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Identification of Phenyl Alanine Ammonia Lyase Gene Involved in the Synthesis of Anacardic Acid in *Anacardium occidentale* L.

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ABSTRACT

Anacardic acids, a class of medicinally and industrially important phenolic compounds is found in a variety of dicotyledonous families chiefly in Cashew (*Anacardium occidentale* L). Phenylalanine ammonia-lyase (PAL) shows a dominant role in the biosynthesis of poly phenolic compounds, which are involved in the defense mechanism in harsh environments related to various stimuli. The current study was conducted to find out the presence of anacardic acid in ethyl acetate extract of young leaves of cashew using high performance thin layer chromatography (HPTLC) method and the presence of phenyl alanine ammonia lyase gene also plays a role in the biosynthesis of anacardic acid in young leaves was also confirmed by cDNA synthesis from a cellular mRNA template connected to the polymerase chain reaction (PCR).

Keywords: *Anacardium occidentale*, Anacardic acid, PAL, HPTLC, PCR.

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INTRODUCTION

Anacardium occidentale is a plantation crop belonging to the family Anacardiaceae. It is native to Brazil, from where it spread widely in the tropical countries from West to East Africa and India. [1] It is a multi-purpose plant. [2] Two most important parts in commercial uses are cashew nut and liquid from nut shell (CNSL). Several groups of phytochemicals have been found in different parts of *A. occidentale*. The phenolic compounds comprising anacardic acid was mainly concentrated in nut shell though it is seen throughout the plant body. [3-4] Anacardic acid is also having very much demand in medical and industrial field. [5] Anacardic acid has very much demand in the international market. [6] In medical field, it has been favorably used for the treatment of various diseases like ringworms, elephantiasis, warts etc. and also used in beauty therapy. It shows antiviral activity, antibacterial activity, antifungal activity, anti-inflammatory activity, antioxidant activity [7], anti-carcinogenic [8] activity etc. In industrially, it is used as a raw material for corrosion resistant varnishes, synthetic resins, brake lining compounds of automobiles, heat and water proof paints, insulating enamels for the electrical industry etc. [9]

Little information concerning the genes involved in the biosynthetic enzymes of the polyketide or acetate pathway for anacardic acid has been published. Phenylalanine ammonia-lyase (PAL) plays an important part in the biosynthesis of poly phenolic compounds, which are involved in the defense mechanism related to different stimuli. This enzyme catalyzes the deamination of phenylalanine which leads to the formation of trans-cinnamic acid and ammonia; it is the initial step in the synthesis of phenolics. [10] Many reports highlight on the relationship between increase in the comparable PAL gene activity and increase in the phenolic compounds in reaction to various external stimuli. [11] The major phenolic lipids such as anacardic acid, anacardols, cardols and their isomers are produced by going through the phenyl propanoid pathway in *Anacardium*. Activity of PAL gene has been determined from many plant species but not from *A. occidentale*. There has been no published report regarding the genes responsible for the production of anacardic acid in cashew. In view of the role of anacardic acid in curing many infections and based on their antioxidant and anticancerous properties, the current study was carried out to give a precised picture on the estimation of anacardic acid in cashew leaves by HPTLC and the detection of phenyl alanine ammonia lyase gene responsible for the production of anacardic acid in young leaves of cashew by polymerase chain reaction (PCR) amplification.

MATERIALS AND METHODS

Source of plant materials

Mature cashew seeds were collected from the mother trees growing at the Kerala State cashew development corporation, Mundakkal, Kollam, Kerala. The explants used in this study were micro leaves, which sprouted from cotyledonary node axils of decapitated seedlings germinated in plastic trays containing sterilized sand.

Preparation of plant extracts for HPTLC

Young leaves of *A. occidentale* were air dried and powdered. It was extracted with ethyl acetate at room temperature for 24 hours at a ratio of 1:100 (g: ml). Samples were then centrifuged at 10,000 rpm for 15 min and supernatants were collected and dried. Residue was dissolved in ethyl acetate and stored at 4-8°C in a refrigerator.

