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# Advances in Breeding Techniques for Genetic Improvement in Ashwagandha [*Withania somnifera* (L.) Dunal]

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**ABSTRACT**

Ashwagandha (*Withania somnifera* (L.) is a medicinal plant, also known as winter cherry or gooseberry. The Indian subcontinent, North-Western and Central parts of Africa, and the Mediterranean region are the native palaces of ashwagandha. The dry and subtropical climate is best suited for its growth and development. Its roots are mainly consumed as a health tonic for fitness, longevity and vitality. It is an important stimulant of the human immune system cells, phagocytes, and lymphocytes: and it is also able to relieve the effects of stress and improve health. It is also utilized to treat COVID-19, flu, insomnia, asthma, bronchitis, emaciation, dementia, inflammation, neurological disorders, and Parkinson's disease. It has 12 types of withanolides, five unknown alkaloids, several free amino acids, glycosides, tannins, chlorogenic acid, glucose, and several flavonoids in the leaves. The availability of Ashwagandha as a natural vegetation is reduced drastically due to its constant uprooting for utilization. Therefore, it is an insistent need to evolve the high yielding varieties of ashwagandha. Even today its cultivation relies on wild, semi-wild plants or primitive cultivars, which have not acquired genes for high productivity. There is enormous scope to improve its yield and quality. Ashwagandha is generally grown under harsh climatic conditions; therefore, identification of the superior genotypes for drought and high-temperature tolerance is essential for genetic manipulation through breeding techniques. Nowadays, plant phenomics is a good technique for analyzing plant biomass, development and growth rate, as well as leaf chlorophyll, carbohydrates, protein, and water content. The male and female parts are present in the same flower and pistil is surrounded by anthers and both mature at same time, which favors self- pollination. But natural out-crossing is also observed in some conditions, thus hybridization is a possible way to utilize heterosis through transgressive segregants. Therefore, any breeding methods may be applied as per suitability and availability of plant genetic resources. In addition, molecular breeding is an excellent way to enhance the specific biomolecules through marker assisted technique, suitable for any kind of genetic improvement related to biotic and abiotic resistance.

***Keywords***: *Withania somnifera*, Ashwagandha, breeding aspects, root yield, biomolecules

## Introduction

Ashwagandha belongs to the family "Solanaceae" and genus "*Withania*" and its botanical name is *Withania somnifera* (L.) Dunal. It is a self-pollinated crop having the chromosome number 2n=48. It is known by several other names viz., winter cherry, Indian ginseng, Asgandh and poison gooseberry. The ashwagandha plants, berries, seeds and roots are presented for identification in Fig. 1. It is a perennial plant species with immense therapeutic uses in traditional as well as modern medicine systems (Datta et al., 2010). Ashwagandha is a "royal herb" because of its potent rejuvenating and life-prolonging effects on the human body (Sharma et al., 2011). This herb

has antioxidant, anxiolytic, adaptogens, anticancer, anti-parkinsonian, anti-venom, and anti-inflammatory properties (Gupta and Rana, 2007). It also acts on various systems of human body including neurologic immune, energy-production, endocrine and on the reproductive system. It has regenerative properties, and thus, useful to treat nervous fatigue, insomnia, potency issues, tiredness, skin issues, coughing, liver tonic, anti-arthritic, resolving reproductive system issues, and other ailments (Arya et al., 2021).

In the Indian ayurvedic medicinal system, it is popularly known as rasayana, or a tonic for fertility and longevity (Singh et al., 2010). Its root contains flavonoids, alkaloids, steroids, and many active functional ingredients (Kumar et al., 2015). It is used for headache treatment, development of the nervous system, heart problems, anesthesia, blood pressure and reduced cholesterol level (Kirti and Arya, 2019). Aslo is utilized in a wide range of clinical activities such as immunomodulation, memory sharpener, stress elevator, anti-epileptic, anti-ageing and antioxidant (Arya et al., 2022a). Besides, it is also effective to cure hypoglycemic, cardiorespiratory and hypocholesterolemia problems (Tiwari et al., 2014). Generally, its roots extracts and powders are available in markets for use as prescribed doses with water, milk, ghee, or honey (Gupta et al., 2006).

***Origin and distribution***

Range of distribution of ashwagandha includes the Canary Islands and the Mediterranean region, as well as Africa, the Middle East, India, Afghanistan, Pakistan Sri Lanka, and China (Fig. 2). It originated from north-western and central India as well as the Mediterranean region of Africa (Kumar et al., 2020). It is a xerophytic plant and found widely adapted to diverse climatic conditions. However, it thrives best under arid and semi-arid climatic conditions which is characterized by sandy loam soil with adequate drainage and warm environment with sufficient rainfall around 400mm (Jana and Charan, 2018). In India, for commercial cultivation it is grown in several provinces and its roots are exported in the international market. In All India coordinated trials the maximum root yield of 1.0-1.1 tons/ha was observed.

