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Detoxification of a carcinogenic paint preservative by *Blumea malcolmii* Hook cell cultures

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ABSTRACT

Phytoremediation is considered as an effective viable alternative to remediate the contaminated sites, industrially hazardous chemicals and other toxic pollutants. This bioremediation option offers a safe, cheap and eco friendly alternative to existing physical and chemical remediation technologies as well as other biological sources. The wall paint preservatives consist of several harmful and carcinogenic compounds causing serious environmental concerns. In the present study, an actively growing *Blumea malcolmii* Hook cell suspensions were established successfully on MS + CM (20%) +2,4-D (5 mg l^{-1}) + Gln (100 mg l^{-1}) + sucrose (3%) and were used to detoxify a paint preservative Troysan S 89 (a mixture of carbendazim, diuron and ochthilinone). FTIR and UV spectral analytical studies revealed the phytotransformation of Troysan S 89 by *Blumea* cell suspension cultures. The non-toxic nature of the products formed after phytotransformation was confirmed by phytotoxicity, cytogenotoxicity while non-carcinogenic nature by Ames tests. The novelty of the present study is effective communal degradation of a mixture of three toxicants in Troysan S 89 by cell suspension cultures of *Blumea*. This work suggested that *Blumea* cell suspensions might be able to contribute to the wider and safer application of phytoremediation.

1. Introduction

The microbial growth is very common in wall paints due to dampness, temperature and humidity [1]; hence, preservatives are generally used to keep the paints free from bacteria, fungi and algae. The most important biocides used as film preservatives are derivatives of urea, isothiazoline-3-one, dithiocarbamates, benzimidazole, triazines, benzothiazole, carbamates, thiophthalimide, sulfenic acids, sulfones, triazoles and pyridine-N-oxide. Troysan S 89, one of the widely used paint preservative, as an algaecide as well as a fungicide, is a formulation of three different chemicals namely carbendazim, diuron and ochthilinone. The percentage by mass, chemical structure and CAS number of these compounds were presented in Table 1. Data have been collected on the direct and indirect effects of constituents of Troysan S 89 on target and non-target organisms, and they are suspected to be genotoxic. The US Environmental Protection Agency (EPA) has classified them as 'known/likely' carcinogen since 1997 [2,3]. Diuron is a substitute phenylurea herbicide and carbendazim is a systematic benzimidazole fungicide and are known to be carcinogenic, mutagenic and teratogenic in experimental animals and animal cell cultures [2,3]. Octhilinone, a mild weedicide, fungicide and bactericide, is reported as neurotoxic and responsible to cause occupational asthma and allergic contact dermatitis, mostly among paint manufacturers [4,5].

Compared to the existing available physical and chemical technologies to degrade harmful paint chemicals [6,7], bioremediation is an alternative, eco friendly, cost effective technique, which has less sludge producing properties and is used for environmental clean up applications in recent years [8]. Biodegradation has been described as the primary mechanism for diuron and carbendazim dissipation in the environment [2,3]; but, yet there is no report on degradation of octhilinone.

Phytoremediation for removal of hazardous chemicals from the environment has been widely reported [9]. For more accurate and precise studies on phytoremediation, aseptic cultures of plants provide a convenient and efficient alternative [10]. The cell suspension cultures proved to be an effective tool for removal of a variety of environmental pollutants such as bisphenol A [11], pesticides [12], explosives [13], and heavy metals [14]. The plant under present study is an annual, wooly herb, belonging to the family Asteraceae and mainly found in tropical and subtropical regions. It has several advantages for phytoremediation as the plant has a deep root system, non-edible nature, non-hazardous and rapid growth. The role of *Blumea malcolmii* plants in bioremediation of textile dyes has

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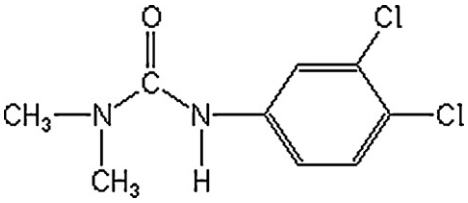
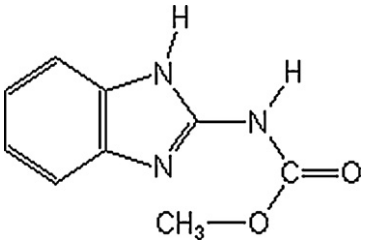
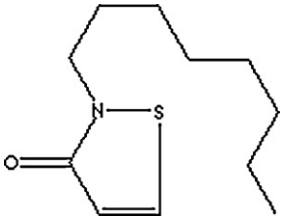
Table 1
Active ingredients of Troysan S 89.

Sr. no.	Name of the active ingredient	Chemical structure	CAS No.	Percentage by mass
1	Diuron (Phenyl urea)		330-54-1	19
2	Carbendazim(Benzimidazole)		10605-21-7	9.9
3	Ochthilinone (Isothiazolone)		26530-20-1	2.25

been demonstrated recently [15]. In this report, we have achieved for the first time cumulative degradation of the three toxicants (carbendazim, diuron and ochthilinone) present in the paint preservative, Troysan S 89 by cell suspension cultures of *B. malcolmii* Hook.

2. Materials and methods

2.1. Chemicals

Troysan S 89 was a generous gift from a local paint industry. 1-Chloro-2,4-dinitrobenzene (CDNB) was obtained from SD Fine Chemicals Limited, India. 2,20-Azinobis, 3-ethylbenzothiazoline-6-sulfonic acid (ABTS) was obtained from Sigma, USA. Tartaric acid was obtained from BDH Chemicals, India. n-Propanol and catechol were purchased from SRL Chemicals, India.

