Biodegradation of Rubine GFL by *Galactomyces geotrichum* MTCC 1360 and subsequent toxicological analysis by using cytotoxicity, genotoxicity and oxidative stress studies

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*Galactomyces geotrichum* MTCC 1360 showed 87% decolorization of the azo dye Rubine GFL (50 mg l\(^{-1}\)) within 96 h at 30 °C and pH 7.0 under static conditions, with significant reduction of chemical oxygen demand (67%) and total organic carbon (59%). Examination of oxidoreductive enzymes, namely laccase, tyrosinase and azo reductase, confirmed their role in decolorization and degradation of Rubine GFL. Biodegradation of Rubine GFL into different metabolites was confirmed using high-performance TLC, HPLC, Fourier transform IR spectroscopy and GC-MS analysis. During toxicological studies, cell death was observed in Rubine GFL-treated *Allium cepa* root cells. Toxicological studies before and after microbial treatment were done with respect to cytotoxicity, genotoxicity, oxidative stress, antioxidant enzyme status, protein oxidation and lipid peroxidation using root cells of *A. cepa*. The analysis with *A. cepa* showed that the dye exerts oxidative stress and subsequently has a toxic effect on the root cells, whereas its metabolites are less toxic. Phytotoxicity studies revealed the less toxic nature of the metabolites as compared with Rubine GFL.

**INTRODUCTION**

Azo dyes are the largest synthetic chemical class of dyes and are characterized by the presence of one or more azo (–N=N–) groups; they are used largely in the textile, pharmaceutical, leather, food, cosmetic, paint and printing industries because of their ease of synthesis and chemical stability. Approximately 10–15% of such compounds are released into the environment during dyeing of different substrates, such as synthetic and natural textile fibres, plastics, leather, paper, mineral oils, waxes, and even (with selected types) foodstuffs and cosmetics. Azo dyes are undesirable in the natural environment, not only because of their colour, but also because many are toxic or mutagenic to life (Chen, 2002; Sponza & Isik, 2005; Liu et al., 2009). Therefore, the treatment of industrial effluents containing such aromatic compounds is necessary prior to their final discharge into the environment.

In the last few decades, several physicochemical treatment methods have been developed, including flocculation combined with flotation, electrofloculation, membrane filtration, electrokinetic coagulation, electrochemical destruction, ion-exchange, irradiation, precipitation and ozonation involving the use of activated carbon and air mixtures, but these technologies are generally expensive, are ineffective in colour removal from textile dyestuffs, are not adaptable to a wide range of dye wastewaters, and produce a large amount of sludge or cause secondary pollution due to excessive chemical usage (Joe et al., 2008; Tamboli et al., 2010a; Saratale et al., 2011; Amoozegar et al., 2011). Therefore, there is a need to develop alternative means of dye decolorization. The use of bioremediation for treatment of dye-containing textile effluent is cost-effective, environmentally friendly and is a better alternative than physicochemical and abiotic methods (Joe et al., 2008). Such bioremediation includes the use of fungal, yeast and bacterial cultures for the removal of dyes from textile effluent (Cervantes et al., 2010; Parshetti et al., 2010; Waghmode et al., 2011a; Jadhav et al., 2011; Yu et al., 2011; Zhang et al., 2011).

In many cases, the metabolites formed after degradation of textile azo dyes are toxic or mutagenic (Jadhav et al., 2011). It is therefore important to measure the toxicity of aromatic pollutants and their metabolites in order to assess the feasibility of the bioremediation technique. Some such bioassays, namely phytotoxicity, microbial toxicity.
cytotoxicity and genotoxicity assays, have been previously reported for pollution monitoring (Dawkar et al., 2010; Jadhav et al., 2010; Phugare et al., 2011).

The present study evaluates the potential of using Galactomyces geotrichum MTCC 1360 in the degradation and detoxification of the azo dye Rubine GFL and investigates possible degradation pathways via enzyme studies, Fourier transform IR spectroscopy (FTIR) and GC-MS analysis. To the best of our knowledge, this is the first report on the degradation of Rubine GFL by a yeast. This study also assesses the toxicity of the dye and its metabolites based on cytotoxicity and genotoxicity assays, and explores the ability of Rubine GFL to generate oxidative stress in Allium cepa root cells.

