**Characterization of Genetic Variability in *Withania somnifera* (L.) Dunal …….. Markers.**

*Withania somnifera* (L.) Dunal is a medicinal plant of wide geographic distribution and immense therapeutic value. The nationwide collection of twenty three accessions of *Withania somnifera* (five cultivated and eighteen wild) were grown and maintained in the germplasm repository of Indian Institute of Integrative Medicine, J&K (India).

1. Morphological characters such as habit, plant height, leaf shape, leaf margin, root yield plant\(^{-1}\), shape of the fruiting calyx and color of berry recorded from fresh material in the field revealed significant differences between wild and cultivated accessions. However, within the wild and the cultivated groups, accessions displayed a high degree of phenotypic variability.

2. Wild and cultivated accessions displayed remarkable variability with regard to three withanolides namely withanolide A, withanone and withaferin A in the leaves and roots. The present results on bio-chemical profiles clearly bring out the biochemical divergence between the wild and cultivated accessions. The cultivated accessions (except AGB025) lack withanone in both leaves and roots. And withaferin A was found to be the major withanolide in the leaves of cultivated accessions, whereas it was available in much lesser quantities in the leaves of the wild accessions. Thus phytochemical data supports the conclusion drawn from the morphological data that cultivated and wild types show genetic divergence.

3. The morphological polymorphism and delimitation of the *W. somnifera* accessions into wild and cultivated groups (morphotypes) based on morphological and biochemical attributes received support from molecular markers.

   (a) **RAPD:** Eighteen RAPD primers used during the present study revealed a high degree of polymorphism. Out of 163 amplification products, only 63 bands were polymorphic with an average percentage polymorphism of 37.82%. The UPGMA dendrogram based on Jaccard’s coefficient and Principal coordinate analysis of the RAPD data clearly discriminated the cultivated and the wild accessions of *W. somnifera* into two major clusters. Thus pooled RAPD data showed concurrence with morphological and biochemical data supporting the grouping of accessions into wild and cultivated morphotypes.
Identification of specific markers for wild and the cultivated accessions was another important finding in the present study. Some RAPD primers (OPB10, OPK08, OPC20 and OPK16) produced monomorphic bands present either in wild or in cultivated accessions. Such markers could be used as diagnostic markers for marking out the cultivated from the wild accessions.

(b) AFLP: AFLP analyses also support the genetic divergence between the wild and cultivated groups. Six primer combinations produced a total of 286 amplification products (bands) with average of 47.6 bands per primer combination. Of these, 127 bands were polymorphic with an average number of 21.1 polymorphic bands per primer combination. Average percentage polymorphism was 44.4%. The average value for Shannon’s index was significantly higher (5.13) with the AFLP marker. The higher value in case of AFLP seems to be the direct result of a larger number of bands obtained with AFLP as compared with the RAPD marker. Values for Shannon’s index correlate well with the percentage polymorphism values obtained with RAPD (r =0.742, p≤0.001) and AFLP (r = 0.876, p≤0.01) markers.

In the UPGMA dendrogram of AFLP data, the group of five cultivated accessions branched out from the wild accessions at a low similarity coefficient of ~0.3 with high bootstrap support at major nodes. Principal Coordinate Analysis (PCO) corroborated the results of cluster analysis. The two groups clustered at the two extremes of the PCO plot did not overlap indicating a high level of divergence between the wild and cultivated groups.

Apart from polymorphic bands, three bands were unique to five cultivated accessions whereas 30 specific bands scored from wild accessions were absent in all the five cultivated accessions. Such bands have diagnostic value for distinguishing the wild from the cultivated types. Thus AFLP has proved to be an efficient tool in discriminating the two groups.

(c) ITS1-5.8S-ITS2: Further support to the distinctness of the wild and cultivated groups of accessions came from amplification and PCR-RFLP of ITS1-5.8S-ITS2 region of rDNA. A single 710 bp amplified product was obtained in all the wild accessions for the entire ITS1-5.8S-ITS2 region, whereas, two ITS bands of 709 bp and 552 bp, named as ITS type A and B, respectively, were amplified in all the cultivated accessions.
(d) **Chloroplast genes:** Apart from ITS1-5.8S-ITS2 region, PCR-RFLP of seven chloroplast genes/intergenic regions namely trnL-trnF, PsaA, trnS-psiC, PsbD, trnH-trnK, trnK and 16S were also carried out in the present study. The amplification and restriction digestions products of these chloroplast gene regions gave identical patterns in the accessions belonging to both the cultivated and the wild groups indicating that the chloroplast genome of cultivated and wild accessions seems to be highly conserved despite significant variations in their nuclear genome.

