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Hairy root induction and phytoremediation of textile dye, Reactive green 19A-HE4BD, in a halophyte, *Sesuvium portulacastrum* (L.) L.

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ABSTRACT

In this study, we report phytoremediation of textile dyes using hairy roots derived through *Agrobacterium rhizogenes* (NCIM 5140) infection of in vitro leaf and stem explants of a halophyte *Sesuvium portulacastrum* (L.) L. Leaf explants showed higher frequency of hairy root induction (70%) than stem explants (30%), and maximum number of roots (leaf 42.3 ± 2.4 and stem 50.3 ± 1.7). Transformed nature of hairy roots was ascertained by amplifying 970 bp region of T-DNA of Ri plasmid. Hairy roots were screened for phytoremediation of various textile dyes and results showed that HRs were able to degrade Reactive green 19A HE4BD upto 98% within 5 days of incubation. Spectrophotometric analysis showed decrease in dye concentration while HPLC and FTIR analysis confirmed its degradation. Seed germination assay demonstrated non-toxic nature of the extracted metabolites. This is the first report on induction of hairy root culture in *Sesuvium portulacastrum* and phytoremediation of textile dyes.

1. Introduction

Hairy root cultures obtained via *Agrobacterium rhizogenes* mediated genetic transformation show distinctive features such as high growth rate, unlimited branching, and biochemical and genetic stability besides possessing differentiated morphology and biosynthetic machinery [1]. Hairy roots also offer as platform for the production of secondary metabolites [2]. Besides, hairy roots (HRs) are also used for screening different plant species for their tolerance, accumulation, and/or removal of environmental pollutants [3,4]. Among the most environmental pollutants that threaten our biodiversity, effluents from dye-based industries pose a serious hazard. The prevalence of even very small levels of dyes in effluent and degradation products of these textile dyes is often highly toxic to the ecosystem [5].

Sesuvium portulacastrum (L.) L., is an important facultative halophyte endowed with salt accumulating ability and as a natural source of 20-hydroxyecdysone (20E), which is an insect molting hormone [5]. Phytoremediation potential of this plant has also been demonstrated for heavy metals like cadmium, lead and arsenic [6–12] and biodegradation of toxic textile dyes [13]. Recent research has demonstrated that halophytes are ideal candidates for phytoextraction or phytostabilization of salinity and heavy metal polluted soils [13,14]. Salinity appears to be a key factor not only for the increased bioavailability of metals in the soils due to reduced soil metal sorption [15,16] but also in the translocation of metals from roots to the aerial parts of the plant [17,18] With this background, we have studied hairy root induction in *Sesuvium* and explored hairy root culture application in the degradation of textile dye Reactive green 19A- HE4BD.

2. Materials and methods

2.1. Dyes, chemicals and tissue culture media

The textile dyes used in this study were procured from local industry of Ichalkaranji, India. While ABTS (2,20-Azinobis,3-

Abbreviations: HRs, hairy roots; ABTS, 2,20-azinobis,3-ethylben-zothiazoline-6-sulfonic acid; BA, benzyl adenine; PCR, polymerase chain reaction; MS, Murashige and Skoog; UV–vis, ultra violet visible; FTIR, Fourier transform infrared spectroscopy.

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Table 1
Hairy root induction from the in vitro leaf and stem segments of *S. portulacastrum*.

Type of explants	Days of culture	No. of explants responded for hairy root induction	%Response	No of roots per explant
Leaf	7	8.3 ± 0.8^{d}	5.5 ± 0.6^{d}	5.3 ± 0.8^{d}
	14	26.6 ± 1.4^{c}	17.7 ± 0.9^{c}	14.6 ± 1.4^{c}
	21	67.6 ± 6.9^{b}	45.1 ± 4.6^{b}	24 ± 1.6^{b}
	28	104.3 ± 4.0^{a}	69.56 ± 2.7^{a}	42.3 ± 24^{a}
Stem	7	3.6 ± 0.3^{d}	7.3 ± 0.6^{d}	3.6 ± 0.6^{d}
	14	10.6 ± 1.4^{c}	21.3 ± 2.9^{c}	11.3 ± 2.0^{c}
	21	18.6 ± 1.2^{b}	37.3 ± 24^{b}	$26. \pm 3.5^{b}$
	28	26 ± 3.3^{a}	$52. \pm 6.4^{a}$	50.3 ± 1.7^{a}

Values in the table are mean ± SE (standard error, $n = 10$). Means with different letters indicate significant differences at $P \leq 0.05$ by one-way ANOVA with Tukey–Kramer comparison test.

ethylben-zothiazoline-6-sulfonic acid) was obtained from Sigma (St. Louis, MO, USA), tartaric acid was obtained from BDH Chemicals (Mumbai, India), dichlorophenol indophenol (DCIP) and Murashige and Skoog (MS) medium from Hi-media (Mumbai, India) and *n*-propanol and catechol were procured from SRL Chemicals (Mumbai, India).

