2018 Small-seeded Verbena TZ Referee

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Purpose:

The Verbenaceae method page for tetrazolium testing was amended in 2017. Two key changes were expected to give better results for small-seeded *Verbena* species (<2mm):

- Recommendation to precondition by soaking the seeds in water for 24 hours.
- Recommendation to cut the seeds laterally on the distal end prior to staining.

Intention:

- Provide an opportunity to TZ test small-seeded *Verbena*
- Not a validation study.
- Participants needed a basic understanding of TZ testing but did not need prior experience testing *Verbena* seeds.

Background:

- *Verbena* seeds (nutlets) germinate very slowly and sporadically even with a variety of dormancy-breaking treatments.

- All *Verbena* nutlets have **woody pericarps**.
  - Difficult to avoid artifacts when cutting seeds for tetrazolium testing.
  - With **larger-seeded *Verbena* species** (>2mm), artifacts are easy to read through once the analyst learns the appearance of such artifacts.
    - Embryos can be removed, turned over and examined more fully.
    - Frequent blade changes also help alleviate this issue.
  - With **small-seeded *Verbena* species** (<2mm),
    - Artifacts created by longitudinal cuts significantly confound evaluation
  - Woody pericarps inhibit or slow imbibition.

Preliminary tests indicated:

- Soaking rather than imbibition by placement on a moist medium improved the test.
- For small-seeded species, a lateral cut on the distal end would limit the artifacts to the cut end and allow most of the embryo to stain undisturbed.

Materials and Methods

- Two small-seeded *Verbena* species: (*V. bonariensis* and *V. hastata)* lots were provided. (Some participants only received V. bonariensis. We had insufficient seed quantity of *V. hastata* for everyone.)

- Participants were given a 2017 amended Verbenaceae page (suggesting 1 day soak, lateral cut and 1 day stain for small-seeded *Verbena* species) and asked to test 400 seeds per sample.

- Part way through the referee, some participants recommended a 2 day soak followed by a lateral cut and a 2 day stain for the *V. hastata* sample.

- An amended page (labeled the 2018 amended page) and a suggestion to try the longer soak with longer stain method was sent out to those who received the *V. hastata* sample.

- After staining, participants were asked to push the embryo out of the laterally cut end for evaluation.

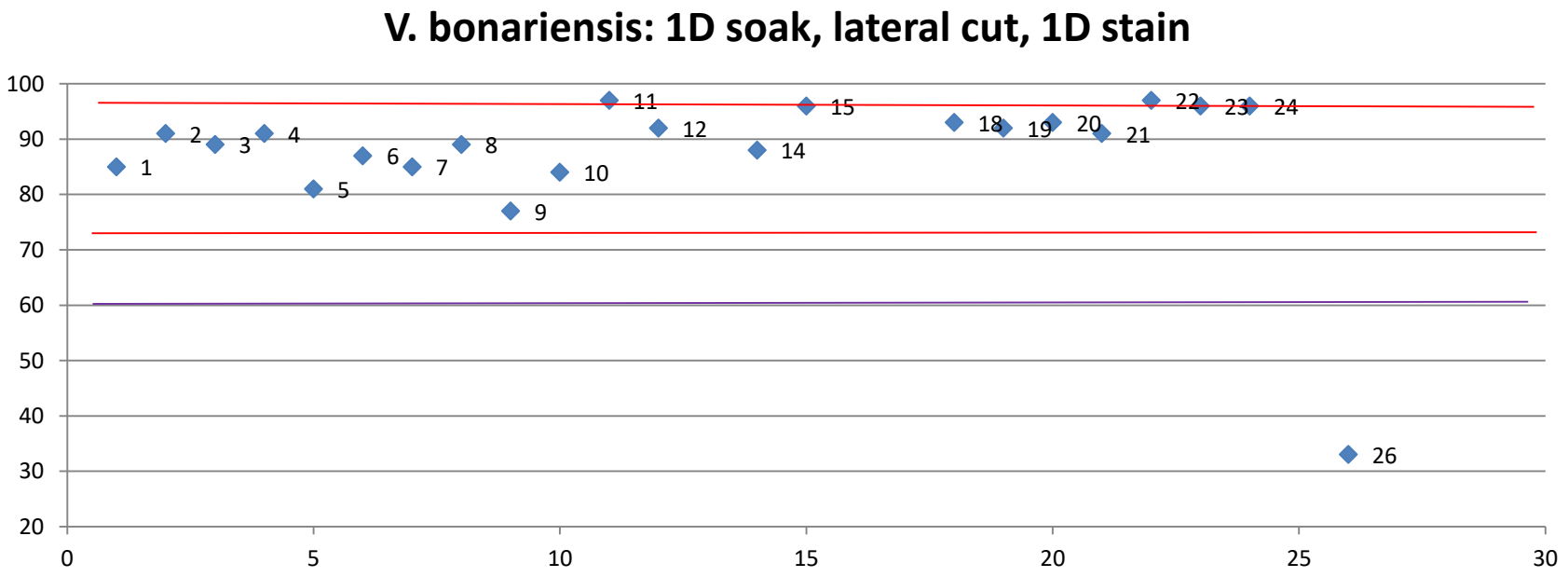
Results:

