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DNA-barcoding, SCoT and SRAP Based Somaclonal Variation in Micropropagated *Withania somnifera* Plantlets

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Abstract

Ashwagandha (Withania somnifera) is one of the recognized plant species that considered of most traditional natural supplements. Tissue culture is an efficient method as fast and affordable in plant propagation. Few studies have discussed the genetic impact of such method on ashwagandha plant. The aim of this research was to identify the genetic stability of micropropagated plantlets and to assess the impact of *in vitro*-propagation on somaclonal variability in ashwagandha using start codon-targeted (SCoT), sequence-related amplified polymorphism (SRAP) and DNA-barcoding assays. SCoT marker assay produced a total number of 132 bands with an average of 11 bands per primer, where scorable PCR fragments were generated from all primers. The phylogenetic tree constructed using SCoT binary data, revealed genetic variability among studied plant samples. SRAP primer combinations showed a total of 78 bands by an average of 11.1 bands / combination, in which all combinations produced scored PCR fragments. Over SRAP assay, one specific band was obtained that was present in different ashwagandha micropropagated plant samples compared to the control (mother plant). This PCR fragments were obtained using me1F/em1R primer combination (287 bp). The phylogenetic tree constructed using SRAP data was successful to differentiate between micro-propagated plants and the control. The DNA-barcoding analysis using chloroplast gene RNA polymerasel (rpoCl) gene was used to detect the soma- clonal variation between control and one micro-propagated plant of ashwagandha. The phylogenetic tree constructed using DNA-barcoding sequences was successful to differentiate between the two samples, where control and micropropagated plantlets were grouped in two different groups. This study suggests the valuableness of using SRAP and DNA-barcoding in detecting soma-clonal variation among micropropagated plantlest of ashwagandha.

Keywords: DNA-barcoding; Genetic stability; Medicinal plants; SCoT; SRAP; Tissue culture; *Withania somnifera.*

1. Introduction

The flora of the kingdom of Saudi Arabia (KSA) is among the richest areas of ecosystems and represents a major valuable genetic resource of plants and medicinal herbs [1]. The KSA is distinguished by its large area, showing climatic variability due to height differences, resulting in broad flora variations. Plants of the Saudi flora are highly known for their use in folk and herbal medicine, particularly those of the Sarat and Hejaz mountains, which are characterized by their highly efficient constituent contents [2]. Ashwagandha **(W. somnifera)** is one of the known plant species that distinguish the Saudi flora. Its root extracts are used to treat candidacies, tuberculosis, asthma, infections transmitted by sex, and cancer [3]. For over 3,000 years it has been a significant herb in the Ayurvedic and indigenous medicinal systems of india [4]. Ashwagandha is familiar for its active biological chemicals such as steroidal lactones, alkaloids, and saponins [5]. Ashwagandha had provided numerous natural antioxidants that are responsible for maintaining the antioxidant liver enzymes. It has been used to strengthen the immune system, improve memory and enhance overall health [6]. The antimicrobial capabilities of such a plant species were recorded extensively in the literature [3].

In vitro propagation of Saudi ashwagandha species through tissue culture is a potential method could be used for plant resources maintenance, through maintaining or grow plant cells, tissues or organs under sterile conditions on a nutrient medium, to produce plant clones those are identical to the chosen genotype [7].

Additionally, tissue culture is one of the potential methods, which could be used to expand species diversity. It can be used to induce somaclonal differences varying from easy-to-detect deviations, especially morphological characteristics, to subtle deviations in intensity, bunch, fruit sizes, and chemical content [8]. Such variations require efficient and quick detection method that can be used in commercial propagation process.

Molecular marker analysis is one of the most efficient technologies used to successfully detect soma-clonal variation in plants [9]. Such technology is reliable, cheap and could be used to detect genetic variability on large

scale. The amplified fragment length polymorphism (AFLP), SCoT, SRAP, and DNA barcoding are the most known molecular marker assays that have been used in studying soma clonal varaitaion in medical plants [10, 11]. SRAP marker assay is an easy and effective marker system with many advantages over other systems: flexibility, acceptable throughput, revealing multiple co-dominant markers and tracking open reading frames [12]. Most significantly, it does not require any prior genomic knowledge and has demonstrated its utility in the study of different species of medical plants [13]. On the other hand, SCoT assay is an emerging and successful molecular assay in recent years [14]. SCoT was developed based on the short standard region flanking the ATG start codon in plant genetic DNA, the use of SCoT markers should be more effective due to high annealing temperatures and longer priming length than other random marker assays in particular. By comparison, the marker design of the SCoT assay does not require comprehensive details on the genome, allowing it to be applied to plants without a genomic reference [15]. The application of SCoT molecular assay has been documented in various plant species such as jojoba [16]. Coconut [17], olive [18], and tomato [19].

DNA barcoding involves the sequencing of a standard DNA region as a method of species identification [20]. Several plastid genome regions such as *matK*, *rbcL*, *ropC1*, *rpoB*, and *trnH-psbA* were extensively assessed in the study of different plant species. The plastide gene ropC1 has been used to explore the genetic variability of Calluna [21]. Gongora [22] and Apocynaceae species [23]. Moreover, it has been successfully applied in somaclonal variation in Olea Euroaea L. [24].

This study was carried out to determine the diversity of Saudi ashwagandha species compared to known plant species. Moreover identify the genetic stability and to assess the impact of *in vitro* propagation on somaclonal variability in ashwagandha.

2. Materials and Methods

2.1. Plant Material

Ashwagandha specimen was obtained as a supplier of explants from El Shafa regions (21°05'26.3"N 40°21'03.5"E) in Taif, Saudi Arabia. The shoot tip explants were cleaned with fresh water, decontaminated with alcohol (70%) for one minute, incubated for 5 minutes in 0.1% mercuric chloride (Hg_2Ch), and then washed with sterilized distilled water.

