ISSN 1021-4437, Russian Journal of Plant Physiology, 2015, Vol. 62, No. 4, pp. 551–556. © Pleiades Publishing, Ltd., 2015.

METHODS

The TTC-Technique Might Not Appropriately Test the Physiological Stage of Plant Tissues[1]

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Received October 18, 2014

Abstract—The 2,3,5-triphenyltetrazolium chloride (TTC) technique has been used during decades to distinguish between dead and alive tissues of perennial grasses. This technique did not consider, however, that dormant (i.e., viable) tissues could exist within those erroneously considered dead tissues, thus being unable to report the true physiological stage of those plant tissues. Development of a procedure able to distinguish between metabolically active or dormant or dead tissues is then critical. This study developed a procedure to classify plant tissues of perennial grasses (*Poa ligularis* (Nees ex Steud.), *Nassella longiglumis* (Phil.) Barkworth, *Amelichloa ambigua* (Speg.) Arriaga & Barkworth, and *Piptochaetium napostaense* (Speg.) Hack.) in a more appropriate physiological stage (i.e., metabolically active, dormant or dead) than the traditional TTC-technique. Perennial grass seeds or meristematic buds were immersed in a TTC solution to obtain metabolically active (red or pink staining) or unstained (either dormant or dead) tissues. TTC-unstained tissues were placed in Evans Blue solution to separate dormant from dead tissues. The combination of TTC and Evans Blue techniques allowed the separation of metabolically active, dormant or dead tissues. Use of Evans Blue on TTC-unstained seeds and buds allowed to determine that some of these tissues were not dead but dormant (i.e., viable). Among the TTC-unstained tissues between 2 to 35% of total grass seeds and from 19.5 to 42% of all evaluated buds were dormant (viable and potentially able to growout) but not dead. Combination of TTC and Evans Blue techniques allowed a better classification of the physiological stages of plant tissues (metabolically active, dormant or dead) than the conventional TTC test.

Keywords: *Poa ligularis, Nassella longiglumis, Amelichloa ambigua, Piptochaetium napostaense*, seeds, axillary buds, dead and alive tissues, TTC-technique, Evans Blue technique

DOI: 10.1134/S1021443715040068

INTRODUCTION

During decades, the 2,3,5-triphenyltetrazolium chloride (TTC) technique has been conventionally used to determine plant and fungi tissue viability (axillary bud meristems [1], fungi spores [2], green tissues [3], pollen [4], and seeds [5]). This method is based on the enzymatic reduction of TTC to insoluble red formazan by metabolically active tissues [6]. Thus, TTC-pink or TTC-red stained tissues have been considered viable, and those unstained with TTC have been evaluated as nonviable [5–8]. This method, however, is unable to distinguish between dead and fully dormant (but viable [9]) tissues which have a very low metabolic activity as to stain with TTC [10]. These viable dormant tissues might still have the potential for future growth under appropriate conditions. For example, Pelton [11] referred seed dormancy as an adaptive trait that causes seeds to germinate at a time or place favourable to the subsequent survival of the seedling and adult plant. Another dye, the Evans Blue, has also been used to determine cell viability [8, 12]. This vital stain is unable to penetrate viable semipermeable membranes such as those of dormant tissues, but will stain whole tissues as dark blue if they are dead [8, 12].

During more than 70 years (since 1951 [10]), a myriad of studies have been done using TTC on various plant life forms to distinguish between viable (red- or pink-colored) and dead (TTC-unstained) tissues (terophytes [13], hemicryptophytes [5, 13], cryptophytes [1], camephytes [14], phanerophytes [15], and orchids [16]). In the light of our results, these studies would need to re-evaluate their results on the effects of any given treatment on plant tissue viability. This is because dormant TTC-unstained tissues (traditionally considered dead) are also viable [9], and might have the potential for growth and development in adequate conditions. Our study is a plea for combining the traditional TTC-unstained tissues with the vital stain Evans Blue to allow a more precise assessment of the physiological

[1] The article is published in the original.

Abbreviations: TTC—2,3,5-triphenyltetrazolium chloride.

state of various plant tissues as dormant or dead. Recently, Hilhorst [9] reported the need of developing a viability test to distinguish dead from dormant seeds.

We hypothesize that TTC-unstained bud or seed tissues might not be dead but dormant (and thus viable). The objectives of this study were (1) to demonstrate that TTC-unstained tissues might not be necessarily dead but dormant, and (2) to combine the conventional TTC-technique with the vital stain Evans Blue to better classify the physiological stage of seed and bud tissues as metabolically active, dormant (but viable) or dead. To test our hypothesis, we used plant tissues of various native perennial grasses of southwestern Buenos Aires, Argentina.

