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DETECTION OF PHENYLALANINE AMMONIA - LYASE ACTIVITY IN DIFFERENT PLANT PARTS OF *ANACARDIUM OCCIDENTALE* L.

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ABSTRACT

Phenylalanine ammonia-lyase (PAL) performs a major role in the biosynthesis of polyphenolic compounds, which are involved in the defense mechanism in harsh environments related to different stimuli. The present study was carried out to detect the activity of phenylalanine ammonia-lyase in different plant parts of *Anacardium occidentale* such as flower, young leaves, shoot tip, mature leaves, raw cashew nut shell, shoot, root and cotyledons. The presence of anacardic acid, a phenolic compound in methanolic extract of young leaves of cashew seedling was also detected using high performance thin layer chromatography (HPTLC) method. The results showed that the highest activity rate of PAL was observed in cotyledon (0.611 ± 0.06 U/ml) and least amount in root (0.161 ± 0.02 U/ml).

KEYWORDS: ***Anacardium occidentale,*** **PAL activity, Anacardic acid, HPTLC.**

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INTRODUCTION

A large number of biologically important phenolic compounds are synthesized by phenylpropanoid pathway.[1] These compounds play important roles in the plant development, mechanical support and disease resistance.[2,3] Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) was first described in 1961 by Koukol and Conn.[4] This enzyme catalyzes the non-oxidative deamination of phenylalanine which results in the formation of *trans*-cinnamic acid and ammonia, it is the initial step in the biosynthesis of phenolic compounds.[5] Many reports highlight on the correlation between the increase in the corresponding PAL gene/protein expression/activity and an increase in the phenolic compounds in response to different external stimuli.[6] *A.occidentale* is a plantation crop belonging to the family Anacardiaceae. It is indigenous to Brazil; from where it spreads widely in the tropical countries from West to East Africa and India.[7] It is a multi-purpose woody plant.[8] Two most important parts in commercial uses are cashew nut for diet and liquid from nutshell (CNSL). Several groups of phytochemicals have been found in different parts of *A.occidentale*. The phenolic compounds comprising anacardic acid was mainly concentrated in nutshell though it is seen throughout the plant body.[9,10] Anacardic acid is a highly valuable natural phenolic lipid widely used in medicine and industry.[11] Anacardic acid has very much demand in the international market.[12] In the medical field, it has been favorably used for the treatment of warts, ringworms and even elephantiasis. The biological properties of anacardic acid include antiviral, antifungal, antibacterial, anti-inflammatory, antioxidant, anti-carcinogenic activities etc.[13,14,15,16] Industrially, it is used as a raw material for heat and water proof paints, brake lining compounds of automobiles, synthetic resins, corrosion resistant varnishes, insulating enamels for the electrical industry, different types of surface coatings and plastic industry.[17] Phenylpropanoid compounds are a forerunner to a wide range of phenolic compounds with a lot of functions in plants.[18] Therefore studying Cashew PAL, the key enzyme of the phenylpropanoid pathway, is helpful in breeding the plant for phenol especially anacardic acid. The most conspicuous phenolic lipids such as anacardic acid, cardol and 2-methylcardol are synthesized by going through the phenylpropanoid pathway in *Anacardium*. The activity of PAL has been determined from many plant species but not from *A.occidentale*. For these reasons, the present study was focused on its PAL. However, there is no investigation about the activity of PAL in different parts of *A. occidentale*. It is also aimed to carry out the detection of anacardic acid, a phenolic compound in young leaves of cashew seedlings by HPTLC analysis.

MATERIALS AND METHODS

Source of plant materials

Mature cashew (*A.occidentale*) seeds and flowers were procured from the mother stock tree grown at the Kerala State Cashew Development Corporation, Kollam, Kerala, India. Flower, cotyledonary tissue and raw cashew nut shell from mature cashew seeds were collected from the above described tree of Kerala State Cashew Development Corporation, Kollam. Young leaves, shoot tip, mature leaves, shoot and roots from seedlings germinated in plastic trays containing sterilized sand as substrate in laboratory situations. The present study material is identified and authenticated by the taxonomist of Department of Botany, Sree Narayana College, Kollam, Kerala, India and plant material is kept in the herbarium of Department of Botany, Sree Narayana College, Kollam. Voucher specimen: Kerala State, Kollam District, 07/09/2015, SIJA.S.L 026 (SNCH).

