Discoloration of dyes by Trametes spp

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Abstract

The dyes used in the textile industry contribute significantly to pollution of water sources as they are disposed, most of the time, without proper treatment. The objective of this work was to test three strains of two species of the genus *Trametes* collected in Brazil against the ability to discolor the indigo carmine dye and to detect the activity of the enzymes laccase, lignin peroxidase and manganese peroxidase. The experiment was carried out in Kirk medium under static, non-sterile condition, at ±28 °C for 120 h. *Trametes lactinea* (URM8350) discolored 81.40% of the indigo carmine dye, *T. lactinea* (URM8354) 85.09% and *T. villosa* (URM8022) 96.11%. Laccase was detected in all specimens. Manganese peroxidase was detected in *T. villosa* (URM8022) and *T. lactinea* (URM8354), while lignin peroxidase was not detected in any of the isolates under the conditions of the experiment. The discoloration rates observed demonstrate the ability of the strains to discolor carmine indigo and the promising use in the discoloration processes in wastewater from the textile segment.

1. Introduction

Population growth allied to industrial development has caused serious environmental problems, such as pollution of soil and water by chemicals (Zhang et al. 2011; Rodríguez-Couto 2017; Choi 2021).

Among the pollutants, the effluents from paper, cellulose, textile and petrochemical industries and from alcohol distilleries contain aromatic, recalcitrant and xenobiotic compounds, responsible for the intense color and toxicity of wastewater (Sharma et al. 2011; Almeida et al. 2017; Chowdhury et al. 2020). The textile sector is considered to be one of the largest sectors in the manufacturing industry. In Brazil alone, the segment is responsible for generating 1.5 million direct jobs, being considered the largest textile chain in the West (Abit 2019). However, its expansion and maintenance cause damage to the environment, since the dyeing and washing processes of the fabric generate a large volume of effluents containing xenobiotic compounds, including dyes (Rodríguez-Couto 2009; Singh 2017).

Synthetic dyes are designed to resist to discoloration, high temperatures and antioxidant chemicals. Therefore, they have a stable chemical structure, usually recalcitrant, highly toxic, mutagenic and carcinogenic (Baughman and Weber 1994; Vacchi et al. 2017; Berradi et al. 2019; Benkhaya et al. 2020). The presence of dyes in water bodies, even in small concentrations, interferes with the trophic chain in aquatic ecosystems, as it prevents the penetration of the light necessary for photosynthesis, causing, thus, serious environmental problems (Kunz et al. 2002; Berradi et al. 2019; Benkhaya et al. 2020; Chowdhury et al. 2020).

The production of dyes reaches 7 x 107 tons per year in the world, of which Brazil accounts for 2.6%. Of this production, 10-20% is transformed into wastewater (Carneiro and Zanoni 2016; Sen et al. 2016; Benkhara et al. 2020). Synthetic carmine indigo belongs to the group of indigoids and has a ketone group

(C = O) in its chemical structure. It is widely used in the food, paper, cellulose and textile industries, being indispensable in dyeing denim (Choi 2020 and 2021; Chowdhury et al. 2020). Considered chemically stable and difficult to remove when discarded in the environment (Guaratini and Zanoni 2000; Choi 2020), it is one of the main causes of wastewater coloring originated from textile effluents. The yarn dyeing process requires large amounts of water: it is estimated that for each kilogram of manufactured product, 80 to 150 liters of water are required, 88% of which wastewater contains a high waste load and more than 10,000 per product as chlorinated compounds, salts, auxiliary chemicals, surfactants and especially dyes (Sen et al. 2016; Almeida et al. 2017; Singh 2017; Choi 2020). There are numerous chemical and physical dye removal strategies implemented over the years. These include adsorption, flocculation, photodegradation, membrane filtration and coagulation (Adenan et al. 2020). The treatment of wastewater from the textile industry, especially discoloration, is expensive and not always effective as it can generate a large volume of sludge and generally requires the addition of other chemical additives dangerous to the environment (Singh 2017). Therefore, the search for low cost biological alternatives is urgent. Biological removal of dyes can occur through three mechanisms: biosorption, bioaccumulation and/or biodegradation (Sen et al. 2016; Singh 2017; Chowdhury et al. 2020). Biosorption involves trapping the dye by binding the dye molecules to the functional groups present on the cell wall. Subsequently, the dyes are accumulated intracellularly in the living cells through a process known as bioaccumulation. The biodegradation process involves the breakdown of dye molecules by enzymes produced by microbial cells, where complete eradication of dyes is possible (Jasińska et al. 2015; Adenan et al. 2020). Mycorremediation emerges as an economically viable and ecologically effective biological alternative, as fungi can adapt to various pH and temperature ranges, in addition to producing extracellular lignolytic enzymes such as laccase (Lac, EC 1.10.3.2), lignin peroxidase (LiP, EC 1.11.1.14) and manganese peroxidase (MnP, EC 1.11.1.13), which can mineralize xenobiotic and recalcitrant compounds (Tien and Kirk 1984; Ellouze and Sayadi 2016; Sen et al. 2016; Singh 2017; Akhtar and Mannan 2020). White rot fungi, mainly

