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A Method of Validating the Tetrazolium Assay for Testing Viability of Dormant Jointed Goatgrass (*Aegilops cylindrica*) Seed[1]

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Abstract. **Rapid biochemical tests developed for measuring viability of nondormant crop seed have been used for measuring dormant weed seed viability. The objective of this experiment was to propose and test a new method for validating one of these assays, the tetrazolium assay, for determining dormant weed seed viability. Subsamples of partially dormant jointed goatgrass seed from the same seed source were periodically subjected to sequential germination and tetrazolium assays as they afterripened until seed were completely nondormant. Changes in percent germinated, percent tetrazolium positive (presumably dormant), and percent tetrazolium negative (presumably dead) seed were graphed versus time, and compared with models of valid and invalid cases. The validation method showed that the tetrazolium assay accurately determined the viability of seed remaining after a 3-wk germination period for jointed goatgrass seed removed from joints at the start, but underestimated the viability for seed afterripened and germinated in joints. The validation method showed that additional research is needed to refine the tetrazolium assay for this latter purpose. Nomenclature: Jointed goatgrass, *Aegilops cylindrica* #[3] AEGCY.**

Additional index words: **Afterripening, dormancy, germination, survival, tetrazolium assay, AEGCY.**

INTRODUCTION

Since the 1930s, seed scientists have evaluated rapid biochemical assays (vital staining) for determining crop seed viability in place of slower germination assays which require 1 to 3 wks to complete (16). Seed technologists define seed viability as the capacity to germinate under suitable conditions (3). The tetrazolium assay is probably the most widely used rapid biochemical assay for measuring crop seed viability, and reproducible standard procedures have been developed for most major crop species (2, 12, 16). In the tetrazolium assay, viable ungerminated seed that have been fully imbibed absorb a colorless dye, 2,3,5-triphenyltetrazolium chloride. Endogenous dehydrogenase enzymes in viable seed embryos convert it to a stable, nondiffusable red chemical, triphenylformazan. This color reaction measures endogenous dehydrogenase enzyme activity present in viable seed but absent in dead seed. Live embryos stain a deep red, whereas dead embryos do not; endosperm staining is not considered part of the tetrazolium assay. The topographic distribution or pattern of staining and the intensity of staining of the embryo indicate viability (5, 12). Viability in the tetrazolium assay is correlated with a seed's ability to germinate (13).

The tetrazolium assay has been used to determine viability of dormant weed seed populations as either a substitute for germination assays or to determine the number of viable dormant (i.e., tetrazolium positive) and dead (tetrazolium negative) seed remaining after germination (5). Viability of seed remaining after various periods of laboratory storage or field burial is often followed using the tetrazolium assay with or without preceding germination assays (4, 14, 18). The tetrazolium assay also has been used to follow the development of viability during weed seed maturation (6).

The tetrazolium assay has several recognized limitations for determining viability of certain types of crop and weed seed. These limitations concern how the tetrazolium assay is conducted and how seed staining is visually evaluated (13). For example, if weed seed are hard seeded or have seed coats which are impermeable to tetrazolium dye uptake, the seed coats must be removed before assaying seed for viability (such as wounding or removal of seed coats). Visual evaluation of staining in the tetrazolium assay also can be somewhat subjective and requires experience (5, 13), especially for small seed or seed that become discolored (i.e., browned or blackened) after extended burial in soil or weathering on the parent plant. Microorganisms contaminating seed can also cause false positive readings for seed viability (3).

Even though the tetrazolium assay is widely used to measure the viability of dormant weed seed (i.e., ability to germinate), there is no commonly accepted method for validating that it does so accurately. Although the tetrazolium assay has been extensively evaluated for use with crop seed, often it has been used on fully or partially dormant weed seed without first determining whether it was performing as expected. A method is needed to determine whether the execution of the tetrazolium assay or visual evaluation of staining is faulty or not for partially dormant weed seed.

The objective of this experiment was to propose and test a method for validating the tetrazolium assay for determining dormant weed seed viability, using jointed goatgrass as an example. Jointed goatgrass seed biology was reviewed recently (9). Freshly shed jointed goatgrass seed in joints (i.e., spikelets) were dormant (11), but seed in joints germinated between 52 and 75% 1 mo after collection (10). Jointed goatgrass seed remained viable after 2 yr of dry storage at laboratory temperatures (15).