2.2. Plant material and explants

The axillary shoots of *S. portulacastrum* were established and maintained on MS media supplemented with 20 μM benzyl adenine (BA) and 3% sucrose as described earlier [19]. The excised leaf and stem explants were cultured in the petridishes containing solidified MS basal medium without plant growth regulators and incubated for two days (pre-culture) before using them for agroinfection. The %induction of hairy roots was calculated by using following formula:

$$\%\text{Induction} = \frac{\text{No. of roots induced}}{\text{Total no. of explants}} \times 100$$

2.3. Bacterial culture and growth conditions

A. rhizogenes NCIM 5140 obtained from the National Chemical Laboratory (NCL), Pune, (India) was used for hairy root induction. Bacteria was grown in YEB liquid medium prepared by dissolving yeast extract (1.5 g l^{-1}), beef extract (1.5 g l^{-1}) and NaCl (5.0 g l^{-1}) in distilled water and medium pH was adjusted to 7.2. The *Agrobaterium* was cultured on agar plates containing solidified YEB medium and incubation was done at 27 °C for 24 h in dark. Single bacterial colony was inoculated in 50 ml liquid YEB medium in a 250 ml conical flask and incubated on a rotary shaker (100 rpm) at 27 °C for 16–18 h. After incubation, optical density was recorded at 600 nm and adjusted to 0.5 with YEB liquid nutrient medium and then used for infection process.

3. Agroinfection and co-cultivation

A. rhizogenes culture ($OD_{600} = 0.5$) grown overnight was centrifuged at 5000 rpm for 3 min at 27 °C. The pellet was re-suspended in 5.0 ml liquid YEB medium and the 1 ml of this bacterial culture was then diluted with addition of 9.0 ml of MS liquid medium. Two-day old pre-cultured leaf and stem explants were immersed in *Agro* suspension and incubated on rotary shaker (30 rpm) for 30 min at 27 °C. The explants were then removed and blotted dry to remove excess bacteria. The agro-infected explants were transferred to fresh culture plates with MS basal medium and were incubated in dark for 48–72 h at 25 ± 2 °C for co-cultivation. After 48–72 h, explants were washed with sterile distilled water and blotted dry on sterile tissue paper. The explants were cultured on MS basal solid medium supplemented with 400 mg l^{-1}cefotaxime and the cultures were incubated for a further period of 3–5 days under controlled conditions. Explant cultures free of *Agrobaterium* were transferred to MS basal solid medium and were maintained under controlled conditions in complete darkness as mentioned earlier.

3.1. Growth of hairy roots

After 20–30 days of culture, observations were made for hairy root induction from the cut edges of explants. Data was taken at an interval of 7 days up to 28 days on the number of explants showing hairy root induction, percent root induction response and number of hairy roots per explants.

3.2. Confirmation of transgenic status of hairy roots

Genomic DNA was extracted from the hairy roots derived from leaf and stem explants using the Mini-prep genomic DNA isolation kit (Sigma–Aldrich, USA) as per manufacturer instructions. Transgenic nature of hairy roots was confirmed using PCR amplification of *rol* gene as described by [20]. Primers (forward:

Table 2
Decolorization performance of *S. portulacastrum* hairy roots for various dyes (30 mg l^{-1}) after 5 days of incubation.

Sr. no.	CI name of the dye	CAS no.	Wavelength (λ_{max})	Decolorization (%)
1	Reactive red 152	71872-80-5	535	86
2	Reactive orange 14	12225-86-4	420	58
3	Reactive pink MB	Not available	535	89
4	Reactive red-2 M5B	17804-49-8	535	82
5	Reactive green 19A-HE4BD	68110-31-6	644	98
6	Remazol yellow 3GL	12237-16-0	420	76
7	Remazol navy blue RGB	147826-71-9	616	60
8	Remazol blue RGB	12225-45-5	605	64
9	Remazol yellow RGB	12237-16-0	420	55
10	Red brown H4R	12225-66-0	535	81

5′-CGGTCTAAATGAAACCGGCAAACG-3′ and reverse: 5′- GGCA-GATGTCTATCGCTCGCACTCC-3′) were used for amplification of 970 bp domain of the T-DNA region of *A. rhizogenes* plasmid.