2.2. Micropropagation

With regard to **in vitro** cultivation, the shoot tip explants of the ashwagandha specimen were grown on a shooting initiation medium for 4 weeks [25]. Using the same (Murashige and Skoag (MS) medium supplemented with 3% (w/v) sucrose, 0.7% (w/v) phytoagar and MS medium without growth regulators was used as a control. And for all *in vitro* culture treatments different concentrations and combinations of cytokinins and auxins were used for **in vitro** culture stages. The culture media pH was adjusted to 5.8 using 1.0 N hydrochloric acid and 1.0 N potassium hydroxide before adding phytoagar. After then were sterilized by autoclave at conditions (20 minutes at temperature 121^{o}C and 1.5 k/cm2 pressure.) Shoot proliferation (10 subcultures, five weeks for each), and elongation periods (four weeks) were achieved. For root induction, a length of 4-5 cm of plant shoots was cultivated on the MS medium. The adaption of plantlets was conducted in controlled greenhouse 16/8h light and in 27-36 ± 2^{o}C through growing in 15cm pots containing sterile soil. All cultured plants were incubated under cool white fluorescent light at 26± 2^{o}C, 3000 Lux light intensity, and 16/8h light / dark period at normal opened growth room raks.

2.3. Isolation of DNA

The total genomic DNA was extracted using DNAeasy Plant Mini Kit (Qiagen, Santa Clarita, CA) as guided by the supplier. Approximately 100 mg of plant tissue obtained from one control (mother plant), and 10 micropropagated plantlets samples of ashwagandha were used for DNA extraction. The quantity of DNA was in around (30 pg) and was assessed by loading the extracted DNA of the control and micropropagated plantlets samples (2 plants) on agarose gel (1%) in comparison to 100bp DNA Ladder and the evaluation of the degree of DNA sample fluorescence with the different DNA size marker bands.

2.4. Molecular Marker Assay

Twelve SCoT, seven combinations of SRAP primers and the **rpoCl** gene region were used in this study (Table 1, 2 and 3). The **rpoCl** PCR program (40 cycles) and gene sequencing procedure was conducted as reported by Phong, *et al.* [26]. The content of the SCoT reaction and the program cycles (40 cycles) for PCR amplification were carried out according to Awad, *et al.* [27]. SRAP primer sequences, PCR reaction and amplification program (40 cycles) were acquired from Li, *et al.* [28], Li and Quiros [12]. The final products of the PCR were preserved at 4^{o}C. Agarose gel (1%) was utilized to separate the fragments of PCR and stained with ethidium bromide using 1 kb DNA Ladder (NEB NEBNext® Ultra™). The Gel Doc XR system (Bio-Rad, Hercules, CA, USA) was used to document PCR results.

In order to detect similar species according to **rpoCl** gene of ashwagandha, the National Center for Biotechnology Information, Basic Local Alignment Search Tool nucleotide (NCBI BLASTn) tool was employed to study the orthological genes of various species [29]. Using the ClustalW tool, orthological genes were associated and phylogenetically analysed [30]. The phylogenetic trees which reflect gene relationships were constructed using the ITOL online tool [31]. In addition, the PCR fragments were scored as present (1) or absent (0), and used for similarity matrix coefficients between separate samples through the construction of dendrograms.

Table-1.The SRAP primers combinations

No.	Combination	Forward seq.	Reverse seq.
A	me1F/em1R	TGAGTCCAAACCGGATA	GACTGCGTACGAATTAAT
B	me1F/em2R	TGAGTCCAAACCGGATA	GACTGCGTACGAATTTGC
C	me1F/em3R	TGAGTCCAAACCGGATA	GACTGCGTACGAATTGAC
D	me2F/em1R	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTAAT
E	me2F/em2R	TGAGTCCAAACCGGAGC	GACTGCGTACGAATTTGC
F	me3F/em1R	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTAAT
G	me3F/em2R	TGAGTCCAAACCGGAAT	GACTGCGTACGAATTTGC

Table-2. The rpoC1 gene primer sequence

Primer Code	Sequence	Product Size
rpoC1-F	5'- GGCAAAGAGGGAAGATTTCG -3'	500bp
rpoC1-R	5'- CCATAAGCATATCTTGAGTTGG -3'	500bp

Table-3. The sequence information of SCoT-PCR primers used in this study

Name	Sequence 5'-3'
SCoT-2	CAACAATGGCTACCACCC
SCoT-3	CAACAATGGCTACCACCG
SCoT-4	CAACAATGGCTACCACCT
SCoT-11	AAGCAATGGCTACCACCA
SCoT-12	ACGACATGGCGACCAACG
SCoT-13	ACGACATGGCGACCATCG
SCoT-14	ACGACATGGCGACCACGC
SCoT-16	ACCATGGCTACCACCGAC
SCoT-16	ACCATGGCTACCACCGAC
SCoT-22	AACCATGGCTACCACCAC
SCoT-23	CACCATGGCTACCACCAG
SCoT-35	CATGGCTACCACCGGCCC
SCoT-36	GCAACAATGGCTACCACC

