Genomic DNA Isolation Protocol for *Aloe Barbadensis* Miller: Using Leaf Gel for Genetic Characterization

Pushpa Deore[*], S. Samantaray[**], S. Maiti [**]

[*]Department of Agricultural Botany, Anand Agricultural University, Anand 388110, Gujarat, India
[**]Directorate of Medicinal and Aromatic Plants Research, Boriavi, Anand

Abstract- Optimization of DNA isolation protocol for genetic characterization of any medicinal plant is the necessary and primary step. Plant leaves of medicinal plants are rich in secondary metabolites, polysaccharides and polyphenols that are problematic during isolation of genomic DNA. Besides, succulent plants freezed under liquid nitrogen turn to stonelike tissues that are difficult to grind. Flowers were not considered as a source of genomic DNA as the case of young leaves, this might be due to the seasonal nature and short half-life feature. Several protocols described for plant DNA isolation from *Aloe barbadensis* Miller using leaf, found difficult and tedious. This plant contains exceptionally high amount of secondary metabolites that interfere with DNA isolation. To address this problem, young leaf gel was used for DNA isolation that gave good quality DNA suitable for molecular biological applications. Cell lyses was carried out in extraction buffer supplemented with cetyltrimethylammoniumbromide and sodium chloride. Inclusion of high concentration of CTAB and PVP removed polyphenols. Chloroform Isoamyl washes followed by precipitation with NaCl efficiently removed high protein and polysaccharide contamination. The yield of pure DNA ranges from 25-50µg per gram of leaf tissues. The RAPD analysis from the isolated DNA was optimized to produce clear amplification in all the germplasm tested.

Index Terms- *Aloe barabdensis*, DNA isolation, medicinal plant, leaf gel

I. INTRODUCTION

Aloe barbadensis Miller is perennial, monocotyledonous, succulent plant belongs to the family 'Liliaceae' and order 'Asparagales' (The wealth, 1985). Genus *Aloe* comprises of 275 species distributed in Africa, Asia and America. In India, only four species occur and of these *Aloe barbadensis* is the most widely naturalized species. *Aloe barbadensis* var. *chinensis* is commonly found in Maharashtra, *A. barbadensis* var. *littoralis* is found in Tamil Nadu. One variety of Jafarabad aloe thrives in Saurashtra coast. Three varieties of aloe are official in the Indian Pharmacopoeia (Kurian and Sankar, 2007). Around the world, the plant has acquired a number of unusual descriptions such as 'Voodoo Juice', 'Fountain of Youth', 'First Aid Plant', and 'Wand of Heaven' (Atherton, 1997). It is popularly known as 'nature's sun screen' (Kurian and Sankar, 2007).

The aloe leaf can be divided into two major parts, namely the outer green rind, including the vascular bundles, and the inner colorless parenchyma containing the aloe gel. Technically, the term 'pulp' or 'parenchyma tissue' refers to the intact fleshy inner part of the leaf including the cell walls and organelles, while 'gel' or 'mucilage' refers to the viscous clear liquid within the parenchyma cells (Ni and Tizard, 2004). These three components of the inner leaf pulp have been shown to be distinctive from each other both in terms of morphology and sugar composition. (Ni *et al*., 2004). The leaves also contain anthroquinone glycosides which are collectively termed as "aloin". The aloin content of *A. barbadensis* is 30 % (Jain, 1998); it has great medicinal importance. Moreover, the polysaccharides in A. vera gel have therapeutic properties such as wound healing, anti-inflammatory effects (Vazquez et al., 1996), immunostimulatory, anti-diabetic (Reynolds and Dweck, 1999), promotion of radiation damage repair, anti-bacterial, anti-viral, anti-fungal, and anti-neoplastic activities, stimulation of hematopoiesis and anti-oxidant effects (Talmadge *et al*., 2004; Ni and Tizard, 2004), anti-cancer activity (Steenkamp and Stewart., 2007). The semisolid gel is used in ointments, cosmetic creams, lotions and shampoos. It is also used as a neutraceutical in form of juice, emulsion and syrup with iron and as jam (Jain, 1998).
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 access 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, color, shape of leaf and seed etc are among the oldest markers used in the evaluation of genetic variability. Biochemical markers like isozymes, are also nonspecific 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.

