

# A Quantitative Bioassay for Necrosis Toxin from *Pyrenophora tritici-repentis* Based on Electrolyte Leakage

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## ABSTRACT

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*Pyrenophora tritici-repentis*, the causal agent of tan spot of wheat, produces a cultivar-specific toxin (Ptr necrosis toxin) closely associated with the disease symptomatology. We initiated studies aimed at the mode-of-action of Ptr necrosis toxin and, as a first step, report here a bioassay for toxin based on electrolyte leakage. Toxin exposures of 4 h or more caused enhanced electrolyte leakage from wheat line ND495 (toxin sensitive), but not cv. Erik (toxin insensitive), relative to water-treated controls. Electrolyte leakage increased with exposure times up to 12 h, the longest time tested, when toxin concentration and leaching time were

held constant. Electrolyte leakage increased with toxin concentration between 1 and 20  $\mu\text{g ml}^{-1}$ , when exposure and leaching times were constant. All toxin-sensitive wheats tested (based on toxin-induced necrosis) showed enhanced electrolyte leakage, and all insensitive wheats did not. A toxin exposure of 4 h permitted completion of the assay in 1 day. We used this rapid assay to demonstrate that incubation of ND495 seedlings at 30°C for 24 h prior to toxin exposure causes a loss of toxin sensitivity. Enhanced electrolyte leakage is the most rapid response to toxin observed to date. However, the toxin exposure times required for leakage to develop (4 h or longer) suggest that effects on the plasmalemma are secondary and occur relatively late in the development of necrosis.

*Additional keywords:* *Triticum aestivum*.

Tan spot, caused by *Pyrenophora tritici-repentis* (Died.) Drechs. (anamorph *Drechslera tritici-repentis* (Died.) Shoemaker), has emerged as one of the most persistent and widespread foliar diseases of wheat (*Triticum aestivum* L.) worldwide (8,14,15). Disease lesions typically possess a necrotic center surrounded by a chlorotic margin (9). Biochemical and genetic evidence indicates that the necrosis is associated with a 13.2-kDa protein (Ptr necrosis toxin) produced by the fungus (1,10,11,21-23). Ptr necrosis toxin is selective for certain cultivars and lines of wheat.

The only bioassay described to date for Ptr necrosis toxin, referred to here as the necrosis bioassay, is based on the reaction of wheat leaves to toxin over a 48- to 72-h incubation period. The necrosis bioassay has been used for many purposes in this system including toxin purification (1,22,23), genetic analysis of host reaction to toxin (5,10,11), effect of temperature on toxin action (12), and identification of potential sources of resistance (17). There have been advances in these areas, but a gap exists in our understanding of the mode-of-action of Ptr necrosis toxin. A major obstacle to progress is the lack of a rapid, simple, and quantitative bioassay for Ptr necrosis toxin.

It has been argued that the physiological basis of a bioassay should reflect the mode-of-action of the toxin in question (24). Little is known of the effect of Ptr necrosis toxin on wheat beyond rapid damage to the plasmalemma detected by transmission electron microscopy (TEM) (6). No primary target for Ptr necrosis toxin has been proposed, but the TEM results suggest that electrolyte leakage might provide a physiological basis for a quantitative bioassay. Electrolyte leakage has been used as a quantitative

bioassay for the host-selective toxins victorin (4), HS toxin (18), and PC toxin (3), even though there is little or no direct evidence that the plasmalemma is the primary target in these systems (16,19). Electrolyte leakage bioassays are rapid, technically simple, and require no special equipment other than a conductivity meter. They have been used to investigate the effect of temperature (3), metabolic inhibitors (7), and competitive inhibitors (13) on the action of host-selective toxins. The purpose of this research was to investigate the possibility of developing electrolyte leakage into a quantitative bioassay for Ptr necrosis toxin.

## MATERIALS AND METHODS

**Wheat cultivars and fungal strain.** All experiments made use of hard red spring wheats obtained from the North Dakota State University breeding program. Ptr necrosis toxin was purified from culture fluids of strain 86-124 of *P. tritici-repentis*, which was obtained from L. Lamari, University of Manitoba. Strain 86-124 has been used extensively by Lamari and coworkers for the production of Ptr necrosis toxin (1,11,12).

