Therefore, both these new genes on chromosomes 7 and 3 of UPRI 95-140TGMS were non-allelic to any known *tms* genes and proposed as *tms6*(*t*) and *tms7*(*t*), respectively.

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Applying intra-specific protoplast fusion in *Streptomyces griseoflavus* to increase the production of Desferrioxamine B

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***Streptomyces griseoflavus* secretes Desferrioxamine B, which is used as a precursor for producing Desferal, a chelator that absorbs additional iron from the blood of thalassaemia patients. In order to obtain a higher rate of Desferrioxamine B synthesis, two UV-mutated strains of *S. griseoflavus*, which were selected upon their resistance to sodium azide or crystal violet, were used in intra-specific protoplast fusion with polyethylene glycol 1000. One of the fusants showed higher production rate compared to the wild type *S. griseoflavus* (81.8%), and was characterized. Desferrioxamine B extracted from mutants and fusants and wild type *S. griseoflavus* was compared with Desferal by paper chromatography to assess the correct identity of the product.**

BLOOD transfusion, which has an important role in the treatment of patients suffering from thalassaemia major, poses several problems to recipients, such as iron overloading of blood[1]. It is necessary to remove the additional iron to prevent its toxic effects on the heart, liver, kidneys, etc.[2].

Currently, the only prescribed medicine, which acts as an iron chelator to absorb and excrete additional iron, is Desferal (Desferrioxamine B mesylate)[3,4], being produced by Ciba-Geigy[5,6] through fermentation of a modified strain of *Streptomyces pilosus*[7]. About 3–4% of Iranians suffer from thalassaemia major[8], and the purchase and import of Desferal to treat such patients is expensive. In order to develop a cost-effective method, *Streptomyces griseoflavus* was modified by intra-specific protoplast fusion to overproduce Desferrioxamine B (Des B) in an economical manner on a large scale. The choice of technique is based on the premise that it is the most efficient recombination technique in the case of *Streptomycetes*, and that the genes involved in Desferrioxamine synthesis and their sequences are not known properly[9]. In protoplast fusion it is essential to have at least one strain or species with a unique screenable marker. Protoplast forma-

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tion, fusion and regeneration are three common steps in every protoplast fusion process and many factors such as age of bacteria, lysozyme and concentration of ions, time of enzyme treatment, osmotic pressure of regenerating media, temperature (during protoplast formation and regeneration), concentration and molecular weight of polyethylene glycol (PEG), exposure time to PEG, and auto-inhibition influence protoplast formation and subsequent regeneration[10,11]. Improved protoplast preparations have been desired by methods that include culturing bacteria in presence of glycine[12,13] and sonication[14] before protoplast formation.

Lyophilized *S. griseoflavus* (PTCC 1130) was purchased from Bacteria and Fungi Collection of Iranian Research Organization for Science and Technology, and was cultured in malt-yeast extract broth (MYB) or agar (MYA) medium[14] containing 1% malt extract, 0.4% yeast extract and 0.4% glucose. Incubation was made at 29°C under shaking at 150 rpm.

To draw the growth curve, the bacterium was cultured in 24 similar flasks containing MYB under the above-mentioned conditions. Samples were drawn every 8 h for 8 days. Contents of one flask were filtered and dried in an oven at 80°C for 1 h. Dry mass of bacteria was measured.

To enhance Des B production, a single bacterial colony was cultured on Des4, a specific solid medium containing 2% dextrin, 0.11 M mannitol, 91 mM L-asparagine, 1.7 mM L-lysine, 0.6 mM L-methionine, 0.8 mM L-threonine, 50 mM $CaCO_3$, 2 mM $MgSO_4$, 2 mM K_2HPO_4, 0.2 mM $ZnSO_4$, and 2% agar. After four days, a piece of Whatman #1 filter paper, soaked in 1% ammonium ferric sulphate [$FeNH_4(SO_4)_2.12H_2O$] in 1% sulphuric acid, was placed on the single colonies. The appearance of a brown or reddish brown halo around the colony in agar after 15 min replicated the presence of Des B[7].

The amount of Des B in culture was measured using spectrophotometery. For this the bacterium was cultured in soybean broth medium (2% soybean flour and 2% mannitol)[15] for 8 day at 29°C under shaking (150 rpm). Every 8 h, 1 ml sample was withdrawn and centrifuged at 4000 rpm (4°C). The supernatant was diluted ten times with distilled water and 5 mg ml^{-1} of ammonium ferric sulphate in 1% sulphuric acid was added to a final concentration of 20%. The absorbance of Des B was read at 430 nm, and its concentration was found by comparing the ODs with the standard curve prepared with different concentrations of Desferal.

