Australian Journal of Basic and Applied Sciences, 4(12): 6570-6574, 2010
ISSN 1991-8178

Studies on Effect of Pcr-rapd Conditions for Molecular Analysis in Asparagus (Satawari) and Aloe Vera" Medicinal Plants.

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Abstract: Molecular marker based analysis of plants relies on high yields of pure DNA samples. This article deals with optimization of DNA isolation and PCR conditions for RAPD analysis of medicinal plants, Asparagus and Aloe vera. The method involves a modified CTAB extraction including addition of PVP, .3M Nacl along with CTAB. The DNA isolated was used for randomly amplified polymorphic DNA (RAPD) analysis. RAPD protocol was optimized using different concentrations of Mg cl $_2$, Taq polymerase, genomic DNA, primer annealing temperature. Reproducible amplifiable products were observed in PCR reactions.

Key words: PCR-RAPD, DNA isolation, CTAB.

INTRODUCTION

Asparagus (Satawari) a medicinal plant belongs to the family *Liliaceae*. Traditionally grown as temperate crop satawari is considered as general tonic and female reproductive tonic. Satawari is the main Ayurvedic rejuvenative tonic for the female, as is Withania for the male. In Ayurveda this amazing herb is known as the "Queen of Herbs" because it promotes love & devotion. Satawari has a strong rejuvenating, nurturing and stabilizing effect on excessive air, gas, dryness and agitation in the body and mind. As such it is traditionally used for nervousness, anorexia, insomnia. Asparagus is having many medicinal properties and so used by many pharmaceutical companies for making drugs. Kamat *et al.,* (2000) reported antioxidant properties of Asparagus racemosus against damage induced by gammaradiation in rat liver mitochondria. Antitussive effect of Asparagus racemosus root against sulfur dioxide induced cough in mice and antibacterial activity has been reported by Mandal *et al.,* (2000). The powedered dried root of A. racemosus has been found to promote gastric emptying in healthy volunteers, Jousilahti *et al.*, (1997).A. racemosus has been mentioned for treatment of ulcerative disorders f stomach and Parinama sula,a clinical entity akin to the duodenal ulcer diseases. The juice of fresh root of A. racemosus has been shown to have definite curative effect in patients of duodenal ulcers, Kamat *et al.,* (2000).The root extract of A. racemosus is prescribed in Ayurveda to increase milk secretion during lactation reported by Galvez *et al.*, (1995) A. racemosus in combination with other herbal substances in the form of 'Ricalex' tablets (Aphali Pharmaceuticals Ltd. Ahemadnagar,India) has been shown to increase milk production in females complainingof deficient milk secretion.

Aloe vera , also known as the medicinal Aloe is a species of succulent plant that probably originated in Northern Africa , Madagascar and Arabian Peninsula. Aloe vera belongs to the kingdom Plantae and family Asphodelaceae and genus Aloe. It is xerophyte and can be grown even in dry lands under the rainfed conditions. Leaves of Aloe vera exude a bitter liquid, which is dried and known as ' bitter Aloe' they also contain a clear gel, which is soothing skin remedy. Clear gel is applied to the skin as first aid for burns. Aloe contains cathartic anthra- glycosides and its active principle ranging from 4.5 to 25% of Aloin. These are extensively used as active ingredients in laxative, anti-obesity preparation,as a moisturizer, emollient, wound healer in various cosmetic and pharmaceutical formulations. It is a drug as well as a cosmetic. Winters *et al.*, (1981) reported that the substances in fluid fraction from fresh leaf sources of Aloe vera markedly promoted attachment & growth of human normal cells. Anitmetastatic properties of success Aloes, contributes to reduction of tumour mass, metastatic foci and metastasis frequency at different stages of tumor progress without affecting tumor growth. ATF 1011, a non mitogenic lectin for T cells purified from Aloe aborescens Mill, bound equally to normal and tumor cells Yoshimoto *et al.*,(1987).

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The use of medicinal plants has a long history in the world. According to the WHO 20,000 species are used for medicinal & aromatic purposes. Today 4000 drugs are widely used and 10% of them are commercially exploited or produced. The acess to new tools for biodiversity prospecting, characterization and data analyses along with change of traditional conservation system towards more use oriented initiatives should be seen as opportunities for revising research goals & partnerships, create greater synergies at national level. Morphological markers like plant height, colour, shape of leaf and seed etc are among the oldest markers used in the evaluation of genetic variability. Biochemical markers like isozymes, are also non specific due to wide variability of biochemical characters which are strongly influenced by an individuals environment. Molecular markers show variability among individuals at the DNA level, which is not influenced by the environment. They are highly informative about genetic variability among individuals, population and cultivars. Their use is universal for all the organisms.

The success of biotechnological tools such as molecular marker and genetic engineering are critically dependent on the development of reliable protocol for isolating superior quality DNA and PCR analyses. The problems encountered in the isolation and purification of DNA specially from medicinal plants include degradation due to endonucleases, coisolation of highly viscous polysaccharides, inhibitor compounds like polyphenols and other secondary metabolites which directly or indirectly interfere with the enzymatic reactions and interfere with DNA amplification involving random primers (Pikkart & Vllieponteau 1993, Mejjad *et al.*, 1994). Thus an efficient protocol for isolation of DNA as well as the optimization of the PCR conditions is required.

