Azo Dye-Mediated Regulation of Total Phenolics and Peroxidase Activity in Thyme (*Thymus vulgaris* L.) and Rosemary (*Rosmarinus officinalis* L.) Clonal Lines

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Thyme (*Thymus vulgaris* L.) and rosemary (*Rosmarinus officinalis* L.) clonal lines, which were previously isolated from a heterogeneous seed population by plant tissue culture techniques, have been targeted as potential plants for phytoremediation of organic pollutants such as azo dyes and related aromatic compounds. Three thyme clonal lines and three rosemary clonal lines were tested for the ability to grow on hormone-free medium containing 0.01% of azo dye Poly S-119. The results showed that dye tolerance was associated with reduced phenolics and enhanced peroxidase activity in these clonal lines. There was a clear inverse correlation between total phenolics and peroxidase activity in these plants in response to Poly S-119. The tolerance of these clonal lines showed variations at different growing stages. These observations suggested that the peroxidase activity was inducible. Because peroxidases are involved in lignification, wound healing, aromatic compound degradation, pathogen defense, and stiffening, the results suggest that azo dye stimulated the defense response of thyme and rosemary clonal plants by increasing the peroxidase activity. Stereomicroscopic observations revealed that the azo dye was sequestered within the growing axis of the plant roots, which may also enhance the polymerization of azo dye onto the cell wall with the help of enhanced peroxidase activity.

Keywords: *Azo dye; peroxidase; phenolics; Poly S-119; Rosmarinus officinalis; Thymus vulgaris*

INTRODUCTION

Bioremediation is the use of biological treatment systems to destroy or reduce the concentrations of hazardous wastes from a contaminated site. Bioremediation has numerous applications, such as cleanup of groundwater, soils, lagoons, sludges, and process waste streams. It is estimated that over the next 30 years, $750 billion will have to be spent in the United States alone to remediate contaminated sites to current legal standards (Cunningham et al., 1996). Bioremediation currently comprises only a small fraction of the very large hazardous waste treatment market, but it is one of the fastest growing sectors in environmental management. As predicted by Caplan (1993), given the current global market of ~$100 million, bioremediation has a long, steep growth curve ahead before leveling out in 10 to 15 years, probably following a pattern similar to that seen in the microcomputer industry during the 1980s.

Phytoremediation, or green plant-based remediation, is one of the most important means of implementing bioremediation at contaminated site matrices. It is defined as the use of plants to remove, contain, or render harmless environmental contaminants. This definition applies to all plant-influenced biological, chemical, and physical processes that aid in the remediation of contaminated substrates (Cunningham et al., 1996). Phytoremediation, although not a new concept, is a relatively new technology. The use of plants in waste treatment originated more than 300 years ago (Cunningham et al., 1993, 1995). Phytoremediation either removes the contaminants from the matrix (phytodecontamination) or sequesters them into the matrix (phytostabilization) (Cunningham et al., 1996). Plants are regarded as solar-driven pumping and filtering systems that have measurable loading and degrading capacities, and their roots are described as exploratory extractors that can find, alter, and/or translocate elements and compounds from the liquid phase (Cunningham et al., 1996). Cost effectiveness is believed to be one of the greatest apparent advantages of phytoremediation. Unlike microbial-based degradation systems, the presence and condition of plants are easily monitored, and they are also more aesthetically pleasing than any other bioremediation techniques (Cunningham et al., 1995).