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[3]Letters following this symbol are a WSSA-approved computer code from Composite List of Weeds, Revised 1989. Available from WSSA, 1508 West University Ave., Champaign, IL 61821-3133.

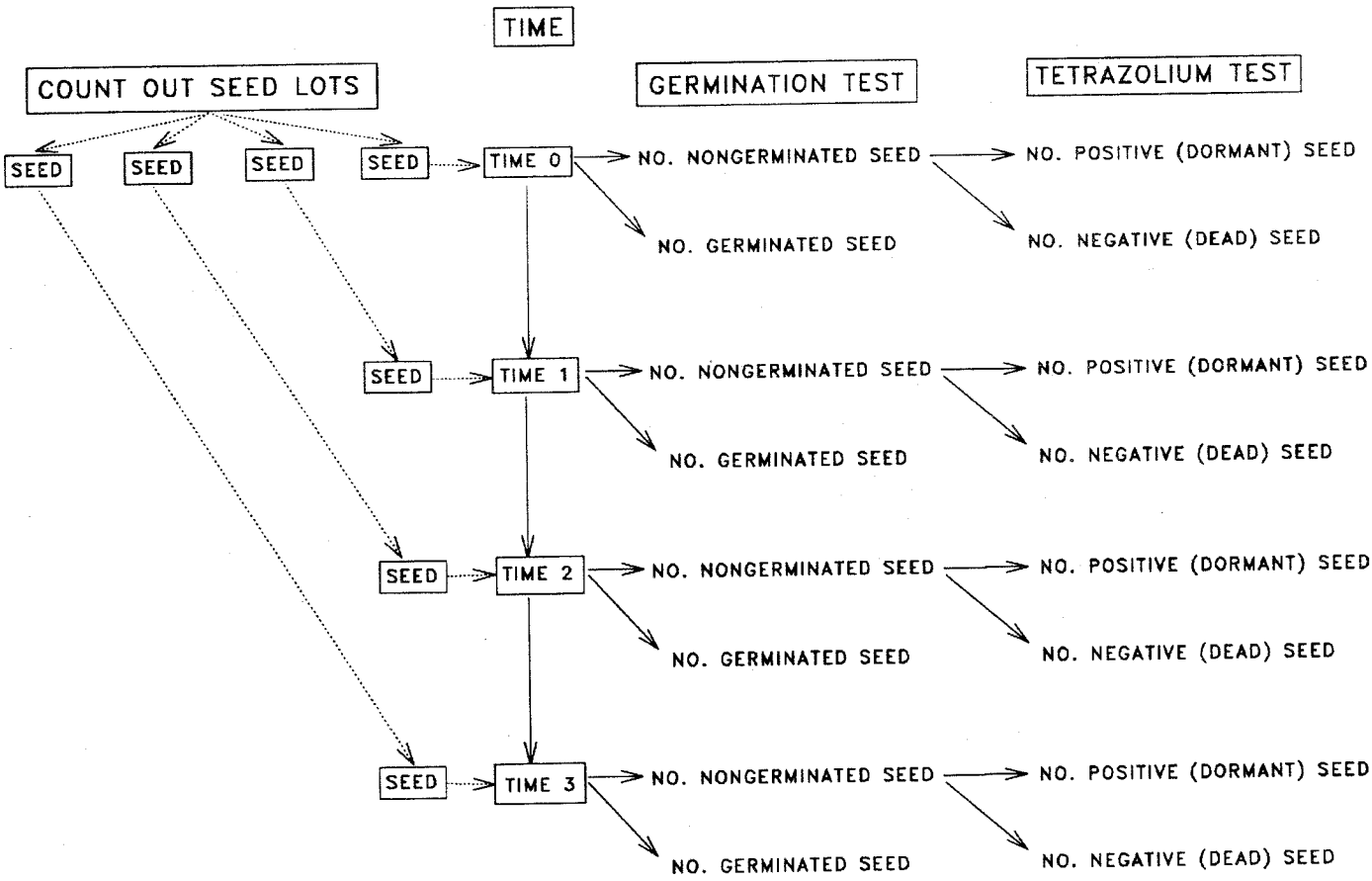

Figure 1. Flow chart of the experimental procedure used for validating rapid biochemical tests of dormant weed seed viability. Seed from a common seed source were counted out into lots. Then, different lots of seed were afterripened for various periods (arbitrary times 0 to 3) (dotted line) before they were subjected to germination assays. Any ungerminated seed remaining after completion of the germination assays were subjected to the tetrazolium assay for determining the number of TP (presumably dormant) seed and tetrazolium negative (presumably dead) seed. The percent germinated (G), percent tetrazolium positive (TP) seed, and percent tetrazolium negative (TN) seed were calculated as a percent of the total number of seed counted.

MATERIALS AND METHODS

Theoretical model cases. The proposed protocol for validating the tetrazolium assay for use on a dormant weed seed population is summarized as a flow chart (Figure 1). Percent germinated seed (G)[4], percent tetrazolium positive seed (TP)[4], percent tetrazolium negative seed (TN)[4], and percent germinated plus tetrazolium positive seed (G + TP) (≤ 100%) were assumed to sum to 100% in equation 1 and were monitored over time:

$$G + TP + TN = 100\% \quad (1)$$

The arbitrary period chosen for validation was assumed to be long enough that a change in percent G could be detected although it was not critical that all seed become nondormant over this period. As the dormant weed seed population afterripened, seed were assumed to become nondormant and capable of germinating under favorable environmental conditions (but not necessarily 100% G). Also, it was assumed that appropriate afterripening periods for validation differed between species. Finally, it was assumed that standard environmental conditions chosen for determining germination were optimum for germination (1). Freshly shed, mature seed would be expected to differ in initial percent germination between different species, seed sources for the same species, locations for the same species, years, afterripening periods, etc.

Logic dictates that graphs of changes in percent G, percent TN, and percent TP seed over time would behave in three hypothetical ways in the validation method (Figure 2). TP seed were assumed to be viable, dormant, and capable of germination, whereas TN seed were assumed to be dead. All three cases also were assumed to obey equation 1 (above) over the study period. The tetrazolium assay is proposed to be valid for

[4]Abbreviations: G = percent germinated seed; TN = percent tetrazolium negative seed; and TP = percent tetrazolium positive seed.

