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ORIGINAL RESEARCH ARTICLE FOR VARIETY REGISTRATION

CIM-Sushil: A high vindoline yielding variety of *Catharanthus roseus* (L.) G. Don

ANIL K GUPTA[1*] • ASHUTOSH K SHUKLA[1*] • POOJA SINGH[1#] • MANEESHA MALL[1#] • SEEMA YADAV[1] • AJIT K SHASANY[1] • KARUNA SHANKER[1] • K BASKARAN[2] • VELUSAMY SUNDARESAN[2] • MOHAMMED TALHA[1] • ABHILASHA SRIVASTAVA[1] • SONI GUPTA[1] • PUJA KHARE[1] • DAYA N MANI[1] • ABDUL SAMAD[1]

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ABSTRACT

Catharanthus roseus produces many terpenoid indole alkaloids (TIAs) but is famous for its scarce and costly anticancer bisindole alkaloids, vinblastine and vincristine that are formed by coupling of monomeric TIAs, catharanthine and vindoline. Biosynthetic potential of the plant for bisindoles is very low due to their high cytotoxicity. The best option for industrial production of bisindoles is their semi-synthesis from the monomers. For this, catharanthine is sourced easily as it is produced throughout the plant and also in cell suspensions. But, sourcing vindoline is the main bottleneck since its biosynthesis is limited to green leaves and requires high level of cellular differentiation, which prevents its bioreactor-based production through cell culture. Vindoline synthesis is non-economical and its production in heterologous systems is still in infancy requiring much optimization. Thus, the plant remains the only major source of vindoline. Improving vindoline availability would therefore significantly reduce the exorbitant cost of bisindoles. Here, we report CIM-Sushil, a new high vindoline yielding C. roseus variety developed through EMS-induced mutation breeding approach. It has dwarf character, spreading/bushy growth (wide canopy) and small dark green leaves. Its leaves have ~0.2% vindoline content and ~5% total alkaloid content (% dry weight basis). Its estimated dry leaf yield potential is ~2418.3 kg/ha, estimated vindoline yield potential is ~4.8 kg/ha and estimated total alkaloid yield potential is ~120.9 kg/ha. It outperformed the check varieties, Nirmal and Dhawal, in content as well as yield of vindoline and total alkaloid. Its vindoline content is comparable to the global benchmark. Additionally, CIM-Sushil will be greatly beneficial for research purpose.

*Equal contribution as first and corresponding author, E-mail: ak.gupta@cimap.res.in (Anil K. Gupta); ashupov@yahoo.com (Ashutosh K. Shukla)
#Equal contribution as second author
[1]CSIR-Central Institute of Medicinal and Aromatic Plants, P.O. CIMAP, Lucknow-226015, Uttar Pradesh, India
[2]CSIR-Central Institute of Medicinal and Aromatic Plants, Research Centre, Allalasandra, GKVK Post, Bengaluru-560065, Karnataka, India

INTRODUCTION

C. roseus produces over 130 terpenoid indole alkaloids (TIAs) but it is significant due to the potent anticancer activities of its bisindole alkaloids, vinblastine and vincristine that are extremely costly and scarce drugs (Shukla and Khanuja, 2013). As a rough estimate, vinblastine currently costs ~US$ 20/mg and vincristine is at least two times costlier (Merck website, 2019). These bisindole alkaloids are composed of two monomeric TIA moieties known as vindoline and catharanthine. As the bisindoles are highly cytotoxic, the plant keeps the monomeric precursors spatially apart, whereby catharanthine is secreted to the leaf surface in wax exudates and vindoline is retained within the leaf cells (Roepke et al., 2010). Due to this compartmentation, the bisindoles are normally present at extremely low levels in the plant (~0.0003% on dry weight basis), and their levels probably rise only when the leaf is damaged due to herbivory, wounding, etc. Clearly, the plant cannot accumulate higher amounts of the highly cytotoxic bisindole alkaloids. The total chemical synthesis of bisindoles and monomeric precursors is known but is economically not viable. The best option for industrial scale production of bisindole alkaloids is their semi-synthesis from the monomeric precursors. For this, catharanthine is obtained relatively easily as it is present throughout the plant (aerial and underground parts) and also in cell suspensions. But, sourcing vindoline is the main bottleneck as its biosynthesis is limited to the green leaves and requires high level of cellular differentiation with presence of well-defined thylakoids, which prevents its bioreactor-based production. Efforts have also been made to produce vindoline in heterologous systems like yeast, but are still in infancy and require much optimization (Qu et al., 2015). Thus, the *C. roseus* plant is still the major source of vindoline and any effort aimed at improving vindoline availability will significantly reduce the exorbitant cost of the bisindoles. The global benchmark for vindoline content is cv. Pacifica Peach, which has been reported to maximally accumulate up to 0.2% (dry weight) vindoline (Chung et al., 2011). The vindoline content in the best Indian varieties, Nirmal and Dhawal (Kulkarni et al., 2003), is less than 50% of that in Pacifica Peach. The third Indian variety, Prabal (Dwivedi et al., 2001) produces lesser vindoline than Nirmal and Dhawal (Pandey et al., 2016).

With this background, the target of the present study was to develop an industrial variety of *C. roseus* that could consistently accumulate 0.2% or more vindoline coupled with high herbage yield. This has fructified in the form of superior strain CIMAP866, which was christened and released as the variety CIM-Sushil. This variety is the outcome of extensive mutation breeding work [on ethyl methanesulfonate (EMS)-induced mutant population] carried out during 2003 to 2019 at CSIR-CIMAP (Fig. 1). The National Gene Bank for Medicinal and Aromatic Plants (NGBMAP) at CSIR-CIMAP, has a rich collection of *C. roseus* genotypes, including mutants originating from seeds of elite variety Nirmal through various types of chemical mutagenesis and contributed by various workers over a period of time (Kulkarni et al., 1999; Rai et al., 2003). The strain CIMAP866 has originated from 0.2% v/v EMS mutagenesis of Nirmal seeds. It is a stable homozygous mutant genotype, which has distinct morphological characters. It was consistently found to contain ~ 0.2% vindoline content and ~5% total alkaloid content in its leaves (on % dry weight basis) (Mall, 2017; Mall et al., 2020, under communication). It was evaluated with the check varieties, Nirmal and Dhawal. The advanced multilocation evaluation trial was conducted at Lucknow (North India) and Bengaluru (South India). We provide here the details of development, evaluation trials and performance data of the variety CIM-Sushil for the purpose of registration of this variety for its commercial cultivation.

