1986). Some toxic compounds like aglycon of cyanogenic glycosides present in plants are public health hazard. This cyanogenic ability of plants has attracted the attention of scientists for nearly two centuries because the hydrocyanic acid (HCN) produced by them is a toxic substance and accounts for numerous cases of acute and chronic cyanide poisoning of animals including man (Poulton, 1990; Schappert and Shore, 2000). HCN is found in the form of cyanogenic glycosides, which are mostly o-β- derivatives of α-hydroxynitriles depending on their precursor amino acids. HCN production in higher plants results from the catabolism of cyanogenic glycosides through enzymic hydrolysis. (Poulton, 1989) In the intact plant, the enzyme and cyanogenic glycosides remain separate but upon tissues disruption, both come together and corresponding cyanohydrin is liberated due to cleaving of glycone moiety from the cyanogenic glycosides (Hosel W, 1981; Seigler, 2002). Intracellular localization and isolation of certain cyanogenic

specific β-glucosidases have been observed with significant activity in the plastids of mesophyll cells (Conn, 1981; Fan *et al.*, 1998). It is now established that the cyanogenic glucosides are present in the epidermal layer, whereas the enzymes are present in mesophyll cells. β-glucosidase present in mesophyll cells is an initial enzyme responsible for the separation of a glucone moiety from the cyanogenic compounds (Hosel, 1981). Despite a large body of phytochemicals, taxonomic and ecological work on cyanogenic species little is known of their frequency in natural plant communities. Screening of cyanogenic species and its biochemical analysis gives distribution pattern of cyanogenic compounds as well as toxicity level in plants (Miller *et al.*, 2006).

In view of the above mentioned reports and the importance of edibility of these three plant species, present study was undertaken to get a clear picture of level of the cyanogenesis and its regulation in natural plant species in tropical regions. Firstly, present communication describes the cyanogenic ability of *M. longifolia* family Sapotaceae, *E. officinalis* family Euphorbiaceae and *D. melanoxylon* family Ebenaceae. Secondly, it is aimed to compare the production of HCN in young plant parts with mature one in relation to free amino acids content observed in above plant species. β-glucosidase activity was also observed to know the developmental loss in cyanogenesis and its role in catabolism of cyanogenic glycosides.

MATERIALS AND METHODS

Field Sites: Tribal zone lies between 220.13-260.45' N latitude and 750.30'-780/45' E longitude. The study area comprises different forest ranges of central India. Six forests ranges namely Shivpuri, Pohari, Pichor, Guna, Ashok nagar and Ghantigaon were selected for screening and cyanogenic analysis in plants. These forest areas are predominantly inhabited by Sahariya tribes whose maximum dependence was found on forest plant species and their products.

Sample collection: Natural plant species of above mentioned sites were collected and an inventory of those plants has been recorded, which are mostly used by economically poor society especially as edible elements and medicinal values. Samples were collected in different seasons of the year. Leaves and fruits after being sampled from the field were segregated according to their sizes (young and old). Uniform sized samples of required stages were weighed prior to their transfer to paper sampling bags.

Hydrogen cyanide analysis: Hydrogen cyanide (HCN) was estimated following the alkaline picrate method of Willianms and Edwards (1980). For the estimation of HCN liberated from cyanogenic compounds of plant samples, 2 g of immature and mature (Commercial maturity) leaf and fruit material was homogenized in 10 ml of phosphate buffer (pH 7.5). The alkaline picrate strip was incubated at constant temperature of 30 $\pm$ 1˚C for 24 hours. Each strip was carefully removed and eluted three times with 2 ml of distilled water. Final volume was made to 10 ml and absorbance of reddish orange color was recorded at 510 nm in lambda - 3 Spectrophotometer (Perkin-Elmer).

β- Glucosidase assay: The method employed for the enzyme extraction and assay was as described by Ali et al. (1995) and modified to our standardization conditions. Fresh tissues of fruits weighing 10 g were homogenized in 20 ml of 0.1 M sodium citrate buffer (pH 4.6) containing 1 M NaCl, 0.5% (w/v) of soluble polyvinyl pyreldone (PVP) and 10 mM 2-mercaptoethanol. The homogenate was left for half an hour with an occasional stirring and the same was centrifuged at 15000g for 30 minutes and the supernatant recovered was used for the enzyme assay. The enzyme activity was calculated as the amount of p-nitro phenol released due to β- glucosidase activity on substrate and expressed as p- nitro phenol g^{-1}fresh wt. $hour^{-1}$.