MATERIAL & METHODS

Plant Material:

Leaf samples of both the plants were taken for DNA extraction. The plants Aloe vera, A. barbadensis and Asparagus racemosus were collected from medicinal park, Gharaunda, Panipat, Haryana.

DNA Isolation:

Extraction buffer consisting different concentrations of CTAB (w/v) 1%, 2%,3%, Tris HCl pH 8.0, EDTA pH 8.0 (0.5M), 3M Nacl, Ribonuclease A (10mg/ml), Chloroform: isoamylalcohol (24:1), Phenol: Chloroform: isoamylalcohol (25:24:1v/v/v), Absolute alcohol, 70% ethanol, TE buffer(Tris HCl 10mM, EDTA 1mM pH 8.0).

DNA Isolation Protocol:

- Preheat the extraction buffer in 50 ml centrifuge tube to 60^0 C in a waterbath.
- Leaf samples were grinded in liquid nitrogen using a mortar and pestle along with 0.1g of PVP. The grinded leaf samples were transferred in centrifuge tube containing the extraction buffer & shaken vigorously by inversion to form slurry. The samples were incubated at 65^0 C for 30 minutes with intermittent shaking. Cool and centrifuge at 8000 rpm for 10 min.
- Add equal volume of phenol : chloroform : isoamylalcohol (25:24:1) to the supernatant and centrifuge at 8000 rpm for 10 min and collect the supernatant in fresh centrifuge tube.
- Repeat the above step once. Extract the aqueous phase with equal volume of chloroform : isoamylalcohol (24:1) to remove residual phenol from solution. Centrifuge at 6000 rpm fir 10 min.
- Supernatant was carefully decanted & transferred to a new tube & was precipitated with equal volume of chilled isopropanol, gently mixed tp produce fibrous DNA & incubated at -20^0 C for minimum of 30 min.
- The samples were centrifuged at 12000 rpm for 15 min. The pellet was washed with 70% ethanol, air dried & suspended in 3 ml of TE buffer.
- RNase treatment was carried out at 37 ^{0} C, remove the RNase using chloroform : isoamylalcohol (24:1) extraction.
- The supernatant was transferred to afresh tube & added equal volume of absolute alcohol &1/10 volume of sodium chloride, incubated at -20^0 C for 30 min followed by centrifugation at 12000 rpm for 15 min.
- Air dried the pellet & resuspended in TE buffer & stored a t4 ^{0} C or -20 ^{0}C for further use.

Optimization of RAPD Reaction:

For optimization of RAPD reaction the genomic DNA was employed for RAPD analysis. Ten decamer oligonucleotide operon Kit Primers were used to perform RAPD PCR reactions. Polymerase chain reaction was performed in a 20 µl reaction mixture containing 0.5 unit Taq DNA polymerase , $Mgcl_2$ (1.5mM), 200mM

dNTPs, (All Bangalore Genei Pvt. Ltd. India), 0.2uM primer, 50ng template DNA.DNA amplification was performed in a thermal cycler (Lark Innovative MG96G) after initial cycle of denaturation at 92^0 C for 2min, followed by 38 cycles of 1min at 94^0 C, 1 min at 37^0 C, extension at 72^0 C for 1 min and final extension at 72^0 C for 10 min. and hold temperature of 4 ^{0} C at end. PCR products were electrophoresed on 2% agarose gels , in 1X TBE buffer at 80 V for three hours. Gels with amplified fragments were visualized and photographed under U.V light using Gel Documentation System (Systems & Control).

RESULTS & DISCUSSION

DNA extraction was improved by modifying some of the steps in the original CTAB DNA isolation protocol (Doyle and Doyle 1987). This modified method was found effective as compared to phenol–chloroform method. Present procedure resulted in extracting, high quality, low polysaccharide genomic DNA from both medicinal plants. DNA isolated from modified method was clear while the latter was partially soluble and formed a gelatinous pellet. DNA was smeared, when agarose gel visualized under U.V light which indicated high levels of protein and polysaccharide impurities in samples isolated from phenol – chloroform method as compared to modified CTAB method. Puchooa. D. (2004) has used similar analysis for interfering the degree of protein and polysaccharide contamination in lychee (Litchi chinensis sonn) . The presence of polyphenols which are powerful oxidizing agents present in medicinal plants can reduce the yield and purity by binding covalently with the extracted DNA making it useless for most research applications (Katterman and Shaltuck, 1983, Peterson *et al.*, 1997). Certain polysaccharides are known to inhibit RAPD reactions they distort the results in many analytical applications and therefore lead to wrong interpretations (Kotchoni *et al.,* 2003, Padmalatha 2006). Several modifications were introduced to CTAB method for removal of impurities, combination of high concentration of CTAB (3%w/v) and sodium chloride (3M) in the extraction buffer along with two successive washes with Phenol- chloroform and final precipitation with salt proved very effective.