Synthetic dyes are an important group among various pollutants that pose environmental problems in both soil and water, which include azo, anthraquinone, and triarylmethane dyes (Rafii et al., 1990). Azo dyes are known as carcinogenic compounds and represent the majority of synthetic dyes. They are characterized by the presence of a chromophoric azo group having nitrogen atoms linked to sp^2-hybridized carbon atoms of the aromatic ring, which in addition may carry a sulfonic acid group(s) (Paszczynski et al., 1992). These compounds are extensively used in textile dyeing, paper printing, and pharmaceutical, food, cosmetic, and other industries (Rafii et al., 1990). More than 0.7 million tons of synthetic dyes are produced annually worldwide, of which azo dyes constitute ~50%. It is estimated that 10−15% of the dyes are lost in the effluent during such dyeing processes (Zollinger, 1987). With the increasing use of synthetic dyes, pollution by dye wastewater is becoming increasingly alarming. Many physical, chemi-

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cal, and biological methods have been attempted in the remediation of synthetic dye-contaminated soil and water (Yeh, 1995; Churchley, 1994; Nigam et al., 1996; Bahorsky, 1998). In our previous research we have found a fungus capable of decolorizing Poly S-119 to a certain degree in liquid systems (Zheng et al., 1999). We also found certain oregano (*Origanum vulgare*) plants that are tolerant to Poly S-119 (Zheng et al., 1998). Several papers reported that the peroxidases from fungi and plants play a critical role in the tolerance or degradation of azo dyes and the related aromatic pollutants (Ollikka et al., 1993; Spadaro and Renganathan, 1994; Klibanov and Morris, 1981; Zheng et al., 1998). The cross-tolerance and ability to degrade azo dye by fungi and plants are also indicators of a common potential to degrade and/or tolerate other aromatic pollutants such as polycyclic aromatic hydrocarbons (Field et al., 1992).

It has been recently revealed that the phenolic content and peroxidase activity in oregano plants were mediated by polymeric dyes and they are closely related to the dye tolerance capacity in these plants (Zheng et al., 1998). Phenolics or phenolic acids are intermediates in phenylpropanoid metabolism and play many important roles in plant cells, tissues, and organs. They are precursors for the synthesis of lignin (Lewis and Yamamoto, 1990), and their deposition into the cell walls after pathogen infection is an important defense mechanism (Bolwell et al., 1985). Phenolics are also involved in the processes of differentiation and organogenesis (Mato et al., 1988). Their level is affected by a wide spectrum of external and internal factors such as phytohormones and growth-regulating substances (Zaprometoy, 1989). Peroxidases are a ubiquitous class of enzymes present in various tissues and cell components in plants, and they are involved in numerous processes, such as lignification, wound healing, antipathogen defense, and stiffening (Djiana et al., 1996). Kwak et al. (1996) reported that peroxidase activity in sweet potato was enhanced by stress-related chemicals. A direct correlation was observed between the variation in peroxidase activity and the resistance to plant pathogen infection in *Hibiscus esculentus* and *Vigna sinensis* cultivars (Leina et al., 1996). The stimulation of peroxidase activity, as a part of the plant defense response to various stresses, is often accompanied by the increased accumulation of phenolics (Van-Loon, 1992). It was observed that the maximum of peroxidase activity coincided with an enhancement in the content of free phenolic acids in alfalfa (Cvikrova et al., 1996).

We have recently found that several thyme (*Thymus vulgaris* L.) and rosemary (*Rosmarinus officinalis* L.) clonal lines were highly tolerant to azo dye Poly S-119 present in their growing environment. The objective of this study was to investigate the effect of azo dye Poly S-119 on the total phenolics and peroxidase activity in these azo dye-tolerant plants. This could be a very important step in understanding the biochemical mechanisms associated with phytoremediation of synthetic dyes and related aromatic pollutants.

MATERIALS AND METHODS

Azo Dye. Poly S-119, a polymeric azo dye (polyvinylamine backbone with azochromophore, orange color, Chemical Abstracts Service Registry No. 68550-82-3), was purchased from Sigma Chemical Co. It is representative of azo dyes and the majority of synthetic dyes (Zheng et al., 1998, 1999).

