Selection of 5-methyltryptophan-resistant callus lines with improved metabolic flux towards terpenoid indole alkaloid synthesis in *Catharanthus roseus*

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Four variant callus lines showing differential tolerance to 5-methyltryptophan (5MT, a tryptophan analogue) were isolated in two accessions of *Catharanthus roseus* ('Nirmal' and 'C-6'), at 0.22, 0.27 and 0.54 mM level of 5MT stress (LD_{50} = 0.04–0.09 mM). The selected callus lines maintained on respective levels of 5MT stress showed a growth index comparable to that recorded for wild-type callus on the stress-free control medium. The line C_6 L-50 showed nearly 1.5-fold increase of its free amino acid pool in the presence of 0.22 mM 5MT stress in comparison to wild-type on the control medium. The lines Nir L-60, C_6 L-50, C_6 L-60 and C_6L-120 also recorded 5–16-fold increase in free tryptophan content in the presence of 5MT stress as compared to their respective wild types. All the selected lines showed higher crude alkaloid content in the presence of the respective 5MT stress. The lines Nir L-60 and C_6 L-120 also indicated the presence of both catharanthine and vindoline in presence of analogue stress, indicating a positive influence of tryptophan accumulation on diverting the metabolic flux towards synthesis of monomeric indole alkaloids.

Keywords: Callus, *Catharanthus roseus*, metabolic engineering, 5-methyltryptophan, tryptophan overproduction.

CATHARANTHUS roseus (L.) G. Don is an important alkaloid-yielding medicinal plant belonging to family Apocynaceae. *C. roseus* produces a wide range of terpenoid indole alkaloids (TIAs) possessing remarkable pharmacological properties; important among them are the antineoplastic (vincristine and vinblastine) and antihypertensive (ajmalicine and serpentine) alkaloids[1]. Terpenoid indole alkaloid pathway in *C. roseus* is one of the most extensively studied secondary metabolic pathways in plants[2,3]. The precursors for the TIA pathway are obtained from the shikimate and mevalonate pathways of the primary metabolic pool which provide tryptophan and secologanin respectively[4]. Several factors such as inherently larger pool of free tryptophan, secologanin abundance, tryptamine transport across the tonoplast and its utilization per se needs to be fine-tuned, if the desired flux towards TIA synthesis is to be achieved[5,6]. Tryptophan is one of the least abundant amino acids (16–80 µM) present in plants and is the sole donor of indole

ring for the synthesis of auxins, glucosinolates, nicotinic acid, phytoalexins and alkaloids[7]. Plant cells do not possess a larger sink capacity for tryptophan and hence it is produced as and when required[8]. Selecting mutants/variants that can overproduce and accumulate tryptophan for its larger availability and multi-flux distribution, is therefore an important step towards metabolic engineering of the TIAs. Here, we report the isolation of 5-methyltryptophan (5MT, a tryptophan analogue) tolerant callus lines in *C. roseus* with a capacity to overproduce tryptophan and divert it towards the TIA pathway, leading to the synthesis of monomeric indole alkaloids–catharanthine and vindoline.

Multiple shoot cultures of four accessions of *C. roseus*, viz. 'Nirmal', 'C-6', 'Red Petal' and 'White Petal' were initiated from axillary bud explants on Murashige and Skoog (MS)[9] basal medium supplemented with 23.23 µM kinetin, 8.87 µM 6-benzylaminopurine (BAP), 4.92 µM indole-3-butyric acid, sucrose 3% (w/v) and agar 0.8% (w/v)[10]. For callus initiation, leaf explants from *in vitro*-grown multiple shoot cultures were placed on MS basal medium supplemented with 4.52 µM 2,4-dichlorophenoxyacetic acid (2,4-D), 2.22 µM BAP, sucrose 3% (w/v) and agar 0.8% (w/v). Callus was multiplied and maintained through regular sub-culturing onto the fresh callusing medium through a 4-week culture cycle. To determine LD_{50} dose, the wild type calluses of the four accessions were plated on callusing medium to which a graded concentration series (0.02, 0.04, 0.06, 0.09, 0.11 and 0.13 mM) of 5MT stress was super-imposed. Screening of putative 5MT-tolerant variants was accomplished via a modified 'Direct Selection Scheme'[11]. Accordingly, 15–20 small callus pieces (200–250 mg) were plated in each petri plate containing the callusing medium (25 ml) with sub- to supra-lethal levels (0.02–0.27 mM) of 5MT stress. Medium devoid of 5MT served as control. Surviving sectors of the analogue challenged calluses were retrieved and sub-cultured onto the same level of 5MT stress for 3–5 subsequent passages to eliminate escapees. The best proliferating sectors growing at different levels of selection pressure were then divided into five parts and transferred accordingly: one part onto the control medium, one part onto the same level of stress, one part onto one level lower than the original level of stress and one part each onto two levels higher than the original level of stress. The selected 5MT-tolerant callus clones of differential tolerance levels were maintained for 25 cell-doubling cycles on stressed as well as control medium before subjecting to characterization studies. The biomass increment in terms of growth index (GI = per cent increase in fresh weight over initial inoculum) of the selected lines was compared with that of non-selected wild type over a 45-day culture cycle on medium containing graded series (0–0.45 mM) of 5MT stress. The variants were also checked for persistence of the acquired trait away from the selection pressure for eight culture cycles. The pH of the culture medium was adjusted to 5.8 ± 0.1 before autoclaving at 121°C, 1.1 kg cm^{-2} for 20 min. Unless otherwise specified,

