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Two-species microbial consortium for growth promotion of *Cajanus cajan*

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We studied the interactions and the importance of a unique relationship in a plant growth promoting consortium comprising two species, *Burkholderia* sp. MSSP and *Sinorhizobium meliloti* PP3. They are rhizospheric isolates with abilities to produce indole-3-acetic acid (IAA) and solubilize inorganic phosphate. The organisms were grown as monospecies or mixed-species culture and studied for growth profile, IAA production and phosphate solubilization. *Burkholderia* sp. MSSP was marked with green fluorescent protein reporter gene to monitor growth in mixed-species culture. The growth rate of PP3 increased in mixed species culture, while that of MSSP remained unaffected. IAA production increased about 50% in mixed-species culture, compared to maximum IAA released in individual trials. The amount of phosphate solubilized was not affected. The two strains were tested on *Cajanus cajan* for their plant growth promoting activities in sterile soil. Inoculation of either MSSP or PP3 resulted in significant increase in seedling length and weight. In accordance with the findings of *in vitro* experiments, exceptional increase in seedling growth was recorded in mixed-species, co-inoculated consortium.

Keywords: *Burkholderia* sp., *Cajanus cajan*, growth promotion, microbial consortium, *Sinorhizobium meliloti*.

BACTERIA live in consortia bound to surfaces such as in biofilms, flocs or granules. Under these conditions the bacteria are positioned in a heterogeneous environment. It is increasingly apparent that in nature, bacteria function less as individuals and more as coherent groups that are able to inherent multiple ecological niches[1]. Populations of bacteria have functional roles within communities that permit their survival. Distinct microbial populations in rhizosphere frequently interact with each other. Syntrophic relationships between different organisms have been demonstrated in several microbial ecosystems. Therefore, mixed inoculants (combination of microorganisms) that interact synergistically are currently being devised, which yield better and quick results[2]. Recently, a microbial consortium for plant growth promotion was suggested[3]. It has been suggested that development of plant growth promoting consortium (PGPC), could be a feasible strategy for increased activity and better viability of plant growth promoting rhizobacteria (PGPR). When these strains are made into an inoculum consortium, each of the constituent strains of the consortium not only out-compete with the others for rhizospheric establishments, but complement functionally for plant growth promotion[4]. Here, we describe the relationship between two distantly related isolates, *Burkholderia* sp. MSSP and *Sinorhizobium meliloti* PP3. We discovered that in combination they promote growth of host plants because of increased indole-3-acetic acid (IAA) production. IAA is a member of the auxin family of the phytohormones that influence many cellular functions in plants and promote plant growth even without concomitant nitrogen fixation, or with heavy nitrogen fertilizers[5]. Phosphate (P) solubilization is another mechanism by which unavailable, immobilized, precipitated phosphorus of applied fertilizers is brought back into the medium by the action of mineral and organic acids produced by bacteria[6]. Both isolates had the ability to solubilize inorganic P and hence P solubilization in mixed-culture was also determined. Both the strains were studied in conjunction to each other as 'two-species' PGPC, for IAA production, P solubilization and effectiveness of this combination for growth promotion of *Cajanus cajan*. Green fluorescent protein (GFP)-based reporter system was utilized to monitor growth in the present study.

Several PGPR were tested in different combinations to observe their effect on the growth of *C. cajan* (data not given). One of the combinations consisting of *Burkholderia* sp. MSSP and *S. meliloti* PP3, was found to enhance growth in pot conditions significantly compared to non-bacterized control or single-species trials (described later). These two bacterial strains were selected for the present study. These

