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FULL LENGTH ARTICLE

DNA Fingerprinting of *Medicinal plant by* RAPD and RFLP of *Piper nigrum*

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ABSTRACT

Herbal medicine has been enjoying renaissance among the consumers through the world. Use of indigenous drugs from plant origin forms a major part of complementary and alternative medicine / traditional medicine (CAM/TM). One of the impediments in the acceptance of herbal formulation is the lack of standardization and quality control profiles. Classical pharmocognosy techniques like morphology, microscopy, phytochemistry, and chromatography are subjective and may not be of use always. Adulteration of market sample remains a major problem in domestic and export market due to confusing nomenclature and lack of correct botanical identification of traded raw drugs. Therefore, application of DNA based marker for authentication is considered as an effective and reliable tool by several research groups. For developing sequence specific markers different methods are RFLP,AFLP,RAPD,SSR sequencing of rDNA-ITS region and DNA bar-coding are used the present study describes the methods involved in developing markers for one of the common medicinal plant of India pepper(piper nigrum) using ITS ,RAPD and RFLP techniques. There are several controversial drugs in trade, this method can be used for other controversial plants as well for species – specific marker development.

KEYWORDS: *CAM/TM, RAPD, RFLP, AFLP, SSR, ITS markers, Phytochemistry, Pharmacognosy, Piper nigrum.*

INTRODUCTION

Herbal medicine has been enjoying renaissance among the customers throughout the world. Use of indigenous drugs from plant origin forms a major part of complementary and alternative medicine/traditional medicine (CAM/TM). One of the impediments in the acceptance of herbal formulations is the lack of standardization and quality control profiles. Due to the complex nature and inherent variability of the chemical constituents of plant-based drugs, it is difficult to establish quality control standards. Adulteration of market samples remains a major problem in domestic and export markets due to confusing nomenclature and lack of correct botanical identification of traded raw drugs. The morphological and microscopic inspection to authenticate is simple and direct, but its accuracy depends heavily on trained botanists, who are sometimes subjective. Chemical fingerprinting too has certain disadvantages. The composition and relative amount of chemicals in a species may vary with growing conditions, harvesting period, post-harvest processing and storage. This variation of chemical composition may hinder the authentication and in some instances, this can be misleading if the samples are deliberately adulterated with a marker compound Therefore, it is necessary to develop sensitive and effective technology for characterization of medicinal plants. Development of DNA based markers for authentication is considered as an effective and reliable tool by several research groups. DNA based molecular markers have acted as very useful tools in various fields like taxonomy physiology, embryology , plant breeding ,ecology, genetic engineering e.t.c DNA based markers have their applications in fingerprinting genotype , determining the seed purity and in phylogenetic analysis by which the conservation of the plant can be made easy. The innovation of polymerase chain reaction (PCR) made the development of DNA based markers easier. Extensive research on molecular markers is in progress in many research institutes all over the world. DNA fingerprinting methods like RFLP, AFLP, RAPD, SSR, sequencing of rDNA-ITS region and DNA bar coding have been used for developing species specific markers for medicinal plants. Hence there are several controversial drugs in our market founds unauthenticated. DNA based molecular marker helps in improvement of medicinal plant species. DNA markers are more reliable because the genetic information is unique for each species and is independent of age, physiological conditions and environmental factors. The molecular marker technique efficiently is based on the amount of polymorphism.

MATERIALS AND METHOD

Black pepper (***Piper nigrum***) is a flowering vine in the family Piperaceae, cultivated for its fruit, which is usually dried and used as a spice and seasoning. The fruit, known as a peppercorn when dried, is approximately 5 millimetres (0.20 in) in diameter, dark red when fully mature, and, like all drupes, contains a single seed. Peppercorns, and the ground pepper derived from them, may be described simply as pepper, or more precisely as **black pepper**. Pepper spirit is used in many medicinal and beauty products. Pepper oil is also used as ayurvedic massage oil and used in certain beauty and herbal treatment. Pepper is used to cure illness such as constipation, diarrhoea, earache, gangrene, heart disease, hernia, hoarseness, indigestion, insect bites, insomnia, joint pain, liver problems, lung disease, oral abscesses, sunburn, tooth decay, and toothaches

MORPHOLOGICAL DIFFERENCE OBSERVED IN THE SAMPLE OF PEPPER WAS:

The sample collected from the KOLLAM (dist) , KERALA is been labeled under the lab identity as (*PAZ).*
The sample collected from the AVALAHALLI, BANGALORE is been labeled under the lab identity as (*PKA).*

Sample *PAZ*	**Sample *PKA***
1. Samples leaves are found to smaller compare to sample *PKA.*	1. sample leaves are found to be bigger in size
2. Petioles of this sample is found to be thinner than compared to the sample *PKA.*	2. petioles of this sample is found to be thicker than the PAZ
3. Taste of the sample is been found to very sharp chilly taste.	3. taste of the sample was not that sharp of the sample PAZ
4. The seed can be removed from the petioles very easily.	4. it was harder to remove the sample seed as it was tightly associated with the petioles
5. The seed is small comparing to the seed collected from AVALAHALLI.	5. Seeds where bigger comparing to the sample collected from Kerala.

