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RESEARCH ARTICLE

Sem Studies of Seed Coat Structures in Mutants of *Catharanthus roseus* (L.) G. Don

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ABSTRACT

The large demand and lower yield of bis indole alkaloids (vincristine and vinblastine), which are present in the leaves of *catharanthus roseus*, promote research effort for the development of superior stocks. The seeds of *C. roseus* have been treated with different doses of gamma rays and ethyl methane sulfonate alone and in combination with more attention towards increased percentage of bis indole alkaloid production to meet current medical demand. As a result of mutagenic treatment, five morphologically and chemically dissimilar mutants were isolated after critical examination upto M_3 generation. These mutants have shown stable agronomic performance in the fields. The scanning electron microscopy of seeds of mutants along with control has revealed extensive variation in seed coat structure. The seed surface have irregular and unevenly arranged elevated area (ridges) and minute depressions of various geometrical shapes (lumina) were present between these elevations. The depressions were decorated with beaded structure.

INTRODUCTION

Plants, having herbal curative properties, are medicinal plants. The medicinal value of plant is due to the presence of chemical compounds of Carbon, Hydrogen, Oxygen and Nitrogen in plant tissues like root, shoot, leaves, flowers, fruits and seeds which produce a definite physiological action on human body (Trivedi and Nehra, 2004). Most plant cells accumulate the secondary metabolites (active principles) in smaller quantities, which exert a profound physiological effect on the mammalian system. A review on the 'Potentials of plant products as anticancer agent', has incorporated the name of *Catharanthus roseus* (L.) G. Don (Nwafor, 2001). The clinical trails by El Lily group deciphered two alkaloids vincristine sulfate (0.0002%) and vinblastine sulfate (0.0005%), were still prescribed by physicians to fight against various form of cancer (Svoboda and Blake 1975, Creasy, 1977). Both vinblastine and vincristine at relatively low doses (1 mg/ day for 10 days) can prolong the life span of leukemia animals by 100% or more. The vincristine sulfate is particularly effective against P1535 leukemia.

But these two alkaloids occur in very low concentrations in plant. However despite concentrated efforts to increase this amount, only minimal improvements has been made (Liu *et al*, 2011). The natural populations of *C. roseus* harbour considerable genetic variability. After thorough assessment of previously published literature on *C. roseus*, it is necessary to emphasize the extensive studies for genetic upgradation of percentage alkaloid present in plants. The mutants of *C. roseus* had been recovered after different concentrations of gamma rays and EMS treatment on the seeds of white flowered variety. The identified mutant were dwarf mutant with obvate leaves (dwob), medium tall mutant with small leaf area (mtsl), nontrichomeous mutant (nt), upright oriented elliptical leaves mutant (upel) and spoon shape leaves mutant (spl) (Roy *et al*., 2008). The SEM studies on seed morphology have been used in elucidating taxonomic and genetic relationship (Heywood, 1971; Rejdali, 1990).

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The SEM study of seed-coat structure of *C.roseus* provides very illuminating information regarding variability among mutants. The apparent variation in plant morphology and seed-coat structure throw light regarding mutation induced variability in a plant.

MATERIALS AND METHODS

The selfed seeds of *C. roseus* (white) were subjected to treatment with gamma rays (5,10,15 and 20 kR) and ethyl methane sulfonate (0.2, 0.4, 0.6%) alone and in combinations. The seeds were sown to raise M_1 generation. The seeds of M_1 generation were bulk harvested (treatment wise) and sown in field to raise M_2 generation. The seeds of selected mutant plant along with control one were grown in randomized block design field to raise M_3 generation. The seeds were harvested from matured plants of M_3 generation after six months of transplantation. Naturally dried mature seeds of different mutants were studied for their external topology by SEM following the procedure of Hayat (1981). Mature dry seeds of all selected mutants were fixed in 2.5% gluteraldehyde in 0.05M phosphate buffer for 12-16 hours. The fixed seeds were washed in 0.05M phosphate buffer for three times. Washed seeds were kept in 1% osmium tetraoxide in 0.05M phosphate buffer for about 3-4 hours till they become black in colour.