MATERIALS AND METHODS

Chemical mutagenesis, screening, selection and herbage improvement

Mutation breeding experiments on *C. roseus* were initiated in 2003-04. The seeds of the widely cultivated elite dieback-resistant variety Nirmal of *C. roseus* were pre-soaked in water for ~ 12h, mutagenised with varying concentrations of EMS

(0.2%, 0.4%, 0.6%, 0.8% and 1.0%) for 6 h taking ~ 500 seeds per dose, and sown to obtain ~2500 M1 plants that in turn produced M2 seeds. The M2 seeds were used to raise the M2 population. Since the number of plants was too high it was practically impossible to evaluate all the M2 plants. A total of 312 distinct and fertile M2 plants (macro-mutants) were taken up for further evaluation of their vindoline content using a HPLC-based screen in 2004-05. It is well known that EMS mutagenesis introduces random mutations throughout a target genome, which can be used to identify loss-or gain-of function mutants through the application of appropriate screening methods (Qu et al., 2018). Here the HPLC-based screen used by us led to the identification of CIMAP866 as a vindoline-rich genotype (originating from 0.2% v/v EMS mutagenesis treatment of Nirmal seeds). It was found to possess a distinct morphology (characterized by dwarf phenotype, spreading/ bushy growth habit, wide canopy and small dark green leaves) and contained ~0.2% vindoline and ~5% total alkaloid content in its leaves (on % dry weight basis) (Mall, 2017; Mall et al., 2020, under communication). After selection of the vindoline-rich mutant genotype in the M2 population it was propagated and maintained vegetatively through terminal stem cuttings for some years. Also, attempts were made to increase its herbage through various breeding interventions as the leaves are the most important plant part for harvesting for vindoline. This was necessary as vindoline biosynthesis is restricted to the green leaves of the plant due to localization of one of the biosynthetic pathway enzymes, *S*-adenosyl-L-methionine: 16-methoxy-2,3-dihydro-3-hydroxy tabersonine-*N*-methyltransferase (NMT), in the thylakoid membrane (De Luca and Cutler, 1987; Shukla and Khanuja, 2013). Further the selected mutant was selfed for many generations to achieve the homozygous true to the type plants and maintaining the stability of the genotype from generation to generation. The major descriptors of *C. roseus* (Mishra et al., 2000) were used to study the morphological characters. The superior CIMAP866 strain was identified on the basis of vindoline and total alkaloid content as well as its

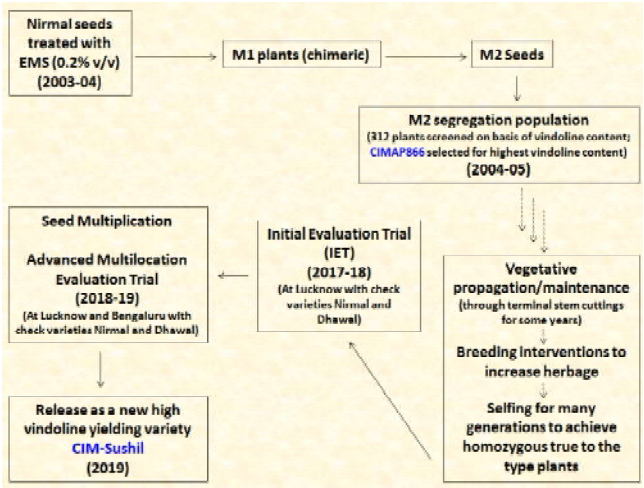

Figure. 1: Origin and scheme of evaluation of the vindoline-rich genotype CIMAP866 leading to the high vindoline yielding variety CIM-Sushil

distinct morphological characters *vis-à-vis* the check varieties, Nirmal and Dhawal (Mall, 2017; Mall et al., 2020, under communication), and was carried forward for evaluation through field trials. The entire breeding strategy adopted in the present study is outlined in Fig.1.

Evaluation trials

The CIMAP866 mutant line was evaluated in the initial evaluation trial (IET) at Lucknow during 2017-18 and advanced multilocation trials at Lucknow (Coordinates 26.8467° N, 80.9462° E; Elevation 123 m; Soil sandy loam) and Bengaluru (Coordinates 12.9716° N, 77.5946° E; Elevation 920 m; Soil red laterite) during 2018-19 along with the check varieties Nirmal [parental genotype] and Dhawal [another familial genotype that originated from Nirmal through mutagenesis by 0.06% N-nitroso-N-ethyl urea; Kulkarni et al., 2003, US Patent No. 6,548,746 B1] employing replicated multi row trials in a randomized block design (RBD) for assessing dry leaf yield as well as vindoline and total alkaloid content and yield. Since the best time to harvest *C. roseus* for recovering maximal vindoline has been found to be in winter and spring (mid-December to mid-April) under North Indian conditions (Mall et al., 2019), all the trials were performed in the non-rainy period (mid-September to mid-June), whereby plants were harvested at ~6 months age. Fertilizer application rate was 100, 40, and 40 kg/ha of N, P_2O_5 and K_2O, respectively and

normal agronomic practices were followed for raising the crop. The data generated from the Completely Randomized Block Design (CRBD) experiments was statistically analyzed and subjected to one way analysis of variance (ANOVA) to test the significance of variation between CIMAP866, Nirmal and Dhawal with the EXCEL® Add-in macro DSAASTAT software ver. 1.101 (Onofri and Pannacci, 2014).

Alkaloid extraction and analysis

Alkaloid extraction was carried out as per the protocol described by Shukla et al. (1997). Shade-dried leaves (1g) were powdered and extracted with 100% methanol (3 x 30 ml) at room temperature. Extraction was done for two hours each for the first two times and for the third time it was kept overnight. The combined methanolic extract was filtered and concentrated to ~10 ml *in vacuo* at 40 °C, diluted with 10 ml of water and further acidified with 10 ml of 3% HCl. Then the acidic aqueous layer was washed with hexane (3 x 30 ml), cooled to 10°C, basified with liquid ammonia to pH 9.0 and, finally extracted with chloroform (3 x 30 ml). The combined chloroform layer was washed with water, dried over anhydrous sodium sulphate and then evaporated to dryness. The residue obtained was dried to a constant weight to get the total alkaloid content. Further it was re-dissolved in 1 ml methanol for HPLC analysis to determine the vindoline content (Mall et al., 2020, under communication). The HPLC analysis was done on an Empower Pro-controlled HPLC system (Waters, USA) equipped with a 600E pump, 717plus autosampler, and a 2996-photodiode array (PDA) detector connected to a C18 column (250 × 4.6 mm, 5 µm; XSelect, Waters, USA). The injection volume was 20 µl. Optimal separation was achieved using a binary linear gradient mobile phase consisting of a mixture of solvent A (100 mM ammonium acetate buffer, pH 7.3) and solvent B (acetonitrile). Initiating the run with a 1.0 ml/min flow rate of the mobile phase (A:B::70:30) for 5 min, it was ramped to A:B::36:64 for the next 5 min with a flow rate of 1.4 ml/min. Subsequently, the composition of the mobile phase was changed to A:B::20:80 in next 5 min with the same flow rate of 1.4 ml/min and maintained for the next 5 min. Finally, the initial elution conditions were achieved in the next 1 min (A:B::70:30) at a flow rate of 1.0 ml/min. The column equilibrium was achieved by keeping the same conditions for the next 9 min. Consequently, the total analysis run took 30 min. The acquired PDA data (200-400 nm) was extracted at 254 nm for quantifying vindoline. The reference standard of vindoline (Sigma-Aldrich, USA) was used for calculating vindoline content (on % dry weight basis) in the *C. roseus* leaves by peak area calculation method.