**METHODS**

**Chemicals.** The Rubine GFL used in this study was obtained from Manpasand Textile Processing Industry. L-Catechol, Evans blue stain, thioarbituric acid (TBA), TCA, peptone, yeast extract and malt extract were purchased from Hi-Media Laboratories. Ascorbic acid, NADH, NADPH and 3,3′-diaminobenzidine tetrahydrochloride were purchased from Sioco Research Laboratory. All chemicals used were of the highest purity available and of analytical grade.

**Micro-organism and culture conditions.** G. geotrichum MTCC 1360 was obtained from the Microbial Type Culture Collection, Chandigarh, India. The pure culture was maintained on malt extract agar slants at 4°C. The composition of malt extract medium used for the decolorization studies was (per litre): 3.0 g malt extract, 3.0 g yeast extract, 5.0 g peptone and 10.0 g glucose.

**Decolorization experiment.** G. geotrichum MTCC 1360 was grown in 100 ml malt extract broth (pH 6.8) for 24 h at 30°C, and this pre-grown culture was routinely used for decolorization. After 24 h growth, Rubine GFL was added (50 mg l⁻¹) and the flask was incubated statically as well as with shaking (120 r.p.m.) at 30°C. Aliquots (4 ml) were withdrawn at different time intervals and centrifuged at 4000 r.p.m. for 15 min, and decolorization was monitored by measuring absorbance at 530 nm using UV–visual spectroscopy (Hitachi U 2800). Reduction in chemical oxygen demand (COD) (APHA, 1998) and total organic carbon (TOC) were measured in the control as well as the degraded sample. TOC was measured using a Hach DR 2700 spectrophotometer (Waghmode et al., 2011a). The ability of G. geotrichum to decolorize Rubine GFL was tested over wide pH (3–11) and temperature ranges (10–50°C). All decolorization experiments were performed in triplicate. Abiotic controls (without micro-organisms) were also included. The percentage decolorization was calculated as reported previously (Jadhav et al., 2011).

**Effects of various carbon and nitrogen sources and agricultural waste on decolorization.** Bushnell Haas medium (BHM) (per litre: 0.2 g MgSO₄, 1.0 g K₂HPO₄, 1.0 g KH₂PO₄, 0.02 g CaCl₂, 0.05 g FeCl₃, 1.0 g NH₄NO₃ and 0.5 g yeast extract) was used to study the effect of carbon and nitrogen sources on the decolorization of Rubine GFL. In addition to synthetic carbon and nitrogen sources (5 g l⁻¹), namely glucose, urea, ammonium chloride, yeast extract and starch, the effects of extracts of different agricultural wastes such as bagasse, rice husks and wood shavings on the decolorization of Rubine GFL were investigated in BHM (5 ml extract of 1% boiled agricultural residue).

**Preparation of cell-free extract.** G. geotrichum was grown in malt extract medium at 30°C for 24 h and the biomass (approx. 2 g wet weight) was collected by filtration through Whatman filter paper no. 1. The mycelium was then suspended in 50 mM potassium phosphate buffer (pH 7.4) and sonicated (Sonics-Vibracell ultrasonic processor) based on a 60 amplitude output, at a temperature below 4°C with 12 strokes of 30 s each at 1 min intervals. After sonication the cell homogenate was centrifuged at 9237 g for 25 min at 4°C to separate cell debris, and the resulting extract was then used as an enzyme source.

**Determination of enzyme activities in G. geotrichum during decolorization of Rubine GFL.** All enzyme activities were assayed in cell-free extract as well as in culture media at room temperature (25°C). Laccase and tyrosinase activities were determined according to previously reported methods (Tamboli et al., 2010b; Waghmode et al., 2011b). One unit of tyrosinase activity was equal to a ΔA₅₃₂₇ of 0.001 min⁻¹ at pH 6.5 and 25°C in a 3.0 ml reaction mixture containing L-catechol and L-ascorbic acid. NADH-dichlorophenol indophenol (DCIP) reductase and riboflavin reductase activities were assayed according to Waghmode et al. (2011b). Azo reductase activity was assayed by using a modified version of the method given by Telke et al. (2010). One unit of enzyme activity was defined as the amount of enzyme required to reduce 1 μmol substrate min⁻¹ (mg protein)⁻¹. All enzyme assays were run in triplicate.

