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Molecular Analysis in Ashwagandha (*Withania somnifera* L.) Influenced by the Mutagenic Effect of Colchicine

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ABSTRACT

The genetic study of the Ashwagandha plant has gained significant importance in recent times. This research aimed to investigate the preliminary effects of colchicine on Ashwagandha and document the findings. Colchicine was applied at concentrations of 0.2%, 0.3%, and 0.5% for durations of 12, 24, and 36 h. The results revealed notable differences across the two generations analyzed. Molecular markers generated using SCoT-PCR produced a total of 260 bands, with an average of 28.89 bands per primer. The phylogenetic tree constructed from SCoT data successfully distinguished treated plants from the control group. Morphological observations, combined with molecular data, demonstrated the effectiveness of SCoT-PCR in detecting the effects of colchicine treatments and identifying beneficial mutants in Ashwagandha. Based on the findings, a 0.5% colchicine concentration is recommended for inducing efficient mutations and achieving genetic improvement in Ashwagandha.

Introduction

Withania somnifera L., commonly known as Ashwagandha, is a significant perennial plant belonging to the Solanaceae family. It is sometimes referred to as "winter cherry" (Tripathi et al., 1996; Andallu and Radhika, 2000) or "Indian Ginseng" in India (Singh et al., 2001). This green, upright, branching evergreen shrub typically grows to a height of 30 to 60 cm and thrives in dry, arid soils (Patra et al., 2004), particularly in acidic conditions (Obidoska et al., 2003). Mutation breeding is a rapid and effective approach for enhancing self-pollinated crops. Locally adapted genotypes undergoing mutation have demonstrated improved agronomic performance and enhanced quantitative traits (Dhulgande et al., 2010). Mutagens are essential tools in crop improvement, as they help induce mutations and measure the frequency and pattern of genetic alterations in selected plants.

Colchicine, a commonly used mutagenic agent, inhibits microtubule formation and is frequently employed to induce polyploidy by doubling chromosome numbers. As a "mitotic poison," colchicine causes significant mutagenic effects, influencing plant function in various ways (Balkanjieva et al., 1980; Castro et al., 2003). This alkaloid is extracted from the seeds or corms of the autumn crocus (*Colchicum autumnale* L.) (Levy et al., 1991; Ben-Chetrit and Levy, 1998) and is widely used in mutation breeding. Notably, colchicine-induced mutations can introduce new traits or restore lost traits following prolonged cultivation (Kharkwal and Shu, 2009). Molecular analysis is a valuable tool for identifying genetic

variation at the molecular level and assessing the mutagenic effects of colchicine (El-Nashar and Ammar, 2016). Molecular markers, which provide reliable and precise measurements of genetic diversity, also help establish phylogenetic relationships among species or variants (Luo et al., 2012). Different molecular marker platforms vary in cost, automation, coverage, repeatability, and reliability (Agarwal et al., 2008).

Several marker systems have been employed to assess genetic variation induced by colchicine. For instance, Random Amplified Polymorphic DNA (RAPD) markers have been used to study genetic variation in colchicine-treated plants (Hassan et al., 2019; Castro et al., 2003). Amplified Fragment Length Polymorphism (AFLP) markers have been applied to investigate genetic diversity and interrelationships among *Withania* species (Negi et al., 2000). Other molecular markers, including Sequence-Related Amplified Polymorphism (SRAP), Start Codon Targeted (SCoT) markers, and DNA barcoding, have been used to explore somaclonal variation in medicinal plants and assess genetic diversity among species and varieties (Roostika et al., 2016; Eloi et al., 2017). This study aimed to evaluate the effects of colchicine treatments on the growth characteristics of *W. somnifera* L., both quantitatively and qualitatively, while also analyzing changes in its chemical composition.

Material and methods

Plant materials

W. somnifera seeds were acquired from the National Research Center in Dokki. The seeds were used for seedling production.