PCR analysis of DNA

DNA isolation was carried out from the plant leaves as described by Khanuja et al. (1999) and its quantification was done on a Nanodrop spectrophotometer. Polymerase chain reactions (PCRs) for random amplified polymorphic DNA analysis were carried out in 25 µl. Each reaction contained 25 ng DNA, 0.2 U *Taq* DNA polymerase (GeNei, Bangalore, India), 100 µM of each dNTP, 1.5 mM $MgCl_2$, and 5 pmol of a decanucleotide primer. For amplifications the thermal cycler was programmed as: 94°C for 5 min; followed by 45 cycles of 94°C for 1 min, 35°C for 1 min, and 72°C for 2 min; and finally at 4°C (Shasany et al., 2005). The amplification products were resolved on a 1.2% agarose gel containing 0.5 µg/ml of ethidium bromide and their image was captured using a gel documentation system. A characteristic PCR profile was generated for CIM-Sushil using selected twenty four decanucleotide primers belonging to the MAP (CSIR-CIMAP, Lucknow) and OPJ (Operon Technologies, Alameda, CA) series (Shasany et al., 2005).

RESULTS AND DISCUSSION

Pedigree of CIM-Sushil

CIM-Sushil (the initial strain CIMAP866) has been developed through application of mutation breeding approach on the seeds of the elite dieback-resistant variety Nirmal of *C. roseus*. It has originated from 0.2% v/v EMS mutagenesis of Nirmal seeds.

Figure 2: Field view of the initial evaluation trial (IET) of CIMAP866 (CIM-Sushil) with check varieties, Nirmal and Dhawal, at Lucknow, during 2017-18

Initial evaluation trial at Lucknow location

The strain CIMAP866 along with the two checks (Nirmal and Dhawal varieties) was evaluated in a two row plot of 1.5 m length (area 1.5 m^2) replicated eight times in RBD (Fig. 2). Observations of fourteen traits - plant height (cm), stem diameter (cm), internodal distance (cm), petiole length (cm), leaf length (cm), leaf width (cm), flower diameter (cm), pedicel/corolla tube length (cm), siliqua length (cm), siliqua diameter (cm), number of seeds per siliqua, 100 seeds weight (g), vindoline content (% dry weight basis), and total alkaloid (% dry weight basis) were recorded (Table 1). The fresh leaf yield, dry leaf yield, vindoline yield, and total alkaloid yield data was also recorded (in g/plot and converted to g/m^2). The respective plot yields were used to estimate the fresh leaf, dry leaf, vindoline and total alkaloid yield in kg/ha (Table 2). The experimental details for IET at Lucknow included - seed sowing: in mid-September; transplanting: in mid-November; leaf harvesting: in mid-March; plot size: two rows of 1.5 m length, (1.5 m^2); replications: eight; row to row distance: 50 cm; plant to plant distance: 30 cm.

Advanced multilocation evaluation trials at Lucknow and Bengaluru locations

The strain CIMAP866 was further evaluated along with the two checks (Nirmal and Dhawal varieties) in a two row plot of 1.5 m length (area 1.5 m^2) replicated eight times (in Lucknow) (Fig. 3) or seven times (in Bengaluru) (Fig. 4) in RBD.

Table 1: Performance of CIMAP866 (CIM-Sushil) in the Initial Evaluation Trial (2017-18) at Lucknow, in two row plot of 1.5 m length (area 1.5 m^2), replications 8

S. No.	Character	CIMAP866 (CIM-Sushil)	Nirmal	Dhawal	Treatment M.S.S. (2)	Error M.S.S. (14)	S.E.M. (±)	SD	CD (5%)	CD (1%)	CV (%)
1.	Plant height (cm)	32.25	81.56	75.69	5804.07**	2.39	0.55	0.77	1.66	2.30	2.45
2.	Stem diameter (cm)	2.04	1.80	1.41	0.81**	0.003	0.02	0.03	0.06	0.09	3.37
3.	Internodal distance (cm)	0.75	2.02	2.18	4.89**	0.003	0.02	0.03	0.06	0.08	3.20
4.	Petiole length (cm)	0.74	0.82	0.81	0.016*	0.003	0.02	0.03	0.06	0.08	6.80
5.	Leaf length (cm)	3.29	4.39	4.26	2.91**	0.002	0.02	0.02	0.05	0.06	1.07
6.	Leaf width (cm)	2.37	2.22	2.20	0.069**	0.004	0.02	0.03	0.07	0.09	2.78
7.	Flower diameter (cm)	2.49	4.26	3.44	6.30**	0.005	0.03	0.04	0.08	0.11	2.12
8.	Pedicel length (cm)	2.02	2.56	2.44	0.65**	0.002	0.02	0.02	0.05	0.07	2.02
9.	Siliqua length (cm)	1.36	2.56	2.31	3.17**	0.002	0.01	0.02	0.04	0.06	1.89
10.	Siliqua diameter (cm)	1.00	1.01	1.00	0.0001	0.003	0.02	0.03	0.06	0.09	5.85
11.	Number of seeds per siliqua	14.38	22.44	19.63	133.97**	0.64	0.28	0.40	0.85	1.19	4.24
12.	100 seeds weight (g)	0.10	0.11	0.10	0.0004**	0.000002	0.0004	0.0006	0.001	0.002	1.18
13.	Vindoline content (% dry wt. basis)	0.21	0.05	0.09	0.06**	0.0002	0.005	0.01	0.01	0.02	11.61
14.	Total alkaloid (% dry wt. basis)	5.25	2.50	3.25	16.17**	0.55	0.26	0.37	0.79	1.10	20.18

