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Variability in alkaloid content and phytochemical profile of periwinkle (*Catharanthus roseus* L.) cv. Local through gamma and ethyl methane sulphonate

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Abstract
The study was conducted to induce variability through mutation breeding using physical and chemical mutagens in periwinklecv. Local. The physical mutagen *i.e.,* gamma radiation dose ranging from 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60 kR and chemical mutagen *i.e.,* EMS ranging from 10, 20, 30,40, 50 and 60 mM. The leaf and root samples are collected from the mutated plants (M_2) having morphological variation plant height, flower color, leaf color and yield characters. The selected mutants of 40kR, 30mM were exposed for alkaloid estimation through High Performance Liquid Chromatography. The putative mutants were selected based on chromatographic pattern with having highest alkaloids are present compared to control. The higher vincristine content was observed in 40 kR (0.37 %), followed by 30mM (0.35%). The physical and chemical mutagens contributed to variabilities in alkaloid contents in periwinkle.The effect of physical mutagen *i.e.,* gamma radiation and on EMS on phytochemical analysis from the mutated periwinkle leaves. The results showed that the different concentrations used for induced mutagenesis in periwinkle 40 kR and 30mM can be highly beneficial to create variability.

Keywords: Gamma rays, Ethyl methane sulphonate, HPLC, periwinkle, phytochemical, alkaloids

Introduction
Periwinkle is an important medicinal plant in India commonly known as Madagascar periwinkle**.** It is widely cultivated in Virudhunagar district of Tamil Nadu due to the availability of congenial soil and temperature for growing crop, the particular pink type is only available in this area. The genus *Catharanthus* have 8 known species, of which 7 are endemic to Madagascar. *Catharanthus roseus* is an evergreen herbaceous plant belongs to the family Apocyanaceae. *Catharanthus* produces more than 100monoterpenoids indole alkaloids (TIA) in different organs of the plants (Barnett *et al.,* 1978) [4]. It is a perennial tropical glycophyte plant, native to Madagascar and from there; it has spread to India, Indonesia, Indo-china, Philippines and subsequently to other parts of the world. However, it is also widely cultivated and naturalized in the tropical and subtropical areas in the world (Lewis *et al*., 1977) [10]. Periwinkle contains dozens of alkaloids, among them vincristine and vinblastine are the major alkaloids play on important role in western medicine as potent as anticancer agents. Vindoline and catharanthine are the major monomer alkaloids as well as biosynthetic precursors for the "dimeric" alkaloids, vinblastine (0.038 % w/w) and vincristine (0.60-0.65%), two anticancer drugs used in the treatment of acute leukemia and Hodgkin's disease. They are naturally extracted from the pink periwinkle plant. Vinca alkaloids are the second-most-used class of cancer drug (Moudi *et al.,* 2013) [12].
Mutation breeding has proven supplement and an effective substitute for conventional breeding methods so as to confer specific improvement in a variety without significantly affecting its acceptable phenotype (Kodym and Afza, 2003, Ashutosh *et al.,* 2013) [8, 3]. The important role of induced mutation in the improvement of crop plants that can best be assessed on the basis of quantitatively inherited characters. Among all radiations like alpha gamma, and beta rays gamma rays are considered to be the most energetic form of radiation used in Periwinkle (Naeem *et al.,* 2015) [13]. The main role for ionizing radiations inducing chromosomal aberration and deletions. Ethyl methane sulphonate is one of the most commonly used mutagens in plants it will causes a high frequency of nucleotide substitutions especially towards guanine, as detected in different genomes. Even though limited work has been done to improve alkaloid production in periwinkle through conventional approaches (Sreevalli *et al.,* 2002, Verma *et al.,* 2008, Bhat *et al.,* 2007) [14, 15, 7].

Therefore, the present study was conducted to induce variability and document the profile based on alkaloid and phytochemical content of periwinkle cv.local was mainly focus for producing high yielding mutants with high alkaloids content using High Performance liquid Chromatography.

