Macromolecular Plant-Wilting Toxins: Artifacts of the Bioassay Method?

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ABSTRACT

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The use of plant cuttings to assay the wilt-inducing ability of potential phytotoxins is subject to misinterpretation. If the molecule being assayed has a molecular weight greater than ~20,000 daltons, it will physically induce wilt. Such a wilt response indicates only the plant's susceptibility to disruption of water movement by macromolecules and should not be confused with any toxic properties of the molecule. Quantitation of the wilt bioassay by visually assessing when wilting occurs is not reliable. Measurement of transpiration of the cuttings is a better method of quantitation, but this also lacks precision. In evaluations of the wilt bioassay, no correlation was found between resistance in alfalfa to *Corynebacterium insidiosum* and wilt induced by the large glycopeptide isolated from cultures of this bacterium.

Additional key word: phytoaggressins.

The bioassay is fundamental to experimental biology. When properly designed and conducted, a bioassay will assess the relative activities of biologically active molecules. If incorrectly understood and used, however, bioassays give artificial responses. The use of plant cuttings in wilt bioassays for phytotoxins is probably the most abused bioassay currently used by plant pathologists.

The current body of literature defines wilt toxins as pathogen-produced molecules that, when applied to plant cuttings in low concentration, will induce wilt in a short period of time. A number of mechanisms of wilting induced by phytotoxins can be invoked (4): interference with water movement through the cuttings, loss of stomatal response to low leaf water potential (Ψ), and loss of membrane semipermeability. The first two mechanisms have been demonstrated for certain wilt-inducing molecules; the last one, while commonly suggested, has not been. A major problem with the wilt bioassay is that it cannot distinguish among these mechanisms.

Previous work in this laboratory has demonstrated that plants are quite susceptible to disruption of water movement through the xylem by very small quantities of macromolecules (12). From this work, it is clear that if the suspected wilt toxin is a macromolecule it will cause wilt by physically interfering with water movement through the cutting, regardless of any other activity it may possess. Unfortunately, few reports of macromolecule wilt toxins have considered the potential of these macromolecules to physically disrupt water transport.

Alternatives to the use of plant cuttings in wilt bioassays are available. Xylem conductivity (11), stomatal aperture (10), or membrane integrity studies (3) can be used to directly determine wilt-inducing mechanisms of molecules: unfortunately, these procedures are infrequently used. The measurement of wilt, using plant cuttings as a bioassay, will probably continue. Thus, it is important to understand how macromolecules can affect this bioassay. The objective of this paper, therefore, is to examine the effect of solutions of macromolecules on the wilt toxin bioassay.

MATERIALS AND METHODS

Alfalfa cuttings for transpiration studies were taken from seedling-grown plants of cultivar DuPuit or from clones (obtained from Donald Barnes, USDA, SEA, St. Paul, MN) with known genetic resistance to *Corynebacterium insidiosum.*

Cuttings were taken from the terminal 8–10 cm of alfalfa shoots just before dawn. The cut was made with a razor blade and then the shoot was recut under filtered, degassed, distilled water 2–3 cm above the original cut. The cuttings were quickly transferred to

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preweighed tubes containing about 5 ml of filtered, degassed, distilled water. Before weighing, the tubes were filled with the water and covered with parafilm (American Can Company, Greenwich, CT 06830). A single cut was made in the parafilm to allow insertion of the cutting without permitting evaporation from the tube. The cuttings were placed in a plant growth chamber (22 C, 30% RH, and under a light intensity of 210 μeinsteins $m^{-2} \cdot sec^{-1}$). After 2 hr, the cuttings were transferred to a second set of preweighed tubes of water. This transfer was to eliminate all cuttings unable to transpire because they had been damaged in the cutting process. After another 2 hr, the cuttings were again transferred, this time to preweighed tubes of the test solutions or the water controls. At the end of the experiment, all tubes were reweighed to determine relative transpiration of the cuttings in each tube. When absolute transpiration values were determined, leaf areas of the cuttings were measured with a leaf area meter (Model LI-3000; Lambda Instrument Company, Lincoln, NE 68504).

The dextrans (purchased from Pharmacia Fine Chemicals, Piscataway, NJ 08854) used in these experiments had the following molecular weights: Dextran 20, 21,800; Dextran T40, 40,000; Dextran T70, 72,500; Dextran T110, 106,000; Dextran T150, 154,000; Dextran T250, 253,000; Dextran T500, 500,000; and blue dextran, 2,000,000. All dextran solutions were dissolved and passed through a 0.45-μm Millipore filter before use.

