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Optimization of genomic DNA isolation protocol in polyphenols and polysaccharides rich *Withania somnifera* for genetic diversity analysis

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ABSTRACT

Withania somnifera, commonly known as Ashwagandha, is an important medicinal plant that has been used in Ayurvedic and indigenous medicine for over 3,000 years. In view of its varied therapeutic potential, it has also been the subject of considerable modern scientific attention. Ashwagandha has been used as a sedative, a diuretic, a rejuvenating tonic, an anti-inflammatory agent, aphrodisiac and an immune booster. This has led to an excessively high demand of this plant products. However, medicinal plants are known to contain high levels of polyphenols and polysaccharides posing a major challenge in the isolation of high quality DNA. DNA was isolated with the present identified method. Purity and amount of DNA was measured by UV spectrophotometer and agarose gel electrophoresis. The spectral quality of DNA as measured by the A260/A280 ratio ranged from 1.73 to 1.85. Isolated DNA can be used for any molecular analysis.

Keywords: *Withania somnifera;* DNA isolation; Gel electrophoresis; Medicinal plants; Secondary metabolites.
Abbreviations: CTAB, Cetyl-Trimethyl Ammonium Bromide; EDTA, Ethylene diamine tetra acetic acid; PVP, Polyvinyl pyrrolidone; NaCl, Sodium Chloride; D/W,Distil water.

INTRODUCTION

Since the origin of human civilization, plants have been used as a source for medicine and curing human diseases, and are a foremost component of Ayurvedic and Unani medicines. WHO (World Health Organization) has listed more than 21,000 plants having medicinal value. Plants have been a rich source of medicines because they produce a host of bioactive molecules, most of which probably evolved as chemical defences against predation or infection (Cox and Balick 1994). In the developing nations, almost 80% people depend on these plants for medicine because of their easy availability and low cost of treatment. The modern allopathic system of medicine is known to produce serious side-effects and resistance against antibiotics which make these drugs non-potent (Pirttila et al. 2001). India being one of the world's 12 mega biodiversity countries needs to conserve its resources where they are being exploited and should be grown commercially to avoid their susceptibility to extinction because of indiscriminate use. *Withania somnifera* is one of the plants which is exploited world wide in both Ayurvedic and Unani Systems of medicine.

Withania somnifera, also known as Ashwagandha, Asgandh, Indian ginseng, Winter cherry, Ajagandha, Kanaje (Hindi), Amukkara (Tami), is a member of Solanaceae or nightshade family. Ashwagandha is found in central Indian states of Madhya Pradesh, Rajasthan, and parts of Punjab, Himachal Pradesh and Uttar Pradesh.

It is cultivated over an area of about 4000 hectares in Madhya Pradesh covering Manasa, Neemuch, and Jawad tehsils of Mandsaur and Ratlam districts and adjoining villages of Rajasthan (Nigam, 1984). Neemuch in Mandsaur district is a wholesale market for this crop.

Ashwagandha prefers a sub-tropical climate. It is planted during the late rainy season and prefers dry weather for its growth. It grows successfully in sandy loam or light red soils with an annual rainfall of 660-750mm and a soil of pH range 7.5 to 8 is ideal for its growth (Singh *et al*., 1996). The flowering season extends from July to September and ripened fruits are available in December.

Withania somnifera (L.) Dunal extensively use as a medicinal herb in the traditional system of medicine as a rasayana and medhya rasayana. Rasayanas are used to promote health and longevity by increasing defence against disease, arresting ageing process and revitalizing the body in the debilitated conditions, while medhya-rasayana are used to promote memory and intellect. Ashwagandha is used to treat various disorders that affect human health including central nervous system (CNS) disorders, particularly in epilepsy, stress and neuro-degenerative diseases like Parkinson's and Alzheimer's disorders, cerebral ischemia, and even in the management of drug addiction.