- 23 participants sent in results for at least one sample.

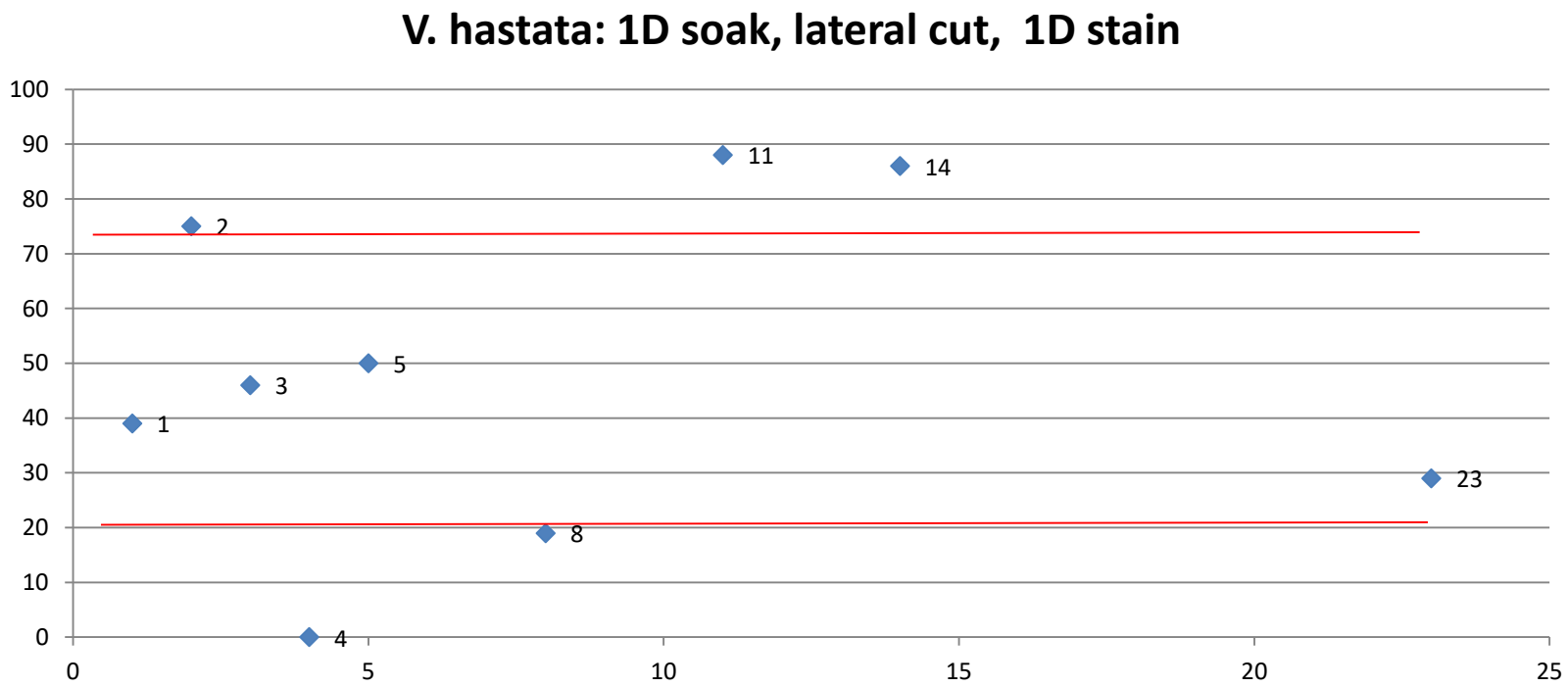
- 9 participants self-reported as “experienced”.