4. Evidence from morphological, biochemical and molecular characterization of cultivated and wild accessions of *W. somnifera* point out distinctness of the cultivated from the wild accessions and strongly support the proposal of Kaul *et al.* (1957) that the cultivated morphotypes be put into a separate species, *W. ashwagandha*.

5. Elite accessions AGB002 (wild) and AGB025 (cultivated) were marked out on the basis of higher root biomass plant\(^{-1}\) and withanolide content respectively. The accession AGB002 had higher root biomass plant\(^{-1}\) with appreciable amount of withanolide A in roots whereas AGB025 had higher withaferin A content in the leaves. These two accessions hold promise in programmes of varietal improvement.

A linear increase in root yield was also observed in these two elite accessions in accordance with development stages with highest root biomass plant\(^{-1}\) at maturity.

6. Biochemical profiling of AGB002 and AGB025 for total proteins, carbohydrate and withanolides (withanolide A, withanone and withaferin A) carried out at seedling, vegetative, pre-flowering, flowering and maturity stages revealed a significantly higher protein content in the leaves compared to roots and a significantly higher carbohydrate content in roots compared to leaves in the two accessions. However, total protein and carbohydrate content showed a uniform increase in roots as well as leaves of both the accessions in step with advancement of developmental stages reaching their maximum values in the flowering stage.

7. The effect of low temperature (5°C) on the elite accessions viz., AGB002 and AGB025 of *W. somnifera* was carried out to study their anti-oxidative potential and its correlation with withanolide elicitation. Both the accessions showed a significant (p≤0.05) increase in superoxide (O\(_2^{-}\)) radicals, cellular damage and lipid
peroxidation on exposure to low temperature but the increase in $O_2^-$ and cellular damage was relatively lower in AGB002 compared to AGB025. An increase in the activities of antioxidant enzymes was recorded. Increase in activities of the SOD and APX were higher in AGB002 compared to AGB025. On the contrary, AGB002 showed 60 % increase in the GR activity as compared to AGB025. The activity of CAT did not show significant differences between the two accessions. Based on these results it appears that AGB002 experienced lesser oxidative stress in response to low temperature as compared to AGB025. The isozyme profile of SOD, GR, APX and CAT analyzed on native polyacrylamide gel showed quantitative (band intensity) increase in response to low temperatures. The expression increased from day first to day seventh and was higher on day seventh when compared to control.

8. Regarding the elicitation of withanolides on low temperature exposure, our data indicated that only leaves show an increased production of withanone and withaferin A reaching highest level on day third and day seven respectively in AGB002. However in AGB025 both these withanolides reached the highest level on day seventh. Hence these withanolides may have a role in combating oxidative stress in the leaves of *W. somnifera*. However in the roots, this withanolide seems to play a lesser role. The other two withanolides show insignificant and irregular fluctuations in content during the period of stress.

9. The elites bear regular pentamorous, actinomorphic, hypogynous and bisexual flowers. The ovary is bilocular and the placentation axile. The pollen: ovule ratio approximated to 817:1 and 1214:1 in AGB002 and AGB025 respectively. The pollen grains are trizonocolporate in both the accessions and the exine pattern was found to be scabrate-granulate under the Scanning Electron Microscope. Reproductive effort was higher in AGB025 which may be attributed to domestication. Proximate placement of the stigma and the anther ensures self pollination leading to selfing. The self compatible nature of the accessions is also evident by significant fruit and seed set in controlled self pollination. Another indicator to selfing is the agreement of percentage fruit set on experimental autogamy and open pollination. Percentage fruit and seed set as also percentage germination on geitnogamy were found to be in the same range as for autogamy and open pollination.
10. Experimental crossing of AGB002 and AGB025 resulted in very low production (up to 6%) of hybrid seed indicating genetic isolation of these types. The hybrids raised from such seeds had intermediate root biomass and withaferin A content in the leaves compared to the parents. Our results also indicate a maternal effect. Yield of root biomass and withaferin A is higher when AGB002 was used as the female parent.

11. Hundred percent callus induction from leaf explants of AGB002 and AGB025 was obtained on MS medium supplemented with 2, 4-D (2 mg l\(^{-1}\)). No differences were observed either in percentage callus induction or its characteristics between the two accessions. Shoot apices cultured on MS medium supplemented with BAP (2 mg l\(^{-1}\)) in both the accessions resulted in proliferative multiple shoot cultures. IBA (2.0 mg l\(^{-1}\)) has been found to be effective for root formation.

12. Withanolide A accumulation was observed in the un-differentiated callus cultures of AGB002 at all the stages whereas it was absent in such cultures of AGB025. However, withanone and withaferin A were not detected in the un-differentiated callus cultures of either of the accessions. Multiple shoot cultures of AGB002 were observed to accumulate more of withanolide A compared to AGB025, whereas those of AGB025 accumulated more of withaferin A. Thus production of withanolides seems to be associated with organogenesis.