PCR reaction mixture (25.0 μl) consisted of 2.5 μl 10× assay buffer, 1.0 U *Taq* DNA polymerase, 10 mM dNTPs, 50 ng each primers, 150 ng template DNA (50 ng μl^{-1}). PCR was carried out using Eppendorf Cycler Gradient with initial denaturation at 94 °C for 3 min, followed by 40 cycles, each with denaturation (94 °C) for 30 s, annealing (55 °C) for 30 s and extension (72 °C) for 1 min 30 sec. Final extension was performed at 72 °C for 10 min followed by hold at 4.0 °C. PCR products were separated on 1.2% agarose gel using 1X TAE buffer and were stained with ethidium bromide for visualization under UV–visible transilluminator.

3.3. Decolorization studies

S. portulacastrum hairy roots (120 mg dry weight) were initially checked for their ability to decolorize various dyes viz. Reactive orange 14, Reactive pink MB, Reactive red 2M5B, Reactive green 19A-HE4BD, Ramazol yellow 3GL, Ramazol navy blue RGB, Ramazol blue RGB, Ramazol yellow RGB and Red brown H4R. Based on initial data, subsequent decolorization experiments were conducted with Reactive green 19A-HE4BD under static condition at 20 °C in sterile MS medium containing 30 mg l^{-1} dye. All the experiments were performed in triplicate and aliquots (3 ml) were taken for determining the residual dye content (%) in the supernatant at specific absorption maxima (Table 2). The decolorization was expressed in terms of percentage and was calculated as follows:

$$\%\text{Decolorization} = \left[\frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}}\right] \times 100$$

The degradation efficiency (%) of hairy roots was studied to see the effect of initial dye concentrations on the decolorization of Reactive green 19A-HE4BD, by the addition of different concentrations of dye (30, 60, 90, 120 and 150 mg l^{-1}) to MS medium and percent degradation was recorded.

3.4. Analysis of degraded metabolites

Spectral analysis of the degraded metabolites was done using Hitachi UV–Vis spectrophotometer (UV 2800) to record changes in its absorption spectrum (160–800 nm). Degradation metabolites of Reactive green 19A-HE4BD were extracted with an equal volume of ethyl acetate. The extract was dried over anhydrous Na_2SO_4 and the solvent was evaporated on a rotary evaporator. The residue thus obtained was dissolved in small volume of HPLC grade methanol. HPLC analysis was performed in an isocratic Waters 2690 system equipped with dual absorbance detector, using C18 column (4.69250 mm) and HPLC grade methanol as a mobile phase. The

