Interaction Between the Phytotoxin Dothistromin and *Pinus radiata* Embryos

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ABSTRACT

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Dothistromin (DOTH) is a fungal toxin occurring in *Pinus radiata* needles infected with *Dothistroma pini*. Dose-dependent toxicity of DOTH toward both *P. radiata* mature seed embryos and meristematic leaf callus was observed. DOTH effected 50 and 100% inhibition of embryo and leaf callus growth after absorbing 3 and 13 nmol of DOTH per g of tissue, respectively. DOTH-binding sites were localized, using immunohistology for light and electron microscopy, within small vesicles in the embryos. A 40-kDa peptide was identified in the particulate fraction of mature embryo extracts that reacted specifically with a DOTH-mouse albumin conjugate.

Dothistromin (DOTH) (2,3,3a,5,10,12a-hexahydro-2,3a,4,6,9-pentahydroxyanthra[2,3-b]furo[3,2-d]furan-5,10-dione) was first isolated from cultures of *Dothistroma pini* Hulbary (5), and its structure has been confirmed using chemical, spectroscopic (5,11), and crystallographic evidence (6). *D. pini* is the causal agent of Dothistroma blight, a disease of *Pinus radiata* Don, which is characterized by red bands through necrotic lesions visible on pine needles (12). Dothistroma blight results in defoliation, retardation of growth, and sometimes death of the infected tree. DOTH also has been identified as a phytotoxic metabolite in some *Cercospora* species (4).

DOTH is a potential human toxin and clastogen at low concentrations, and concern has been expressed with regard to its potential risk to forest workers in situations in which significant levels of DOTH on or at the forest floor and in the surrounding environment result from needle fall (9). DOTH also inhibits the growth of *Chlorella pyrenoidosa* (13,22) and *Bacillus megaterium* (13) and induces necrotic and red band symptoms when administered artificially to *P. radiata* needles at concentrations 10 to 1,000 times less than the concentration found in natural *D. pini* lesions (22).

Although DOTH has been shown to initiate lesions when administered in vitro to pine needles, its mode of action has not been elucidated. Recently (16), monoclonal antibodies (MAbs) specific for DOTH have been prepared and used to develop an immunoassay to quantitate the toxin in *P. radiata* tissue.

In this paper, we describe the use of DOTH-specific MAbs to investigate the toxicity of DOTH to *Pinus* tissue and, in combination with immunohistological techniques, the identification of DOTH-binding sites in these tissues. We also identify a putative receptor protein for DOTH.

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MATERIALS AND METHODS

Bovine serum albumin (BSA), ovalbumin (OVA), RPMI culture media, hypoxanthine-aminopterin-thymidine selection additive, fetal calf serum (Hybrimax), L-glutamine, sodium pyruvate, 2,4,10,14-tetramethyl-pentadecane (Pristane), Tween 20, horseradish peroxidase, peroxidase-labeled sheep anti-mouse immunoglobulin (Ig) G (gamma chain specific), *o*-phenylene diamine, phosphatase-labeled sheep anti-mouse IgG, nitro blue tetrazolium (NBT), bromochloro-indolyl phosphate (BCIP), and succinimidyl pyridyl-dithio propionic acid (SPDP) were obtained from Sigma Chemical Co. (St. Louis). Mouse albumin (MSA) was obtained from Cappel-Organon Teknika (Durham, NC). Cell culture plasticware and micro–enzyme-linked immunosorbent assay (micro-ELISA) flat bottom F16 modules (Maxisorb) were obtained from Nunc (Roskilde, Denmark). Thin-layer chromatography (TLC) silica gel plates (Merck Art 5554) were obtained from Biolab Scientific (Auckland, NZ). Dynabeads were obtained from Dynal International (Oslo, Norway). All other reagents and solvents were of analytical grade or better.

Isolation of DOTH. DOTH was purified from cultures of *D. pini* as described previously (16). DOTH was crystallized from pyridine (23,25) and purified from the minor anthraquinones contained in the pyridine filtrates by preparative TLC (20 × 20 cm plates, 0.5-mm-thick silica gel G) developed with chloroform/methanol/formic acid (50:3, vol/vol, plus 1% formic acid). DOTH formed a red band at R_f 0.36. The identity of DOTH was confirmed by comparison with published mass-spectral data (11), and the compound was stored at 4°C in sealed dark bottles in the presence of silica gel as a drying agent.

Synthesis of DOTH-protein conjugates. DOTH contains active hydrogens (positions C7 and C8) adjacent to aromatic hydroxyl groups on the anthraquinone skeleton. These hydrogens can undergo a Mannich condensation reaction with free amines on carrier proteins in the presence of formaldehyde. Briefly, DOTH (2 ml; 1 to 20 mg ml^{-1} in 50% aqueous dimethyl sulfoxide [DMSO]) was added to the ice-cooled protein solution (2 ml; 10 mg ml^{-1} in phosphate buffered saline [PBS]/DMSO, 1:1, vol/vol) in stirred reactivials (Pierce Ltd., Rockford, IL) followed by dropwise addition of formaldehyde (1 ml; 18.5% formaldehyde in 50% DMSO).