Plant methods

Three concentrations of colchicine (0.2%, 0.3%, and 0.5%) were used for mutagenizing Ashwagandha seeds, alongside a control group. The seeds were exposed to the treatments for three durations: 12, 24, and 36 h. The experiment was conducted during the summer season. Seeds were initially planted in trays using a mixture of well-rotted yard manure and agricultural clay soil. One month after sowing, the seedlings were transplanted into the field. Fertilizer containing NPK (25-25-0) (Desai et al., 2017) was applied at intervals of 60 d throughout the growing season. The study followed a completely randomized design to ensure robust results. The collection and handling of plant materials adhered to all relevant institutional, national, and international guidelines and legislation.

DNA extraction

DNA was isolated from the young leaves of the different samples using the CTAB method (Saghai-Maroof et al., 1984).

PCR analysis

In the present investigation, nine start codon targeted (SCoT) polymorphism markers were employed to characterize the different samples at the DNA level (Xanthopoulou et al., 2015; Bhawna et al., 2017). The primers were synthesized by Macrogene Corp, USA (Table 1).

The PCR reaction was performed in a 20 µL volume containing the following components: 1 µL of DNA template, 1 µL of primer DNA (20 µM), 10 µL of Taq DNA polymerase mix (including 20 mM Tris buffer, 100 mM KCl, 0.1 mM EDTA, 1 mM DTT, glycerol, $(NH_4)_2SO_4$, KCl, dNTPs, gel loading dyes, and $MgCl_2$), and 8 µL of sterile distilled water. The SCoT PCR amplification protocol was as follows: an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 50 s, annealing at temperatures ranging from 49 °C to 53 °C for 1 min, and an extension at 72 °C for 2 min. The final extension step was carried out at 72 °C for 5 min. The amplified PCR products were resolved on a 1.2% agarose gel containing ethidium bromide (0.5 mg mL^{-1}) and visualized under UV light using a transilluminator. Banding patterns obtained from ten SCoT primers were analyzed using a Gel Analyzer software version 3 to record the presence (1) or absence (0) of bands. The resulting binary data were processed using the Multi-Variate Statistical Package (MVSP) software version 3.22. A dendrogram illustrating the relationships among different samples was constructed using the UPGMA method, based on Nei and Li's coefficient (Nei and Li, 1979).

Table 1. The code and sequence of the nine primers used in the SCoT detection of polymorphism and their corresponding annealing temperatures.

Primer name	The sequence from 5' to 3'	Temperature of annealing °C	Primer	The sequence from 5' to 3'	Temperature of annealing °C
SCOT13	ACGACATGGCGACCATCG	52	SCOT61	CAACAATGGCTACCACCG	49
SCOT14	ACGACATGGCGACCACGC	50	SCOT70	ACCATGGCTACCAGCGCG	51
SCOT26	ACCATGGCTACCACCGTC	50	SCOT71	CCATGGCTACCACCGCCG	50
SCOT31	CCATGGCTACCACCGCCT	50	SCOT77	CCATGGCTACCACTACCC	53
SCOT52	ACAATGGCTACCACTGCA	50			

Results

Polymorphism as detected by SCoT analysis

In this study, SCoT markers were used for evaluating genetic changes at the molecular level in Ashwagandha mutants treated with colchicine. Genetic variability was assessed using nine SCoT markers, selected for their ability to rapidly detect mutations. SCoT markers target the short conserved regions of plant genes surrounding the ATG translation initiation codon, making them particularly suitable for identifying DNA alterations induced by colchicine. The nine SCoT primers produced a total of 260 bands, with the number of bands per primer ranging from 20 (SCoT 31) to 38 (SCoT 71) (Fig. 1, Table 2). The highest number of bands was observed with SCoT 71. Of the 260 bands, 244 were polymorphic, with the number of polymorphic bands per primer ranging from 18 to 35, and an average of 27.11. The overall polymorphic percentage was 93.85%, varying between 85% for SCoT 26 and 100% for SCoT 13, SCoT 14, SCoT 52, and SCoT 70 (Table 2). This high polymorphism percentage highlights the effectiveness of SCoT markers in detecting colchicine-induced genetic variations.