all *in vitro* cultures were incubated at $25 \pm 4°C$ under 16 : 8 h light : dark photoperiod maintained through cool white fluorescent tube lights of 32 $\mu mol\ m^{-2} s^{-1}$ intensity.

Free amino acid pool and tryptophan content in the selected lines and wild type were measured after 45 days of growth. Freshly harvested callus (100–200 mg) was homogenized in 80% (v/v) cold ethanol (5 ml), centrifuged at 15,000 rpm for 20 min (X3) and pooled supernatant used as test solution for estimation of total free amino acids[12]. For estimating free tryptophan content, fresh callus tissue (2–8 g) was homogenized twice each in methanol : chloroform : water (MCW 12 : 5 : 3) mixture (10 ml) and 80% (v/v) cold ethanol (10 ml) and centrifuged at 15,000 rpm for 15 min[13]. Chloroform (5 ml) and distilled water (7 ml) were added to the combined MCW extract and centrifuged. Top layer was mixed with pooled ethanol extracts and volume reduced to 10 ml on a flash evaporator. Concentrated extract was loaded onto a DOWEX 50 X-2 column (1.0 × 15.0 cm) and allowed to stand for 30 min before fractionating out @ 1.0 ml min^{-1}. The column was washed with distilled water (25 ml) and tryptophan was eluted from the column using 0.3 N NH$_4$OH (3 × 20 ml). Pooled ammonia fractions were evaporated to dryness on a water bath (80°C) and the residue dissolved in 2.0 ml distilled water. For estimation of free tryptophan content, 0.5 ml of this sample was used[14].

For terpenoid indole alkaloid estimation, callus from the selected lines and wild-type was harvested after 45 days, oven-dried, ground to a fine powder (0.7–1.0 g) and extracted with methanol (3 × 30 ml). Pooled methanolic extract was filtered, concentrated *in vaccuo* at 40°C to 10 ml, diluted with distilled water (10 ml), acidified with 3% HCl (10 ml) and extracted with ethylacetate (3 × 30 ml). Aqueous extract was cooled (10°C), made alkaline with NH$_4$OH (pH 8.5) and extracted with chloroform (3 × 30 ml)[15]. Combined chloroform extract was washed with distilled

water, dried over Na$_2$SO$_4$ and air-dried to a constant weight to obtain crude alkaloid yield. HPLC analysis was done using Water's Modular HPLC system[16].

The LD$_{50}$ values for wild type calluses of the four accessions of *C. roseus* when challenged against a graded series of 5MT stress (0.02, 0.04, 0.06, 0.09, 0.11 and 0.13 mM) were as follows: 'C-6': 0.06–0.09 mM; 'Nirmal': 0.04–0.06 mM; 'Red Petal' and 'White Petal': 0–0.02 mM (Figure 1). None of the callus tissues could survive beyond 0.13 mM 5MT. The degree of inherent tolerance towards 5MT stress in terms of callus survival was in the order of 'C-6' > 'Nirmal' > 'Red Petal' ≥ 'White Petal', indicating a strong genotypic influence on the sensitivity towards analogue stress, as has been reported for selection of amino acid analogue tolerance in many plant species[11–17]. To ascertain that 5MT actually behaved as a true analogue of tryptophan in callus cultures of *C. roseus*, tryptophan was co-fed at 0.2 and 0.5 mM levels to callusing medium supplemented with 0–0.36 mM 5MT (Figure 2). Tryptophan at 0.2 mM level was sufficient to reverse the growth inhibition caused by 5MT up to 0.36 mM level, but with 1–1.5-fold less biomass accumulation compared to wild type in the absence of analogue stress. At 0.5 mM tryptophan feeding, no depression in growth was observed till 0.36 mM 5MT stress, suggesting that 5MT behaved as a true analogue of tryptophan. In the presence of excess amount of tryptophan, the growth inhibitory effects of 5MT are counter-balanced through a dilution effect, as suggested earlier[18,19].