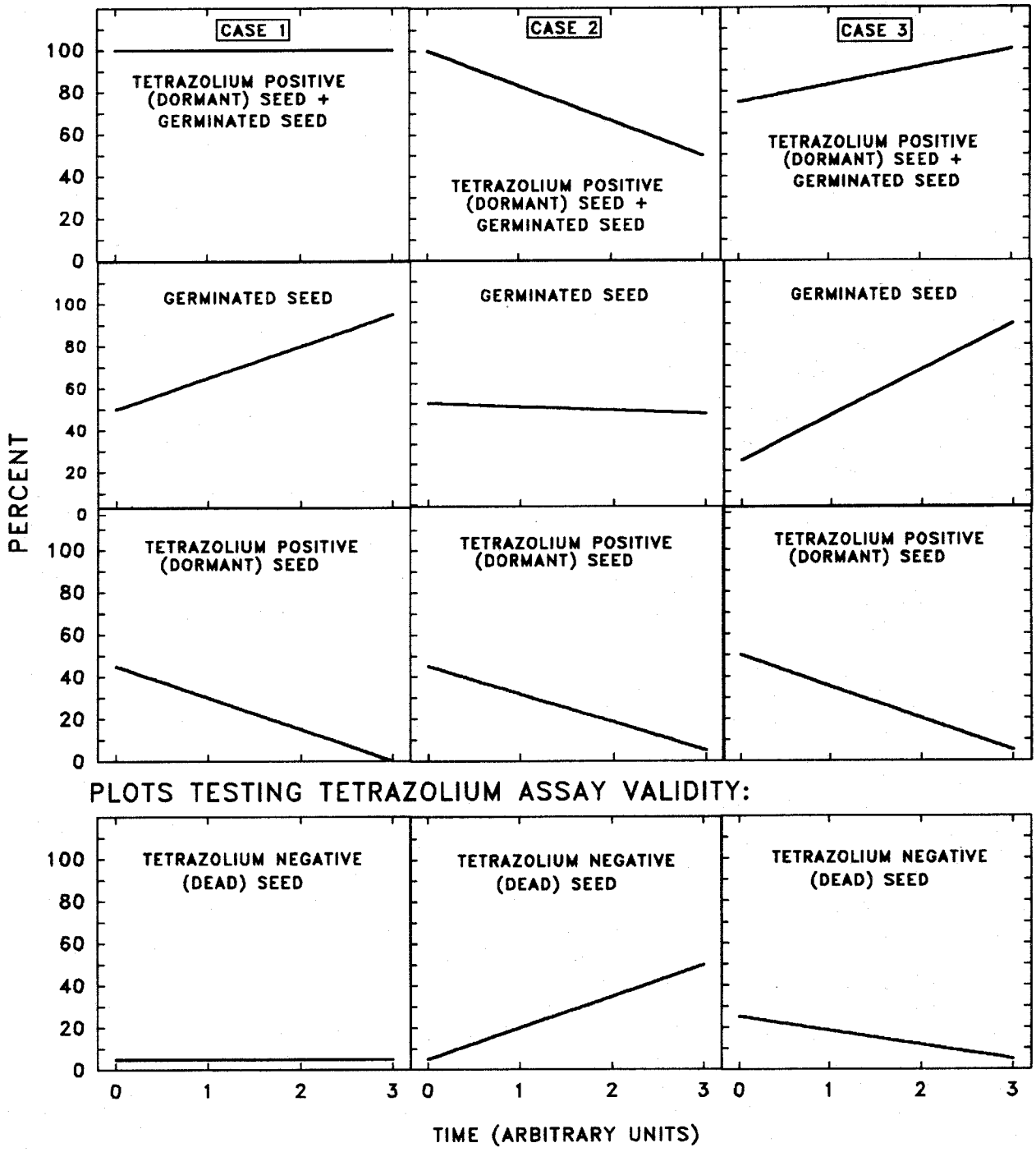

Figure 2. Logical theoretical model cases expected for valid (Cases 1 and 2) and invalid (Case 3) responses of totally or partially dormant weed seed over time in the tetrazolium assay. Only graphs of percent tetrazolium negative seed over time are needed for validation of the tetrazolium test. Note that the percent tetrazolium negative (apparent dead seed (D)) decreased with time in invalid Case 3, but either remained the same over time in valid Case 1 or increased over time in valid Case 2. See Materials and Methods for further explanation.

Cases 1 and 2, but invalid for Case 3 for the reasons described below.

Case 1 represents a hypothetical situation in which percent TN seed remain constant over time (Figure 2). Percent TP seed decrease over time to 0%, and percent G seed increase to 100% (Figure 2). In Case 1, the sum of percent TP plus G seed was assumed to remain constant over time after harvest at maturity. Thus, partially dormant seed were assumed to afterripen over

time and eventually become completely nondormant and capable of germinating under favorable conditions.

In Case 2, percent TN seed increase over time (Figure 2). Percent TP plus G seed was assumed to either decrease continuously over time or increase and then decrease over time to 0% (Figure 2). Case 1 seed populations may exhibit Case 2 behavior after a sufficiently long afterripening period because seed may die over time. Case 2 behavior is likely to be more common than Case 1 behavior.

In Case 3, percent TN seed were assumed to decrease over time. The only way percent TN seed could decrease over time would be if dead seed spontaneously became viable, a logical impossibility assuming that the tetrazolium assay was properly conducted and accurately estimated seed viability. Thus, if Case 3 occurred, the tetrazolium assay probably was conducted improperly and additional research would be needed to determine the cause and correct for it if the tetrazolium assay was to be used for determining viability of dormant weed seed of particular species. Case 3 behavior represents a high number of false negatives (i.e., tetrazolium assay fails to detect viable seed that are actually present). Percent TP plus G was assumed to increase over time in Case 3.