To prove that the synthesized Desferrioxamine (Des) was B type, Des secreted into a six-day culture in soybean broth was extracted using Gaeumann's method[16], and run on a chromatography paper along with Desferal. Chromatography solvent contained 40% (v/v) *n*-butanol and 10% (v/v) glacial acetic acid in water[16]. For developing the spots, the paper was dried in an oven at 70°C; and then soaked into 0.2% ninhydrin in acetone and baked at 105°C for 4 min. Purple spots confirmed the presence of Des B.

To assess the resistance of inhibitor substances, plates of MYA containing 0.0005–0.01% (w/v) sodium azide or 0.0001–0.001% (w/v) crystal violet were inoculated using 200 spores of *S. griseoflavus* per plate and incubated at 29°C. To reach the dose of UV irradiation in which 1–5% of cells could survive, plates of MYA were inoculated with 200 spores per plate and irradiated for 1–10 s. After incubation at 29°C, colonies on plates were counted and the percentage of survived cells was calculated. Following this, 20,000 spores distributed on each plate of MYA containing appropriate concentration of either inhibitors, and irradiated with suitable dose of UV, were exposed. Colonies that appeared on plates were sequentially cultured on inhibitor-free and inhibitor-containing MYA in order to obtain resistant mutants.

The most appropriate concentration of glycine in the medium is that in which bacteria grow up to half as fast as they do in glycine-free medium[17]. To optimize this concentration, 20 ml MYB containing 0–5% (w/v) glycine was inoculated by 1×10^6 spores. After 24 h incubation at 29°C and 150 rpm, the dry mass of each culture was weighed and compared with that of glycine-free medium.

The most suitable period for sonication represents most cuts in mycelia with least damage to the cell structure. To determine the exposure time, some 24-h-old mycelia of each strain, which had been cultured in MYB containing appropriate concentration of glycine, were exposed to 76 W ultrasound waves for 5–20 s and observed under microscope. When cells were exposed to waves for more time, the cell walls broke and the clear culture became turbid.

Bacteria were cultured in MYB containing glycine and incubated till they reached the transition phase (determined by growth curve of each strain). The mycelia were sonicated for protoplast formation, which was performed according to Matsushima and Baltz[17]. Already cut mycelia were centrifuged at 1060 *g* at 10°C for 10 min and washed three times with 20 ml P (preservative) solution (containing 1.4 mM K_2SO_4, 1 mM $MgCl_2$, 3.7 mM K_2HPO_4, 0.19 M $CaCl_2$, 0.3 M sucrose, and 0.25 M Tris pH 7.2). Mycelia were resuspended in 20 ml P solution containing 1 mg ml^{-1} lysozyme and incubated at 27°C with gentle shaking till most of the mycelia were transformed to protoplasts. Protoplast formation was followed under microscope after sampling and staining with methylene blue. To remove the lysozyme, mycelia were washed three times with 20 ml P solution resuspended in another 20 ml P solution and kept at room temperature until further use. To calculate the frequency of regenerating protoplasts, the number of colonies formed on selective R2 media[17] was divided by the number of cells that were counted before protoplast formation. To calculate the frequency of regenerating non-protoplast cells, the number of colonies formed on non-hypertonic selective MYA was divided by the number of cells that were counted before protoplast formation.

Fusion and regeneration were performed using the method described by Matsushima and Baltz[17]. Protoplasts of both the strains were mixed well and spun down at 1060 *g* for 10 min. The pellet was resuspended in 200 µl of P solution, and mixed with fresh 1.8 ml of P solution containing 50% (w/v) PEG

1000, and stored at room temperature for 1 min. Then the protoplasts were washed with 10 ml P solution, resuspended and diluted in appropriate amount of P solution and divided into tubes so that each tube contained 200 protoplasts. Three ml of 45°C modified R2 medium (containing 0.3 M sucrose, 55 mM glucose, 1 g/l casamino acid, 15 mM L-asparagine, 1.4 mM K_2SO_4, 50 mM $MgCl_2$, 2.2% (w/v) agar, 0.15 M $CaCl_2$, 3.7 mM KH_2PO_4 and 10% 0.25 M Tris pH 7.2) was added to each tube, mixed gently, and immediately poured onto modified R2 plates, which contained appropriate concentration of both sodium azide and crystal violet.