***Genetic variability and related species***

Genetic improvement of any crop depends on the available genetic variability for yield and its contributing traits as well as quality parameters. A number of researchers reported sufficient genetic variability and genetic divergence in the ashwagandha using conventional methods (Kumar et al., 2007; Yadav et al., 2008; Kumar et al., 2012; Joshi et al., 2015; Sukh et al., 2015; Patel and Desai, 2017; Srivastawa et al., 2018; Ekka et al., 2021; Venugopal et al., 2021) and modern molecular techniques (Chaurasiya et al., 2009; Arif et al., 2010; Parita et al., 2018; Koli 2022). When sufficient genetic variability or a particular gene of interest is not found within the species, it may be transferred from the related species. In the case of ashwagandha, there are about 61 related species have been reported throughout the world; these belong to the genus *Withania*. Out of these, the most important are *Withanis somnifera* (L.) Dunal, *W. coagulans* (Stocks) Dunal, *W. ashwagandha*, *Withania frutescens* (L.) Pauguy, and *W. begonifolia* (Roxb.) Hunz (Srivastava et al., 2020).

***Bioactive compounds***

Bioactive compounds of ashwagandha such as Withaferin-A, Withanone, Withanoside IV, Withanolide-A, and Sitoindosides VII-X play an important role alone or in combination as potential therapeutic agents (Kaur et al., 2017). Twelve withanolides, five unidentified alkaloids, several free amino acids, chlorogenic acid, glycosides, glucose, condensed tannins, and flavonoids are the bioactive compounds found in the plant (Indian chemotype) (Khare, 2007). Proteins like glycoprotein and withania lectin protein have medicinal properties. The main alkaloids belonging to ashwagandha are withanolide, somniferine, somniferinine, somine, withanine, pseudowithanolides, withanonine and withasomine (Covello and Ciampa, 1960) and (Patel and Desai, 2017). Like ginseng, ashwagandha was also found to be effective against COVID-19. Young (2022) revealed the potential preventive and therapeutic roles of ginseng in COVID-19 based on its regulatory role in inflammasome initiation.

Ashwagandha plants are also well known to trigger the immune system cells, namely, lymphocytes and phagocytes, which also assist in controlling the effects of stress and promote general wellness and other problems (Singh et al., 2001). Phyto-chemicals and root extracts have antiviral activity and may be efficient in controlling viral infections (Balkrishna et al., 2020). Now-a-days, an immunity boosting plant, is playing a very vital role to fight against coronavirus (Koli et al., 2021) Hoffmann et al. (2020) observed that Withanone and Withaferin-A interact with transmembrane protease serine 2 (TMPRSS2) and block entry of SARS-CoV2 into cells. Kumar et al. (2020a) also reported inhibitory potential of three natural compounds, Wi-A, Wi-N and CAPE for TMPRSS2 besides its known inhibitor Camostat mesylate. Sangwan and Sangwan (2013) studied the secondary metabolites and reported that the withanolides are C28 steroidal structures based on an ergostane system having oxidation at C22 and C26

location to form a lactone ring. The tri-terpenoid source pathway is used to construct withanolides.

Sivananthan et al. (2014) explained the biosynthesis of major and minor withanolides in *Withania somnifera* and found that the highest total withanolides detected withanolide A (7606.75 mg), withanolide B (4826.05 mg), withaferin A (3732.81 mg), withanone (6538.65 mg), 12 deoxy withanstramonolide (3176.63 mg), withanoside IV (2623.21 mg), and withanoside V (2861.18 mg)] were obtained in the combined treatment of chitosan (100 mg/l) They reported higher concentrations of total withanolides in shake-flask culture (2.13-fold) and bioreactor culture (2.13-fold) as compared to control temperature (1.66-fold). Generally, the bioactive compound content of ashwagandha increases when plants are in stressful conditions. It is important for plant adaptation and protection against adverse environmental conditions (Ramakrishna and Ravishankar, 2011). Bioactive compounds are formed as a protection mechanism in response to abiotic and biotic stress conditions. Biochemical and morphological variations highly depend on the environment and interaction with the environment (Arya et al., 2022b).

Earlier, the medicinal plants were easily available in forest areas and mountain regions, but due to continuous utilization at large scale reduced their availability. Therefore, it is need of hour to develop the high yielding improved varieties of important medicinal plants to produce the good quality raw drugs to fulfill the local demands of ayurvedic medical practitioners as well as the global demands of drug manufacturing industries (Arya et al., 2022a).

**A. *Conventional Methods***

Conventional breeding methods are, although time consuming and labour intensive but still effective and useful to develop new varieties in ashwagandha. Most used conventional methods are discussed here:

***Pure line breeding method***: Ashwagandha is predominantly a self-pollinated plant species due to self-compatible pollination behavior is mainly accomplished due to proximity of stigma and anthers (Mir et al., 2012). Therefore, a pure line breeding method is used for its quick genetic improvement from the past several decades. In this method, the most desirable plants are selected based on root yield contributing traits and quality parameters (Fig. 3). The efficacy of selection generally depends on the heritable variations available in the base population (Koli et al., 2022). Manivel et al. (2017) studied a set of ashwagandha 328 (DWS1 to DSW327) pure lines derived from JA 134. According to Srivastava (2018) high heritability in conjunction with high genetic advance was observed for fresh root weight, 12 deoxywithastramonolide in roots, and plant height, which indicated that selection could be effective for these traits. Kujur (2021) reported strong heritability and high genetic advance due to additive gene action, and hence selection particularly for yield and quality parameter would be effective.

