

An Agar-Based Method for Determining Mechanisms of *Striga* Resistance in Sorghum



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Abstract This chapter describes an agar-based method for co-culturing sorghum and *Striga* that can be used to determine *Striga* germination stimulant activity, haustorial initiation activity and other pre-attachment and early post-attachment resistance mechanisms in sorghum.

Keywords *Striga* · Sorghum · Bioassay · Mechanisms of resistance · GR24

Introduction

Having evidence of gained *Striga* resistance in mutagenized cereals through field and pot studies, it is useful to determine what mechanism underlies the resistance reaction. This is particularly important in the context of sustainability and integrated *Striga* management, whereby the crop resistance is combined with other control methods, e.g., soil nutrient enhancement through fertilizers, water conservation practices, post-emergent weed control practices or with other resistance traits (Rich 2020). Extended reliance on a singular host plant resistance trait may lead to selection of virulent *Striga* populations that are able in a few generations to overcome the acquired resistance from mutagenized crop varieties. Determining the underlying mechanism leading to the reduced number of parasites emerging around field and pot grown plants is most easily done in a medium in which the *Striga*-host interactions can be observed at their earliest stages with repeated, non-destructive observations.

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Protocols

This section tells how to prepare plates of *Striga* seed embedded in 0.7% agar beginning with surface sterilization and conditioning of the *Striga* seed. It builds on an agar gel assay for determining *Striga* germination stimulant activity published decades ago by Hess et al. (1992). In this assay, since one is observing a limited number of *Striga* seeds, it is important to choose a weed seed source that germinates fairly well, at least 30% in 10^{-8} M GR24. Ideally, the source of *Striga* seed should come from the target environment in which the resistant cultivar is to be grown. The following procedures are intended for determining sorghum reactions to *Striga asiatica* or *S. hermonthica*. They may be adaptable to maize or rice but modifications to suit screening of those crops is not covered in this chapter.

Conditioning *Striga* seed. Prepare a Benomyl (a benzimidazole fungicide, Sigma-Aldrich) 100× stock solution by dissolving 0.15 g Benomyl wettable powder in 10 mL DMSO. Heat (50–70 °C) to completely dissolve the fungicide in the solvent. Store at room temperature in a brown glass bottle for up to three months.

Prepare Metricide 28[®] solution (2.5% glutaraldehyde; Metrex Research Corp.). A small volume can be activated by adding 1.2 mL of activator to 100 mL of the bulk Metricide if only a few batches of *Striga* will be conditioned over the period. In a biosafety cabinet or on a lab bench, but outside the (laminar flow) transfer hood, remove an aliquot of cleaned (dry but sieved to remove plant material other than seed) *Striga* seed from bulk to a 50 mL flask. This should be done on a heavy white blotter soaked with bleach to contain any dry seed which might miss the flask during the transfer. The normal amount for this size flask is usually six smidgen scoops, enough for about 100 plates. You should be able to get about 15–18 100 mm plates for each smidgen of clean weed seed. Less can be used, but if you use more than six scoops, you will need to perform the surface sterilization steps in a 125 mL flask, doubling the volumes of sterilants (ethanol and Metricide), washes and conditioning solution. Add 25 mL 75% ethanol to the seed in the flask. Wash the outside of flask with bleach. Move the flask with seeds in alcohol to a sterile (laminar flow) transfer hood (also onto a light colored blotter soaked with bleach) and with a sterile glass pipette equipped with an amber bulb, agitate for 2 min by sucking the solution and weed seed up and down in the pipette repeatedly. Let the seed settle in the flask. If sand or heavy debris is visible at the bottom of flask, use the pipette to suck that up. You will also unavoidably suck seed into the pipette with the sand but once the sand settles in the pipette, the sand will be at the bottom and you can remove it by squeezing over the waste flask filter funnel until you reach the seed layer. Add this back to the flask of seed in the 75% ethanol. Let the seed settle again and then slowly pour the liquid off into the waste flask filtered funnel until mostly seed remains. Use the pipette to suck off the remaining liquid by squeezing the bulb before putting into the seed slurry and pressing the tip against the inside bottom of the flask, then slowly releasing the bulb. Properly done, this will suck up mostly liquid and very little seed.