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were root nodule isolates of *Mimosa pudica* and *Trifolium foenograecum*, isolated according to standard procedure. The isolates were identified as *Burkholderia* sp. MSSP and *S. meliloti* PP3 respectively, in earlier studies[7,8]. Strains were grown on yeast extract mannitol (YEM) medium, pH 7.2 at 28 ± 1°C, unless otherwise mentioned. When required, antibiotics were added at final concentrations of 50 µg/ml for gentamycin and 50 µg/ml for chloremphenicol. *Escherichia coli* S17-1 (hs dR *pro rec* containing RP4-2 Tc :: Mu integration into chromosome) having suicidal plasmid pED S15 containing *Tn* 5 fused with the promoterless GFP and carrying chloremphenicol and gentamycin markers was used for tagging purpose[9]. It was grown in Luria Bertini (LB) medium (tryptone 1%, yeast extract 0.5%, NaCl 0.3%, glucose 0.1%, agar 2% and pH 7). MSSP was marked with GFP reporter gene. Colonies of wild-type MSSP and PP3 do not show fluorescence when exposed to UV radiation. Suicidal plasmid pED S15 containing *Tn 5-gfp* was transferred from *E. coli* to MSSP by biparental patch mating[10]. *E. coli* S17-1 (pED S15) containing promoterless *gfp* was mated with *Burkholderia* sp. MSSP resistant to aziocillin (100 µg/ml). Transconjugants were observed for fluorescence under ultraviolet radiation on a medium containing chloremphenicol (50 µg/ml), gentamycin (50 µg/ml) and aziocillin (100 µg/ml). Mutants containing *Tn 5* insertion were observed at a transposition frequency of 2.4 ± 10^{-5} and frequency of *gfp* fusions was 3.3×10^{-5}. A colony with maximum fluorescence (*Burkholderia* sp. $MSSP^G$) was selected for further studies. To assure that $MSSP^G$ retains the desirable characteristics of wild-type MSSP, growth rate, physiological characteristics and plant growth promoting attributes (described later) of mutant $MSSP^G$ were checked and compared with wild-type MSSP, which were found to be similar.

Growth curve of two isolates was determined by viable cell count method. Growth profile of either *Burkholderia* sp. $MSSP^G$ or *S. meliloti* PP3 was determined by inoculating early exponential phase culture in 50 ml of YEM broth. In mixed culture, 25 ml of early exponential phase cultures of *Burkholderia* sp. $MSSP^G$ and *S. meliloti* PP3 were mixed aseptically. Samples were withdrawn after every 4 h. Suitable dilutions were plated on solid medium and CFU per ml enumerated later. Fluorescence in *Burkholderia* sp. $MSSP^G$ was utilized to differentiate between the two strains (Figure 1). Mean growth rate constant (K) was calculated using the formula: $K = 3.322\ (\log Z_t - \log Z_0)/\Delta t$; where Z_0 and Z_t are the initial and final cell populations, while Δt is difference in culture time.

IAA production was detected by inoculating the strains in YEM broth (pH 7.2) supplemented with 0.01% tryptophan and incubated for 24 h at 120 rpm at 27°C. Exponential phase culture was centrifuged at 8500 rpm for 10 min at 4°C to collect the supernatant. Two drops of orthophosphoric acid was added to 2 ml of supernatant. Appearence of pink colour confirmed the production of IAA[11]. The amount of IAA (µg/ml) was determined quantitatively by adding 800 µl of Salkowski's reagent (1 ml of 0.5 M $FeCl_3$ in 50 ml of 35% $HClO_4$)[12] to 200 µl of culture supernatant, collected after various time intervals. Absorbance was measured at 535 nm after 20 min. Uninoculated YEM broth with Salkowski's reagent was utilized as reference. For the determination of IAA production in two-species consortium, strains were grown and treated as described above.

P-solubilizing ability of isolates was checked on Pikovaskaya's agar[13] based on clear zone of solubilization formed around bacterial growth. *Burkholderia* sp. MSSP and *S. meliloti* PP3 were found to be hyperactive strains based on the size of the clearing zone (data not given). P-solubilization was quantitatively determined by estimating phosphorus released by bacterial action as soluble phosphate[14]. Pikovaskaya's broth was inoculated separately with 1% (v/v) exponential phase cells of overnight-grown culture of the two respective strains, and incubated at 27°C under shaking conditions. The culture samples were withdrawn aseptically after every 24 h. After centrifugation at 8500 rpm for 10 min at 4°C, the supernatant was collected and estimated for P. All glassware were rinsed thoroughly with ethanol and distilled water to eliminate phosphorus during complete experiment. P solubilization in two-species consortium was estimated as described above, except that the Pikovaskaya's medium was inoculated instead of the YEM broth.