Analytical methods: Estimation of total amino acids was done by the method of Sadasivam and Manikam (1992).

Statistical analyses: Every estimation was done with five replicates and the data, thus procured were subjected to statistical analyses using student "t" test of significance. "P" values were calculated at 0.5% level of significance.

RESULTS

Cyanogenic activity: The cyanogenic activity (expressed as the hydrogen cyanide evolved from the fresh tissues of leaves and fruits) was higher in young leaves than the mature leaves in *M. longifolia*, *E. officinalis* and *D. melanoxylon*. The activity was maximum in young leaves of *D. melanoxylon* (86.36mg kg^{-1} fresh weight) followed by *M. longifolia* and *E. officinalis.* These data are shown in Table 1. Similarly, fresh fruits of all the three plants produced more HCN when young than the mature ones and highest HCN production was recorded in the young fruits of *M. longifolia* (84.64 mg kg^{-1} fresh weight). The mature fruits of *Emblica* however, showed lowest activity (21.33mg kg^{-1} f w) of Cyanogenesis (these data are shown in Table 1). Further, to see the effect of drying on the reduction rate of hydrogen cyanide (HCN), data were analyzed on the dry weight basis as the HCN actually produced and also values directly computed. Interestingly, it was observed that the actual values showed some losses only in the young leaves of these three plants, when compared to the computed values of HCN activity. Whereas, mature leaves and fruits at both stages of development depicted an additive content of HCN production in actual than the computed values. The reduction in amount of HCN was as high as 19% in the young leaves of *D. melanoxylon* followed by young leaves of *M. longifolia* and *E. officinalis* (For further details see Table 2). Rest of the samples showed gain in the level of HCN production and was observe 21% in matured leaves of *Emblica.* In case of fruits, all the samples were found with gain in cyanogenic activity and the highest additive value was observed in the mature fruits of *Emblica* (16%). It is however; additive values were higher in mature fruits in comparison to young fruits (as shown in Table 2).

Total free amino acids: Total free amino acids content of tissues was analyzed on percent fresh weight basis in leaves and fruits sample of these natural plant species. Amino acids content was higher in young leaves than in the mature leaves of *M. longifolia*, *E. officinalis* and *D. melanoxylon.* Content of amino acids was found maximum (0.042% fresh weight) in young leaves of *D. melanoxylon*. Similarly, in the case of fruits highest content of amino acids was recorded in young fruits of *D. melanoxylon* (0.045% fresh weight), followed by *M. longifolia* (0.034%) *and E. officinalis* (0.032%). In any case, fruits samples have higher content of free amino acids in comparison to leaves (Table 3).

Figure : 1 Correlation between Prussic Acid and free Amino Acid content in leaves of *M. longifolia. E. officianalis* and *D. melanoxylon*