The validation method. One way to distinguish between these three cases and test them would be to regress percent TN seed versus afterripening time. For the hypothetical cases considered, changes in percent TN seed (as well as percent G, TP, and G + TP seed) have been represented as linear functions of time for simplicity (Figure 2). However, these variables may be nonlinear functions of time for actual weed seed. Nonlinearity and variation in the shape of the curves over time would not be expected to compromise the proposed validation method. In fact, in its simplest form a t-test could be conducted on percent TN seed at the beginning and end of an afterripening period. To establish Case 3 behavior, all that is needed is a statistical test demonstrating that the percent TN seed decreased over an arbitrarily long afterripening period. If a decrease or an increase in percent TN seed is detected with a before-and-after assay, no further testing would be required. But if no change is detected with a before-and-after assay, additional testing over time would be warranted until all seed reach 100% germination or either a decrease or increase in percent TN seed was detected.

Experimental test of theoretical model cases. Freshly shed, fully mature, air-dried, partially dormant jointed goatgrass seed were gathered from potted plants grown in the greenhouse, as previously described (7). Seed were gathered just before the experiment was started in April and May, 1982 for trials 1 and 2, respectively.

Treatments were time for afterripening after harvest at maturity and the presence or absence of the joint covering the seed. Seed in intact joints or seed removed from joints at the start of each trial were afterripened for 0, 1, 2, and 3 mo in darkness at 25 C (range from 23 to 26 C) and 16 to 18% relative humidity. When seed were removed from the joints covering them, tweezers were used to pull the endosperm end of the seed to detach the seed from the base of the joint. This avoided physical damage to the embryo although some endosperm cells may have been crushed or damaged. Visible damage to the endosperm was not obvious when dry seed were removed from joints at the start, but some damage to the endosperm occurred when seed were removed from joints after they had been imbibed for the 3-wk-long germination period (Figure 1).

Germination assay. After various periods of afterripening, seed in joints or seed removed from joints at the start were wrapped between layers of standard germination paper subirrigated with deionized water under uniform conditions in a germination cabinet in darkness at 20 C and 100% relative humidity for 3 wk (8). The germination temperature of 20 C was shown to be optimum for jointed goatgrass germination (17). Brief exposure to laboratory light did not influence germination of dormant jointed goatgrass seed. The number of germinated seed was counted at the end of 3 wk and the remaining ungerminated seed were removed from the joints and subjected to the tetrazolium assay to determine the number of TP (presumably dormant) seed and TN (presumably dead) seed (Figure 1). Then, the percent of seed in each category was calculated.

Tetrazolium assay. After the 3-wk-long germination assay, the remaining ungerminated seed were removed from joints if they had not been removed at the start. To conduct the tetrazolium assay, ungerminated seed were transferred to petri dishes containing filter paper and 2,3,5-triphenyltetrazolium solution. A 1% aqueous solution of tetrazolium chloride was prepared and the pH was adjusted to 7, if it was not between pH 5.8 and 7 (2). Then, seed were soaked for 24 h at 20 C before embryos were examined for red staining.

Experimental design and statistical analysis. A completely randomized design was used with 10 seedheads per treatment in trial 1 and 20 seedheads per treatment in trial 2. Percent germination of seed was greater in trial 2 than trial 1, presumably because afterripening was greater on parent plants. Seedheads were considered to be replicates and there were 20 to 25 seed per seedhead in both trials. Percentage data calculated per seedhead were subjected to analysis of variance and nonlinear regression analysis with and without arc sin square root transformation using SPSS-PC+ software[5]. Tablecurve software[6] was used to model either linear or nonlinear least squares regression equations for percent G, TP, TN, or G plus TP seed versus time for each trial. The Tablecurve software used the Levenburg-Marquardt algorithm to iteratively fit nonlinear equations by adjusting the equation parameters to minimize the goodness of fit of the chi-square parameter (19). The algorithm identified convergence when the coefficient of determination (R^2) remained unchanged for five consecutive iterations. The R^2 represents the proportion of variability due to the independent variable in the regression equation. The magnitude of the analysis of variance F-value, residual mean square error, and R^2, and inspection of plots of

[5]SPSS, Inc., 444 N. Michigan Ave., Chicago, IL 60611.