Seeds of *C. cajan* var. Manak with uniform shape and size were surface-sterilized with 95% alcohol for 30 s followed by 0.1% $HgCl_2$ for 1–2 min and then washed with sterile distilled water (SDW) 5–6 times. Seeds were dried overnight under sterile air stream. Seed bacterization was done as described earlier[15]. The isolates were grown in YEM broth and late log phase cells were collected by centrifugation (7100 *g* 15 min at 4°C). Supernatant was discarded, pellets were washed with SDW and resus-

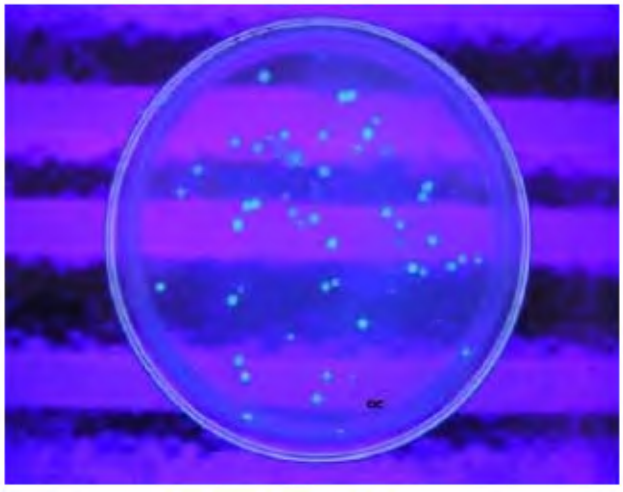

Figure 1. Fluorescent colonies (β) of *Burkholderia* sp. $MSSP^G$ (due to expression of *gfp* gene) differentiated with non-fluorescent (α) *S. meliloti* PP3 over ultraviolet radiation.