Materials and methods

Collection of materials

The seedmaterial of periwinkle seeds were collected in the January 2014 from Rajapalayam local type, from Virudhunagar district during the year 2014 at an altitude of latitude of 9.4218°N and Longitude 77.83 °E. Uniform sized seeds (1 kg) with 95 percent germination were collected for the experimental trial.

Mutagenic agents

Gamma treatment was given from the gamma chamber installed at the orchard of Horticultural College and Research Institute, Tamil Nadu Agricultural University, Coimbatore. Gamma ray source was cobalt - 60 in 1000 Ci, emitting 5000 Rads per minute at the time of irradiation. Ethyl Methane Sulphonate (CH_3 SO_2 OC_2 H_5) with a molecular weight of 124.16g and specific gravity of 1.20 from the sigma Aldrich corporation chemical company, U.S.A. was used for treating the periwinkle seeds. Treated seeds were sown in the nursery area, then the mature plants were transplanted in the main field. After six months M_1 generation plants were harvested, seeds were collected then the seeds were used for raising M_2 generation. The individual M_2 generation plants seeds were sown in the every plots. Based on the variability characters identified the individual plants *viz*., flower color, leaf color, etc, leaves and roots were collected. The collected plant samples were dried and then sieved and used for this experiment. The M_2 plant samples (leaves and Roots) are used for estimation of qualitative characters using High performance Liquid Chromatography.

Standards preparation

Vincristine sulfate and Vinblastine sulfate of sigma Aldrich corporation chemical company, U.S.A. used as reference standards. The above standards were dissolved in mobile phase (Acetonitrile – 0.01M diammonium hydrogen ortho-phosphate (75:25) Vincaleukoblastine (VLB) 300 µg ml^{-1} and Vincristine sulphate (VCR) 350 µg ml^{-1}(Arularasu, 2008) [2].

HPLC analysis

Quantitative determination of periwinkle leaf alkaloids was carried out by RP-HPLC using a Bondapak C_{18} column. The mobile phase consisted of Acetonitrile, 0.01 M diammonium hydrogen ortho-phosphate (75:25) and a linear gradient program was used. AOmniscribe series B-5000 liquid chromatograph, equipped with M6000A solvent delivery system, U6K septumless injector and model 440 absorbance detector set up at 254nm and 280 nm with a chart speed of 0.5 cm/min. at a flow rate of 1 ml min^{-1}.

The temperature of 27°C and injection volume of 10 µl for vindoline rich fraction at 0.5 AUFS, 100 µl at 0.5 AUFS and 2.0 AUFS were used for vinca-leukoblastine and vincristine respectively (Arularasu, 2008) [2].

Extract preparation

The economically mutated plant samples were selected and washed with running tap water, the samples were rinsed using distilled water. High amount of moisture should be reduced from the selected plant samples, shade dried and powdered. The powdered samples are used for preparation of extract.

Aqueous extraction

The 10 g powdered plant samples were dissolved in 100 ml of distilled water for 24hrs in rotator shaker. Then the samples is filtered with filter paper and used for phytochemical analysis.

Methanol extraction

Weighed 10 g powdered plant samples were dissolved in 100 ml of methanol for 24hrs in rotator shaker. Then the samples is filtered with filter paper and used for phytochemical analysis. The Qualitative analysis of phytochemicals in periwinkle was carried out based on procedures followed by Kabesh *et al*., 2015 [9].

Test for Alkaloids

To the 1 mL of extract added 1 mL of Mayers reagent and few drop of Iodine solution. Formation of yellow colour precipitate indicates the presence of Alkaliods.

Test for Terpenoids

To the 1 mL of crude extract added 1 mL of concentrated H_2SO_4 and heated for 2 minutes. A grayish colour indicates the presence of terpenoids.