2.2. Source of plant material and establishment of callus cultures

The seeds of *B. malcolmii* collected from the plants grown in the campus of Shivaji University, Kolhapur, India during the month of February were used as source of plant material for the establishment of cultures. The seeds (~500) were aseptically germinated on half strength MS basal medium as per the protocol described earlier [15]. The internodal stem segments (8–10 mm) were excised from 6-week-old *in vitro* germinated seedlings and used as a source of explants for initiation of the callus cultures. The explants were cultured on MS medium supplemented with 3% (w/v) sucrose and variable concentrations of plant growth hormones such as BA (1.0–5.0 mg l^{-1}), 2,4-D (1.0–8.0 mg l^{-1}) and IAA (1.0–5.0 mg l^{-1}) alone or in combinations. The pH of the medium was adjusted to 5.8 ± 0.05 and solidified with Clarigel (0.2%, w/v) prior to autoclaving at 1.06 kg cm^{-2} and 121 °C for 15 min. The cultures were incubated under controlled conditions such as 25 ± 2 °C temperature, 16/8 h (light/dark) photoperiod provided with diffuse light (40 μmol m^{-2} s^{-1}). After 4 weeks of culture incubation, the compact and friable callus induced from internodal stem was separated and maintained on MS medium supplemented with 3% (w/v) sucrose, 2,4-D (5 mg l^{-1}), CM (20%) and Gln (100 mg l^{-1}) with frequent sub-culturing at an interval of 4 weeks for the period of six months.

2.3. Establishment of cell suspension culture

For the establishment of cell suspension culture, the friable calli (~2 g) were transferred in to 250 ml Erlenmeyer flask containing 50 ml liquid callus maintenance medium (MS containing 3% sucrose and 5 mg l^{-1} 2,4-D, CM (20%) and Gln (100 mg l^{-1}). The cultures were incubated on a gyratory shaker at 100 rpm under controlled conditions as mentioned earlier. After 3 weeks of incubation period, the calli in the liquid medium dispersed into small clumps of calli as well as individual cells. The suspension cultures containing calli clumps and individual cells were filtered aseptically through four layers of muslin cloth to separate out the suspension of individual cells. The established cell suspension was regularly sub-cultured at an interval of 10 days for the period of two months on freshly prepared cell suspension medium (callus maintenance medium without Clarigel) and maintained under controlled conditions as described earlier to maintain the cells in actively growing stage.

2.4. Growth kinetics of cell suspension culture

When 10 ml of *B. malcolmii* cell suspension was regularly subcultured to 50 ml of cell suspension medium, 10 ± 0.500 g FW of cells was obtained routinely after 8 days of incubation under controlled conditions and there was neither any alteration in the growth characters nor any decline in growth rate of cells resulting into a stable cell line. After the stable establishment of cell suspension, the growth kinetics was studied in terms of fresh and dry weight (FW and DW) of cells at the end of each week for the period of 4 weeks. For growth kinetics, the packed cell volume of 15% was inoculated as initial biomass in the Erlenmeyer flasks (250 ml capacity)

containing 50 ml cell suspension medium. The culture flasks were incubated under control conditions as mentioned earlier. At the end of each week, three flasks from individual treatments were harvested and subjected for the measurement of FW and DW. The cells from each flask were separated individually using Whatman No. 1 filter paper and Buchner's funnel. FW (g) was measured after complete removal of medium from the cell biomass, whereas for DW measurement, the cells separated on filter paper were dried in the hot air oven at 60 °C for 24 h to obtain the constant DW (g).

2.5. Experimentation for biotransformation of Troysan S 89

Actively growing cells (3 ± 0.500 g FW) from cell suspension cultures were inoculated aseptically in to 100 ml Erlenmeyer flask containing 20 ml of cell suspension medium and with (Test) or without (Control) addition of 0.5% of Troysan S89. The cultures were incubated on the gyratory shaker at 100 rpm speed for the period of 10 d under controlled conditions as mentioned earlier. About 2 ml of sample from both test and control cultures was harvested periodically at the interval of 2 d for the period of 10 d and centrifuged at 5000 rpm for 10 min. The separated supernatant was analyzed for ability of the cells to detoxify Troysan S89.

2.6. Measurement and in situ visualization of cell mortality

To assess the effect of initial concentration of Troysan S 89 on the growth of *Blumea* cells and phytoremediation process, the relative growth of cells and UV spectra of culture supernatant have been observed at various initial concentrations of Troysan S 89. Actively growing *Blumea* cells (6 ± 0.234 g FW) from cell suspension cultures were inoculated aseptically in to 250 ml Erlenmeyer flask containing 50 ml of cell suspension medium and with addition of various concentrations (0.02–1.02%) of Troysan S 89. Cultures were incubated for 8 days under controlled conditions as mentioned above and the number of cells survived were observed and counted. To assess the acute toxicity of Troysan S 89 to *Blumea* cells, cell mortality at various concentrations of Troysan S 89 was estimated by Evan's Blue staining method [16], where control and cell suspensions treated with Troysan S 89 were stained with 0.25% (w/v) aqueous solution of Evan's Blue (Himedia, India) for 5 min. Briefly, cell suspension (100 μl) was mixed with 100 μl of Evan's Blue staining solution and then spread on to the slide, covered with cover slip and observed under microscope (Lowrence and Mayo, India). Viable cells appeared unstained, whereas the dead cells appeared blue-stained. At least 200 cells were counted per sample. The observed responses fitting to regression model were evaluated using Probit analysis (SPSS 8.0 for Windows Student version) and 8 days LC_{50}, no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) were calculated using the standard probit procedure [17].

The linear model we used can be written as:

$$Y = \alpha + \beta X \quad (1)$$

while the parabolic model we used can be written as:

$$Y = \alpha + \beta X + \gamma X^2 \quad (2)$$

where Y is cell mortality in probit unit and X is natural log of concentration of Tyoysan S89 (values of α, β, and γ could be calculated).

The flasks from individual treatments were harvested and subjected for the measurement of FW and DW and the relative growth of *Blumea* cells was calculated as the ratio of observed growth of cells to the initial mass of cells.

2.7. Analytical procedures

The supernatant of both test and control samples as well as the cell suspension medium containing 0.5% Troysan S 89 without addition of cells were subjected for spectral analysis using UV–vis double beam spectrophotometer (UV-2800, Hitachi, USA). Absorption spectra of the samples were detected within range of 200–400 nm wavelength.