***Pedigree method***: In this method, two genetically diverse parents are crossed, and the pedigree record of segregating generation is maintained (Fig. 4). Using this, superior and desirable traits are combined in one parent and sometimes the transgressive segregants to be evolved due to synergistic effect of different gene combinations (Koli and Arya, 2022). Dhuri et al. (2017) reported high heterosis for root yield and other traits. Thus, there is possibility to obtain the desirable transgressive segregants for root yield and its quality parameters. The Bulk method is the modification of Pedigree method, and it is commonly used to evolve the population having resistance or tolerance to biotic and abiotic stress. In this scheme, plants are grown in the field to face all the natural calamities and plants surviving in natural conditions are harvested in bulk up to $F_6$ generations. Then, individual plants were selected and harvested separately and tested in trials for their performance.

***Back cross method***: This method is used when a most promising variety becomes susceptible to disease or insect pests and a source of resistance is available (Fig. 5). The procedure of gene transfer will depend on inheritance of genes whether recessive or dominant. In case of recessive gene transfer, one generation selfing is required to identify the high yielding desirable plant with resistance. This may be utilized to improve the quality parameters if high heritability is available. Medicinal plants are generally cultivated under harsh climatic conditions; therefore, identification of the superior genotypes for high temperature tolerance is essential for effective manipulation through breeding techniques (Arya et al., 2022b). Therefore, the newly developed elite genotypes of Ashwagandha to be evaluated for heat stress under semi-arid conditions.

According to Arya et al. (2022b) the breeding among high yielding genotypes (HWS 105 and HWS 1333) and stress tolerant genotypes (JA 134 and RAS-16) may lead to development of transgressive segregants, having the potential of high root yield along with heat tolerant characteristics. Kumar et al. (2012) studied different growth phases and low temperature stress - and concluded that the enhancement of marker secondary metabolites is not a direct consequence of plants' phenology, but low temperature also acts an important factor. Econometrics based on root biomass yield and content of secondary metabolites in various growth phases recommended 180 days after plantation to be the most suitable time for root harvesting.

**B. *Modern Techniques***

The development of useful and cost-effective modern techniques is needed in the present era, for rapid development of new high yielding and quality cultivars of ashwagandha. Therefore, these would be utilized in the ashwagandha breeding programs. The plant phenomics, speed breeding, double haploids and molecular markers once identified and established could offer consistent and remunerative results as described below:

***Plant phenomics*:** It is a good technique to analyze plant features such as plant biomass, its development & growth rate, leaf pigments (chlorophyll and anthocyanin), and deposits of lignin, carbohydrates, starch, and protein content in the plant and water content. It empowers the plant breeders to quickly assess ashwagandha plants for drought tolerance, salt tolerance, heat tolerance, nutrient use efficiency and improvement in herbage and root yield and its quality parameters (Fig. 6). Drought is a most severe and unpredictable abiotic stress, faced at any stage of growth and affecting crop yields. Therefore, it is necessary to develop drought tolerant cultivars to ensure sustainable yield production in an ever-changing climate (Joshi et al., 2021). Now-a-days, to overcome the issues of global warming, the climate resilient varieties are in more demand. With the help of plant phenomics techniques, climate resilient varieties could be developed for its commercial cultivation. In ashwagandha, erect plant with 1-3 main branches having single prominent tap root supported with minor secondary roots and root hairs seems to be the most desirable phenotype which, can tolerate drought stress and accumulates maximum alkaloids.

The plant phenomics technique constitutes a modern glasshouse with headhouse, laboratory space, growth chambers and a state-of-the-art, automated, high-throughput phenotyping system. The phenotyping system houses have a large pot carriers on a 250 m conveyor belt system for automated watering, weighing and high- throughput digital imaging with the help of two cameras, visible (RGB) and hyperspectral (Surya Kant et al., 2023 personal communication). It is an excellent technique to speed up the assessment of the available germplasm for further utilization in development of new varieties of ashwagandha.

According to Banerjee et al. (2020) the precise, efficient, and timely measurement of traits in crop plants is essential in the evaluation of the breeding lines. in breeding trials, assessment of biomass is difficult, as reproductive and senescence stages do not relate to reflectance spectra, and multiple growth stages occur concurrently in diverse genotypes. Moreover, vegetation indices (Vis) saturate at high canopy coverage, and vertical growth profiles are difficult to capture using Vis. A new program was implemented involving a fusion of complementary spectral and structural information, to calculate intermediate metrics such as crop height model (CHM), crop coverage (CC) and crop volume (CV), which were found useful to calculate dry weight and fresh weight of above-ground plants. As we know, roots are underground plant parts and responsible for absorbing the water and nutrients from the soil. Therefore, identification of important root traits responsible for consumptive use of soil moisture and nutrients particularly under the stress environment is essential. But the roots vary as per the growing conditions, and it becomes tough to record the observations on roots. Therefore, Kennedy et al. (2022) suggested the different growing techniques, imaging cameras and analysis software programs for recording the better observation on important root traits.

***Double haploid technique***: This is the most successful technique to obtain the 100% homozygous lines in one year in self-fertilized plant species otherwise it takes nearly 6-7 years to get the homozygous lines (Fig. 7). According to Lemos et al. (2022) double haploid lines may be achieved through *in vitro* anther or pollen culture of $F_2$ plants and followed by chromosome doubling through colchicine application. For the development of double haploids, a standard protocol is required to be developed for ashwagandha. It is already developed in some of the solanaceous plant species i.e. in Datura by Maheshey et al in early 1970s and in tobacco by Kasperbauer and Collins in 1974.