Agaricomycetes, have been identified as a potentially efficient biological tool in the removal of synthetic dyes from textile effluents (Wesenberg et al. 2003; Ali 2010). Some studies have demonstrated the efficacy of *Trametes* species in the degradation of phenolic compounds in effluents from the paper industry, degradation of pentachlorophenol and synthetic dyes in textile effluents (Pinedo-Rivilla et al. 2009; Rodrigues-Couto 2009; Pandey et al. 2017). However, in Brazil, which has a high mycodiversity (Forzza et al. 2010; Maia et al. 2015), little is known about the potential for degradation and discoloration of the species collected in the country (Balan e Monteiro 2001; Lyra et al. 2009; Motato-Vasquéz et al. 2016; Araújo et al. 2020). Thus, the aim of the present study was to test three strains of two species of *Trametes* collected in Northeast Brazil for the ability to remove the indigo carmine used in the customization of denim and to quantify lignolytic enzymes (Lac, LiP and MnP) produced after the experiment.

2. Material And Methods

2.1 Microorganism: collection and cultivation conditions

Specimens of *Trametes lactinea* (Berk.) Sacc. were collected on the campus of the Universidade Federal de Pernambuco (08°03'07"S 34°56'59"O, Atlantic Forest domain) in November 2019, while *T. villosa* (Sw.) Kreisel was collected in the Chapada Diamantina National Park (13°14'31”S, 41°40'7” O, Caatinga domain) in March 2015. For culture, three fragments with diameter of 5 mm were removed from the basidiomata and transferred to Petri dishes containing 2% malt extract supplemented with chloramphenicol (20 mg L-1). The plates were kept at 28 °C for 7 days or until mycelial development (Cavalcanti 1972; Stalpers 1978; Motato-Vásquez et al. 2016). The cultures obtained were deposited in the Collection of Cultures Micoteca URM of the Department of Mycology of the Center Biosciences of the Federal University of Pernambuco under registration numbers URM8350 (*T. lactinea*), URM8354 (*T. lactinea*) and URM8022 (*T. villosa*).

2.2 Microorganism: identification

The morphological identification of the basidioma and DNA analyses followed the usual for this group

(Gomes-Silva et al. 2010; Verma et al. 2018; Xavier et al. 2020). The resulting ITS and LSU sequences

were subjected to BLASTn search in NCBI to verify the closest identification match.

2.3 Qualitative tests for phenoloxidases

The qualitative analysis of phenoloxidase activity was verified using the Bavendamm method, which allows observing the production of cellular oxidase such as laccase, lignin peroxidase and manganese

peroxidase, in addition to tyrosine and catechol oxidase (Nobles 1965; Melo and Azevedo 2008). In our assay, an agar block with diameter of 5 mm was removed from colonies with 7 days of cultivation and transferred to the center of the Petri dishes with diameter of 90 mm containing solid malt agar medium plus tannic acid (0.5%). The control was prepared under the same conditions without tannic acid. All procedures were performed under aseptic conditions. After 3 days of incubation, the formation of a brown halo was observed in the colony reverse, indicating a positive reaction to produce phenoloxidases. These halos were measured with the aid of a digital caliper. The enzyme index was measured through the relationship between the average diameter of the degradation zone and the average diameter of the colony, expressed in millimeter (Hankin and Anagnostakis 1975; Silva et al.2019).