The metabolites produced after biodegradation of carbendazim, diuron and ochthilinone from Troysan S 89 using cell cultures of *B. malcolmii* were detected using Fourier Transformed Infra Red (FTIR) spectroscopy. The samples were extracted with equal volumes of n-butanol (SRL Chemicals, India). The extracts were air-dried in Petriplates and the residue obtained for each sample was provided for TLC and FTIR analysis. For TLC, the residues of each sample were redissolved separately in small amounts of HPLC grade methanol and the degraded metabolites of Troysan S 89 were analyzed by loading the samples on silica gel (60F-254, 4 cm × 10 cm, thickness 0.2 mm) using mobile phase methanol:water (2:1). The dark brown spots observed for degraded metabolites of Troysan S 89 on TLC plate after iodine staining were used for the calculation of Rf values.

For FTIR analysis, the residue (∼5 mg) was mixed with potassium bromide (KBr, spectroscopic grade) in the ratio of 5:95 and fixed in a sample holder. The change in percent transmission (*T*%) of Toysan S 89 and their degraded metabolites was observed using FTIR (PerkinElmer, USA) spectrophotometer. The spectra obtained after biodegradation were compared with Troysan S 89 in the mid IR region of 400–4000 cm^{-1} with 16 scan speed.

2.8. Carcinogenicity and toxicological studies

Ames test was assayed to evaluate the carcinogenecity of Troysan S 89 and its degradation metabolites, while *Allium* root chromosomal aberration assay and phytotoxicity assays were carried out to determine their toxic nature. *Allium* root chromosomal aberration assay was conducted with respect to cytotoxicity by using *Allium cepa* bulbs. Phytotoxicity assay was executed by using a very commonly consumable seeds of *Triticum aestivum* and *Ervum lens* Linn and Ames test was performed with *Salmonella typhimurium* NCIM 2501 (histidine-requiring) as a test organism.

2.8.1. Ames test

The *Salmonella*/microsome reversion assay was conducted using the plate incorporation procedure described earlier [18,19], for assaying the carcinogenicity/mutagenesis of the metabolites produced after degradation of Troysan S 89. *S. typhimurium* was grown on nutrient agar medium plates containing Troysan S 89 and metabolites, respectively, with individual concentrations of 20, 40, 100, 500, and 1000 ppm. Plates were incubated at 37 °C for 48 h and the culture obtained on nutrient medium containing metabolites was then continuously transferred to Vogel–Bonner *E* medium (1.5% agar, 2% glucose, without histidine).

2.8.2. Allium root chromosomal aberration assay

Small equal sized bulbs of *A. cepa* were chosen, of which, the loose outer scales were removed and the dry bases were scraped to expose the root primordial. Thus, before use, the bulbs were germinated initially in water for development of roots. The prepared bulbs were grouped in to three sets (each set containing three bulbs) and each set was treated individually with water, Troysan S 89 (1000 ppm) and its degradation metabolites (1000 ppm) for 5 days. After exposure, the bulbs were removed, washed thoroughly under running tap water and used for further cytotoxicity and genotoxicity studies as per previous reports [20,21].

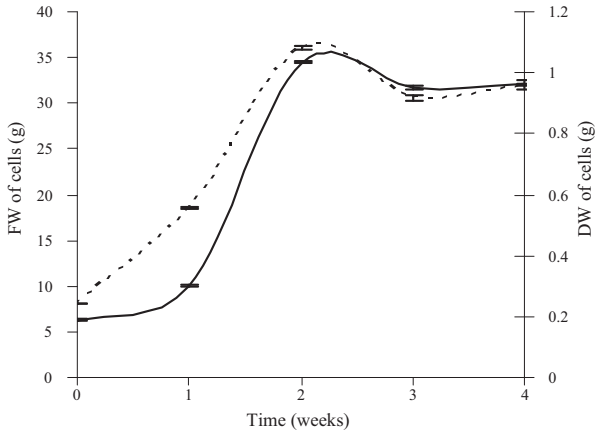


Fig. 1. Growth curve of *Blumea malcolmii* Hook cells. Dotted line represent dry weight (g) and solid line represent fresh weight (g).

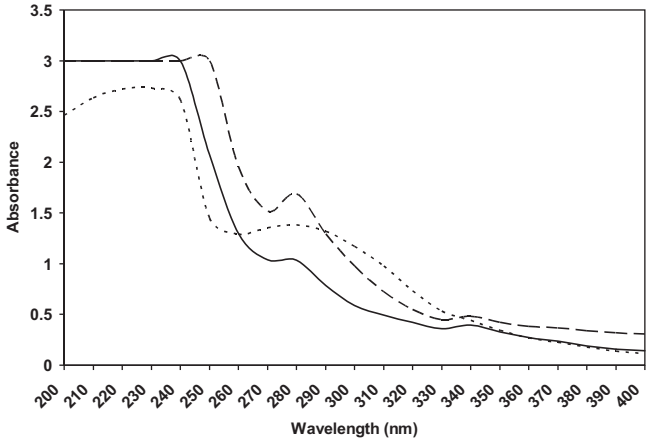


Fig. 2. UV–vis spectral analysis of Troysan S 89 before and after degradation by *B. malcolmii* cells. Troysan S 89 before degradation – – –; Troysan S 89 after degradation —; *Blumea* cells in control medium - - -.

2.8.3. Phytotoxicity tests

The pot experiments were carried out in plastic pots having diameter 5.5 cm and height 6 cm. 100 g of thoroughly washed and then oven dried sand was taken in each pot and 20 seeds each of *T. aestivum* and *E. lens* Linn were added separately in the pots. The seeds were watered for 14 days with Troysan S 89 (1000 ppm) and its degradation metabolite (1000 ppm). The length (cm), wet and dry weight (g) of plumule and radical were recorded after 14 days of seed germination.