[6]TableCurve ver. 3.0. Jandel Scientific, 65 Koch Rd., Corte Madera, CA 94925.

residuals versus the independent variable were used to evaluate the adequacy of regression models. The slopes and intercepts of selected regression models are presented ± standard errors. All slopes and intercepts were significantly different from zero at $P \leq 0.01$.

Results for seed that were removed from joints at the start were averaged over the trials because trial by treatment interactions were nonsignificant. Data for seed afterripened in joints were presented by trial because there was a significant trial by treatment interaction. The interaction was probably caused by differences between trials in initial percent germination. All regression equations were statistically significant ($P \leq 0.0001$), as were slopes and intercepts ($P \leq 0.05$).

RESULTS AND DISCUSSION

Jointed goatgrass seed removed from joints at the start (see bottom panel of Figure 3) exhibited Case 2 behavior (i.e., TN seed increased over time) (see Materials and Methods and the bottom panel of Figure 2). Percent TN (presumably dead) seed increased and percent TP (presumably dormant) seed decreased over 3 mo, whereas percent G seed increased before peaking in month two for jointed goatgrass seed that were removed from joints (Figure 3). After the percent G seed peaked, the percent TN (presumably dead) seed increased. The net result was that the percent G plus TP seed decreased slightly toward the end of the experiment. Seed behavior shown in Figure 3 is expected and is consistent with a properly conducted tetrazolium assay.

Seed afterripened in intact joints exhibited Case 3 behavior in both trials, although the change over time differed between trials (see bottom panels of Figure 4). In Case 3 behavior, the percent TN (presumably dead) seed decreased over time, a logical impossibility if the tetrazolium assay is accurately distinguishing between TP (dormant) and TN (dead) seed. Case 3 behavior indicated the tetrazolium assay was faulty for use with intact joints as described here and suggested that the tetrazolium assay would need to be modified if it is to supply valid information on the viability of jointed goatgrass seed afterripened and germinated in intact joints.

Jointed goatgrass seed that were removed from joints at the start of the experiment before afterripening began (Figure 3) behaved differently in the validation method than did jointed goatgrass seed afterripened in intact joints and imbibed in intact joints for 3 wk to determine percent germination before conducting the tetrazolium assay (Figure 4). Both treatments (afterripening as isolated seed or seed in joints) employed seed from the same seed source which were afterripened under identical environmental conditions. Seed in joints and seed removed from joints also were imbibed for a 3-wk-long germination period before being subjected to the tetrazolium assay. The major differences between treatments is that seed removed from joints at the start were dry (Figure 3), whereas seed afterripened in intact joints were imbibed for a 3-wk-long germination period before being removed from joints for the tetrazolium assay (Figure 4). Why relatively more seed would be tetrazolium negative at the start in the latter case is unknown. This response may represent excision-induced mechanical damage and death of dormant seed early in the experiment, but not later in the experiment after most seed had become nondormant and had germinated. This possibility remains to be proven.