**Analysis of biodegradation metabolites.** The culture supernatant was centrifuged at 9000 r.p.m. for 25 min after 96 h of decolorization. The culture supernatant containing metabolites formed after degradation was extracted with equal volumes of ethyl acetate, concentrated in a rotary evaporator and dried over anhydrous Na₂SO₄; the resulting metabolite residue was dissolved in HPLC-grade methanol and directly used for further analysis. High-performance TLC (HPTLC) analysis of the control dye and its metabolites was performed using a CAMAG system (Waghmode et al., 2011a). The composition of the mobile phase used for HPTLC analysis was toluene/ethyl acetate/methanol (7 : 2 : 1 by volume). FTIR analysis was carried out using a spectrophotometer (Shimadzu 8400S) in the mid-IR region of 400–4000 cm⁻¹ with a scan speed of 16. HPLC analysis was performed in an isocratic system (Waters 2690) equipped with a dual absorbance detector, using a C₁₈ column (4.6 × 250 mm) and HPLC-grade methanol as the mobile phase at a flow rate of 1 ml min⁻¹. Identification of the metabolites formed after degradation was carried out using a QP2010 gas chromatograph coupled to a Shimadzu mass spectrometer.

**Toxicological studies of the dye and its metabolites.** The toxicity of Rubine GFL and its metabolites obtained after dye degradation was investigated using an A. cepa test and a phytotoxicity test using Phaseolus mungo and Sorghum vulgare. The A. cepa test included various toxicity parameters, namely cell death, antioxidan enzyme status, lipid peroxidation, protein oxidation and genotoxicity.

**A. cepa test.** Small bulbs of A. cepa of equal size and shape were initially exposed to water for root development. The root-developed bulbs were then grouped into three sets: control (a) (distilled water treatment), metabolites obtained after dye degradation (b) (1000 p.p.m.) and treatment with dye (c) (1000 p.p.m.) (Jadhav et al., 2011). In each case the bulbs were exposed to the respective treatment for 72 h.

**Cell death assay.** Loss of cell viability or cell death was evaluated using an Evans blue staining method (Achary et al., 2008). After treatment, freshly harvested roots were stained with 0.25% (w/v) aqueous Evans blue (HiMedia) for 15 min. After washing with distilled water for 30 min, 10 root tips of equal length (10 mm) were excised and soaked with 4 ml N,N-dimethylformamide (Merck) for
1 h at room temperature. The absorbance of released Evans blue was measured at 600 nm by using a UV–visible spectrophotometer (Hitachi U 2800).

**Genotoxicity.** The genotoxicity of Rubine GFL and of its metabolites obtained after degradation was assessed by determining the mitotic index (MI) and chromosomal aberrations in *A. cepa* root cells. After washing, root tips of equal length (1 cm) from the respective treatments were excised, and slides for the evaluation of MI and chromosomal aberrations were prepared by following the protocol described by Jadhav et al. (2011). Mean values for root length, MI and number of cells undergoing division were examined and scored to assess the induction of various chromosome and nuclear aberrations. The experiments were done in triplicate to establish the reproducibility of the results.

**Determination of antioxidant enzyme activities, protein oxidation and lipid peroxidation in *A. cepa*.** Various antioxidant enzymes, namely catalase (CAT; EC 1.11.1.6), guaiacol peroxidase (GPX; EC 1.11.1.7) and superoxide dismutase (SOD; EC 1.15.1.1) were analysed spectrophotometrically (Achary et al., 2008). Spectrophotometric assays were initiated by adding aliquots of enzyme extracts containing 50 μg protein to 3 ml reaction mixture. Protein oxidation and lipid peroxidation were assayed according to published protocols (Achary et al., 2008; Phugare et al., 2011). The reaction of carboxyls with 2,4-dinitrophenylhydrazine (DNPH) was used to determine the amount of protein oxidation. Carboxyl concentration was calculated from the difference in absorbance between the test and the blank using the molar absorption coefficient of DNPH (ε = 22 mM⁻¹ cm⁻¹) and is expressed in nanomoles of DNPH incorporated per milligram of protein. Lipid peroxidation was measured as the amount of malondialdehyde (MDA) produced by the TBA reaction. The concentration of MDA was calculated using the molar absorption coefficient (ε = 155 mM⁻¹ cm⁻¹) and is expressed in nanomoles per gram fresh weight.

