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Micropropagation and apical cell morphology study of flowerless plant of *Catharanthus roseus* (L.) G. Don

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Abstract

A flowerless plant of *Catharanthus roseus* (L) G. Don was isolated by treating seeds with 0.4% NMU. The flowerless plant was medium tall, leaves curved clustered at the tip of stem, flowers absent. These variant was unable to set fruits and seeds. Therefore, multiple shoot were induced through direct organogenesis from the shoot tips of flowerless plant. The combination of 0.2 BAP, 1.0 kinetin and 0.05 mg/L NAA showed higher shoot induction and growth index. The roots were induced in MS medium supplemented with 0.5 mg/L IAA. Apical cell morphology study showed lack of differentiation of SAM into IAM. This depression caused by strong alkalyting action of NMU. A model explaining the phenomenon of flowering and nonflowering constructed, based on longitudinal section of stem going through apical dome.

Keywords: *Catharanthus roseus*, NMU, flowerless plant, multiple shoots

Introduction

Natural resources, like medicinal plants which contain a wide range of secondary metabolites are the most important source of life saving drugs for majority of the world's population. Global attention has been shifted towards detecting new phytochemicals from plants which help in the development of new drugs. The plant tissue culture technique offers an integrated approach to select, multiply and conserve the critical genotypes of medicinal plants and production of phyto-pharmaceutical of standard quality through mass-production of consistent plant material (Nisbet *et al*.1997) [20]. The Madagascar periwinkle (*Catharanthus roseus*) is a short-lived perennial; a valuable medicinal plant belonging to the family Apocynaceae produces many terpenoid indole alkaloids (TIAs), such as vindoline, ajamlicine, serpentine, catharanthine, vincristine and vinblastine (Goyal *et al*. 2008) [11]. More scientific evidences have proved the potential health benefits of individual phytochemicals extracted from this plant (Nejat *et al*. 2015) [19]. The vinblastine and vincristine from *C. roseus*, and their synthetic analogues, have been used in combination with other cancer chemotherapeutic drugs for treating advanced testicular cancer, breast and lung cancers (Cragg and Newman 2003) [6]. However, the yield of indole alkaloids are low in the wild-type plants, and the total chemical synthesis at large scale is extremely difficult due to high-cost and their complicated structures. Thus, there is a need for developing whole plant and/or single cell genotypes that will hyper-synthesize and accumulate pharmaceutically important alkaloids. Periwinkle organogenesis was first reported in late 1970s by Dhruva *et al*. (1977) [7] followed by Ramavat *et al*. (1978) [22] and Abou-Mandour *et al*. (1979) [1] where the rate of shoot regeneration was low. Although *Agrobacterium* infection or particle bombardment techniques had been established and studied intensively but these transgenic cell lines do not produce alkaloids in a stable manner and their ability to accumulate TIAs is gradually declined in long-term cultures (Wang *et al*. 2012) [28]. The mutilple shoot regeneration of *C. roseus* on all concentrations of BAP and NAA were experimented by Haq *et al*. 2013 [12]. The tip of shoot cultures on the 2 mg/L of BA supplemented medium showed significantly increased number of shoots as well as dry and fresh weight (Al-oubaidi *et al*. 2014) [2]. A study was undertaken to increase the yield of alkaloids by application of EMS and gamma rays alone and in combination on the seeds of *C. roseus* through conventional breeding method by Roy *et al*. (2013) [24]. A recent study describes the antioxidant, antibacterial and photocatylatic activities of silver nanoparticles synthesized using flowers of six varieties of *C. roseus* (Kandiah and Kavishadi, 2021) [16]. The object of the present work is to optimise the growth medium (*in vitro*) and study of floral development in flowerless plant.