Tissue Culture of Thyme and Rosemary. Three shoot-based thyme clonal lines, T-12, T-16G, and M-3, and three rosemary clonal lines, R-1, R-8, and R-16, were each generated via adventitious shoot formation from individual heterozygous seedlings following germination of a heterogeneous seed population (Shetty et al., 1996; Yang et al., 1997). They were subcultured and maintained on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962). The MS medium contained 1 mg of 6-benzylaminopurine (BAP), 30 g of sucrose, 1 mL of Nitsch and Nitsch (1956) vitamin solution (diluted from a 1000× stock, Sigma), and 3 g of phytogel (Sigma Chemical Co.) per liter with an adjusted pH of 5.8, before being autoclaved at 121 °C for 15 min. Each Petri plate contained seven apexes, and each apex had four lateral leaves below it. These Petri dishes were incubated at 20 °C under continuous light of 40 μmol·m^{-2}·s^{-1}. The shoot apex explants generated several more apexes through axillary bud proliferation during incubation. All clonal lines were maintained by subculturing the shoots at 30-day intervals.

Transferring the shoot apexes to half-MS hormone (BAP)-free medium resulted in their rooting. To test their tolerance to azo dye, thyme and rosemary clonal lines were transferred and cultured on half-MS hormone-free medium supplemented with 0.01% of Poly S-119. The half-MS hormone-free medium without azo dye was used as control. The total phenolics and peroxidase activity were measured on days 15, 30, 45, and 60 following subculture.

Total Phenolics Assay. The total phenolics of thyme and rosemary tissues were determined by an assay modified from that of Chandler and Dodds (1983) and described by Shetty et al. (1995). Approximately 50 mg of shoot tips was placed in 2.5 mL of 95% ethanol and held at 0 °C for 48 h. Each sample was then homogenized with a Tissue Tearor (Biospec Products, Inc., Racine, WI) and centrifuged at 13000*g* for 10 min. One milliliter of the supernatant was transferred to a test tube and mixed with 1 mL of 95% ethanol and 5 mL of distilled water. To each sample was added 0.5 mL of 50% Folin−Ciocalteu reagent. After 5 min, 1 mL of 5% Na_2CO_3 was added and the reaction mixture was allowed to stand for 60 min. The absorbance was read at 725 nm using a Genesys spectrophotometer (Milton Roy, Inc., Rochester, NY). Standard curves were established for each experiment using various concentrations of gallic acid in 95% ethanol. Absorbance values were converted to milligrams of phenolics per gram of tissue fresh mass. Each value reported in this study was an average of three replicate assays of three separate samples.

Peroxidase Assay. The extraction and assay procedures for peroxidase were adapted from the methods described by Biles et al. (1997) and Laloue et al. (1997). One hundred milligrams of plant tissue were ground at 4 °C for 2 min in the presence of 5 mL of extraction buffer using a pestle and mortar. The extraction buffer was 0.1 M potassium phosphate buffer (pH 7.5) containing 2 mM EDTA and 1% of poly(vinylpyrrolidone) to remove phenolics and polysaccharides. The extracts were centrifuged at 13000*g* for 15 min, and the supernatants were diluted to $\times 10^{-1}$ and used for enzyme assay. Peroxidase assays were conducted at 25 °C in a reaction mixture containing 50 mM potassium phosphate buffer (pH 6.5), 2 mM hydrogen peroxide, and 20 mM guaiacol in a total volume of 2 mL. The reaction was initiated by the addition of 0.1 mL of enzyme extract. Spectrophotometric readings at 470 nm, which measure the oxidation product of guaiacol, were taken within 5 min after addition of the enzyme extract. One unit of peroxidase activity is defined as micromoles of guaiacol oxidized per minute of reaction. The molar extinction coefficient for oxidized guaiacol of $\epsilon_{470} = 26.6$ mM^{-1}·cm^{-1} was used to convert absorbance measurements to molar units (Laloue et al., 1997; Djiana et al., 1996). Enzyme activities were expressed as units per gram of fresh plant tissue. Each value reported in this study was an average of three replicate assays of three separate extracts.