Preparation of plant extracts for PAL activity

One gram of each fresh sample was homogenized in 3 ml of ice-cold 0.1M trisodium borate buffer (pH 8.5) containing 1.4mM 2-mercaptoethanol and 0.1g of insoluble polyvinylpyrrolidone. The extract was filtered through cheesecloth and centrifugation was carried out at 16000 rpm for 15 min. The supernatant served as the enzyme source. [19]

Preparation of plant extracts for HPTLC

Young leaves of cashew seedlings were washed and air dried. The dried samples were ground to fine powder form. Powdered samples were extracted with methanol at a ratio of 1:100 (g: ml) at room temperature for 24 hours by the process of maceration. Supernatants were collected after centrifuging the homogenized samples at 10,000rpm for 15 min. They were transferred into evaporating dishes and dried at room temperature. The residue thus obtained was dissolved in methanol and stored at 4-8°C in a refrigerator for HPTLC analysis.

Enzymatic assay of phenylalanine ammonia-lyase

PAL activity was analyzed as the rate of conversion of L-phenylalanine into *trans*-cinnamic acid [(E)-cinnamic acid] at 270nm in a UV-Vis spectrophotometer.[19] Samples containing 0.1 ml of enzyme extract were treated with 0.5 ml of 0.1M trisodium borate buffer (pH 8.5) and 0.5 ml of 12 mM L-phenylalanine in the same buffer.The volume of reaction mixture was made up to 3ml with deionized H_2O and kept for incubation at 30°C for 30 minutes. Immediately mixed by inversion and recorded the increase in absorbance at 270nm for approximately 5 minutes. The samples were prepared in triplicate for each analysis and the mean value of the ΔA_{270nm}/minute was obtained using the maximum linear rate for both the Test and Blank. One unit will deaminate 1.0 µmole of L-phenylalanine to *trans*-cinnamate and ammonia per minute at of 30°C at pH 8.5. The amount of *trans*-cinnamic acid synthesized was calculated by using its extinction coefficient of 9630 M^{-1} cm^{-1}.[19] Enzymatic activity of samples was calculated using the following expression.

$$\text{Units/ml enzyme} = \frac{(\Delta A_{270nm}/\text{min Test} - \Delta A_{270nm}/\text{min Blank})\ (3)\ (df)}{(19.73)(0.1)}$$

Where ΔA is the change in absorbance per minute, 3 is the total volume (in milli liters) of assay, df is the dilution factor (1), 19.73 is the milli molar extinction coefficient[20] of trans-cinnamate at 270nm and 0.1 is the enzyme volume.

HPTLC detection of anacardic acid

HPTLC analysis was carried out on Camag HPTLC. Using Linomat V applicator (Hamilton, USA), methanol extracts were transferred on pre-coated silica gel plate $60F_{254}$ (Merk, Germany). The standard compound used was anacardic acid (Sigma-Aldrich) (100µg/ml).Twin trough chamber was used for separation using the solvent system as mobile phase [chloroform: ethyl acetate (9:1)] [25]. The bands were applied over the HPTLC plate with following settings: Band length 8 mm, the distance between track 15 mm, slit dimension - 6.00 mm × 0.45 mm × micro, scanning speed- 20 mm/s; data resolution- 100 µm/step. Qualitative analysis was carried out by TLC scanner 3 Camag HPTLC systems at wavelength 305 nm by comparing the peak area values of methanol extracts of the sample with that of the standard using the Wincats software.

Data analysis

Data were expressed as means ± standard deviation (SD) of triplicate determinations. All statistical analysis was carried out using an SPSS (Chicago, IL) statistical software package (SPSS for Windows, ver.20). To determine whether there were any differences among the means, one-way analysis (ANOVA) and the Duncan's New Multiple range test were applied to the result at 0.05 level of significance ($p < 0.05$).