Materials and Methods

Induction of Mutation and Morphological and Histological Study

Air-dried mature seeds of *C.roseus* (var. NIRMAL) having moisture content of about 10-12% were used for mutagenic treatment under room temperature during last week of February. The seeds were soaked in freshly prepared buffered aqueous solution of 0.2%.and 0.4%N-methyl-N-nitrosourea (NMU) following the method of

Kulkarni *et al*. (2005) [17]. The treated seeds were thoroughly washed in running water to remove the excess mutagen from seed surface. The seeds were then blotted and sown in the field immediately after treatment along with control. Bulk seeds from mutagenic populations of M_1 generation were collected from mature pods of healthy plants. These bulk seeds were sown in raised nursery beds along with control to grow M_2 generation separately for both mutagenic treatments. After one month of transplantation, all plants have undergone rigorous selection and rejection process to identify and isolate variants *in vivo*. After isolation of flowerless variant, morphometric observations like plant height, number of branches, leaf area, flowering percentage and harvest index in compare to control were measured following the method Roy *et al*. 2013 [24]. The apical portion of the stem of control and flowerless plants were critically studied by fine longitudinal section going across apical dome, mounted in a drop of 10% glycerine and observed under 100X magnification of bright field microscope (Zeisis).

Micropropagation of Flowerless Plant

For initiation of culture and shoot regeneration through direct organogenesis, the shoot tips were first washed in 2% (v/v) Teepol solution (Qualigens, India) for five minutes and were washed thoroughly in running tap water for 3 hours. After thorough washing, explants were surface sterilized with 0.1% $HgCl_2$ (mercuric chloride) for three minutes and rinsed with sterilized distilled water four times in front of laminar air flow cabinet for maintaining aseptic conditions. The explants were inoculated into Murashige and skoog's (MS) basal medium supplemented with 3% sucrose solidified with 0.6% agarose as well as with various combinations and concentrations of 6-Benzylamino purine (BAP) (0.2- 1.0 mg/l), Kinetin (kn) (0.5-1.0 mg/l) and α-Naphthalene acetic acid (NAA) (0.05 mg/l) in 250 ml Erlenmeyer flask. The pH of the medium was adjusted to 5.6 prior to addition of agar and autoclaving. All cultures were incubated at 22 ± 2°C under 10 hours photoperiod in randomised block on culture rack. After 30 days of primary inoculation, sub-culturing on the same medium composition was repeated. The emergence percentage and length of leafy shoots were observed and responses of phytohormones critically evaluated after 60 days. The growth index was calculated according to the formula of Hirata *et al* (1989) [13]. The regenerated shoots were excised from parent culture and were transferred to half and full strength of MS semi-solid medium with different concentrations of IAA (0.5- 1 mg/L) for the induction of roots. The *in vitro* grown plants with well-developed root systems were first washed under running tap water and then were transferred to plastic cups containing sterile sand-soil mixture (1:1) with adequate water and small amount of half strength MS salts. The surviving plants were transplanted to the soil in earthen pots in net house for gradual acclimatization and finally transplanted to fields. All experimental data were analysed by one-way ANOVA test, followed by the DMRT comparison at 0.05 significance level using SPSS (version 16) software package. Statistical analyses were made on the arcsine transformed values of the data. The chemicals used for preparation of MS medium were procured from Merck (AR), PGR from Himedia and NMU from Sigma chemicals.

Results and Discussion

A flowerless plant was isolated from 0.4% NMU dose in a population of 150 germinated plants of M_2 generation. The flowerless plant was medium tall plants having semi-erect growth habit, stem moderately thin and greyish green, leaves ovate, mucronate, oblique, entire with smaller leaf area. Leaves were present at the apical portion of the stem where flowers are absent, while control plants were tall (70-75 cm), leaves obvate, opposite-decussate and flowers white in colour (Fig 1). Previously flowerless mutants of *Catharanthus roseus* have been reported by Belsevich and Bishop (1989) which exhibited abnormal characters such as modification of flowers into vegetative structures, transformation of petals and sepals into green leaves, stamens into staminodes and ovary into leafy structure. Venkateswarlu *et al.* (1983) [27] recovered three mutants that exhibited abnormal characters such as modification of flowers into vegetative structures after 18 hour treatment with 0.01% hydroxylamine. Thus, from the previous and present research it can be assumed that application of chemical mutagens may affect flowering phase ultimately causing high sterility in plants. The application of NMU produced point mutations in DNA, ultimately affecting the morphology of plant in general. The point mutation is less detrimental than large chromosomal rearrangements (Gilchrist and Haughn 2010) [10].