Add 25 mL of activated Metricide. As before, agitate for 2 min by sucking seeds and sterilant in and out of the pipette under the surface of the liquid. Remove the

bubbles from the surface of the Metricide solution with the pipette before emptying the liquid to the waste flask in the same manner as the alcohol was removed, pouring off most, then sucking remainder off with the pipette. Rinse 2× with sterile distilled deionized water (ddH₂O), each time agitating with the pipette for about a minute then removing the liquid as before.

Add 25 mL ddH₂O and 25 μL of the 100× Benomyl stock to flask. This is the conditioning solution. Cover the mouth of the flask with Aluminium foil and put in darkness in an incubator set at 29 °C. **Change Benomyl solution after 1 day** and then every 2–3 days after until the seed is embedded in agar.

Pouring the plates. You may embed the *Striga* seed in agar after 5 days in the conditioning solution and allow the conditioning to finish in the agar. It is usually most convenient to do this a day or two before planting the sorghum. **Do not use the seed until it has been in a combination of solution and agar for a total of 10 days.** Germinability is maximal from 10 to 15 days after the conditioning start date.

Prepare agar (0.7%) to embed *Striga* seeds (six smidgen scoops of seed should yield about 100 plates). Prepare bottles of agar in 1 L Pyrex bottles, each containing 900 mL water + 6.3 g BactoAgar. Each bottle should be enough to pour twenty-five 15 × 100 mm plates (35 mL/plate) or fifteen 25 × 100 mm plates (60 mL/plate). Autoclave the bottles of agar on a liquids cycle with 15–20 min sterilization time, then allow to cool in a water bath to 50 °C (turn on water bath when autoclave cycle starts).

To distribute the seed properly and uniformly on each plate takes some practice. Ideally you want around 20 *Striga* seeds per square cm (more if germinability is low). Use a glass Pasteur pipette, the same kind used during the initial surface sterilization and changing of Benomyl. After changing the Benomyl solution, suck as much seed into the pipette from the conditioning flask as possible with the aid of an amber bulb and let it just settle to the narrow tip. Under sterile transfer hood, set out 20 plates (or whatever amount conveniently fits in the front of your hood) and open them. Add a drop of settled *Striga* seed from your pipette by touching it to the center of the opened plate bottom and barely squeeze the bulb to deliver an amount of wet seed about the size of a sorghum seed to each plate, trying to be consistent between plates. The amount of *Striga* is the same whether you are using 15 or 25 mm Petri dishes.

After distributing seed to all plates in a set, add more of the conditioning solution from the flask of conditioned seed to dilute the drop of seed. Compare the density of seed in each drop and add or remove seed from plates until all look to have a similar amount of seed (Figs. 1 and 2). Be sure not to let any seed dry out during this distribution process. After seed has been put in all the plates in roughly equal portions, remove the bottle of sterile agar cooled in a water bath to 50 °C, spray the outside with 75% ethanol and then bring it under the hood and open it. In a smooth and steady manner, pour the liquid agar directly over the *Striga* seed keeping the mouth of the bottle as close to the plate as possible and not interrupting the flow until just after the agar reaches the sides of the plate. The volume of agar should be about 35–40 mL for 15 × 100 mm plates or 60 mL for 25 × 100 mm plates (the level of the agar in the plate should appear to be about 2/3–3/4 full, Fig. 3) and the *Striga* seeds distributed evenly across the plate (Fig. 4). Using the deeper plates will allow



Fig. 1 Try to distribute *Striga* seed equally among plates. Keep the seeds always covered with liquid

the sorghum shoot more space to grow if you plan on keeping them long enough for early post-attachment observations (21 days or longer after plating sorghum). Allow plates to cool 10–15 min before covering. Continue this process until all the plates you need for that set are poured.