Furthermore, techniques such as amplified fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) are useful in studying plant diversity, genetic transformation and clonal fidelity determination of micropropagated plants. The isolation of high molecular weight genomic DNA is a pre-requisite for these molecular techniques. However, high amounts of polysaccharides, polyphenols and various secondary metabolites such as alkaloids, flavonoids and tannins in tree species usually interfere with DNA isolation (Mishra *et al*., 2008). Similarly, Diadema *et al*. (2003) observed that genomic DNA extraction from succulent plants is difficult. These authors reported that the difficulty could be attributed to

small cell density in succulent tissues and high levels of contaminants (polyphenols or polysaccharides) that co-precipitate with DNA.

Polyphenols released from the vacuoles during the cell lysis process are oxidized by cellular oxidases and undergo irreversible interactions with nucleic acids causing browning of the DNA (Varma *et al*., 2007). The presence of gelling polysaccharides prevents complete dissolution of nucleic acids and imparts a viscous constituency to the DNA making it stick to the wells during gel electrophoresis (Barnell *et al*., 1998; Diadema *et al*., 2003; Varma *et al*., 2007). According to these authors, residual polyphenols, polysaccharides and secondary metabolites interfere with the activity of several biological enzymes like polymerases, ligases and restriction endonucleases. Though several successful genomic DNA isolation protocols for high polyphenol and polysaccharide containing plant species have been developed, none of these are universally applicable to all plants (Varma et al., 2007). Researchers often modify a protocol or blend two or more different procedures to obtain DNA of the desired quality (Varma *et al*., 2007; Jabbarzadeh *et al*., 2009). A good isolation protocol should be simple, rapid and efficient; yielding appreciable levels of high quality DNA suitable for molecular analysis. Our objective was to develop a reliable protocol for achieving larger quantity and highly purified DNA in *A. barbadensis* that could be used for molecular biology work.

II. Materials and Methods

Plant material

Young leaves of 38 accessions (IC310611, IC310617, IC310618, IC310609, NR127, IC1112521, IC1112532, IC1112527, IC283671, NMRM2, IC 285630, NR72, IC285626, IC310623 IC310619, MP1, GUJ2, NR63, NR57, IC283655, NR74, GUJ1, NR61, IC310906, IC310903, IC310904, IC310908, IC310614, IC112531, IC112517, K98, GUJ6, IC285629, N129, GUJ3, RAJ1, RAJ2, IC283670) of *A. barbadensis* were collected from field gene bank of National Research Centre of Medicinal and Aromatic Plant (Directorate of Medicinal and Aromatic Plants Research), Boriavi, Anand, Gujarat, India; these accessions were collected from diverse locations of Gujarat, Madhya Pradesh, Rajasthan and NBPGR, New Delhi

Reagents and solutions

- Extraction buffer: 4% (w/v) CTAB (Cetyl trimethyl ammonium bromide), 5 M NaCl, 20mM EDTA (pH 8.0), 100mM Tris- HCl (pH 8.0), 2% PVP (Polyvinylpyrrolidone) and and 2% β-mercaptpethanol
- Chloroform-isoamyl alcohol (IAA) (24:1; v/v)
- 3 M sodium acetate (pH, 4.8)
- Isopropanol (-20ºC)
- Absolute ethanol (-20ºC)
- 70% ethanol (-20ºC)
- 5 M NaCl
- TE buffer (10 mM Tris, 1mM EDTA)
- PCR reaction buffer (Bangalore Genei, India)
- Primers Operon Technologies Inc. Almeda CA, USA
- Taq DNA polymerase (Bangalore Genei, India)
- Deionized autoclaved water

Preparation of Extraction buffers

The extraction buffer used for the homogenization contained 100 mM Tris (pH, 8.0), 20 mM EDTA (pH, 8.0), 5M NaCl. The extraction buffer was autoclaved and 2% β-mercaptoethanol, 4% CTAB, 2% PVP (40,000) was added immediately before use; care was taken at the time of addition to avoid precipitation.