RESULTS AND DISCUSSION

Estimation of Phenylalanine ammonia - lyase activity

In the present study, eight different extracts were explored for the enzymatic assay of Phenylalanine ammonia-lyase. The samples were processed in triplicate for each analysis and the mean value of the change in absorbance at 270nm/minute was attained using the maximum linear rate for both the Test and Blank (Table 1). Cotyledon segment showed 0.723 ± 0.04 change in absorbance at 270nm/minute whereas root showed 0.427 ± 0.01. Phenylalanine ammonia-lyase activity values were ranked as: cotyledon (0.611 ± 0.06 U/ml) > shoot tip (0.475 ± 0.04 U/ml) > flower (0.442 ± 0.09 U/ml) > young leaves (0.334 ± 0.06 U/ml) > shoot (0.332 ± 0.04 U/ml) > raw cashew nut shell (0.328 ± 0.08 U/ml) > mature leaves (0.173 ± 0.04U/ml) > root (0.161 ± 0.01 U/ml) (Fig.1). The results of the present study showed that PAL activity of cotyledon differs significantly ($p < 0.05$) from other plant parts (Fig.1). The results also revealed that cotyledon had the highest PAL activity (0.611 ± 0.06 U/ml) while the root showed the least PAL activity (0.161 ± 0.01 U/ml). The activity of PAL is highly sensitive to an environmental condition, as it played a major role in controlling the biosynthesis of polyphenolic compounds. A strong correlation was reported between PAL activity and phenolic compounds in leaves of hybrid maize under drought stress.[21] The suitability of a quantitative spectrophotometric determination for phenylalanine ammonia-lyase activity in Buckwheat, Barley and Pea seedlings were also investigated.[22]

Table 1
The mean value of ∆A at 270nm/minute of investigated plant extracts of A.occidentale.

Plant parts	ΔA/min (OD at 270 nm)
Blank	0.231 ± 0.002
Cotyledon	0.723 ± 0.04[d]
Shoot tip	0.634 ± 0.03[c]
Flower	0.612 ± 0.06[c]
Young leaves	0.541 ± 0.04[b]
Shoot	0.540 ± 0.03[b]
Raw cashew nut shell	0.537 ± 0.05[b]
Mature leaves	0.435 ± 0.03[a]
Root	0.427 ± 0.01[a]

For each treatment the means within the column by different letters are significantly different at p < 0.05. Each value is expressed as the means ± SD (n=3).

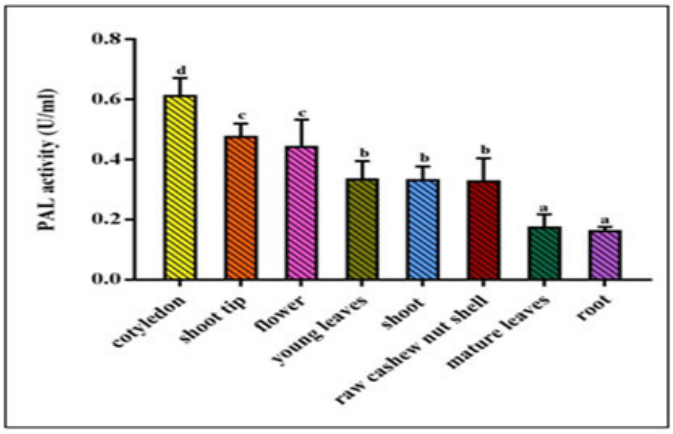

Figure 1
Phenylalanine ammonia-lyase activity (PAL) (U/ml) of different plant parts of A.occidentale; values with different letters are significantly different (p<0.05), (n=3, error bars represent standard deviation)

Detection of anacardic acid by HPTLC

Qualitative evaluation of anacardic acid was done by HPTLC using solvent systems. The mobile phase used was chloroform: ethyl acetate (9:1) (v/v). Aluminum-backed TLC plates pre-coated with 0.2mm layer of silica gel $60F_{254}$ (20 cm × 10 cm) was used as stationary phase. The present study revealed that young leaves from seedling showed the presence of anacardic acid. By winCATS software, regression analysis and statistical data were automatically generated. Calibration curve and HPTLC chromatogram of standard anacardic acid were represented in Fig.2 (a,b). HPTLC chromatogram of young leaves of *A.occidentale* was also obtained (Fig.2c). HPTLC has become a potential tool for identification, authentication and quality control of phytochemicals.[23,24] The presence of anacardic acid in cashew nut shell liquid, cashew nut and cashew fruit was also reported.[25,26,27,28]