MATERIALS AND METHODS

Bud viability. During spring 2013, *Poa ligularis* (Nees ex Steud.), *Nassella longiglumis* (Phil.) Barkworth, and *Amelichloa ambigua* (Speg.) Arriaga & Barkworth plants were randomly collected at the field (40°39′49.7″ S, 62°53′6.4″ W, 40 m.a.s.l.). These cool-season perennial grass species are native to the Monte Phytogeographical Province [17]. Five axillary buds were obtained per plant to determine their degree of metabolic activity after dissecting tillers. After dissection, each stem base was cut longitudinally with a razor blade, leaving entire tiller buds on each side of the cut. Both halves were immersed in test tubes containing 25 mL of 0.6% (w/v) TTC ("Santa Cruz Biotechnology Inc.", USA) in phosphate buffer (0.05 M Na_2HPO_4–KH_2PO_4, pH 7.4) with a 0.05% (v/v) wetting agent (Tween 20), and incubated in darkness at 30°C for 15 h [6]. Thereafter, both halves were rinsed using distilled water and observed again under a stereoscopic microscope ("Nikon", 10×). TTC staining indicates enzymatic reduction of the tetrazolium salt to insoluble red formazan by living, respiratory active tissues [10]. The TTC accepts electrons from the electron transport chain of the mitochondria, and as a result it is converted to formazan within viable cells with fully active mitochondria [18].

Bud was considered metabolically active when the apex stained pink or red. Buds that remained unstained (either dormant or dead) after incubation with the TTC solution and were not visibly necrotic, were tested using the vital stain Evans Blue ("Santa Cruz Biotechnology, Inc."; color index number is 23860; 0.25% w/v). Evans Blue leaks through the ruptured membranes and stains the contents of dead cells [8]. Longitudinal sections of TTC-unstained buds were soaked in Evans Blue for 30 min at room temperature (20°C). Excess dye was rinsed from the sections, which were then mounted in water and examined under a microscope (Leica ICC50). Bud tissues which remained uncolored with Evans Blue were classified as dormant but viable [8, 9], while those which stained dark blue were considered dead. Immersion of some buds in boiling water assured us that those buds were dead.

Seed viability. These determinations were made on another native perennial grass of central Argentina—*Piptochaetium napostaense* (Speg.) Hack., also a cool-season grass. Seeds from six plants were collected in the Caldenal, within the Espinal Phytogeographical Province during 1997, 2000, 2001, 2003, 2006, 2010, 2012, and 2013. Seed collection was always made at the end of the growing season (late spring, early summer: December) to make sure that seeds reached full maturity. Plant seeds were maintained in paper bags at laboratory temperature. Twenty seeds were sampled from each bag and immersed in distilled water during 24 h previous to their cutting. Seeds of this species have innate dormancy imposed by their glumes [19]. They were removed from the seeds, which were cut longitudinally under a stereoscopic microscope. Each half was exposed to the staining procedure with TTC and Evans Blue which has already been mentioned for bud tissues above.

Statistical analysis. Bud and seed percentages in the various viability categories were transformed to integral arcsin(x)dx prior to analysis, and all data were checked for normality. Two-way analysis of variance (ANOVA, $p < 0.05$) was used to compare treatment effects [20]. If ANOVA showed significant effects, Fisher test (LSD, $p < 0.05$) was used to determine differences between treatments. All mean comparisons were performed with the statistical package "INFOSTAT" [21]. Untransformed data are presented in figures.

RESULTS

Combining Use of TTC and Evans Blue Techniques to Determine the Appropriate Physiological Stage of Plant Tissues

As expected, metabolically active buds or seed embryos stained red or pink after immersion in TTC (Fig. 1a, buds; Fig. 2c, seed embryos). Those buds or seeds killed by boiling water remained uncolored in TTC (Fig. 1a, buds; Fig. 2b, seed embryos). When dead, TTC-unstained bud tissues were checked for viability using Evans Blue, they stained uniformly dark blue with the nuclei staining darker than the protoplast (Fig. 1c). Tissues with such an appearance were considered dead. Similarly, dead TTC-unstained seed embryos (killed by boiling water) stained uniformly dark blue after exposure to Evans Blue (Fig. 2e).