The leaves and roots of the plant have medicinal properties. It is known to possess anti -tumour activity, which is mainly because of withanolides present in its roots (Uma devi *et al*., 1993, 1996). Five polyphenolics (gallic, syringic, benzoic, p-coumaric and vanillic acids) and three flavonoids (catechin, kaempferol, and naringenin was found in high concentrations especially in the leaves part, confirming the antioxidant potential and health benefits of *W. Somnifera (*Nadia Alam *et al.*,2011**) (**Saidulu *et al*.,2014**).** The secondary metabolites including polyphenols and flavanoids are not completely removed using common extraction protocols and remain as contaminants in the final DNA preparations (S. Ghaffariyan, 2012). The methods employed for extracting DNA from fresh and dried parts of medicinal plants are however time consuming and generally yield DNA in lesser quantity(Ajmal Iqbal 2013).

The advents of molecular markers have revolutionized the entire scenario of biological sciences. DNA based molecular markers have application in various fields like taxonomy, DNA fingerprinting, genetic diversity analysis, gene tagging, marker assisted selection etc. They can be successfully used to study the genetic diversity among naturally occurring populations and also in those populations where diversity could be conserved. The diversity evaluation has been tremendously empowered by invoking biomolecular techniques like polypeptide and DNA polymorphism profiling which facilitate direct and reliable measurement of genetic divergence. The present objective of our research was to optimize a CTAB based protocol for the extraction of genomic DNA for *Withania somnifera* which is rich in polyphenols and polysaccharides.

MATERIALS AND METHODS

Plant Material and seed sterilization

In the present investigation seeds of Forty six genotypes of *W. somnifera* (L.) Dunal were procured from 'Botanical Garden of Chimanbhai Patel College Of Agriculture', Saradarkrushinagar, Dantiwada Agricultural University, Saradarkrushinagar which were originally collected from different research stations.The details of forty six Ashwagandha germplasms are given below in the table 1.

S.No.	Geno type	origin
1	MWS 311	Mandsaur (M.P.)
2	MWS 316	Mandsaur (M.P.)
3	MWS 226	Mandsaur (M.P.)
4	MWS 205	Mandsaur (M.P.)
5	MWS 322	Mandsaur (M.P.)
6	MWS 302	Mandsaur (M.P.)
7	MWS 201	Mandsaur (M.P.)
8	MWS 217	Mandsaur (M.P.)
9	MWS 329	Mandsaur (M.P.)
10	MWS 309	Mandsaur (M.P.)
11	MWS 101	Mandsaur (M.P.)
12	MWS204	Mandsaur (M.P.)

13	MWS208	Mandsaur (M.P.)
14	RAS 18	Udaipur (Rajasthan)
15	RAS 16	Udaipur (Rajasthan)
16	RAS 21	Udaipur (Rajasthan)
17	RAS 23	Udaipur (Rajasthan)
18	RAS 15	Udaipur (Rajasthan)
19	RAS 33	Udaipur (Rajasthan)
20	RAS 67	Udaipur (Rajasthan)
21	RAS 11	Udaipur (Rajasthan)
22	RAS 29	Udaipur (Rajasthan)
23	RAS 32	Udaipur (Rajasthan)
24	RAS57	Udaipur (Rajasthan)
25	RAS 55	Udaipur (Rajasthan)
26	RAS 65	Udaipur (Rajasthan)
27	IC 286632	NBPGR (New Delhi)
28	IC 283662	NBPGR (New Delhi)
29	IC 283942	NBPGR (New Delhi)
30	IC 283966	NBPGR (New Delhi)
31	IC 310595	NBPGR (New Delhi)
32	IC 310620-A	NBPGR (New Delhi)
33	IC 310320-B	NBPGR (New Delhi)
34	K-86	NA
35	JA-134	Released seed varied
36	JA-20	Cultivated variety
37	MPAS-2	NA
38	MPAS-3	NA
39	MPAS-4	NA
40	MPAS-5	NA
41	MPAS-6	NA
42	MPAS-7	NA
43	MPAS-10	NA
44	MPAS-12	NA
45	MPAS-15	NA
46	MPAS-16	NA

Table 1: List of Ashwagandha germplasm
(NA- Not Available)

Seeds of *Withania somnifera* (L.) Dunal were surface sterilized with 10% ethanol for 1 minute, followed by a 10 minute treatment with aqueous sodium hypochlorite solution and finally three rinses with sterile distil water. For initial germination seeds were grown in petri dish on moist filter paper. After two weeks seeds were transferred in a plastic pot containing sterilized sand.