3. Results

3.1. SCoT Molecular Marker Assay

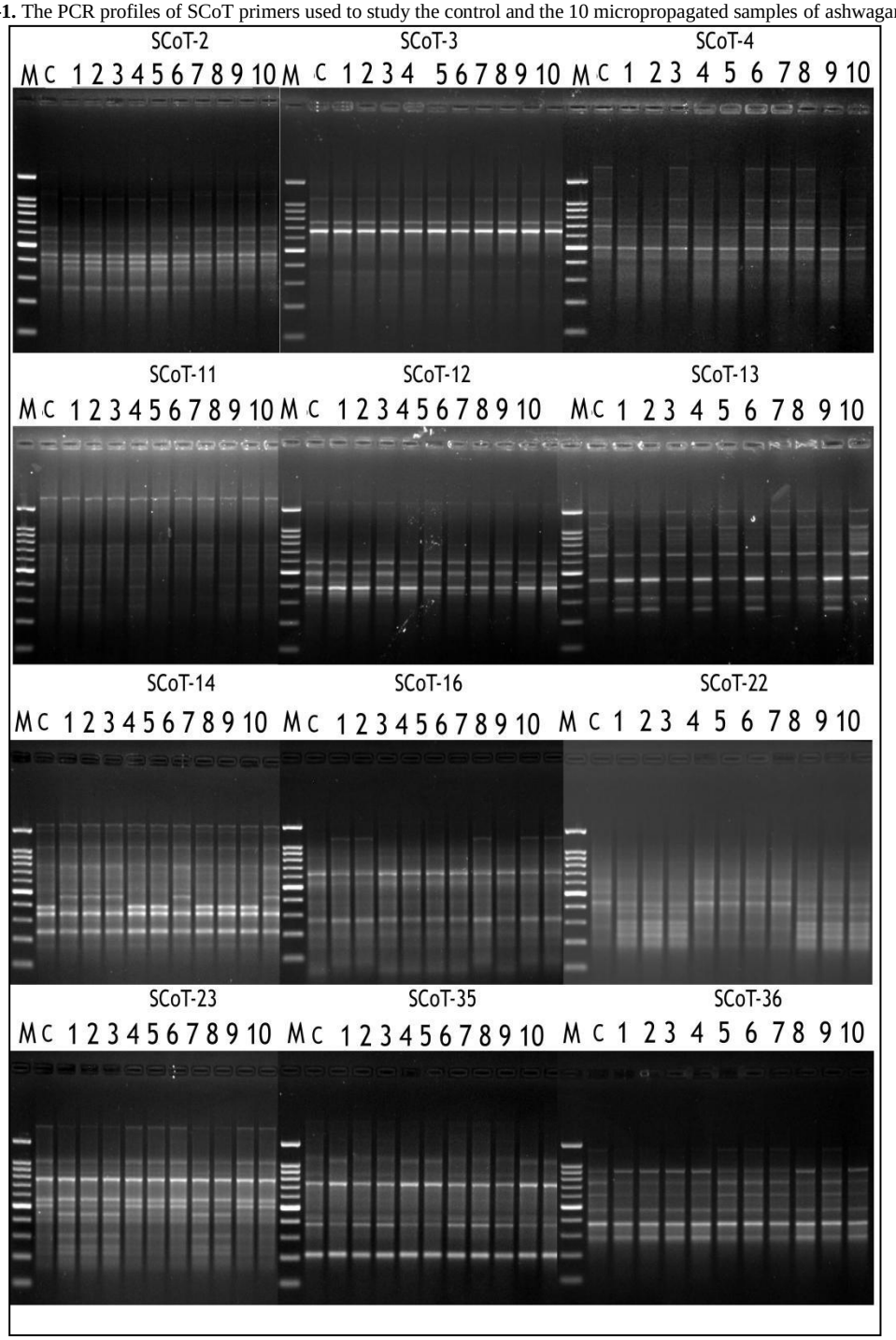
In this study, SCoT marker assay produced a total number of bands of 132 with an average of 11 bands per primer, where scorable PCR fragments were generated from all primers (Fig. 1 and Table 4). The SCoT-PCR bands ranged from 7 (SCoT-3) to 16 (SCoT14). About 8 polymorphic bands has been collected using SCoT primers, of which SCoT-14 produced two band, with a polymorphism percentage of 0.12% (Table 4).

Table-4. The primer name (PN), total number of PCR bands (TB), monomorphic bands (MB), polymorphic bands (PB), and the (PP) polymorphism percentage

PN	TB	MB	PB	PP	PN	TB	MB	PB	PP
SCoT2	9	9	0	0.00%	SCoT35	9	9	0	0.00%
SCoT3	7	7	0	0.00%	SCoT36	9	9	0	0.00%
SCoT4	11	10	1	9.00%	me1F/em1R	12	9	3	25.00%
SCoT11	10	9	1	10.00%	me1F/em2R	9	9	0	0.00%
SCoT12	11	10	1	9.00%	me1F/em3R	13	12	1	8.00%
SCoT13	14	13	1	7.00%	me2F/em1R	14	14	0	0.00%
SCoT14	16	14	2	12.00%	me2F/em2R	12	11	1	8.00%
SCoT16	11	10	1	9.00%	me3F/em2R	8	8	0	0.00%
SCoT22	12	11	1	8.00%	Com-8	10	8	2	20.00%
SCoT23	13	13	0	0.00%					

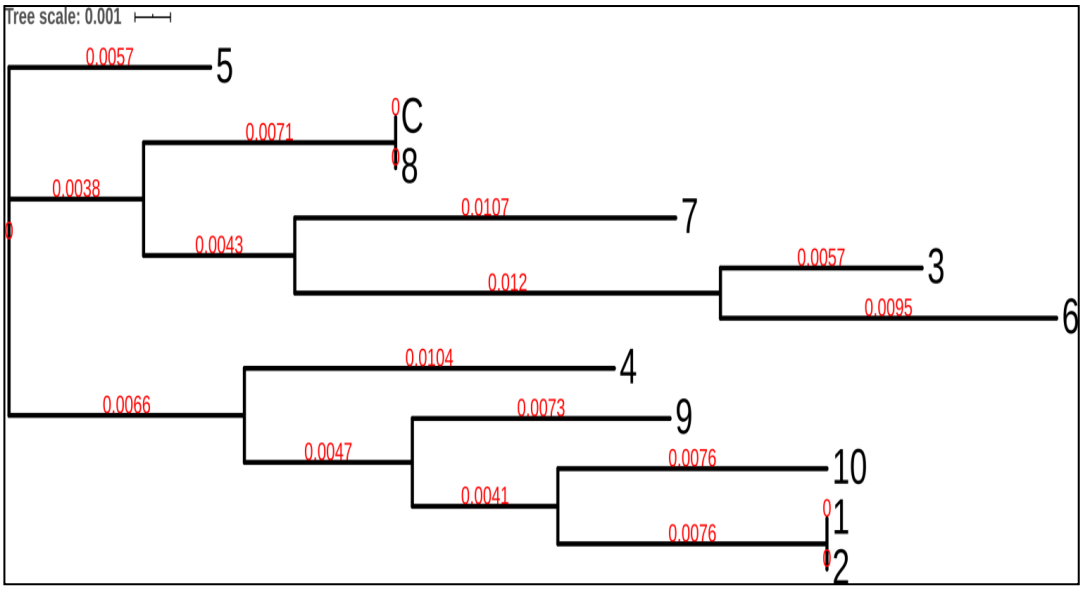
Through SCoT assay, no unique bands were retrieved in different micropropagated plantlets ashwagandha samples compared to the control (Fig. 1).