Sample 1: *Verbena bonariensis*

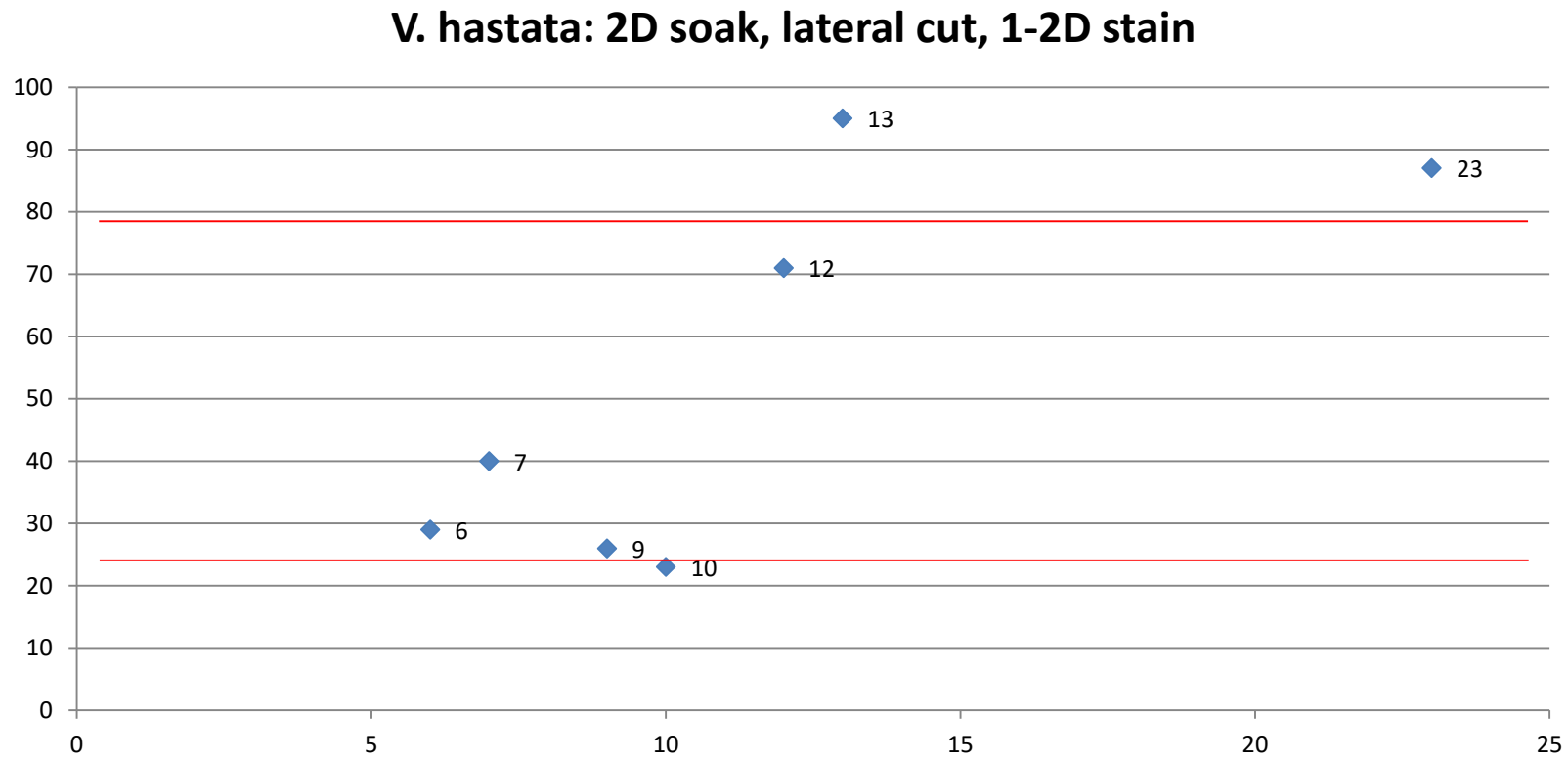
- 22 participants
- Mean: 87%
- Standard deviation: 13 (red line)
- 2x standard deviation: 26 (purple line)
- 21 of 22 participants were within one standard deviation of the mean.
- The participant that was not within 2 standard deviations of the mean, self-reported as “inexperienced.”
- Y axis is % viability.
- Numbers on the data points are the assigned participant numbers



- **Sample 2: *Verbena hastata:* 1D soak, lateral cut, 1D stain**
- 9 participants
- Mean: 48% High: 86% Low: 0%
- Standard deviation: 30%
- Y axis is % viability.
- Numbers on the data points are the assigned participant numbers.