The reaction was allowed to proceed for 22 h at 37°C, and the reaction mixture was dialyzed first against 50% aqueous DMSO (2 × 1 liter) and then exhaustively against distilled water. The conjugate was separated from any remaining free DOTH by fractionation on Sephadex G-25 (Pharmacia Ltd., Uppsala, Sweden). Aliquots (50 µl) of the DOTH conjugates were stored at –20°C.

Preparation of MAbs. MAbs against DOTH (hybridoma 10C12 [16]) were prepared from ascitic tumors as described previously (18). MAbs were purified using ammonium sulfate fractionation followed by affinity chromatography on protein A sepharose (8). Protein concentration was determined by absorbance at 280 nm using an extinction coefficient of 14.49 for a 1% solution of mouse gamma globulin. MAbs were labeled with peroxidase (Sigma type VII) using the heterobifunctional reagent SPDP as described previously (17).

Micro-ELISA procedures. DOTH was quantitated using the competitive micro-ELISA developed previously (16). Briefly, microtiter wells were coated with DOTH conjugate (DOTH-MSA; 2.5 µg of MSA per ml) in PBS (100 µl per well). OVA (1%) in PBS was used to block remaining protein-binding sites on the microwell surface. DOTH samples and standards (200 µl) were preincubated with labeled MAb 10C12 (200 µl; 400 ng ml^{-1} in PBS containing 0.1% Tween 20 [PBST]) for 1 h at 37°C. The reactants (100 µl) were added in triplicate to micro-ELISA wells for 3 h at 37°C. Bound peroxidase-labeled MAbs were developed with substrate and quantitated as described previously (16).

Preparation of pine tissue. Open-pollinated seeds of *P. radiata* were sterilized in a 50% aqueous hypochlorite solution, hydrated for 48 h at 4°C, and sterilized before use with peroxide (21). Whole embryos were excised and collected into sterile liquid ½ LP medium (LP5 with half-strength major, minor, and iron components [1]). Embryos were used either for histological studies or for toxicity studies as described below.

Needle callus was initiated from secondary needles of *P. radiata* shoots maintained in sterile culture. Vegetative buds from 4-year-old trees were collected and induced to form shoots (14). Excised secondary needles were placed on a modified Lepoivre solid medium (1) containing 2,4-D (1 mg/liter) and 6-benzylaminopurine (1 mg/liter) to initiate callus formation. Proliferation of cells at the needle base was observed within 1 week. Callus was maintained under low light intensity as a green friable cell mass by weekly transfer to fresh media.

Toxicity of DOTH to pine embryos and meristematic callus. Pine embryos were exposed, in the absence of light, to sterile solutions (2 ml) of DOTH in ½ LP. Thirty embryos (approximately 0.17 g) were used per treatment, and three independent treatments were used in each experiment. Concentrations of DOTH ranged from 0.050 to 20 µg ml^{-1} and exposure times ranged from 5 to 60 min. After exposure to DOTH, samples of media were removed aseptically to determine DOTH concentration, and the embryos were drained on sterile filter paper. The embryos were transferred to solid medium containing the modified Lepoivre medium described by Aitken-Christie et al. (1) for the development of meristematic tissue and placed under low light intensity at 22°C. Toxicity was assessed after 3 weeks in culture by measuring inhibition of development of meristematic tissue using the visual assessment method (1).

Meristematic leaf callus (3 g per treatment) was suspended in 50 ml of ½ LP liquid medium (1) containing DOTH at 0, 0.1, 0.3, 0.5, and 1 µg ml^{-1} for 1 h. In a second experiment, callus (3 g) was suspended in medium containing DOTH (0.5 µg ml^{-1}; 50 ml), and the suspension was sampled every 5 min for a total of 30 min. Aliquots of suspension (1.5 ml) were removed for DOTH analyses and determination of callus viability. Callus viability was determined by collecting the callus cells on Nybolt cloth, culturing on solid medium as described above for 2 weeks, and measuring the increase in the weight of callus.

Immunohistochemistry. *Light microscopy.* Pine embryos were fixed in 2% paraformaldehyde in PBS for 4 h at room temperature. The fixed embryos were washed twice with PBS, transferred to 6.8% sucrose in PBS, and agitated for 16 to 20 h at 4°C. The embryos then were washed twice in deionized water and dehydrated in a progressive acetone series (40 to 95%, 15 min each step) and 2 × 15 min in 100% acetone. The dehydrated embryos were first infiltrated for 24 h at 4°C and then embedded in Technovit 8100 (Heraeus Kulzer GmbH, Wehrheim, Germany). Sections (3 µm) were cut on a rotary microtome, air-dried for 2 h, and stored over silica gel at –20°C until used.