SD- Standard deviation, CV- Coefficient of variation, SEM- Standard Error of the Mean, CD (5%) and CD (1%)- Critical difference at 5% and 1% level of significance, respectively. * and ** denote significance at 5% and 1%, respectively

Table 2: Yield performance of CIMAP866 (CIM-Sushil) in the Initial Evaluation Trial (2017-18) at Lucknow, in two row plot of 1.5 m length (area 1.5 m²), replications 8

S. No.	Yield Parameter	CIMAP866 (CIM-Sushil)	Nirmal	Dhawal	Treatment M.S.S. (2)	Error M.S.S. (14)	S.E.M. (±)	SD	CD (5%)	CD (1%)	CV (%)
1.	Fresh weight leaves (g/plot)	2328.25	1809.50	1410.75	1693212.5**	1437.83	13.41	18.96	40.66	56.44	2.05
2.	Fresh weight leaves (g/m²)	1552.17	1206.33	940.50	752538.89**	639.04	8.94	12.64	27.11	37.63	2.05
3.	Estimated fresh weight leaves (kg/ha)	15521.67	12063.33	9405.00	75253888.89**	63903.70	89.38	126.40	271.09	376.26	2.05
4.	Dry weight leaves (g/plot)	379.25	308.50	247.00	35037.17**	268.21	5.79	8.19	17.56	24.38	5.26
5.	Dry weight leaves (g/m²)	252.83	205.67	164.67	15572.07**	119.21	3.86	5.46	11.71	16.25	5.26
6.	Estimated dry weight leaves (kg/ha)	2528.33	2056.67	1646.67	1557207.41**	11920.63	38.60	54.59	117.09	162.51	5.26
7.	Vindoline (g/plot)	0.80	0.15	0.22	0.996**	0.001	0.01	0.01	0.02	0.03	5.91
8.	Vindoline (g/m²)	0.53	0.10	0.15	0.44**	0.0002	0.01	0.01	0.02	0.02	5.91
9.	Estimated vindoline (kg/ha)	5.31	1.03	1.48	44.25**	0.02	0.05	0.08	0.17	0.23	5.91
10.	Total alkaloid (g/plot)	19.91	7.71	8.03	386.80**	0.43	0.23	0.33	0.70	0.98	5.52
11.	Total alkaloid (g/m²)	13.27	5.14	5.35	171.91**	0.19	0.15	0.22	0.47	0.65	5.52
12.	Estimated total alkaloid (kg/ha)	132.74	51.42	53.52	17191.24**	19.11	1.55	2.19	4.69	6.51	5.52

SD- Standard deviation, CV- Coefficient of variation, SEM- Standard Error of the Mean, CD (5%) and CD (1%)- Critical difference at 5% and 1% level of significance, respectively. * and ** denote significance at 5% and 1%, respectively

Observations of fourteen traits - plant height (cm), stem diameter (cm), internodal distance (cm), petiole length (cm), leaf length (cm), leaf width (cm), flower diameter (cm), pedicel/corolla tube length (cm), siliqua length (cm), siliqua diameter (cm), number of seeds per siliqua, 100 seeds weight (g), vindoline content (% dry weight basis), and total alkaloid (% dry weight basis) were recorded at Lucknow (Table 3) as well as Bengaluru (Table 5). The data for fresh leaf yield, dry leaf yield, vindoline yield, and total alkaloid yield was also recorded (in g/plot and converted to g/m^2). The respective plot yields were used to carry out estimation of fresh leaf, dry leaf, vindoline and total alkaloid yield in kg/ha at Lucknow (Table 4) as well as Bengaluru (Table 6). The experimental details for advanced

Table 3: Performance of CIMAP866 (CIM-Sushil) in the advanced evaluation trial (2018-19) at Lucknow, in two row plot of 1.5 m length (area 1.5 m²), replications 8

S. No.	Character	CIMAP866 (CIM-Sushil)	Nirmal	Dhawal	Treatment M.S.S. (2)	Error M.S.S. (14)	S.E.M. (±)	SD	CD (5%)	CD (1%)	CV (%)
1.	Plant height (cm)	33.56	84.59	76.44	6011.97**	1.23	0.39	0.55	1.19	1.65	1.71
2.	Stem diameter (cm)	2.03	1.79	1.40	0.81**	0.003	0.02	0.03	0.06	0.08	3.21
3.	Internodal distance (cm)	0.76	2.03	2.16	4.80**	0.002	0.02	0.02	0.05	0.07	2.96
4.	Petiole length (cm)	0.75	0.81	0.81	0.010*	0.002	0.02	0.02	0.05	0.07	5.65
5.	Leaf length (cm)	3.28	4.38	4.29	3.02**	0.002	0.02	0.02	0.05	0.06	1.08
6.	Leaf width (cm)	2.36	2.21	2.19	0.07**	0.003	0.02	0.03	0.06	0.08	2.39
7.	Flower diameter (cm)	2.50	4.25	3.46	6.13**	0.002	0.02	0.02	0.05	0.07	1.47
8.	Pedicel length (cm)	2.01	2.55	2.45	0.65**	0.001	0.01	0.02	0.03	0.05	1.37
9.	Siliqua length (cm)	1.35	2.55	2.33	3.26**	0.001	0.01	0.02	0.04	0.05	1.74
10.	Siliqua diameter (cm)	1.01	1.00	1.00	0.0001	0.002	0.01	0.02	0.04	0.06	4.06
11.	Number of seeds per siliqua	14.94	23.00	19.81	131.91**	0.33	0.20	0.29	0.62	0.86	3.01
12.	100 seeds weight (g)	0.10	0.11	0.10	0.0003*	0.00001	0.001	0.001	0.003	0.004	2.35
13.	Vindoline content (% dry wt. basis)	0.20	0.04	0.08	0.06**	0.0002	0.01	0.01	0.02	0.02	13.84
14.	Total alkaloid (% dry wt. basis)	5.00	2.75	3.50	10.5**	1.17	0.38	0.54	1.16	1.61	28.8

SD- Standard deviation, CV- Coefficient of variation, SEM- Standard Error of the Mean, CD (5%) and CD (1%)- Critical difference at 5% and 1% level of significance, respectively. * and ** denote significance at 5% and 1%, respectively

Table 4: Yield performance of CIMAP866 (CIM-Sushil) in the advanced evaluation trial (2018-19) at Lucknow, in two row plot of 1.5 m length (area 1.5 m²), replications 8