Therefore, by using the available literature for the development of double haploids in datura or tobacco and tissue culture techniques for micropropagation in ashwagandha double haploids could be developed. Generally, double haploid production procedure has five steps as depicted in Fig. 7. Das et al. (2011) reported *in vitro* and seed propagated plants of elite genotypes with high performing recommended varieties Poshita and Jawahar 22 of ashwagandha for cytomorphological parameters and chemical contents. The results demonstrated the reliability of *in vitro* raised plants, highlighting the value of biotechnological approaches in the development of planting materials. This is one of the fastest techniques to breed new genotypes/ varieties. Kaul et al. (2009) described that *in vitro* procedure could be well utilized for rapid amplification of a selected genotype and hybrid line.

Nagella and Murthy (2010) studied cell suspension cultures for the development of withanolide A and reported that 10 g $L^{-1}$ of inoculum on a fresh weight basis, full strength MS medium, 3% (w/v) sucrose, a

four-week culture cycle, and an initial medium pH of 5.8 were the best for biomass accumulation. These findings are beneficial in the scaling-up phase. Sivanandhan et al. (2014) studied the impact of seaweed extracts on biomass and withanolides accumulation in shoot suspension culture. Supplementing 40 % G. edulis extract in MS liquid medium for 24 hours resulted in the highest biomass accumulation [62.4 g fresh weight and 17.82 g dry weight (DW)] and withanolide production (withanolide A 0.76 mg/g DW; withaferin A 2.80 mg/g DW; withanolide B 1.66 mg/g DW; and withanone 2.42 mg/g DW) after 35 days of culture. For optimal biomass and withanolide processing, this naturally available G. edulis extract-treated multiple shoot suspension culture protocol was found as a viable alternative to shake-flasks. Recently, Hancock et al. (2015), Ma et al. (2020) and Lemos et al. (2022) used the double haploid production technology successfully in tobacco.

***Marker Assisted Selection***

The molecular markers are useful to study the genotypes of ashwagandha for the genetic distinctness, uniformity, discrimination, so that the identified desirable genotypes could be utilized in further varietal developmental programs. Molecular markers are more advantageous as they are not influenced by the environment and large samples can be tested quickly and accurately. Several researchers used different molecular markers and some of them are discussed here. In recent years, nucleotide gene sequencing is permitted for simple and fast recognition of any genotypic mutation such as SNP, SSR, and Insertion/Deletion (IN/DEL) in full-length genomes of different genotypes. This technique gives more remuneration at low costs as well as rapid identification of molecular markers. Diverse DNA markers like RFLP, RAPD, STS, SSR, ISSR, ETS, DArT and SNP analyses are being developed for differentiation, identification and selection of promising genotypes.

*Restriction fragment length polymorphism (RFLP)*: It is based on differences in the length of DNA fragments obtained when DNA from different genotypes is digested with restriction enzymes that recognize specific DNA sequences. Although RFLP is an excellent technique for genome mapping and genetic fingerprinting, it requires a large amount of sample DNA and probe labeled DNA sequence that hybridizes with one or more fragments (Jo et al., 2017).

*Random Amplified Polymorphic DNA (RAPD)*: The RAPD markers are comparatively simpler and easier than RFLP and to identify polymorphic DNA fragments. It requires a small quantity of DNA, thus making it a more economical method. However, RAPD is a dominant marker and only useful in advance generations assessments. It is reported that the classification of ashwagandha species by comparing interspecies and intraspecies mutational relations by means of polymorphic bands of RAPD. Based on the genetic diversity, it is possible to do genetic improvement in the cultivars of ashwagandha. Chaurasiya et al. (2009), Arif et al. (2010) and Dharmar and De Britto (2011) studied divergence in ashwagandha through RAPDs, isoenzymes, polypeptide polymorphism, and withanolide profiles. Chauhan et al. (2022) used RAPD and reported its simplicity for the selection of high-yielding bioeconomic varieties that could be utilized to improve ashwagandha breeding programs. RAPD generates nonspecific amplicons depending on experimental and ambient conditions; therefore, it is difficult to ensure reproducibility. To overcome the problem, an analysis of a sequence-characterized amplified region (SCAR) marker was found effective in terms of convenience and reproducibility.

*Sequence-tagged site (STS)*: STS markers involve analysis of a sequence for specific clones followed by construction of diverse primers for genetic analysis. A DNA library requests to be recognized for the construction of specific STS primer sets. Recently, methylation filtering (MF) enables removal of repetitive DNA and is thus useful for creating a library of genetic regions. RAPD and PCR-RFLP markers explain insufficient numbers of suitable loci within the genome and have poor reliability, sensitivity, discriminatory ability, and reproducibility, posing difficulties in differentiation in varieties. In contrast, an STS marker with simplicity allows for easy verification of outcome with high reproducibility and, therefore, useful for analysis of massive genetic resources or varieties.