After osmication, the seeds were washed again in ordinary tap water for 2-3 times. Washed seeds were dehydrated in graded series of ethyl alcohol (15%, 30%, 50%, 70%, 80% and 95% for 30 minute each). Then seeds were dehydrated in 100% alcohol thrice and each time for 10 minutes. The seeds were then kept in moisture free absolute alcohol for some time and in acetone for few minutes. These specimens were subjected to critical point drying at 31°C and 7.3X 10^6 pa (Hall and Hawes, 1991) to remove the remaining fluid as vapour in a critical point drier. The dried specimens were mounted on the round aluminum stubs with the help of double sided aluminum tape. The mounted specimens were then coated by Sputter model balter 5CD005 using gold coating of thickness around 200°A for variable time at 10 KVA. The coated seeds were finally observed under the SEM Jeol JSM T330A at 10 KV photographed using 35 mm 100 ASA film. We adopted the technical terms used by Stearn (1966) for describing seed surface patterns revealing through SEM.

RESULTS

The seed coat colour of the isolated mutant along with control were varied from brown to blackish–brown. All seeds have convex face on one side while other face have convex periphery with concave mid region, where hilum was present. The seed shape has shown considerable variation among themselves (Table 1).

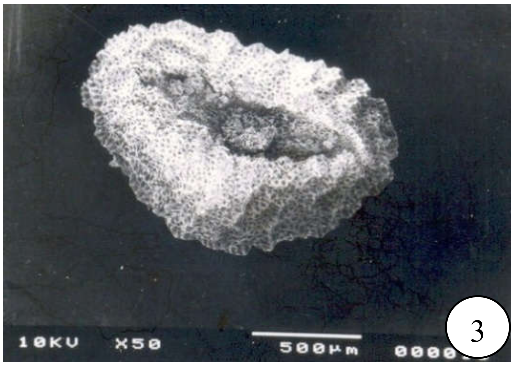
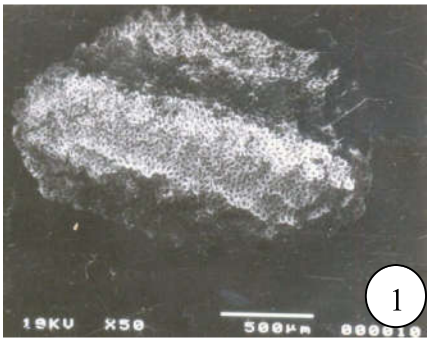
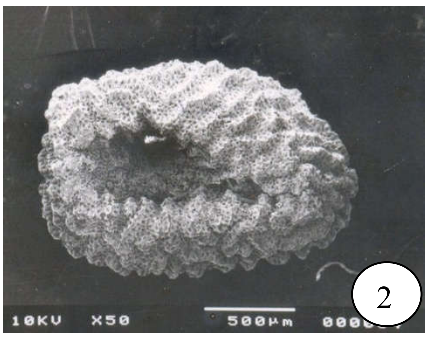


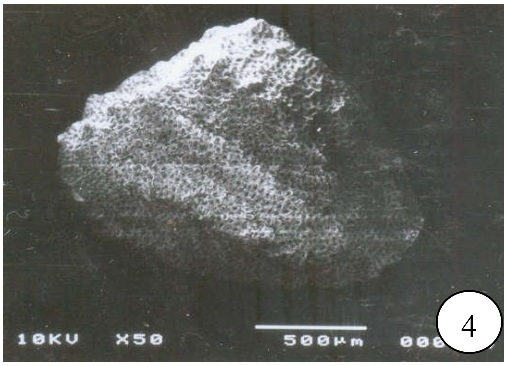
Table 1. A comparative account of seed shape and colour, seed length, hilum length and lumina length, percentage vuncristine and vinblastine of control and mutant of *C. roseus*