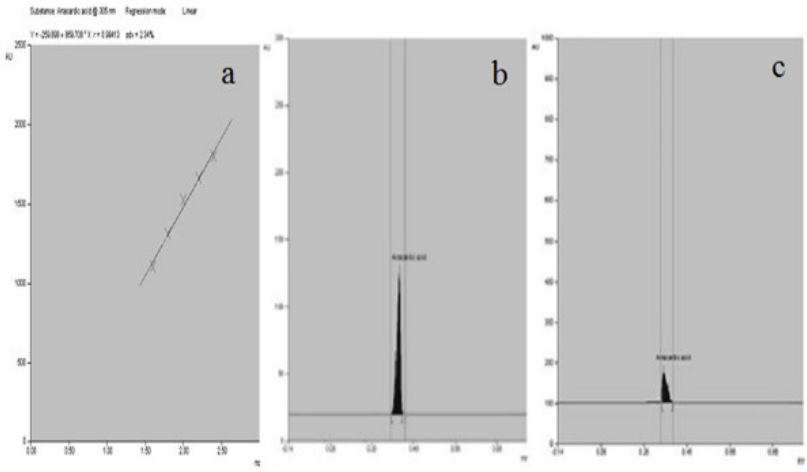

Figure 2
a) Calibration curve of standard anacardic acid; b) HPTLC chromatogram of standard anacardic acid; c)HPTLC chromatogram of young leaves

CONCLUSION

An enhancement in the activity of PAL can be treated as a biochemical marker and it provides defense mechanism in plants against environmental stresses, given that this enzyme is the key factor for the synthesis of phenolic compounds. The present investigation proved the activity of PAL in different plant parts of *A.occidentale* and also detected the presence of medicinally and industrially important polyphenolic compound, the anacardic acid in young leaves of cashew by HPTLC technique.

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CONFLICT OF INTEREST

Conflict of interest declared none.