*Simple Sequence Repeats (SSR)*: It uses the fabrication of primers for repeated onomeric, dimeric, trimeric, and early selective strategies tetrameric DNA sequences. This method is costly and time consuming for construction of a genomic library and for primer invention; however, it is a codominant marker and has very high reproducibility. Paramar et al. (2015) derived SSR markers from cross transferability are less polymorphic than ESTs-SSR, because there is a significant conservation of markers among genera. A total of 13 alleles were detected in 10 loci, with an average of 1.3 per locus. The applicability of cross-genera amplification of solanaceous SSR provides a good opportunity for studying *W. somnifera*. The new set of six polymorphic EST–SSR loci will enable the characterization of population genetic diversity and structure throughout the species in conjunction with cross-transferable SSRs for which till date no

information about EST-derived as well as genomic SSR is available. (Paramar et al., 2015). Molecular characterization with the help of SSR markers, the promising genotype HWS 8-18 was also compared with other varieties/genotypes (Table 1).

It was found unique and different from all other genotypes/varieties. The genotype HWS 8-18 is differentiated based on presence of CAMS 34 primer 50 kb band which was absent in HWS-205, HWS-205 and HWS 12-12. In addition to this, it was differentiated from HWS 1203 and JA-20 due to absence of CAMS 34 primer 400kb band, as this was present in HWS 8-18 (Koli and Arya 2022). Parita et al. (2018) studied 36 ashwagandha genotypes using SSR primers. Amplicons' sizes ranged from 130 to 1652 bp, with an overall polymorphism of 94.71 percent. For the polymorphic SSR primers, the PIC value ranged from 0.219 (EMS-5) to 0.865 (EMS-12). Thirteen SSR primers revealed the dissimilarity indices, ranged from 0.069 (between MWS-205-3-2 and MWS-322-1-2) to 0.846 (between RAS23-2-1 and MPAS-15-3-1). A dendrogram was drawn on DARWIN 6.0.15 PC-based software and grouped in three clusters.

*Single Nucleotide Polymorphism (SNP)*: SNPs are used to identify the genetic variations among the genotypes. Each SNP represents a difference in a single DNA building block, known as a nucleotide. Arpan et al. (2014) investigated whether heterotic positive QTL alleles based on hybridization can be introgressed in a fine quality good agronomic genetic base using marker assisted selection. Via RNA sequencing, they developed a wide resource of *Withania* specific genomic microsatellite markers and SNP. The expression of 101245 unigenes in parents was quantified using an RNA-seq technique. Thirty percent of transcripts had different speech levels between parents, with the majority having more than 1.5 fold changes. RNA sequencing was found helpful in marking the Withanolide biosynthesis pathway and identifying SNPs that imparts functional polymorphism in pathway genes, allowing for the guess of heterosis potential.

*Inter-Simple Sequence Repeat (ISSR)*: It showed considerable diversity between the genotypes. The combined unweighted pair group technique with arithmetic mean (UPGMA) dendrogram of morphological, biochemical, and molecular markers grouped all 25 genotypes of ashwagandha into two main clusters at 0.61 coefficient value. In addition to this, secondary metabolite profiling by high-performance liquid chromatography (HPLC), there were high variations for withanolide B (WL-B), withanoside-V (WS-V), wedelolactone (WDL), withanoside-IV (WS-IV), and withaferin A (WF-A) content between different genotypes. For the total alkaloid and withanolide concentration in the roots and leaves, high heritability with an increased genetic gain was observed, indicating that selection based on these traits could be an effective method in breeding programs. Furthermore, the path coefficient analysis showed a direct positive impact of the total root fiber, WL-B (leaves), WF-A (leaves), WS-IV (roots), WDL (roots), and the total alkaloid content on the dry root yield. High content of WDL, a high-quality bioactive withanolide, was also described for the first time in the genotype UWS23. These properties can further be exploited to improve the dry root yield in ashwagandha genotypes. The outcomes of the present study also provide an essential foundation for the selection of high-yielding bioeconomic varieties that could be utilized to improve Ashwagandha breeding programs (Chauhan et al., 2022).

Koo et al., (2022) conducted simultaneous analysis of transcriptomes and metabolomes from adventitious roots of two tetraploid species (*Panax ginseng* and *P. quinquefolius*) and two diploid species (*P. notoginseng* and *P. vietnamensis*) revealed the diversity of their metabolites and related gene expression profiles.

## Results

The transcriptome analysis showed that 2,3-OXIDOSQUALENE CYCLASEs (OSCs) involved in phytosterol biosynthesis are upregulated in the diploid species, while the expression of OSCs contributing to ginsenoside biosynthesis is higher in the tetraploid species. In agreement with these results, the contents of dammarenediol-type ginsenosides were higher in the tetraploid species relative to the diploid species. They indicated that the accumulation pattern of ginsenosides correlates with the expression level of various OSC genes and with polyploidy level in *Panax* species, suggesting that dammarane-type and oleanane-type ginsenoside biosynthesis pathways are upregulated in tetraploid *Panax* species, likely due to the genome duplication event.

*CRISPR Technology:* This technology is the latest one used for genetic improvement in crop plants. It may be utilized in ashwagandha, as it is already used by Choi et al. (2022) in ginseng. They designed two sgRNAs (single guide RNAs) for target mutations in the exon sequences of the two PPT synthase genes (both PPTa and PPTg sequences) with the CRISPR/Cas9 system. Transgenic ginseng roots were generated through Agrobacterium-mediated transformation. The mutant lines were screened by ginsenoside analysis and DNA sequencing. Result: Ginsenoside analysis revealed the complete depletion of PPT-type

ginsenosides in three putative mutant lines (Cr4, Cr7, and Cr14). The reduction of PPT-type ginsenosides in mutant lines led to increased accumulation of PPD-type ginsenosides. The gene editing in the selected mutant lines was confirmed by targeted deep sequencing. They have established the genome editing protocol by CRISPR/Cas9 system in *P. ginseng* and demonstrated the mutated roots producing only PPD-type ginsenosides by depleting PPT-type ginsenosides. Because the pharmacological activity of PPD-group ginsenosides is significantly different from that of PPT-group ginsenosides, the new type of ginseng mutant producing only PPD-group ginsenosides may have new pharmacological characteristics compared to wild-type ginseng. This is the first report to generate target-induced mutations for the modification of saponin biosynthesis in *Panax* species using CRISPReCas9 system.