Record the conditioning start date and the date the seed was embedded in agar for each batch of poured plates. Stack 18–20 plates, making sure lids of each are securely fitted. They can be left in the laminar flow hood so long as the blower is turned off. The blower will dry them out. They can also be left on a lab bench if they will be used within a day or two. If they were poured early and won't be used for a few days, they should be closed inside a plastic bag, most conveniently the sleeve in which the new plates were originally packaged, to maintain high humidity during the remaining conditioning period and placed in a dark 29 °C incubator until they are ready to use (between 10 and 14 days from the conditioning start date of the *Striga* seed).

Surface sterilization of sorghum seed. Determine which accessions you will test according to how many *Striga*-in-agar plates you have prepared and what you can reasonably read at each observation period. Always include at least one check entry with known high *Striga* germination stimulant activity (most important), and another with low *Striga* germination stimulant activity. These will allow you to compare between sets of experimental entries (accessions) as you screen your collection. I



Fig. 2 In a drop of conditioning solution with a 1 cm diameter, the seed density should look similar to this



Fig. 3 The agar level should fill the bottom of the petri dish 2/3–3/4 full

usually try to keep the number of accessions to about 30 in a set so that with three plates per accession, I am not overwhelmed on observation days and I can fit all 90 or so plates on a large tray on a single shelf in our incubator.

Deglume sorghum seeds and remove any debris or immature, uncharacteristic or damaged kernels. Record seed source (year and field row) and assign each entry a simple number. Count out 10–20 seeds and place in a 6 dram glass vial, 15 mL Falcon® tube or similar glass or plastic container. Having them all together in a rack is a convenient way to handle multiple entries. Once all entries are placed in vials, add approximately 5 mL of a freshly prepared 50% bleach/0.2% Tween® 20 (a polysorbate-type nonionic surfactant, Sigma-Aldrich) solution from a squirt bottle (for 500 mL, combine 250 mL household bleach [5.25% NaOCl] with 250 mL ddH₂O and add to this 1 mL of Tween 20, use this within 1–2 days). Cap the vials. Shake them either individually or together in the rack.



Fig. 4 If poured properly, seed will be evenly distributed in the agar at about this density. Ideally the seed density will average about 20 seeds/cm²

Soak seeds in this solution for about 30 min (but don't leave more than 60 min). Shake them occasionally (two or three times) during this surface sterilization to assure uniform contact of bleach solution and break surface tension. Seeds which do not sink to the bottom may be bug eaten or damaged and will likely not germinate so they should be discarded. After the 30 min soak, move the vial(s) to a sterile transfer hood. Pour off the bleach solution into a waste container, a large beaker or better yet, a sidearm flask fitted with a funnel and lined with filter paper. Be careful not to pour out the seeds. If any are still floating, you will likely lose them but that's okay since any that float are probably not viable. After pouring off the bleach, fill the vial with sterile ddH₂O from another squirt bottle. Pour this off and repeat this water wash two more times to remove the bleach solution. Proceed with these washes for each vial of seeds. Leave the caps off.

Prepare a 5% w/v Captan 50 W (a phthalimide fungicide) solution by adding 25 g Captan powder to 500 mL sterile ddH₂O in a squirt bottle (use within 1 week). Wear gloves and a mask when weighing out Captan powder. The powder doesn't really dissolve in the water but rather forms a slurry. It is therefore important to shake the bottle often to keep the slurry at the appropriate 5%. Wear gloves when handling the Captan solution. Add approximately 5 mL of this Captan slurry to each vial, remembering to agitate the squirt bottle between vials. Recap the vials and let the seeds soak in this solution overnight to imbibe. They can be removed from the transfer hood as long as they are capped.