S. No.	Yield Parameter	CIMAP866 (CIM-Sushil)	Nirmal	Dhawal	Treatment M.S.S. (2)	Error M.S.S. (14)	S.E.M. (±)	SD	CD (5%)	CD (1%)	CV (%)
1.	Fresh weight leaves (g/plot)	2274.75	1765.75	1508.75	1215848**	3540.95	21.04	29.75	63.81	88.57	3.22
2.	Fresh weight leaves (g/m²)	1516.50	1177.17	1005.83	540376.89**	1573.76	14.03	19.84	42.54	59.05	3.22
3.	Estimated fresh weight leaves (kg/ha)	15165.00	11771.67	10058.33	54037688.89**	157375.66	140.26	198.35	425.42	590.47	3.22
4.	Dry weight leaves (g/plot)	362.75	291.75	240.00	30382.17**	148.83	4.31	6.10	13.08	18.16	4.09
5.	Dry weight leaves (g/m²)	241.83	194.50	160.00	13503.19**	66.15	2.88	4.07	8.72	12.11	4.09
6.	Estimated dry weight leaves (kg/ha)	2418.33	1945.00	1600.00	1350318.52**	6614.81	28.76	40.67	87.22	121.06	4.09
7.	Vindoline (g/plot)	0.73	0.12	0.19	0.88**	0.0003	0.01	0.01	0.02	0.02	4.85
8.	Vindoline (g/m²)	0.48	0.08	0.13	0.39**	0.0001	0.004	0.01	0.01	0.02	4.85
9.	Estimated vindoline (kg/ha)	4.84	0.78	1.28	39.17**	0.012	0.04	0.06	0.12	0.17	4.85
10.	Total alkaloid (g/plot)	18.14	8.02	8.40	263.02**	0.23	0.17	0.24	0.51	0.71	4.15
11.	Total alkaloid (g/m²)	12.09	5.35	5.60	116.90**	0.10	0.11	0.16	0.34	0.47	4.15
12.	Estimated total alkaloid (kg/ha)	120.92	53.49	56.00	11689.57**	10.17	1.13	1.59	3.42	4.75	4.15

SD- Standard deviation, CV- Coefficient of variation, SEM- Standard Error of the Mean, CD (5%) and CD (1%)- Critical difference at 5% and 1% level of significance, respectively. * and ** denote significance at 5% and 1%, respectively

Table 5: Performance of CIMAP866 (CIM-Sushil) in the advanced evaluation trial (2018-19) at Bengaluru, in two row plot of 1.5 m length (area 1.5 m²), replications 7

S. No.	Character	CIMAP866 (CIM-Sushil)	Nirmal	Dhawal	Treatment M.S.S. (2)	Error M.S.S. (12)	S.E.M. (±)	SD	CD (5%)	CD (1%)	CV (%)
1.	Plant height (cm)	30.43	79.43	77.86	5428.43**	3.54	0.71	1.01	2.19	3.07	3.01
2.	Stem diameter (cm)	1.97	1.87	1.84	0.032*	0.005	0.03	0.04	0.08	0.12	3.82
3.	Internodal distance (cm)	0.64	1.74	1.69	2.68**	0.01	0.05	0.06	0.14	0.20	8.81
4.	Petiole length (cm)	0.51	0.57	0.56	0.006	0.002	0.02	0.03	0.06	0.08	8.76
5.	Leaf length (cm)	3.39	4.83	4.49	3.98**	0.03	0.06	0.09	0.19	0.26	3.76
6.	Leaf width (cm)	2.36	2.17	2.11	0.11*	0.03	0.06	0.09	0.19	0.26	7.31
7.	Flower diameter (cm)	3.24	3.79	4.01	1.10**	0.02	0.06	0.08	0.18	0.25	4.21
8.	Pedicel length (cm)	2.16	2.73	2.77	0.82**	0.004	0.03	0.04	0.08	0.11	2.61
9.	Siliqua length (cm)	1.96	2.73	2.66	1.27**	0.01	0.05	0.06	0.14	0.20	4.95
10.	Siliqua diameter (cm)	1.31	1.21	1.24	0.02*	0.005	0.03	0.04	0.08	0.11	5.44
11.	Number of seeds per siliqua	18.00	27.29	22.00	151.86**	4.19	0.77	1.09	2.38	3.34	9.13
12.	100 seeds weight (g)	0.12	0.12	0.12	0.00004**	0.000002	0.001	0.001	0.002	0.002	1.13
13.	Vindoline content (% dry wt. basis)	0.21	0.05	0.10	0.047**	0.0004	0.01	0.01	0.02	0.03	16.47
14.	Total alkaloid (% dry wt. basis)	5.14	2.43	3.29	13.48**	0.70	0.32	0.45	0.97	1.36	23.09

SD- Standard deviation, CV- Coefficient of variation, SEM- Standard Error of the Mean, CD (5%) and CD (1%)- Critical difference at 5% and 1% level of significance, respectively. * and ** denote significance at 5% and 1%, respectively

Table 6: Yield performance of CIMAP866 (CIM-Sushil) in the advanced evaluation trial (2018-19) at Bengaluru, in two row plot of 1.5 m length (area 1.5 m²), replications 7

S. No.	Yield Parameter	CIMAP866 (CIM-Sushil)	Nirmal	Dhawal	Treatment M.S.S. (2)	Error M.S.S. (12)	S.E.M. (±)	SD	CD (5%)	CD (1%)	CV (%)
1.	Fresh weight leaves (g/plot)	1869.14	2357.71	2227.14	447900.76**	26607.87	61.65	87.19	189.97	266.33	7.58
2.	Fresh weight leaves (g/m²)	1246.1	1571.81	1484.76	199067.01**	11825.72	41.1	58.13	126.65	177.55	7.58
3.	Estimated fresh weight leaves (kg/ha)	12460.95	15718.10	14847.62	19906700.53**	1182572.13	411.02	581.27	1266.48	1775.52	7.58
4.	Dry weight leaves (g/plot)	410.00	512.29	477.14	18906.48**	1197.59	13.08	18.5	40.3	56.5	7.42
5.	Dry weight leaves (g/m²)	273.33	341.52	318.10	8402.88**	532.26	8.72	12.33	26.87	37.67	7.42
6.	Estimated dry weight leaves (kg/ha)	2733.33	3415.24	3180.95	840287.83**	53226.10	87.2	123.32	268.69	376.68	7.42
7.	Vindoline (g/plot)	0.86	0.26	0.48	0.66**	0.001	0.01	0.02	0.04	0.06	6.82
8.	Vindoline (g/m²)	0.57	0.17	0.32	0.29**	0.0006	0.01	0.01	0.03	0.04	6.82
9.	Estimated vindoline (kg/ha)	5.74	1.71	3.18	29.14**	0.06	0.09	0.13	0.28	0.39	6.82
10.	Total alkaloid (g/plot)	21.07	12.45	15.70	132.84**	1.11	0.40	0.56	1.23	1.72	6.41
11.	Total alkaloid (g/m²)	14.05	8.30	10.47	59.04**	0.49	0.27	0.37	0.82	1.15	6.41
12.	Estimated total alkaloid (kg/ha)	140.49	82.99	104.65	5903.79**	49.19	2.65	3.75	8.17	11.45	6.41