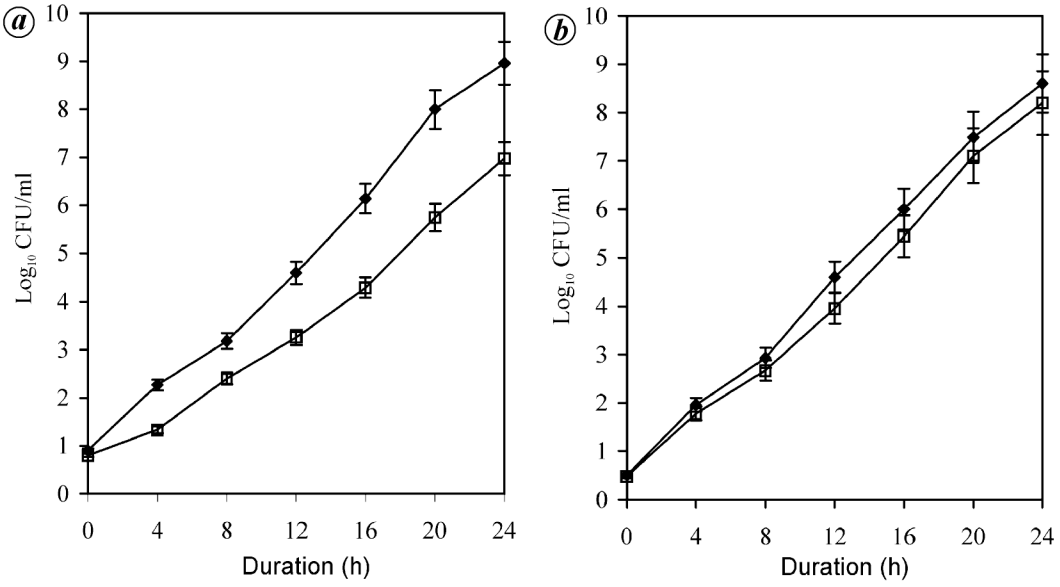

Figure 2. Time-course analysis of growth of *Burkholderia* sp. MSSP^G (♦) and *Sinorhizobium meliloti* PP3 (□), where the strains were established either as monospecies culture (***a***) or as mixed species consortium (***b***). CFU was enumerated on YEM plates containing appropriate antibiotics and strains were screened over ultraviolet radiation. Error bars indicate standard error of mean; where error bars are not visible, they are smaller than the marker.

pended in SDW to obtain a population density of 1×10^8 CFU ml^{-1}. This suspension was mixed with 1% carboxymethylcellulose (CMC) and the slurry thus obtained was coated onto pigeon pea seeds. The seeds were allowed to air-dry overnight under aseptic condition. Care was taken to avoid clumping of seeds. Seeds coated with CMC slurry, otherwise unbacterized, served as control. For standardizing the inoculum, the bacterized seeds were sampled and CFUs were counted on YEM agar. The population of MSSP and PP3 was recorded as 3.6×10^7 and 3.0×10^7 CFU g^{-1} seed by dilution plate technique. The same method was adopted for co-inoculation of seeds, but 1×10^4 CFU ml^{-1} of each isolate was mixed to achieve 1×10^8 CFU ml^{-1} of total population density before preparing the slurry. The seeds were sown in pots having sterile soil. Surface-sterilized bacterized and non-bacterized seeds were sown in separate pots. The experiment was performed with four sets of treatment: (i) MSSP bacterized seeds, (ii) PP3 bacterized seeds, (iii) MSSP + PP3 bacterized seeds and (iv) non-bacterized seeds (control). Pots were watered routinely with sterile water. Seedlings were uprooted after 40 days and total plant length, shoot length, root length and fresh weights of root and shoot were measured. For statistical analysis, *t* scores were determined and contrasted to confidence level of 0.05.

Burkholderia sp. MSSP^G and *S. meliloti* PP3 were grown in monoculture (Figure 2 *a*) and mixed species consortium (Figure 2 *b*). Both isolates were fast growing. *K* value of *Burkholderia* sp. MSSP and *S. meliloti* PP3 was 1.11 ± 0.03 and 0.85 ± 0.05 h^{-1}, respectively, in single-species cultures. When grown as two-species mixed-culture, *K* value of *S. meliloti* PP3 increased, while that of *Burkholderia* sp. MSSP remained almost unchanged. *K* value in mixed species consortium was 1.11 ± 0.02 and 1.06 ± 0.03 h^{-1} for *Burkholderia* sp. MSSP and *S. meliloti* PP3 respectively.

Significant amount of IAA production was observed in the case of *S. meliloti* PP3. It produced maximum 80 µg/ml IAA after 168 h of incubation. MSSP was a better IAA producer compared to PP3. Maximum 100 µg/ml of IAA was released by MSSP after 120 h. However, in two species consortium, IAA production increased considerably. About 159.5 µg/ml of maximum IAA was released in the mixed-species culture. The IAA production profile of PGPC had a declining trend with a maximum at 24 h, which kept falling subsequently thereafter. The yield was approximately 1.5 times higher in consortium compared to maximum amount of IAA produced individually by the two strains. Moreover, maximum value was achieved immediately after 24 h of incubation in co-inoculated culture. Results are given in Figure 3.

P solubilization for the two isolates was monitored up to 8 days in Pikovaskaya's broth (pH 7). Maximum solubilization of P was achieved on the eighth day by MSSP. PP3 appeared to be a better P solubilizer compared to MSSP. For PP3, level of soluble P gradually increased up to 168 h, with a maximum value of 10.95 mg/ml. Both strains were found to lower the pH of the growth medium (data not shown). Decrease in pH indicates the production of acids, which is considered to be responsible for P solubilization[6]. In co-inoculated consortium, the maximum value of P released was achieved after 96 h. The maximum amount of soluble P was comparable with PP3 alone. Results are given in Figure 4.