***Multi Location evaluation***

Once the promising genotypes are developed and identified as promising at one location, they are further evaluated along with checks for its yield performance over multiple locations for three years to judge for consistency in yield as well as for quality parameters (Arya et al., 2022). Stability is one of the most important requirements of any breeding program for yield and quality traits in targeted locations. The variations in genotypes mainly depend on the locations and interaction of genotypes with locations. Explaining such variations is biased upward by the fact that all genotypes generally don't react in the same way as change in circles and the two locations do not have exactly the same environmental conditions. Therefore, combined analysis of any variance is calculated that can measure GxE interaction and identify prime components, though it is not sufficient to declare the G x E interaction effectiveness. In Ashwagandha, AMMI1 biplots and simultaneous selection index statistics identified SKA-11 as the most desirable genotype for root branches and length while SKA-26 and SKA-27 for root diameter (Kumar et al., 2020b).

## Conclusions

The information on conventional as well as modern breeding methods in ashwagandha will be able to stimulate the medicinal plant breeders to develop new high root yielding varieties with high root quality for the utilization for domestic use as well as for commercial marketing. A breeder can choose any of the above-described methods for varietal development in ashwagandha.

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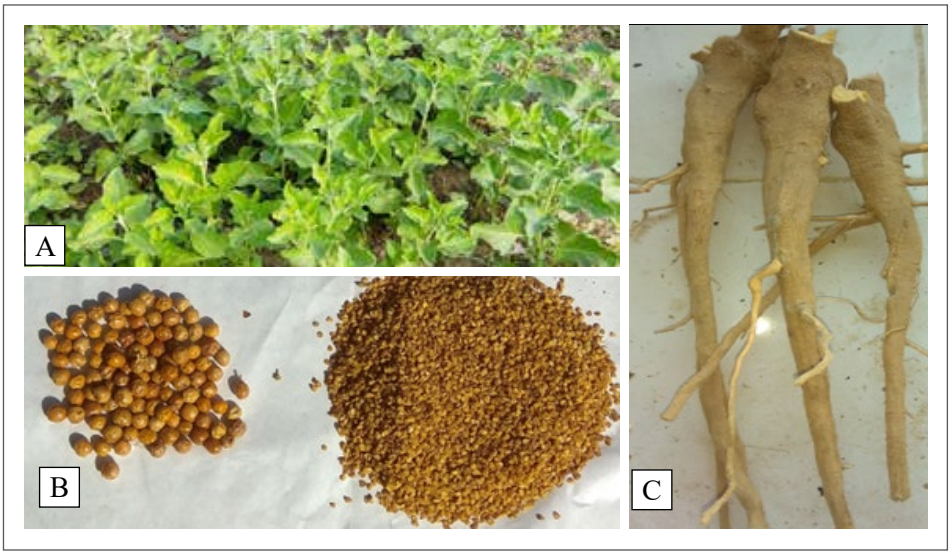


Figure 1. A. Ashwagandha plants, B. Ashwagandha Berries and Seeds, and C. Ashwagandha Roots. (Original)