Surprisingly, TTC-unstained buds or seed embryos obtained from grass plants growing at the field, considered dead by the conventional TTC-technique, showed to be either viable (dormant: viable tissues remained unstained; Fig. 1b, buds; Fig. 2d, seed embryos) or dead (tissues of both buds and seed embryos stained dark blue; Fig. 1c, buds; Fig. 2e, seed embryos) after exposure to the Evans Blue stain. Even more, Evans Blue was unable to penetrate into viable TTC-stained metabolically active tissues (Fig. 2f). Thereafter, use of TTC and Evans Blue techniques allowed to classify buds and seeds in three categories of physiological stages: metabolically active or dormant (but viable), or dead.

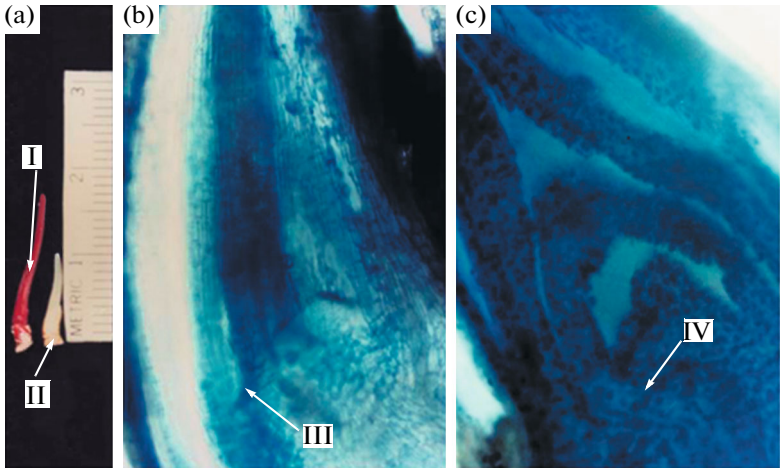

Fig. 1. Photographs of *P. ligularis* stem and bud tissues at different physiological stages. a—stem bases (10×) showing metabolically active (I, stained red with TTC) and inactive buds (II, unstained, light-yellow, dead). Tissue was killed using boiling water. Solid arrows indicate the buds or stems within the stem base; b—TTC-unstained (40×), dormant (i.e., viable) bud tissue after incubation in Evans Blue. Damaged cells after cutting the buds (to observe bud tissues under microscope) are shown as light blue (III); c—TTC-unstained (40×), dead bud tissue (IV) after incubation in Evans Blue; note the darkened nuclei within each cell.