Reagents and chemicals

DNA Extraction buffer **(**200 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 3.5% CTAB, β-mercaptoethanol (60µ/100 milligram) (v/v) (added immediately before use) and 2% PVP (w/v) (added immediately before use)**,** Wash buffer (100 mM Tris-HCl (pH 8.0), 1% PVP)**,** Phenol: chloroform: isoamyl alcohol (25:24:1)**,** chloroform: Isoamyl alcohol (24:1)**,** Isopropanal**,** 5M NaCl, 70% ethanol**,** High salt TE buffer (1 M NaCl, 10 mM Tris-Cl (pH 8.0) and 1 mM EDTA). TE buffer (Tris HCl (pH 8.0)1M EDTA (disodium, pH 8.0) 0.5 M), Nuclease free water and RNase 2mg/ml.

The chemicals used for Agarose Gel Electrophoresis are 1X TBE buffer-pH 8.0 (Tris 10.8g, Boric Acid 5.5g, EDTA 0.75g (add small pellet of NaOH till it completely dissolve) for one Litre. 6X Loading dye (Bromophenol blue 0.25g, 30% Glycerol, 30ml, D/W 70 ml, Loading dye solution was stored at 4^0C in the refrigerator.

All the chemicals and reagents used in the present investigation for DNA extraction and electrophoresis were of high purity analytical molecular biology grade of HiMedia.

Genomic DNA Isolation

Genomic DNA extracted from medium size leaves from one month old seedlings of *W. somnifera* using CTAB extraction method as reported by Doyle and Doyle (1990) with minor modification. This modification was essential because plant encloses exceptionally high amount of polyphenols, polysaccharides, tannins and other secondary metabolites such as alkaloids, flavanoids, phenols, terpenes and quinines which could interfere in DNA isolation protocol.

Fresh leaves were collected washed with D/W and surface sterilize with Triton X-100 and blotted with tissue paper to remove water. Midrib, vein were removed and chopped into 3-5mm small pieces. Around 1 gram chopped leaf was taken and ground (using prechilled morter and pestle) with 1.5 ml freshly prepared wash buffer and 60µl β mercaptoethanol. The samples were transferred into 2ml autoclaved Eppendorf tubes and centrifuged at 12,000 RPM for 3 min at 40c. The upper aqueous phase was discarded and 1.5 ml wash buffer was added to the pellet,

mixed well and centrifuged as above. The above process was repeated twice with wash buffer. In the next step, 1.5 ml extraction buffer (preheated at 650c) was added along with 0.06gram PVP and 7.5 µl β mercaptoethanol to the remaining pellet and mixed well. The samples were kept for incubation into water bath at 650c for 1 hrs. After incubation tubes were kept at room temperature for 5 min to cool down. The incubated samples were centrifuged at 12,000 RPM for 10 min at room temperature and aqueous phase was transferred into fresh autoclaved tubes. Then equal amount of Phenol: chloroform: isoamyl alcohol (25:24:1) solution was added to the transferred aqueous phase, gently mixed for 1 minute by inversion and centrifuged at 12,000 rpm for 10 min at 40c. Again the aqueous phase was transferred into a fresh autoclaved tube containing equal volume of chloroform: Isoamyl alcohol (24:1) and mixed well for 1 min by inversion. The samples were centrifuged at 12,000 rpm for 10 min at 40c and the aqueous phase was transferred into a fresh autoclaved Eppendorf tube having 5 µl of 5M NaCl and 0.6 volume of prechilled isopropanol. The samples were kept for overnight at -200c for better precipitation. These precipitated samples were centrifuged at 15,000 rpm for 10 min at 40c. The aqueous phase was discarded and the remaining pellets were air dried at room temperature for 3-4 hrs. Add 100 µl of high salt TE buffer and 10 µl of RNase. Keep at 370c for 30 min followed by chloroform: Isoamyl alcohol (24:1) extraction and ethanol precipitation in presence of 3M sodium acetate (pH 5.2). The samples were centrifuged at 12,000 RPM for 10 min at 40c. Extracted pellet was washed using 70% ethanol and centrifuged at 5,000 RPM for 5 min at 40c. The pellet was air dried for 3-4 hrs and 100 µl TE buffer was added and stored at -20 0c for further use.