3. Results and discussion

3.1. Establishment of callus and cell suspensions, and growth curve of B. malcolmii cells

The seeds subject for germination on half strength MS medium revealed 65% germination after 3 weeks of culture incubation. Among the various concentrations of plant growth hormones (BA, 1.0–5.0 mg l^{-1}; 2,4-D 1.0–8.0 mg l^{-1}; and IAA 1.0–5.0 mg l^{-1}) used for the induction of callus from internodal stem explants, the medium supplemented with 5.0 mg l^{-1} 2,4-D was found most suitable for maximum callus induction (175 mg DW per culture). Lower concentrations of 2,4-D induced poor callus, whereas, the media containing different concentrations of BA or IAA alone or in combinations did not induce callus.

The MS medium supplemented with 2, 4-D (5 mg l^{-1}), L-glutamine (100 mg l^{-1}), coconut milk (20%, v/v) and sucrose (3%) was found suitable for development of a friable callus after four weeks. Cell suspension cultures showed exponential growth phase up to the end of 2nd week followed by stationary phase extended up to 2nd to end of 4th week (Fig. 1). Cell suspension cultures gave highest 691 ± 2.517 g l^{-1} of FW and 21.774 ± 0.589 g l^{-1} of DW of biomass on the 2nd week.

3.2. Analysis of biotransformation of Troysan S 89 by B. malcolmii cells

As per the UV–vis spectral analysis (Fig. 2), change in absorbance after every 2 days incubation showed the decrease in absorption spectra in the test medium indicating decrease in concentration of Troysan S 89. The gradual decrease in the absorbance of Troysan S 89 in the media after a regular time period suggested its removal from the media. After 10 days of incubation of toxicants with *B. malcolmii* cells, Troysan S 89 was significantly removed from the test medium.

Dose–response assessment has popularly been used in toxicology to quantitatively reveal toxicity tolerance of living organisms to toxicants. Reports led growth rate measurement (pepper growth bioassay) and cell mortality determination by cells' staining could be obliging as phytotoxicity bioassays [22,23]. Upon 8 days exposure of *Blumea* cells to carbendazim, cell disintegration leading to relative growth inhibition was observed, and the frequency and severity of which is increased with increasing concentration of the test material (Fig. 3). Survival rates at the respective Troysan S 89 concentrations were as follows: 98.885% at 0.02%, 94.809% at 0.1% and 1.115% at 1%. All of the *Blumea* cells were died at or above 1.02% exposure concentration of Troysan S 89. The empirical NOEC, LOEC and LC_{50} for Troysan S 89 determined by probit analysis were 0.04%, 0.05% and 0.498%, respectively. The values of α, β and γ were calculated and the Eqs. (1) and (2) could be derived as:

$$Y = 3.662 + 1.146X \quad (3)$$

$$Y = 3.152 + 0.79X + 0.362X^2 \quad (4)$$

From the derived equations and the plotted graphs, it is apparent that parabolic approximation gave better estimates than the linear trend.

After 8 days of culture incubation, relative growth of *B. malcolmii* cells for control and highest concentration of Troysan S 89 used

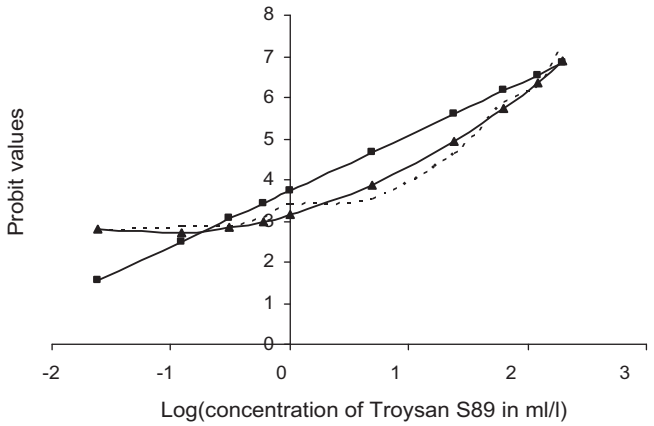


Fig. 3. Effect of initial concentration of Troysan S89 on *B. malcolmii* cell mortality. Cells were exposed to Troysan S89 for 8 days at 25 ± 2 °C and cell mortality was estimated by Evan's Blue staining method. Dotted line represents empirical probit, line with squares represents linear regression, while line with triangles represents parabolic regression.

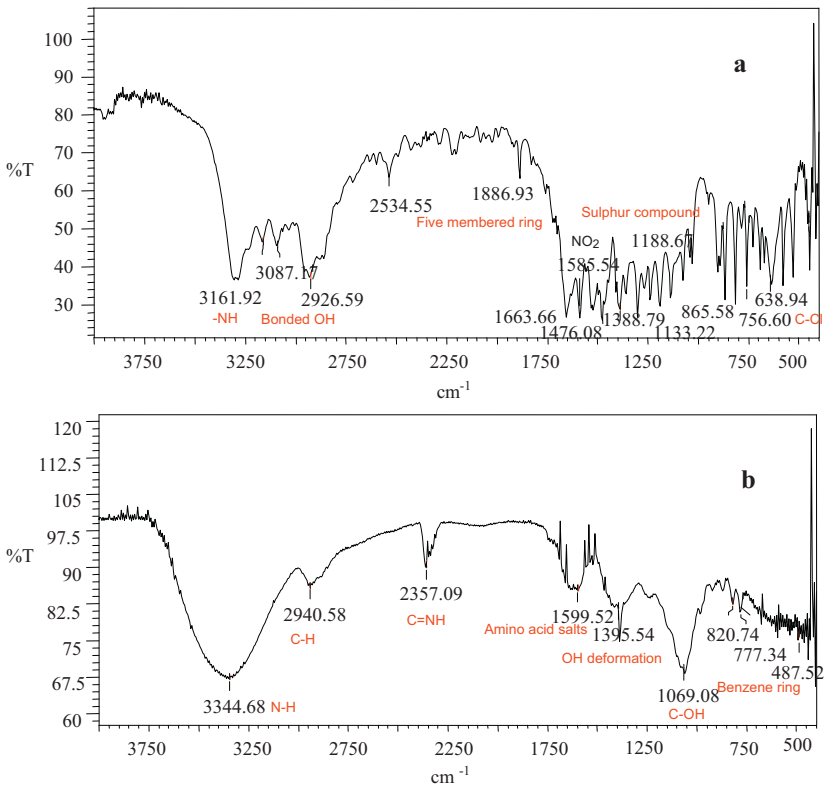


Fig. 4. FTIR analysis of carbendazim, diuron and ochthilinone from Troysan S 89 (a) before and (b) after degradation by *B. malcolmii* cells.