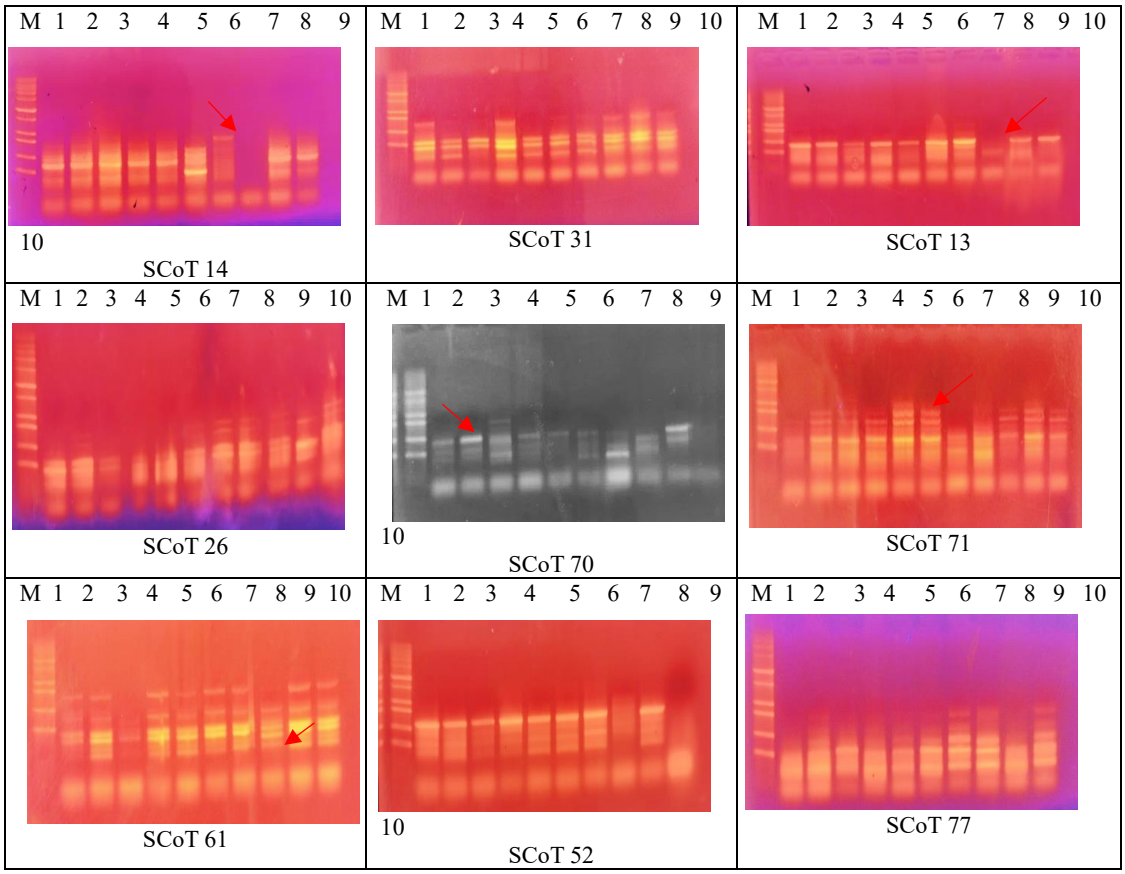

Fig. 1. SCoT profiles of ten Ashwagandha treatments amplified with SCoT primers (lane M = 1 kb marker, 1 = control, 2, 3, 4 = 0.2% colchicine/12, 24, 36 h. 5, 6, 7 = 0.3% colchicine/12, 24, 36 h. 8, 9, 10 = 0.5% colchicine/12, 24, 36 h).

