ABSTRACT

Induced Chemical Mutagenesis (Ethyl methane sulphonate and Diethyl sulphate) in *Andrographis paniculata* (Burm.F.) Ness (Family: Acanthaceae)

Submitted by:

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Regn. No. Ph.D/Regn./N.Rgl./Sc-75/Bot./BKG/2011

In partial fulfillment for submission of Ph.D. thesis under new regulations

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The possibility of improvement (to raise desirable plant type(s) with specific marker trait effective for efficient breeding; plants with enhanced leaf yield and with elevated amount of andrographolide content are most desirable) through induction of mutation (an essential tool to create genetic variations in a short span of time within the existing germplasm) of \textit{Andrographis paniculata} (commonly known as kalmegh; family: Acanthaceae; an important medicinal herb) has been attempted and the consequences of induced (EMS and dES) mutagenesis are explored in the present investigation. Presence study deals with morphological, anatomical, cytological, palynological and biochemical aspects of \textit{A. paniculata} as well as embodies mutagenic sensitivity, mutagenetic efficiency and effectiveness (EMS and dES), screening of macromutants and their cytogenetic behaviour, mode of inheritance of mutant trait(s) and assessment of quantitative parameters of the mutant in relation in control at M$_4$. Further, quantitative and qualitative (SDS-PAGE) estimation of seed protein and genetic polymorphism using RAPD markers were also made in the mutants in relation to control. All the mutant bred true at M$_4$. A concise account of the findings is presented below.

1. Morphological, anatomical (T.S. of root and stem), palynological (SEM analysis) and cytological (meiotic analysis) studies were performed in the germplasm under investigation.

2. For induction of mutation, dry and filled seeds of \textit{A. paniculata} (moisture content: 11.55\%) were treated with ethyl methane sulphonate (EMS) and diethyl sulphate (dES). Treatments were performed at the concentration of 0.25, 0.50, 1.00 per cent solution of the chemical mutagens for 2 and 4 hour durations. Each treatment consisted of 100 seeds (pH adjusted to 6.8; room temperature 35°C ± 1°C). Seed dipped in distilled water under laboratory condition(s) serve as control.

3. Control seeds showed 100.0\% germination under petriplate conditions. Germination frequency in EMS treated sample varied from 24.0\% to 60.0\%; while, it ranged from 10.0\% to 48.0\% in dES treatments. In mutagenic treatments germination frequency was mostly dose dependent. Prolonged treatments (4 hours compared to 2 hours) affected germination frequency.
4. In control seedling length was noted to be 45.0 mm ± 3.36 and in treatments it varied from 29.0 mm ± 5.76 to 45.43 mm ± 2.81 in EMS treated samples; while, in dES treatments it ranged from 12.8 mm ± 2.87 to 50.57 mm ± 2.34. Although seedling length reduced mostly in treatments as compared to control, it was only significant in 0.25%, 2h EMS and 1.0%, 4h dES treatments.

5. LD₅₀ was noted between 0.50%, 2h and 1.0%, 2h EMS treatments; while, all the employed doses in dES manifested less than 50% reduction in germination frequency and therefore LD₅₀ could not be ascertained.

6. Effect of physical (chilled water, warm water, scarification of seeds and induction of light) and chemical (KNO₃, thiourea, GA₃ and H₂SO₄ at different concentration) treatments (24 and 48 hours duration) was analyzed on germination frequency and seedling growth under petriplate conditions. As compared to control (100.0%), frequency of germination seems to be commensurable (75.0% to 100.0%) in different treatments excepting H₂SO₄ (complete inhibition was noted). In relation to respective controls, seedling length enhanced as well as reduced significantly in different treatments.

7. About 80.0% germination was studied in control under field conditions at M₁ and it varied from 22.0% to 48.0% in EMS and 5.0% to 48.0% in dES treatments. However, all M₁ germinating seedlings survived till maturity.

8. Pollen sterility in control plants at M₁ was noted to be 14.4% and it varied from 10.1% to 24.1% in EMS and 7.1% to 23.7% in dES treatments. Pollen sterility recorded in treatments was not dose dependent.

9. Seed yield/plant in control plants at M₁ was recorded to be 1.14 gm ± 0.44 and it ranged from 0.91 gm ± 0.14 to 1.36 gm ± 0.80 in EMS and 0.90 gm ± 0.13 to 3.10 gm ± 0.76 in dES treatments. Seed yield was noted to be enhanced in 1.0%, 2h and 0.50%, 4h EMS as well as in 0.50%, 2h and 1.0%, 2h dES treatments.

10. Selfed seeds of each M₁ treated plant were harvested separately and grown in plant to row along with control to raised M₂ generation. The control plants showed 12.78% germination at M₂ and in treatments it varied from 8.14% to 15.35% in EMS and 8.33% to 16.67% in dES. All the germinated plants (100.0%) survived till maturity.
11. Fourteen macromutants (all were viable) affecting plant ideotype (affecting branching nature, stem and leaf characteristics, seedling color and maturity) were spotted at M
2. From the estimated mutation frequency over the mutagen treated population it appears that the mutants are in the following order: lax branching > unbranched II > dark green leaf = drooping leaf II > viridis = bushy = broad leaf II = narrow leaf II = dwarf = early maturity > unbranched I = broad leaf I = narrow leaf I = drooping leaf I.