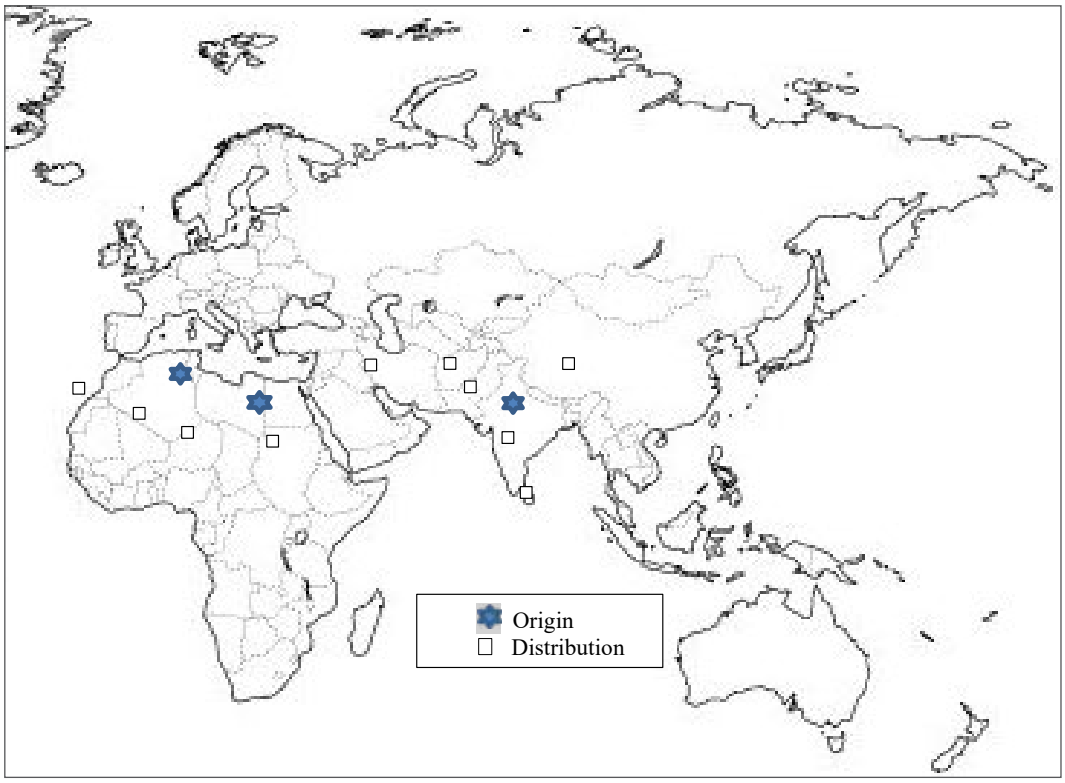


Figure 2. Origin and distribution of ashwagandha.

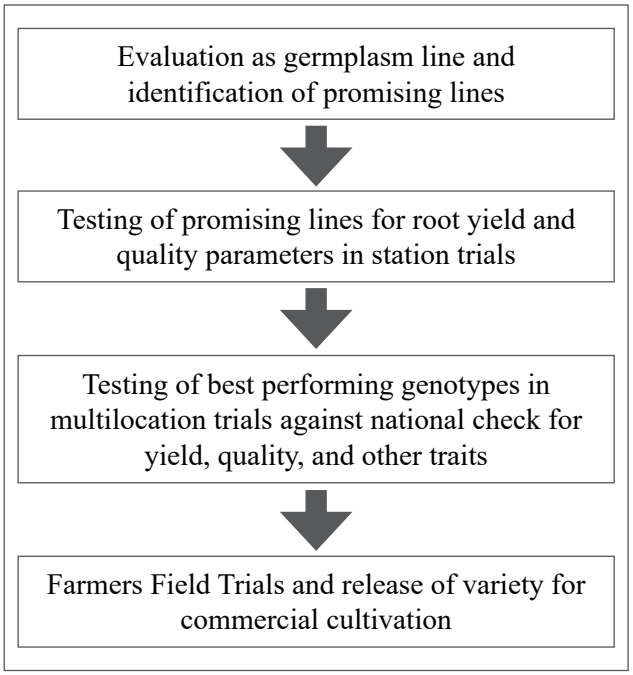


Figure 3. Schematic diagram of Pure line breeding method.

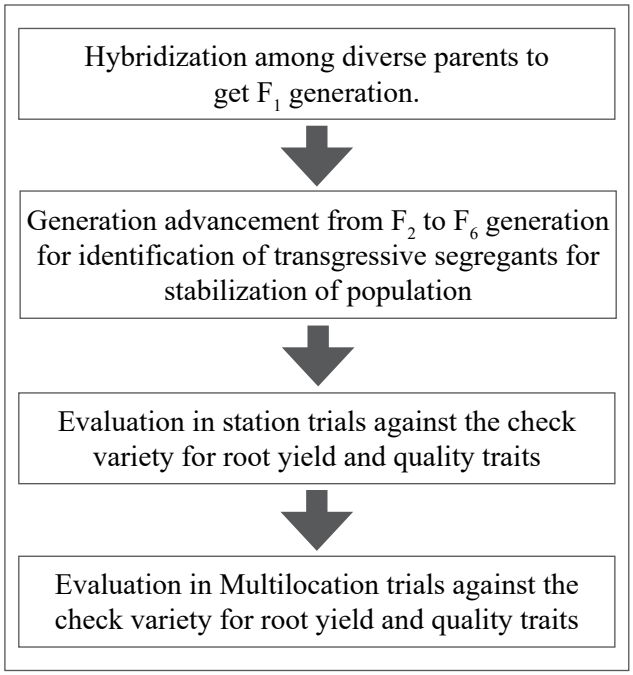


Figure 4. Schematic procedure of Pedigree method in Ashwagandha.