Protocol for genomic DNA Isolation

- Take 10 ml of extraction buffer and pre-warm to 65ºC.
- Approximately, 5g of young fresh leaf gel was homogenized with a mortar and pestle in liquid nitrogen. The homogenized mixture was transferred in a centrifuge tube using a pre-cooled spatula and add the prewarmed isolation buffer. Vortex thoroughly and place the tube at 65ºC for 1h and vortex at every 10-15 min during the incubation.
- Centrifuge the tube at 10,000 rpm for 20 min at RT.
- Transfer the upper phase into a new tube and add 2.5 ml of 5 M NaCl and mix by inverting the tube carefully.
- Add double volume chilled ethanol and mix gently.
- Thread like structure/precipitation will appear.
- Drain out the solution and wash DNA with 70% ethanol twice.
- Allow the DNA to air dry and then dissolve it in 1 ml T10E1 buffer.
- Add (60 µg/ml) RNase and incubate for 1 hour at 37ºC with continuous shaking.
- Add 1 ml of chloroform: isoamyl alcohol (24:1) and shake for 10 min.
- Centrifuge at 10,000 rpm for 10 min at room temperature.
- Take supernatant and add 1/10th volume of 3M sodium acetate followed by addition of 2.5 ml chilled ethanol. Mix properly.
- A mass of white thread like structure will appear.
- Drain out all the solution and wash the DNA with 70% ethanol twice.
- Dissolve the DNA in minimum quantity of T10E1 buffer.
- Place the tube at -20ºC for further use.
- Check the quality and concentration of the DNA with a spectrometer and on a 0.8% agarose gel

Notes:

1. The homogenized mixture should not be allowed to thaw at any point before adding the isolation buffer. By keeping the temperature below 0ºC, the oxidizing enzymes are inactivated during this step. If larger amounts of plants are processed simultaneously, the powder can be stored in a -20ºC freezer.
2. The tube should not be shaken vigorously because the DNA is very vulnerable to fragmentation at this step.
3. Care should be taken so that the pellet is not dried excessively, making it difficult to dissolve.

Optimization of RAPD reaction (Randomly Amplified Polymorphic DNA)

4. For the optimization of RAPD reaction using DNA extracted from various accessions, oligonucleotide primers from A and J series (OPA09; 5'GGGTAACGCC3', OPJ09; 5'TGAGCCTCAC3') were used for amplification to standardize the PCR conditions.
5. PCR amplification was performed in 25 µl reaction volumes containing 2.5 µl of 10X assay buffer (100 mM Tris-Cl; pH 8.3, 500 mM KCl, 15 mM MgCl2), 0.2 mM of each dNTPs (dATP, dCTP, dGTP and dTTP) (MBI Ferment Inc., Maryland, USA) 5 pg of primer, 1.0 unit of Taq DNA polymerase and 30ng of template DNA. The amplification reaction was carried out in a DNA Thermal Cycler (Eppendorf AG, Hamburg, Germany) programmed for 44 cycles as follows: 1st cycle of 5 min at 94ºC followed by 43 cycles each of 1min at 92ºC, 1min at 37ºC, 2 min at 72ºC. The final step consisted of one cycle of 7 min at 72ºC for complete polymerization. After completion of the PCR, 2.5 µl of 6X loading dye (MBI Ferment Inc., Maryland, USA) was added to the amplified products and were electrophorsed in a 1.5% (m/v) agarose (Bangalore Genei, Bangalore, India) gels with 1X TAE buffer, stained with ethidium bromide and photographed and documented by a gel documentation system (Syngene, Cambridge, UK) . The sizes of the amplification products were estimated by comparing them to standard DNA ladder (O'Gene Ruler 1.0 kbp DNA ladder; MBI Ferment Inc., Maryland, USA). All the reactions were repeated three times.

III. Results and Discussion

In this study, DNA extraction was improved by major modifications of CTAB method (Doyle and Doyle, 1987). Firstly, DNA was isolated from leaf (excluding the gel like mesodermal region). But it was found that the DNA extracted was viscous and difficult to dissolve and pipette out. Also, it required several purification steps and tedious one. So it necessitated the modification to obtain good quality and quantity DNA. Then, four types of plant materials were used shown in Table No.1 for DNA extraction. It was found that DNA extracted from young rind with gel and old rind without gel was poor in quantity and quality. This could be due to the presence of other impurities like protein. Proteins associated with the isolated DNA could not be removed with phenol. The presence of such proteins may have retarded the migration rate decreasing the quality of the isolated DNA. Among these DNA extraction from young leaf gel was found to be effective.