SD-Standard deviation, CV - Coefficient of variation, SEM - Standard Error of the Mean, CD (5%) and CD (1%) Critical difference at 5% and 1% level of significance, respectively. * and ** denote significance at 5% and 1%, respectively

Figure 3: Field view of the advanced evaluation trial of CIMAP866 (CIM-Sushil) with check varieties, Nirmal and Dhawal, at Lucknow, during 2018-19

Figure 4: Field view of the advanced evaluation trial of CIMAP866 (CIM-Sushil) with check varieties, Nirmal and Dhawal, at Bengaluru, during 2018-19

multilocation evaluation at Lucknow and Bengaluru included - seed sowing: in mid-September; transplanting: in mid-November; leaf harvesting: in mid-March; plot size: two rows of 1.5 m length, (1.5 m²); replications: eight at Lucknow and seven at Bengaluru; row to row distance: 50 cm; plant to plant distance: 30 cm.

Statement of distinction, uniformity, stability and PCR profile

CIM-Sushil possesses a distinct morphology [mainly dwarf character, spreading/bushy growth (wide canopy) and small dark green leaves] (Fig. 5) and has ~ 0.2% vindoline content and ~5% total alkaloid content in its leaves (on % dry weight basis). It has demonstrated stability and uniformity for its distinct morphological characters and higher vindoline (Fig. 6) and total alkaloid content and yield in comparison to check varieties, Nirmal and Dhawal, during its evaluation. No variants have been observed during its multiplication and testing and the plant is maintaining the improved characters upon being propagated through selfed seeds. A characteristic PCR profile has been generated for CIM-Sushil using 24 decanucleotide primers (Fig. 7).

Figure 5: A representative *C. roseus* CIM-Sushil plant growing in the CSIR-CIMAP farm at Lucknow in 2019

Description of the variety CIM-Sushil

The recorded values for the distinguishing morphological characters (Table 7) and yield performance (Table 8) of CIM-Sushil (initially strain CIMAP866) along with the check varieties are provided. All the characters were recorded at the time of harvest. The CIM-Sushil plant has a dwarf character, spreading/bushy growth (wide canopy), smaller dark green leaves (lesser length, more width), shorter internodes, petioles and siliquae, smaller flower diameter and pedicel, lesser number of seeds in each siliqua and greater stem diameter as compared to the check varieties. It has ~0.2% vindoline content and ~5% total alkaloid content in its leaves (on % dry weight basis). The estimated dry weight of leaves produced by CIM-Sushil is ~2418.3 kg/ha, which is achieved within a short span of ~180 days. It provides an estimated vindoline yield of ~4.8 kg/ha and an estimated total

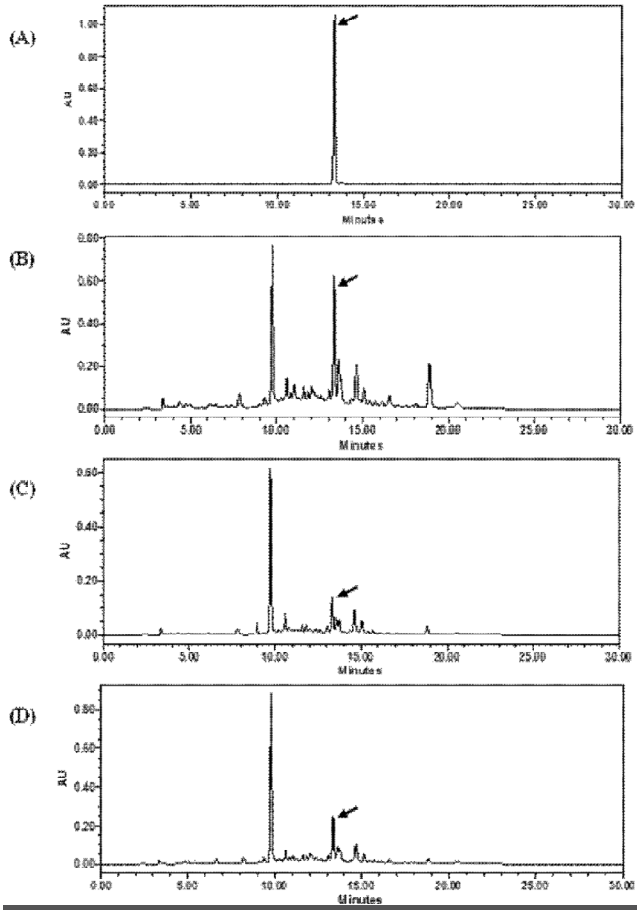

Figure 6: Representative HPLC chromatograms. A. Authentic vindoline standard; B. CIM-Sushil leaf extract; C. Nirmal leaf extract; and D. Dhawal leaf extract. The vindoline peak is marked with an arrow in each case

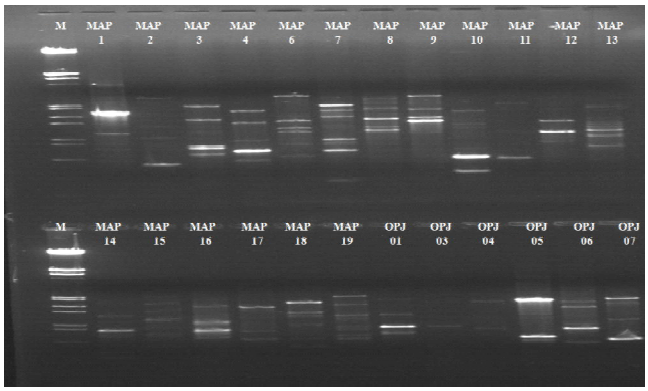

Figure 7: Characteristic PCR profile of CIM-Sushil with twenty four decanucleotide primers. M = DNA molecular weight marker (λDNA/*Eco*RI+*Hind*III fragments; 21226, 5148/4973, 4268, 3530, 2027, 1904, 1584, 1375, 947, 831, and 564 bp)

Table 7: Summary of distinctive morphological characters of CIM-Sushil vis-à-vis check varieties*