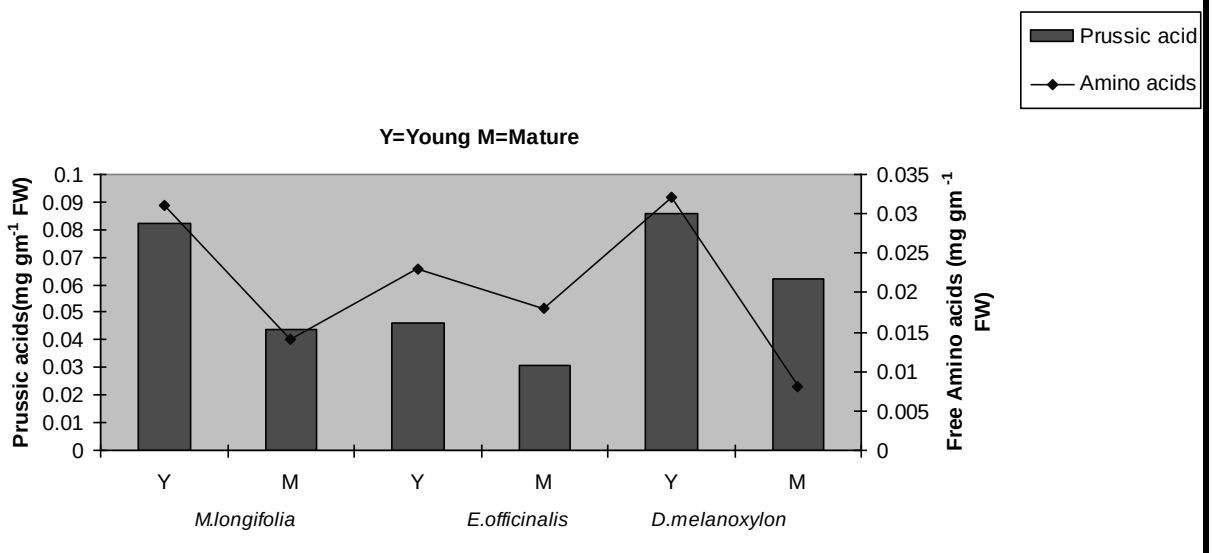

Figure : 2 Corrletaion of Prussic Acid and FreeAmino acid content in the fruits of *M.longifolia,E.officinalis* and *D.melanoxylon*