2.4 Discoloration tests

The indigo carmine dye was purchased from Sigma-Aldrich Corporation, St. Louis, Missouri, USA and used at a concentration of 50 mg L-1. The experiment was carried out in Erlenmeyer flasks (250 mL) containing 50 mL of Kirk medium without sterilization (Kirk and Farrell 1987) plus 5 disks of the fungal mycelium with diameter of 5 mm grown in 2% malt extract after 7 days. The vials were kept for 120 h at ± 28 °C under static condition; 2 mL aliquots were removed from the broth and centrifuged at 1500 rpm for 15 min at 4 °C. The percentage of discoloration of the supernatant was measured in a spectrophotometer (Hitachi U.5100) at a wavelength of 610 nm, obtained from scanning the dye, where the highest

absorbance peak was observed at 610 nm. As a control, Kirk medium was used with the dye without fungal inoculum. The experiments were carried out in triplicate. The percentage of discoloration was calculated according to equation:

$$D = \frac{Abscontrol - Abstest}{Abscontrol} X100$$

D % = percentage of discoloration, Abscontrol = absorbance of the control at 610 nm and Abstest =absorbance with fungal treatment at 610 nm. The discolored broth was used to quantify the production of the enzymes laccase, manganese peroxidase and lignin peroxidase.

2.5 Enzymatic assays

The enzymatic activity of the laccase was verified by measuring the oxidation of ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) 0.5 mM in 100 mM sodium acetate buffer (pH 5) plus the enzyme broth. The final volume of the reaction was 1 mL (800 µL of ABTS + 100 µL of sodium acetate buffer + 100 µL of crude extract). Activity was calculated based on ABTS molar absorptivity (ε420nm = 36,000 M-1. Cm-1) (Bourbonnais et al. 1997; Boran 2019). The activity of lignin peroxidase was verified through the oxidation of the mixture composed of 375 µL of 0.25 M sodium tartrate buffer at pH 3.0; 125 µL of 10 mM veratryl alcohol; 50 µL of 2 mM hydrogen peroxide and 500 µL of enzymatic extract. The reaction was monitored on a spectrophotometer at a length of 310 nm (ε310nm = 9,300 M-1. Cm-1) (Tien and Kirk 1984). The reaction mixture for manganese peroxidase (1mL) was composed of 100 µL of phenol red (0.01%), 100 µL of sodium lactate (25 mM), 50 µL of MnS04 (100 mM), 200 µL of egg albumin (0.5%), 50 µL of H202 (100 µM) in 20 mM sodium succinate buffer (pH 4.5) and 500 µL of enzymatic extract. The reactions were carried out at 30 °C for 5 minutes and stopped with the addition of 40µL of 2N NaOH. The absorbance was monitored at 610 nm (Kuwahara et al. 1984). A unit of enzymatic activity was defined as 1 µmol of the product formed per minute. All tests were performed in triplicate.

2.6 Statistical analysis

The decolorization test was carried out in triplicate. The data were analyzed using analysis of variance (ANOVA) with the software Statistical Package for the Social Sciences (SPSS) version 24.0. The Tukey-Kramer multiple comparison test (honestly significant difference, HSD, $P < 0.05$) or paired t test ($P < 0.05$) was also performed to obtain statistical significance between the mean values.

3. Results And Discussion

3.1 Morphological and molecular identification

The specimens were morphologically identified as *Trametes lactinea* (URM8350, URM8354) and *T. villosa* (URM8022). DNA analyses resulted in one ITS sequence for each specimen *Trametes lactinea* URM8350 (MW578797), *Trametes lactinea* URM8354 (MW578798) and *T. villosa* URM8022 (MW578795) and LSU

sequences for both *Trametes lactinea* URM8350 (MW553720), *Trametes lactinea* URM8354 (MW553721) and *T. villosa* URM8022 (MW553718). BLASTn Search confirmed the original identifications.