S. No.	Character	CIM-Sushil	Nirmal	Dhawal
1.	Plant height (cm)	33.56	84.59	76.44
2.	Stem diameter (cm)	2.03	1.79	1.40
3.	Internodal distance (cm)	0.76	2.03	2.16
4.	Petiole length (cm)	0.75	0.81	0.81
5.	Leaf length (cm)	3.28	4.38	4.29
6.	Leaf width (cm)	2.36	2.21	2.19
7.	Flower diameter (cm)	2.50	4.25	3.46
8.	Pedicel length (cm)	2.01	2.55	2.45
9.	Siliqua length (cm)	1.35	2.55	2.33
10.	Number of seeds per siliqua	~15	~23	~20

* As observed in the advanced evaluation trial (2018-19) at Lucknow (Table 3)

Table 8: Summary of comparison of CIM-Sushil with the check varieties for yield performance*

S. No.	Character	Lucknow			Bengaluru		
		CIM-Sushil	Nirmal	Dhawal	CIM-Sushil	Nirmal	Dhawal
1	Vindoline content (% dry weight basis)	0.20	0.04	0.08	0.21	0.05	0.10
2	Total alkaloid (% dry weight basis)	5.00	2.75	3.50	5.14	2.43	3.29
3	Estimated fresh weight leaves (kg/ha)	15165.00	11771.67	10058.33	12460.95	15718.10	14847.62
4	Estimated dry weight leaves (kg/ha)	2418.33	1945.00	1600.00	2733.33	3415.24	3180.95
5	Estimated vindoline (kg/ha)	4.84	0.78	1.28	5.74	1.71	3.18
6	Estimated total alkaloid (kg/ha)	120.92	53.49	56.00	140.49	82.99	104.65

* As observed in the advanced evaluation trial (2018-19) at Lucknow (Tables 3 and 4) and Bengaluru (Tables 5 and 6).

alkaloid yield of ~120.9 kg/ha. Like the check varieties, Nirmal and Dhawal, CIM-Sushil is also white flowered.

Potential for cultivation and commercial viability

The *C. roseus* crop is cultivated in about 3000 ha in India, mainly in the states of Tamil Nadu, Karnataka, Andhra Pradesh, Gujarat, Madhya Pradesh and Assam. Commercial cultivation of *C. roseus* is carried out in the Deccan region of India (Rajeswara Rao et al., 2012). If properly disseminated, around 50 % of this area may be covered by the new industrial variety within the next 5 years. New areas in the northern states like Uttar Pradesh and Bihar may also be brought under cultivation of CIM-Sushil. In due course of time, the cost of semi-synthetic production of anticancer bisindole alkaloids (vincristine and vinblastine) could be reduced significantly. The USA is globally the largest user of *C. roseus* as a raw material and consumes over 1000 MT of leaves annually. India is a major exporter of the *C. roseus* herb as per the data from the Directorate General of Commercial Intelligence and Statistics, Ministry of Commerce and Industry, Govt. of India. *C. roseus* is among 178 plant species in India that are traded in high volume (>100 MT) (Ravikumar et al., 2018). Exporters from Tuticorin in Tamil Nadu, India, exported 400 MT of *C. roseus* leaves during 2005-06 (Ravikumar et al., 2018), and the demand has grown tremendously since then. During the ten year period 2005-06 to 2014-15, the average export volume of *C. roseus* (earlier known as *Vinca rosea*) was 735 MT and the average export value was Rs. 4.6 crore (Goraya and Ved, 2017). As per the 2014-15 survey of 40 odd major herbal mandis (markets) in India, the trade volume of *C. roseus* leaves was 200-500 MT/year (Goraya and Ved, 2017). The higher yield of vindoline achieved through the new variety (CIM-Sushil) in the Indian export material would be able to fetch a premium price in the market and earn higher amount of crucial foreign exchange. In India, the cost of cultivating *C. roseus* is ~Rs 40000/ha and going by a conservative estimate (dry leaves sold @ Rs. 50/kg), CIM-Sushil is expected to give a net profit of ~Rs 80000 under North Indian conditions (Lucknow) and ~Rs 95000 under South Indian conditions (Bengaluru), whereby the dry leaf yield is ~2418.33 kg/ha and ~2733.33 kg/ha, respectively. This variety will also be greatly beneficial for research purpose, providing an excellent resource for prospecting genes related to the high vindoline trait.

Availability of nucleus planting material

CIM-Sushil is homozygous and stable, making it suitable for commercial cultivation. It can be propagated by seeds obtained through self-pollination and also through terminal stem cuttings. Seeds of CIM-Sushil are available for commercial cultivation at CSIR-CIMAP, Lucknow, and CSIR-CIMAP Research Centre, Bengaluru. Its NGBMAP Accession Number is CIMAP866.

CONCLUSION

CIM-Sushil meets the four essential criteria of novelty, distinctiveness, uniformity and stability. The vindoline and total alkaloid content as well as yield of CIM-Sushil are the highest reported so far in India. In fact its vindoline content is comparable to the global benchmark. Its herbage was found to be consistently superior to that of Nirmal and Dhawal at Lucknow although it was slightly lesser than that of the other two genotypes at Bengaluru. Nevertheless, CIM-Sushil was reproducibly found to be superior to Nirmal and Dhawal varieties in terms of vindoline and total alkaloid content and yield, at both Lucknow and Bengaluru. It was also found to be at par with its parental variety Nirmal for its resistance to die back disease. Interestingly, all the *C. roseus* varieties performed better at Bengaluru as compared to Lucknow, in terms of vindoline and total alkaloid yield, which reinforces the suitability of South India over North India for commercial cultivation of *C. roseus*. The new variety, CIM-Sushil, provides a superior natural source of vindoline with ~0.2% vindoline content (on % dry weight basis), which is higher than the levels reached in other existing varieties of *C. roseus* by the application of biotic factors like endophytes (Pandey et al., 2016; Tiwari et al., 2013). In future, efforts will be made to disseminate the industrial variety CIM-Sushil for commercial cultivation.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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NOTE

CIM-Sushil, developed by CSIR-CIMAP, was released for commercial cultivation on the occasion of CSIR Foundation Day 26 September, 2019. It is named in honour of Dr. Sushil Kumar, Former Director, CSIR-CIMAP, who has made an immense contribution to research on *Catharanthus roseus*.