Germination of sorghum seed. Next day (or after at least 5 h of soaking in Captan) under a laminar flow hood, pour off Captan slurry (into the Captan waste container) after shaking the tube to resuspend the Captan. Take care not to dump the seeds. Add 5 mL sterile distilled water. Pour seeds and water into labelled sterile 100 mm petri dishes, each containing two sterile 90 mm germination paper circles (#SD3-3/8 from www.anchorpaper.com) in the bottom and another wetted one in the lid. Replace the plate cover (lined with germination paper). You may use autoclaved glass petri dishes so that they can be reused. If the seed are clumped together, spread them across the filter paper with gentle shaking or use forceps cleaned with alcohol to move them. Stack plates inside a plastic box with a loose fitting lid to retain high humidity but also allow air flow. Add wetted paper towels to the box to help maintain high humidity. Place this box in a warm dark place (30 °C incubator) overnight or until the seed has germinated and the radicle is around 1 cm long. Unfortunately, this varies among accessions so you may have to adjust the time in the incubator. One cm is ideal, but seedlings with roots as long as 2 cm and as short as 0.5 cm are still okay.

Plating sorghum into agar with embedded Striga. Next day, move plates containing germinated seeds to the laminar flow hood. Remove 100 mm plates containing *Striga* in agar (at least 8 days after conditioning start date, but not more than 18 days—optimal is 10–12 days). Remove atomizer containing 10^{-5} M GR24 (see inset “*How to prepare and keep a clean and active GR24 solution for applying to agar plates*”) from refrigerator so that it can come to room temperature. Open each plate (under the hood) and wipe out any condensation that may have formed from inner lid surface. Label agar plates at the edge on both the top and bottom with the appropriate sorghum entry number. Use alcohol wiped forceps to puncture the agar and split it gently apart enough to accommodate the sorghum seed and radicle. Make this “planting hole” over the place where you marked the entry number. Gently pick up the germinated sorghum with the forceps, holding it by the kernel so as to not damage the emerging shoot or root (be especially careful with longer roots, as they can easily bend and break). Plant it into the gap you made in the agar such that the root reaches beneath the agar to where the *Striga* seeds are embedded and that it points toward the center of the plate. The kernel should be at least 1 cm from the edge of the plate. Use the forceps to make the split in the agar close over the remaining root, again being very careful not to damage it. Done properly, the root will grow across the plate among the *Striga* seed and the shoot will grow in the space between the top surface of the agar and the lid. Prepare at least three plates per sorghum accession being tested, more if the accession is heterogeneous (like a landrace or segregating population). I prefer to prepare four plates of each from which the best three best will be used for measurements. Wash forceps in 100% ethanol between each accession. Leave one plate with the same batch(es) of *Striga* blank (no sorghum) to test the germinability of each *Striga* batch. After all accessions are plated, move in stacks of three on trays to shelves in the 29 °C incubator where they will remain for 3–4 days. After returning other plates to the incubator, open the blank plate (no sorghum) and lean it against the back of the hood. Get the atomizer containing 10^{-5} M GR24 (that you removed from the 4 °C earlier, it should now be at room temperature, see inset “*How to prepare and keep a clean and active GR24 solution for applying to agar*”

plates”), swirl it to mix and spray the exposed agar with two firm squeezes (three if you are spraying a 25 mm deep plate) of the bulb. Make sure you are holding the spray bottle upright so that the bottom of the atomizer reservoir is parallel to the surface of the transfer hood when you squeeze the bulb. This should deliver around 35 μL of the solution so that once it diffuses through the 35 mL (or 60 μL to 60 mL in deep plates) of agar, gets diluted to around 10^{-8} M. Return spray bottle to the 4 °C refrigerator. Replace lid on the sprayed blank plate and return to incubator, stacking it along with those planted with sorghum no higher than three plates to a stack. Check the plates in three days. As long as the roots of the sorghum have grown across the plate, most being at least 5 cm long, they are ready to read.