Table 2. Primer name, the total number of bands, number of monomorphic bands, number of polymorphic bands and the percentage of polymorphism as revealed by nine SCoT primers among the treatments of colchicine.

No.	Primer name	Total number of bands	Monomorphic bands	Polymorphic bands	Polymorphism (%)
1	SCoT13	21	0	21	100%
2	SCoT14	29	0	29	100%
3	SCoT26	28	4	24	85%
4	SCoT31	20	2	18	90%
5	SCoT52	33	0	33	100%
6	SCoT61	27	3	24	88.88%
7	SCoT70	28	0	28	100%
8	SCoT71	38	3	35	92.10%
9	SCoT77	36	4	32	88.88%
	Total	260	16	244	93.85%
	Average	28.88	1.77	27.11	

As presented in Table 3, a total of eight unique SCoT markers were identified across the ten treatments. The 0.3% colchicine concentration with a 36-h exposure produced the highest number of unique markers (three bands), followed by the 0.2% concentration with a 24-h exposure, which yielded two bands. Additionally, one unique band was identified in the treatments 0.5%/12 h, 0.3%/12 h, and 0.3%/24 h. The molecular weights of these unique bands ranged from 0.394 to 5.293 kbp. Notably, only five of the treatments produced unique markers, with the 0.3%/36-h treatment standing out as the most distinctive, producing three unique markers. In contrast, four treatments did not exhibit any unique markers (Fig. 1).

Table 3. Treatment name, primer names, marker band size, and total number of markers per treatment for the ten Ashwagandha treatments characterized by unique SCoT markers.

Treatment name	Primer name	marker band size	Number of bands
0.3/36	SCoT13	4.846, 3.764,	3
	SCoT14	1.666	
0.3/24	SCoT14	0.782	1
0.5/12	SCoT61	2.052	1
0.2/24	SCoT70	5.293, 4.757	2
0.3/12	SCoT71	5.223	1

Across the treatments, the highest genetic similarity (0.845) was revealed between the 0.3/12 and 0.2/24 h treatments. Meanwhile, the lowest genetic similarity (0.5) for SCoT was between 0.5/12 h and the control (Table 4). This similarity matrix was used for generating a dendrogram using the UPGMA method.

Table 4. Similarity matrix from the nine SCoT analysis.

	control	0.2/12	0.2/24	0.2/36	0.3/12	0.3/24	0.3/36	0.5/12	0.5/24
0.2/12	0.789								
0.2/24	0.710	0.712							
0.2/36	0.776	0.769	0.754						
0.3/12	0.719	0.827	0.727	0.845					
0.3/24	0.685	0.810	0.720	0.825	0.831				
0.3/36	0.620	0.683	0.575	0.718	0.747	0.810			
0.5/12	0.500	0.563	0.516	0.687	0.656	0.685	0.648		
0.5/24	0.710	0.712	0.625	0.783	0.788	0.800	0.630	0.581	
0.5/36	0.554	0.711	0.537	0.667	0.783	0.718	0.632	0.646	0.746

As shown in Figure 2, the dendrogram revealed distinct clustering among the treatments. The 0.5% colchicine concentration with a 12-h exposure (0.5/12 h) formed a separate cluster, distinct from the other nine treatments. The second cluster, containing the remaining nine treatments, was further divided into two main subclusters. The first subcluster included only the 0.5/36 h treatment. The second subcluster was divided into two groups: the first group contained the 0.3/36 h treatment as a separate branch, while the second group was further divided into two subgroups. The first subgroup separated the 0.2/24 h treatment, while the second subgroup distinguished the control group from two additional classes. One class contained only the 0.5/24 h treatment, while the other was further divided into two subclasses. The first subclass grouped three treatments (0.3/24 h, 0.3/12 h, and 0.2/36 h), and the second subclass contained the 0.2/12 h treatment as a distinct branch.