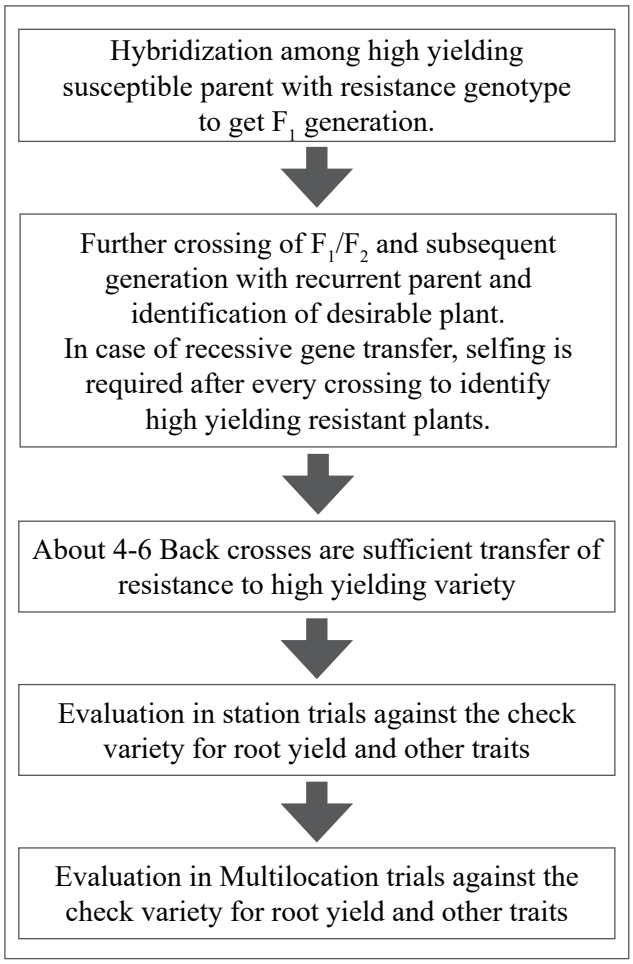


Figure 5. Schematic procedure of Backcross method in Ashwagandha.

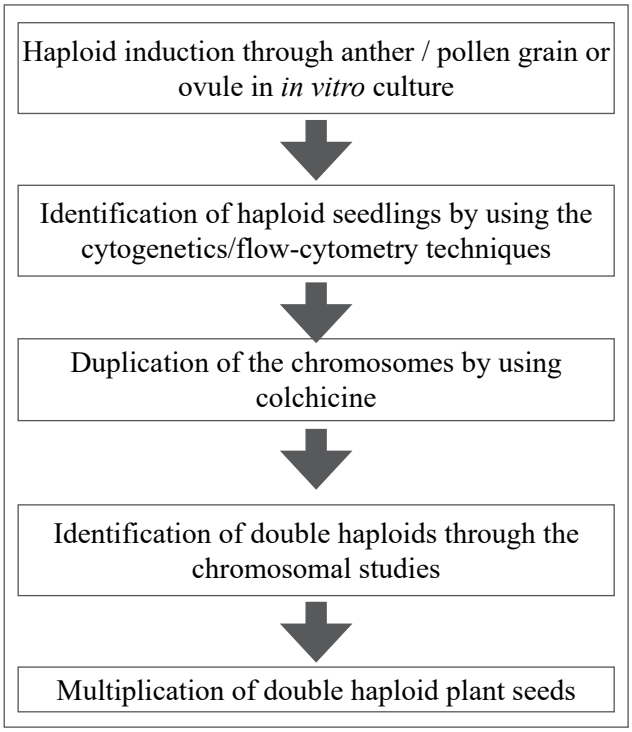


Figure 7. Schematic diagram of Pure line breeding method.

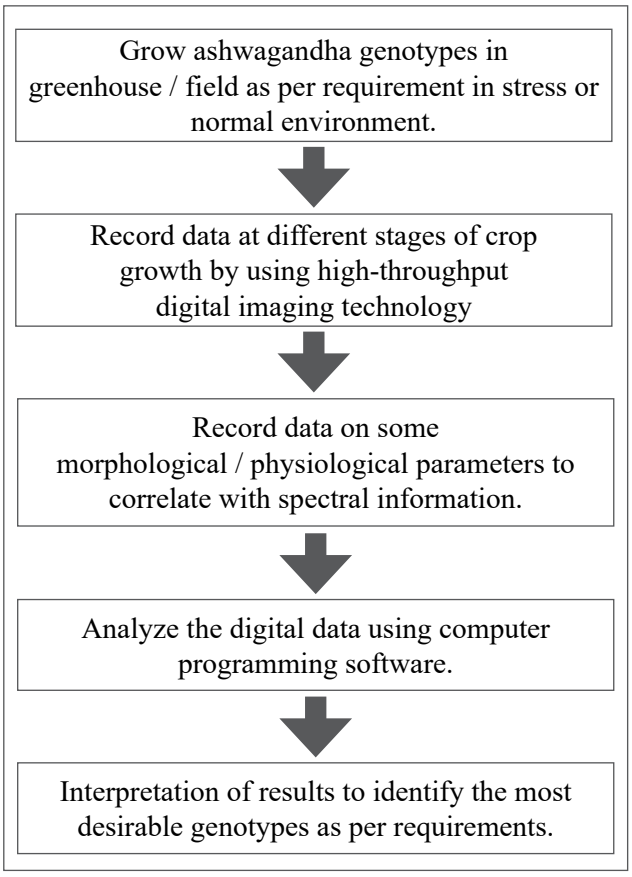


Figure 6. Schematic diagram of Pure line breeding method.

Table 1. Ashwagandha SSR Markers profile of some important genotypes and varieties.

| SN | Marker | CAMS 34 | | | CAMS 351 | | | CAMS 376 | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Allele Size | | | | | | | | | |
| | Genotype | 50 kb | 200 kb | 400 kb | 50 kb | 130 kb | 420 kb | 130 kb | 170 kb | 200 kb | 350 kb |
| 1 | HAG-1 | - | + | + | - | - | + | - | + | + | + |
| 2 | HWS 1203 | + | - | - | - | - | - | + | - | - | - |
| 3 | HWS 8-18 | + | + | + | - | - | + | - | - | - | - |
| 4 | HWS-205 | - | + | + | - | - | + | - | + | - | - |
| 5 | HWS-222 | - | + | + | - | - | + | - | + | - | - |
| 6 | JA - 20 | + | - | - | + | + | - | - | + | + | + |

Source: Koli and Arya, 2022

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