Sections were stained with Johansen's quadruple stain (15) to determine morphological detail. Prior to immunostaining, sections were brought to room temperature and air-dried for 15 min; all subsequent incubations were performed at room temperature. Sections were rehydrated in PBS for 15 min and then preincubated with a solution of 1% goat serum and 1% BSA in PBS for 15 min. All dilutions of reagents were made in PBS containing 0.1% goat serum and 0.1% BSA. Dilutions of the MAbs were made in the above dilutent but also included 0.1% mouse serum. Tissue sections were incubated for 1 h with a 1:200 dilution of DOTH-MSA conjugate (10 µg ml^{-1}), washed extensively with PBS, and incubated with anti-DOTH MAb 10C12 (50 µg ml^{-1}) for 1 h. Sections were washed in PBS, and bound MAb was detected either by silver-enhanced gold or fluorescent labeling.

Gold labeling was performed by incubation with a 15-nm colloidal gold–goat anti-mouse Ig(Fc) conjugate (Auroprobe RPN 444, Amersham, Buckinghamshire, England) diluted 1:200, and bound gold label was visualized by silver enhancement (SEKL 15, British BioCell Int., Cardiff, Wales). After counterstaining with 0.1% aqueous crystal violet, sections were viewed under oil immersion using a Zeiss Axioplan photomicroscope (Carl Zeiss, Inc., Thornwood, NY). Photomicrographs were taken using Ektachrome 64T color reversal film (140 9937, Eastman Kodak Co., Rochester, NY).

Immunofluorescent labeling was performed by incubation with a biotinylated sheep anti-mouse antibody (RPN 1001, Amersham) diluted 1:200. The sections were washed and stained with a streptavidin-phycoerythrin cytochemical reagent kit (RPN 1172, Amersham) following the protocol recommended by the manufacturer. Sections were viewed under a Ziess epi-fluorescence system (filter set 09); photomicrographs were taken using Ektachrome Elite 200 ASA (366 4372, Eastman Kodak).

Electron microscopy. Pine embryos were fixed in 2% paraformaldehyde and 0.1% glutaraldehyde on 0.1 M phosphate buffer, pH 7.2, for 1 h. The embryos were washed in buffer dehydrated in an ethanol series and embedded in LR White resin (London Resins, London).

Ultrathin sections of the leaf initials were cut and mounted on carbon-coated formvar nickel grids. The grids and sections were blocked for 15 min in 1% OVA in PBS. The sections were treated with DOTH-MSA and MAb as described for light microscopy. The grids were incubated for 1 h in 10-nm gold-labeled goat anti-mouse IgG (Sigma) diluted 1:50 in PBST containing 5% fetal bovine serum. Grids were washed in PBST and water and stained for 5 min in saturated aqueous uranyl acetate and for 1 min in lead citrate. Sections were viewed on a JEOL (Tokyo) JEM-1200EX 2 transmission electron microscope at 80 kV.

Control incubations (for both electron and light microscopy) were carried out by: (i) omitting DOTH-MSA, (ii) replacing DOTH-MSA with MSA, (iii) replacing MAb 10C12 with an unrelated MAb of the same subclass as 10C12, and (iv) using mouse serum. All experiments were repeated at least three times, and representative data are given.

Identification of DOTH-binding molecules in pine embryos. Dynabeads (1 ml of M-450 Dynabeads coated with sheep anti-mouse IgG) were reacted with MAb 10C12 (100 µg ml^{-1} in PBS for 16 h at 4°C) with gentle shaking. The Dynabeads were collected with a Dynal MPC magnet, the liquid phase was discarded, and the beads were washed four times in PBS containing 0.1%