**Phytotoxicity analysis.** Phytotoxicity studies were carried out using *P. mungo* and *S. vulgare* seeds at room temperature with watering with 5 ml of Rubine GFL (1000 p.p.m.) or its metabolites (1000 p.p.m.). The control used distilled water (daily 5 ml watering). Germination (%) and shoot and root lengths were recorded after 7 days. Percentage germination was calculated as: (number of seeds germinated/number of seeds sown) × 100.

**Statistical analysis.** Data were analysed by ANOVA with the Tukey–Kramer multiple comparisons test.

## RESULTS AND DISCUSSION

### Decolorization experiment and physicochemical parameters

*G. geotrichum* showed 87 % decolorization of Rubine GFL (50 mg l⁻¹) in malt extract medium within 96 h under static conditions, with a 67 and 59 % reduction in COD and TOC respectively, whereas less decolorization was observed under shaking conditions (data not shown). The optimum pH and temperature for Rubine GFL decolorization were pH 7.0 and 30 °C (data not shown).

### Effects of various carbon and nitrogen sources and agricultural waste on decolorization

The biodegradation activity of *G. geotrichum* towards Rubine GFL varied greatly according to the type of carbon and nitrogen source added to BHM. In BHM (control), 30 % decolorization was observed in 7 days. In an attempt to enhance decolorization in the control medium, the medium was supplemented with extra carbon and nitrogen sources and extracts of agricultural residues. Rice husks, wood shavings and bagasse showed 64, 63 and 46 % decolorization, respectively, indicating that certain components present in these residues might be acting as electron donors for the reduction of the azo dye. Saratale et al. (2010) reported that addition of rice husks to synthetic medium increased decolorization of the reactive dye Green HE4BD. Their data indicated a positive effect of the agricultural residue components on growth of the micro-organism as well as decolorization of the dye. Glucose (76 %) and urea (50 %) showed increased decolorization both individually and in combination (81 %). Niebisch et al. (2010) observed increased degradation of Reactive Blue 220 by *Lentilus critinus* when glucose was used in combination with urea in culture media. Also, ammonium chloride showed 56 % decolorization when used in combination with glucose as compared with its use alone (19 %). This suggests that the higher decolorization of Rubine GFL could be due to the combined action of carbon and nitrogen sources on the growth as well as on the decolorization efficiency of *G. geotrichum*. Handayani et al. (2007) reported that the addition of glucose as carbon source enhanced the growth of *Enterococcus faecalis* and also enhanced the decolorization of Acid Red 27. Finally, yeast extract and starch showed 74 % decolorization when used individually.

### Enzyme activities in *G. geotrichum* during decolorization of Rubine GFL

The oxidative and reductive enzymes responsible for dye degradation were studied. The induction of different enzymes during decolorization gives additional insights into the mechanism of decolorization and also supports the active role of *G. geotrichum* in the biodegradation process. Enzyme analysis of *G. geotrichum* showed induction of laccase (314 %) after 96 h of degradation as compared with the control (Table 1). Saratale et al. (2010) discussed the role of laccase in the degradation of textile dyes. The activities of tyrosinase, DCIP reductase, riboflavin reductase and azo reductase were significantly reduced after 96 h of degradation as compared with the control (before degradation). The presence of these oxidoreductive enzymes during the degradation of Rubine GFL indicates their role in the degradation process.