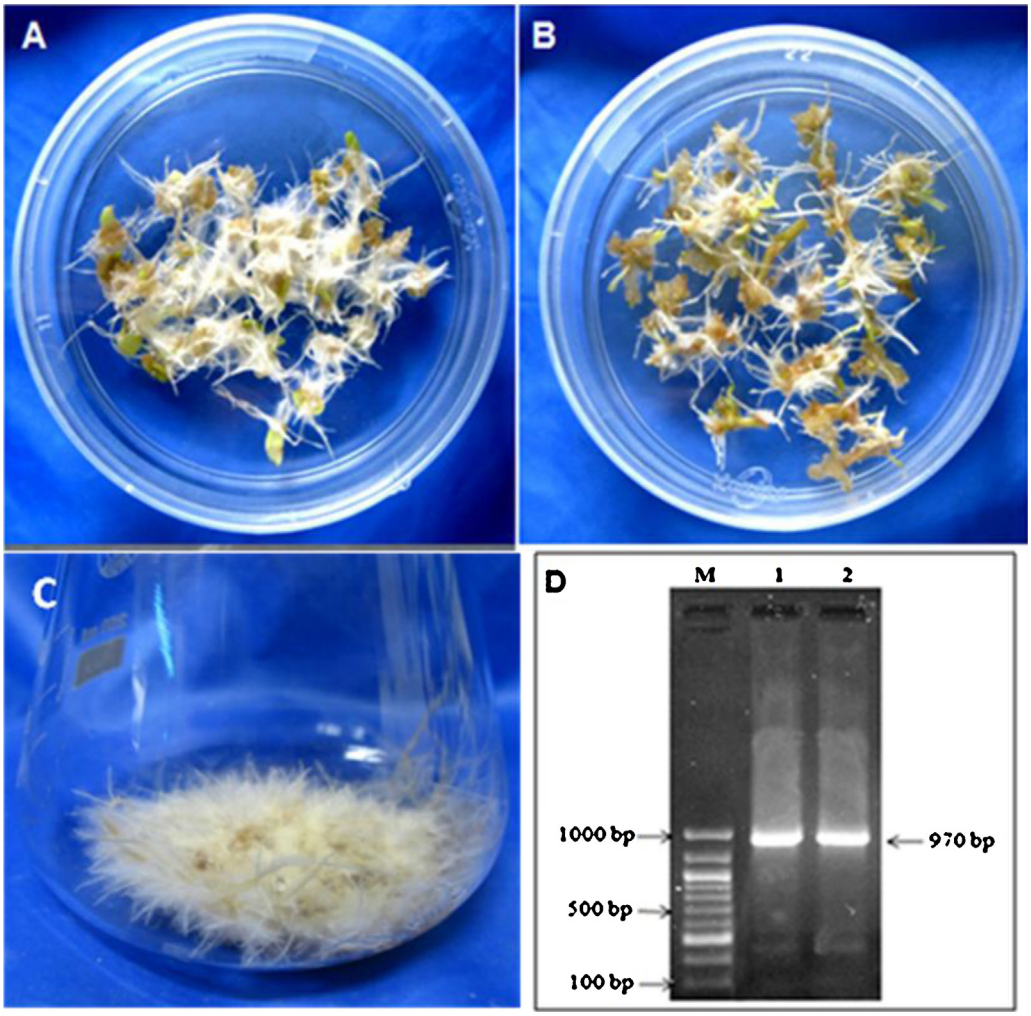

Fig. 1. (A and B) Hairy root induction, (C) biomass of hairy roots, (D) PCR confirmation of transformed nature of hairy roots after 28 days of culture. M: 100 bp DNA ladder, 1: PCR product from hairy root DNA of leaf explants and 2: PCR product from hairy root DNA.

FTIR analysis was done using Perkin Elmer 783 spectrophotometer in the mid-IR region of 400–4000 cm^{-1} with 16 scan speed and comparison was with control dye. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95 and the pellets were fixed in sample holders for the analyses.

3.5. Phytotoxicity assay

Phytotoxicity of the dye and its degraded products was carried out using germination assay with seeds of *Phaseolus mungo* L. The Reactive green 19A-HE4BD degraded product was extracted in ethyl acetate, was dried and dissolved in water to a final concentration of 700 ppm for phytotoxicity study carried out at room temperature (23 ± 2 °C). Ten seeds were watered separately with 5 ml per day of 700 ppm Reactive green 19A-HE4BD dye and at similar concentration with degradation products. Control set was maintained using plain distilled water. Length of plumule, length of radicle and % germination was recorded after 7 days of seedling growth.

3.6. Experimental design and statistical analysis

The experiments were done in completely randomized design (CRD), with three independent biological replicates. The data were analyzed by one-way analysis of variance (ANOVA) using General Linear Model procedure (SPSS 10.0 statistical package). The means were compared by using the post hoc least significant difference (LSD) test. Means differing significantly were compared with Duncan's multiple Range Test (DMRT) at $P \leq 0.05$ probability level. Data was expressed as the mean ± SE.

4. Results

4.1. Induction of hairy roots

Leaf and nodal explants transformed with *A. rhizogenes* strain NCIM 5140 showed induction of hairy roots after 15–20 days of co-cultivation. Swelling followed by hairy root induction was observed at the cut ends of explants. The extent of hairy root induction varied among the explants (Fig. 1; Table 1). The leaf explants showed higher root induction (70%) than stem explants (50%) and the number of explants showing hairy root induction was also higher in leaf explants (104 ± 4) than stem explants (26 ± 3), while roots per explant were higher in stem explants (50 ± 1.7) than leaf explants (42.3 ± 2.4) (Table 1). The PCR amplification of genomic DNA of hairy roots showed product of 970 bp size.