Figure-1. The PCR profiles of SCoT primers used to study the control and the 10 micropropagated samples of ashwagandha



The phylogenetic tree constructed using SCoT binary data, revealed genetic variability among studied plant samples (Fig. 2).

Figure-2. The Dendrogram constructed using SCoT molecular assay data of ashwagandha control and 10 micropropagated plantlets samples

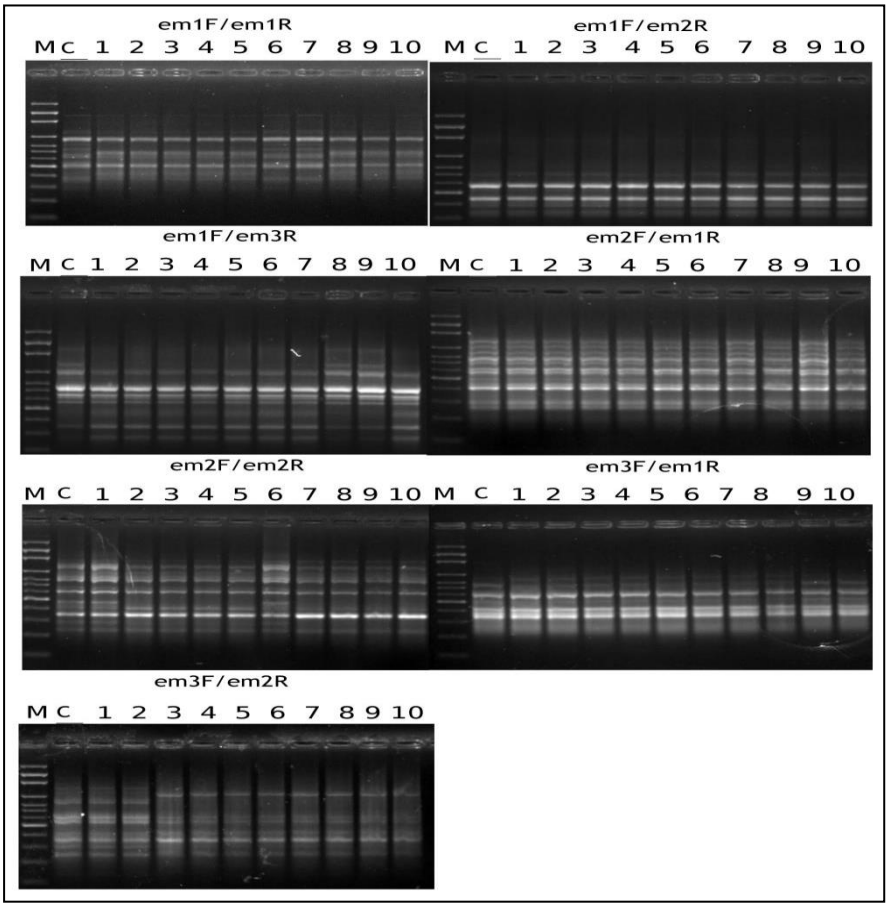


The Dendrogram was divided into three distinct clusters, where sample 5 is divided into one branch. Control, 8, 7, 3 and 6 samples were clustered together, while samples remaining were clustered in a separate cluster. According to SCoT assay, low variation has been detected among studied samples, which indicate low effect of micropropagation on the genetic content of ashwagandha or the low ability of SCoT assay to detect genetic variation.

3.2. SRAP molecular marker assay

SRAP primer combinations showed a total of 78 bands by an average of 11.1 bands every combination, in which all combinations produced scored PCR fragments (Fig. 3).

Figure-3. The PCR profiles of SRAP primers used to study the control and the 10 micropropagated plantlets samples of *Withania somnifera*



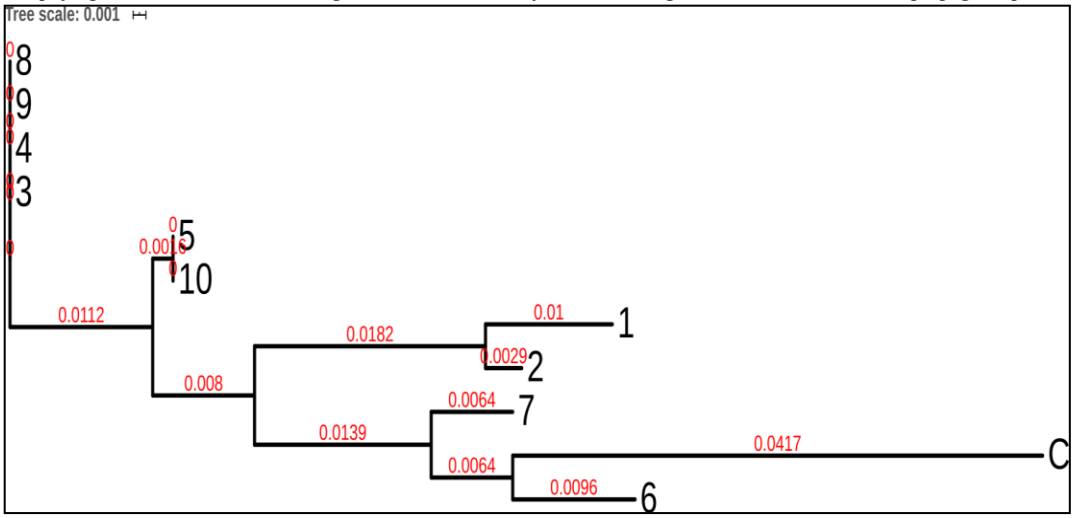
The primer combination me3F/em2R generated the minimum amount of PCR bands (8), so that the maximum was produced by the me2F/em1R (14). Seven polymorphic bands were collected using SRAP primers, of which me2F/em1R, me3F/em2R and me1F/em2R combinations did not produce polymorphic bands and me1F/em1R

produced 3, with a polymorphic ratio of 25% (Table 4). Over SRAP assay, we could obtain one specific band that is present in different ashwagandha micropropagated plantlets samples and is absent on control. This PCR fragments were obtained using me1F/em1R primer combination (287 bp).