### Analysis of biodegradation metabolites

Spectrophotometric analyses of control and culture supernatants obtained after decolorization showed a significant reduction in absorbance (after decolorization) as compared with the control (data not shown). HPTLC analysis of dye metabolites showed different degradation patterns with different *Rf* values as compared with the control. The difference in *Rf* value of the control dye (0.79) and the
metabolites formed (Fig. 1) revealed nine peaks (0.06, 0.10, 0.16, 0.27, 0.32, 0.39, 0.52, 0.65 and 0.78), indicating the biodegradation of Rubine GFL. HPLC of control Rubine GFL showed one major peak at a retention time of 1.554 min, whereas the metabolites formed after degradation of Rubine GFL showed two peaks with retention times of 2.826 and 2.950 min, suggesting further conversion of Rubine GFL into various metabolites. Comparison of the FTIR spectra of the control dye and the metabolites formed after degradation indicated biodegradation of Rubine GFL by \textit{G. geotrichum} (Fig. 2). The FTIR spectrum of Rubine GFL showed a peak at 2933.34 cm\(^{-1}\) for CH stretching in alkanes, 2242.81 cm\(^{-1}\) for C=\textit{N} stretching in \textit{\alpha} and \textit{\beta} unsaturated alkyl compounds, 1595.18 cm\(^{-1}\) for N=\textit{N} stretching in azo compounds and N=O stretching in nitrites, 1518.51 cm\(^{-1}\) for NH deformation and C=\textit{N} stretching in acyclic compounds, 1339.12 cm\(^{-1}\) for NO symmetrical stretching, 1203.14 and 1142.38 cm\(^{-1}\) for CH deformation in alkanes, 1073.38 cm\(^{-1}\) for ring CH deformation in pyridines, 914.29 cm\(^{-1}\) for C–N stretching vibrations in nitrates, 833 cm\(^{-1}\) for CH deformation in alkanes and NO stretching in nitrites, 734.76 cm\(^{-1}\) for a benzene ring with five adjacent free H atoms and 629.30 cm\(^{-1}\) for C–N stretching in acyclic compounds (Fig. 2a). By contrast, the product formed after degradation (Fig. 2b) had no peak at 1595.18 cm\(^{-1}\) (cleavage of the azo bond), indicating colour removal, a peak at 3203.39 cm\(^{-1}\) indicating formation of amino acid salts (NH\textsubscript{2} stretch), alkanes at peaks of 2877.41 and 2960.83 cm\(^{-1}\), a peak at 2223.52 cm\(^{-1}\) (presence of a C–\textit{N} group in the product), NO\textsubscript{2} stretch at 1516.10 and 1679.95 cm\(^{-1}\), C–H deformation at 822.19 and 1460.64 cm\(^{-1}\), and C–N vibration at 1329.0 cm\(^{-1}\). These differences support the degradation of Rubine GFL by \textit{G. geotrichum}.

GC-MS analysis identified the probable metabolites produced during the biodegradation of Rubine GFL. The structures of the detected compounds were assigned on the

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>0 h (before degradation)</th>
<th>96 h (after degradation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laccase†</td>
<td>0.07±0.05</td>
<td>0.29±0.00**</td>
</tr>
<tr>
<td>Tyrosinase (intracellular)†</td>
<td>1408±451</td>
<td>480±50</td>
</tr>
<tr>
<td>Tyrosinase (extracellular)†</td>
<td>1217±111</td>
<td>210±2.00</td>
</tr>
<tr>
<td>NADH-DCCIP reductase‡</td>
<td>183.9±11.21</td>
<td>53.3±1.99*</td>
</tr>
<tr>
<td>Riboflavin reductase§</td>
<td>14.3±0.29</td>
<td>6.05±0.16</td>
</tr>
<tr>
<td>Azo reductase</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

†Values shown are enzyme units min\(^{-1}\) (mg protein\(^{-1}\)).
‡Values shown are µg DCIP reduced min\(^{-1}\) (mg protein\(^{-1}\)).
§Values shown are µg riboflavin reduced min\(^{-1}\) (mg protein\(^{-1}\)).
||Values shown are µmol Methyl Red reduced min\(^{-1}\) (mg protein\(^{-1}\)).