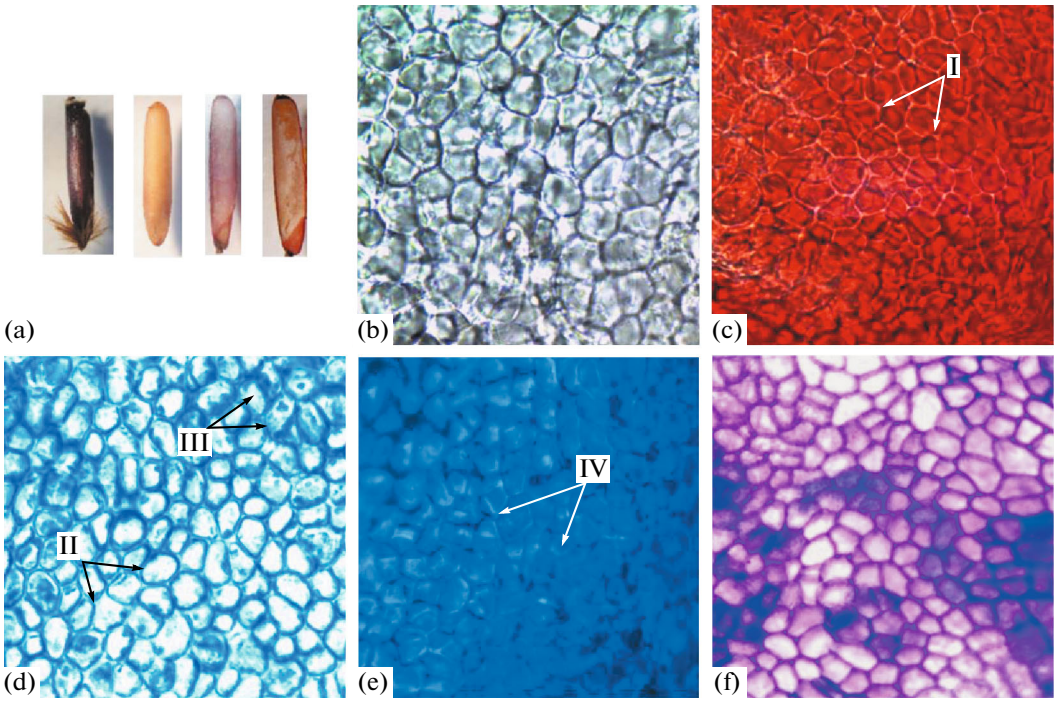

Fig. 2. Photographs of *Piptochaetium napostaense* seeds. a—intact seed and seeds without glumes (10×); b–f—microscopic photography (1000×) of longitudinal cuts of seeds; b—cells were killed using boiling water, and thereafter did not stain with TTC; c—cells showed high metabolic activity, staining red with TTC (i.e., viable, I); d—cells remained unstained with TTC and thereafter remained uncolored with Evans Blue indicating dormancy (i.e., viability, II); damaged cells are shown as light blue (III); e—cells stained dark blue (IV) in Evans Blue indicating whole death; f—cells stained with TTC and remained unstained in Evans Blue indicating viability.

Physiological Stage of Tissues in Perennial Grass Species

Buds. There were no significant differences ($p > 0.05$) between species and categories of bud viability in *P. ligularis, N. longiglumis*, and *A. ambigua*. The percentage of metabolically active buds was significantly greater ($p \leq 0.05$) than that of dead buds in all three species (Fig. 3). The percentage of dormant (i.e., viable) buds was as high as that of metabolically active or dead buds in all three perennial grass species (Fig. 3).

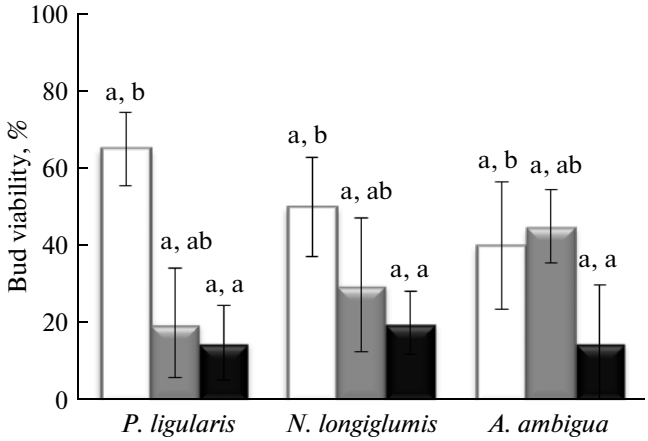

Fig. 3. Percentage of metabolically active (white column), dormant (grey column), or dead (black column) buds of *Poa ligularis*, *Nassella longiglumis*, and *Amelichloa ambigua*. Different letters before comma indicate significant differences ($p \leq 0.05$) between species, while those after comma indicate significant differences ($p \leq 0.05$) among bud viability categories. Histograms are means of four replicates ±SE.

Seed embryos. We found significant differences ($p \leq 0.05$) between years and categories of seed viability in *P. napostaense*. The percentage of metabolically active seeds increased ($p \leq 0.05$) and that of dormant and dead seeds decreased ($p \leq 0.05$) from 1997 to 2013, until reaching 100% of metabolic activity in 2013 (Fig. 4).

DISCUSSION

Use of TTC [7] and Evans Blue methods on bud and seed tissues allowed a much better expression of their physiological state. This is, TTC-unstained either buds or seed embryos were not necessarily dead, as indicated by use of the traditional TTC-technique, but either dormant (i.e., viable [9]) or dead. This is especially important when the magnitude of dormant tissues is at least as high as that of metabolically active or dead tissues (Fig. 3). Dormant tissues might still have the potential of becoming metabolically active given appropriate conditions. Our results confirm our hypothesis that TTC-unstained tissues might not necessarily be dead but dormant.

Germination of seed and emergence of seedlings from the soil are primary considerations for the rancher, whether in regard to the reseeding of desirable plants or the control of unwanted species. This emphasizes the importance of the development of new methods which appropriately can classify seeds in their adequate physiological stages (metabolically active, dormant or dead). Of the several aspects of germination, the retention of seed viability with considerable age (longevity) has been rather extensively studied (seeds may remain dormant during many years in the soil seed bank [22, 23]). It is generally understood that after several years of storage seed viability declines, the rate of this decline being mainly patterns of the species and storage conditions [24]. Accordingly, the percentage of metabolically active seeds of *P. napostaense* decreased while that of dormant and dead seeds increased with increasing age. These results agree with those for other plant species [25–27], and are the most likely the result of seed storage conditions [24].