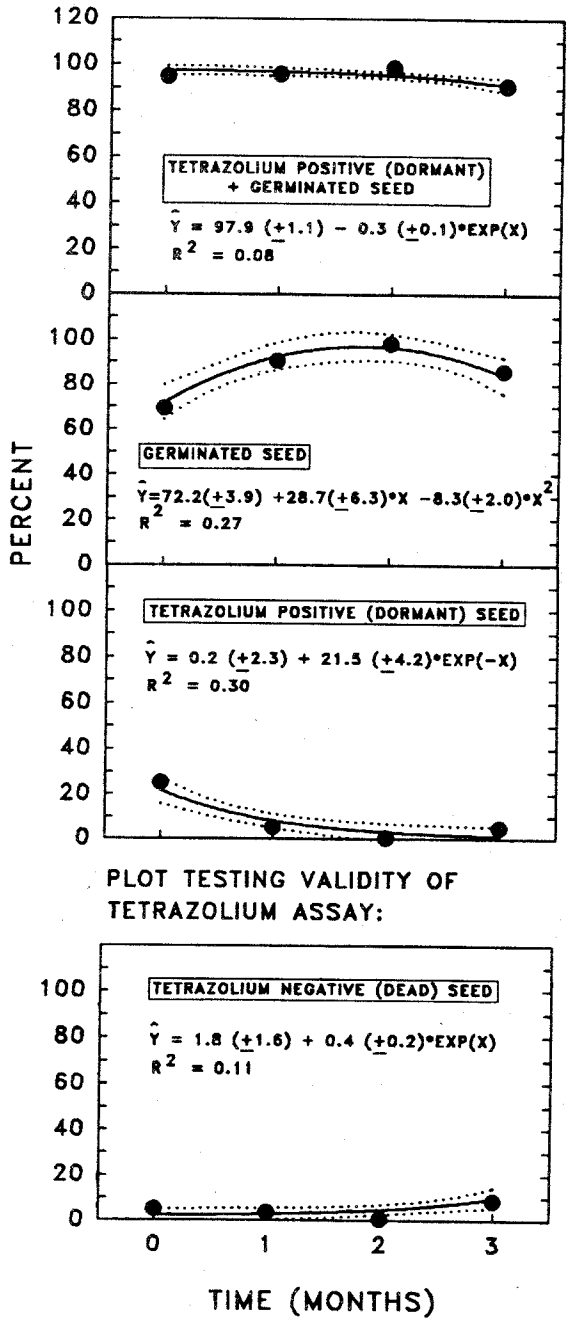

Figure 3. Changes in percent germinated, tetrazolium positive, tetrazolium negative, and germinated plus tetrazolium positive seed over time for jointed goatgrass seed removed from joints at the start. Coefficients (± standard errors) of slopes and intercepts of regression equations, and predicted response (solid line) and 95% confidence interval (dotted lines) from the equation are presented. Data for two trials were averaged and means (solid dots) are graphed.