(1.02%) was 1.489 ± 0.085 and 0.97 ± 0.081, respectively (Suppl. Fig.) Cell mortality evaluated by Evan's Blue staining method indicated disintegration/damage of cell wall with various extents by Troysan S 89 at 0.02–1.02%. The 8 days LC_{50} concentration of Troysan S 89 for *B. malcolmii* cells was near to 0.5% and therefore 0.5% of Troysan S 89 has been used through out the phytoremediation studies. The significant cell mortality was observed above the 0.5% concentration of Troysan S 89 which affected phytoremediation process considerably.

The TLC analysis of control Troysan S 89 showed Rf values 0.68, 0.57, 0.48 while for the degraded metabolite it showed different Rf values (0.77, 0.61) suggesting possibility of biotransformation of Troysan S 89 by *B. malcolmii* cells. The FTIR analysis of Troysan S 89 (Fig. 4a) displayed peak at 3161 cm^{-1} for bonded NH of secondary amides, peaks at 3087, 2926 and 2534 cm^{-1} for bonded OH, peak at 1886 cm^{-1} for conjugated 5 member ring; peak at 1663 cm^{-1} for cyclic α β C=N stretching; peak at 1585 cm^{-1} for NO_2 stretching of primary and secondary nitro compounds and nitramines; peak at 1476 cm^{-1} for aromatic homocyclic compounds, 1388 and 1188 cm^{-1} for presence of sulphur compounds; peak at 1133 cm^{-1} for C-H deformation of substituted benzenes; 865 cm^{-1} for benzene ring containing two adjacent H atoms; peaks at 756 and 638 cm^{-1} for C–Cl stretching of chlorides. This confirmed the presence and structures of carbendazim, diuron and ochthilinone in Troysan S 89 (Table 1). The FTIR spectrum of degradation metabolite of Troysan S 89 (Fig. 4b) displayed peaks at 3344 cm^{-1} for N–H stretching of amino acids, peak at 2940 cm^{-1} for C–H stretching of alkanes, peak at 2357 cm^{-1} for $C{=}NH^{+}$ stretching of charged amines, peak at 1599 cm^{-1} for amino acid salts, peaks at 1395 and 1069 cm^{-1} for OH deformation and C–OH stretching of primary alcohols respectively; peaks at 820 and 777 cm C–H deformation of benzene rings containing two and three adjacent H atoms respectively. Peaks observed for carbendazim, diuron and ochthilinone were confirmed by their structures (Table 1) while peaks observed for degradation metabolites probably determine simpler products like amino acids, carboxylic acids, primary alcohols etc. None or minimum number of peaks of degradation metabolites were detected similar to that of peaks of carbendazim, diuron and ochthilinone indicating their transformation.

3.3. Carcinogenecity and toxicological studies

Evaluation of the ecological and genetic impact of carbendazim, diuron and ochthilinone and their metabolites produced after biodegradation on plant populations is of great importance as plants are important commercial products and are consumed routinely by people. Present study demonstrated the toxicological effects of toxicants from Troysan S 89 and their degradation metabolites by using *Allium* test and phytotoxicity bioassay. Microbial toxicity assay was performed by using Ames test, which could reveal mutagenic, in turn, carcinogenic characters of the toxicants.

Ames test was previously employed to determine the non-carcinogenic characters of degradation metabolites of textile dye [19]. Mutagenic (or carcinogenic) nature of ingredients of Troysan S 89 has been previously discussed in the text, but the conversion of these ingredients into non-mutagens (or non-carcinogens) due to degradation by *Blumea* cells is an important issue. *S. typhimurium* was observed to grow on a nutrient agar plate containing Troysan S 89 up to 500 ppm and found inhibited at 1000 ppm concentrations of Troysan S89. Within the used concentrations range, metabolites did not affect the growth of the organism. *S. typhimurium* grown on nutrient medium containing degradation metabolites of Troysan S 89 was further transferred to an agar plate lacking histidine (Vogel–Bonner *E* medium). All the times *S. typhimurium* failed to grow without histidine, which indicated no mutations,

Table 2
Effect of carbendazim, diuron and ochthilinone from Troysan S 89 and their phytotransformed metabolites on root length, mitotic index (MI), number and frequency chromosomal aberrations obtained for the *Allium* tests.

Analysis	Treatment		
	Water	Troysan S 89 (1000 ppm)	Degradation metabolites (1000 ppm)
RL (cm)[a]	8.033 ± 0.172	3.366 ± 0.147***	5.3 ± 0.109***
MI[a]	11.609 ± 2.660	9.428 ± 1.217***	9.627 ± 3.987***
TA	0	15	0
TCA	234	233	238
Frequency of TA	0	0.081 ± 0.012	0

RL, radical length; MI, mitotic index; TCA, total number of cells analyzed; TA, total number of alterations (chromosomal aberrations).
[a] Values are mean of three experiments ± SEM.
*** Significantly different from treatment with water at $P < 0.001$.

in turn showed non-mutagenic and/or non-carcinogenic nature of metabolites produced after degradation of Troysan S 89 by *B. malcolmii* cells.