Table 1: Comparison of *A. barbadensis* DNA isolation from different plant parts

Accession used: N129

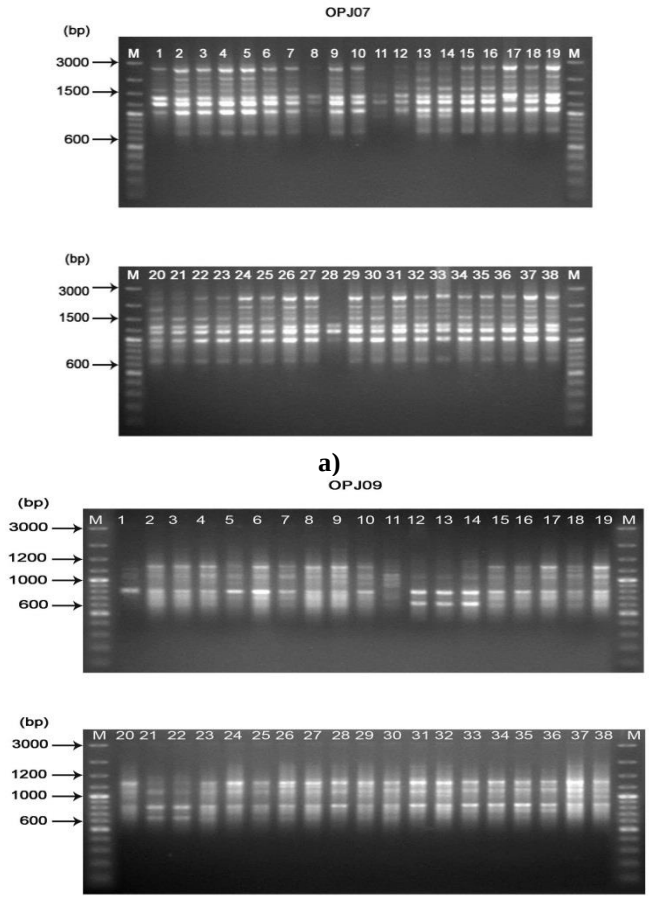
Observations	Young leaf gel	Old leaf gel	Adult leaf rind and gel	Adult leaf rind
Supernatant	Clear	Clear	Mucilaginous	Mucilaginous
Purification steps	1	3	More than 3	More than 3
DNA dissolution	Easy	Quiet easy	Difficult	Difficult
% DNA observed	100	70	20	0

High yield of total cellular DNA in many plants was obtained by increasing CTAB and NaCl concentrations in the extraction buffer (Syamkumar *et al*., 2003). Polysaccharides contaminations are particularly problematic as they can inhibit the activity of many commonly used molecular biology enzymes, such as polymerases for which increased concentrations of NaCl more than 1.5 M can be used for removal of polysaccharides for manipulating DNA (Fang *et al*., 1992). Several methods on removal of polysaccharides from DNA have been extensively studied (Sarwat *et al*., 2006; Harini *et al*., 2008; Doosty *et al*., 2012; Samantaray *et al*., 2009). The presence of polyphenols which are powerful oxidizing agents can reduce the yield and purity by binding covalently with the extracted DNA making it useless for most of the molecular biological works (Katterman and Shattuck, 1983; Peterson *et al*., 1997). Besides, also polyphenol contamination of DNA makes it resistant to restriction enzymes as also shown in other taxa where polyphenols copurify with DNA (Katterman and Shattuck, 1983) and interact irreversibly with proteins and nucleic acids (Loomis, 1974). This phenomenon is mainly due to the oxidation of polyphenols to quinines and quinones by polyphenol oxidase followed by covalent coupling or by oxidation of the proteins by the quinines. During homogenisation, polyphenols are released from vacuoles and then they react rapidly with cytoplasmic enzymes. Addition of PVP along with CTAB to extraction buffer may bind to the polyphenolic compounds by forming a complex with hydrogen bonds and may help in removal of impurities to some extent. Use of high- molecular- weight PVP (40,000) was recommended by a number of workers instead of using lower ones. (Stewart and Via, 1993; Porebski *et al*., 1997; Zhang and Stewart, 2000). Similarly, addition of ethanol instead of isopropanol in the last step of DNA isolation enhances the DNA yield and purity (Samantaray *et al*., 2009). It was observed that incorporation of 2% PVP and 5M Nacl to CTAB buffer produced high quantity and purified DNA observed by RAPD markers (Fig. 1a, 1b).