Fig 1: Apical portion of a branch of *Catharanthus roseus* L. (G.) Don A. flowerless plant B. Control plant

The comparative morphometric observations like plant height, number of leaves and branches, leaf area, flowering and harvest index between flowerless and control plants of M_2 generation have been summarized in Table 1. The flowering percentage recorded hundred percent inhibition. The absence of flowering phase may be the result of cumulative effect of meiotic abnormality in M_1 generation combined with some physiological and genetic damage induced by NMU (Jaybalan and Rao, 1987) [15]. NMU is a strong alkalyting agent indicating a high degree of sterility due to gene mutation and cytologically undetectable cryptic structural changes (Prasad, 1972) [21].

Table 1: Morphometric variation of traits under study in control and flowerless plant

Traits	control	flowerless
plant height (cm)	70.05	45.225
number of branches	21.675±1.68	11.225±1.01
leaf area $(cm)^2$	6.275±0.36	4.225±0.29
flowering %	55.325±1.57	0.000±0.00
harvest index %	15.45	11.1

The flowerless plant was not able to set flowers, fruits and seeds. Therefore, the multiple shoot cultures were induced from the shoot tips through direct organogenesis, a suitable *in vitro* protocol for rapid propagation of flowerless (Fig. 2). The time taken for initiation of shoots from the primary explants ranged from 11.2 ± 0.06 to 15.6 ±0.09 days (Fig 3).The occurrence of the leafy shoots regenerated from explants depends upon the plant growth regulators supplemented in the medium. The concentration of NAA remains constant in the experiment which fails to exhibit much beneficial effect on leafy shoot emergence. However, the varying concentrations of BAP and kinetin play key role in determining the fate of explants. The addition of NAA alone in the medium exhibited low frequency of leafy shoots which reflects inactiveness of the explants due to lack of adequate endogenous growth regulators (Roy and Banerjee 2000) [23]. The best response was achieved through the application of 0.2 BAP, 1 kinetin and 0.05 mg/L NAA which implies the synergistic action for enhancing shoot emergence (Table 2).

Table 2: Response of shoot tip culture of flowerless variant on MS media supplemented with different growth regulators after 60 days of inoculation

PGR combinations with medium	Leafy shoot emergence%	Length of leafy shoot	Growth Index (%)
0.0 BAP+0.0 Kn+0.05 NAA	$16.325±1.205^{b}$	$1.165±0.057^{b}$	$12.953±2.48^{a}$
0.1 BAP+0.5 Kn+0.05 NAA	$25.65±1.245^{c}$	$1.855±0.04^{c}$	$29.90±1.82^{b}$
0.2 BAP+1.0 Kn+0.05 NAA	$36.3±1.363^{d}$	$2.675±0.26^{d}$	$54.90±2.10^{d}$
0.3 BAP+1.5 Kn+0.05 NAA	$24.875±0.796^{c}$	$1.3725±0.23^{b}$	$45.925±2.46^{c}$
0.4 BAP+2.0 Kn+0.05 NAA	$0.935±0.127^{a}$	$0.63±0.09^{a}$	$31.525±1.39^{b}$

The data are given as mean ± SE. Means having the different superscript letter in a column were significantly different at 0.05 significance level.