12. Mutation frequency was not dose dependent in either EMS (0.79% to 5.0%) or dES (0.56% to 10.0%) treatments. EMS treatments induced relatively higher (3.12%; spectrum 1 to 4; 640 plants scored) frequency of mutation than dES (2.46%; spectrum 1 to 3; 528 plants estimated).

13. Mutation affecting branching nature was most predominant (1.88%) in EMS treatments; while, leaf mutation were prevalent in dES treatments.

14. Concomitant trait(s) associated with the macromutants persisted in all (M
2 - M4) the generations studied. Unbranched I was associated with late flowering; broad leaf II with dark green leaf; narrow leaf II with early flowering and drooping leaf II with pigmented stem. Pleiotropic gene action for some of the mutant trait(s) has been predicted.

15. EMS seems to be relatively more effective than dES in all doses of 2 hour treatments; while, it was reversible for 4 hour treatments. The most effective doses were 1.0%, 2h EMS and 0.25%, 4h dES. Based on injury, 0.25%, 4h EMS, 1.0%, 4h EMS and 0.25%, 2h dES were the most efficient doses.

16. Inheritance of the mutant trait(s) was assessed from the self-segregation of M
2 macromutant seeds sown at M3. \( \chi^2 \) - test analysis revealed that the mutant trait segregated into either 1:1 or 3:1 ratio, thereby indicating possible monogenic inheritance of the trait(s). Viridis showed digenic mode of inheritance.

17. Control plant showed 14.67% germination at M4; while, in the mutant plant types germination frequency varied from 4.67% (early maturity) to 12.0% (narrow leaf II).

18. Meiotic analysis performed in M4 plant types (control and 14 mutant) revealed the formation of \( 2n=50 \) chromosomes always. Mean chromosome association at metaphase I was 24.92\% + 0.15\% in control and in mutant it varied from 25\% (dwarf Induced Chemical Mutagenesis (Ethyl methane sulphonate and Diethyl sulphate) In Andrographis paniculata (Burm.f.) Nees (Family: Acanthaceae)
ABSTRACT

and early maturity) to 24.45II + 1.10I (dark green leaf). Predominant chromosomal association studied in the plant types was 25II. The AI chromosome segregation in the plant types was always equal (25/25).

19. Pollen fertility (using 1.0% acetocarmine stain) in control was studied to be 80.63% and it varied from 21.28% (broad leaf I) to 89.25% (narrow leaf I) in mutant plant types. Dark green leaf (25.64%), broad leaf I (21.28%) and early maturity (26.13%) plant types showed considerably low pollen fertility in relation to control plants. Pollen viability percentage was assessed following the use of different stain test (amido black, aniline blue, lugol’s iodine, methylene blue and X-gal) both in control and in M₄ mutant plant types. In comparison to control, pollen viability was observed to be lower in the mutant plant types; however, bushy, lax branching, broad leaf II, narrow leaf I and II, drooping leaf II and dwarf mutant demonstrated relatively higher pollen viability. Dark green leaf, drooping leaf II and broad leaf I showed low pollen fertility and viability than control. Abnormal pollen nuclei composition (0v+1g, 1v+0g, v-dispersed and 1v+2-5g) was found to be higher in drooping leaf II (40.04%) mutant than the other plant types (control-21.25%, dark green leaf-23.17%, broad leaf I-25.79%). Seed set per capsule and seed yield per plant were significantly lower in drooping leaf II mutant than control; while, seed yield per plant enhanced in broad leaf I.

20. Comparison between (C.D. at 5 % level) the mean values of control (selfed lines) and mutants (14 true breeding macromutants) has been made following assessment of 8 quantitative parameters (plant height, no. of primary branch/plant, leaf yield/plant, capsule length, seed/capsule, 100 seed weight, fresh root weight and root length) of M₄ plant types grown in 3 replications. Results of this study indicated that none of the mutants has demonstrated superiority over control for all the traits under study but few of them exhibited betterment than control in some traits.

21. Analysis of variance (F-test) performed among 15 plant types for 8 different quantitative parameters revealed significant variations for all traits at 0.01 probability level.

22. Estimation of population mean, genotypic and phenotypic variance and heritability (broad sense) was made for 8 different quantitative characters over 15 genotypes.
ABSTRACT

(control and 14 macromutants of M₄) to assess the selection criteria. Results obtained have been discussed.

23. The yield of andrographolide estimated by HPTLC was found to be significantly (t=2.92, p<0.05 DF 4) higher (3.41%) in mature leaves of untreated control plants than young leaves (0.93%). Andrographolide content was noted to be 2.33% in **viridis**, 3.45% in **lax branching**, 3.99 in **bushy**, 0.40% in **unbranched I**, 0.37% in **unbranched II**, 0.30% in **dark green leaf**, 3.76% in **broad leaf I**, 3.84% in **broad leaf II**, 0.11% in **narrow leaf I**, 1.26% in **narrow leaf II**, 0.64% in **drooping leaf I**, 0.02% in **drooping leaf II**, 0.03% in **dwarf** and 1.28% in **early maturity**. **Bushy** (t=26.85, DF=4, p<0.001), **broad leaf I** (t=14.71, DF=4, p<0.001) and **II** (t=19.91, DF=4, p<0.001) mutants were with significantly higher amount of andrographolide content than normal plants.