We propose that TTC-unstained tissues of rangeland grass species should not be considered necessarily dead in future research (see Figs. 1 and 2). Their viability should be further tested with dyes (i.e., Evans

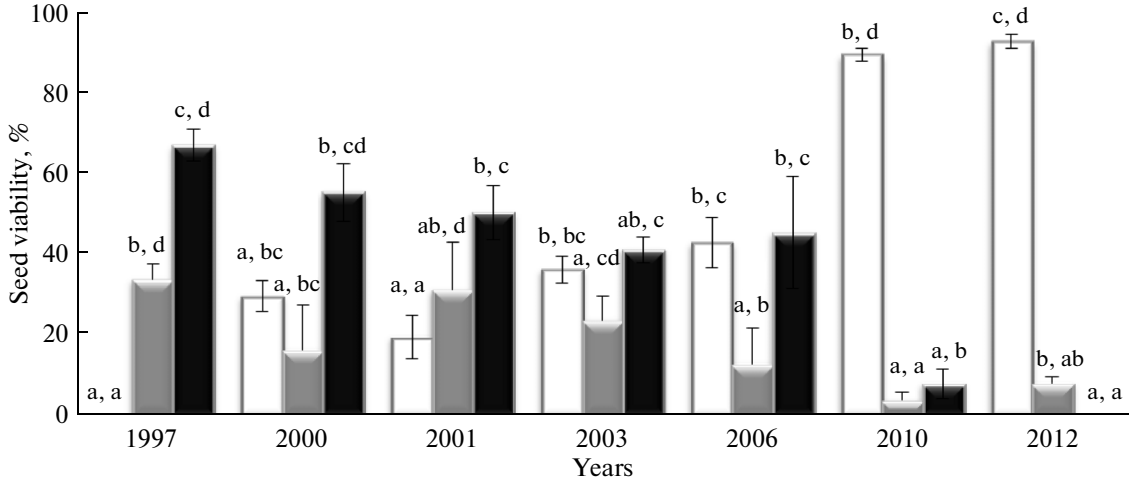

Fig. 4. Percentage of metabolically active (white column), dormant (grey column), or dead (black column) seeds of *Piptochaetium napostaense*. Within each year, different letters before comma indicate significant differences ($p \leq 0.05$) between categories of seed viability. Seeds were harvested at the end of the growing season (late spring, early summer) to make sure that they reached full maturity. Within each seed viability category, different letters after comma indicate significant differences ($p \leq 0.05$) between years. Histograms are means of six replicates ±SE.

Blue) that do not penetrate intact semipermeable membranes, indicating tissue viability (i.e., dormancy [9]). Castro-Concha et al. [8] reported several dyes that can be used to assess cell membrane integrity. Usually, the traditional TTC-method for evaluating tissue viability is used [28]. As a result hundreds of papers appeared using the conventional TTC-method to evaluate tissue viability (either metabolically active or dead) [e.g., 28]. These reports should be re-evaluated in the light of our findings, as an accurate assessment of cell viability is a fundamental factor to consider since it is connected to plant productivity [8].

A major need for our research was to show the limitations of the traditional TTC-method [7]. This is because partially of the various applications that an appropriate determination of tissue viability directly relates to agro-ecosystem services such as seed harvesting, processing, production, and commerce.

We acknowledge that our method was first briefly outlined in 1989 [29], and on subsequent papers [30] of our ecology team. However, it was not taken into account by the other scientists as a method itself. As a result the scientists used the traditional 2,3,5-triphenyltetrazolium technique to determine the viability of plant tissues, when this method might be inadequate. So, it might not test the correct physiological stage of plant tissues because it just classifies them as either alive or dead only. It does not distinguish between dormant or dead tissues; it considers them altogether.

Our method, however, allows to classify plant tissues as either metabolically active, dormant (and then viable, having the potential capacity of growing out into new individuals under appropriate conditions), or dead. Using tissues of perennial grass species in our paper (buds and seeds), we showed that the magnitude of error of the traditional TTC-method can be as high as 75% (on buds), determining dead tissues when they are in fact alive (i.e., dormant). In this paper, we propose that the conventional TTC-technique is not necessarily the most appropriate, and it should be replaced at once by ours, which combines two stains (2,3,5-triphenyltetrazolium + Evans Blue), for a more appropriate determination of the physiological stage of plant tissues.

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