**Toxin production and purification.** Ptr necrosis toxin was produced on Fries medium supplemented with 0.1% yeast extract as described by Ballance et al. (1). Details of toxin purification are described elsewhere (25). Culture fluids containing toxin were filtered through Whatman filter paper, and the filtrates were centrifuged for 20 min at 35,000  $\times g$ . The supernatant was filtered through nylon (0.45  $\mu\text{m}$ ), and the resulting filtrate was dialyzed overnight against 20 mM sodium acetate buffer (pH 4.8) in tubing with a 3,500-molecular weight cutoff. The dialyzed extract was loaded onto a S-sepharose Fast Flow cation exchange column (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), and then was washed with four column volumes of 20 mM sodium acetate buffer (pH 4.8). Ptr necrosis toxin was eluted with a linear gradient (0 to 300 mM) of sodium chloride. Active fractions were

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concentrated under vacuum at room temperature and fractionated by fast protein liquid chromatography (FPLC) on a HiLoad Superdex 30 FPLC size exclusion column (Pharmacia LKB Biotechnology Inc.). Chromatography was in a buffer of 20 mM sodium acetate (pH 4.8) and 150 mM NaCl. Toxin-containing fractions were desalted and loaded onto a cation exchange high-pressure liquid chromatography column (Sperogel TSK 5P-5P-5PW; Beckman Instruments Inc., Fullerton, CA). Toxin was eluted as a sharp peak with a linear gradient of NaCl (0 to 300 mM). No contaminating proteins were revealed when purified toxin was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with either silver or Coomassie brilliant blue (25). Protein was quantified by the method of Bradford (2).

**Toxin infiltration and bioassays.** Plants were grown in plastic cones (Stuewe & Sons, Inc., Corvallis, OR) in the greenhouse for approximately 17 to 21 days with a 16-h photoperiod until the third leaf was one-half expanded. Wheat seedlings were transferred to a growth chamber (20°C) 24 h prior to infiltration. Water or toxin in water was infiltrated into the middle portion of the second true leaf as described (5). All experiments made use of purified toxin.

The necrosis bioassay was as described (5). Seedlings used for electrolyte leakage experiments were incubated in the growth chamber for a defined toxin exposure period. After the toxin exposure, a razor blade was used to obtain a single 2.5-cm leaf section from the infiltrated region of each leaf. The cuts were made so that the leaf section contained the infiltration site. Unless otherwise stated, each treatment made use of 10 leaf samples divided into two replicates of five 2.5-cm leaf sections each. Leaf sections of each replicate were wrapped in cheesecloth and vacuum-infiltrated with distilled water for 15 min. Leaf sections were removed from the cheesecloth, gently blotted, and placed in 15 ml of distilled water for leaching of electrolytes. Conductivity of the ambient water was determined at the beginning of the leaching time and at various intervals thereafter with an Orion (model 160; Orion Research, Inc., Beverly, MA) conductivity meter. Each data point is the average conductivity of the two replicates. Each experiment was repeated at least two times with similar results.

**Effect of temperature on necrosis and electrolyte leakage.** Greenhouse-grown ND495 plants were separated into groups that were preincubated at 20 or 30°C for 24 h prior to infiltration with water or toxin (7  $\mu\text{g ml}^{-1}$ ). Plants subjected to the necrosis bioassay were returned to their preincubation temperature after infiltration of experimental solutions and evaluated after 72 h of incubation.

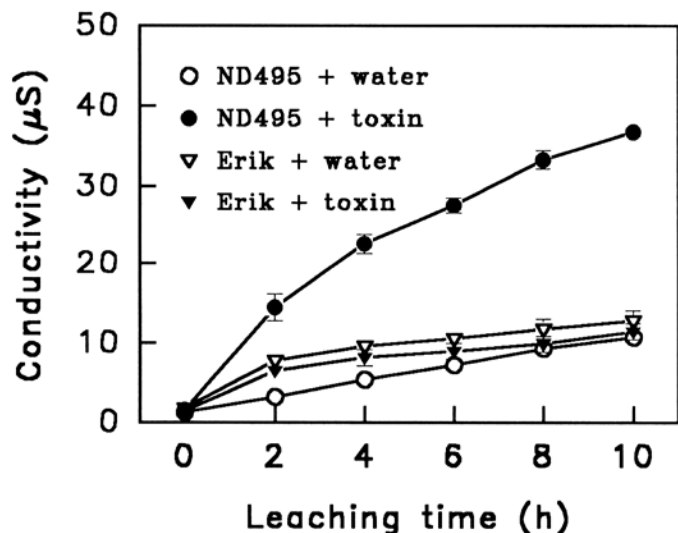


Fig. 1. Electrolyte leakage from ND495 and Erik as a function of leaching time after 12 h of exposure to water or toxin at 7  $\mu\text{g ml}^{-1}$ . Standard deviations, in some cases smaller than the symbols, were plotted for each point.

tion. For experiments on the effect of temperature on electrolyte leakage, seedlings were preincubated at the two temperature regimes for 24 h, and then were infiltrated at 20°C. Exposure was for 4 h at 20°C, regardless of the preincubation temperature, and conductivity was determined as described above. Plants were not incubated after infiltration at the elevated temperature in the conductivity experiment.