In Ashwagandha SRAP molecular marker assay the combinations of SRAP primer showed a total of 78 bands. The primer combination me3F/em2R generated the minimum amount of PCR bands (8), so that the maximum was produced by the me2F/em1R (14). Seven polymorphic bands were collected using SRAP primers, and me1F/em1R produced 3, with a polymorphic ratio of 25%. Over SRAP assay, we could obtain one specific band that is present in different ashwagandha micropropagated plantlets samples and is absent on control. This PCR fragments were obtained using me1F/em1R primer combination (287 bp). Additionally, SRAP molecular marker assay has been successfully utilized for studying somaclonal variation in micropropagated Banana and used for its variants but with greater number of amplified fragments of about 1463 total fragments were amplified during the analysis.

This study revealed that, the phylogenetic tree constructed using SRAP data was successful to differentiate between micro-propagated plants and mother plant (Fig. 4).

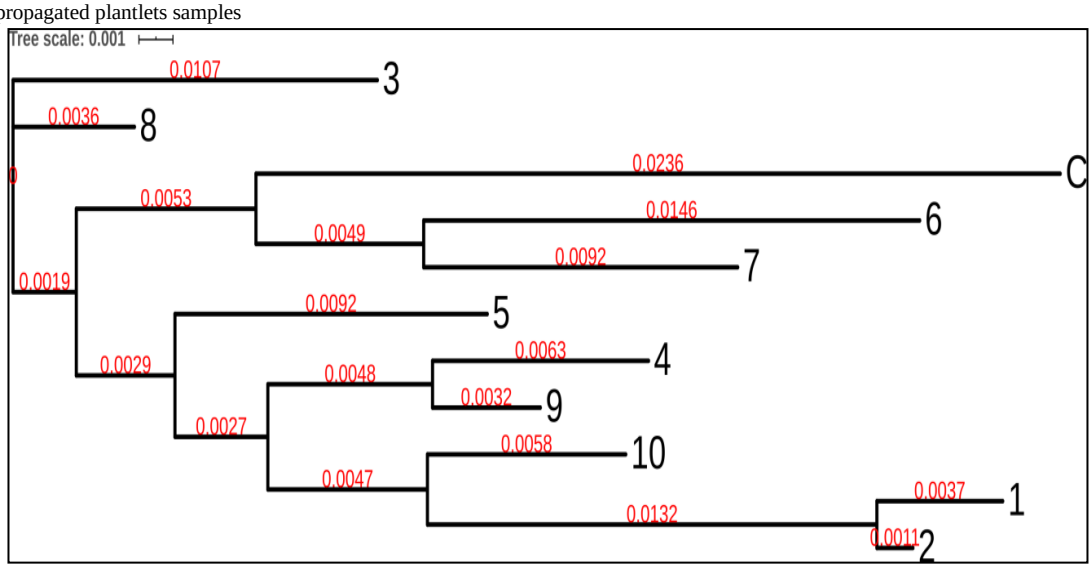
Figure-4. The phylogenetic tree constructed using SRAP molecular assay data of ashwagandha control and 10 micropropagated plantlets samples



The control (mother plant) differed with 0.0417 from other studied micropropagated plantlets samples. The phylogenetic tree was also divided into two clusters, where samples 8, 9, 4 and 3 were grouped into one cluster and some other plants in another (Fig. 4). Such structure could indicate a high ability of SRAP assay to detect somaclonal variation among ashwagandha samples.

Combined data of SCoT and SRAP: The binary data of SCoT and SRAP assays were combined in order to construct more efficient phylogenetic tree (Fig. 5). The tree of combined data was more effective in differentiating between control and micropropagated plantlets compared with the phylogenetic tree built solely using data of SCoT and SRAP (Fig. 5).

Figure-5. The phylogenetic tree constructed using the combined data of SRAP and SCoT molecular assays of ashwagandha control and 10 micropropagated plantlets samples



The plant control, 6, and 7 samples were grouped in one cluster by the phylogenetic tree, which is closer to the tree root (Fig. 5). Such result could indicate the need for more than one molecular assay in detecting micro-propagated ashwagandha plants.

3.3. DNA-Barcoding Analysis Using rpoCl gene

The DNA-barcoding analysis using **rpoCl** gene was used to detect the soma-clonal variation between control and one micro-propagated plantlets of ashwagandha. The NCBI-BLAST results indicate that both sequences are of high similarity to **W. coagulans** plant species (Fig. 6 and 7).

Figure-6. The NCBI-BLAST result of the rpoC1 gene sequence recovered from the ashwagandha control plant.(A) sequence information, (B) similar species and blast result, (C) shared sequences region, (D) sample sequence alignment between ashwagandha and similar species