The quantity of DNA is also dependent on the amount of grinded tissue harvested and collection of the supernatant (Moyo *et al.*, 2008). For this, 3gm, 5gm and 10gm of leaf gel were used for study. It was found that 5gm of young leaf gel of *A. barbadensis* yielded high quantity good quality DNA. The isolated DNA had normal spectra in which the A260/A280 ratios were 1.6-1.7 (Pich and Schubert, 1993). Agarose gel electrophoresis also demonstrated no evidence of protein and RNA contamination and confirmed that the DNA was of high molecular weight compared with undigested λ DNA the yield range was 25-50 µg per gram of leaf tissue. The DNA isolated from 38 accessions of *A. barbadensis* using this method was suitable RAPD experiments using OPJ 07 (Fig. 1a) and OPJ 09(Fig. 1b).

The present study on development of protocol for isolation of high purity DNA and optimization of RAPD conditions from leaf gel is the first report for *A. barbadensis*. This will form a strong beginning for future molecular characterization and genetic improvement works in this promising medicinal plant. This work

represents a novel method that does not require ultracentrifugation to isolate DNA.



b)

Figure1: RAPD banding patterns of 38 accessions of *A. barabdensis*, (a) amplified with primer OPJ 07 (5'CCTCTCGACA3') and (b) OPJ 09 (5'TGAGCCTCAC3'); M- 100bp DNA ladder, Lane 1-38: leaf gel DNA isolated from 38 accessions of *A. barabdensis*.

ACKNOWLEDGEMENTS

The authors gratefully acknowledge the financial assistance of NRCMAP (DMAPR), Boriavi, Anand, Gujarat for providing necessary facilities. The authors are also thankful to Dr. (Ms.) K.A. Geetha, Senior Scientist (Plant breeding) for providing the plant materials for this study.

REFERENCES

[1] A. Kurian, A. Sankar,. Medicinal Plants: Vol.2: Horticulture Science Series, 2007, NIPA, New Delhi.

[2] A. Varma, H. Padh, N. Shrivastava, Plant genomic DNA isolation: an art or a science. Biotechnology Journal, 2007, vol.2:pp.386–392.

[3] B. Doosty, R. Drikvand, E. Salahvarzi, H. Amiri, J. Hadian, Comparative Analysis and Optimization of Different DNA Extraction Protocols in Satureja khuzistanica. International Journal of Biology, 2012, vol4: pp.4.

[4] B. Vazquez, G. Avila, D. Segura, B. Escalante,. Anti-inflammatory activity of extracts from Aloe vera gel, J. Ethnopharmacol, 1996, vol55: 69-75.

[5] C.N. Stewart, L.E. Via, A rapid CTAB DNA isolation technique useful for RAPD fingerprinting and other PCR applications. Biotechniques, 1993, vol19: 394-504.

[6] D.G. Peterson, K.S. Boehm, S.M. Stack, Isolation of Milligram Quantities of Nuclear DNA from Tomato (Lycopersicon esculentum), A Plant Containing High Levels of Polyphenolic Compounds. Plant Mol. Biol. Reptr. 1997, vol15 (2):pp.148-153.

[7] FRH. Katterman, VI Shattuck, An effective method of DNA isolation from the mature leaves of Gossypium species that contain large amounts of phenolic terpenoids and tannins. Prep. Biochem. 1983, vol13:pp.347-359.

[8] G. S. Fang, S. Hammer, R. Grumet, A quick and inexpensive method for removing polysaccharides from plant genomic DNA. Biotechniques. 1992, vol13: pp52-57.

[9] J. Talmadge, J. Chavez, L. Jacobs, C. Munger, T. Chinnah, J.T. Chow, D. Williamson, K. Yates, Fractionation of Aloe vera L inner gel, purification and molecular profiling of activity, Int. Immunopharmacol, 2004, vol4: pp.1757-1773.

[10] J. Zhang, J.M. Stewart, Economical and rapid method for extracting cotton genomic DNA. The Journal of Cotton Science, 2000, vol4: pp193-201.

[11] J.J. Doyle, J.L. Doyle, Isolation of plant DNA from fresh tissue. Focus, 1990, vol12: pp.13-15.

[12] K. Diadema, A. Baumel, M. Lebris, L. Affr, Genomic DNA isolation and amplification from callus culture in succulent plants, Carpobrotus species (Aizoaceae). Plant Mol. Biol. Reptr, 2003, vol21:pp.173a–173e.