Reading Striga germination at Day 3. With the blank plate(s), that is a plate run in the same batch as the ones being read that contains only *Striga* with no sorghum sprayed with GR24, randomly position the plate in at least ten different areas under a dissecting microscope, counting both the number of *Striga* seeds and the number of germinated *Striga* in individual 2×2.5 cm fields. If the *Striga* seed density is ideal, you will be counting about 100 seeds in each 5 cm² field. These counts may be taken later by imaging the ten fields. Observe all plates through the bottom of the plate because the root and *Striga* seed are closest to this surface and you won’t have to look through the layer of agar. Record each of these counts on the data sheet. Aim at counting around 1000 seeds. Calculate the *Striga* batch germination rate for this set of plates by adding all the counts (from each of the ten field counts) for no. germinated *Striga* and dividing this by the total number of *Striga* seeds (in the ten fields). This should be at least 30%. If it is less, the *Striga* seed is not responsive enough to give you a meaningful measure of *Striga* germination stimulant activity on your sample (sorghum) plates and so you shouldn’t waste more time reading those.

Provided you have > 30% germination on your blank plates, the plates containing sorghum are checked under the dissecting microscope after 3 days. Before taking them to the microscope, wipe any condensation inside the plate lids with clean paper towels (plates should only be opened under the transfer hood). Select the best three plates out of the four prepared for each entry (more if you’re looking at a heterogeneous group) for microscopic observations. Selections should be based on health of the host plant and whether its root has properly penetrated the agar. Assign each a number (– 1, – 2, – 3) and mark it on the top and bottom of each plate next to the entry number marked when planting the sorghum. Record the condition of each host plant on the data sheet (Fig. 5). Use the descriptor “G” (good) for normal looking sorghum or “F” (fair) if the root or shoot is especially small or there is some other problem. Record any problems with host condition in the notes column of the data sheet. If the sorghum seedling is damaged or didn’t grow, and its main root is < 3 cm long, return it to the incubator if you have less than three plates for that entry. Check it again in a day or two and use it only if the root exceeds 3 cm.

Observe plates at about 10 \times magnification (again through the bottom of the plate). Look for germinated *Striga* along the sorghum root beginning 2 cm from the kernel. Mark a line at the basal 2 cm and only search for germinated *Striga* beyond this mark (toward the root apex). Mark a small dot on the plate over the seed coat of the three germinated *Striga* furthest from each host root (Figs. 6 and

Striga hermionhica SH9P5 Striga seed batch germination rate = 60% (These further germinated seeds on plate 1 were from distance 10mm to 15mm)

Conditioning started 10/27/21; Striga embedded in agar 11/2; Sorghum plated 1/5; GR24 plate applied 1/5; MGD read and plates imaged 1/18 (6/3)