Test for Phenol and Tannins

To the 1 mL of crude extract added 1 mL of $FeCl_3$. A blue green or black colour indicates presence of tannins.

Test for reducing Sugar

To the 1 mL of extract added 1 mL of Fehling's A solution and 1 mL of Fehling's B solution. Formation of red color indicates the presence of sugar.

Test for Saponins

To the 1 mL of extract added 2 mL of distilled water, shaken well and formation of 1 cm layer of foam indicates presence of saponins.

Test for Flavonoids

To the 1 mL of extract added few fragments of magnesium ribbon and added few drops of concentrated HCl drop wise. Appearance of pink scarlet colour confirmed the presence of flavonoids.

Test for Quinines

To the1 mL of extract added 1 mL of 1% NaOH and mixed well. Appearance of blue green or red indicates presence of Quinines.

Test for Protein

To the1 mL of extract added few drop of mercuric chloride. Formation of yellow colour indicates the presence of protein.

Test for Steroids

2 mL of extract mixed with 2 mL of chloroform and followed by 2ml concentrated H_2SO_4 sidewise. A red colour presence at the lower chloroform layer indicates presence of steroids.

Symbols used

+ = indicates presence of phytochemicals

- = indicates absence of phytochemicals.

++ = shows moderate concentration.

+++ = shows high concentration.

Results and Discussion

Compared to untreated samples the treated samples are significantly affected the vincristine and vinblastine content by physical and chemical mutagenic treatments. Based on High performance Liquid Chromatography method vincristine and vinblastine content are analysed in M_2 generation treated plants. The vincristine sulphate ranged from 0.03 to 0.37 similarly the vinblastine sulphate ranged from 0.037 to 0.085 (Fig 1 and 2). The maximum vincristine sulphate percentage was observed in T_1 (40 kR) i.e., 0.37 followed by T_5 (30 mM) i.e., 0.31. The minimum vincristine sulphate percentage was observed T_3 (40 kR) i.e., 0.03 followed by control. The maximum vinblastine sulphate percentage was observed in T_1 (40 kR) i.e., 0.085 followed by T_4 (30 mM) i.e., 0.077. The minimum vinblastine sulphate percentage was observed T_6 (30 mM) i.e., 0.037 followed by control. The experiment has exposed to presence of phytochemical constituents from periwinkle extracted leaves the graphical presentation of nine selected phytochemical *viz.*, alkaloids, terpenoids, phenol, sugar, saponins, flavonoids, quinines, protein, steroids.

Table 1: Effect of Gamma and EMS on Vincristine and Vinblastine content in leaves and roots of M_2 generation plants of *Catharanthusroseus* L.cv. local.

S. No	Treatments	Vincristine sulphate (%)	Vinblastine sulphate (%)
1	$T_{1\ (40\ kR)}$	0.37	0.085
2	$T_{2\ (40\ kR)}$	0.30	0.056
3	$T_{3\ (40\ kR)}$	0.03	0.044
4	$T_{4\ (30\ mM)}$	0.35	0.077
5	$T_{5\ (30\ mM)}$	0.31	0.054
6	$T_{6\ (30\ mM)}$	0.24	0.037
7	Control	0.10	0.014
Mean		0.24	0.05

The phytochemical constituents from periwinkle M_2- 30mM generation extracted leaves the 30mM terpenoids, saponins, alkaloids were higher in concentration while phenol and protein in medium concentration and sugar, flavonoids, quinines, steroids in lowest concentration or absent. Extracted from leavesM_2-40 Kr periwinkle leaves quinines, alkaloids were higher concentration, while saponins, phenol in medium concentration and steroids, flavonoids, sugar in lowest concentration or absent (Fig, 3,4, and 5).The conventional breeding methods induced mutation is an important complementary method for the creation of genetic variability for specific characters in a crop. Based on the present mutagenic treatment studies the quality parameters like vincristine and vinblastine content was influenced. Doses of 40 kR and 30 mm showed good mutability with enhanced effect for high vincristine and vinblastine content in periwinkle. The maximum vincristine content in leaves was observed in physical mutagen (40 kR – 0.37 per cent) and vincristine content in chemical mutagen is (30 mm- 0.35 percent), The maximum vinblastine content in leaves was observed in physical mutagen (40 kR – 0.085 percent) and vinblastine content in chemical mutagen is (30 mm- 0.077 per cent).