- **Sample 2: *Verbena hastata:* 2D soak, lateral cut, 1-2D stain**
- 7 participants
- Mean: 53% High: 95% Low: 23%
- Standard deviation: 31%
- Y axis is % viability.
- Numbers on the data points are assigned participant numbers.



Comments:

- The soak preconditioning step improved results.
- For the *V. hastata*, One person did: 2D soak (rm temp) + 3D soak 5°C prior to cutting; with excellent staining after just 24 hours in TZ solution.
- Some participants wrapped their seeds in filter paper before placing in the water to soak to ensure total immersion. The rough texture of the seeds can cause the seeds to float on the meniscus of water in the vial.
  - A question arose as to whether some participants with low results for *V. hastata* did not achieve good immersion for preconditioning and/or staining steps.

- One person reported difficulty pushing the embryos out of the laterally cut end without damage. (If this is the case, one can always cut the seeds longitudinally after staining.)
- Most participants thought the *V. hastata* seeds needed more staining time.
- One analyst reported some success using lactic acid to clear the seed coat after staining.

Discussion:

- For this *V. bonariensis* sample, one day preconditioning soak followed by a lateral cut and one day of staining is sufficient.
- Most analysts were able to achieve results that were within one standard deviation and the standard deviation was reasonably low.
- For the *V. hastata* sample, some participants had difficulty achieving good results with either method.
- Although the results for *V. hastata* are wide ranging, the fact that some analysts were able to achieve good results suggests that technique issues are the cause.

Conclusions:

- Good tetrazolium testing results are achievable for small-seeded *Verbena* species. The following are best practices suggested by the results of this referee:
  - **Preconditioning:** Small-seeded Verbena will stain well if given extra preconditioning time (24-48+ hours) and full immersion in water. (Wrap seeds in filter paper to ensure good immersion.)
  - **Cutting:** Problems with artifacts that occur with the longitudinal cut can be alleviated by using a lateral cut on the distal end.
  - **Staining:** Laterally cut small-seeded verbena will stain well if given extra staining time and full immersion in the TZ solution is ensured (wrap in filter paper).
  - **Evaluation:** Learning the appearance of cutting artifacts is an important part of evaluation. If the embryo is difficult to push out from the base, cut the seed open longitudinally to examine the entire embryo. If staining is inadequate, use a longer stain time. If the seeds have been generously preconditioned (at least 48 hours full immersion), the seeds should not need more than 48 hours to stain.

- These suggestions will be added to the Verbenaceae page. The amended page will be labeled “amended 5/2018” and posted to the TZ website.

FAMILY: VERBENACEAE

Genera: *Glandularia*, *Lantana, Verbena*

1. PRECONDITIONING:

METHOD	TIME (h)	TEMP (°C)
Soak in water	16-48*	20-25

Note: Push seeds down to immerse or wrap in filter paper and submerge. If seeds continue to float after soaking overnight, they may be empty. *Referee tests on one sample of *V. hastata* showed that 48 hours of imbibition time (soaking) was necessary.

Morphology

Fig 1 External

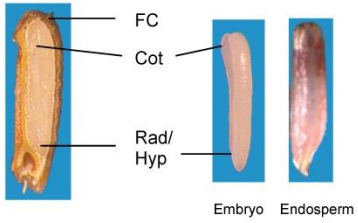


Fig 2 Embryo

Note: A thin layer of endosperm surrounds the embryo. *Small-seeded *Verbena* (<2mm).

2. PREPARATION AND STAINING:

METHOD	TZ Conc (%)	TIME (h)	TEMP (°C)
Cut laterally at the distal end, just enough to see the embryo. (recommended for small-seeded *Verbena* <2mm).	1.0	16-48*	30-35
Cut longitudinally, leaving fruit coat or distal end intact to keep both halves together	0.1	4-16	30-35

Note: Rigid woody fruitcoat will cause artifacts at the cut site. Change blades frequently to reduce artifacts.
Seed may release an oily substance into TZ solution when staining.
A lateral cut will restrict artifacts to the distal end. Longitudinally cut seeds sometimes fail to stain either because of artifacts or seed constituents released from the cut that may interfere with the tetrazolium reduction reaction.
Ensure immersion of small-seeded *Verbena* in TZ solution by wrapping seeds in filter paper.
*Referee tests on one sample of *V. hastata* showed that 48 hours of staining time was necessary.