Table 1. Effect of *Burkholderia* sp. MSSP and *Sinorhizobium meliloti* PP3 on vegetative growth of *Cajanus cajan* after 40 days of seed germination

Strain	Growth measurement*				
	Seed germination (%)	Total plant length (mm)	Shoot length (mm)	Fresh root weight (mg)	Fresh shoot weight (g)
Control	70	125a	101a	401a	2.15a
MSSP	100	188b	150b	835b	3.18b
PP3	90	142a, b	111a	560a	3.47b
MSSP + PP3	100	312c	242c	750c	6.06c

*Mean value was calculated from three replicates of each treatment. Results were analysed by Student's *t* test and there was no significant difference between treatments followed by the same letters in each column at $P \geq 0.05$.

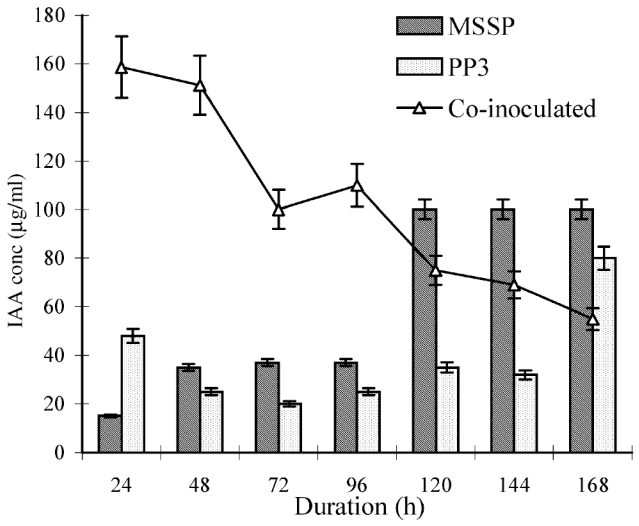

Figure 3. Effect of mixed-species consortium (△) on IAA production compared to monospecies culture of *Burkholderia* sp. MSSP[G] and *S. meliloti* PP3.

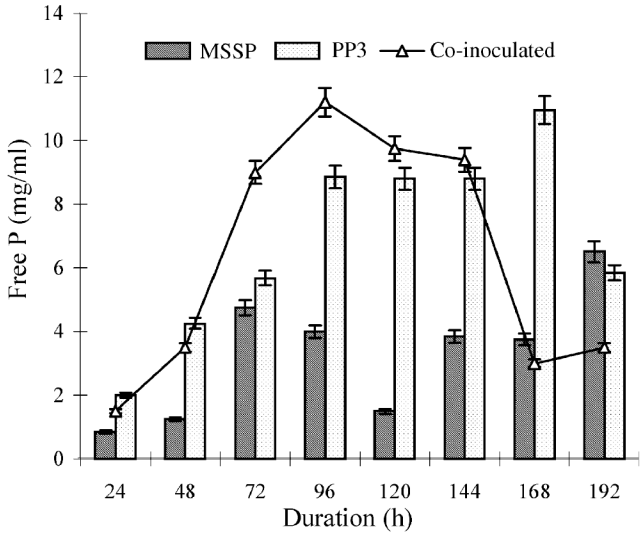

Figure 4. Effect of mixed-species consortium (△) on phosphate solubilization compared to monospecies culture of *Burkholderia* sp. MSSP[G] and *S. meliloti* PP3.