Stereomicroscopic Observation. The shoots and roots of thyme and rosemary clonal lines grown on half-MS hormorne-free medium containing azo dye Poly S-119 in Petri

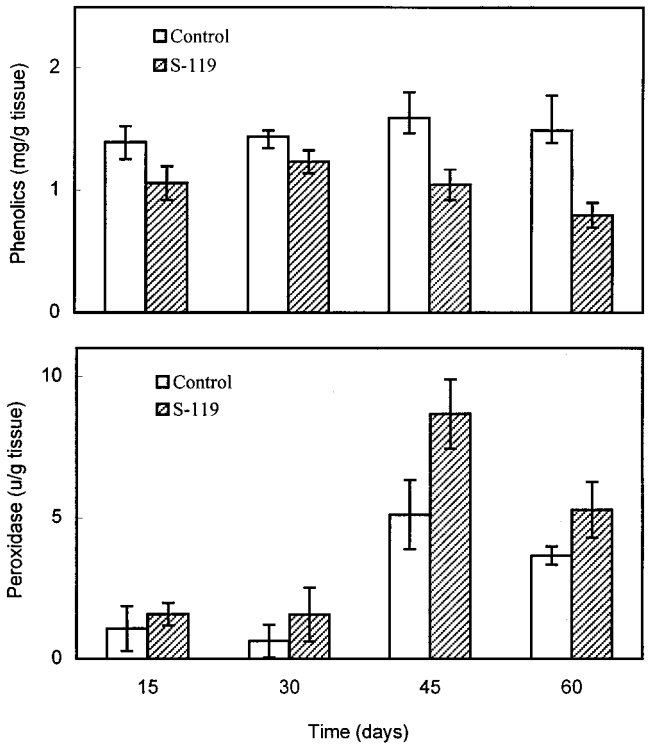

Figure 1. Effect of azo dye Poly S-119 on total phenolics and peroxidase activity in thyme clonal line T-12.

plates were observed under 25× magnification using a stereomicroscope (Olympus, Inc., Tokyo).

RESULTS AND DISCUSSION

Analysis of total phenolics and peroxidase activity was done on days 15, 30, 45, and 60 in three thyme clonal lines T-12, T16G, and M-3 in response to azo dye Poly S-119. As shown in Figure 1, the average phenolic content in thyme T-12 decreased over time on azo dye-containing media, whereas there was no significant change in control plants. In contrast, the average peroxidase activity in thyme T-12 was higher on Poly S-119-containing media than on control medium, and the difference became more evident at later stages, especially on day 45, suggesting that the peroxidase activity in thyme T-12 was probably induced by azo dye Poly S-119.

Thyme clonal lines T-16G and M-3 showed a very similar trend in both phenolics and peroxidase changes in responses to Poly S-119 (Figures 2 and 3).

It is known that peroxidases are involved in numerous processes in plant tissues and cells, such as lignification, wound healing, antipathogen defense, and stiffening (Djiana et al., 1996). Increases in peroxidase activities in host plants following treatments with stress-related chemicals and pathogens were well documented (Kwak et al., 1996; Leina et al., 1996). On the other hand, the stimulation of peroxidase activity, as a part of the plant defense response to various stresses, is often accompanied by an increased accumulation of phenolics (Van-Loon, 1986; Cvikrova et al., 1996; Zheng et al., 1998). Peroxidase is known to be responsible for the cross-linking of phenolic moieties during the biosynthesis of lignins in the plant cell wall (Morales and Barcelo, 1997). There is evidence that an increased peroxidase activity in bean plants correlated with lignification in response to pathogen defense (Milosevic and Slus-