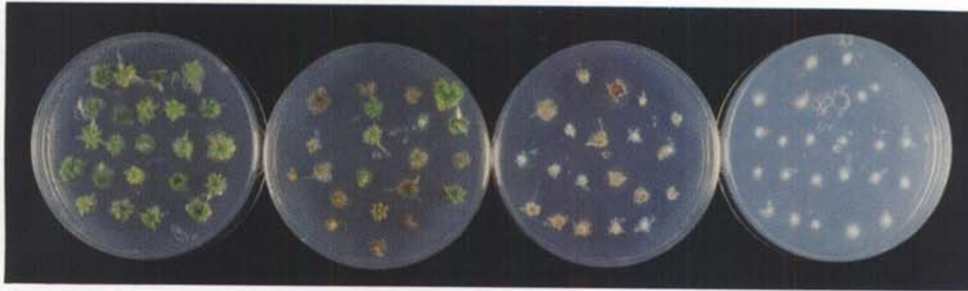
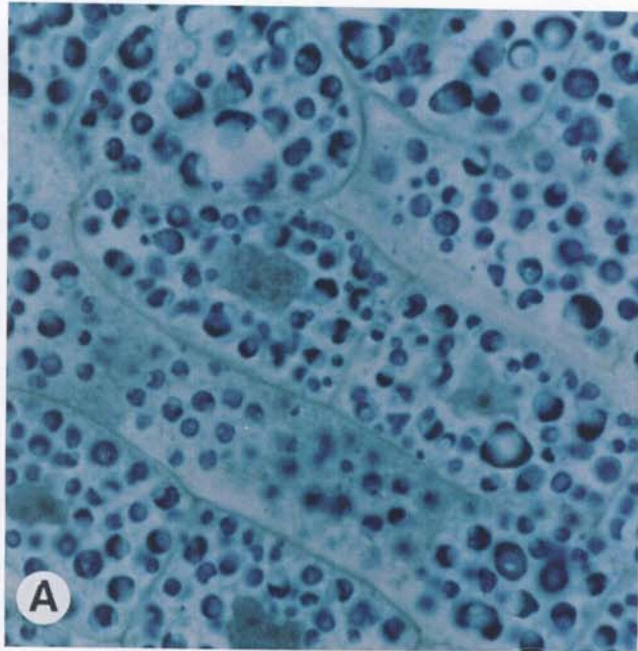
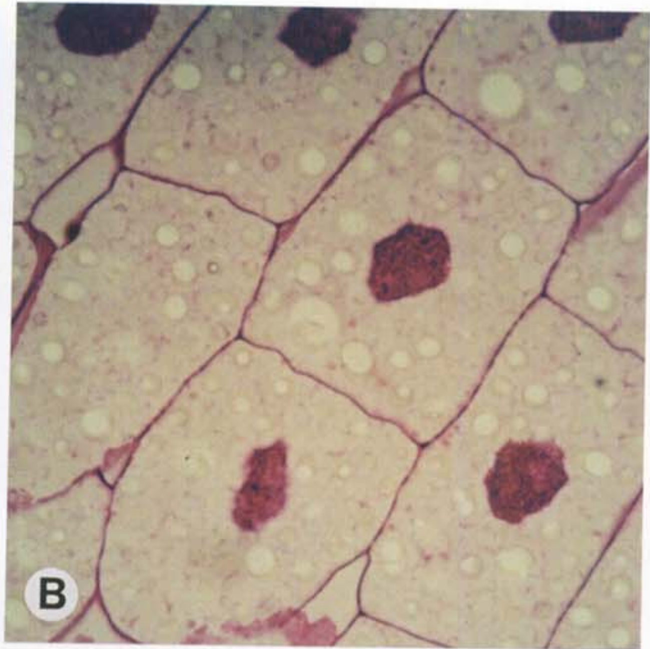
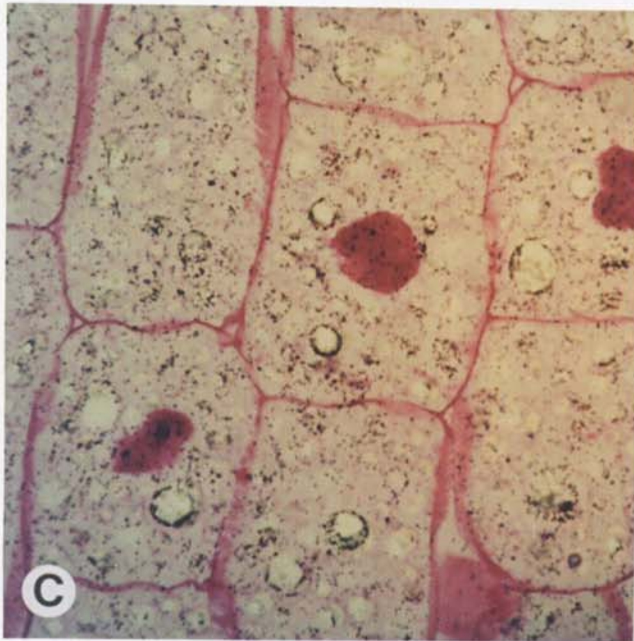


Fig. 1. Inhibition of germination of meristematic callus from mature pine embryo by dothistromin (DOTH). Mature pine embryos were exposed to DOTH for 1 h in the absence of light and then were placed on meristematic-growth medium for 3 weeks. From left to right: control (no DOTH), 0.1, 0.5, and 1 μg of DOTH per ml of medium, respectively.