Plant collection;

Fresh seed sample of pepper plant is been collected from Kerala and from Avalahalli of Bangalore Genomic DNA extraction from ***P. Nigrum.***

Total genomic DNA was extracted following protocol described as follow:

CTAB isolation of total DNA

Approximately 100-200 mg leaf sample was ground to fine powder using liquid nitrogen. A pinch of Polyvinyl Pyrrolidone (PVP) was added to the ground powder. This was transferred to 2ml microfuge tubes and about 0.7 ml pre-warmed (60˚C).CTAB extraction buffer with 2M µl was added; and mixed gently by inversion. Approximately 1.4µl of β mercaptoethanol was added to each tube. This mixture was then, incubated at 60ºC for 1 hour. The tubes were removed from water bath and allowed to cool to room temperature .An equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added and mixed gently for 10 minutes. Then, they were centrifuged at 9000 rpm for 10 minutes at 4°c.The upper aqueous layer was transferred to a new tube and steps 5 & 6 were repeated. To the separated aqueous layer, an equal volume of Chloroform: Isoamyl alcohol (24:1), was added and mixed gently for 5 minutes, and centrifuged at 9000 rpm for 10 Minutes. The aqueous layer was collected in a fresh tube and two volumes of ice-cold ethanol were added. They were mixed gently by inverting the tube several times to precipitate DNA. The tubes were incubated on ice for 30 minutes. DNA was then pelleted by centrifuging the tubes at 9000 rpm for 15 minutes at 4°C. The supernatant was discarded and the pellet was washed with 0.5 ml of ice-cold wash buffer and left at room temperature for 5 minutes. The DNA was resuspended in 50 to 100 µl of 1X TE buffer and incubated at 65 ºC for 1 hr with periodic. Gentle mixing, followed by centrifugation at 9000 rpm for 10 minutes. The aqueous Layer was transferred to a fresh tube without disturbing the pellet and stored at 4ºC.

AGAROSE GEL ELECTROPHORESIS:

To prepare 50 ml of 1.5 % agarose gel.

Measure 0.75 g agarose in a glass beaker or flask and add 50ml 1X TAE buffer. Heat the mixture on a microwave or hot plate or burner. Swirling the glass beaker/flask occasionally, until agarose dissolves completely. Allow the solution to cool to about 55-60^{o}C. Add 2 µl Ethidium bromide (10 mg/ml). Mix well and pour the gel solution into the gel tray. Allow the gel to solidify for about 30 minutes at room temperature. Load 5 µl of ready to use DNA Marker into the well 1. Load 30 µl of each DNA samples (reference samples) onto wells 2, 3 and Load 30 µl of test DNA sample well. Connect the power cord to the electrophoresis at 100-120 volts and 90 mA current until dye markers have migrated an appropriate distance, depending on the size of DNA to be visualized. Switch off the power supply once the tracking dye from the wells reaches 3/4th of the gel which takes approximately 45 - 60 minutes. Observe the gel under a UV transilluminator.

GEL PURIFICATION

The DNA fragment from the agarose gel was excised with a clean, sharp scalpel. The gel slice was weighed in a colorless tube and 3 volumes Buffer QG to 1 volume gel
(100 mg ~ 100 µl) were added. It was incubated at 50°C for 10 minutes. The solution was vortexes every 2–3 minutes to
help dissolve gel. One gel volume of isopropanol was added to the sample and mixed. A QIAquick Spin Column was placed in a 2 ml Collection Tube provided by the kit. To bind DNA, the sample was applied to the column and centrifuged for 2 minutes at
13000 rpm.0.5 ml Buffer QG was then added and centrifuged for 1 minute at 9000 rpm.The column was washed by adding 0.75 ml Buffer PE and centrifuging for 1 minute at 9000 rpm.The column was placed into a clean 1.5 ml micro centrifuge tube.DNA was eluted by addition of 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) to the center of the QIAquick membrane and allowed to stand for 30 minutes. Finally it was centrifuged for 1 minute at 9000 rpm and stored at 4ºC.

POLYMERASE CHAIN REACTION (PCR)/RAPD

Before starting the experiment, crush ice and place the vials containing DNA samples, TAQ Polymerase enzymes, and Dntp, $MgCL_2$, Primer and assay buffers onto it.In this experiment two DNA sample test sample are made to amplify using PCR.