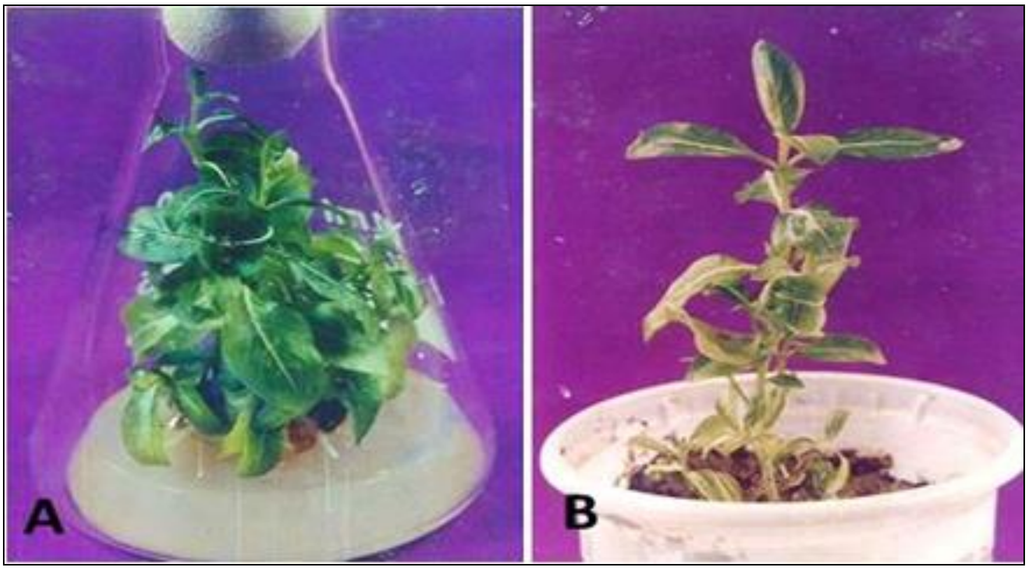

Fig 2: A. *in vitro* culture, B. Hardening of flowerless plant

However, the higher concentration of BAP and kinetin with NAA (0.4+2.0+0.05 mg/L) suppressed the shoot formation potential and growth index.. The exogenously supplied hormone in coordination with endogenous hormone present in tissue showed better growth in optimum conditions as observed in Table 2.

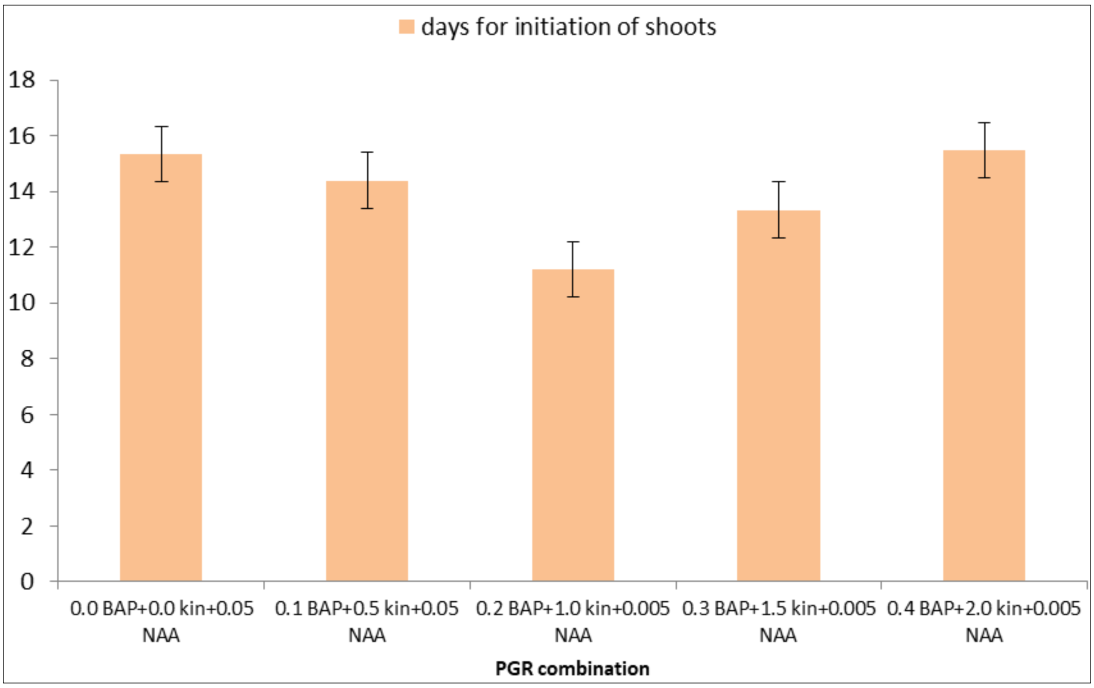

Fig 3: Days of *in vitro* initiation of shoots in flowerless

Table 3: Regeneration of roots after sub-culture of flowerless variant on full strength and half strength MS media supplemented with IAA

PGR combinations with medium	Root emergence%	Length of root	Days of rooting
MS+ 0.5 IAA	33.475±0.84 ^{c}	2.425±0.15 ^{c}	17.675±0.55^{a}
MS+ 1.0 IAA	25.9±0.36^{b}	1.6±0.14^{b}	22.4±1.029^{c}
1/2MS+ 0.5 IAA	18.8±0.76^{a}	0.725±0.13^{a}	25.875±0.87^{d}
1/2MS+ 1.0 IAA	36.8±0.57 ^{d}	1.8±0.16 ^{b}	19.525± 0.59^{b}

The data are given as mean ± SE. Means having the different superscript letter in a column were significantly different at 0.05 significance level.