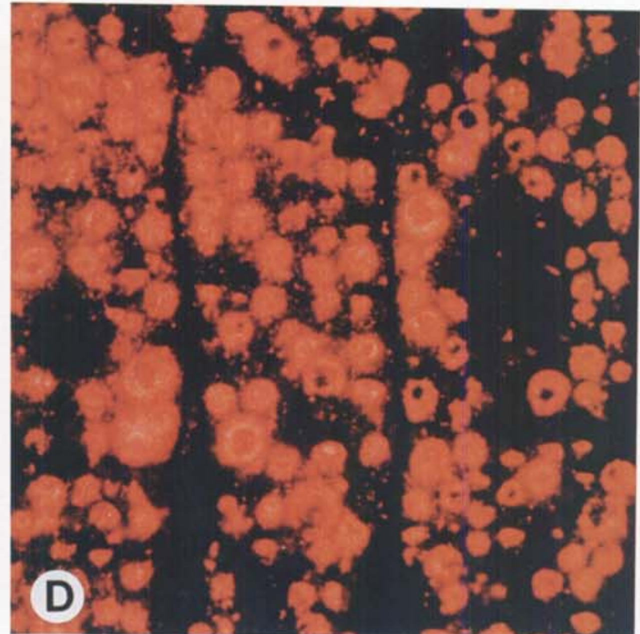


Fig. 2. Immunohistochemical localization of dothistromin (DOTH) binding to paraformaldehyde-fixed sections of mature pine embryos. Sections were blocked with 1% bovine serum albumin and 1% goat serum in phosphate buffered saline, probed with DOTH-mouse albumin (MSA) and then with either anti-DOTH monoclonal antibody (MAb) 10C12 or an unrelated MAb. Bound mouse antibody was detected with either a gold-labeled goat anti-mouse immunoglobulin (Ig) G antibody or a biotinylated sheep anti-mouse antibody followed by a phycoerythrin-streptavidin complex. **A,** Mature embryo stained with Johansen's quadruple stain showing a central nucleus and many densely stained protein bodies. **B,** Section probed with DOTH-MSA and an unrelated MAb followed by gold-labeled anti-mouse IgG and counterstained with crystal violet. No gold deposits are visualized. **C,** As in **B**, except the section was probed with anti-DOTH MAb 10C12. Heavy gold staining is seen in both large and small vesicles. **D,** As in **C**, except that bound mouse antibody was detected by an anti-mouse–biotin-streptavidin-phycoerythrin complex. Intense fluorescence is seen in the vesicles; the nuclei are not stained. Original magnification ×630.

OVA. The beads were suspended in 1 ml of PBST with 0.1% OVA and 2 mg of DOTH-MSA conjugate (0.125 mg of DOTH) for 16 h at 4°C. The beads were washed six times in PBST, resuspended in 1 ml of PBST containing 0.1% OVA and 0.01% thimersol, and stored at 4°C.

Pine embryos (0.56 g; 100 embryos) were macerated in PBST (2 ml) using glass/glass homogenizers. DOTH-MSA–coated Dynabeads (0.1 ml; 4×10^7 beads) were suspended in either the embryo extract (0.9 ml) or in PBST (0.9 ml) for 2 h at room temperature. The beads were collected and washed 10 times in PBST, two times in water, and resuspended in 0.1 ml of sodium dodecyl sulfate (SDS) sample buffer (19), and the suspension was boiled for 5 min. The beads were removed, and the supernatant was collected and analyzed by Western blotting (18). Western blots were either stained for protein with Coomassie Blue R or, after blocking protein-binding sites on the membrane with 1% OVA in Tris-buffered saline (TBS) for 2 h at room temperature, were probed for DOTH-binding proteins. The membrane was incubated sequentially in DOTH-MSA (0.625 µg of DOTH per ml), MAb 10C12 (1 µg ml^{-1}), and phosphatase-labeled sheep anti-mouse IgG (1:1,000 dilution). All reagents were diluted in TBS containing 0.1% Tween 20 (TBST) and 1% OVA, and incubations were performed for 1 h at room temperature. Between each incubation, the membranes were washed three times for 10 min in TBST containing 0.1% OVA. The blots were developed using NBT/BCIP phosphatase substrate as described previously (18). Blots also were stained using MSA in place of DOTH-MSA and substituting an unrelated MAb for 10C12 as described above. Similar results were obtained in three independent affinity-enrichment experiments.

RESULTS

Preparation of DOTH-MSA conjugates. Conjugates of varying molar ratios of DOTH to MSA were produced. The molar ratio of DOTH per mol MSA increased from 2:1 to 20:1 as the DOTH in the reaction mixture increased from 1 to 12 mg ml^{-1}. Higher concentrations of DOTH resulted in precipitation of DOTH-MSA conjugates. Using DOTH/MSA, 1:2, by weight, resulted in high yields of the conjugates (>95% MSA as soluble protein). These conjugates were suitable for immunoassay and immunohistology. The molar ratio of DOTH/MSA was 10:1 (DOTH estimated by competitive ELISA and absorbance at 500 nm).

Toxicity of DOTH to pine tissue. Mature pine embryos initially were exposed to concentrations of DOTH ranging from 0.050 to 20 µg ml^{-1} for 1 h, and toxicity was observed at concentrations greater than 100 ng ml^{-1}. Meristematic development after exposure to 0, 0.1, 0.5, and 1 µg ml^{-1} is shown in Figure 1. The DOTH concentration needed to inhibit tissue development by 50% was 300 ng ml^{-1}, and total inhibition was observed at 1 µg ml^{-1}. A lack of greening was observed as an early (within 48 h of culture) symptom of toxicity by DOTH.

The minimal dose of DOTH required to inhibit germination of embryos was determined by exposing embryos to DOTH, at initial concentrations of 0, 0.3, 0.5, and 1 mg ml^{-1}, for varying lengths of time (5 to 60 min). Ten-minute exposure resulted in 50, 80, and 100% growth inhibition, and the uptake of DOTH, measured by ELISA, was 0.94, 2.0, and 4.9 µg g^{-1} for initial DOTH concentrations of 0.3, 0.5, and 1 µg ml^{-1}, respectively. No inhibition of meristematic development was observed when embryos were exposed to DOTH and MAb 10C12 in equimolar concentrations for 10 min (DOTH at 20 µg ml^{-1}, MAb 10C12 at 50 mg ml^{-1}).