3.2 Detection of phenoloxidases

All strains tested showed a dark amber halo in three days of the experiment, evidenced by the degradation of tannic acid and the production of phenoloxidases: diameter of 80 mm for *T. villosa* (URM8022), diameter of 90 mm for *T. lactinea* (URM8350) and diameter of 80 mm for *T. lactinea* (URM8354). According to Bavendamm (1928), these amber-colored diffusion zones around the fungal colony are the result of the oxidation of phenolic acid produced by extracellular phenoloxidases. The detection of phenoloxidases in microorganisms is used as a way to select promising strains with the potential for degradation of complex compounds to be used in studies of degradation of recalcitrant compounds. The production of phenoloxidase complex enzymes is associated with the discoloration of synthetic dyes due to the similarity in the chemical structure of the dyes and the components of lignin (Melo and Azevedo 2008; Arora and Sharma 2010; Sen et al. 2016; Singh 2017).

3.3 Discoloration of indigo carmine

The percentages of discoloration detected were 81.40% for *T. lactinea* (URM 8350), 85.09% for *T. lactinea* (URM8354) and 96.11% for *T. villosa* (URM8022) (Fig. 1). Our results were better that those of Lopes et al. (2014), who obtained 44.74% of discoloration of the indigoid dye using *T. versicolor* and similar to those of Uribe-Arizmendi et al. (2020), who observed discoloration of 90% at 100 mg L-1, 91% at 150mg L-1 and 93% at 200 mg L-1 of indigo carmine by *T. polyzona* in 21 days of experiment in a reactor under agitation and without aeration.

The results obtained in the experiments referring to the percentage of discoloration detected by the treatment with the 3 distinct fungal strains were submitted to analysis of variance (ANOVA) and the effects were considered significant for $p < 0.05$. All groups showed values of F (26.60) greater than the values, indicating that there is a significant difference in all experiments performed in the present work. Species of *Trametes* are well studied for discoloration of various synthetic dyes: *T. trogii* discolored 7% of the remazol brilliant blue (Zouari-Mechichi et al. 2006), 8% of indigo carmine (Grassi et al. 2011) and 69% of Janus Green and 6% of Poly R-478 (Levin et al. 2010); *T. hirsuta*, 94% indigo carmine, 85% of Bromophenol Blue, 41% of Methyl Orange and 47% Poly R-478 (Rodríguez-Couto et al. 2006); *T. membranacea*, 99.2% of bromophenol blue and 71.8% of methylene blue (Lyra et al. 2009); *T. pubescens*, 59% of Bemaplex Navy M-T and 50% of Bezaktiv Blue BA (Rodríguez-Couto et al. 2014); *T. versicolor*, 93.5% of Remazol Brilliant Yellow 3-GL (Asgher et al. 2016); and *T. ljubarskyi*, 97.7% of reactive violet 5 (Goh et al. 2017); *T. villosa*, 93.8% of acid orange 142 (Ortiz-Monsalve et al. 2019); *T. polyzona*, 90% at 100 mg L-1, 91% at 150mg L-1 and 93% at 200 mg L-1 of indigo carmine (and Uribe-Arizmendi et al. 2020). However, *T. lactinea* has not been tested before for discoloration of dyes, while *T. villosa* has not been tested for discoloration of indigo carmine.Also, studies of discoloration of this dye using species, not only of *Trametes*, collected in Brazil are scarce.

Balan and Monteiro (2001) tested *Phellinus gilvus* and *Pycnoporus sanguineus*, probably collected in the Atlantic forest, *Phanerochaete chrysosporium* ATCC24725, and *Pleurotus sajor-caju,* commercially produced, to remove the indigo carmine in liquid culture medium. *Phellinus gilvus* discolored 100% of the dye after 4 days of experiment, followed by *Pl. sajor-caju* (94%), *Py.sanguineus* (91%) and *Ph. chrysosporium* (70–75%). Lyra et al. (2009) found that *T. membranacea* collected in the Atlantic Forest was able to discolor 99.2% of the bromophenol blue and 71.8% of the methylene blue in 10 days, while Ortiz-Monsalve et al. (2019) observed that *T. villosa*, also collected in the Atlantic Forest, discolored 93.8% of acid orange 142 in 264 h of incubation. To date, our study is the first report of discoloration of indigo carmine and quantification of lignolytic enzymes using species of *Trametes* collected in Brazil.