Table 1. Enzyme assay for \textit{G. geotrichum} MTCC 1360: 0 and 96 h degradation of Rubine GFL

Values are the means of three experiments ± SEM. Asterisks indicate values significantly different from controls: *\(P<0.01\), **\(P<0.001\) (one-way ANOVA with Tukey–Kramer comparison test).
basis of the fragmentation pattern and \( m/z \) values. Based on the presence of various enzymes during degradation and on MS analysis, the possible biodegradation pathway adapted by \textit{G. geotrichum} is illustrated in Fig. 3. Initial cleavage may begin with reduction of the azo bond via the action of azo reductase, followed by oxidative and reductive cleavage with the help of various oxidative and reductive enzymes. According to our scheme, azo reductase catalyses initial reductive cleavage of the azo bond, leading to the formation of two intermediates. One of these was identified as 2-(aminomethyl)-4-nitroaniline (\( m/z \) 168). This then undergoes deamination to produce 1-(3-nitrophenyl)methanamine (\( m/z \) 154). The intermediate (III) formed in the first step of azo bond cleavage undergoes demethylation to produce \( N\)-(3-aminopropyl)benzene-1,4-diamine (\( m/z \) 168).

**Toxicological studies of the dye and its metabolites**

Assessment of the toxicological impact of Rubine GFL and its metabolites on food plants is clearly of importance. \textit{A. cepa} was used here in this regard. Cytogenetic analysis showed a strong genotoxic effect of Rubine GFL on root cells of \textit{A. cepa} (Table 2). This analysis was based on a cell death assay (\( \text{OD}_{600} \)), MI and chromosomal aberration in \textit{A. cepa} root cells (Fig. 4). In the control set, cell viability (optical density 0.041) was higher as compared with cells treated with the dye and its metabolites, whereas the cell viability was higher in cells treated with metabolites (optical density 0.076) as compared with cells treated with Rubine GFL (optical density 0.142). Living cells retain the ability to release Evan’s blue stain, whereas damaged cells do not (Achary \textit{et al.}, 2008). MI thus serves as an important parameter of cytotoxicity in environmental biomonitoring studies (Table 2). The MI of samples exposed to 1000 p.p.m. Rubine GFL was 15.67 %. By contrast, the MI of the samples exposed to 1000 p.p.m. of the metabolites formed after degradation was 13.72 %, close to that of the control (12.42 %). The decrease in MI in the presence of the metabolites as compared with the parent dye showed the potential of \textit{G. geotrichum} to remove textile pollutants as well as to maintain normal MI in common crop plants.

An increased MI in cells treated with Rubine GFL may be
associated with tumour formation and uncontrolled cell division, or with other detrimental effects on the cells (Phugare et al., 2011). Different kinds of chromosomal aberration were also observed, the most common in all treatments being laggard formation, chromosome breaks, anaphase bridges, stickiness in metaphase and micronuclei. The percentage of aberrant mitotic cells due to the genotoxicity of Rubine GFL was significantly different from that of the control (Tables 2 and 3). The frequencies of total alterations in control and metabolite-treated samples were 0.042 and 0.148, respectively, but were much higher in dye-treated samples: up to 0.389. Root length in control and metabolite-treated samples was 4.71 and 4.42 cm, respectively, whereas in dye-treated samples root length was decreased (3.51 cm). The number and frequency of cellular aberrations were significantly reduced after biodegradation of Rubine GFL. Previous studies have shown that chromosomal aberrations provide an important measure of the genotoxicity potential of textile dyes and effluents (Carita & Marin-Morales, 2008; Jadhav et al., 2010, 2011). Phugare et al. (2011) reported similar results for chromosomal aberration and cell division alteration caused by the textile dye Red HE3B. The results presented here collectively suggest that Allium has great sensitivity towards Rubine GFL and can be used as an environmental monitoring agent. Samples treated with biodegradation metabolites showed significantly less alteration in chromosomal DNA than samples treated with Rubine GFL, with values almost matching those of controls. G. geotrichum was therefore found to be effective not only for degradation of Rubine GFL but also for its detoxification.