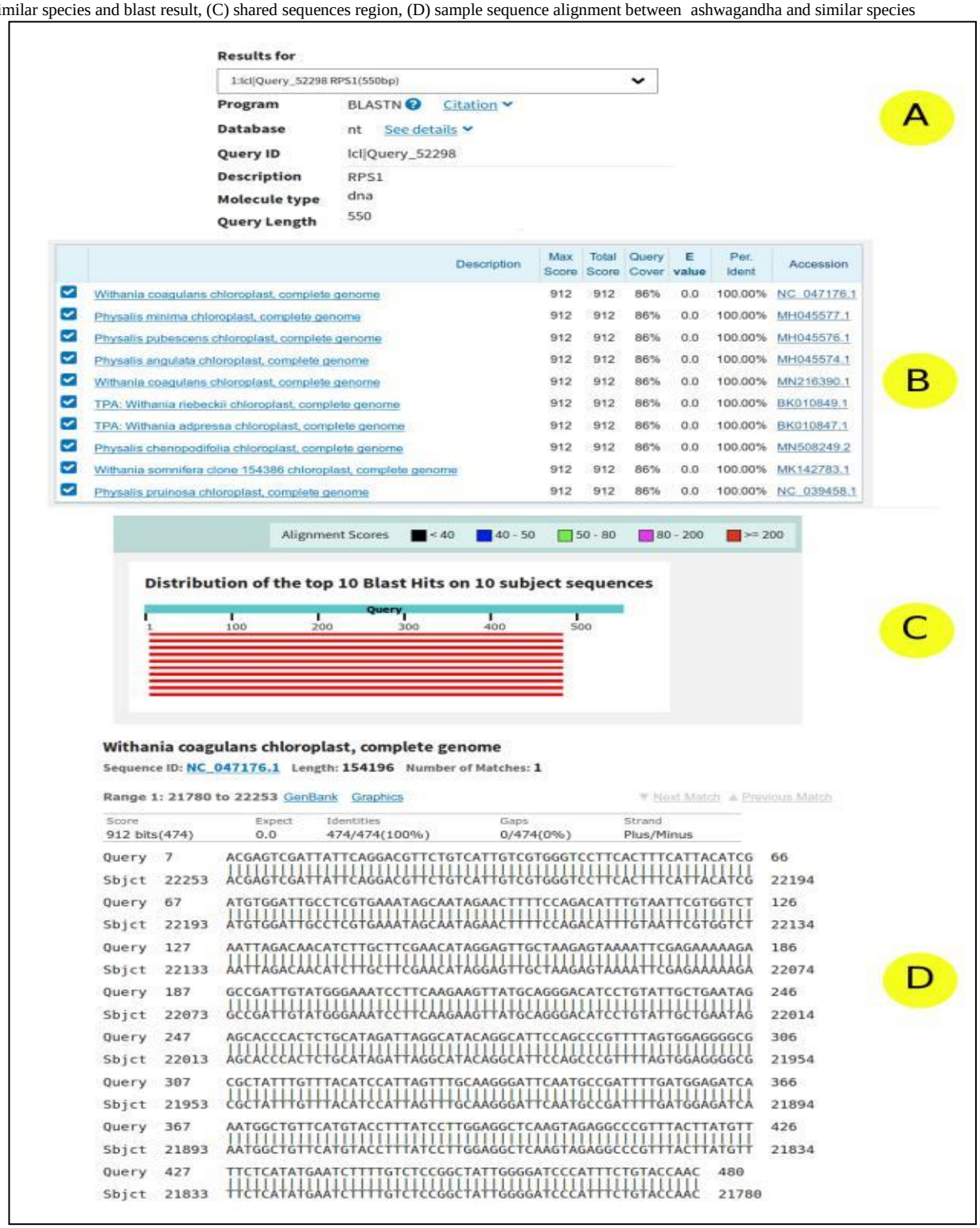
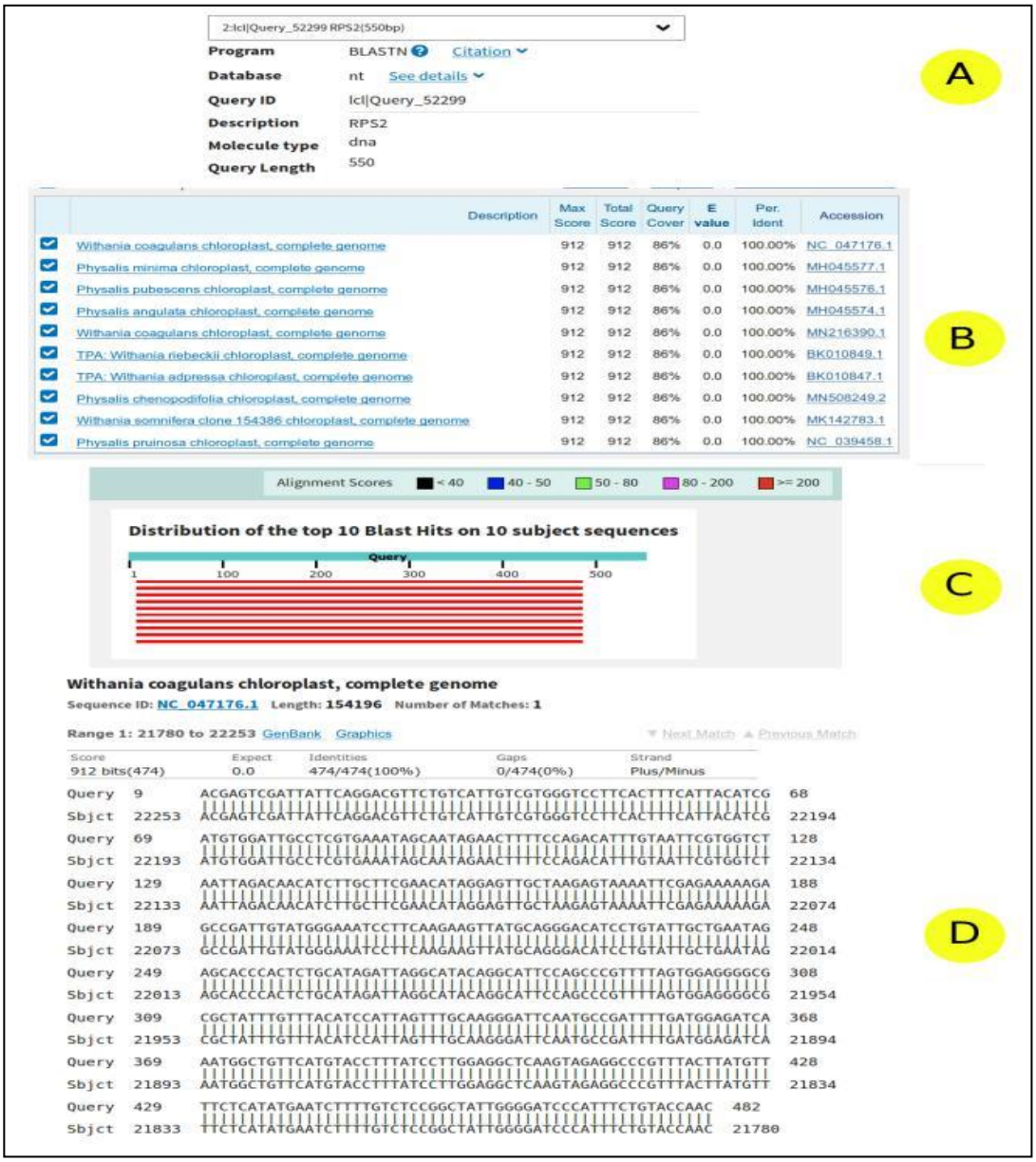
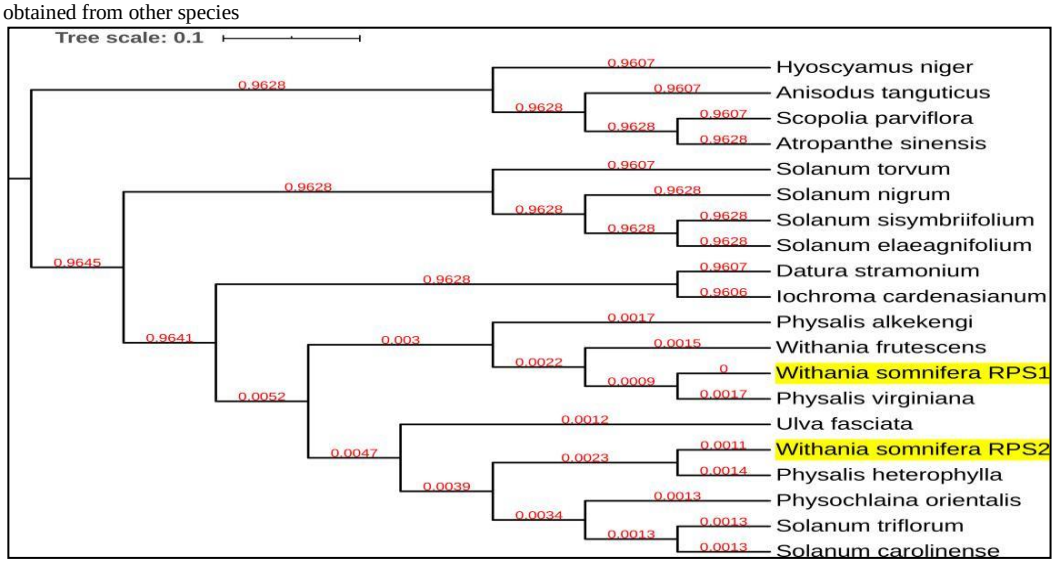