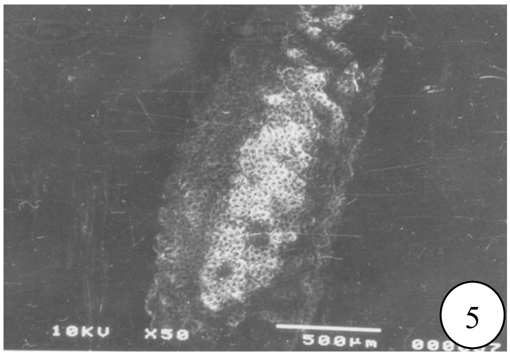
Sl. No.	Plants	Treatment Gamma rays+ EMS	Seed Shape	Colour	Seed length (mm)	Hilum Length (mm)	Surface ornamentation	Lumina Length (µm)	Vincristine %	Vinblastine %
1	control	nil	Ovoid	Brown	1.96±0.03	0.28±0.006	Reticulate	6 - 8	0.007±0.0003	0.01±0.003
2	Spl	10 kR	Ellipsoidal ovoid	Brown	1.95±0.04	0.27±0.003	Ligulate	14 -32	0.006±0.0001	0.026±0.0004
3	Dwob	20kR+0.4%	Pyriform	Blackish brown	1.53±0.22	0.15±0.003	Colliculate	14 -16	0.014±0.0009	0.046±0.0012
4	Mtsl	15kR+0.6%	Triangular	Blackish brown	1.61±0.13	0.22±0.011	Rugose	6 - 38	0.012±0.0005	0.029±0.0024
5	Nt	0.4%	Ellipsoidal oblong	Black	2.04±0.05	0.36±0.002	falsifoeveate	22 - 34	0.008±0.0008	0.017±0.0002
6	upel	15kR+0.4%	Linear oblong	Black	2.06±0.09	0.31±0.001	Mix of colliculate and scorbiculate	14 - 30	0.007±0.0001	0.0021±0.0004

The seed length of control (untreated, 1.96±0.03 mm) (Fig.1) and spoon shaped leaves mutant (1.95±0.04 mm) (Fig.2) have nearly same seed length with ovoid and ellipsoidal ovoid shape respectively. The dwarf mutant with obvate leaves (1.53±0.22 mm) (Fig.3) smallest length with pyriform shape. But medium tall plant with smaller leaf area mutant (1.6±0.13) (Fig. 4) have slightly larger length with triangular shape. The seeds of upright oriented elliptical leaf mutant (2.06±0.05 mm) and nontrichomeous mutant (nt 2.04±0.09) ((Fig.5) have much larger seed length than with linear oblong and ellipsoidal oblong seed shape respectively. The hilum length varied between 0.16±0.004 mm to 0.22±0.017 mm reflects negligible variation. The hilum shape were circular in control and spl mutant, triangular in dwob and mtsl mutant, semilunar in nt and upel mutant. The seed coat were rough and uneven and decorated with ridges.