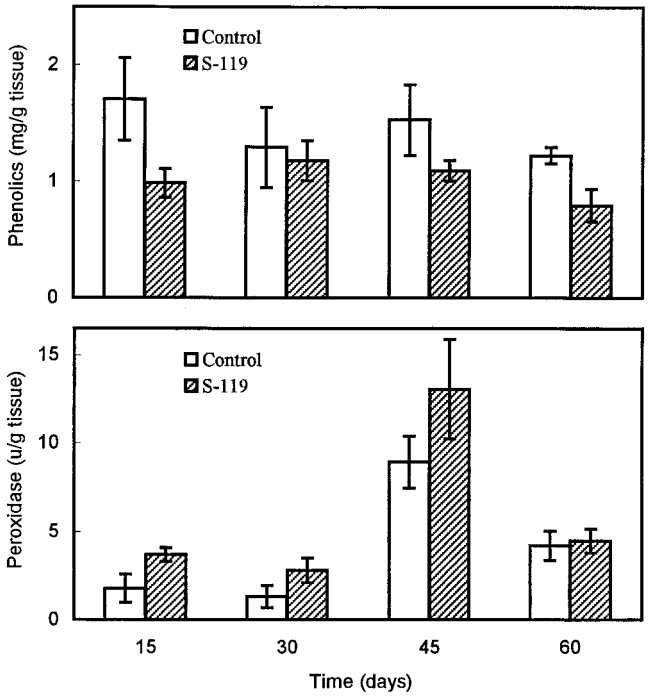

Figure 2. Effect of azo dye Poly S-119 on total phenolics and peroxidase activity in thyme clonal line T-16G.

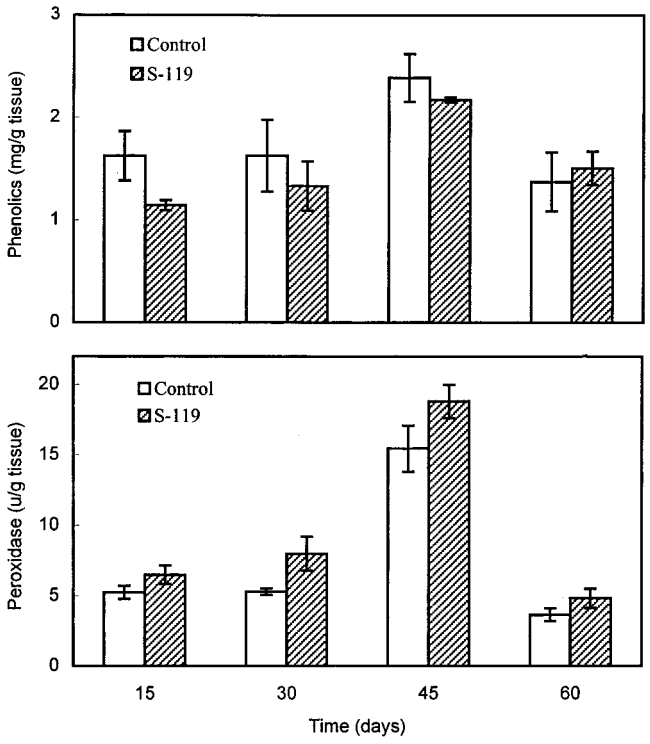

Figure 3. Effect of azo dye Poly S-119 on total phenolics and peroxidase activity in thyme clonal line M-3.

arenko, 1996). Our observation on thyme plants in response to azo dye also confirms the conclusion based on the observations on oregano plants that polymeric dyes including azo dye induced peroxidase activity and reduced the total phenolics at the same time (Zheng et al., 1998).

Phenolics are intermediates in phenylpropanoid metabolism, and they are precursors for the synthesis of lignin (Lewis and Yamamoto, 1990). The deposition of free phenolics in plant tissues into the cell walls after stress-related infection or stimulation is an important