DOTH-MSA conjugates showed toxicity to pine embryos, although these were less effective than DOTH. DOTH-MSA (10 mol DOTH per mol MSA) inhibited meristematic tissue development by 50% after a 10-min exposure at a DOTH concentration of 5 µg ml^{-1} (MSA at 80 µg ml^{-1}). No inhibition was observed using MSA at 1 mg ml^{-1}.

DOTH was toxic to meristematic leaf callus when tissue was exposed for 1 h to DOTH at concentrations greater than 0.1 µg ml^{-1}, reducing growth to approximately 12.5% of control (no DOTH) at 0.3 µg ml^{-1} and to no growth at 0.5 µg ml^{-1}. Using an initial DOTH concentration of 0.5 µg ml^{-1}, the uptake of DOTH by meristematic leaf callus (4.1 µg g^{-1} in 5 min) was faster than the uptake of DOTH by embryos (4.1 µg g^{-1} in 60 min), presumably due to the larger surface area per unit weight of callus tissue.

Localization of DOTH-binding sites. Pine embryo tissue ap-

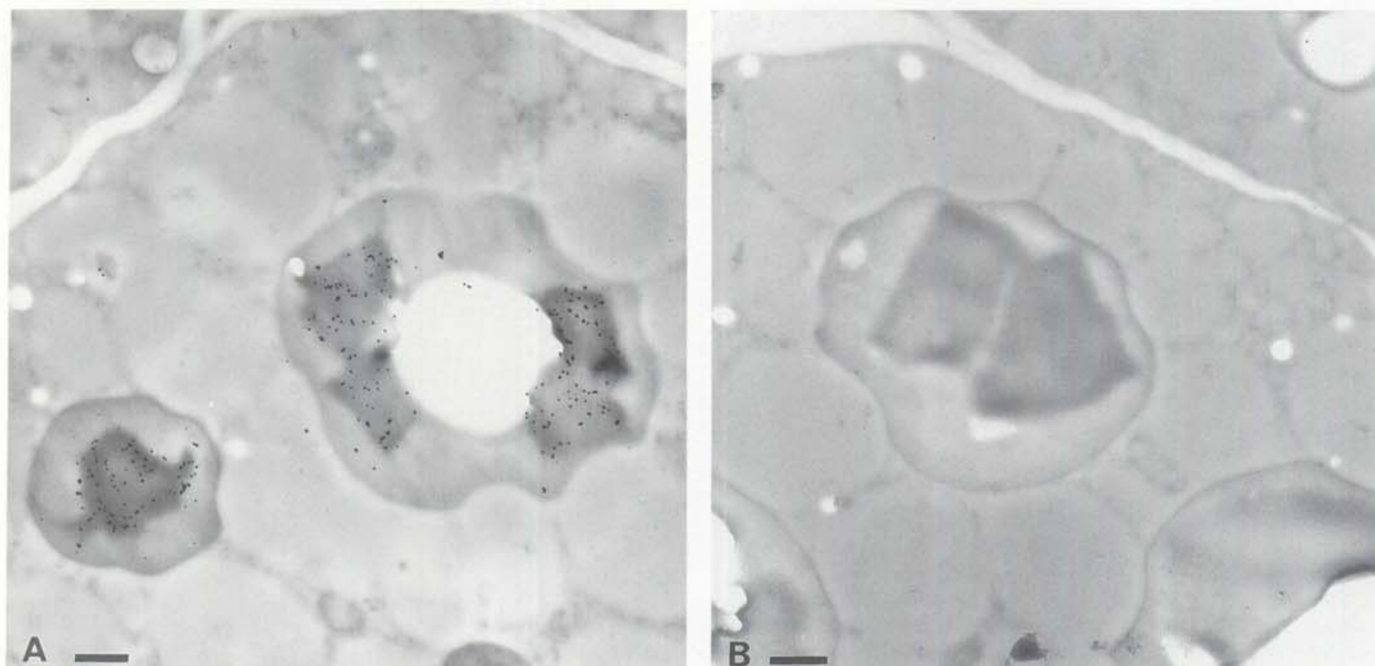


Fig. 3. Electron microscopic localization of dothistromin (DOTH) binding sites in vesicles of mature pine embryos. Sections were blocked with 1% ovalbumin in phosphate buffered saline and probed with DOTH-mouse albumin and then with **A,** anti-DOTH monoclonal antibody (MAb) 10C12 or **B,** an unrelated MAb. Bound antibody was detected by incubation with gold-labeled anti-mouse immunoglobulin G. Sections were examined by transmission electron microscopy. Positive staining is indicated by electron dense particles. Bars = 500 nm.