## RESULTS

**Effect of Ptr necrosis toxin on ND495 and Erik wheats.** Toxin-treated ND495, but not Erik, developed tan necrosis by 3 days after infiltration with Ptr necrosis toxin at concentrations ranging from 1 to 20  $\mu\text{g ml}^{-1}$  (data not shown). The time course of tan necrosis development was similar to that reported by other groups (1,22,23).

**Effect of Ptr necrosis toxin on electrolyte leakage.** Preliminary experiments aimed at detecting electrolyte leakage from toxin-treated ND495 were modeled after those of victorin, PC toxin, and HS toxin (3,4,18). These experiments, which involved vacuum infiltration of toxin (7  $\mu\text{g ml}^{-1}$ ) into excised leaf sections and an exposure time of 1 h, revealed no enhanced electrolyte leakage from ND495 over water-treated controls (data not shown). However, when Ptr necrosis toxin was infiltrated into the leaves of intact seedlings, as is done for the necrosis bioassay, and exposure was for 12 h, electrolyte leakage increased over a 10-h leaching period in a cultivar-specific manner (Fig. 1). Conductivity increased from ND495, but not Erik, with toxin concentrations between 1 and 20  $\mu\text{g ml}^{-1}$ , the highest concentration tested, with 12 h of exposure to toxin and a leaching time of 6 h (Fig. 2). Cultivar specificity was also demonstrated with electrolyte leakage. All wheat cultivars and lines that developed necrosis in response to Ptr necrosis toxin exhibited enhanced electrolyte leakage, and all wheats that failed to develop necrosis did not show enhanced electrolyte leakage (Table 1).

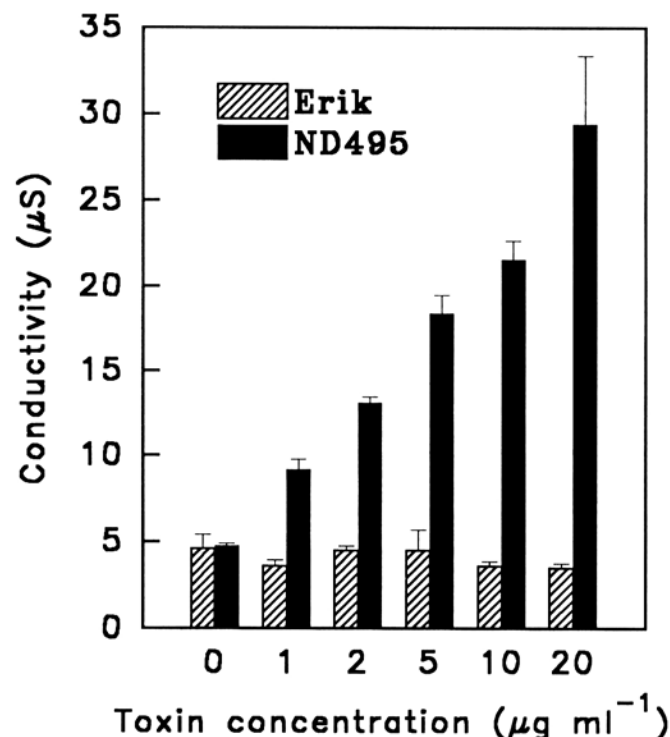


Fig. 2. Electrolyte leakage from Erik and ND495 as a function of toxin concentration. Seedlings were given 12 h of exposure to water or toxin at the concentrations indicated. Conductivity values were obtained after 6 h of leaching in distilled water. Toxin concentration 0 = water control. Error bars = standard deviation.