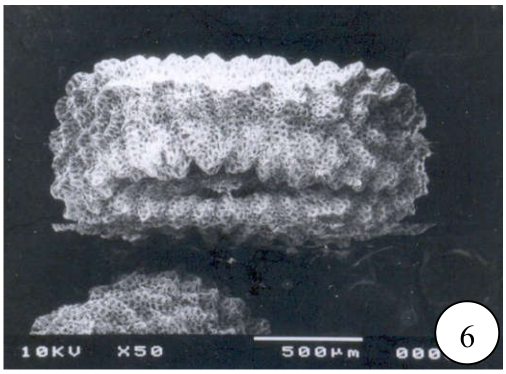
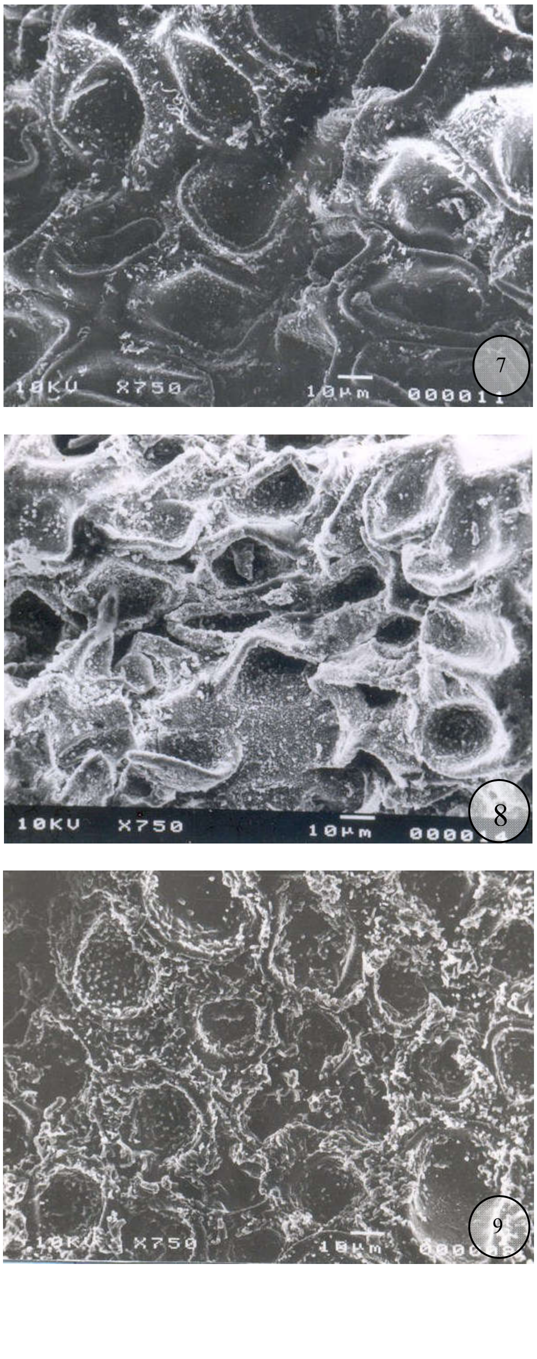


Plate 1. Seeds of *C.roseus* at 50X magnification. Fig. 1) control, Fig.2) spl, Fig.3) dwob, Fig.4) Fig. mtsl, 5) Fig. nt, Fig. 6) upel

These ridges have shown different ornamentations at higher magnifications (750X).The seed coat of control (Fig.7) have reticulate appearance with clear cut ridges and luminas. These luminas have well defined muri of (strong and stout walls) 6-8 µ wide. The spl mutant (Fig.8) have ligulate appearance of seed coat, where one end was broad pointed and other end was narrow pointed. The luminas were 14-32µ wide and dotted with beads.The ridges were 6-8 µ wide clearly demarcated while pits were shallow and less beaded. The dwob mutant (Fig.9) have somewhat colliculate look, having broad rounded elevations closely spaced covering the entire seed coat .The luminas were encircled by flat obliterated irregularly placed muri. The higher magnification showed that lumen floor was filled with higher density of beads giving granular appearance to seed coat. The seed coat of mtsl mutant (Fig.10) have obliterted non-granular muri (thin walls) giving rugose appearence. The luminas were shallow and less beaded. The nt mutants (Fig.11) have falsifoveate seed coat. The pits did not have uniform depth and shape. The pits were separated by muri of prominent ridges of 4-6 µ wide and highly beaded. The upel mutant (Fig.12) have mix appearance of colliculate alike dwob mutant and scorbiculate seed coat. The separating walls or muri were well defined circularly 2-4 µ wide and luminas of colliculate highly beaded while scorbiculate less beaded.