Plant growth promoting activity of isolates was determined with seed inoculation experiment in sterile soil with *C. cajan*. Both strains proved to be effective when used individually. About 48.5 and 9.99% increase in shoot length was recorded with seeds inoculated with either *Burkholderia* sp. MSSP or *S. meliloti* PP3 respectively, compared to control. The findings are given in Table 1. Results with co-inoculation were even more promising and supported the *in vitro* findings of IAA production and P solubilization by the two-species consortium. Plant length increased by 149% in co-inoculated condition. Similarly, 47.9 and 61.4% increase in shoot weight was recorded with *Burkholderia* sp. MSSP and *S. meliloti* PP3 respectively, in individual trials. However, 181.86% increase in shoot weight was recorded when both were inoculated together. No nodule was detected with any of the treatments.

Rhizosphere is a major soil ecological environment for plant–microbe interactions involving colonization of different microorganisms in and around the roots of growing plants. Distinct microbial populations frequently interact with each other in the rhizosphere in an integrated manner. These symbiotic relationships are in fact beneficial in the global context, because they act to maintain ecological balance[16]. Here we report cooperation between two rhizobacteria which belong to two distant genera – *Burkholderia* sp. and *S. meliloti*. Rhizobia are established plant growth promoting bacteria due to their nitrogen-fixing ability. However, *Burkholderia* spp. are less known as bio-fertilizers[17]. *Burkholderia* sp. MSSP and *S. meliloti* PP3 showed cooperation while growing together *in vitro*, which indicates their common ecological niche. Both the organisms improved seedling growth. However, one curious attempt revealed that seedling growth was further improved when both the organisms were applied together. This encouraged us to study their behaviour in consortium.

Growth profile of MSSP was similar in monospecies and mixed-species cultures. About 25% increase in mean growth rate was recorded for *S. meliloti* PP3 when grown in mixed-species, two-species culture with respect to single-species culture. This shows that while the remaining species are unaffected, MSSP favours the growth of PP3. Data represent commensalism between the two isolates. This

interaction also indicates that in soil, association with *Burkholderia* sp. MSSP favours *S. meliloti* PP3 as an adaptation of high rate of reproduction – a well-known strategy that enables organisms to successfully survive and maintain themselves in communities[18]. Earlier, microbial studies performed without plants indicated that some combinations allow the bacteria to interact with each other synergistically, provide nutrients, remove inhibitory products, and stimulate each other through physical and biochemical activities that may enhance some beneficial aspects of their physiology[2].

Both PP3 and MSSP isolates were found to release good amount of IAA. IAA synthesis has been reported with rhizobia[19] and several other rhizobacteria. In mixed culture, IAA production almost increased by 50% with respect to monospecies cultures. This further extended the findings of growth dependence between the two isolates. Increased phytohormone production when grown in mixed culture has been reported with *Azospirillum* under *in vitro* condition[20]. However, in mixed-species culture, maximum release of IAA in the initial hours of incubation was observed, which remains unexplained.

Seed or soil inoculation with P-solubilizing bacteria is known to improve solubilization of fixed soil phosphorus and applied phosphates resulting in higher crop yield. P-solubilization root-nodulating bacteria, e.g. *Rhizobium* and *Bradyrhizobium*, have been earlier reported[21]. In our study we found that in co-inoculated culture, maximum P-was solubilized at the beginning relative to monospecies culture. The results suggest that the process of P-solubilization, which is governed by a complex group of mechanisms, is substantially affected by a mixed culture. Although there was no appreciable increment in maximum soluble P level, maximum soluble P was released much earlier in the two-species consortium.

Seneviratne[3] has mentioned that co-inoculation and co-culture of microbes have been observed to perform the tasks better than the individual microbes. Both the isolates resulted in improved seedling growth in individual trials, suggesting their role as PGPRs. Besides, result of co-inoculated seeds was better, where approximately three times higher shoot weight was recorded with respect to control in co-inoculated treatment. Similarly, 150% increase in seedling length was recorded. Data were higher with respect to control, as well as individual trials. This supported the *in vitro* findings of IAA production and P solubilization in the two-species consortium. In the present investigation, the two isolates were studied with the possibility of a consortium, as effective bioinoculant formulation. While the centre of attention of most co-inoculation/co-culture studies with rhizobia had been nitrogen fixation[22], the present study exploits IAA production ability of the two isolates. Being an incompatible symbiont, *S. meliloti* did not form nodules in *C. cajan* as anticipated. This excluded the role of nitrogen fixation in co-inoculated trials.