Four stable variant callus lines (i.e. Nir L-60 of accession 'Nirmal'; C$_6$ L-50, C$_6$ L-60 and C$_6$ L-120 of accession 'C-6') were isolated over a period of twelve subculture cycles of 4 weeks each. The wild type calluses of 'Red Petal' and 'White Petal' accessions failed to yield any stable variant lines. Growth characteristics of the stable 5MT-tolerant callus lines selected at supra-lethal levels of stress were monitored over a graded series of 5MT (0–0.45 mM) stress
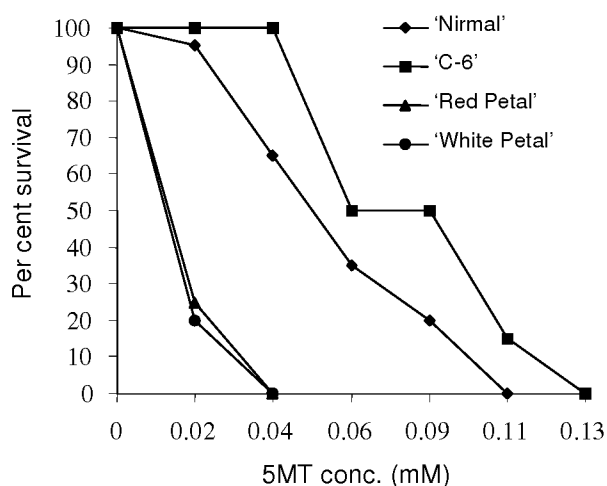

Figure 1. Dose-response of wild type callus cultures of four accessions of *Catharanthus roseus* against 5MT stress.
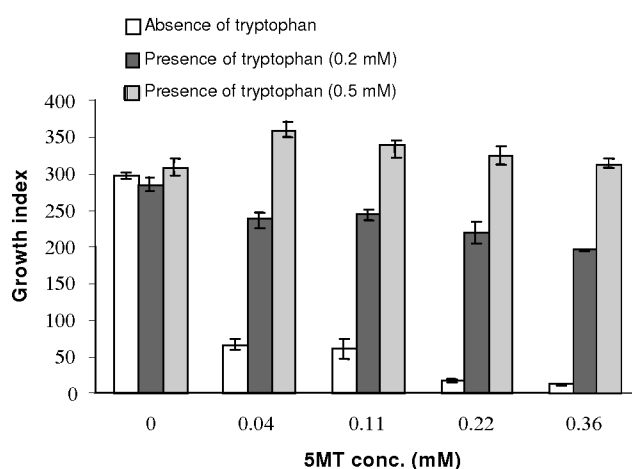

Figure 2. Counterbalance of growth inhibition caused by 5MT stress due to tryptophan supplementation in wild type callus cultures of *C. roseus* ('Nirmal').

Table 1. Growth characteristics of some of stable 5MT-tolerant callus lines of *Cartharanthus roseus* in comparison with their respective non-selected wild types

| | Growth index* | | | | |
| | 'Nirmal' | | 'C-6' | | |
5MT conc. (mM)	Wild type	Nir L-60	Wild type	C_6 L-50	C_6 L-60
0.0	557.52 ± 10.33	501.08 ± 1.07	417.81 ± 12.94	876.20 ± 4.38	516.82 ± 12.03
0.22	17.92 ± 2.36	510.34 ± 2.02	18.72 ± 0.6	662.30 ± 15.41	502.20 ± 14.11
0.27	14.10 ± 3.26	520.95 ± 14.49	12.26 ± 1.72	544.80 ± 15.70	499.42 ± 10.05
0.36	11.68 ± 0.71	430.32 ± 14.19	9.86 ± 3.54	419.96 ± 14.11	475.28 ± 5.38
0.45	9.56 ± 3.04	340.14 ± 10.53	8.22 ± 2.30	110.86 ± 7.16	310.58 ± 7.48

Data presented as mean ± SE (n = 3), *Per cent fresh biomass increment over initial inoculum (200 mg).