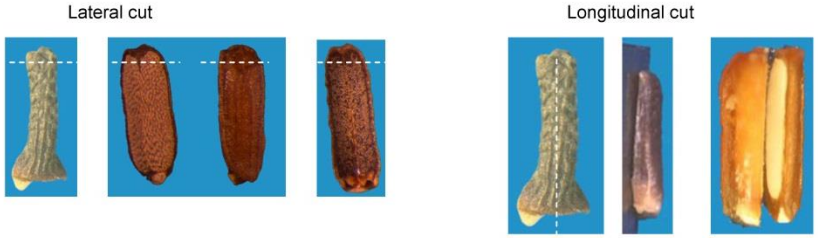
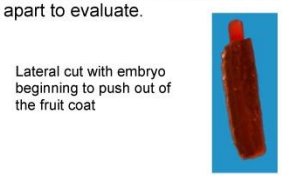


Fig 3 Preparation method

AOSA/SCST TETRAZOLIUM TESTING HANDBOOK, 2010 Edition
revised 5/2018

FAMILY: VERBENACEAE
Genera: *Glandularia*, *Lantana, Verbena*

Post-staining notes: If prepared with a lateral cut, either push the embryo out of the end by squeezing the base or cut longitudinally. Pull halves of longitudinally cut seed apart to evaluate.

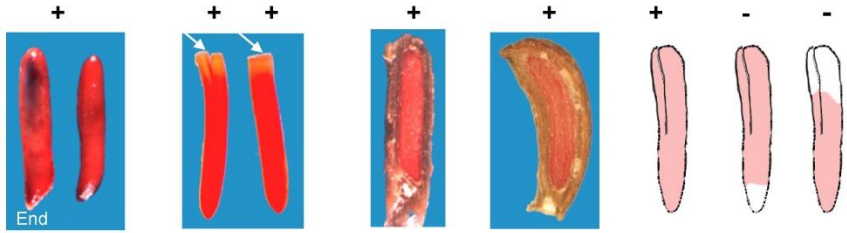


3. EVALUATION:

VIABLE (NORMAL STAINING)
- entire embryo evenly stained

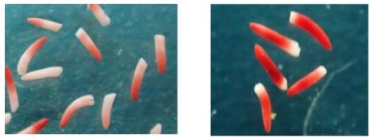
NON-VIABLE (ABNORMAL OR NO STAINING)
- any part of embryo unstained
- empty or partially filled seed units.

Notes: Seed is subject to artifact damage (see sections 14.2 and 15.1.3). Surface pathogens will often cause germination results to be lower than TZ test results. Endosperm surrounding the embryo is living and will stain; however, it is not included in evaluation. For laterally cut seeds, the tissue may be white at the cut site.



Endosperm, left Embryo, right. Endosperm is a thin sheath around the embryo.

Embryos with white artifact at cut surface (distal end of cotyledons)



V. hastata

1 day imbibition, 1 day stain

2 day imbibition, 2 day stain

Fig 4 Seed stain evaluation

Photos:
Arnold Larsen, Fort Collins, CO: external view, *G. x hybrida*
Annette Miller, USDA/ARS NLGRP: Fig. 1 External *V. bonariensis, V. hastata.* Fig. 2 Embryo, endosperm, Fig 3. cut with razor, Post stain: lateral cut view, Fig. 4 three evaluation views at left, drawings and *V.hastata* stained embryo images.
Sarah Dammen, SGS, Brookings SD: Fig. 3 right view of cut seed, Fig. 4 fourth evaluation view from left,.

Thank you!

Participants were from the following labs:

IL Crop Improvement , Incotec, KY Seed Lab, MN Dep't of Ag., USDA/ARS NLGRP (3), NM State Seed Lab, OR State Seed Lab, PA Dept of Ag., Ransom Seed Lab, SGS North America (6), SODAK, TX - Giddings Seed Lab (3), UT State Seed Lab, WY State Seed Lab.