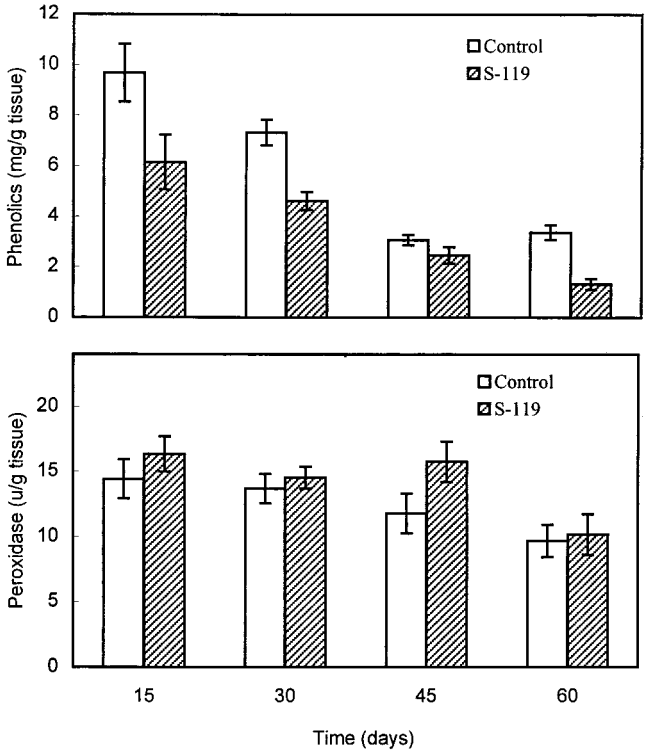

Figure 4. Effect of azo dye Poly S-119 on total phenolics and peroxidase activity in rosemary clonal line R-1.

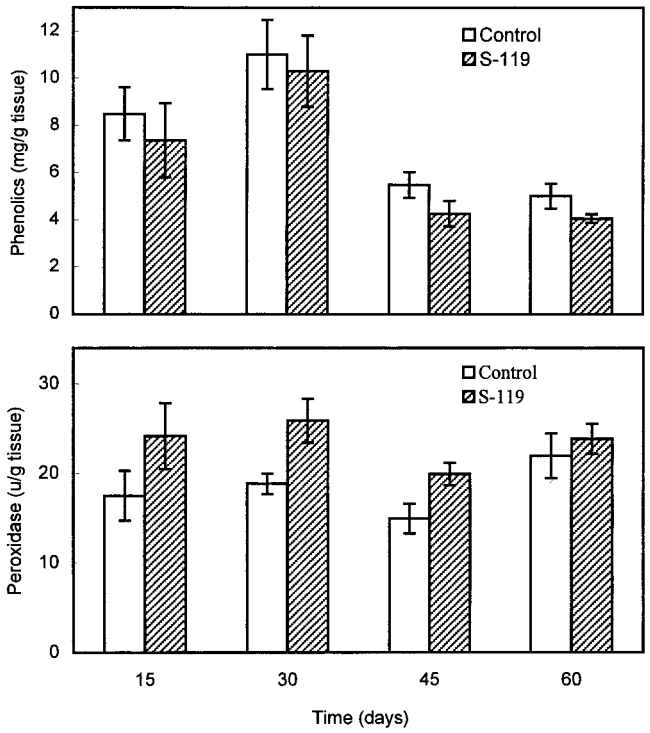

Figure 5. Effect of azo dye Poly S-119 on total phenolics and peroxidase activity in rosemary clonal line R-8.