The glycopeptide produced in culture by *C. insidiosum* was a gift of Gary Strobel, Department of Plant Pathology, Montana State University, Bozeman. This glycopeptide was also isolated in our laboratory from cultures of a strain of *C. insidiosum* obtained from Strobel. Purification procedures were as described (8) except that we found it necessary to add 10 μg/ml $CuSO_4$ to the medium for production of the reported blue glycopeptide.

RESULTS AND DISCUSSION

Dextrans (α-D (1-6) glucans) were used as model macromolecules since they are carbohydrates, as are most reputed macromolecule wilt toxins, and are readily available in a wide range of defined molecular weight classes. The glycopeptide produced in culture by *C. insidiosum* is of interest since it has been reported to have host-selective properties. Alfalfa cultivars most susceptible to the wilt pathogen are reported to be more susceptible to wilt induced by the glycopeptide than are resistant cultivars (9). Therefore, this macromolecule (5×10^6 daltons) is of particular interest in our wilt bioassay studies.

Effects of macromolecules on transpiration. The effects of two different-sized macromolecules on transpiration of alfalfa cuttings over a period of 3 hr are illustrated in Fig. 1. This set of data was collected by first allowing the cuttings to transpire in water for 2 hr (0 time values) and then transferring them to the test solutions. After each hour, one third of the cuttings were removed and transpiration was determined. During the first hour in the test solutions, the transpiration of all cuttings declined. After the first hour, cuttings in water, but not those in solutions of macromolecules returned to their previous transpiration rates. The greatest effects of the macromolecules on transpiration occurred during the first hour, particularly those of the 5×10^6 dalton glycopeptide. During that hour, in which transpiration was greatly reduced and wilt occurs, the cuttings took up an average of 50 pmol of the glycopeptide per square centimeter of leaf area. Considering that most cuttings had approximately 6.5 cm^2 of leaf area, very little of the glycopeptide (250 $\mu g/cm^2$) was required to induce wilt. The 5×10^5 dalton dextran also reduced transpiration during the first hour, although not to the extent that the larger molecule did.

The effect of molecular weight of a molecule on transpiration of cuttings is better illustrated in Fig. 2. An identical relationship between molecular weight of molecules and their effect on xylem conductivity was reported earlier (12). Molecules with molecular weights of 7×10^4 dalton and less had no effect on transpiration of cuttings after 2 hr. After 8 hr, however, these smaller molecules significantly reduced transpiration and frequently caused wilt. Transpiration of cuttings in water was only slightly more reduced after 8 hr than at 2 hr. These results were not unexpected and probably only reflect the site of blockage. The molecules with molecular weights greater than 7×10^4 daltons reduce xylem

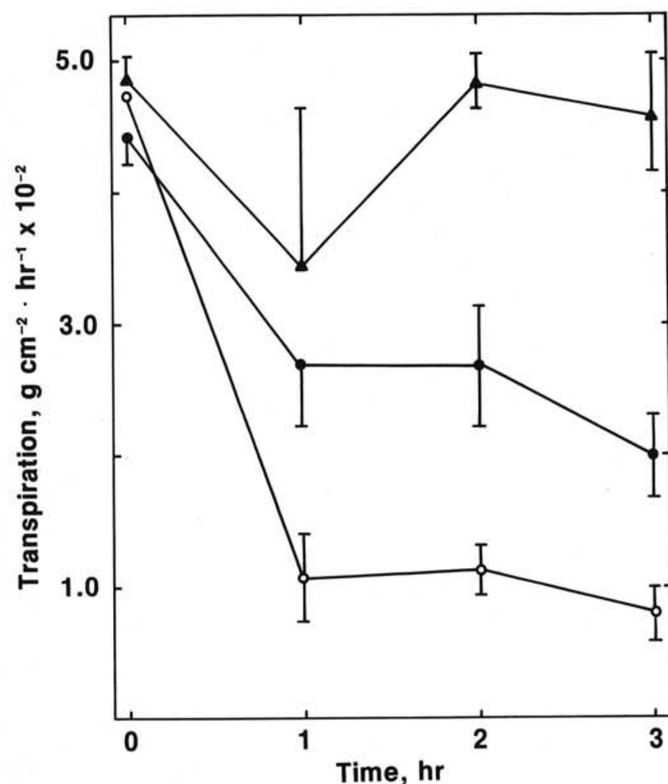

Fig. 1. Transpiration of alfalfa cuttings in water (–▲–), 5×10^5 dalton dextran (–●–), and the 5×10^6 dalton glycopeptide from *Corynebacterium insidiosum* (–o–), as influenced by time. The macromolecules were at a concentration of 200 μg/ml. The cuttings were placed in water for 2 hr before transferring the test solutions. The 0 time transpiration was that of the cuttings in water. The bars represent twice the standard error.