4.2. Screening of different textile dyes for biodegradation

Sesuvium hairy root culture exhibited degradation of all the reactive textile dyes after 5 days of incubation. The maximum decolorization was observed in case of Reactive green 19A-HE4BD (98%), while minimum decolorization was observed in case of dye Ramazol yellow RGB (55%) (Table 2). Hairy roots showed varied decolorization efficiency from 55 to 98% with increasing concentrations of dyes. The efficiency of decolorization was affected by the concentrations of the dye (30 up to 150 mg l^{-1} of Reactive green 19A-HE4BD). The concentration of 30 mg l^{-1} was decolorized up to 98% within 5 days, whereas at higher concentrations (150 mg l^{-1} dye) decolorization efficiency was reduced up to 87% (Fig. 2). At lower concentrations of 30 mg and 60 mg dye, there was no statistically significant difference, however, above this range, efficiency of decoloration was reduced significantly.

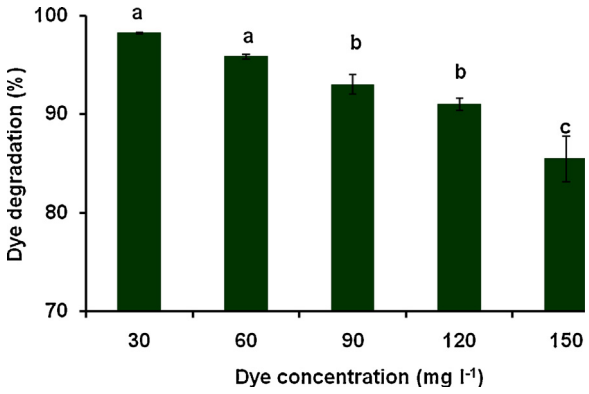

Fig. 2. Effect of dye concentration on dye degradation in *S. portulacastrum* hairy roots.
Different letters on bar indicate significant differences at $P \leq 0.05$ by one-way ANOVA with Tukey–Kramer comparison test.

4.3. Decolorization and biodegradation analysis

UV–vis spectral analysis of the dye Reactive green 19A-HE4BD showed maximum absorbance at 644 nm whereas absorbance was reduced in samples after decolorization. The HPLC elution profile of Reactive green 19A-HE4BD indicated a major and minor peak at 2.09, 1.68 and 3.53 retention times, respectively. The dye degradation metabolites gave additional five peaks at retention times 2.36, 2.47, 2.61, 2.81, 3.08 min (Fig. 3). Comparison of the FT IR spectra obtained between Reactive green 19A and its decolorization products, confirmed biodegradation of the dye into different metabolites. The FTIR spectrum of extracted metabolites showed significant deviations in the peak position as compared to the spectrum obtained with the dye. The FT IR spectra of the dye (Reactive green 19A) indicated sulfonic group and azo groups. The peak showed S—S stretching at 547.75 cm^{-1} for sulfonic groups, CH stretching at 2923 cm^{-1} and N—H stretching at 3463 cm^{-1}.

Post incubation with HR, the degradation products showed peak at 1458 cm^{-1} for CH stretching (of alkynes). The biodegradation of dye was shown by the absence of disulfide group and azo group. The spectra of dye showed peaks at 1573 cm^{-1} for NH bend, 1735 cm^{-1} for C=O stretching, 2933 cm^{-1} for CH stretching alkanes, while the degraded sample showed peaks at 2731 cm^{-1} for OH stretching (Fig. 4). Taken together, our results suggested substantial changes in the peak position in comparison to the tested dye.

4.4. Phytotoxicity studies of degradation products

To study phytotoxicity of degraded metabolites, *Phaseolus mungo* seeds were treated with 700 ppm solution of Reactive green 19A-HE4BD dye and degraded metabolites. The later showed no effect on seed germination while in the former solution, seed germination was reduced significantly (35%) as compared to control (Table 3). The radical length and plumule length was significantly affected in both degraded metabolites solutions and dye solutions as compared to control. In dye solution, plumule length was reduced by almost 3 fold (4 ± 0.1) while in degraded metabolite solution it was decreased by 1.2 fold (10 ± 0.2) as compared to control (12 ± 0.1). Radical length was highly affected by dye solution; it was decreased almost 7 fold (1 ± 0.08). Also in the degraded metabolite solution, radical length was decreased significantly by 1.2 fold as compared to control (7 ± 0.1). Although in both treatments plumule and radical length was decreased, but treatment with degraded metabolite showed improvement as compared to dye treatment (Table 3).