Growth curve of *S. griseoflavus* (PTCC 1130) was drawn based on dry mass of bacteria in MYB medium (Figure 1). Log phase of growth started at 16 h post-inoculation and reached a stationary phase around 90 h. This is a critical stage in the life cycle of the bacteria, because most of the regenerative protoplasts of *S. griseoflavus* can be obtained from the mycelia at this stage[18].

Des B production was the highest at the middle of the stationary phase (Figure 2) and remained constant hours after the death phase had started. Thus, middle of the stationary phase is the ideal time for estimation of Des B. To make sure that the produced Desferrioxamine was B-type, it was extracted from the original *S. griseoflavus* and paper chromatography was performed using extracted materials alongside Desferal (Figure 3). Retardation factor (Rf) of extracted Des (Rf = 0.462) was comparable to that of Desferal (Rf = 0.457) and that described by Gaeumann for Des B (Rf = 0.44)[15]. An exposure to UV irradiation for 7 s resulted in 3% survival (data not shown). The most tolerable concentrations of crystal violet and sodium azide were 0.0003% (w/v) and 0.003% (w/v) respectively (data not shown). To obtain marked mutant strains, spores of *S. griseoflavus* were exposed to UV irradiation for 7 s and spread on MYA plates containing either 0.005% (w/v) of sodium azide or 0.0005% (w/v) of crystal violet. Colonies were sequentially cultured on inhibitor-free and inhibitor-containing MYA plates, wherein 17 strains resistant to sodium azide and 9 to crystal violet were tested for growth and sporolation rate, Des B synthesis, and resistance to only one of the inhibitors. Among these, two strains, S7011and C7031, were selected, which showed the highest rates of Des production (data not shown). An increase of 33.2 and 52.3% in Des production in strains S7011 and C7031 was not unexpected, because UV irradiation causes many different mutations on genes, some of which are related to Des synthesis. In addition, due to changes which occur in the cell wall and plasma membrane,

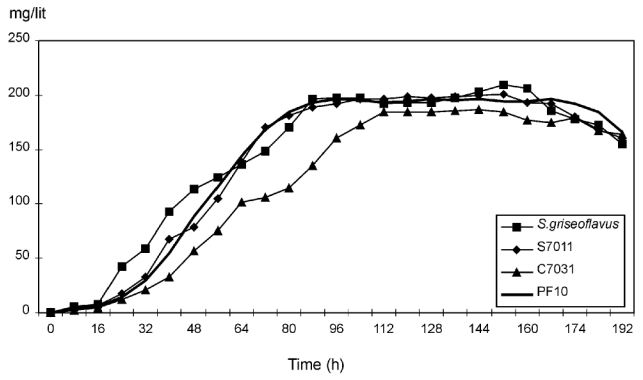

Figure 1. Growth curve of *Streptomyces griseoflavus* (PTCC 1130), mutants S7011 and C7031, and fusant PF10 during a period of 192 h. Cultures were performed in MYB medium at 29°C and 150 rpm. Sampling was done every 8 h and filtered bacterial masses were weighed after drying in an 80°C oven for 1 h.

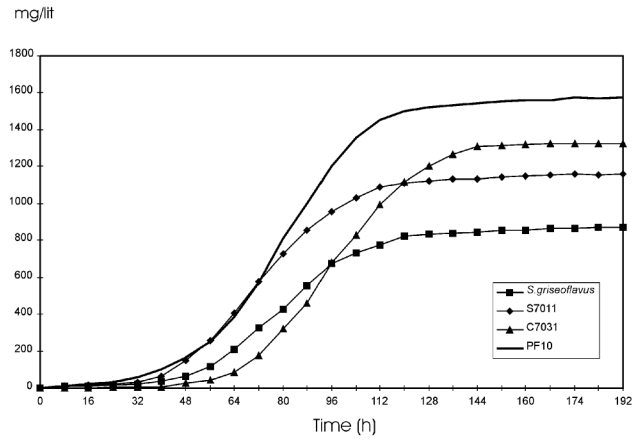

Figure 2. Des B production curve of *S. griseoflavus* (PTCC 1130), mutants S7011 and C7031, and fusant PF10 during a period of 192 h. Culturing was in soybean medium. Sampling was done every 8 h and samples were centrifuged to remove the mycelia and the supernatant was diluted 10 × with distilled water. Next 5 mg/ml ammonium ferric sulphate in 1% sulphuric acid was added to the solution to a final concentration of 20%. Optical density of Des B was read in 430 nm and concentration was calculated by comparing the ODs with that of the standard Desferal and its related concentration.