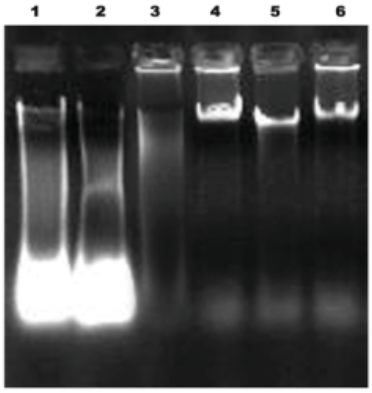

Fig. 1(a): Electrophortic pattren showing smear & fire type bands in the DNA samples isolated using phenol-chloroform method. Lane 1-6 represents Aloe vera, Asparagus racemosus, A. Barbadensis

Fig. 1(b): Electrophoretic pattren of DNA samples showing sharp, distinct & clear bands in samples isolated using modified cTAB method. Lane 1-3 represents Aloe vera, Asparagus racemosus, A.barbadensis.

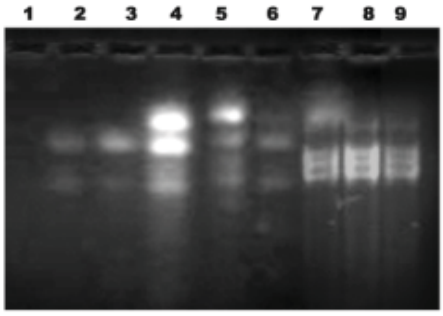

Fig. 2: Electrophoretic pattren showing concentration of DNA for amplification. Lane 1-3 (less than 50ng) lane 4-6 (50ng) lane 7-9 (more than 50ng)

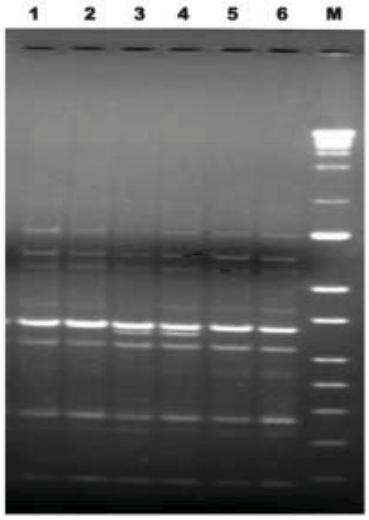

Fig. 3: Electrophoretic pattren of RAPD products generated with primers 1&2. Lane 1-3 represents Asparagus racemosus, A.barbadensis, Aloe vera & lane 4-6 represent Aloe vera, Asparagus racemosus, A.barbadensis.

Addition of PVP along with CTAB may bind to polyphenolic compounds by forming a complex with hydrogen bonds and may help in removal of impurities. Two consecutive washes with Phenol- chloroform-isoamylalcohol excluded protein impurities (Dipanker et al., 2006, Harni *et al.*, 2008). Several methods on removal of polysaccharides from DNA have been extensively reviewed (Lodhi et al., 1994, Maryam Sarwat *et al.*, 2006) of which salt precipitation has been recommended to be most effective(Tamsyn *et al.*, 2003). Salts when in precipitation increase the solubility of polysaccharides in ethanol thus preventing its coprecipitation with DNA. In the present study reprecipitation of dissolved DNA with salt at end of process ensured complete removal of residual polysaccharide in the sample (Fig 1a &b).We found these modified steps necessary to standardize and increase the quality and quantity of genomic DNA. DNA isolated by this method yielded strong and reliable amplification products showing its compatibility for RAPD –PCR using random decamer primers (Fig 1b). The parameters for RAPDs like concentration of genomic DNA, primers dNTPs, Taq DNA Polymerase, $Mgcl_{2,}$ denaturation temperature and time, annealing temperature, no. of cycles were optimized by trying different concentrations and amounts. The different concentrations of DNA (ng) used in the present study were 30,40,50,70,100 for amplification out of these conc. of 50ng genomic DNA was found optimium for PCR amplification. Lower than 50ng showed no amplification and presence of smear was found at higher conc. (Fig 2). Optimum concentration of $Mgcl_{2,}$ was found to be 1.5mM out of 1.0, 1.5, 2.0 mM concentrations tried in present study, excess/ lower conc. increases the non specifity and yield of the product. dNTPs at 200mM were found optimum, increased conc. reduces the free magnesium ions interfering with the enzyme

and reduced conc. of dNTPs showed lack of reproducibility. Primer conc. was optimized at 0.5 µM lower and higher then optimized concentration lead to absence of amplification and primer dimer formation. 0.5 U of Taq polymerase was found optimum for PCR amplification, annealing temperature of 37^0 C for 1 min. was found to be optimum lower / higher annealing temperature resulted in non specific amplicons. Out of different no. of cycles tried 38 cycles were found to be standard for PCR amplification. The optimized reaction conditions produced clear, scorable amplified products suitable for RAPD analysis (Fig 3) in the 10 primers tested . The present study on the development of protocol for isolation of high purity DNA and optimization of RAPD conditions may serve as an efficient tool for further molecular studies.

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