The two isolates, *Burkholderia* sp. MSSP and *Sinorhizobium meliloti* PP3 have the ability to produce IAA and solubilize inorganic P. They enhanced the growth of pigeon pea in individual trials. Besides, plant growth further improved when both were applied together. Considering the plant growth promoting abilities of these two isolates, a non-specific, two-species PGPC for bioinoculant preparation is possible.

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Chemical profiling of *Nothapodytes nimmoniana* populations in the Western Ghats, India for anti-cancer compound, camptothecin

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Camptothecin (CPT) is a potent anti-tumour isoquinoline alkaloid used extensively in the treatment of several cancers. Among several plant species known to contain the compound, by far the highest concentration of about 0.3% (w/w) has been reported from *Nothapodytes nimmoniana*, a small tree distributed in the Western Ghats, South India. With no synthetic sources of this alkaloid and with an increasing global demand, there has been a heavy dependence on naturally existing populations of *N. nimmoniana*. Prospecting for populations and or individuals of the species for higher yields of the alkaloid could potentially help in establishing high-yielding clonal orchards and in developing *in vitro* production systems and thereby relieving the pressure on natural populations.

Towards this end, we have chemically profiled 148 individuals from 11 populations of *N. nimmoniana* in the Western Ghats, a mega-diversity hotspot in South India. CPT was estimated in stem and root bark of individual trees. There was significant variation in CPT content, both in stem and root bark samples, among the populations. Differences in CPT content among individuals did not seem to be related to either their size (age) or their geographical origin. Of the 148 individuals assayed, 23 yielded more than 1% CPT. These estimates are nearly three to eight-fold more than what has been reported hitherto in the literature. Subject to further confirmation, these 'elite' trees could be focused for conservation and judicious utilization through clonal multiplication, as also for deriving tissue material for *in vitro* production systems.

Keywords: Camptothecin, chemical profiling, conservation, Icacinaceae, *Nothapodytes nimmoniana*, Western Ghats.

CAMPTOTHECIN (CPT), isoquinoline alkaloid is one of the most promising anti-cancer drugs of the twenty-first century[1–6]. Several water-soluble derivatives of CPT are currently being used for treating colorectal and ovarian cancer[7–9]. The projected global demand for CPT in 2002 was valued at US$ 4045 million[10]. CPT was first discovered in the Chinese deciduous tree, *Camptotheca acuminata* (Nyssaceae)[11]. Later, the alkaloid has been reported from several plant species, with by far the highest concentration (about 0.3% on a dry weight basis) from *Nothapodytes nimmoniana*[12]. *N. nimmoniana*, formerly known as *Nothapodytes foetida* Sleumer and *Mappia foetida* Meirs, is a small tree, naturally distributed in many parts of the Western Ghats, South India, some parts of Assam, the Himalayan foothills, Sri Lanka, Myanmar and Thailand.

In the absence of synthetic sources, the global demand for this alkaloid is being met by the extraction of naturally existing populations of *N. nimmoniana* from the Western Ghats, India. Consequently in the last decade alone, over 20% of the population of the species has been lost from the Western Ghats[13,14]. In fact due to the extremely high pressure, the species has been declared as endangered[15]. In recent years, several independent groups have addressed the need to conserve the species and to explore the possibilities of identifying high-yielding individuals or populations for the development of *in vitro* production systems[16–22]. Padmanabha *et al.*[23] reported patterns of accumulation of the alkaloid in *N. nimmoniana* with respect to age, sex and seasonality. They found significant variation in CPT content among individuals and emphasized the need for chemically screening more populations in order to identify high-yielding sources of the alkaloid. In this study, we present

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