[13] K.M. Mishra, S.N. Rani, A.S. Ram, H.L. Sreenath, A simple method of DNA extraction from coffee seeds suitable for PCR analysis. African Journal of Biotechnology, 2008, vol7:pp.409–413.

[14] M. De la Cruz, R. Whitkus, L. Mota-Bravo, Tropical tree DNA isolation and amplification. Mol Ecol, 1995, vol4: pp.787-789.

[15] M. Loomis, Overcoming problems of phenolics and quinones in the isolation of plant enzymes and organelles. Methods Enzymol. 1974, vol 31: pp.528-545.

[16] M. Moyo, S.O. Amoo, M.W. Bairu, J.F. Finnie, J. Van Staden, Optimizing DNA isolation for medicinal plants. South Afr J Bot, 2008, vol74:pp.771-775.

[17] M. Sarwat, M.S. Negi, M. Lakshmikumaran, A.K. Tyagi, S. Das, P.S. Srivastava, A standardized protocol for genomic DNA isolation from Terminalia arjuna for genetic diversity analysis. Ele J.of Biotechnology. 2006, vol9:1:86-91.

[18] M.J. Pikkart, B. Villeponteau, Suppression of PCR amplification by high levels of RNA, Biotechniques. 1993, vol14: pp.24-25.

[19] P. Atherton, The Essential Aloe Vera. Newport Pagnell: Mill Enterprises, 1997.

[20] P. Barnell, A.N. Blanchard, J.A. Bryant, N Smirnoff, A.F. Weir, Isolation of DNA from the highly mucilaginous succulent plant Sedum telephium. Plant Mol. Biol. Reptr, 1998, vol.16:pp.133–138.

[21] S. Jain, Handbook of medicinal plants,1988. Pointer, India.

[22] S. Porebski, L.G. Bailey, B.R. Baum, Modification of a CTAB DNA extraction protocol for plants containing high polysaccharide and polyphenol components. Plant Mol. Biol. Reptr, 1997, vol15 (1): pp.8-15.

[23] S. Samantaray, K.P. Hidayath, S. Maiti, An isolation protocol of genomic DNA from Commiphora wightii (Arnott.) Bhandari: An endangered medicinal plant. Int. J. Integr. Biol. 2009, vol6:3pp.127.

[24] S. Syamkumar, B. Lowarence, B. Sasikumar, Isolation and amplification of DNA from rhizomes of turmeric and ginger. Plant Mol. Biol. Reptr, 2003, vol21:pp.171a-171e.

[25] S.S. Harini, M. Leelambika, M.N. Shiva Kameshwari, N. Sathyanarayana, Optimization of DNA isolation and PCR-RAPD methods for molecular analysis of Urginea indica Kunth, Int. J. Integr. Biol, 2008, vol2: pp.138-144.

[26] T. Reynolds, A.C. Dweck, Aloe vera leaf gel: a review update. J. Ethnopharmacol. 1999, vol68: pp.3-37.

[27] The wealth of India- Raw materials, CSIR, New Delhi, 1985, Vol.1A pp. 191-193.

[28] V. Steenkamp, M.J. Stewart, Medicinal applications and toxicological activities of Aloe products. Pharm. Biol, 2007 vol4:pp.411-420.

[29] Y. Ni, D. Turner, K.M. Yates, I. Tizard, Isolation and characterization of structural components of Aloe vera L. leaf pulp. Int. Immunopharmacol, 2004, vol4: pp.1745-1755.

[30] Y. Ni, I.R. Tizard, Analytical methodology: the gel-analysis of aloe pulp and its derivatives. In Aloes the Genus Aloe; Reynolds, T., ed.; CRC Press: Boca Raton 2004, pp.111-126.

[31] Z. Jabbarzadeh, M Khosh-Khui, H Salehi, A Saberiv, Optimization of DNA extraction for ISSR studies in seven important rose species of Iran. American-Eurasian Journal of Sustainable Agriculture, 2009, vol3(4): pp.639-642.

AUTHORS

First Author – Pushpa Deore, Department of Agricultural Botany, Anand Agricultural University, Anand 388110, Gujarat, India
Second Author – S. Samantaray, Directorate of Medicinal and Aromatic Plants Research, Boriavi, Anand
Third Author – S. Maiti, Directorate of Medicinal and Aromatic Plants Research, Boriavi, Anand