Table 2. Concentration of total free amino acids in some stable 5MT-tolerant lines of *C. roseus* in comparison to their respective non-selected wild types

| | 'Nirmal' | | | 'C-6' | | | |
| | Free amino acids (mg gf wt^{-1}) | | | Free amino acids (mg gf wt^{-1}) | | | |
5MT conc. (mM)	Wild type	Nir L-60	5MT conc. (mM)	Wild type	C_6 L-50	C_6 L-60	C_6 L-120
0.0	5.36 ± 0.2	2.65 ± 0.2	0.0	9.07 ± 0.6	9.08 ± 0.7	10.26 ± 1.9	5.57 ± 0.1
0.27	3.90 ± 0.1	2.79 ± 0.1	0.22	4.28 ± 0.3	15.45 ± 0.7	–	–
			0.27	3.80 ± 0.5	–	10.21 ± 2.2	–
			0.54	1.10 ± 0.8	–	–	9.12 ± 0.85

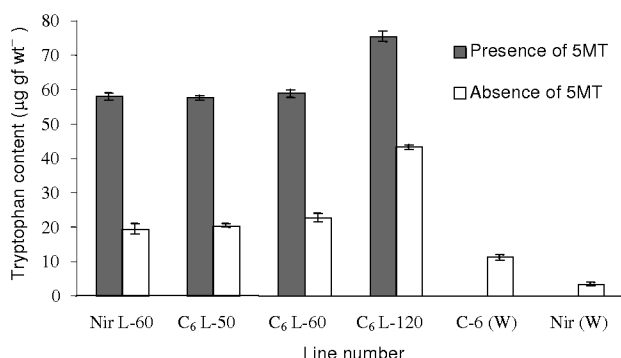'–' indicates transfers were not made; Data presented as mean ± SE (n = 3).

Figure 3. Tryptophan content in some 5MT-tolerant callus lines of *C. roseus* in absence and presence of analogue stress for which the lines were selected.

(Table 1). The lines Nir L-60 and C_6 L-60 grew well up to 0.45 mM 5MT, whereas line C_6 L-50 showed 50% growth inhibition at 0.36 mM 5MT stress. Line C_6 L-60 recorded a comparable growth index of 475.28 and 516.82 in the presence (0.36 mM) and absence of 5MT respectively. Its growth performance was almost equal to that on 0.27 mM level of stress for which it was originally selected (GI = 499.42). Line Nir L-60 also exhibited a remarkable capacity to gradually build up its tolerance up to 0.45 mM 5MT stress (GI = 340.14). C_6 L-50 on the other hand, completely collapsed at this supra-lethal level of 5MT stress (GI = 110.86).

Free amino acid pool size of wild type and selected lines is given in Table 2. The selected line Nir L-60, though had twofold less content of the free amino acids than the wild line callus, could maintain this level in the presence of 0.27 mM analogue stress, at which the amino acid pool of the wild line got reduced by more than 1.5-fold. The enlargement of the amino acid pool under respective 5MT stress was more pronounced in lines C_6 L-50 and C_6 L-120 (>1.5-fold). Line C_6 L-60 could maintain its high amino acid content even when the analogue stress was withdrawn. In comparison to the selected variant lines, the wild-type callus of the accession C-6 registered a steady decline in its free amino acid content in the presence of increasing concentration of the analogue in the medium.

The selected lines and non-selected wild type were also prospected for increment in tryptophan content in the presence and absence of 5MT stress (Figure 3). On the stress-free control medium, the wild type of accession 'Nirmal' had 3.25-fold more tryptophan than that of genotype C-6 after 35 days of growth on the control medium. All the selected 5MT-tolerant lines showed higher accumulation of free tryptophan, and this increment varied from 5 to 16-fold compared with the tryptophan level in the wild line calluses maintained on 5MT-free medium. The highest tryptophan accumulation occurred in line C_6 L-120 (75.44 μg gf wt^{-1}) in the presence of 0.54 mM 5MT. The selected lines having tolerance levels of 0.22 and 0.27 mM analogue stress had almost similar tryptophan

Table 3. Crude alkaloid content and quality profile of some 5MT-tolerant lines of *C. roseus* in presence and absence of analogue stress

| Line number | 5MT conc. (mM) | Crude alkaloid content* (% dry wt) | Alkaloid quality (% dry wt)** | |
			Catharanthine	Vindoline
'Nirmal'				
Wild type	0.0	0.343	ND	ND
Nir L-60	0.0	0.293		
	0.27	0.501	0.0072	0.0052
'C-6'				
Wild type	0.0	0.255	0.0016	ND
C_6 L-50	0.0	0.178		
	0.22	0.341	ND	0.0010
C_6 L-60	0.0	0.279		
	0.27	0.381	0.0046	ND
C_6 L-120	0.0	0.363		
	0.54	0.404	0.0080	0.0052

*Values represent mean of five replicates; **Combined crude extracts of all replicates growing in presence of 5MT stress only subjected to HPLC analysis; ND, Not detected.

concentration. This was 2–2.5-fold more in the presence of respective 5MT stress than on the control medium. These results clearly indicate that the overproduction of free tryptophan was an inducible effect of the analogue stress.