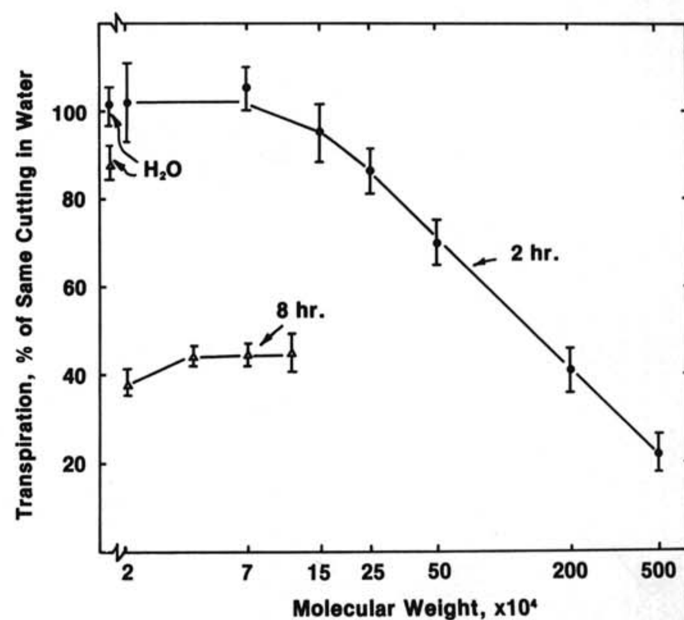

Fig. 2. Transpiration of alfalfa cuttings in solutions of macromolecules of different molecular weights. Data are expressed as the transpiration rate of a cutting in the test solution divided by the transpiration rate of the same cuttings in water multiplied by 100. One set of data (–●–) was obtained using macromolecule concentrations of 200 μg/ml and transpiration times of 2 hr. The second set of data (–Δ–) was obtained using concentrations of 400 μg/ml and transpiration times of 8 hr. All macromolecules were dextrans, with the exception of the 5×10^6 dalton glycopeptide from cultures of *Corynebacterium insidiosum*. The bars represent twice the standard error.

conductivity (12). The dextrans with molecular weights of 7×10^4 daltons or less probably accumulate on primary cell walls in the leaf mesophyll, interfering with water movement from leaf veins to the evaporative surfaces. Carpita et al (2) demonstrated that dextrans with molecular weights as low as 6,500 daltons could not move through primary cell walls of plants. If these molecules cannot pass through the apoplastic sites of water movement, they will accumulate and eventually interfere with normal water transport. The longer times required for these smaller molecules to affect transpiration probably indicates that more molecules are required for such blockage than are required for xylem blockage by larger molecules.

The effects of different concentrations of three macromolecules on transpiration after 2 hr are shown in Fig. 3. The general shape of the curve relating concentration to transpiration is similar to that of time to transpiration. Data from either Fig. 1 or 3, when replotted as a relationship between weight of macromolecule taken up by the cutting versus transpiration, yield the same shape of curve. All of these relationships indicate that, as molecules are introduced into the transpiration stream, they reduce water movement to a level that appears to be unique for each size of molecule. Further significant reductions of water movement do not occur within 2–3 hr no matter how much more of the molecule is added. The best model for explaining this behavior has the macromolecules plugging holes in the xylem, with large molecule(s) being able to plug more holes than small ones.

Bioassay methods. The above data describe important parameters for measuring the effects of macromolecules on transpiration of plant cuttings. We have found that measurements of transpiration rates, either relative (Figs. 2 and 3) or absolute (Fig. 1) are the best means of quantitating the wilt bioassay. The traditional visual assessment of wilt lacks quantitation. Attempts to quantify data from wilt bioassays have ranged from using a "wilt-o-meter" (7) to measure relative plant turgidity, to using a measure of the time required to induce wilt (5). The problem with many wilt assays is that they provide no meaningful data concerning a plant's water relations and, in fact, can be misleading. Table 1 compares two methods of obtaining quantitative data on the effects of the *C. insidiosum* glycopeptide on alfalfa cuttings. It is clear from these data that visually assessing the number of plants that have wilted after 2 hr could give misleading results. Although plants in 50–200 μg/ml glycopeptide wilted within 2 hr, only some of the cuttings in 400–800 μg/ml glycopeptide wilted. The transpiration data show that, as expected, cuttings in the highest concentrations of the glycopeptide transpired the least. The results of the visual assessment were undoubtedly the result of cuttings in the highest concentrations wilting quickly and then regaining turgor by the time the assessment was made.