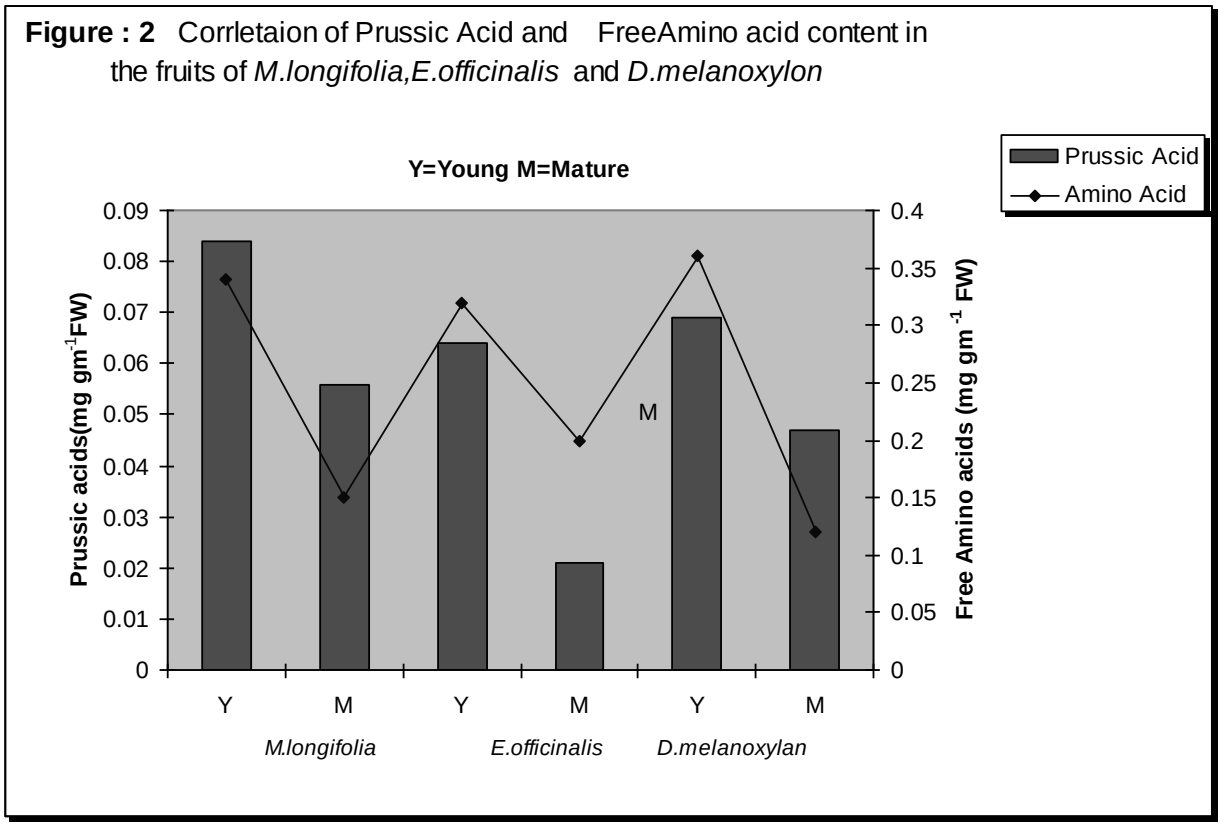

Table 1: Cyanogenic activity (mg HCN Kg^{-1} FW) in the young and mature leaves and fruits of *M. longifolia, E. officinalis* and *D. melanoxylan*

Plant	Leaf		Fruit	
	Y	**M**	**Y**	**M**
M.longifolia	**82.27(±5.22)**	**44.66(±4.74)**	**84.64(±8.56)**	**56.06(±5.21)**
E. officinalis	**46.63(±4.66)**	**31.46(±2.65)**	**64.66(±4.96)**	**21.33(±3.63)**
D. melanoxylon	**86.36(±7.16)**	**62.35(±7.13)**	**69.05(±5.14)**	**47.37(±5.17)**

Data are represented as means ± SE (n = 5)
Y = Young, M = Mature

Table 2: Percentage loss or addition of HCN production on dry matter basis from actual to computed values in the young and mature leaves and fruits of *M. longifolia, E. officinalis* and *D. melanoxylan*

Plant		Leaf			Fruit		
		Actual Value	Computed Value	Loss / Add (%)	Actual Value	Computed Value	Loss/ Add (%)
M. longifolia	**Y**	**26.30**	**29.30**	**-11.74**	**39.84**	**37.90**	**+04.96**
	M	**20.04**	**23.40**	**-04.65**	**44.67**	**40.71**	**+09.72**
E. officinalis	**Y**	**22.31**	**23.40**	**-04.65**	**44.67**	**40.71**	**+09.72**
	M	**15.66**	**12.90**	**+21.39**	**12.44**	**10.70**	**+16.26**
D.melanoxylon	**Y**	**18.23**	**22.55**	**-19.15**	**37.13**	**35.99**	**+04.76**
	M	**13.39**	**11.36**	**+17.86**	**13.27**	**11.64**	**+14.01**

Data are represented as means ± SE (n = 5)
Y = Young, M = Mature

Table 3: Content of free amino acids in leaves and fruits tissues as equivalent weight of leucine (Percent Fresh Weight).

Plant		Leaf	Fruit
M. longifolia	Y	0.031 ± 0.002	0.024 ± 0.002
	M	0.014 ± 0.001	0.015 ± 0.0007
E. officinalis	Y	0.023 ± 0.002	0.032 ± 0.004
	M	0.018 ± 0.001	0.020 ± 0.002
D. melanoxylon	Y	0.042 ± 0.001	0.045 ± 0.003
	M	0.008 ± 0.0003	0.012 ± 0.001

Data are represented as means SE ± (n = 5)
Y = Young, M = Mature

Table 4: β- **glucosidase activity in the young and matured fruits of *M. longifolia, E.officinalis* and *D. melanoxylon***

(Mg-p-nitrophenolhr^{-1}freshwt)

Sample	Young	Mature
M. longifolia	32.7 ± 4.37	21.43 ± 2.73
E. officinalis	78.46 ± 6.21	52.33 ± 5.78
D. melanoxylon	46.66 ± 4.33	24.42 ± 2.66

Data are represented as means ± SE (n = 5)
Y = Young, M = Mature

Correlation between cyanogenic activity and amino acids content: To study the effect of free amino acids content on the degree of cyanogenic activity, a positive correlation has been recorded between the production of prussic acid (HCN) and free amino acids content of plant tissues. Data were also demonstrated a concomitant increase in cyanogenic activity of younger leaves and fruits with higher amino acids content, which receded when amino acid content lowered during maturity (These data are shown in Figure1 and 2). Young leaves of *D. melanoxylon* observed with higher amino acids content showed highest cyanogenic activity, whereas in matured leaves, content of amino acids was sharply reduced with reduction in HCN production. Similar trend was visible in fruit samples, and highest cyanogenic activity was found in young fruits of *M. longifolia*. In contrast, the level of HCN (prussic acid) content appeared significantly low in *D. melanoxylon* and *E. officinalis*, which receded with the reduction in free amino acids content during maturity as shown in Figure 2.