Figure-7. The NCBI-BLAST result of the rpoC1 gene sequence recovered from the ashwagandhamicropropagated plantlets . (A) sequence information, (B) similar species and blast result, (C) shared sequences region, (D) sample sequence alignment between ashwagandha and similar species



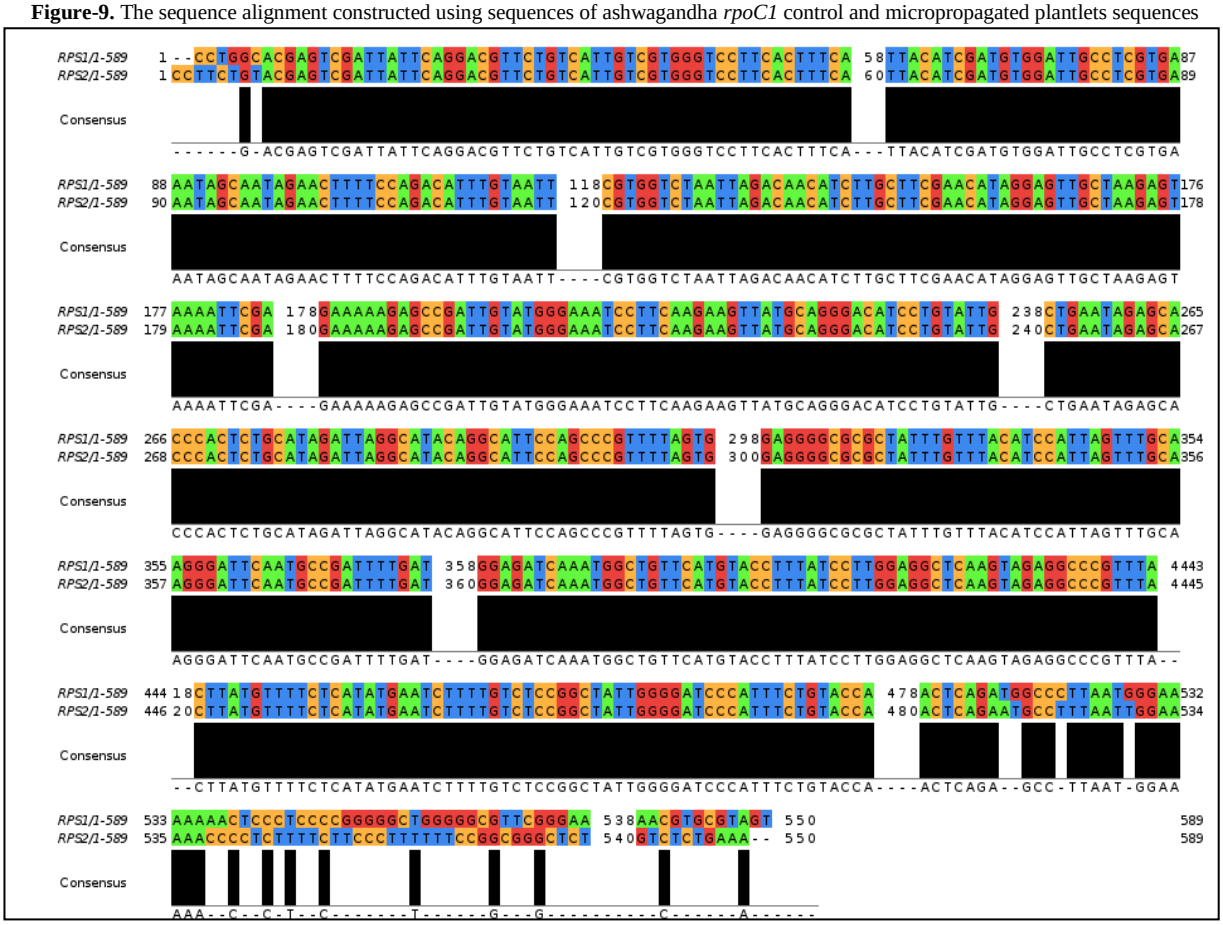
The phylogenetic tree constructed using ashwagandha **rpoCl** sequences and the most similar sequences obtained from other species (Fig. 8). The phylogenetic tree was successful in differentiated between the two samples, where control and micropropagated plantlets were grouped in two different groups (Fig. 8).