AUTHOR CONTRIBUTIONS

AKG was the principal breeder of the variety, who carried out the original planning and execution of field experiments under his overall supervision. AK Shukla contributed in planning, G x E evaluation and analysis. PS and MM were involved in field data collection, compilation and analysis. SY contributed in field trials at Lucknow. AK Shasany contributed in the screening and selection. KS and PK contributed in chemical analysis. KB and VS contributed in conducting the trial at Bengaluru. MT carried out the statistical analysis of the data. A Srivastava and SG helped in data compilation and data analysis. DNM helped in plant maintenance at Lucknow. A Samad contributed in evaluation for biotic stresses as well as in planning and guidance for variety development. AKG and AK Shukla prepared the manuscript that was read and approved by all the authors.

REFERENCES

Chung I-M, Kim E-H, Li M, Peebles CAM, Jung W-S, Song H-K, Ahn J-K, San K-Y. 2011. Screening 64 cultivars *Catharanthus roseus* for the production of vindoline, catharanthine, and serpentine. *Biotechnol Prog* **27**: 937-943.

De Luca V, Cutler AJ. 1987. Subcellular localization of enzymes involved in indole alkaloid biosynthesis in *Catharanthus roseus*. *Plant Physiol* **85**: 1099-1102.

Dwivedi S, Singh M, Singh AP, Singh V, Uniyal GC, Khanuja SPS, Kumar S. 2001. Registration of a new variety Prabal of *Catharanthus roseus*. *J Med Arom Pl Sci* **23**: 104-106.

Goraya GS, Ved DK. 2017. Medicinal Plants in India: An assessment of their demand and supply. National Medicinal Plants Board, Ministry of AYUSH, Government of India, New Delhi and Indian Council of Forestry Research and Education, Dehradun.

Khanuja SPS, Shasany AK, Darokar MP, Kumar S. 1999. Rapid isolation of DNA from dry and fresh samples of plants producing large amounts of secondary metabolites and essential oils. *Plant Mol Biol Rep* **17**: 74.

Kulkarni RN, Baskaran K, Chandrashekara RS, Kumar S. 1999. Inheritance of morphological traits of periwinkle mutants with modified contents and yields of leaf and root alkaloids. *Plant Breed* **118**: 71-74.

Kulkarni RN, Baskaran K, Chandrashekara RSR, Khanuja SPS, Darokar MP, Shasany AK, Uniyal GC, Gupta MM, Kumar S. 2003. 'Dhawal', a high alkaloid producing periwinkle plant. U. S. Patent No. 6,548,746 B1.

Mall M. 2017. Molecular studies related to key terpenoid indole alkaloid biosynthesis in *Catharanthus roseus*. Ph.D. thesis, Jawaharlal Nehru University, New Delhi, India.

Mall M, Singh P, Kumar R, Shanker K, Gupta AK, Khare P, Shasany AK, Khatoon S, Sundaresan V, Baskaran K, Yadav S, Shukla AK. 2020. Phenotypic, genetic and expression profiling of a vindoline-rich genotype of *Catharanthus roseus*. (Under Communication).

Mall M, Verma RK, Gupta MM, Shasany AK, Khanuja SPS, Shukla AK. 2019. Influence of seasonal and ontogenic parameters on the pattern of key terpenoid indole alkaloids biosynthesized in the leaves of *Catharanthus roseus*. *S Afr J Bot* **123**: 98-104.

Merck website. 2019. https://www.sigmaaldrich.com/

Mishra P, Singh M, Dwivedi S, Kumar S. 2000. Descriptors of periwinkle *Catharanthus roseus*. *J Med Arom Pl Sci* **22**: 268-272.

Onofri A, Pannacci E. 2014. Spreadsheet tools for biometry classes in crop science programmes. *Communications in Biometry and Crop Science* **9**: 43-53.

Pandey SS, Singh S, Vivek Babu CS, Shanker K, Srivastava NK, Shukla AK, Kalra A. 2016. Fungal endophytes of *Catharanthus roseus* enhance vindoline content by modulating structural and regulatory genes related to terpenoid indole alkaloid biosynthesis. *Sci Rep* **6**: 26583.

Qu Y, Easson MLAE, Froese J, Simionescu R, Hudlicky T, De Luca V. 2015. Completion of the seven-step pathway from tabersonine to the anticancer drug precursor vindoline and its assembly in yeast. *Proc Natl Acad Sci USA* **112**: 6224-6229.

Qu Y, Thamm AMK, Czerwinski M, Masada S, Kim KH, Jones G, Liang P, De Luca V. 2018. Geissoschizine synthase controls flux in the formation of monoterpenoid indole alkaloids in a *Catharanthus roseus* mutant. *Planta* **247**: 625-634.

Rai SP, Luthra R, Kumar S. 2003. Salt-tolerant mutants in glycophytic salinity response (GSR) genes in *Catharanthus roseus*. *Theor Appl Genet* **106**: 221–230.

Rajeswara Rao BR, Syamasundar KV, Rajput DK, Nagaraju G, Adinarayana G. 2012. Potential species of medicinal plants for cultivation in Deccan region. *J Pharmacognosy* **3**: 96-100.

Ravikumar K, Noorunnisa Begum S, Ved DK, Bhatt JR, Goraya GS. 2018. Compendium of traded Indian medicinal plants. Foundation for Revitalization of Local Health Traditions (FRLHT), Bengaluru, India.

Roepke J, Salim V, Wu M, Thamm AMK, Murata J, Ploss K, Boland W, De Luca V. 2010. Vinca drug components accumulate exclusively in leaf exudates of Madagascar periwinkle. *Proc Natl Acad Sci USA* **107**: 15287–15292.

Shasany AK, Darokar MP, Dhawan S, Gupta AK, Gupta S, Shukla AK, Patra NK, Khanuja SPS. 2005. Use of RAPD and AFLP markers to identify inter- and intraspecific hybrids of *Mentha*. *J Hered* **96**: 542-549.

Shukla AK, Khanuja SPS. 2013. *Catharanthus roseus*: The metabolome that represents a unique reservoir of medicinally important alkaloids under precise genomic regulation. In: Barh D, ed. OMICS Applications in Crop Science. Boca Raton: CRC Press (Taylor & Francis Group), 325–384.

Shukla YN, Rani A, Kumar S. 1997. Effect of temperature and pH on the extraction of total alkaloids from *Catharanthus roseus* leaves. *J Med Arom Pl Sci* **19**: 430-431.

Tiwari R, Awasthi A, Mall M, Shukla AK, Satya Srinivas KVN, Syamasundar KV, Kalra A. 2013. Bacterial endophyte-mediated enhancement of *in planta* content of key terpenoid indole alkaloids and growth parameters of *Catharanthus roseus*. *Ind Crops Prod* **43**: 306– 310.