Estimation of Quantity and Quality of genomic DNA of *Withania somnifera:*

Quality and quantity of DNA was estimated by UV spectroscopy and agarose gel electrophoresis.

Quantity of DNA by UV-Spectrophotometric Estimation

An aliquot of DNA sample was properly diluted and absorbance (A) was determined at 260 nm and 280 nm wavelength in U.V-spectrophotometer. Using the relationship of 1.0 O.D. at 260 nm equivalent to 50 µg DNA per ml, the quantity of DNA was estimated from the following formula:

Concentration of DNA (µg/ml) = A260 x 50 x dilution factor.

Quality of DNA

Quality of DNA samples was checked both by UV-spectrophotometer and on agarose gel electrophoresis. Using spectrophotometer the ratio of the absorbance at 260 nm and 280 nm was noted. Samples with a ratio of 1.8 to 2.0 were considered of good quality.

A260/A280 = 1.8 (pure DNA)

Quality of DNA preparations was also tested by submerged horizontal agarose (0.8%) gel electrophoresis. Appearance of high molecular weight band near the wells and no smear on gels indicated a quite good quality DNA. DNA quantity was also determined on the basis of band intensity as compared with the lambda DNA marker (used to determine the concentration) on agarose gels.

Agarose Gel Electrophoresis

Gel casting tray was washed, air dried and its ends were sealed with the tape. Agarose was melted by boiling in 1X TBE buffer, cooled to 50-55ºC and ethidium bromide at a concentration of (5mg/ml) was added. Melted agarose was poured into gel casting tray containing with an appropriate comb (based on the required number of wells and size of the well). Gel was allowed to solidify for 30 min. After solidification, sealing tapes were removed to allow conduction. Plate was submerged in 1x TBE buffer and comb was removed gently. Samples were prepared by adding 2µl of 6x loading dye and were spin briefly in a micro-centrifuge for proper mixing. DNA samples were loaded in the wells and electrophoresis was carried out at a constant voltage (3V/cm of gel)

till bromophenol blue/loading dye migrated to other end of the gel. The gel was visualized on a UV- transilluminator and photographed.

RESULTS AND DISCUSSION

Published methods of DNA isolation including those of Doyle and Doyle (1990), Rogers and Benedict (1985) and Dellaporta *et. al.* (1983) proved relatively less successful and reliable for *Withania somnifera* (L.) Dunal. The DNA acquired was dirty yellow in appearance and with high viscosity. This is due to high levels of polysaccharides, polyphenols and secondary metabolites like alkaloids, flavonoids, phenols, terpenes etc, present in the medicinal plants which meddle with the DNA isolation and purification procedure.

Secondary metabolites and polysaccharides interfere with total DNA isolation procedures and PCR based downstream applications. The removal of such contaminants needs complicated and time-consuming protocols (Khumallambam Devala Devi, 2013). Polysaccharide contaminations are particularly problematic (Scott and Playford, 1996) as they can inhibit the activity of many commonly used molecular biological enzymes, such as polymerases (Fang et al. 1992), ligases and restriction endonucleases. This is because nucleic acids form tight complexes with polysaccharides creating a gelatinous pellet and the embedded DNA is inaccessible to the enzymes (Sharma et al. 2002). During homogenization, polyphenols are released from vacuoles and they then react rapidly with cytoplasmic enzymes.