peared to be poorly differentiated. The cells contained a central nucleus, and in the cytosol, there were numerous protein-filled vesicles that were densely stained by the Fast Green component of Johansen's quadruple stain (Fig. 2A). No reactivity was observed in the sections exposed to DOTH-MSA followed by incubation with the unrelated control MAb prior to either immunogold (Fig. 2B) or fluorescence (results not shown) detection. Figure 2B also clearly shows the presence of intracellular vesicles, the protein contents of which have not been stained by the post-immunogold crystal violet counterstain. When sections were first exposed to MSA followed by MAb 10C12, no reactivity was observed (results not shown). After exposure to DOTH-MSA and subsequent incubation with MAb 10C12, immunogold (Fig. 2C) and immunofluorescent (Fig. 2D) detection of the MAb revealed that specific DOTH-MSA binding was associated with the protein-filled residues. Prior blocking of the sections with MSA yielded identical results, indicating that there was no interaction between MSA and pine tissue. A competitive experiment was performed on the section to demonstrate the specificity of the DOTH interaction with the protein-containing vesicles. DOTH-MSA binding was abolished (results not shown) when sections were first incubated with 50 or 100 µg of free DOTH per ml and substantially reduced at 10 µg of free DOTH per ml. This further indicates the specificity of DOTH binding and rules out the possibility of nonspecific hydrophobic interactions producing the observed staining.

Because of the association of DOTH binding with the protein-filled vesicles, these vesicles were examined at higher magnification to ascertain where the observed DOTH binding was occurring. Transmission electron micrographs of pine embryo sections stained with DOTH-MSA, MAb 10C12, and gold-labeled anti-mouse IgG identified the site of DOTH-MSA binding as the protein bodies contained in the small vesicles (Fig. 3A), whereas no reactivity was observed when MAb 10C12 was replaced by an unrelated antibody (Fig. 3B).

Identification of DOTH-binding molecules in pine embryos. Affinity enrichment ("panning") of embryo extracts with DOTH-coated Dynabeads revealed three peptides with molecular masses of 50, 40, and 24 kDa on SDS polyacrylamide gels (Fig. 4, lane 4). The heavy chain of MAb 10C12 (band at 54 kDa) also was faintly visible in SDS extracts of both the control bead (Fig. 4, lane 3) and the embryo-treated bead (Fig. 4, lane 4). A band at 28 kDa corresponding to the light chain of MAb 10C12 was visible to the naked eye in these lanes but did not photograph. When MSA-coated beads were used instead of DOTH-MSA–coated beads, no peptide bands were visible on SDS gels. When a Western blot of these panned embryo extracts was probed with DOTH-MSA, MAb 10C12, and phosphatase anti-mouse IgG, only the 40-kDa peptide showed positive staining (Fig. 4, lane 5). No specific staining was observed when MSA was used in place of DOTH-MSA, when DOTH-MSA was omitted, or when MAb 10C12 was replaced with an unrelated mouse antibody.

Extracts of embryos were centrifuged, and soluble and particulate fractions were solubilized in SDS sample buffer, fractionated on 15% polyacrylamide gel, and the proteins were subjected to Western blotting. Immunoprobing of the Western blot, as described above, indicated that the 40-kDa peptide was associated with the particulate fraction (results not shown).

DISCUSSION

DOTH is the major phytotoxin produced by the fungus *D. pini* (5). Other minor anthraquinones (7), which differ from each other and DOTH primarily in the structure of the furan moiety, are not as toxic as DOTH to *P. radiata* tissue (P. M. Debnam, *unpublished data*), suggesting that the furan structure in DOTH could be important in its toxicity. This contention is supported further by this study in that DOTH conjugated to MSA through the anthraquinone moiety was still toxic to pine embryos. MAbs with high affinity for DOTH recently have been prepared (16). The bifuran structure of DOTH was an important feature of the epitope recognized by these antibodies, and one MAb (10C12) has been used to develop a competitive ELISA for the quantitation of the compound.

Embryos exposed to solutions of DOTH become orange, and this apparent uptake of DOTH by the embryos was determined using an immunoassay to measure disappearance of DOTH from solution. Approximately 13 nmol DOTH per g of tissue produced 100% inhibition of growth in pine embryo and leaf callus, a level similar to the lower end of the range that produces artificial lesions in pine needles (10,22). Other studies (3,20,24) have shown DOTH to be toxic to plant tissues. In all these cases, tissue was exposed to higher concentrations of DOTH than were used in this work, and no data on uptake of DOTH by plant tissue was reported. Stoessl et al. (24) reported that DOTH inhibited radicle elongation of freshly germinated seeds of fenugreek (*Trigonella foenum-graecum*), but no inhibition was observed for germinating seeds of wheat, tomato, or pea. We also have noted the absence of inhibition of growth of meristematic tobacco leaf callus at DOTH concentrations in excess of 2.6 µM DOTH per g of tissue (V. L. Mett, W. T. Jones, and P. H. S. Reynolds, *unpublished data*).