Discussion

Colchicine is a mutagen commonly used on aromatic and ornamental plants to develop new cultivars with distinct phenotypic and cytogenetic traits (Cabahug et al., 2022). In this study, molecular data analysis revealed substantial genetic diversity among the colchicine treatments. The observed appearance and disappearance of bands are likely linked to DNA changes or mutations induced by colchicine. SCoT markers, known for their ability to assess genetic diversity, have previously been applied to evaluate colchicine-induced diversity in chamomile, revealing significant variations between plants exposed to different concentrations (Soubra et al., 2018). Similarly,

Ismail et al. (2022) analyzed somaclonal variation in micropropagated *W. somnifera* plantlets using three molecular markers: DNA barcoding, SCoT, and SRAP. The study concluded that SRAP and DNA barcoding were more efficient than SCoT in detecting somaclonal variability in Ashwagandha. Despite this, SCoT markers remain valuable tools for genetic diversity analysis due to their high polymorphism and repeatability across the genome (Zeinullina et al., 2023). Colchicine has also been shown to induce changes in *W. somnifera* at the morphological level and in its alkaloid and phenolic content, with results correlating to colchicine concentration (Mahdy et al., 2024). In our study, molecular data analysis using SCoT markers confirmed significant genetic diversity in *W. somnifera* exposed to varying colchicine concentrations. The polymorphic percentage among treatments ranged from 85% to 100%, demonstrating the efficacy of colchicine in inducing genetic variability.

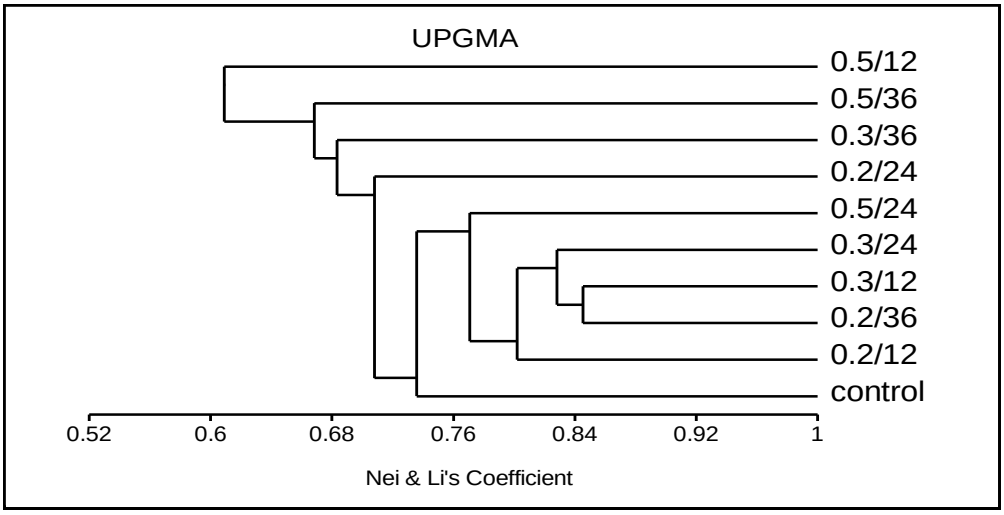

Fig. 2. Dendrogram for the ten Ashwagandha treatments constructed from the SCoT data using the UPGMA and similarity matrices computed according to Nei and Li's coefficient.

Conclusions

Our research showed that colchicine can produce greater results in *W. somnifera* L. plants. Colchicine is clearly able to cause a mutation, especially at a concentration of 0.5%, which generates new possibilities of studying the effect of colchicine on other aspects of the plant. Thus, our study is important in the process of discovering colchicine-induced mutations.

Author contributions

This study was done in collaboration with all authors. DR, AND, and AA designed this study. DR drafted the manuscript. BH, AA, and AS critically revised the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors indicate no conflict of interest in this work.

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