HPTLC estimation of anacardic acid

HPTLC analysis was carried out on Camag HPTLC. Anacardic acid (Sigma Aldrich) was used as the standard (100µg/ml). Twin trough chamber was used for separation using the solvent system as mobile phase [chloroform: ethyl acetate (9:1)] and the HPTLC settings were - Band length 8 mm, distance between track 15 mm, slit dimension - 6.00 mm × 0.45 mm × micro, scanning speed- 20 mm/s; data resolution-100µm/step. Qualitative analysis was carried out by TLC scanner 3 Camag HPTLC systems by comparing the peak area values of sample with that of standard using the Wincats software at wavelength 305 nm.

Molecular detection of phenyl alanine ammonia lyase gene

mRNA Isolation

Total RNAs were isolated using the RNeasy Plant MiniKit (Qiagen Science, Maryland USA). With a mortar and pestle 100 mg of sample was homogenized in 450µl of RLT buffer. The homogenate was poured into the QIA shredder column (purple) and centrifuged at 10,000 rpm for 1 min. The supernatant were pooled and mixed with 0.5 volumes (225µl) of absolute ethanol. The resulting mixture was applied onto the RNeasy column (pink) and centrifuged at 10,000 rpm for 1 min. The supernatant were discarded. The column was washed with 450µl RW1 buffer by centrifugation at 10,000 rpm for 1 min, and followed by 450µl RPE buffer as the same manner. The total RNAs was eluted with 30µl Rnase-free water by applying on top of the column and left for one min, then centrifuged at 10,000 rpm for 1 min. The eluted RNAs were further used for cDNA synthesis.

cDNA Synthesis and PCR Amplification

A cDNA was synthesized using QIAGEN one-step RTPCR kit. The total 20µl reaction was prepared by aliquot 1µl of total RNAs solution, 1µl of 20µM forward primer, 1µl of 20µM reverse primer, 4µl of 5X RT-buffer, 0.8µl of 10mM dNTPs, 0.8µl of enzymes and 11.4µl Rnase-free water. Synthesis of cDNA was performed on the Biometra thermalcycler (Germany) at 50°C for 30 min and followed by denaturation at 95°C for 15 min.

For the PCR reaction the forward primer used for the PAL gene was AATGGCTCCCCAAAAAATAGAAA and the revers primer sequence was GTTGGAACAAATGCGGGTCT. Each of forward and the revers primers were used to amplify about 350bp of the cDNA. [11] The forward and revers primers were designed using the gene bank (ncbi.Org). Master Mix (DreamTaq TM green PCR Master Mix (2x) containing (DNA polymerase + optimized green buffer + $MgCl_2$ and dNTPs). The PCR amplification was carried at the initial denaturation at 94°C for 3 min, 35 cycles of denaturation 94°C for 30 sec, annealing at 55°C for 30 sec extension at 72°C for 30 sec, and a final extension at 72°C for 5 min.

Gel Electrophoresis of PCR Products

The amplification was confirmed on 1% agarose gel by gel electrophoresis. A 1kb + ladder (New England Biolabs, Inc., MA,USA) was loaded along with the PCR products. An aliquot of 5µl from 20µl PCR reaction volume was mixed with 3µl of loading buffer and then loaded to each gel. The gels were run in a mupid geltang at 100V for 40 min. Gels were stained with ethidium bromide (0.5µl/ml) for 10 min and washed with water for few second. DNA was visualized using UV transilluminator (UPV, CA, USA).