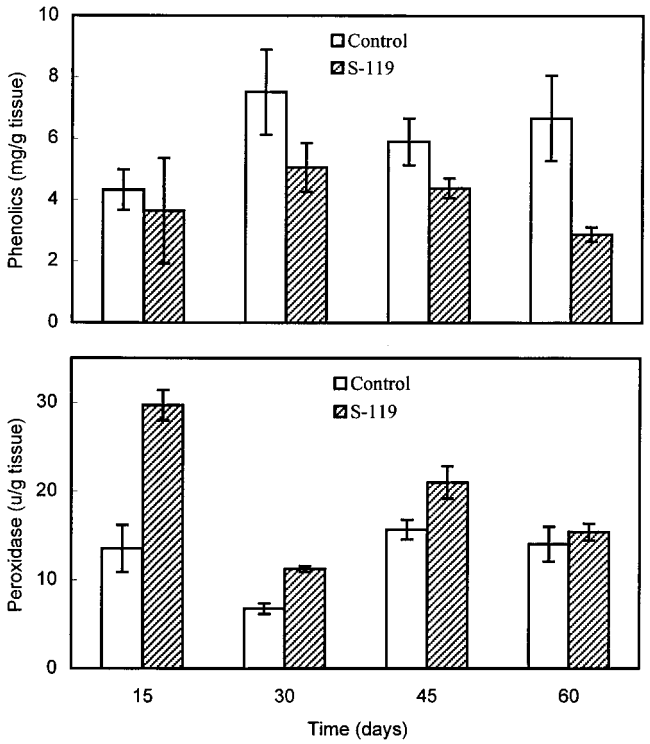

Figure 6. Effect of azo dye Poly S-119 on total phenolics and peroxidase activity in rosemary clonal line R-16.

defense mechanism (Bolwell et al., 1985). Their level is affected by many external and internal factors (Zaprometoy, 1989). On this basis, our observations suggested that azo dye may have stimulated the synthesis of lignins by converting free phenolics into polymerized compounds through the action of peroxidases in thyme clonal lines. Concurrently, it is possible that azo dye is also linked to the cell wall by peroxidases. Because azo dye strongly stimulated peroxidase activity in thyme clonal lines, the rate of polymerization of free phenolics to lignins and their precursors by peroxidase in thyme tissues likely increased. This is an important defense reaction by plants to protect themselves from unfavorable environments. Increased phenolic content in response to a nonpathogenic *Pseudomonas* spp. was also observed in thyme clonal lines (Kwok and Shetty, 1996).

The azo dye-mediated regulation of phenolics and peroxidase activity in thyme varied at different stages of growth. The total phenolics in both azo dye-treated plants and untreated control plants remained relatively stable during the test period, but, in general, they are apparently lower in azo dye-treated plants than in controls (Figures 1−3). The peroxidase activity in thyme clonal lines was unstable during the test period, and this may be because peroxidase is inducible and dependent on many factors including lignification and other processes which occurred at different rates at different stages. Although peroxidase activity was high on day 45 in both azo dye-treated plants and controls, its level was substantially increased in all thyme clonal lines in response to azo dye (Figures 1−3).

Compared to thyme, rosemary contained much higher levels of phenolics. In response to azo dye, rosemary clonal lines also exhibited decreased phenolics levels and increased peroxidase activity (Figures 4−6). The changes in total phenolics and peroxidase activity in response to azo dye at different stages, however, were different in different clonal lines. The total phenolics level in both azo dye-treated and untreated R-1 clonal line decreased over time, but the overall level of phenolics in azo dye-treated plants was much lower than in control plants (Figure 4). The overall peroxidase activity in both treated and untreated plants was stable over time, but its level in azo dye-treated plants was lower than in control (Figure 4). Similar results were observed in rosemary clonal line R-8 (Figure 5). The total phenolics in rosemary clonal line R-16 decreased significantly in response to azo dye at a later stage (day 60), whereas