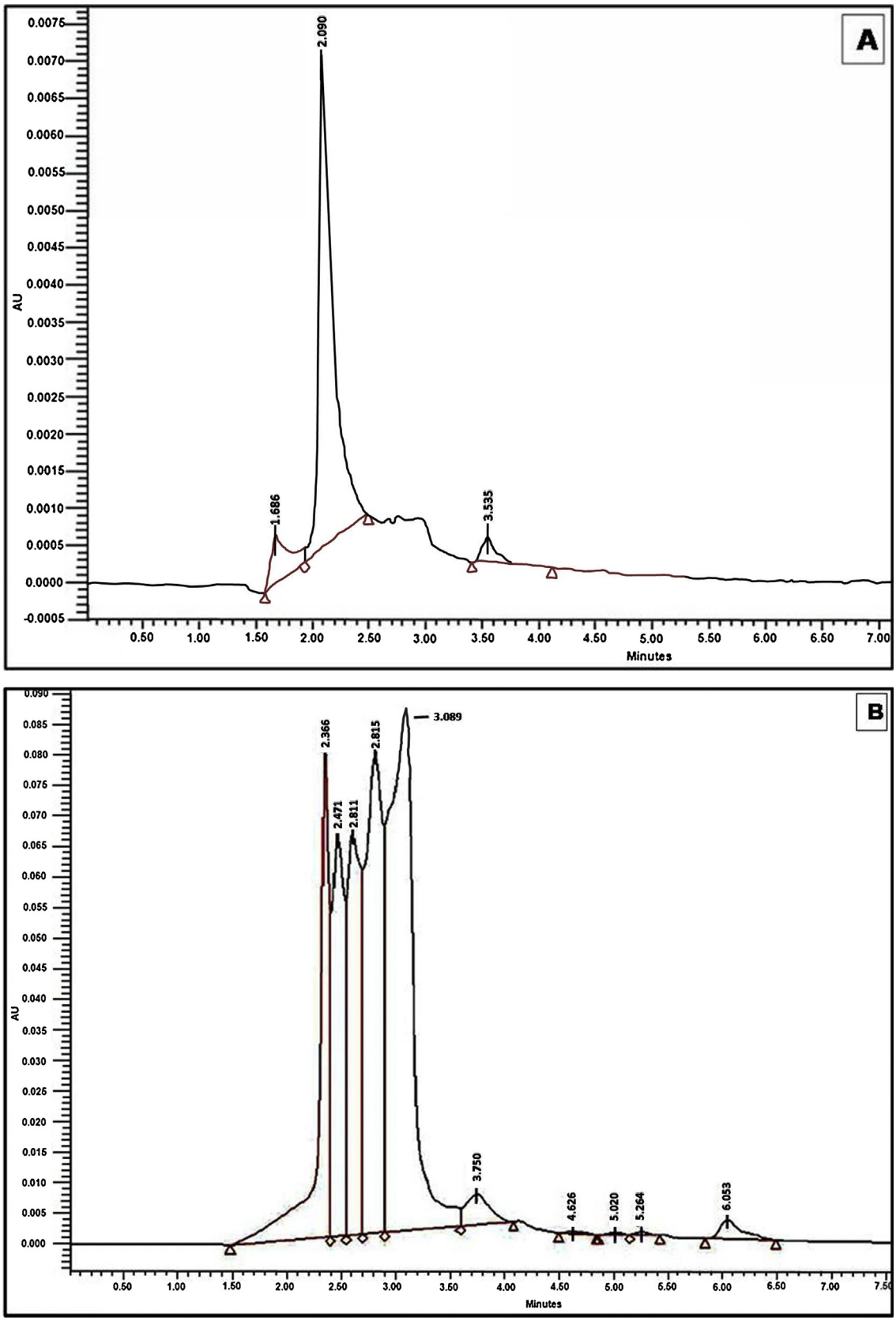

Fig. 3. HPLC elution profile of the dye and its degradation products. (A) Reactive green 19A-HE4BD dye. (B) Metabolites formed after degradation of dye by *S. portulacastrum* hairy roots.