The time required for initiation of roots after sub-culturing ranged from 17.3 ± 0.4 to 26.4 ± 0.22 days. Table 3 showed that rooting was initiated better in the lower concentration of IAA in full strength of MS medium. The higher concentration of IAA put inhibitory effect on root formation. The ratio of cytokinin to auxin seemed critical for shoot regeneration. However, the naturally occurring hormones in periwinkle tissues failed to induce *in vitro* shoot formation. Addition of exogenous PGRs to the medium was necessary for plant regeneration of periwinkle (Swanberg and Dai 2008). The regenerated *in vitro* plants were well established in the field. Longitudinal section of flowerless passing through apical region demonstrated the traces of floral buds in contrast to fully developed floral buds of control plant (Figs. 4)). In the control plant, with the onset of reproductive phase, the shoot apical meristem (SAM) turned into inflorescence apical meristem (IAM) and there upon developed a pair of opposite leaves at axil of each stem for the formation of floral meristem(s). However, in the axils of flowerless variant, SAM did not turn into IAM. The lateral region of SAM had opposite pair of leaves.

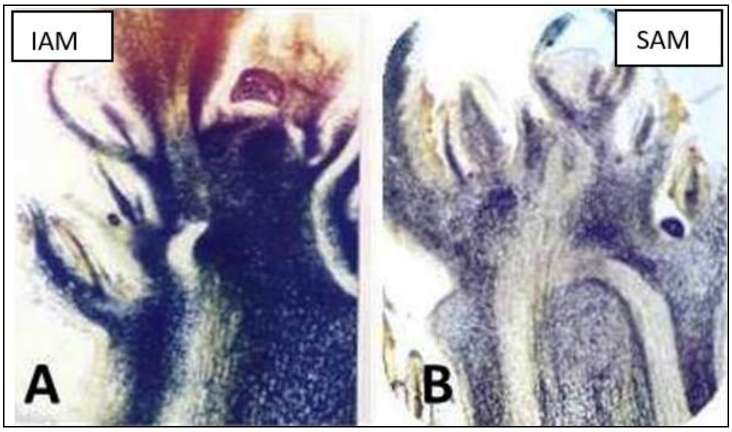

Fig 4: Longitudinal section of apical region of A. control B. flowerless plants

Apical cell morphology revealed the stimulation of cell division and change in size and shape in various parts of the shoot apex which are known to be common features of the floral transition in many plant species (Francis and Herbert 1993) [9]. The shoot apical meristem had a tunica/ corpus organization exihibiting the classical separation into three zones like central, peripheral and rib zones. The peripheral zone surrounding the central zone produced leaf primordial and rib zone lying below the central zone produced central pith of stem axis (Lydon 1998). An increase in cell division in shoot apical meristem leads to subsequent morphological changes at the SAM that are required for floral transition and initiation of first reproductive structure (Fletcher 2002) [8]. The action of mutagen hindered the complete transition of SAM into IAM resulting in traces of floral buds in flowerless variant. Plants homozygous for mutant allele produce either a small number of flowers or none due to undifferentiated out-growth of apical meristem (Bowman *et al*. 1989) [4]. However the failure of flower formation in flowerless variant has been explained by Smyth *et al*. (1990) [25]. During early flower development, a primordium forms on the flanks of the apical inflorescence meristem and adopts floral fate. The cells at the distil end of floral primordial gave rise to flower. Alternatively mutants which fail to form distal structure resulted in the formation of flowerless pedicels on peduncle. A model (Fig 5) has been deciphered for study of apical cell morphology following the interpretation of Chen *et al*. (1999) [5].