Table 2: Phytochemical analysis of M_2 generation periwinkle leaves from -control, gamma rays (40 kR) and EMS.

Phytochemical constituents	Control (L)		M_2 - 40 kR (L)		M_2 - 30mM (L)	
	Water extract	Methanol extract	Water extract	Methanol extract	Water extract	Methanol extract
Alkaloids	++	++	+++	++	++	+++
Terpenoids	+	+	+	+	+++	++
Phenol	++	+	++	+	+	+
Sugar	-	-	-	-	-	-
Saponins	++	++	+	+++	++	+
Flavonoids	-	-	-	-	-	-
Quinines	-	+	+++	-	-	-
Protein	++	+	+	+	+	+
Steroids	-	-	-	-	-	-

Higher doses of mutagen were directly proportional to the quality parameters of periwinkle. Enzymes involved in different stages biosynthesis of vincristine might be due to promotive or inhibiting influence exerted by gamma and ems at different doses causing altered physiological and biochemical reactions in enzymes biosynthesis. The mutants would have enhanced protein synthesis and synthesis of cellulose hydrolyzing enzymes which might have triggered the secondary metabolites involved in different stages of biosynthesis of phytochemical compounds, similar results were obtained by Anandhi *et al.*, 2013 [1] and Barnett *et al.*, 1978 [4]. The selection of plant types rich in alkaloids and phytochemical can be easily achieved by simple selection on the basis of a per se performance of the M_2 generation, selection in the early generation followed by confirmation of the enhanced trait in M_3 has been reported by many researchers (Mishra and Momin, 2004) [11]. Similar trend was also revealed by Baskaran *et al.*, 2013 [5] in which two EMS induced macro-mutants (‘necrotic leaf’ and ‘nerium leaf) of periwinkle with enhanced contents of total root and leaf alkaloids and anticancer leaf alkaloids, vincristine and vinblastine than the parental variety was being reported. Increasing in alkaloid biosynthesis in plant can be stimulated by various means, including radio-mutagenesis confirmation of results needs to be drawn from field experiments by inducing mutations and then making a survey of subsequent generations to select mutant lines richest in respective alkaloids content and other desired characteristics (Benslimani and Khelifi, 2014).

Conclusion

Induced mutation is one of the amenable approaches to

generate genetic variability in medicinal plants. The vincristine and vinblastine content was influenced by physical and chemical mutagenic treatment. The mutants with enhance synthesis of protein and triggered secondary metabolites. The identification of variant and mutant with high alkaloid content in periwinkle will be highly useful to propose the future prospect for the isolation of the secondary metabolites which can be used in pharmaceutical industries.

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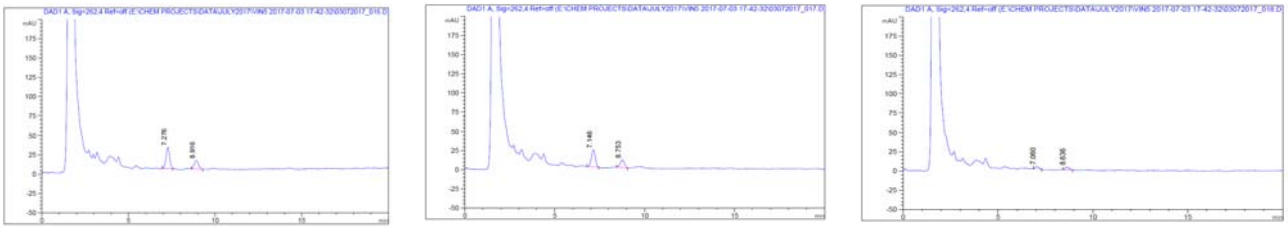