Experiments reported heretofore made use of a 12-h exposure time to toxin coupled to leaching times of 6 h. In an effort to make the assay more rapid, we investigated the effect of reduced toxin exposure time. Cultivar-specific increases in electrolyte leakage were detected with toxin exposures as short as 4 h (Fig. 3). Four-hour exposures to toxin permitted completion of the assay in 1 day, but reduced the magnitude of leakage (Fig. 3). Two-hour exposures to toxin gave variable results and were considered unreliable (data not shown).

**Effect of preincubation temperature on ND495.** Necrosis failed to develop in ND495 seedlings held at 30°C for 24 h prior to and 3 days after toxin exposure. In comparison, necrosis developed within 3 days when the necrosis bioassay was conducted at 20°C (data not shown). Conductivity measurements for plants preincubated at 20°C and treated with water were  $5.0 \pm 0.9 \mu\text{S}$ ; measurements from toxin-treated plants at this temperature were fourfold higher at  $20.8 \pm 0.1 \mu\text{S}$ . However, there was no significant difference in electrolyte leakage from plants preincubated at 30°C and exposed to water or toxin; conductivity measurements were  $6.6 \pm 0.1$  and  $6.9 \pm 0.5 \mu\text{S}$ , respectively.

## DISCUSSION

We have provided the basis for a rapid and quantitative bioassay for Ptr necrosis toxin based on electrolyte leakage. In developing this procedure, electrolyte leakage was related to the variables of wheat genotype, toxin exposure time, toxin concentration, and leaching time after toxin exposure. Electrolyte leakage clearly increased during a 10-h leaching period after a 12-h exposure to toxin (Fig. 1). An important attribute of a reliable bioassay is that the plant response, in this case electrolyte leakage, increases in magnitude with the concentration of toxin (24). Electrolyte leakage was shown to increase with toxin concentrations between 1 and  $20 \mu\text{g ml}^{-1}$ , when toxin exposure and leaching times were constant (Fig. 2). This experiment, which covered only a 20-fold range in toxin concentrations, did not detect a saturation effect of toxin. It is possible that saturation could have been found had toxin concentrations covering two or three orders of magnitude been used (24). However, the toxin concentrations used are comparable with those reported in other studies where necrosis was studied in a dose/response manner (1,22,23). We detected a slight enhancement of electrolyte leakage with toxin at  $1 \mu\text{g ml}^{-1}$  (approximately 75 nM). Other groups have reported that the minimum toxin concentration for necrosis development is in the

TABLE 1. Electrolyte leakage and Ptr necrosis sensitivity in selected *Triticum* genotypes

| Wheat line <sup>b</sup> | Necrosis <sup>c</sup> | Conductivity ( $\mu\text{S}$ ) <sup>a</sup> |       |
|-------------------------|-----------------------|---|-------|
|                         |                       | Water                                       | Toxin |
| BH1146                  | Yes                   | 2.2   | 28.4  |
| ND495                   | Yes                   | 3.5   | 28.6  |
| Grandin                 | Yes                   | 2.6   | 20.4  |
| Columbus <sup>d</sup>   | Yes                   | 3.8   | 12.6  |
| Glenlea                 | Yes                   | 2.7   | 38.0  |
| Amidon                  | Yes                   | 3.0   | 33.2  |
| Bergen                  | No                    | 2.4   | 2.3   |
| W-7976                  | No                    | 2.0   | 3.0   |
| Salamouni               | No                    | 2.0   | 2.2   |
| Erik                    | No                    | 3.4   | 2.5   |
| 6B365                   | No                    | 2.6   | 2.5   |
| 4B1149                  | No                    | 3.1   | 1.5   |

<sup>a</sup> Toxin exposure was 12 h and leaching time was 6 h. Samples consisted of six leaves divided into two replicates of three leaves each.

<sup>b</sup> The second leaf of 17-day-old seedlings was infiltrated with either water or toxin ( $7 \mu\text{g ml}^{-1}$ ).

<sup>c</sup> Seedlings were evaluated 3 and 5 days after infiltration. No seedlings developed necrosis in response to water. All genotypes that developed necrosis in response to toxin did so by day 3.

<sup>d</sup> Reported as heterogenous for reaction to Ptr necrosis toxin (10,17).

range of 60 to 90 nM (22,23). Thus, the assay is sensitive to relatively low levels of toxin.

All toxin-sensitive lines tested developed electrolyte leakage in response to toxin, and all insensitive lines did not (Table 1). This indicates that the procedure has wide applicability and suggests that similar processes may be affected in toxin-sensitive lines. The data in Table 1 also suggest that the electrolyte leakage bioassay can quickly identify wheats insensitive to Ptr necrosis toxin, and this may facilitate breeding efforts.