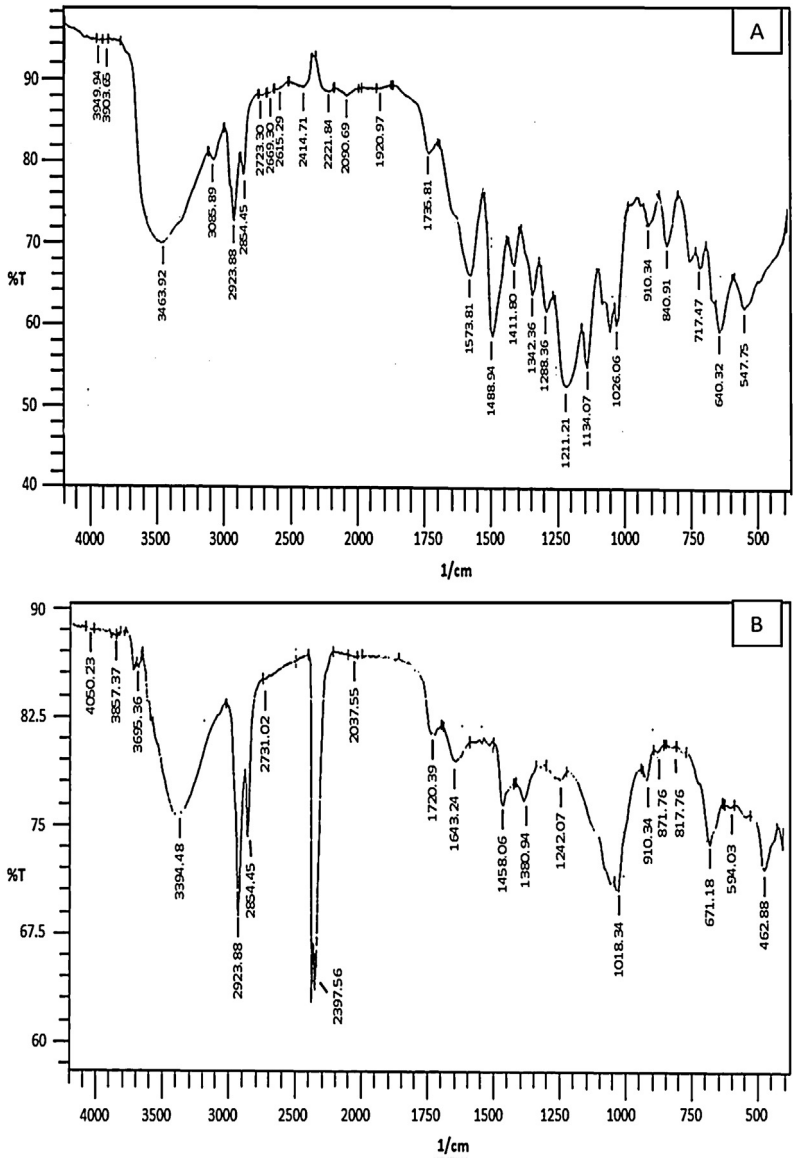

Fig. 4. FT IR spectrum of the dye and its degradation products. (A) Reactive green 19A-HE4BD dye and, (B) degraded product.

5. Discussion

In the present study, we have shown hairy root induction in *S. portulacastrum* L. by using *A. rhizogenes* (NCIM 5140) and further demonstrated application of hairy roots in the degradation of textile dye, Reactive green 19A-HE4BD. In general, hairy roots are genetically stable and synthesize secondary metabolites normally present in plant organs [1,21]. In this study, hairy root induction in leaf and stem explants varied significantly in terms of frequency and number of roots suggesting that different explants of *Sesuvium* can be used for the induction of the hairy roots and that physiological state of explant tissue can affect the transformation efficiency. In a preliminary study, *Sesuvium* leaf explants transformed with the *A. rhizogenes* strain ATCC 15834 showed hairy root induction [22]. In the present study, the highest rate of transformation and hairy root induction from leaf and stem explants was observed using bacterial strain NCIM 5140. The differential transformation efficiency and variation in hairy root induction could possibly be due to the difference in the virulence of different *A. rhizogenes* strains tested [23]. In *Linum flavum*,*A. rhizogenes* strains LBA 9402 and TR 105 induced hairy roots with 50% frequency from leaf discs whereas low induction was observed with strains 15834 and A4 [24]. Variable degree of transformation efficiency was also observed in *Beta vulgaris* transformed with different *Agrobacterium* strains [25] and from root segments of *Maytenus senegalensis* using strains LBA 9402 and A4T [26]. Besides strain virulence, susceptibility of the plant species is also critical for achieving high frequency of hairy root induction [27]. In the present investigation, the rate of hairy root induction was found different in leaf and stem explants of *Sesuvium*. The rate of hairy root induction as well as the growth was increased significantly

Table 3
Phytotoxicity of Reactive green 19A and its metabolites using *Phaseolus mungo* seeds.