24. Total andrographolide content was found to be 5.43% in control and it ranged from 1.05% (**drooping leaf II**) to 6.13% (**bushy**) in the mutants.

25. TLC profile of total andrographolide was prepared from matured leaves of control plants using methanol and ethanol as extraction solvents. Petroleum ether: propanol (7:3); ethyl acetate: ethanol: water (75:15:10) and ethyl acetate: toluene (95:5) were used as mobile phases. A total of 33 spots were detected. Spots were (excepting: Rf 0.4 and 0.69 - spots were bluish in nature) pinkish in nature under UV. Upon comparison with reference, presence of andrographolide and andrographoside were identified.

26. Seed viability test was performed from harvested M₄ plants using 1% tetrazolium chloride. Control showed 30.0% viability and in mutants it ranged from 10.0% to 24.0%.

27. Seed protein content was estimated in control and in 11 macromutant from M₄ harvested seeds. Protein content could not be analysed in **dwarf**, **unbranched I** and **drooping leaf II** mutants due to extreme paucity of seeds. Protein content was 3.86% in control and it varied from 2.87% (**bushy**) to 4.60% (**lax branching**) in the mutants.

28. Electrophorograms obtain from Polyacrylamide Gel Electrophoresis (SDS-PAGE) using seed protein (M₄ harvested seed; protein of 12 genotypes- control and 11 mutants) resulted in 55 polypeptide bands with Rf values ranging from 0.106 to 0.972.
ABSTRACT

Molecular weight of the polypeptide bands varied from 121.28 kc to 14.69 kd. The total number of detectable bands in different plant types ranged from 23 to 35. The bands were grouped into high (70 kd to 122 kd), medium (30 kd to 69 kd) and low (<30 kd) molecular weights. Heterogeneity in banding pattern was noted and the results obtained have been discussed.

29. Cluster analysis by UPGMA of the polypeptide band profile revealed 3 major clusters. Cluster 1 (upper to lower) showed closeness among early maturity, drooping leaf I and broad leaf I and II mutant; while, cluster 2 comprises of drooping leaf I and viridis. Rest of the plant types are in cluster 3, where control and bushy, lax branching and dark green leaf, unbranched II and narrow leaf I and lax branching and unbranched II were closely related. Cluster 2 and 3 were sub-clustered together. Result obtained has been significant as it provided scope for intermatting between/among germplasms, which may provide scope of enhancing genetic diversity in the plant species.

30. RAPD (OPB-01 to 10) band profile studied acrossed the germplasms (control and 10 macromutants) revealed a total of 90 reproducible bands out of which 39 were polymorphic (43.33%). Per cent polymorphism was maximum in OPB-04 (71.43%) followed by OPB-06 (66.67%), OPB-10 (54.55%) and OPB-09 (50.00%). PIC values was much higher in OPB-03 (0.9917) and OPB-04 (0.4723) compared to other primers.

31. Dendogram constructed by UPGMA from proximity matrix revealed close relatedness between/among the plant types (lax branching, broad leaf I and II; narrow leaf I and II and unbranched II; and early maturity and drooping leaf I). Control, dark green leaf and bushy plant types were distinctly apart from the 3 major clusters; however, all the plant types were subsclustered to one another.

Although kalmegh (A. paniculata) is an economically important medicinal herb, there have been no attempts for its genetic improvement and therefore the present investigation has been designed primarily to create genetic variations using the tools of induced mutagenesis. The plant types evolved seems to be in the direction of the objective underlined and corresponds closely to the ideotype being looked for in the plant species.
Bushy and broad leaf I showed enhanced amount of andrographolide content than control notwithstanding the utmost significance of early maturity mutant plant type. The other mutant plant types screened may be directly used in Andrographis breeding programme as genetic/morphological markers. The yielding ability in the mutant may be better with improved agronomic management. Further, inter-mating among the mutant lines followed by rigorous selection may give rise to desirable plant types. Thus, variations released through induced mutagenesis have enriched genetic diversity in the species which did not exist in natural population. Further, the present work will be helpful in indexing of A. paniculata germplasms.

The concern area in the present investigation is that the germination frequency and seed viability of the raised plant types (control and mutants) were extremely low. Direct sowing of seeds may be a hindrance to germination. In such case, seeds may be sown in seed-beds and seedlings of appropriate maturity could be transplanted to field for better results. Rigorous selection procedure(s) may also be helpful for enhancing seed viability. Shattering of seeds and asynchronous maturity are also major problems in the plant species. Plant type mutation in this direction will be worthwhile. Appropriate care must be taken to collect matured seeds as well as seeds should be dried properly prior to plantation.