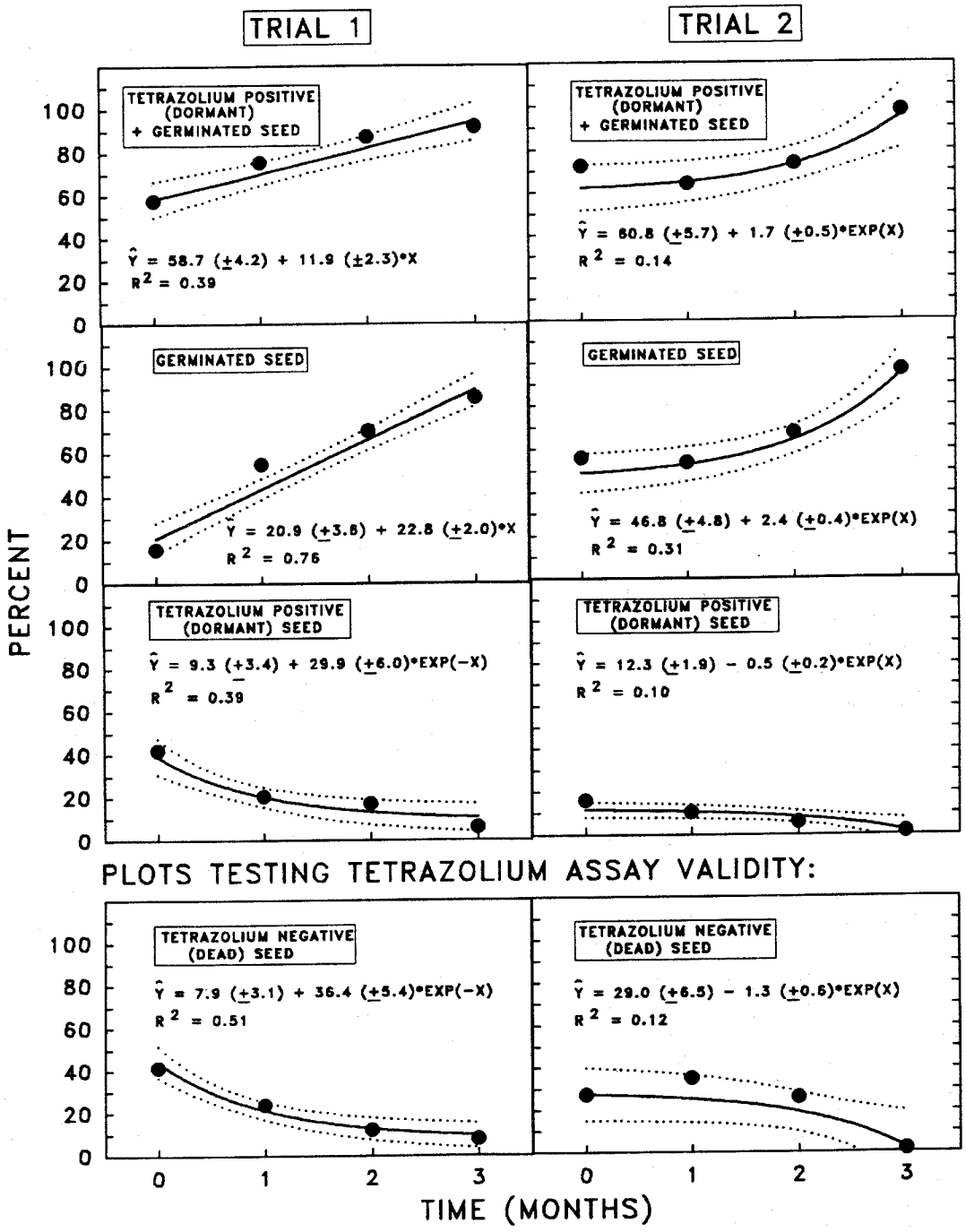

Figure 4. Changes in percent germinated, tetrazolium positive, tetrazolium negative, and germinated plus tetrazolium positive seed over time for jointed goatgrass seed afterripened in joints. Coefficients (± standard errors) of slopes and intercepts of regression equations, predicted response (solid line), and 95% confidence interval (dotted lines) for the equation are presented. Data from two trials are presented separately and means (solid dots) are graphed.

The validation method showed that the tetrazolium assay accurately determined viability for jointed goatgrass seed removed from joints at the start (Figure 3), but underestimated viability when performed on jointed goatgrass seed afterripened in joints (Figure 4). The results suggest that if the tetrazolium test is to perform accurately, jointed goatgrass seed must be removed from joints when dry before conducting either germination or tetrazolium assays on them.

The objective of the validation method was to detect a decrease in percent TN seed over time, not to model or gain detailed

information on error (confidence intervals) in modeling the change in percent TN seed over time. Use of regression analysis in the method for data presentation was arbitrary and other simpler statistical methods could have been used, as suggested in Materials and Methods. Relatively small R^2s values observed for nonlinear regression equations (Figures 3 and 4) were probably caused by the data variability and R^2s values could probably be increased by increasing replications per sampling time and increasing the number of sampling times.

The proposed validation method (graphing percent TN seed over time) has several advantages and disadvantages. It has the advantage that it can be applied to either fully or partially dormant weed seed. Moreover, not all seed must reach 100% germination to use the validation method. This validation method also has value before conducting long-term seed persistence studies. Assumptions of the validation method listed in Materials and Methods are its chief limitations and should be evaluated before using the method. The validation assay must be conducted over a sufficiently long period that most seed can germinate to allow a change in the TN seed over time to be detected statistically. Often, afterripening periods are not known ahead of time and have to be determined empirically.

This research fills a void by providing a new method for validating the tetrazolium assay for determining viability of fully or partially dormant jointed goatgrass seed. This validation method can be used to identify procedural flaws in using the tetrazolium assay to determine viability of dormant weed seed, such as jointed goatgrass seed in intact joints. The validation method may be applicable for identifying faults in using the tetrazolium assay for determining seed viability of fully or partially dormant seed of other weed species and with other biochemical assays. Further research is needed to establish the general utility of the suggested validation method for biochemical assays of dormant weed seed viability. However, the goal of this paper was to suggest a new research method, not to prove its general validity for all biochemical assays for detecting viability of dormant seed of all higher plants.

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