β- Glucosidase activity: Tissues and organs of fruits of *M. longifolia*, *E. officinalis* and *D. melanoxylon* were analyzed at different stages to determine changes in enzymic activities during their development. The enzyme β-glucosidase activity (mg p-nitropehnol h^{-1} gm^{-1} fresh weight) was found to be always higher in young developing fruits in relation to mature and developed fruits. The maximum value of β-glucosidase activity was observed in young fruits of *E. officinalis* (78.46 mg gm^{-1} fresh weight) followed by *D. melanoxylon* and *M. longifolia*, while, lowest enzyme activity was recorded in the matured fruits of *M. longifolia* (21.43 mg gm^{-1} fresh weight). In any case with ripening, the enzyme activity of fruits extract receded significantly (see Table 4).

DISCUSSION

The production of free HCN (hydrocyanic acid) or prussic acid in the leaves and fruits of *M. longifolia*, *E. officinalis* and *D. melanoxylon* is not well documented. However, cyanogenic level in both immature and mature stages of plant and plant parts is well corroborated in other plants (Miller *et al.*, 2006; Poulton, 1990). In the present study, it has been observed that there is a concomitant increase in the cyanogenic activity of younger leaves and fruits with higher amino acids content. Whereas, the degree of cyanogenic activity decreased with maturity of the leaves and fruits, when the content of free amino acids lowered at maturity of plant parts. This possible correlation between cyanogenic activity and amino acids content is either due to the cyanogens (linamarrin or lataustralin) presents in these plant species or may be due to their subsequent synthesis from L-valine and L-isoleucine. Our current observation appears to support some earlier report on the biosynthetic mechanism of cynogenic glycosides (Conn, 1981; Selmer, 1988). It

seems that the cyanogenic glycosides are synthesized into the leaves and transported to the site of accumulation through linaustalin (another glycone of linaustralin) pathway (Du L *et al.*, 1995). This may be possible either by precursor-product relationship or by the balance between sink and source.

High level of cyanogenesis is reported to be due to enzymic hydrolysis of cyanogenic compounds because β-glucosidases were analyzed for rapidity of HCN evolution in plants (Selmer et al. 1989). Present investigation also supports the above contention pertaining to the promotive effects of β-glucosidase in relation to HCN (Hydrocyanic acid) production in these three plants. β-glucosidase may regulate the cleaving of glucone moiety from the cyanogenic glucosides, especially in young fruits where substrate and enzyme containing green tissues are maximum, thus they provide aglycone nitrile fractions for the further degradation and release of HCN from the cyanogonic nitriles (Poulton, 1990; Conn, 1981). Since, presence of cyanogonic activity was shown by the fruits as already discussed in this study, the β-glucosidase activity was estimated to ascertain its presence and also if, it had any association with the presence of cyanogonic activity especially in the fruits which are edible. When the data were analyzed on the basis of dry weight, the HCN actually produced (degree of toxicity) and values directly computed, showed low cyanogenic activity due to the drying effect. Similar report has been reported for cassava roots (Cooke & coursey 1981, Selmer et al. 1989). Although the experiments show consistency and reproducibility on three replications but this off routine observation is difficult to understand. A possible answer for this interaction between two factors (loss and gain of HCN) may be a failure of HCN detoxification or inactivation/degradation of enzyme responsible for the further conversion of cyanogenic glucosides to aspargnine used in further nitrogen metabolism (Conn, 1981; Poulton, 1990). The present report shows the presence of toxic compounds (cyanogenic) and reduction of its toxicity level in natural plant species used for the food and feed purposes. Young parts of plants have higher amount of HCN while, in matured parts cyanogenic activity was reduced significantly due to developmental loss. However, interaction between forest plant species and tribal population needs more biochemical analyses along these lines.

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