RESULTS AND DISCUSSION

Estimation of anacardic acid by HPTLC

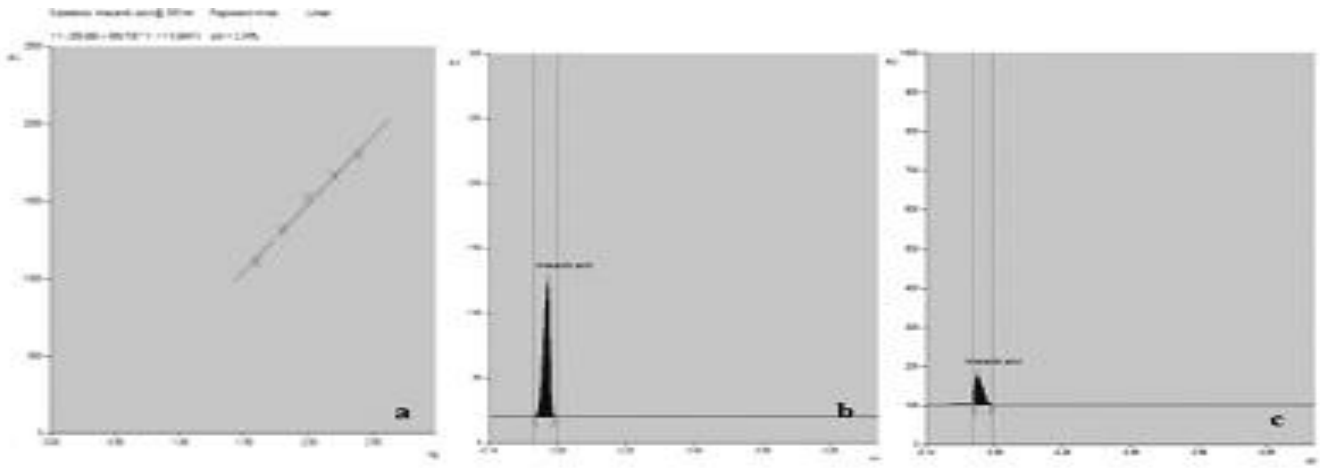

Fig. 1: a) Calibration curve of standard anacardic acid; b): HPTLC chromatogram of standard anacardic acid; c) HPTLC chromatogram of young leaves

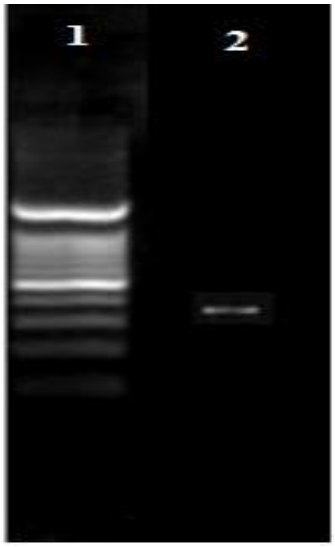

Fig. 2: Agarose gel electrophoresis of PCR amplified products.
Lane 1:- 1 kbDNA ladder; Lane 2:- sample (cashew leaves)

Qualitative evaluation of anacardic acid was done by HPTLC. Chloroform: ethyl acetate (9:1) (v/v) was used as the mobile phase. The current study showed that the presence of anacardic acid in young leaves from seedling. Calibration curve and HPTLC chromatogram of standard anacardic acid were conferred in fig. 1 (a, b). HPTLC chromatogram of young leaves of *A. occidentale* was also obtained (Fig. 1c). HPTLC technique is one of the best tools for identification and authentication of plant secondary metabolites. [12] It was also reported the presence of anacardic acid in cashew nut shell liquid, cashew nut and cashew fruit. [13-16]

Molecular detection of phenyl alanine ammonia lyase gene

mRNA was isolated from the young leaves of cashew using RTPCR. cDNA was synthesized from cellular mRNA template. Presence of phenyl alanine ammonia lyase gene in cashew leaves was confirmed by PCR amplification using the primer [Forward Primer Name:
F: AATGGCTCCCCAAAAAATAGAAA
Reverse Primer Name:
R: GTTGGAACAAATGCGGGTCT]

Gel electrophoresis revealed that the bands are found between 300 and 400 bp. This is consistent with the 350 bp. Plant phenolics are the secondary metabolites produced from either the shikimate pathway or the malonate pathway. [17] Molecular cloning, characterization and expression of the phenylalanine ammonia-lyase gene from some tree species like *Juglans regia* and *Eucalyptus robusta* were also reported. [18-19]

The present investigation proved the presence of medicinally and industrially important anacardic acid in young leaves of cashew by HPTLC technique and also confirmed the presence of phenyl alanine ammonia lyase gene carried out in the synthesis of anacardic acid in the tender cashew leaves by cDNA synthesis from a cellular mRNA template connected to the polymerase chain reaction (PCR).

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