Exposure of ND495 to toxin for 4 h or more resulted in electrolyte leakage greater than controls (Fig. 3). This indicates that the assay can be varied to suit the needs of a particular experiment. Exposure times as short as 4 h allow the experiment to be completed in 1 day and provide quantitative differences between cultivars. However, longer exposure times increased the amount of leakage in ND495 (Fig. 3).

Lamari and Bernier (12) demonstrated that high temperature ( $\geq 27^\circ\text{C}$ ) caused wheat plants to lose susceptibility to *P. tritici-repentis*, which was correlated with the loss of sensitivity to Ptr necrosis toxin at the restrictive temperature. Those experiments relied on the necrosis bioassay, and this made it impossible to discern whether the loss of toxin-sensitivity at the restrictive temperature was because of an effect on the plant or on the toxin molecule (12). The usefulness of the electrolyte leakage bioassay was demonstrated by examining this question. In our conductivity experiments, ND495 seedlings were preincubated at 20 or 30°C for 24 h, and then were infiltrated with water or toxin at 20°C and given a 4-h exposure at 20°C to toxin or water, regardless of their preincubation temperature. Thus, toxin was never incubated at the higher temperature. Toxin-induced electrolyte leakage developed in ND495 preincubated at 20°C, but not at 30°C, indicating that high temperatures cause the plant to lose sensitivity to toxin. This suggests that either toxin perception or a signaling pathway downstream of the toxin perception is temperature sensitive, as has been demonstrated with other host-selective toxins (3,20).

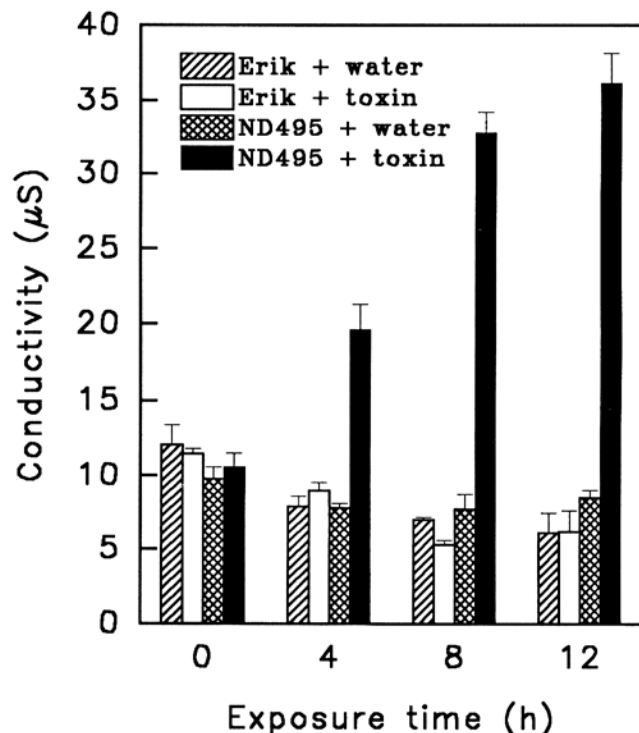


Fig. 3. Electrolyte leakage as a function of toxin exposure time. ND495 and Erik were exposed to water or toxin ( $7 \mu\text{g ml}^{-1}$ ) for the times indicated. Conductivity was determined after 6 h of leaching in distilled water. Error bars = standard deviation.

The electrolyte leakage bioassay will permit additional quantitative studies on various aspects of this toxin system. For example, Tuori et al. (23) found multiple peaks of toxin activity from culture fluids of the fungus. The electrolyte leakage bioassay may detect quantitative differences in the potency of various forms of Ptr necrosis toxin once they are purified. Further, the procedure should permit an examination of processes in the plant leading to necrosis. Electrolyte leakage is the most rapid physiological response to Ptr necrosis toxin observed to date, yet it requires a toxin exposure of at least 4 h. This suggests that the effects on the plasmalemma may be secondary to other events in the host. Thus, electrolyte leakage may be preceded by other toxin-induced processes in the plant such as signaling and active metabolism associated with toxin action (e.g., respiration, transcription, and translation). The bioassay will permit exploration of these possibilities, since any manipulation (e.g., inhibitors of metabolism or signaling) that interferes with toxin action should result in a reduction of electrolyte leakage.

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