Accession name	entry #	plate	Sorghum seedling fitness			MGD (mm) Plate 1			MGD (mm) Plate 2			MGD (mm) Plate 3			mean all plates	std all plates	stage germination without activity			
			1	2	3	1	2	3	1	2	3	1	2	3						
PP023 Normal	28	G	G	G	G	161	149	144	151	6	6	111	111	100	113	13.2	2.8	high		
PP050 Bmr1-2-ref	29	G	G	G	G	151	141	100	13.0	17	12	11	13.3	14	11	9	13.3	12.6	1.1	high
PP290 Bmr1-2-18	30	G	G	G	G	199	188	166	17.7	24	18	17	19.7	18	16	15	16.6	17.9	1.7	high
PR1810000 Normal	31	G	G	G	G	251	248	198	191	300	229	22.7	12	13	100	113.0	200.4	8.4	high	
PR183999 Bmr1-2-ref	32	F	short branched root	F	short root (short)	200	153	144	16.6	12	12	12	12.0	18	14	13	14.2	3.1	high	
PR183999 Bmr1-2-ref	33	G	G	G	G	151	12	151	12.7	22	20	20	20.7	10	9	9	9.3	14.2	3.8	high
Sudan Zera Zera Normal	34	F	short root	F	short root	142	132	111	11.1	7	7	5	6.3	6	7	6.8	6.2	2.7	low	
Sudan Zera Zera Bmr1-2-ref	35	G	G	G	G	7	7	5	6.3	15	14	13	14.0	22	21	21.0	13.9	7.6	high	
Sudan Zera Zera Bmr1-2-18	36	G	F	branched root	F	short root	200	29	24	26.0	19	17	16	17	24	17	21.7	23.8	4.5	high
PL12188 Normal	37	G	G	G	G	191	18	17	18.8	26	22	18	22.6	21	18	20.1	20.2	2.0	high	
PL12188 Bmr1-2-ref	38	G	G	G	G	205	200	21.7	24	24	24	24.0	19	14	13	15.5	20.3	4.5	high	
PR12188 Bmr1-2-18	39	F	short root	F	short root	6	6	5	5.7	15	15	13	13.7	13	13	10.0	12.0	10.4	4.2	high
PR12188 Normal	40	G	G	G	G	131	6	8	8	131	12	13	13	14	18	18	20.0	14.1	5.7	high
PR12188 Bmr1-2-ref	41	G	G	G	G	14	14	12	13.3	22	22	19	21.0	18	18	15	17.0	17.1	3.8	high
PR12188 Bmr1-2-18	42	G	G	G	G	200	15	13	16.0	25	22	19	22.0	20	24	22	25.0	23.0	4.6	high
PR12188 Normal	43	F	short root	F	short root	161	9	8	11.0	16	16	13	13.7	16	7	15.0	12.6	2.7	high	
PR12188 Bmr1-2-ref	44	G	G	G	G	22	17	17	18.7	30	29	28	29.0	21	22	20.1	23.1	5.3	high	
PR12188 Bmr1-2-18	45	G	G	G	G	16	15	15	15.3	12	12	12	12.0	19	14	12	15.0	14.3	1.8	high
PR12188 Normal	46	G	G	G	G	27	26	25	26.0	18	16	15	16.7	25	24	23	25.0	22.0	5.0	high
PR12188 Bmr1-2-ref	47	G	F	short root	F	short root	28	24	25	23	31	9	14.6	31	28	25	26.0	22.6	7.5	high
BT423 Normal	48	G	G	G	G	19	17	15	17.0	17	16	12	15.0	15	15	13	14.3	18.4	1.4	high
BT423 Bmr1-2-ref	49	G	F	short root	F	short root	50	27	24	24	10	10	11.3	21	20	16	17.0	18.7	6.5	high
BT423 Bmr1-2-18	50	G	G	G	G	11	11	10	10.5	9	9	9	9.0	17	14	13	14.7	14.6	2.9	high
PR1508478 (Bmr1-2-18, sp1)	51	F	short root	F	short root	2	0	0	0.7	0	0	0	0.0	6	5	2	4.3	1.7	2.5	low

Fig. 5 Example data sheet for recording MGD

7). Try to avoid areas within 2 cm of the kernel if the main root happens to curl back toward it. Using a transparent ruler, record the distances of these marks (to the nearest 0.5 mm) to the nearest sorghum root (main root or branch). If less than three germinated *Striga* are seen, record this on the sheet. Maximum germination distance (MGD) is the average of these three measurements on each seedling. If less than three germinated weed seeds are observed, enter “0” in the calculations for average. For example, if you observe only one germinated *Striga* in the measuring zone and the distance from that to the nearest root is 1 mm, then the MGD is 0.3 mm [(1 + 0 + 0)/3]. MGD for the accession is the average of at least three good seedlings. Also calculate the standard deviation between seedling MGDs of a given accession to give an indication of the variability between them. The MGD is a measure of *Striga* germination stimulant activity. Generally, a sorghum seedling with an MGD > 10 mm has high *Striga* germination stimulant activity. A seedling with an MGD < 5 mm has low *Striga* germination stimulant activity. Those with MGDs between 5 and 10 are borderline lows and may indicate influence of germination inhibitors (see next section). All measures should be considered in the context of the standard high and low checks run with the set of plates. Sometimes the 10 mm threshold will need adjusting slightly, particularly for those borderline accessions or with low responsive *Striga* seed sources. A sorghum line truly having the resistance mechanism of low *Striga* germination stimulant activity should consistently show MGDs below 10 mm from set to set.