REFERENCES

1. Liu R, Xu S, Li J, Hu Y, Lin Z. Expression profile of a PAL gene from *A.membranaceus* var. mongholicus and its crucial role in flux into flavonoid biosynthesis. Plant Cell Rep. 2006; 25: 705-710.
2. Chen AH, Chai YR, Li JN, Chen L. Molecular cloning of two genes encoding cinnamate 4-hydroxylase (C4H) from oilseed rape (*Brassica napus*). J. Biochem. Mol. Biol. 2007; 40: 247-260.
3. Harakava R. Genes encoding enzymes of the lignin biosynthesis pathway in *Eucalyptus*. Genet. Mol. Biol. 2005; 28: 601-607.
4. Mac Donald MJ, Cunha GB. A modern view of phenylalanine ammonia lyase. Biochem. Cell Biol. 2007; 85: 273-282.
5. Hahlbrock K, Scheel D. Physiology and molecular biology of phenyl-propanoid metabolism.Annu. Rev. Plant Physiol. Plant Mol. Biol. 1989; 400: 347-369.
6. Boudet AM. Evolution and current status of research in phenolic compounds. Phytochemistry. 2007; 68: 2722-2735.
7. Mitchell JD, Mori SA. The cashew and its relatives (*Anacardium*: Anacardiaceae). Mem. New York Bot. Gard. 1987; 42: 1-76.
8. Togun A. A review of the prospect of cashew industry. Cocoa Research Institute of Nigeria. 1977; 100-110.
9. Ojezele MO, Agunbiade S. Phytochemical constituents and medicinal properties of different extracts of *Anacardium occidentale* and *Psidum guajava*. Asian Journal of Biomedical and Pharmaceutical Sciences. 2013; 3 (16): 20-23.
10. Paramashivappa R, Phanikumar NP, Vithayathil PJ, Sreenivasa RA. Novel method for isolation of major phenolic constituents from cashew (*Anacardium ocidentale*L.) nut shell liquid. J. Agri. Food Chem. 2003; 149: 2251-2548.
11. Pillai CKS, Prasad VS, Sudha JS, Bora SC, Menon AAR. Polymeric resins from renewable resources. J. Applied Sci. 1990; 4: 2487-2501.
12. Rodrigues FHA, Feitosa JPA, Ricardo NMPS, de Franca FCF, Carioca JOB. Antioxidant activity of cashew nut shell liquid (CNSL) derivatives on the thermal oxidation of synthetic cis-1, 4-polyisoprene. J Braz Chem Soc. 2006; 17: 265-271.
13. Goncalves JLS, Lopes RC, Oliveira DB, Costa SS, Miranda MMFS, Romanos MTV, Santos NSO, Wigg MD. *In vitro* anti rotavirus activity of some medicinal plants used in Brazil against diarrhea. J Ethno pharmacol. 2005; 99(3): 403-407.
14. Schmourlo G, Mendonca FRR, Alviano CS, Costa SS. Screening of antifungal agents using ethanol precipitation and bioautography of medicinal and food plants. J. Ethnopharm. 2005; 96: 563-568.
15. Abas F, Lajis N, Israf D, Khozirah S, Umi Kalsom Y. Antioxidant and nitric oxide inhibition activities of selected Malay traditional vegetables. Food Chemistry. 2006; 95(4): 566-573.
16. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidant by means of Folin-Ciocalteu reagent. Method. Enzymol. 1999; 299: 152-178.
17. Menon ARR, Pillai CKS, Sudha JD, Mathew AG. Cashew nut shell liquids, its polymeric and other industrial products. J. Sci. Ind. Res. 1985; 44: 324-338.
18. Huang J, Gu M, Lai Z, Fan B, Shi K, Zhou YH, Yu GQ, Chen Z. Functional analysis of the Arabidopsis PAL gene family in plant growth, development and response to environmental stress. Plant Physiol. 2010; 153: 1526-1538.
19. Dickerson DP, Pascholati SF, Haagerman AE, Butler LG, Nicholson RL. Phenylalanine ammonia-lyase and hydroxy 1 cinnamate: CoA ligase in maize mesocotyls inoculated with *Helminthosporium maydis* or *Helminthosporium carbonum*. Physiology Plant Pathology. 1984; 25: 111-123.
20. Hodgins DS. Yeast Phenylalanine ammonia lyase: Purification, properties and the identification of catalytically essential dehydroalanine. Journal of Biological Chemistry. 1971; 246: 2977-2985.
21. Hura TK, Hura, Grzesia S. Contents of total phenolics and ferulic acid and PAL activity during water potential changes in leaves of maize single-cross hybrids of different drought tolerance. J.Agron.Crop.Sci. 2008; 194: 104-112.
22. James A, Saunders, Jerry W, Mcclure. The suitability of a quantitative spectrophotometric assay for Phenylalanine ammonia-lyase activity in Barley, Buckwheat, and Pea Seedlings. Plant Physiol. 1974; 54: 412- 413.
23. Harish CA, Vijay KP. High Performance Thin Layer Chromatography (HPTLC): A modern analytical tool for biological analysis. Nature and Science. 2010; 8(10): 58-61.
24. Shailajan S, Shah S, Sayed N. HPTLC method development and validation of a secondary metabolite -β- sitosterol from *Caesalpinia bonduc* (Linn.) Roxb. emend. dandy & exell. seeds. Int J Pharma Bio Sci. 2010; 1(3): 1-10.
25. Sabna PS, Potty VP. Thermal stability of CNSL by TGA and HPTLC. International Journal of Biotechnology and Biochemistry. 2011; 7 (5): 607-616.
26. Praveen Kumar SN, Bhadre Gowda DG, Vathsala DC, Mantelingu K, Rangappa KS. Development and validation of a normal phase HPLC method for separation of anacardic acid isomers in cashew nut shell liquid. J Chem Pharm Res. 2013; 5 (5): 369-373.
27. Trevisan MTS, Pfundstein B, Haubner R, Wurtele G, Spiegelhalder B, Bartsch H, Owen RW. Characterization of alkyl phenols in cashew (*Anacardium occidentale*) products and assay of their antioxidant capacity. Food Chem Toxicol. 2006; 44(2): 188-197.
28. Kubo I, Masuoka N, Ha TJ, Tsujimoto K. Antioxidant activity of anacardic acids. Food Chem. 2006; 99: 555-562.