Parameters studied	Water	Reactive green 19A	Metabolites
Seed germination (%)	100^{a}	35^{b}	100^{a}
Plumule (cm)	12 ± 0.14^{a}	4.1 ± 0.12^{c}	10 ± 0.23^{b}
Radicle (cm)	7.9 ± 0.14^{a}	1 ± 0.08^{c}	6 ± 0.13^{b}

Values in the table are mean ± SE (standard error, $n = 10$). Means with different letters indicate significant differences at $P \leq 0.05$ by one-way ANOVA with Tukey–Kramer comparison test.

with increase in the days of culture incubation and with explants: leaf explants (70%) as compared to stem explants (52%). In *Helicteres isora* L., hairy roots were induced on the cut-edges of leaves and at the point of agroinfection on the leaf fragments, but not on nodal explants [28]. The variable degree of hairy root induction from different types of explants for variable days of culture incubation has also been observed in other plants such as *Gmelina arborea* [29], *Centella asiatica* [30], *Gossypium hirsutum* and *G. barbadens* [31] and *Maytenus senegalensis* [26]. Hairy root cultures have been demonstrated to have significant potential for phytoremediation [32–34] In the present study, we have applied hairy roots system of a halophyte *Sesuvium* sp. for biodegradation of ten structurally different textile dyes. The efficiency of hairy roots to degrade these dyes ranged from 55 to 98%. Such differential degradation response could be due to certain inhibitory groups such as $—NO_2$ and $—SO_3$ in the dyes [26] or their structural difference and molecular weight [35]. We have also found that decolorization and biodegradation were time dependent as time required for dye degradation is directly proportional to the dye concentration. The higher concentration of dye also decreases the decolorization rate [36] which may be due to toxicity of the dye altering growth, metabolic activity, and in some cases, insufficient mass of culture may affect the uptake of higher concentration of dyes. In *Sesuvium* which is a halophyte, it has been observed that the decolorization potential of in vitro plantlets improved with increase in the culture incubation period upto 7th day in 200 mM NaCl in comparison to decolorization of GHE4B in the absence of NaCl [13] In *Tagetes patula*, Patil et al. [37] hairy roots were able to remove dye concentrations up to 110 mg l^{-1} and the hairy roots were successively used for at least five consecutive decolorization cycles. The hairy roots of *Tagetes* also decolorized six different dyes, viz. Golden Yellow HER, Methyl Orange, Orange M2RL, Navy Blue HE2R, Reactive Red M5B and Reactive Red 198. This study suggests superiority of hairy root cultures over shoot cultures in degradation ability and use for several cycles. Moreover, although cultures derived from differentiated tissues such as roots, shoots and embryos can be used, they are limited by the methods requiring exorbitant cost of raising and maintaining large scale cultures [38] and growth is often slow in case of root cultures requiring continuous exogenous growth regulator supplementation. On the contrary, HRs are stable and do not require any growth hormones except nutrient supply and can be scaled up [21]. Patil et al., [37] correlated increased production of intracellular and extracellular enzymes with dye degradation. The enzymes like Azoreductase, NADH-DCIP reductase and laccase were proved to be key players in biotransformation of toxic dyes into non toxic compounds [39–41]. These enzymes remove toxic Azo and Sulphon groups from dyes and degrade them into non toxic compounds. The HPLC and FT IR data confirmed this phenomenon of biotransformation in *Sesuvium*.

S. portulacastrum has several advantages for the protection of the environment due to its salt accumulating, drought and heavy metal stress tolerant nature [5]. The present report on the detoxification of GHE4B using hairy root system suggests that such an approach could find application for the removal of toxic azo dyes into less toxic metabolites in the locality of textile industries. This also has bearing as the dyes get released in the form of effluents and contaminate the natural ecosystem including water bodies and arable lands [42]. *Sesuvium* hairy roots offer as an effective system for dye degradation as it can degrade dye more than 85% even at higher concentration (150 mg l^{-1}). The non-toxic nature of degradation metabolites of Reactive green 19A-HE4BD with respect to germination and growth of *P. mungo* indicates detoxification of the dye. Further detailed analysis of degraded products is essential to better understand biodegradation. Elicitors/stress conditions can be employed to enhance biodegradation efficacy. To our knowledge, this work is the first report on efficient degradation of textile dye, Reactive green 19A-HE4BD using hairy roots of *S. portulacastrum* L. The results thus indicate potential of hairy root cultures for phytoremediation of textile dyes.

Compliance with ethical standards

This study was funded through the departmental funds provided by Savitribai Phule Pune University, Pune, India.

Conflict of interest

Authors of this manuscript hereby state that there are no conflicts of interest.

Research involving human participants and/or animals: This research does not involve human participants and/or animals.

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