3.4 Quantification of detected enzymes

In the present study, the production of enzymes was detected (Table 1). The low enzyme indicesobserved for laccase may be related to the presence of the dye, as found by Novotny et al. (2001) in which the presence of dye decreased the detection rates of laccase and manganese peroxidase in alineage of *Irpex lacteus*, as well as the mycelial development of the fungus. Trombini and Obara-Doi(2012) obtained 99.97% of discoloration using *Ganoderma* sp. and low laccase indices, showing theaction of another enzyme or other mechanisms involved in the discoloration process. Dye discolorationprocess may involve the participation of enzymes as well as the association of other mechanisms suchas adsorption involved in the discoloration process (Novotný et al. 2001; Couto and Sanromán 2004;Srinivasan and Viraraghavan 2010). Several studies indicate that laccase acts as the enzymeresponsible for discoloration (Couto and Sanromán 2004; Rodríguez-Couto et al. 2006; Levin et al.2004; Zeng et al. 2011; Yuan et al. 2012; Younes et al. 2015; Orzechowski et al. 2018;Uribe-Arizmendi et al. 2020). However, the participation of manganese peroxidase has also been observed in some discoloration studies (Eichlerová et al. 2007; Grassi et al. 2011; Li et al. 2015; Zhang et al. 2016).

Table 1 Production of lignolytic enzymes after the discoloration of the indigo carmine dye.

Fungi	Lac (U/L)	LiP	MnP (U/L)	% Discoloration
T.villosa (URM8022)	27.833± 0.031	-	3.408.065±31.70	96.11±0.86
T.lactinea (URM8350)	0.250± .002	-		81.40 ±3.40
T.lactinea (URM8354)	0.750±0.003	-	3.677.125±25.36	85.09±2.73

In the present study, the indices of discoloration of indigo carmine were well above the rate observed

by Lopes et al. (2014, 44.74% *T. versicolor*), Rodríguez-Couto et al. (2014, 59% *T. pubescens*), Levin et al. (2010, 69% *T. trogii*) and Grassi et al. (2011, 8% *T. trogii*).

Also, the discoloration time observed in the present study was relatively good if compared to other studies. Uribe-Arizmendi et al. (2020) carried out their experiments in 21 days (*T. polyzona,* 90% at 100 mg L-1, 91% at 150 mg L-1 and 93% at 200 mg L-1 of indigo carmine), Ortiz-Monsalve et al. (2019) in 264

h (*T. villosa*, 93.8% of acid orange 142), Lyra et al. (2009) in 10 days (*T. membranacea*, 99.2% of bromophenol blue and 71.8% of methylene blue), and Zouari-Mechichi et al. (2006) after three weeks (*T. trogii*, 97% of the remazol brilliant blue). Generally, studies that report good results of dye discoloration in a shorter time are those that use optimization of the enzymes of interest with addition of the dye after enzymatic production, commonly laccase and/or manganese peroxidase (Campos et al. 2001; Rodrigues-Couto et al. 2001; Rodríguez-Couto et al. 2006; Li et al 2015; Wang et al. 2019; Xu et al. 2020). The chemical treatment processes of indigo carmine generate potentially dangerous by-products and sludge, causing serious environmental pollution.

The treatment with indigoids using the enzymatic arsenal of fungal species has been considered a promising strategy at an environmental and economic level (Nyanhongo et al. 2007; Mugdha and Usha 2011; Li et al. 2015). Species belonging to the genus *Trametes* can produce multiple isoforms of Lac and MnP expressed under different cultivation conditions. However, LiP, when observed, is produced in low quantities (Choi 2021). Lacase contains copper polyphenoloxidases, produces four free electrons that react with phenolic and non-phenolic molecules and is one of the few enzymes capable of catalyzing the reduction of four electrons of molecular oxygen to water and even produced in small quantities can act in the degradation of recalcitrant compounds. The catalytic efficacy of Lac and MnP in the removal of recalcitrant compounds is due to the high redox potential, activity and stability of these enzymes, whether in a raw or purified state. However, other enzymes may be involved in the discoloration process (Nyanhongo et al. 2007; Campos et al. 2016; Zheng et al. 2017; Xu et al. 2020; Choi 2021).