To ascertain the possible influence of higher tryptophan availability on diverting the metabolic flux towards TIA synthesis, the crude alkaloid content estimation and quality profiling were carried out in the selected lines and non-selected wild type (Table 3). The crude alkaloid level in the wild type of accessions 'Nirmal' and 'C-6' grown on 5MT-free medium was 0.343 and 0.255% on dry weight basis respectively. The selected lines, i.e. Nir L-60, C_6 L-50, C_6 L-60 and C_6 L-120 showed higher crude alkaloid content compared to their respective wild types. However, all the 5MT-tolerant lines showed increment in crude alkaloid content only in the presence of 5MT stress, probably because of larger tryptophan pool existing under stress. HPLC analysis of the crude alkaloid extract of the wild type of accession of 'C-6' showed the presence of low amounts of catharanthine (0.0016% dry wt). Amongst the selected lines, Nir L-60 and C_6 L-120 having light-green coloured and nodular callus morphology showed the presence of both catharanthine and vindoline under 5MT stress. Nir L-60 had 0.0072% dry wt catharanthine and 0.0052% dry wt vindoline, while C_6 L-120 had 0.0080% dry wt catharanthine and 0.0052% dry wt vindoline. In contrast, no such increment in indole alkaloids was recorded in previous studies on 5MT-tolerant cell lines of *C. roseus*[18–20].

The present study provides a new avenue for the utilization of amino acid analogue tolerance in the metabolic engineering of medicinal herbs, where the precursors for the synthesis of the desired metabolites are drawn from the free amino acid pool.

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Species diversity contributes to productivity – Evidence from natural grassland communities of the Himalaya

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The impact of species diversity on ecosystem functioning has generated considerable research and tremendous debate in view of the accelerated depletion of biodiversity worldwide. A number of recently conducted experiments based on synthetic assemblages of plant species indicated that ecosystem productivity declines with loss of species. The problem with acceptability of this hypothesis is that in spite of best efforts, conditions created in the experiments fall short of natural conditions. The present study, which was carried out in alpine grasslands of Himalaya, is from natural ecosystems to lend support to the above hypothesis. It emphasizes that with the depletion of biodiversity, we are going to lose some of the life-supporting ecosystem services.

Keywords: Biodiversity, ecosystem, grassland, Himalaya.

IN recent years, concern for the extinction of species and populations due to human activities has stimulated a number of observational and experimental studies on the relationships between species richness and ecosystem functioning. In observational studies, the impact of species richness on ecosystem function was examined by comparing different ecosystem types varying in species richness or similar ecosystems distributed at different locations[1,2]. The problem with these comparisons is that the ecosystems differing in species richness also differ in environmental conditions, such as precipitation and soil type. Clearly, in these situations, the response to the variation in species diversity cannot be separated from the response to environmental variation. To address this problem, a number of experimental studies were carried out in which species richness was designed by investigators as the sole independent variable, holding the physico-chemical environmental factors constant[3,4]. A number of these experiments conducted in North America and Europe showed that productivity increased with increase in species richness[5–7]. These investigators emphasized that with more species comes more complete harvesting of resources and, hence, more production[8]. A species-rich ecosystem would have more species in complementary roles (more niche differentiation and facilitation), and therefore, more niche space occupied than one with fewer species[7].

Serious weaknesses have been pointed out in the design and interpretation of experiments with synthesized model species assemblages[9,10]. Units of vegetation differing in species richness were synthesized by making random draws from a species pool, thus making it difficult to establish the causes of increase in productivity. In some experiments, more species communities that were created contained more productive species that were absent from the experimental assemblages of less species richness[9]. Furthermore, at least in some cases, the device employed to cause variation in species richness affected the result itself. For example, in a Minnesota grassland experiment[11], a supply of nitrogen was used to bring about changes in species richness. Consequently, it was not possible to ascertain whether the low stability in the species-poor sample was because of the low species richness or because the species adapted to high nitrogen levels were vulnerable to drought, the response to which was used as a measure of stability.

Another flaw was that it was not established whether the experiments were carried out under environmental conditions in which the component species naturally occur[10]. In specific cases, soils were sterilized and sand/fertilizer mix was used in place of soil.

There is a need for studies based on natural ecosystems, involving comparative approaches that control variation of all factors except diversity[12]. It is challenging to combine the positive features of both observational and experimental studies, in order to remove confounding effects of the physio-chemical environment in the observational studies and remove problems associated with the constitution of synthetic species assemblages of experimental studies. In the present study, we addressed the question whether species