DNA isolation protocols generally use CTAB to avoid co-purifying polysaccharides from plant tissues. Therefore, DNA extraction procedure was standardized by using different concentrations of CTAB (2%, 3%, 3.5% & 4%) and PVP (1% and 2%). Use of 3.5 %CTAB, 2% PVP, 1.4 M NaCl and 1 hour incubaton time was found to be most appropriate. Highly purified genomic DNA was obtained when the optimised protocol described in "Materials and Methods" was used. A sufficient amount of clean genomic DNA was obtained with this method (Figure 1). The yield was ranged from 20-50 µg of DNA per gram of leaf sample. The A260/280 nm ratio was around 1.840 (Table 2).

genotype	Quantity (µg/ml)	Quality (A260/A280)
JA-20	1950	1.75
JA-134	1950	1.80
MWS-226	2050	1.81
MWS-205	1950	1.83
MWS-322	1950	1.76
MWS-302	1700	1.82
MWS-201	2000	1.83
MWS-217	1750	1.78
MWS-329	1850	1.79
MWS-309	1700	1.84
MWS-101	1900	1.77
MWS-204	1950	1.80
MWS-208	1250	1.85
RAS-18	2050	1.75
RAS-16	1950	1.83
RAS-21	1800	1.76
CI-286632	2250	1.81
CI-283662	1900	1.78
CI-283942	1.83	1.82
MPAS-15	1950	1.79

Table-2. Estimation of Quantity and quality of DNA

Figure-1. Gel picture of isolated DNA using CTAB method

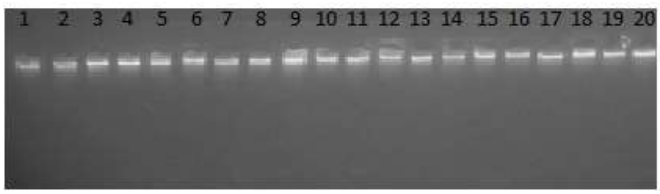

Figure-2. Gel picture of isolated DNA using CTAB modified method.
(JA-20, JA-134, MWS-226, MWS-205,MWS-322,MWS-302,MWS-201,MWS-217,MWS-329,MWS-309,MWS-101,MWS-204,MWS-208,RAS-18,RAS-16,RAS-21,CI-286632,CI-283662,CI-283942, MPAS-15)position from left to right.

Our protocol involves initial repeated washing steps with wash buffer containing PVP. PVP has been used to remove polyphenols from mature, damaged and improperly stored leaf tissues (Rogers and Bendich, 1985; Doyle and Doyle, 1987, Howland et al., 1991). PVP forms complex hydrogen bonds with polyphenolic compounds which can be separated from DNA by centrifugation (Maliyakal, 1992).

Complete digestion with restriction endonucleases and amplification in PCR indicate the absence of polysaccharides. Polysaccharides are difficult to separate from DNA (Murray and Thompson, 1980). The problem arising from the presence of high levels of polysaccharides was overcome by using NaCl at a higher concentration. These compounds are easily identifiable in the DNA preparations as they impart a sticky, viscous consistency to the DNA preparations dissolved in TE buffer. Polysaccharides interfere with several biological enzymes such as polymerases, ligases and restriction endonucleases (Shioda et al., 1987; Richards, 1988). This problem was overcome by using 5M NaCI and high salt TE buffer.

CONCLUSION

Plants are rich source of natural products or bioactive substances produces large amount of secondary metabolites and are valuable for human being. Thus, while working with a plant samples enriched with secondary metabolites it is common to encounter problems arising from the polysaccharides, polyphenols and other secondary metabolites in the DNA preparations. The secondary compounds may hamper DNA isolation. In our experiments we encountered difficulties related to low DNA yield or poor PCR amplification. This protocol provides good quality of genomic DNA which was not reported earlier. This indicated that the isolated DNA was amenable to further processing in cloning experiments as well as DNA finger printing. The method described here for the extraction of genomic DNA will be useful for molecular, genetic diversity, and transgenic studies in *Withania Somnifera*.

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