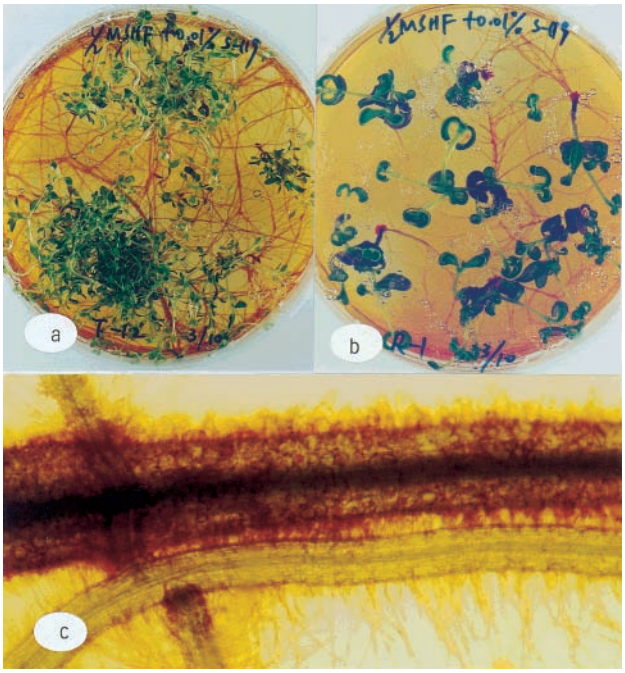

Figure 7. Observations of azo dye-tolerant plants growing on half-MS hormone-free medium containing 0.01% of Poly S-119: (a) thyme clonal line T-12; (b) rosemary clonal line R-1; (c) stereomicroscopic view of azo dye uptake by roots of thyme clonal line T-12.

the peroxidase activity was induced substantially at an early stage (day 15) by azo dye (Figure 6). The differences among individual clonal lines in the changes in total phenolics and peroxidase activity in response to azo dye could be due to the complexity of the defense systems in rosemary, and there may be other mechanisms regulating the phenolic synthesis, lignification, and dye polymerization processes. The poor and ambiguous responses of the R-1 and R-8 lines but not the R-16 line correlate well with *Pseudomonas*-induced phenolics stimulation previously observed for the same clones (Yang et al., 1997). In general, however, there is a clear inverse correlation between peroxidase activity and total phenolics in most thyme and rosemary plants in response to azo dye Poly S-119. These observations are consistent with the idea that the increased peroxidase was used to convert more free phenolics to form lignin and other dye-polymerized products in both thyme and rosemary plants. This may have also contributed to the polymerization of azo dye onto the cell wall.

Oxidation of phenolic compounds, the naturally occurring substrates in the plant cell, is one of the functions of peroxidases. The fact that the changes in total phenolics in some plants were in the inverse direction from the changes in peroxidase activity was also supported by evidence obtained by Mato et al. (1988), who found a similar correlation between changes in phenolics and accompanying changes in peroxidase activity during root formation in grapevine (*Vitis*) species. A similar inverse correlation between phenolics level and peroxidase activity was also shown in oregano plants in response to polymeric dyes (Zheng et al., 1998). The azo dye-mediated regulation of plant metabolism may have allowed the conversion of free phenolic compounds into lignins with the help of enhanced peroxidase activity in these thyme and rosemary clonal lines. This lignification process could have also helped to polymerize the azo dye onto the cell wall.

Observations of thyme clonal line T-12 and rosemary clonal line R-1 growing on a half-MS hormone-free medium supplemented with 0.1% of Poly S-119 are shown in Figure 7a,b. Both clonal lines are azo dye-tolerant plants. The root growth in dye-containing medium was normal, and the dye was concentrated and sequestered around and along the growing root axis (Figure 7c).

Chivukula et al. (1995) reported that sulfonated azo dyes were oxidized by a lignin peroxidase from a white-rot basidiomycete fungus. The enhanced peroxidase activity in thyme and rosemary clonal lines in response to Poly S-119 was one of the defense responses in plant tissues, and the activated peroxidase was very critical for those plants in dealing with the azo dye pollutants in the medium. Consequently, the azo dye tolerance mechanism was activated in the plant tissues. Although we do not yet know whether the induced peroxidase activity in thyme and rosemary plants is responsible for degradation of azo dye, it is clear that the activated azo dye tolerance mechanism in these plants is necessary for survival in such a contaminated environment, and this would lay the foundation for further bioremediation of azo dyes. Therefore, understanding the azo dye tolerance responses in plants is a very important step in the development of phytoremediation systems or for further development of effective rhizospheres for supporting microbial degradation of similar and related aromatic pollutants.

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