Fig 1: HPLC chromatogram of Vincristine and Vinblastine content in Physical mutagen (40kR)

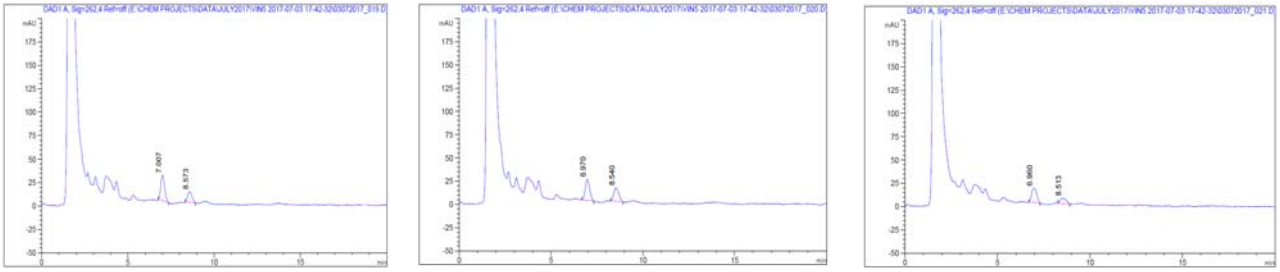

Fig 2: HPLC chromatogram of Vincristine and Vinblastine content in Physical mutagen (30 mM)

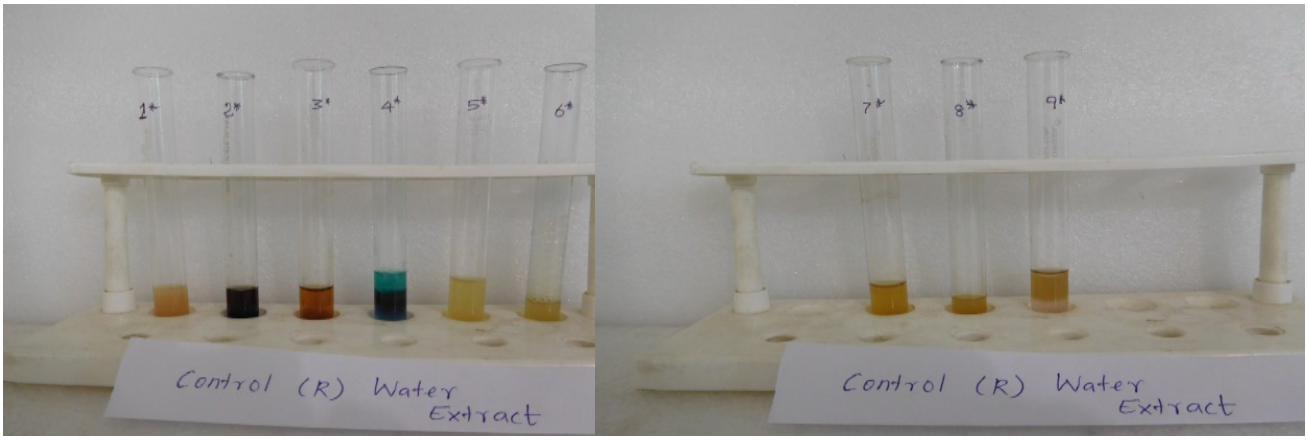

Fig 3: Phytochemical analysis of *Catharanthus roseus* leaf samples from water extract (M_2 control)

Fig 4: Phytochemical analysis of *Catharanthus roseus* leaf samples from methanol extract (M_2 40- kR)

Fig 5: Phytochemical analysis of *Catharanthus roseus* leaf samples from water extract (M_2 30 mM)

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