Figure 3. Paper chromatography of extracted Des. from *S. griseoflavus* (PTTC 1130) (2) and fusant PF10 (3) along with Desferal (1). Rfs of 0.457, 0.462, and 0.469 for spots nos 1, 2, and 3 respectively, show that the produced Des in *S. griseoflavus* and the fusant were B-type.

the absorption of substances and the secretion of metabolites are known to increase growth curves of both the mutants (Figure 1). This was necessary, since mycelia, which are at the transition stage (the intermediate stage between log and stationary phase), are in the best conditions to yield the most regenerative protoplasts. It is due to their relatively thin cell walls, which results in least exposure to sonication and lysozyme treatment. This exposure time for strain S7011 was 70–90 h and for strain C7031 it was 96–120 h post-inoculation. No noticeable difference in growth and sporulation rate among mutants and wild type-strain was observed. Type of Des was also identified in both the mutants to make sure that they were B-type. After paper chromatography of extracted Des from both mutants, Rf values were 0.461 and 0.463 for S7011 and C7031 respectively which were comparable with that of Desferal (Rf = 0.456).

Ultrasound waves cut the mycelia of bacteria into pieces, and unfold the hank of *Streptomycetes* colonies. In this application, glycine helps in sonication by loosening the junctions between cells. Glycine substitutes D- or L-alanine in the structure of bacterial cell wall. Furthermore, holes formed in the bacteria cell wall after sonication, are gates for lysozyme to penetrate into the cell wall faster and more efficiently than that without sonication. Furthermore, glycine helps the enzyme by weakening peptidoglycan bonds and increases wall permeability for lysozyme[12,18,19]. In this manner, exposure time to enzyme is shortened and cells can sustain least damage from lysozyme.

The best concentration of glycine is that at which growth of bacteria is half as fast as its growth in the same medium without glycine. For both mutant strains, 0.5% (w/v) was suitable. Mycelia treated with glycine were longer and thinner than usual, and colonies were loose. The optimum time for sonication that could properly cut mycelia, was determined as 50 s for S7011 and 70 s for C7031 at 70 W power.

Strains S7011 and C7031 were cultured in MYB containing 0.5% (w/v) glycine for 40 and 60 h, respectively. Mycelium of S7011 and C7031 was sonicated for 50 and 70 s, respectively, and treated with 1 mg ml^{-1} lysozyme for 40 and 25 min respectively, to yield enough entire cell wall-free protoplasts. The optimum temperature for lysozyme treatment was 27°C.

Only a portion of cells can regenerate and form colonies, while some of these colonies result from cells that, more or less, carried some parts or the whole of their cell wall. To identify the percentage of real protoplasts, which could regenerate, and the percentage of regeneration, certain number of each strains, protoplasts were cultured on hypertonic (regenerative) and non-hypertonic (regular MYA) media. Then the number of formed colonies on both types of plates was counted and divided by the number of plated protoplasts. It was distinguished that in S7011, 47.5% of cells could regenerate, while 31.7% of them were real protoplasts. It meant that 66.7% of cells yielded real protoplasts and could regenerate successfully. The figures for strain C7031 were 39.4, 28 and 71.7% respectively. The average output of protoplast formation was (66.7 + 71.7%)/2 = 68.9%. As soon as the protoplasts were prepared, they had to be fused. The most common reagent for fusion of *Streptomycetes* protoplasts is PEG, whose various molecular weights have been used in different concentration. Among them, PEG 1000 at a concentration of 50% (w/v) is the most recommended and efficient one[17,20,21].

The optimum time for protoplast to be exposed to PEG, has been suggested[10,12,17] as 30–60 s. Therefore, in these experiments, protoplasts were treated with 50% PEG 1000, and after 1 min, a large amount of P medium was immediately added to dilute the PEG[17]. Then the mixed protoplasts were distributed on double-selective (containing sodium azide and crystal violet) and non-selective regeneration plates. After incubation period 58 colonies appeared on all ten regeneration plates (5.8 colonies per plate as average), while the average number of colonies on non-selective plates was 132. Among 58 recombinant colonies, only 17 (1.7 colonies per plate) could keep growing on double selective media.