DOTH combined with equimolar concentrations of MAb 10C12 did not show any toxicity to pine embryos. This protection could have resulted either from decreased uptake of DOTH, due to the size of the DOTH/MAb complex, or from possible detoxification of DOTH by binding of the MAb to the biologically active part of the DOTH molecule. Similarly, the decreased toxicity of DOTH-MSA conjugates would be explained either by decreased uptake due to size or by reduced toxicity of the conjugated molecule.

Identification of molecules (receptors) that bind toxins has been achieved either by radiolabeling the small molecule or by using anti-idiotypic antibodies that mimic the toxin. For example, re-

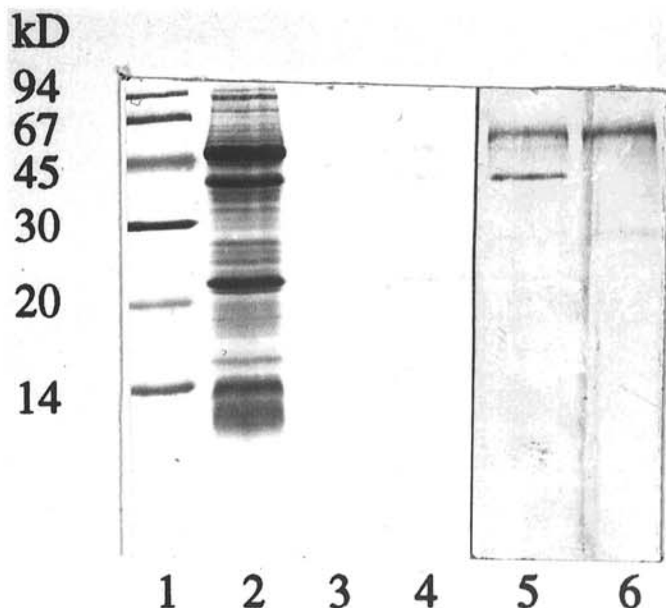


Fig. 4. Western blot of dothistromin (DOTH)-binding protein in mature pine embryo extracts. Embryos were macerated into phosphate buffered saline containing Tween 20 (PBST) (0.5 g ml^{-1}). Dynabeads (4×10^7) coated consecutively with anti-DOTH monoclonal antibody (MAb) 10C12 and DOTH-mouse albumin (MSA) were incubated in either the embryo extract (test beads) or PBST (control beads), washed and suspended in sodium dodecyl sulfate sample buffer (0.1 ml), boiled for 5 min, and the supernatant was recovered. Proteins were separated on a 15% polyacrylamide gel and transferred to poly(vinylidene fluoride) membrane. The membrane was either stained for protein (lanes 1–4) or probed with DOTH-MSA, MAb 10C12, and phosphatase anti-mouse immunoglobulin G and developed with nitro blue tetrazolium/bromochloro-indolyl phosphate substrate (lanes 5–6). Lane 1: molecular weight markers; lane 2: embryo macerate (10 µl); lanes 3 and 6: control beads; lanes 4 and 5: test beads.

ceptor molecules for the toxin victorin, produced by the fungus *Cochlibolus victoriae* in *Avena sativa*, have been identified by iodination of victorin (26) or by using anti-idiotypic mimics of victorin (2). These approaches have been unsuccessful for DOTH since iodination destroys DOTH and the anti-idiotypic antibodies produced so far have been of too low an affinity to be of use (W. T. Jones, *unpublished data*).

Therefore, we have developed a novel approach, using MAb 10C12 in combination with DOTH-MSA as a probe, to localize the binding sites of DOTH to small vesicles in pine embryos. Multiple DOTH molecules on mouse albumin allowed for reaction with DOTH-binding sites in tissue, leaving available multiple non-tissue–bound DOTH sites to which the MAb 10C12 could bind and provide an amplified signal following the reaction with a labeled anti-mouse antibody. This approach allowed immunological visualization of DOTH-binding sites without the need for an anti-idiotypic antibody. Light microscopy, using either silver-enhanced gold labeling or phycoerythrin fluorescence, identified DOTH binding to small vesicles in pine embryos. Electron microscopy, using immunogold labeling, showed that these DOTH-binding sites were associated with protein bodies within the vesicles.

Based on the immunoprobe format for microscopy, a method which relies on DOTH immobilization on magnetic beads was developed to "pan" DOTH-binding proteins from a pine embryo extract. A protein complex, consisting of three individual peptides with molecular masses of 24, 40, and 50 kDa, was isolated, and the 40-kDa peptide was identified by Western blotting as the DOTH-binding peptide. This is the first report of localization of DOTH-binding sites in any plant tissue and may represent isolation of a component of a specific DOTH receptor system.

Preliminary results with mature pine needles (W. T. Jones, D. Harvey, S. D. Jones, P. W. Sutherland, M. J. Nicol, N. Sergejew, P. M. Debnam, N. Cranshaw, and P. H. S. Reynolds, *unpublished data*), using the histological probes described above, indicated the presence of DOTH-binding sites associated with chloroplasts. Future studies will investigate the relationship between the putative embryo DOTH receptor and the chloroplast-associated DOTH binding observed in mature pine needles to understand the basis of the toxicity caused by DOTH.

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