The cell wall and plasma membrane constitute the outermost layers of the plant cell, ensuring cellular protection from abiotic and biotic environmental stresses. Cellular protection is often mediated by oxidative mechanisms involving reactive oxygen intermediates such as O$_2^-$, H$_2$O$_2$ and ·OH. Previous studies have shown that environmental pollutants such as textile dyes and aluminium can induce the production of reactive oxygen intermediates in plant and animal cells, thereby inducing oxidative stress and causing oxidative damage to lipids, proteins and DNA (Meriga et al., 2004; Achary et al., 2008; Kumar et al., 2009; Mihaljević et al., 2009; Jadhav et al., 2010). Dye-induced DNA damage was presumed to be the consequence of direct attack by oxygen free radicals on DNA strands. A similar observation was made in the case of transgenic plants in the presence of aluminium (Ezaki et al., 2000). Under certain environmental conditions plants may experience oxidative stress due to increased levels of reactive oxygen intermediates and decreased activities of antioxidant enzymes (Jadhav et al., 2011). Plant systems possess their own defence mechanisms against such oxidative stresses, including antioxidant enzymes such as CAT, GPX and SOD, which are responsible for the conversion of reactive oxygen intermediates into harmless products.

### Table 2. Effects of untreated and treated (biodegraded) Rubine GFL on root length, MI, number and frequency of micronuclei, and chromosome breaks obtained for the A. cepa test

Values are the means of three experiments ± SEM. The asterisk indicates a value significantly different from the control (roots germinated in water) at P<0.01 (one-way ANOVA with Tukey–Kramer multiple comparison test). The frequency of the total number of alterations was calculated as follows: total number of alterations × 100/total number of cells analysed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sample</th>
<th>Rubine GFL metabolites†</th>
<th>Rubine GFL†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root length (cm, mean ± SEM)</td>
<td>Control</td>
<td>4.71 ± 0.151</td>
<td>4.42 ± 0.026</td>
</tr>
<tr>
<td>MI (mean ± SEM)</td>
<td></td>
<td>12.42 ± 0.517</td>
<td>13.72 ± 0.672</td>
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<tr>
<td>Number of micronuclei</td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Number of chromosome breaks</td>
<td></td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Total number of alterations</td>
<td></td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>Total number of cells analysed</td>
<td></td>
<td>2500</td>
<td>2687*</td>
</tr>
<tr>
<td>Frequency of total number of alterations</td>
<td></td>
<td>0.042 ± 0.001</td>
<td>0.148 ± 0.08</td>
</tr>
</tbody>
</table>

†At 1000 p.p.m.

![Fig. 4. Different types of aberration induced by Rubine GFL in A. cepa tips: (a) normal anaphase cell, (b) sticky metaphase, (c) vagrant chromosome in telophase, (d, e) anaphase with bridge, (f) altered anaphase.](http://mic.sgmjournals.org)
intermediates into simpler molecules. Here, we studied the status of antioxidant enzymes in *A. cepa* root cells to gain an insight into the role of Rubine GFL and its metabolites in inducing oxidative stress. The results summarized in Table 4 show that Rubine GFL induced SOD and GPX activities and suppressed the activity of CAT; the metabolites formed after degradation of Rubine GFL induced less SOD and GPX, and the activity of CAT was suppressed as compared with the parent dye. The metabolites thus had a less toxic effect than the parent dye on root cells of *A. cepa*. Also, Rubine GFL increased the rate of protein oxidation and lipid peroxidation compared with its metabolites. Levels of lipid peroxidation and protein oxidation were increased in dye-treated samples, whereas metabolite-treated samples showed levels similar to those of the control. The formation of reactive oxygen intermediates can lead to a lipid peroxidation chain reaction and protein oxidation, which are stronger indices of oxidative stress than alterations in antioxidant enzyme levels. Similar observations have been made in *A. cepa* with aluminium and the textile dye Red HE3B (Achary et al., 2008; Phugare et al., 2011). Our studies with metabolites formed after degradation showed that antioxidant enzyme activities almost matched those of the control or were less than in dye-treated samples, indicating reduced toxicity. This clearly underlines the less toxic nature of the metabolites obtained after degradation compared with Rubine GFL. Both this dye and aluminium thus play possible roles in the generation of oxidative stress in valuable crop plants. Phytotoxicity assessment of Rubine GFL and its metabolites was carried out using two common agricultural plants, *P. mungo* and *S. vulgare*, which showed 100 and 90 % seed germination, respectively, in the presence of the metabolites, as compared with 80 and 10 % seed germination with the parent dye. Also, shoot/root lengths of *P. mungo* and *S. vulgare* were reduced in dye-treated samples (10.90/0.6 and 1.80/0.25 cm, respectively), as compared with treatment with metabolites (13.23/3.46 and 3.20/5.31 cm, respectively), the former values almost matching those of controls (seeds treated with distilled water). The metabolites obtained after degradation of Rubine GFL by *G. geotrichum* MTCC 1360 showed a less toxic effect than the parent dye on root cells of *A. cepa*.