Precision of bioassay. One frustration in using the wilt bioassay is its lack of precision, which becomes particularly troublesome when subtle differences are sought, such as in the response of different cultivars or clones to wilt-inducing macromolecules. The procedures outlined in the methods section reduce as many variables as possible in this bioassay. Nevertheless, difficulties remain, such as those illustrated in Table 2 and large standard errors of the data presented in the figures. Table 2 reports the use of dextran (2×10^6 dalton) to determine whether genetically different clones would respond differentially to the dextran in the wilt bioassay. With four replications (Exp. I and II) differential responses were detected. They were, however, different in each experiment. A combined analysis of four different experiments of four replications each showed no differences between the responses of clones to the dextran. Many replications are clearly needed when using the wilt bioassay.

Host-selective wilt toxins. Differential cultivar responses in a wilt bioassay have been reported for the glycopeptide of *C. insidiosum* (9) and the macromolecule "amylovorin" produced by

TABLE 1. Comparison of two wilt toxin bioassay methods to measure the effects of a glycopeptide from *Corynebacterium insidiosum* on alfalfa cuttings

Concentration of glycopeptide (μg/ml)	Cuttings wilted after 2 hr (no./total)	Transpiration[a] (%)
0	0/4	92.5 ± 8.9
50	4/4	47.2 ± 4.3
100	4/4	33.5 ± 3.9
200	4/4	22.1 ± 2.8
400	2/4	21.3 ± 4.7
800	3/4	14.1 ± 2.3

[a] Transpiration percentage is determined by first measuring the basal rate of transpiration of a cutting in water. That cutting is then transferred to one of the glycopeptide solutions or water controls and transpiration rate again determined. The second transpiration rate is then expressed as a percentage of the first transpiration rate.

TABLE 2. Replication numbers required for wilt bioassay. Bioassay of 2×10^6 dalton dextran

Alfalfa clone	Transpiration[a] (%)		
	Four replications		Sixteen replications
	Exp. I	Exp. II	
B12	79.25 A	78.75 AB	69.63 A
B10	67.50 AB	54.25 BC	61.00 A
B15	61.00 ABC	55.25 BC	59.19 A
A12	60.00 ABC	61.25 BC	62.25 A
B14	59.25 ABC	68.00 BC	67.75 A
B13	58.75 ABC	71.50 ABC	64.63 A
A13	51.00 BC	46.75 BC	54.25 A
A10	47.25 BC	62.75 BC	54.75 A
A14	42.50 BC	94.00 A	68.94 A

[a] Transpiration of alfalfa cuttings with stems immersed for 2 hr in a solution containing 100 μg of 2×10^6-dalton dextran per milliliter. Data are expressed as the transpiration rate of a cutting in the dextran solution divided by the transpiration rate of the same cutting in water multiplied by 100. Means in the same column followed by a common letter do not differ significantly, $P = 0.05$, by the LSD test.

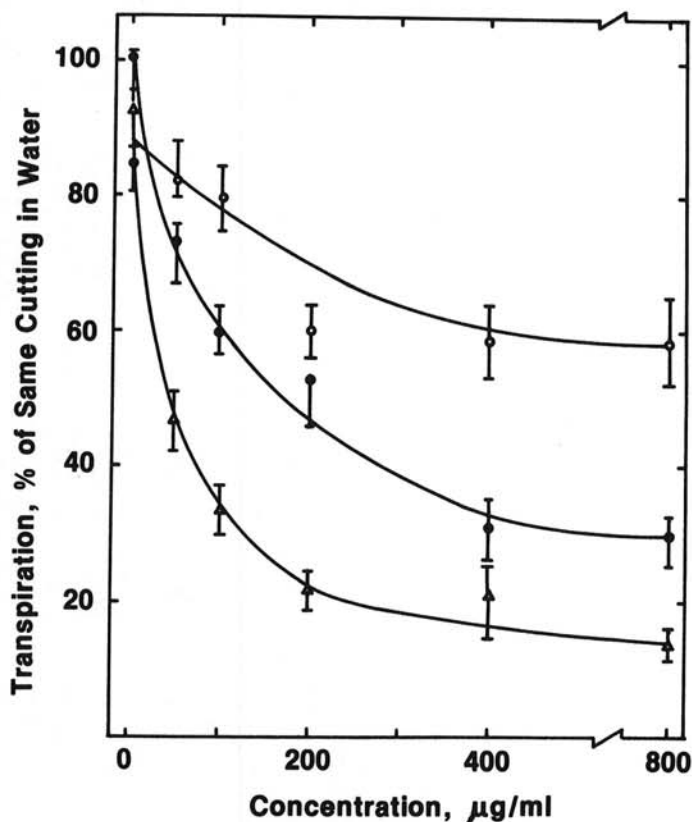

Fig. 3. Transpiration of alfalfa cuttings for 2 hr in different concentrations of 5×10^5 dalton dextran (–o–), 2×10^6 dalton dextran (–•–), and 5×10^6 dalton glycopeptide from *Corynebacterium insidiosum* (–Δ–). Data are expressed as in Fig. 2. The bars represent twice the standard error.