The *Allium* test was carried out for *in situ* biomonitoring the cytotoxicity of carbendazim, diuron and octhilinone from Troysan S 89 before and after treatment with *Blumea* cells. The *Allium* root chromosomal aberration assay is an established plant bioassay validated by the International Programme on Chemical Safety (IPCS, WHO) and the United Nations Environment Programme (UNEP) as an efficient and standard test for the chemical screening and *in situ* monitoring for genotoxicity of environmental substances [24]. Cytotoxicity analysis is determined on the basis of increase or decrease in the mitotic index (MI). MI serves as an important parameter of cytotoxicity studies in environmental biomonitoring [25]. The analysis showed strong cytotoxic effect of carbendazim, diuron and octhilinone from Troysan on the root cells of *A. cepa* (Table 2, Fig. 5). MI of *Allium* bulbs exposed to 1000 ppm each of Troysan S89 and its degradation product were 9.428% and 9.627%, respectively, which were significantly different from MI of *Allium* bulbs in water (11.609%). Though MI of root cells of *Allium* bulbs exposed to Troysan S 89 is comparable with MI of root cells of bulbs exposed to degradation product, there was no (or very less) observed chromosomal aberration(s) in case of root cells from bulbs treated with degradation product. Besides mitotic index, different kinds of chromosomal aberrations were observed in of root cells of *Allium* bulbs exposed to Troysan S 89; the most common chromosomal abnormalities being c-mitosis, laggards, chromosome breaks, sticky-ness and micronuclei. The total number of observe chromosomal alterations were 15 out of 233 root cells examined in *Allium* bulbs exposed to Troysan S 89 with alteration frequency of 0.081 ± 0.012. Thus the study indicated non-cytotoxic nature of the degradation product.

Though many methods have been previously described, seed germination and plant growth bioassays are a few of the most common used techniques [26,27], to evaluate phytotoxicity. Many researchers have described the pot experiments for studying various toxicity parameters in a variety of plants [28,29]. In the present study, the toxicity of carbendazim, diuron and ochthilinone from

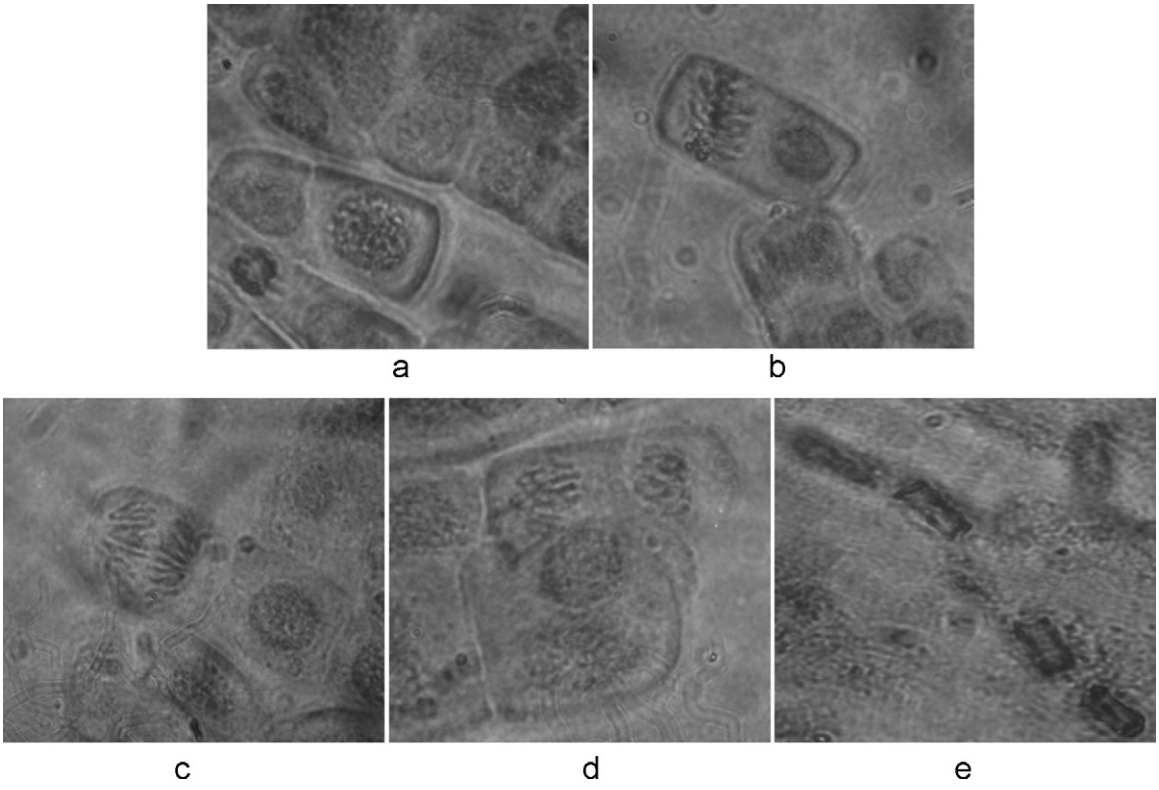


Fig. 5. Normal mitotic cells of *Allium cepa* (a) prophase (b) metaphase (c) anaphase (d) telophase and (e) chromosomal aberrations due to cytotoxic effects of carbendazim, diuron and ochthilinone from Troysan S 89.

Table 3
Phytotoxicity studies.

Parameters studied	*Triticum aestivum*			*Ervum lens* Linn		
	Water	Troysan S 89 (1000 ppm)	Extracted metabolites (1000 ppm)	Water	Troysan S 89 (1000 ppm)	Extracted metabolites (1000 ppm)
Percentage germination	100	70	95	100	45	90
Plumule (cm)	16.720 ± 0.580	$6.275 \pm 1.664^{***}$	$13.680 \pm 1.171^{\$\$\$}$	23.520 ± 0.535	$7.735 \pm 0.500^{***}$	$13.495 \pm 0.651^{\$\$}$
Radical (cm)	8.580 ± 1.170	$3.030 \pm 0.929^{***}$	$6.930 \pm 0.979^{\$\$\$}$	8.193 ± 0.360	$1.990 \pm 0.081^{***}$	$4.190 \pm 0.212^{\$\$\$}$
Wet weight of plumule (g)	0.101 ± 0.005	$0.033 \pm 0.009^{***}$	$0.097 \pm 0.010^{\$\$\$}$	0.110 ± 0.002	$0.061 \pm 0.009^{**}$	$0.010 \pm 0.001^{\$\$}$
Dry weight of plumule (g)	0.071 ± 0.007	$0.032 \pm 0.008^{***}$	$0.833 \pm 0.012^{\$\$\$}$	0.011 ± 0.001	$0.002 \pm 0.001^{***}$	$0.010 \pm 0.001^{\$\$\$}$
Wet weight of radical (g)	0.014 ± 0.001	$0.006 \pm 0.001^{***}$	$0.014 \pm 0.000^{\$\$\$}$	0.077 ± 0.012	$0.042 \pm 0.005^{***}$	0.050 ± 0.007
Dry weight of radical (g)	0.021 ± 0.002	$0.003 \pm 0.001^{***}$	$0.029 \pm 0.001^{\$\$\$}$	0.009 ± 0.001	$0.003 \pm 0.000^{***}$	$0.007 \pm 0.000^{\$\$}$