Some optional measures—extending the agar assay. Near host germination rates can also be recorded at Day 3 to get another measure of *Striga* germination stimulant activity. A standard area is selected for all observed seedlings so that comparisons of



Fig. 6 Example of MGD measures at Day 3. Blue dots were marked on bottom of plate on what appear to be the furthest germinated *Striga* seeds. Distances of the three furthest to the nearest root are measured and averaged for the MGD [in this case, $(8 + 8 + 10)/3 = 8.7$ mm]

Striga germination stimulant activity as well as any possible germination inhibition and influence on haustorial formation can be made. This measure generally correlates well with MGD except in cases where germination inhibitors are present in the sorghum root exudate. It is also useful for comparing to in vitro germination assays using collected sorghum exudates. To prepare for these counts, cut a template from a transparent ruler that is 2×2.5 cm. Use this or another ruler to make a small mark 2 cm from the sorghum kernel along the main root. Use the template to trace a rectangle on the bottom of the plate, keeping one of the narrow edges (a 2 cm side of the rectangle) on the 2 cm mark along the main root you made from the kernel. Try to keep the main root in the center of this rectangle so that it splits it in half, that way the rectangular space you will observe under the microscope will cover a 5 cm^2 area along the main root from 2 to 4.5 cm from the kernel and roughly 1 cm on either side of the main root. After marking all your plates with these rectangles, look at them each under the microscope, setting the magnification to around the 1.4 mark, or there about, such that the edges of your drawn rectangle match the edges of your viewing field. Once you've set this field of view for the first plate and focused and set the illumination to clearly see the main sorghum root and surrounding *Striga*, you shouldn't have to change it for the other plates. Snap a photo of each plate, keeping track of which image goes with which plate. Make sure you always orient each plate



Fig. 7 Another example of MGD measure at Day 3, this time on a sorghum seedling with low *Striga* germination stimulant activity [$(0 + 0 + 0.5)/3 = 0.2 \text{ mm}$]

as you image them so that the basal end of the main root (toward the kernel) is always to the right of the captured image. These images contain all the *Striga* within about 1 cm on each side of the main root in this zone and from these counts of germinated and total number of *Striga* seed can be made later. Record these on the data sheet (Fig. 8). Because germinability varies between conditioned batches of *Striga* seed, it is a good idea to correct germination rates counted in your images by dividing the germination rates by the batch germination rate determined from the blank plate that you treated with GR24 at Day 0 and from which you imaged 20 random 5 cm² fields on Day 3. This “corrected” germination rate allows you to more meaningfully compare plates run in separate batches or with different *Striga* seed sources, as it tells you the proportion of viable seeds that responded to the sorghum root germination stimulants.

To overcome differences in *Striga* germination stimulant activity between sorghum accessions for post-germination measures, you can spray all the plates (after imaging) with 10⁻⁵ M GR24 (final conc. = 10⁻⁸ M, after diffusing through the agar) as you did earlier for the blank plate, under the hood and return them to the incubator for two more days.

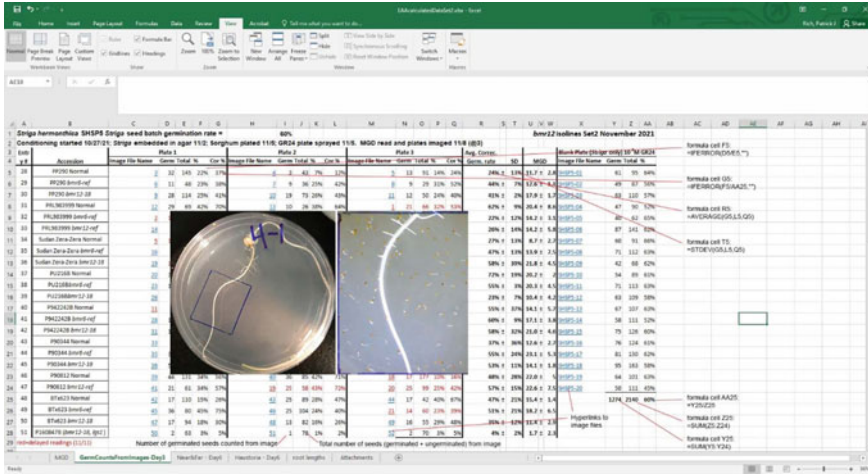


Fig. 8 Example data sheet for recording Day 3 measures of germination rates. Germination rates are corrected for germinability of *Striga* seed source used