These 17 fusants (PF1–PF17) showed a diversity of Des production among them, three fusants had almost similar production rates to C7031, four were like S7011, four were like wild type *S. griseoflavus*, five had higher rates than those of the three parental strains, and 1 lost its ability to produce Des. Out of five fusants with higher rate of production, PF10 and PF9 had 81.8 and 68.3% respectively increase in comparison to *S. griseoflavus*. PF10 was chosen as the main yield of the fusion. Paper chromatography of extracted PF10, Des alongside Desferal yielded Rf = 0.469, thus proving that Des B is synthesized (Figure 3). PF10 did not have any noticeable difference with its parents regarding properties such as morphology, sporolation and growth speed (Figure 1). In fact, its growth curve was similar to that of strain S7011. Des production behaviour of PF10 showed a combination of behaviour of its two parents, S7011 and C7031.

MYB and MYA were used for regular culture of *S. griseoflavus* and its derivatives, as well as for drawing growth curves, because these bacteria could grow well in MYA and MYB. Also, since MYB medium did not have any non-dissolvable part, it could not affect the dry weight of bacteria mass. But, as bacteria refused to produce enough Des B in the media, soybean (broth) and Des 4 (agar) media were used for Des B assessment. Soybean broth could not be used for drawing growth curve because soybean flour disappeared 8 days post-inoculation.

Paper chromatography of extacted Des from *S. griseoflavus* and its derivatives showed that the most of Des was B-type. Therefore, it seems reasonable to work on this strain and its fusants, and improve them for further Des B production.

In this study, mutants resistant to inhibitors such as sodium azide and crystal violet and not to antibiotics were generated. This is because *S. griseoflavus* like other *Streptomycetes* is

able to synthesize some of the antibiotics and is resistant to high dosage of most of them.

Three different methods were tested for protoplast preparation. Okanishi's protocol yields a high number of completely cell wall-free protoplasts[19] that are stable for several months if kept in –70°C. However, it takes more than 30 h to complete the process. Sagara's protocol is very fast (about 5 h), but protoplasts can hardly regenerate[18]. The method of Matsushima and Baltz[17] yields many stable free-free protoplasts in a reasonable time (7–8 h) and was the chosen method.

As mentioned earlier, the percentage of real protoplasts which could successfully regenerate, was 66.7 for S7011 and 71.7 for C7031, out of all cells that were counted before spreading on plates. The mean of these two percentages gives the average efficiency of protoplast formation and regeneration (68.9%) which is comparable to results of other studies[10,12,18–20,22].

After incubation 58 colonies appeared on all 10 regeneration plates (5.8 colonies per plate as average), while the average of colony number on non-selective plates was 132. As mentioned above, only 68.9% of colonies resulted from protoplasts. Thus $132 \times 68.9\% = 91$ colonies on non-selective plates originated from protoplasts. Therefore, primary recombination frequency would be $(5.8\%/91) \times 100 = 6.4\%$.

Frequency of recombination via protoplast fusion is always impressed by 'heterocolony' phenomenon. A 'heterocolony' is a colony formed by an initial temporary diploid cell. During protoplast fusion, in addition to cytoplasm, chromosomes of the two cells join together[23]. The two chromosomes are cut into pieces and randomly reassembled to form a new haploid chromosome. The other pieces of chromosomes will be eliminated[12,20,21]. Until these steps are completed, heterocolonies can survive on double-selective media. After that, depending on which marker gene is kept, the haploid colony can grow only on the related selective medium. Therefore, frequency of real recombination can be calculated using the average number of stable recombinant colonies (1.7) and the average number of colonies on non-selective plates (91), as follows: Frequency of real recombination = $(1.7/91) \times 100 = 1.87\%$. Although this frequency is not high, it agrees well with other results[10,12,24]. Every gene that is responsible for bacterial proliferation, morphological characteristics, sporolation rate and/or metabolite synthesis and secretion, could be recombined after protoplast fusion. However, since the fusants or recombinant progenies were chosen based on their survival on double selective media, only cells that carried recombination between marker genes were screenable. Fusants PF9 and PF10, which yielded 68.3 and 81.8% increase in Des B production respectively, along with three other fusants with 65, 40.1 and 38.6% respectively, and increase in another fusant that lost its ability to produce Des B, were 35.3% of total recombinant fusants. In other words, 35.3% of fusants did not show the same rate of Des B production as those of their parents, while three fusants (17.65%) had almost the same rate of production as C7031, four fusants (23.53%) showed the same rate as their S7011 parent, and the last four (23.53%) were similar to the original *S. griseoflavus*. It means that if there was any possibility to screen all fusants regardless of their ability to grow on a double-selective plate, the extent of selection based on Des B production would be 35.3%, and might result in a better chance to find a more productive fusant. Further mutation and fusion can be done on PF9 and PF10 to increase Des B production.