Phytotoxicity study of Troysan S 89 and its extracted metabolites produced after phytodegradation, values are mean of germinated seeds of three experiments ±SEM, significantly different from the control (seeds germinated in water) at $^{**}P<0.01$, $^{***}P<0.001$, while significantly different from Troysa S 89 treated group $^{\$\$}P<0.01$, $^{\$\$\$}P<0.001$, calculated by One-way ANOVA with Tukey Kramer multiple comparison test.

The present work is the first report on cell suspension cultures of *Blumea malcolmii* Hook for detoxification of carcinogenic compounds carbendazim, diuron and ochthilinone present in the paint preservative, Troysan S 89. Phytoremediation can be an ecofriendly, inexpensive and efficient new alternative (when compared to other chemical and physical technologies) to the existing pollutant treatments, as the plants are nonpathogenic, easiest source available and having immense potential to remediate pollutants. The present work will open up new avenues for remediation of hazardous chemicals using plant cells under precise aseptic growth conditions.

Troysan S 89 was confirmed by the decrease in growth of plumule and radical. The relative sensitivities towards Troysan S 89 and its degradation metabolite in relation to *T. aestivum* and *E. lens* Linn seeds were presented in the Table 3. Troysan S 89 showed highest toxicity, while the metabolites extracted after degradation of S 89 were found less toxic.

Hence, the study demonstrated detoxification of carbendazim, diuron and ochthilinone from Troysan S 89 by a safer biological source *i.e. B. malcolmii* cells.

Cell suspension cultures provide simple, sensitive and rapid method for biochemical and metabolic research; as these cultures grow very fast, available on demand and free from genetic variability; which help to improve the reproducibility of the results. Besides, the method provides a model system to carry out rapid screening of plants, toxicity tolerance study and effects of various medium additives on biotransformation capacity of the cells. Because of the reduced amount of starch, chlorophyll and other pigments in cultured plant cells compared with whole plants, isolation of reaction products from cell cultures is easier, require fewer purification steps and yield samples of higher purity than from intact plants [30–32]. Also, plant cell cultures provide early and rapid method for isolation, purification and identification of enzymes and intermediate metabolites; which in turn may help to understand metabolic pathways.

However, the underlying mechanisms of phytoremediation have still remained unanswered and needed further experimentation and opening up a new era of bioremediation. Further, plant cells do not pose any environmental threat problems thus avoiding stringent regulations; unlike microbes which have a potential threat of escaping of mutants into the environment and requires stringent regulations of containment. Also, the microbial systems have low potentiality to degrade toxic compounds [2,3] implying the superiority and safety of plant cells to detoxify toxic pollutants in comparison with microbial systems.

4. Conclusion

In our study, we have demonstrated the potentiality of *B. malcolmi* cells to degrade a toxic paint compound, Troysan S 89. The cells established in cultures, grew rapidly, and could degrade Troysan S 89 within 10 days into a non-toxic product. As far as we know, this might be the first report on degradation of three toxicants existing in Troysan S 89 using a single biological source of *Blumea* cells. The plant cell culture gives an opportunity to identify and study the intrinsic enzymatic capacity and offers experimental manipulations and rapidity to carry out uptake, localization, metabolic, toxicity and tolerance studies by eliminating translocation barriers, such as leaf wax, bark, cuticles, epidermis and endodermis, under aseptic conditions. Therefore, it could be model system to obtain useful information to guide and direct subsequent plant trials as well as to exploit plant-microbe (rhizospheric) partnership and additionally to develop transgenic plants with improved phytoremediation capabilities.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhazmat.2011.04.055.