Reaction mixtures for PCR are as follows:

DNA sample	– 9.0 µl
10X Assay Buffer	– 3.0 µl
Milli Q water*	– 9.0 µl
Primer	– 1.5 µl
DNTP	– 6.0 µl
$MgCl_2$	_ 1.5 µl
Total	30 µl

*Molecular biology grade water is recommended. Mix the components by gentle pipetting and tapping. Incubate the tubes at 37 ^{0}C for 1 hour. After incubation at room temperature run the PCR.Run the samples on agarose gel.

RESTRICTION FRAGMENT LENGTH POLYMORPHISM (RFLP)

Before starting the experiment, crush ice and place the vials containing DNA samples, restriction enzymes and assay buffers onto it. In this experiment three reference DNA samples and the test sample are digested simultaneously with two restrictio0n enzymes Eco RI and Pst I. Set up four separate reaction mixtures as follows:

DNA sample	– 15.0 µl
10X Assay Buffer	– 3.0 µl
Milli Q water*	– 10.0 µl
Eco RI	– 1.0 µl
PST I	– 1.0 µl
Total	30 µl

Molecular biology grade water is recommended after preparing the four reaction tubes; mix the components by gentle pipetting and tapping. Incubate the tubes at 37^{o} C for 1 hour. After incubation, immediately add 5 µl of 6X Dye to each tube.

RESULT AND DISCUSSION

GENOMIC DNA EXTRACTION

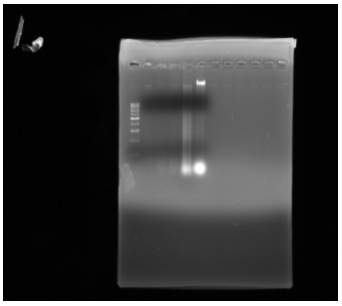

Fig 1. Is of the gel which is been run after the DNA extraction from the sample where the DNA is been found in the 5th & 6th well having PKA & PAZ respectively and is been found with lots of RNA contamination which can be observed in a round shape in the well number 6. And the 1st well consists of 100 base pair ladder.

The genomic DNA is been extracted from the Pepper sample and is observed under UV illuminator and gel docked.

POLYMERASE CHAIN REACTION (PCR)/RAPD

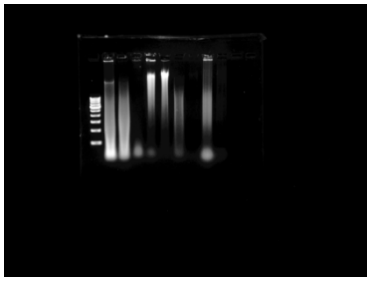

Fig 2 is the result for the RAPD analysis. The first well consists of 500 base pair ladder. And the other well consisting of different Pepper sample PKA & PAZ in alternative wells respectively. The sample PKA in 2nd well is been observed at 365 BP and the sample PAZ which in 5th well is observed 412 BP.

The sample **PKA & PAZ** are genetically different as the bands are observed at different base pair.

RFLP (RESTRICTION FRAGMENT LENGTH POLYMORPHISM)

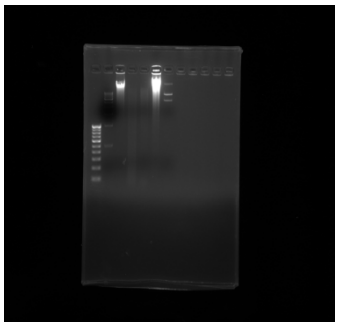

Fig 4 is of RFLP analysis where 1st well consists of 500 BP ladder and the 2nd & 3rd well consisting of cut and uncut PKA samples respectively. Whereas the 6th & 7th well consisting of uncut and cut PAZ sample respectively. The cut sample of PKA is observed at 467bp, 405bp, 380bp and 253bp. PAZ is observed at 457bp, 385bp, and 295 BP .

As the cuts are observed at different base pairs the sample resembles to be genetically different under RFLP analysis**.**

CONCLUSION

The markers developed using the following techniques can be further used by pharmacy industries for checking the originality of the sample which is difficult to identify by looking into its morphology. DNA fingerprinting, apart from identifying alterations in the genotypes of plant species, is also used for the betterment of drug-yield by tissue culturing. DNA of interest can be stored as germplasm, which is then

used for future cultivation. In addition, germplasm can be used for the conservation of selected plant species, which are endangered such as Rauwolfia serpentine (Snake Root). DNA fingerprinting of herbal drugs, though still in its early years, seems to be a promising tool for the authentication of medicinal plant species and for ensuring better quality herbs and nutraceuticals.

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