Figure-8. phylogenetic tree constructed using sequences of ashwagandha rpoC1 control, micropropagated plantlets and the most similar sequences obtained from other species



The sequence alignment of the two sequences showed several single nucleotide mutations between control and micropropagated **rpoCl** sequences (Fig. 9).

Figure-9. The sequence alignment constructed using sequences of ashwagandha *rpoC1* control and micropropagated plantlets sequences



Such results could indicate the efficacy of the DNA barcoding assay in the differentiation between control and micropropagated plantlets and its ability to detect genetic variability caused by the micropropagation protocol.

4. Discussion

The mirco-propagation system could produce soma-clonal variability between all the subclones from one plant source. In the **in vitro** regenerated plants there were some somaclonal variations [32]. Variations occurrence during **in vitro** clonal production depends upon the pathway of regeneration and the source of explants [33]. The most commonly used method to produce clonal plants is mass multiplication from meristematic tissues and now is well-established fact [34]. It has been reported that plantlets obtained from **in vitro** cultivation can have soma-clonal variations which are sometimes inherited [35]. The genetic stability of the tissue cultured-raised plants could be assessed by number of molecular markers. However, the genetic fidelity of regenerated plants could not be completely guaranteed by one kind of marker analysis alone [36]. Palombi and Damiano [37], also reported using more than one DNA marker analysis for detecting genetic fidelity or variability usefulness. Therefore SCoT, SRAP and *rpoCI*-barcoding techniques were used to investigate the implications of somaclonal variation produced through the **in vitro** propagation of ashwagandha. These molecular marker assays are used to evaluate the genetic stability of micropropagated ashwagandha plantlets.

In Ashwagandah case, SCoT marker assay produced a total number of bands of 132 with an average of 11 bands per primer, where scorable PCR fragments were generated from all primers. The SCoT-PCR bands ranged from 7 primers. About 8 polymorphic bands has been collected using SCoT primers, of which SCoT-14 produced two band. Through SCoT assay, no unique bands were retrieved in different micropropagated plantlets ashwagandha samples compared to the control.

At the same trend studying the genetic stability in micropropagated **Cleome gynandra** using SCoT analysis provided a total of 65 fragments using SCoT 15 primers with an average of 4.3 varying from 27 per primer. Additionally, there was no polymorphism in regenerated plants and in the mother plant, which indicates the genetic consistency of plantlets grown **in vitro** [9].Similarly, utilizing SCoT assay in studying the genetic homogeneity of micropropagated **Alhagi maurorum** revealed that the PCR products were monomorphic through all studied micropropagated plants and mother plant [38]. While, during the analysis of genetic stability among micropropagated **Ansellia africana** using SCoT markers, provided a total of 70 fragments those generated by sixteen producible SCoT primers, five of which were found to be polymorphic with a polymorphism value of 7.14% [39]. In this study, the phylogenetic tree constructed using SCoT binary data, revealed genetic variability among studied plant samples. The phylogenetic tree was divided into three distinct clusters, where sample 5 is divided into one

branch. Control, 8, 7, 3 and 6 samples were clustered together, while samples remaining were clustered in a separate cluster. According to SCoT assay, low variation has been detected among studied samples, which indicate low effect of micropropagation on the genetic content of ashwagandha or the low ability of SCoT assay to detect genetic variation.

Somaclonal Banana variants using 17 SRAP primers. Some SRAP markers were able to differentiate a few of the somaclones plants [40].SRAP markers were used to determine the genetic stability of some micropropagated **Musa** species. Four SRAP primers amplified 16 bands, where all the bands produced were monomorphic in micropropagated plants relative to the mother plants; with the exception of the me1+em2 combination, which generated one polymorphic [41]. Additionally, through investigating the somaclonal variation of geranium the genetic differences between new clones and the mother plant were identified through pairs of SRAP primers [42].

The phylogenetic tree constructed in this study that revealed, using SRAP data was successful to differentiate between micro-propagated plants and mother plant. The control (mother plant) differed with 0.0417 from other studied micropropagated plantlets samples. The phylogenetic tree was also divided into structure could indicate a high ability of SRAP assay to detect somaclonal variation among ashwagandha samples.

4.1. Combined data of SCoT and SRAP

The binary data of SCoT and SRAP assays were combined in order to construct more efficient phylogenetic tree. The tree of combined data was more effective in differentiating between control and micropropagated plantlets compared with the phylogenetic tree built solely using data of SCoT and SRAP. The plant control, 6, and 7 samples were grouped in one cluster by the phylogenetic tree, which is closer to the tree root. Such result could indicate the need for more than one molecular assay in detecting micro-propagated ashwagandha plants.

4.2. DNA-Barcoding Analysis Using rpoCl Gene

The DNA-barcoding analysis using **rpoCl** gene was used to detect the soma-clonal variation in micro-propagated plantlets of ashwagandha. The NCBI-BLAST results indicate that both sequences are of high similarity to **W. coagulans** plant species. The phylogenetic tree constructed using ashwagandha **rpoCl** sequences and the most similar sequences obtained from other species. The phylogenetic tree was successful in differentiated between the two samples, where control and micropropagated plantlets were grouped in two different groups. The sequence alignment of the two sequences showed several single nucleotide mutations between control and micropropagated **rpoC1** sequences**.**

5. Conclusion

The genetic impact of the micropropagation process on the variability of ashwagandha was examined. Such soma-clonal variation required the use of more advanced molecular assays. SCoT, SRAP and DNA-barcoding have shown their usefulness in tracking such genetic variability, both SRAP and DNA barcoding assays were more efficient than SCoT in the identification of somaclonal variability. The present study; suggests the advantage of using SRAP and DNA-barcoding in detecting soma-clonal variation among ashwagandha explants.

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