References

[1] J. Gillatt, The use of biocides and fungicides in wood coatings and preservatives, Surf. Coat. Int. 7 (1998) 337–341.
[2] S.R. Sorensen, C.N. Albers, J. Aamand, Rapid mineralization of the phenylurea herbicide diuron by *Variovorax* sp. strain SRS16 in pure culture and within a two-member consortium, Appl. Environ. Microbiol. 74 (2008) 2332–2340.
[3] Z. Wang, Y. Wang, F. Gong, J. Zhang, Q. Hong, S. Li, Biodegradation of carbendazim by a novel actinobacterium *Rhodococcus jialingiae* djl-6-2, Chemosphere 81 (2010) 639–644.
[4] J. Arning, R. Dringen, M. Schmidt, A. Thiessen, S. Stolte, M. Matzke, U. Bottin-Weber, B. Caesar-Geertz, B. Jastorff, J. Ranke, Structure-activity relationships for the impact of selected isothiazol-3-one biocides on glutathione metabolism and glutathione reductase in the human liver cell line Hep G2, Toxicology 246 (2008) 203–212.
[5] J. Thormann, Contact dermatitis to a new fungicide, 2-n-octyl-4-isothiazolin-3-one, Contact Dermat. 8 (2008) 204.
[6] M.C. Edelahi, N. Oturan, M.A. Oturan, Y. Padellec, A. Bermond, K. El Kacemi, Degradation of diuron by the electro-Fenton process, Environ. Chem. Lett. 1 (2004) 233–236.
[7] P. Mazellier, E. Leroy, J. De Laat, B. Legube, Degradation of carbendazim by UV/H_2O_2 investigated by kinetic modeling, Environ. Chem. Lett. 1 (2003) 68–72.
[8] S. Singh, S.H. Kang, A. Mulchandani, W. Chen, Bioremediation: environmental clean-up through pathway engineering, Curr. Opin. Biotechnol. 19 (2008) 437–444.
[9] E. Arthur, P.J. Rice, T.A. Anderson, A. Todd, et al., Phytoremediation—an overview, Crit. Rev. Plant Sci. 24 (2005) 109–122.
[10] P.M. Doran, Applications of plant tissue cultures in phytoremediation research: incentives and limitations, Biotechnol. Bioeng. 103 (2009) 60–76.
[11] H. Hamada, R. Tomi, Y. Asada, T. Furuyati, Phytoremediation of bisphenol A by cultured suspension cells of *Eucalyptus perriniana*—regioselective hydroxylation and glycosylation, Tetrahedron. Lett. 43 (2002) 4087–4089.
[12] R. Karmakar, R. Bhattacharya, G. Kulshrestha, Comparative metabolite profiling of the insecticide thiamethoxam in plant and cell suspension culture of tomato, J. Agric. Food. Chem. 57 (2009) 6369–6374.
[13] R. Podlipna, A. Nepovim, P. Soudek, M. Vagner, T. Vaněk, Soapwort oxidoreductase is involved in trinitrotoulene detoxification, Biol. Plant. 51 (2007) 367–371.
[14] R.A. Gomes-Junior, P.L. Gratao, S.A. Gaziola, P. Mazzafera, P.J. Lea, R.A. Azevedo, Selenium induced oxidative stress in coffee cell suspension cultures, Funct. Plant. Biol. 34 (2007) 449–456.
[15] A.N. Kagalkar, U.B. Jagtap, J.P. Jadhav, V.A. Bapat, S.P. Govindwar, Biotechnological strategies for phytoremediation of the sulfonated azo dye Direct Red 5B using *Blumea malcolmii* Hook, Bioresour. Technol. 100 (2009) 4104–4110.
[16] C.J. Baker, N.M. Mock, An improved method for monitoring cell death in cell suspension and leaf disc assays using Evan's Blue, Plant Cell Tissue Organ Cult. 39 (1994) 7–12.
[17] D.J. Finney, Probit Analysis, third ed., University Press, Cambridge, UK, 1971.
[18] B. Ames, F. Lee, W. Durston, An improved bacterial test system for the detection and classification of mutagens and carcinogens, Proc. Natl. Acad. Sci. U.S.A. 70 (1973) 782–786.
[19] V.V. Dawkar, U.U. Jadhav, M.U. Jadhav, A.N. Kagalkar, S.P. Govindwar, Decolorization and detoxification of sulphonated azo dye Red HE7B by *Bacillus* sp. VUS, W. J. Microbiol. Biotechnol. 26 (2010) 909–916.
[20] J.P. Jadhav, D.C. Kalyani, A.A. Telke, S.S. Phugare, S.P. Govindwar, Evaluation of the efficacy of a bacterial consortium for the removal of color, reduction of heavy metals, and toxicity from textile dye effluent, Bioresour. Technol. 101 (2010) 165–173.
[21] S.S. Phugare, D.C. Kalyani, A.V. Patil, J. P. Jadhav, Textile dye degradation by bacterial consortium and subsequent toxicological analysis of dye and dye metabolites using cytotoxicity, genotoxicity and oxidative stress studies, J. Hazard. Mater. (2010), in press, doi:10.1016/j.jhazmat.2010.11.049.
[22] M. Singh, W. Mersie, R.H. Brlansky, Phytotoxicity of the fungicide metalaxyl and its optical isomers, Plant Dis. 87 (2003) 1144–1147.
[23] C. Lina, B. Fugetsua, Y. Sub, F. Watari, Studies on toxicity of multi-walled carbon nanotubes on *Arabidopsis* T87 suspension cells, J. Hazard. Mater. 170 (2009) 578–583.
[24] G. Rani, K. Kaur, R. Wadhwa, S.C. Kaul, A. Nagpal, Evaluation of the anti-genotoxicity of leaf extract of Ashwagandha, Food Chem. Toxicol. 43 (2005) 95–98.
[25] R. Carita, M.A. Marin-Morales, Induction of chromosome aberrations in the *Allium cepa* test system caused by the exposure of seeds to industrial effluents contaminated with azo dyes, Chemosphere 72 (2008) 722–725.
[26] A. Kapanen, M. Itavara, Ecotoxicity tests for compost applications, Ecotoxicol. Environ. Saf. 49 (2001) 1–16.
[27] T. Xuan, T. Eiji, T. Hiroyuki, M. Mitsuhiro, T. Khan, I. Chung, Evaluation on phytotoxicity of Neem (*Azadirachta indica*, *A. Juss*) to crops and weeds, Crop. Protect. 23 (2004) 335–345.
[28] U. Shedbalkar, R. Dhanve, J. Jadhav, Biodegradation of triphenylmethane dye cotton blue by *Penicillium ochrochloron* MTCC 517, J. Hazard. Mater. 157 (2008) 472–479.
[29] M.A. Khan, S.S. Shaukat, M.A. Khan, Growth, yield and nutrient content of Sunflower (*Helianthus Annus* L.) using treated wastewater from waste stabilization ponds, Pak. J. Bot. 41 (2009) 1391–1399.
[30] G.L. Lamourreux, J.M. Gouot, D.G. Favis, D.G. Rusness, Pentachloronitrobenzene metabolism in peanut cell suspension cultures, J. Agric. Food Chem. 29 (1981) 996–1002.
[31] R.O. Mumma, G.H. Davidsonis, Plant tissue culture and pesticide metabolism, in: D.H. Hutson, T.R. Roberts (Eds.), Progress in Pesticide Biochemistry and Toxicology, Wiley, Chichester, 1983, pp. 255–278.
[32] B. Schmidt, Metabolic profiling using plant cell suspension cultures, in: J.C. Hall, R.E. Hogland, R.M. Zablotowicz (Eds.), Pesticide Biotransformation in Plants and Microorganisms, American Chemical Society, Washington DC, 2001, pp. 40–56.