4. Conclusion

Species of Agaricomycetes that cause white rot have an arsenal of degradable lignolytic enzymes that can be used in bioremediation processes. These enzymes are expressed according to the composition of the substrate and the lineage used. The interest in identifying promising strains has been increasing as an effort to minimize and/or treat environments polluted or degraded by anthropic action. Knowing the enzymatic potential of neotropical species is essential in view of the fungal megadiversity in these still unexplored, but threatened environments. The strains *T. lactinea* (URM8350), *T. lactinea* (URM8354) and *T. villosa* (URM8022), collected in Northeastern Brazil, showed significant percentage of discoloration of indigo carmine in a short time and at a low cost and their Lac and MnP were efficient in discoloration of the dye. The present work allowed the identification of promising strains of the genus *Trametes* that can be used in the treatment of textile effluents, with *T. lactinea* being the first study to report discoloration of a dye. Further studies will be necessary to verify the toxicity level of the discoloration product.

Declarations

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Data availability statements: All data generated or analysed during this study are included in this published article (and its supplementary information files).

Featured article: The discoloration rates demonstrate the potential use of the tested strains in removing indigo carmine and as an ecologically correct and economically viable alternative, as well as show the paucity of studies with tropical species for this purpose. Thus, species of the genus *Trametes*, under suitable conditions, can be explored in the process of environmental bioremediation against several recalcitrant compounds, among which dyes.

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Figures

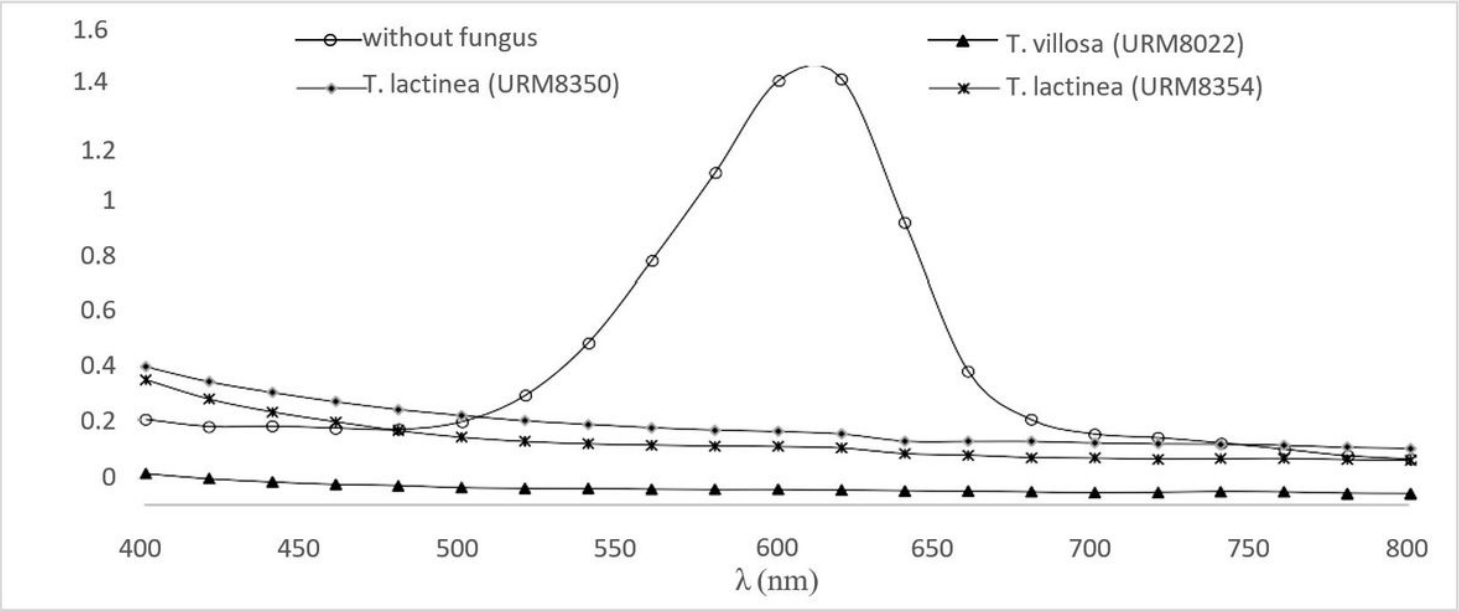

Figure 1

Effect of discoloration of the indigo carmine dye (50 mg L-1) by fungal treatment.