### Table 3. Effect of untreated and treated (biodegraded) Rubine GFL on the number and frequency of chromosome aberrations obtained for *A. cepa* tests

Values are means of three experiments ± SEM. The asterisk indicates a value significantly different from the control (root meristems germinated in water) at *P*<0.001 (one-way ANOVA with Tukey–Kramer comparison test). The frequency of the total number of cells with alterations was calculated as follows: total number of alterations × 100/total number of mitotic cells analysed or observed.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Anaphase with bridge</td>
<td>3</td>
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<tr>
<td>Binucleated cell</td>
<td>0</td>
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<td>Laggard chromosome</td>
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<td>Metaphase with loss</td>
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<tr>
<td>Total number of cells with alterations</td>
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<tr>
<td>Total number of mitotic cells observed</td>
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</tr>
<tr>
<td>Frequency of total number of cells with alterations</td>
<td>1.23 ± 0.452</td>
</tr>
</tbody>
</table>

†At 1000 p.p.m.

### Table 4. Analysis of antioxidant enzyme activities (CAT, GPX and SOD), protein oxidation and lipid peroxidation from root cells of *A. cepa* exposed to Rubine GFL and its metabolites obtained after degradation with *G. geotrichum* MTCC 1360

Values are mean of three experiments ± SEM. Asterisks indicate significant differences from the control at *P*<0.001 (one-way ANOVA with Tukey–Kramer comparison test).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Rubine GFL metabolites†</th>
<th>Rubine GFL‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT activity [nmol H₂O₂ utilized (mg protein)⁻¹]</td>
<td>37.74 ± 0.45</td>
<td>29.01 ± 0.17*</td>
<td>20.41 ± 0.06*</td>
</tr>
<tr>
<td>GPX activity [mol tetraguaiacol formed (mg protein)⁻¹ min⁻¹]</td>
<td>32.45 ± 0.02</td>
<td>46.16 ± 0.08*</td>
<td>54.14 ± 0.03*</td>
</tr>
<tr>
<td>SOD activity [inhibition of NBT reduction by 50% (mg protein)⁻¹ min⁻¹]</td>
<td>3.24 ± 0.02</td>
<td>4.43 ± 0.02*</td>
<td>9.61 ± 0.10*</td>
</tr>
<tr>
<td>Protein oxidation [nmol carbonyl (mg protein)⁻¹]</td>
<td>6.17 ± 0.02</td>
<td>7.56 ± 0.03*</td>
<td>9.79 ± 0.01*</td>
</tr>
<tr>
<td>Lipid peroxidation [nmol MDA produced (g fresh weight)⁻¹]</td>
<td>0.52 ± 0.05</td>
<td>0.62 ± 0.09*</td>
<td>0.85 ± 0.09*</td>
</tr>
</tbody>
</table>

†At 1000 p.p.m.
G. geotrichum were therefore less phytotoxic than the parent dye. This study reveals the importance of bioremediation of textile dyes with respect to the growth of common agricultural plants.

Conclusion

G. geotrichum showed good decolorization potential for the removal of the azo dye Rubine GFL. This bioremediation of textile dyestuffs could prove to be better than existing physicochemical methods. The involvement of dye-decolorizing enzymes systems suggests that they have a role in biodegradation. The dye was found to induce overall oxidative stress in root cells of A. cepa, and this stress was reduced in the presence of the metabolites obtained after degradation by G. geotrichum. Biodegradation of Rubine GFL by G. geotrichum diminished its toxicity towards A. cepa, P. mungo and S. vulgare.

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References


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