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Paradox of leaf phenology: *Shorea robusta* is a semi-evergreen species in tropical dry deciduous forests in India

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***Shorea robusta*, widely distributed in moist and dry forests in the tropics, has been paradoxically described as deciduous, semi-deciduous or evergreen species. To assess this contradiction, quantitative documentation of leaf dynamics, flowering and fruiting (by monthly counts on tagged twigs) was made in marked individuals of *Shorea* in a tropical dry deciduous forest. Annual leaf exchange seems to be a survival strategy in *Shorea* during the period of seasonal drought; it replaces all old leaves of differing longevity with new leaves to reduce water loss due to transpiration, and simultaneously supports asynchronous flowering. During March (the transitional month for the leafing phenophase) four phenological variants, reflecting considerable functional diversity in conspecific trees, were recorded. These were: variant a, leaf fall completed and leaf flush begins; variant b, leaf fall and leaf flush overlapping; variant c, leaf fall completed but leaf flush delayed, short leaflessness; variant d, leaf fall incomplete (old leaves persist) and leaf flush delayed. Individuals of *Shorea* responded variously (leaf exchange or evergreenness to leaflessness or deciduousness, but ≤ 1 year leaf lifespan) to microsite conditions, making it essentially a semi-evergreen species. It is suggested that semi-evergreenness in *Shorea*, an indicator of high adaptability, permits its extensive distribution in the tropics, from moist to the dry regions.**

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SHOREA robusta Gaertn. f. (sal) is one of the dominant tree species in tropical deciduous forests (moist as well as dry types) in India[1]. The nature of *S. robusta* in terms of leaf phenology has been questionable due to evergreen vs deciduous paradox[2]. While some workers have described *S. robusta* as a deciduous species[3–5], or semi-deciduous species[6], others have viewed it as an evergreen species[7–9]. Joshi[10] has variously described *S. robusta* as deciduous or as border line between evergreen and deciduous. The lifespan of leaves is important in that it reflects several eco-physiological attributes[11]. For example, compared to deciduous species, evergreen species generally show longer leaf lifespan, deeper root system, earlier leaf flushing during the dry season, higher stem water potential and greater ability to rehydrate the stem during the dry season, lower resource requirement to support leaf turnover, and longer duration of photosynthetic activity at lower rates[12–15]. Thus, the phenological terms 'deciduous' and 'evergreen' have different connotations in tree physiology and should be applied to a species with great care.

In tropical trees, leaf phenology is important because it reflects the influence of evolution and environment on plant characteristics, and in turn has substantial implications for plant functioning[16]. Contrary to the deciduous tree species which are generally summer-flushing (vegetative bud breaks in hot-dry summer, May–June), *S. robusta* is a spring-flushing species (vegetative bud breaks around spring equinox, March–April) in Indian dry tropical forests[17]. The period around spring equinox is important because of the overlap of leaf fall, leaf flushing, and flowering/fruiting in *Shorea*. Occurrence of annual leaf exchange in *Shorea* in tropical dry deciduous forests raises several questions. Whether this species is evergreen or deciduous? Why does leaf exchange occur always during the mid dry season? Whether conspecific trees show similar phenological response? There is a need to precisely document the time and duration of various phenological events (like leaf flush and leaf fall) in this species and to quantify the extent of leafless period, if any, in conspecific trees. The objectives of the present quantitative phenological study on *S. robusta*, carried out in a tropical dry deciduous forest in the Vindhyan region, were to document the seasonal pattern of phenological events, especially the leaf dynamics, flowering and fruiting, including conspecific asynchrony, with a view to determining the leaf phenological nature of the species.

This study was carried out in Hathinala Forest (24°18′N, 83°6′E; elevation 315–485 m asl), spread over the Vindhyan plateau (ca. 150 km away from Varanasi), in the Sonbhadra