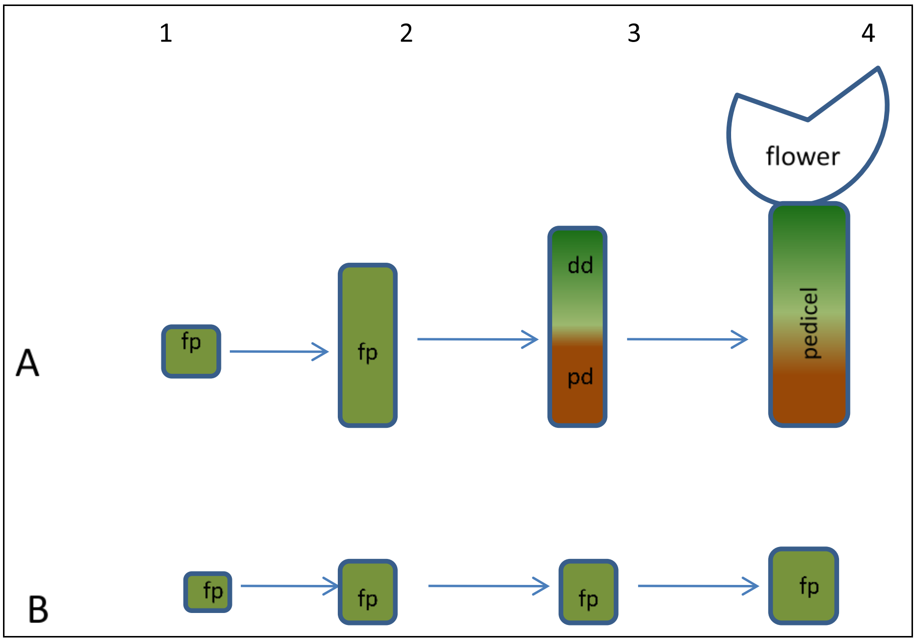

Fig 5: Floral primordia development model in *Catharanthus roseus* A. Control plant 1) IAM, 2) enlarlegement of IAM, 3) differentiation into proximal (pd) and distal (dd) end, 4) formation of pedicel at proximal end and flower on distal end. B. Flowerless variant IAM, 2) slight enlarlegement of IAM, 3) & 4) No differentiation in IAM

During early flower development in *Arabidopsis*, a primordium forms on the flanks of apical inflorescence meristem and adopts floral fate. The cells at the distal end of the floral primordium gave rise to a flower and proximal end differentiates into pedicel leading to establishment of a proximal-distal (P-D) axis within floral primordia (Chen *et al*. 1999) [5].

The floral primordium enlarges giving rise to a pedicel at its proximal end and at the distal end of a flower of the control plant. The flowerless plant has floral primordium without any differentiation into proximal and distal end. In flowerless plant, mutagenic treatment caused depression of cell division, which in turn hinders the initiation, and development of flowers.

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Conflict of Interest

The authors declared that they have no conflicts of interest with this submitted work.

References

1. Abou-Mandour A, Fischer S, Czygan F. Regeneration of intact plants from haploid and diploid callus cells of *Catharanthusroseus*. Z. Pflanzen-physiolgy Botany,1979:91:83-88.
2. Al-oubaidi HKM, Mohammed-Amin AS. Effect of Benzyl Adenine on multiplication of *Catharanthus roseus in Vitro*. World J of Pharmacy and Pharmaceutical Sciences,2014:3(6):2101-2107.
3. Balesvich J, Bishop JG. Distribution of catharanthine, vindoline and 3’,4’-anhydrovinblastine in the aerial parts of some *Catharanthus roseus* plants and the significance thereof in relation to alkaloid production of

cultured cells. Primary and secondary Metabolism of Plant Cell Cultures Ed. WGW Kurz Springer-Verlag Berlin Heideilberg, 1989, 150-153.