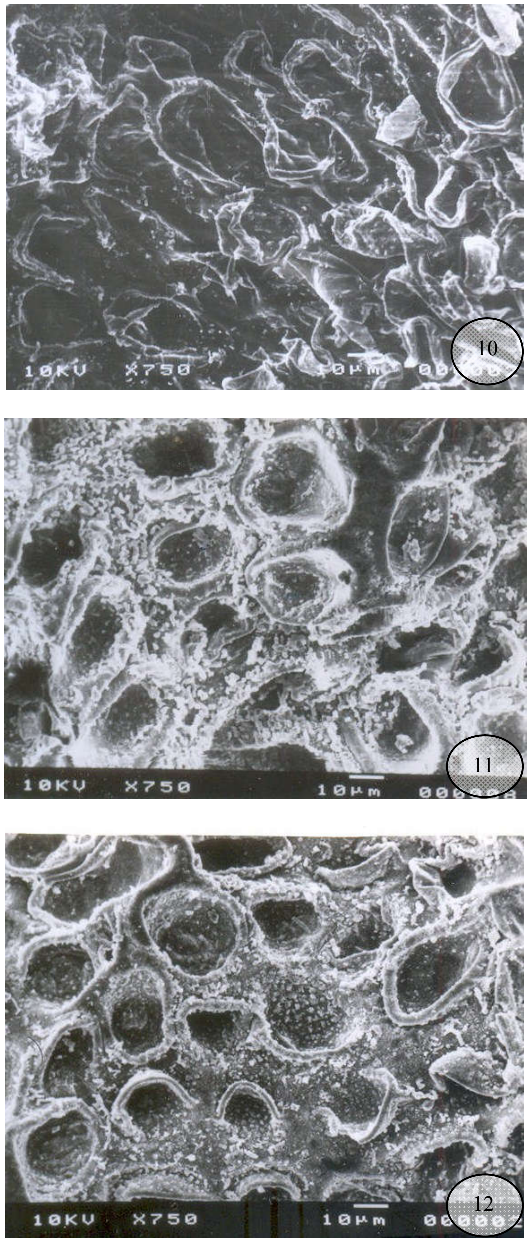


Plate 2. Scanning Electron Micrographs showing seed coat patterns of *C.roseus* at 750X magnification Fig.7) control, Fig.8) spl, Fig.9) dwob Fig.10) mtsl, Fig.11). nt, Fig.12) upel

DISCUSSION

Seed morphological studies with emphasis on the seed coat surface ornamentation in mutants of *C. roseus* with control showed variation, as a landmark in demarcating one from another. The SEM study of seeds of mutants is a measure for deciding structural diversity induced after chemical mutagenesis and also facilitate information about identity of specific mutant. Here difference in seed size and shape, hilum size and shape and seed coat ornamentation varied from one macromutant with another prove that mutagenic treatments induced not only chemical variations as well as seed micromorphological variations. The variation in seed shape and size inferred that EMS treatment penetrate deep at morphogenetic and biochemical level enhancing variability to generate a new morphotype or ideochemovars of *C. roseus*. The seed coat is rough, uneven and consists of continuous ridges of various geometrical shape. The higher doses of mutagens in dwob and mtsl mutants cause irregular, unclear ridges but lower doses in nt mutants produced prominent ridges and lumina. In *Cassia* L. species shape size and position of the lens in each species is genetically constant serves as an additional parameter in taxa identification after through SEM study (Sahai,1999). The seeds of *Phyllanthus* revealed that surface possessed ridges and troughs, which are joining at hilar, ends (Singh, 2001) same as ridges are ending at hilar region in *C. roseus*. The SEM studies of seed and seed surfaces of *Lycoperiscon* have shown thickened longitudinal bands of hair, these hair and pores can be used as cultivar marker (Chakrabati *et al*, 2003). The seed characterization of *Opuntia ficus* have shown variation in hilum position and presence of aril to characterize their species (Degano *et al*, 1997) as contrast to *C. roseus* where aril absent and fixed hilum position . The seeds of *Angelonia* sp. were small ovate with reticulate-crested exotesta as revealed by SEM (Fabiola *et al*, 2001) like control and spl mutant. Dinesh kumar and Rangaswamy (1984) have prepared a ready reference table of technical terms to explain seed surface pattern while working on *Vigna* spp and this table helped in describing seed coat pattern in mutants of *C. roseus*. The seeds were green in colour at immature stage but turn to black or brown in colour due to pigmentation of the pigment cell layer in seed. The seed colour is also correlated with seed surface thickness because organelles and biochemical pathway mediate the synthesis of secondary metabolite which may enhances the change in pigment cell layer (Talebi *et al*, 2012).

Conclusion

This work is a first attempt to reveal substantial variability, which exist among induced mutants were sorted out through seed coat structures. The study proves an alternative of advance molecular method to decipher divergence between mutants which form preliminary base for study depend on SEM of seed coat as morphological marker. The lower doses of EMS did not effect adherently with clear cut ridges as compare to higher doses of mtsl. whereas higher doses cause minutely increase in the VCR and VLB percentage.

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