TABLE 3. Resistance of alfalfa clones to bacterial wilt and to disruption of transpiration by the glycopeptide produced by *Corynebacterium insidiosum*

Alfalfa clone	Mean transpiration[a] (%)	Infected plant phenotypic score[b]	Proposed plant genotype[c]
B14	69.5 A[d]	3	BBbbCccc
B13	60.7 AB	2	BBBBCccc
A14	59.8 BC	5	aaaa
B12	59.7 BC	2	BBBbCCcc
A13	56.1 BC	2	Aaaa
B11	55.9 BC	1	BBBbCCCc
A10	55.9 BC	1	AAaa
A11	54.1 BC	2	Aaaa
B15	52.5 BC	4	bbbbCccc
A12	50.2 CD	2	Aaaa
A15	42.5 DE	5	aaaa
B10	38.9 E	1	BBBBCCCc

[a] Transpiration of alfalfa cuttings for 2 hr in a 50-μg/ml solution of *C. insidiosum* glycopeptide. Data are expressed as the transpiration rate of a cutting in the glycopeptide solution divided by the transpiration rate of the same cutting in water multiplied by 100.

[b] A scale of 1–5 in which 1 represents resistance and 5 complete susceptibility to *C. insidiosum* (13).

[c] Resistance is dominant; susceptibility is recessive (13).

[d] Means followed by a common letter do not differ significantly, $P = 0.05$, by the LSD test.

Erwinia amylovora (5). In each case, it was reported that disease-susceptible cultivars were more susceptible to wilt in the bioassay than were resistant cultivars. These claims concerning amylovorin were challenged by Beer and Aldwinckle (1), who found no relationship between amylovorin and disease susceptibility.

Table 3 reports the results of a comparison between the effect of the glycopeptide of *C. insidiosum* on transpiration of cuttings of alfalfa clones and the susceptibility of the clones to the bacterium. The known genetics for resistance of these clones (13) are indicated in the table. The transpiration data represent the mean of 12 replications. The bioassay detected a differential response to the glycopeptide by the clones. This response was not correlated with expression of disease resistance by any of the three genes for resistance to the disease. These results confirm the report of Straley et al (9) that alfalfa cultivars react differentially to the glycopeptide; our data, however, do not support their report that the glycopeptide can be used to select disease-resistant cultivars of alfalfa among those with the three genes for resistance that we tested.

The data presented illustrate the difficulties that can result from misapplications of the wilt bioassay. Perhaps the greatest problem lies with misinterpretations of the results of the assay. Many macromolecules have been described as phytotoxins primarily because they are produced by pathogens, and in low concentrations cause plant cuttings to wilt. Such criteria for including wilt-inducing molecules as phytotoxins are clearly no more valid than considering *Leuconostoc mesenteroides* a plant pathogen because it produces copious quantities of wilt-inducing macromolecules (dextrans).

It is entirely possible, and in fact probable, that the susceptibility of plants to disruption of their water transport system by macromolecules is exploited by pathogenic microorganisms or insects. Such exploitation of the plant's susceptibility must be demonstrated, however, before we can assign wilt-inducing roles to pathogen-produced extracellular macromolecules.

If such a role is found for these macromolecules, they would be better termed phytoaggressins, as Graniti (6) proposed, than phytotoxins. This would avoid confusing these molecules, which affect plants by physical means, with those that directly affect cellular biochemistry or structure. A macromolecule could have both phytotoxin and phytoaggressin activities. From this study, it should be clear that molecules larger than approximately 20,000 daltons can cause plants to wilt. The work of Carpita et al (2) suggests that dextrans as small as 6,500 daltons could also induce wilt. Such macromolecules will have phytoaggressin activity in a wilt bioassay that must be distinguished experimentally from any other activity it may possess. The wilt bioassay as described in this paper will not distinguish between such activities. This assay should be used only after the mode of action of a wilt-inducing molecule has been determined. Obviously, it is unsuitable for any macromolecule that has phytotoxic activity.

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