4. Bowman JL, Smyth DR, Meyerowtiz EM. Genes directing flower development in *Arabidopsis*. Plant Cell,1989:1:37-52.
5. Chen Q, Atkinson A, Denichir O, Christensen T, Reynolds L, Drews GN. The *Arabidopsis* Filamentous flower formation gene is required for flower formation. Development,1999:126:2715-2726.
6. Cragg GM, Newmen DJ. Plant as a source of anticancer and anti HIV agents. Annual Applied Biology,2003:143:127-133.
7. Dhruva B, Ramakrishnan T, Vaidyanthan C. Studies in *Catharanthus roseus* callus cultures, callus initiation and differentiation. Current Science,1977:46:364-365.
8. Fletcher JC. Shoot and floral meristem maintenance in *Arabidopsis*. Annual Review of Plant Biology,2002:53:45-66.
9. Francis D, Herbert RJ. Regulation of cell division in the shoot apex. In: Ormodm J.C. and Francis, D. Dordrecht (editors). Molecular and cell biology of the plant cell cycle.: Kulwer Academic Publishers, 1993, 201-210.
10. Gilchrist E, Haughn G. Reverse genetics techniques: engineering loss and gain of gene function in plants, 2010. PMID: 20081218 DOI: 10.1093/bfgp/elp059
11. Goyal P, Khanna A, Chauhan A, Chauhan G, Puroshotam K. *In vitro* evaluation of antibacterial activity. International Journal of Green Pharmacy,2008:2(3):176-181.
12. Haq R, Naz S, Aslam F, Manzoor F. Comparison of *in Vitro* response of Micrppropagation and callogenesis of Medicinal plants, *Vinca rosea* L. Journal Agriculture Research,2013:51(1):9-17.
13. Hirata K, Kobayashi M, Mjamoto K, Hoshi T, Okazaki M, Muira Y. Quantitative determination of VLB in tissue culture of *Catharanths roseus* by radio immunoassay. Planta Medica,1989:55:262-264.
14. Hirata K, Miyamoto K, Muira Y. *Catharanthus roseus* L. (Periwinkle): Production of Vindoline and Catharanthine in multiple shoot cultures Biotechnology in Agriculture and Forestry Vol. 26 In: YPS Bajaj (editor) Medicinal and Aromatic Plants Vol. VI by Springer – Verlag, Berlin, 1994, 47-55
15. Jaybalan N, Rao GR. Effect of NMU and EMS on chromosomal behaviour of *Lycopersicon esculentum* Mill. during meiosis. Cytologia,1987:52:813-819.
16. Kandiah M, Kavishadi NC. Green synthesis of silver nanoparticles using *Catharanthus roseus* flower extract and the determination of their antioxidant, antimicrobial and photocatalytic activity. Journal of Nanotechnology, 2021, 1-18. http://doi.org/10.1155/2021/15512786
17. Kulkarni RN, Sreevalli Y, Baskaran K. Allelic differences at two loci govern different mechanisms of intraflower self-pollination in self-pollinating strains of periwinkle. Journal of Heredity,2005:96(1):71-77. doi: 10.1093/jhered/esi004
18. Lyndon RF. The shoot apical meristem. Cambridge University Press, Cambrigde, UK, 1998.
19. Nejat N, Valdiani A, Cahill D, Tan YH, Maziah M, Abiri R. Ornamental Exterior versus Therapeutic Interior of Madagascar Periwinkle (*Catharanthusroseus*): The Two Faces of a Versatile Herb. Scientific World Journal, 2015. :982412doi: 10.1155/2015/982412
20. Nisbet LJ, Moore M. Will natural product remain an important source of drug research for the future? Current Opin Biotechnology,1997:8:708-712.
21. Prasad MVR. A comparison of mutagenic effectiveness and efficiency of gamma rays, EMS, NMU and NG. Indian J of Genetics and Plant Breeding,1972:32(3):360-367.
22. Ramavat K, Rajbhansli R, Arya H. Shoot formation in *Catharanthus roseus*(L.)G. Don callus cultures. Current Science,1978:77:93-94.
23. Roy J, Banerjee N. Rhizome and shoot development during *in Vitro* propagation of *Geodorum densiflorum* (Lam.) S Chtlr. Scientia Horticulturae,2000:94:181-192
24. Roy AM, Banerjee N, Mandal S. Studies on alkaloid yield of *Catharanthus roseus* (L.) G. Don. through character correlation and path analysis. Elixir Applied Botany,2013:64:19193-19198.
25. Smyth DR, Bowman JL, Meyerowitz EM. Early flower development in *Arabidopsis*.Plant Cell,1990:2:755-767.
26. Swanberg A and Dai W. 2008. Plant regeneration of Periwinkle (*Catharanthus roseus*) via organogenesis. Horticulture Science,1990:43:832-836.
27. Venkateswarlu M, Kumar P, Kashinath J, Mogil T, Ramesh Babu CH. Sterile mutant induced by hydroxylamine in *Catharanthus roseus* (L.) G. Don. Indian Botanical Reporter,1983:2:82.
28. Wang KT, Wang Q, Xing S, Pan Q, Yuan F. Development of efficient Catharanthus roseus regeneration and transformation system using Agrobacterium tumefaciens and hypocotyls as explants, 2012. http://www.biomedcentral.com/1472-6750/12-34