How to prepare and keep a clean and active GR24 solution for applying to agar plates

1. Prepare a GR24 “stock solution”

Dissolve 0.024 g GR24 (a synthetic strigolactone, molecular wt. = 298.3) in 10 mL DMSO

$$0.024 \text{ g}/0.01 \text{ L} = 2.4 \text{ g/L} \approx 8 \times 10^{-3} \text{ M (in 100\% DMSO)}$$

Keep this frozen in a brown glass bottle at $-20\text{ }^{\circ}\text{C}$ when not in use. Thaw thoroughly before using. Stored properly, this solution will last several years

2. Prepare the GR24 “application solution”

Add 250 μL “stock solution” to 100 mL ddH₂O in a clean brown glass bottle $8 \times 10^{-3} \text{ M GR24} \times 0.25 \text{ mL}/100 \text{ mL} = 2 \times 10^{-5} \text{ M (in 0.25\% DMSO)}$

Use this solution to fill the atomizer (15-RD glass atomizer with plastisol bulb, De Vilbiss Healthcare LLC) which holds about 25 mL. Keep the remainder (not put in the atomizer) frozen at $-20\text{ }^{\circ}\text{C}$ when not in use. Thaw thoroughly before filling the atomizer and shake after thawing to redissolve any GR24 which may have precipitated. Keep atomizer in a refrigerator ($4\text{ }^{\circ}\text{C}$) when not in use. Bring to room temperature before spraying plates

3. Spraying the plates

Open agar plates only under a laminar flow hood to avoid surface contamination. GR24 is usually applied after measuring MGD and imaging for germination counts 3 days after plating the sorghum in the agar embedded with *Striga*.

Only open a few plates at a time when spraying to avoid drying them out under the airflow of the hood. Lean them at the back of the hood so that the surface of the agar is perpendicular to the work surface. Take care not to damage the sorghum shoot which may fall from the bounds of the plate edges. Holding the glass reservoir of the atomizer in one hand such that the delivery nozzle is 10–15 cm from the exposed agar surface, give the bulb two firm squeezes with the other hand. This should deliver about 35 μL ($\approx 17 \mu\text{L}$ per squeeze) to the agar. If you are using 25 mm deep plates containing 60 mL agar, spray with three firm squeezes of the bulb. It is important to keep the bottom of the atomizer reservoir parallel to the work surface of the hood. Tilting it excessively will not deliver the proper volume. Assuming the volume of the agar is 35 mL (60 mL in deep plates), the final concentration of GR24, once it diffuses through to the embedded *Striga* seed, will be in the range of 10^{-8} M, which is ideal to germinate all germinable weed seed. The final DMSO concentration is negligible and will not harm the plants. Replace the lids on the sprayed plates promptly, returning them to a horizontal position. Carefully guide the sorghum shoot back into the confines of the plate if it came out during spraying. Make sure not to pinch it between the bottom plate sides and lid. Wait two days before imaging for post-GR24 germination counts. Return atomizer to refrigerator. A full atomizer reservoir should be enough to spray 100 plates

Two days after spraying the plates with GR24 (Day 5), mark a second 2×2.5 cm rectangle on the bottoms of the plates using the template you used on Day 3 (Fig. 9). This time, however, choose an area as far from any roots (especially the main root) as possible on the plate, avoiding that area within 2 cm of the kernel. Use a different color for this rectangle than what was used on Day 3. Where you place this second rectangle will vary from plate to plate, the idea being that you observe *Striga* seeds minimally influenced by any sorghum roots so that you can compare these to that *Striga* within 1 cm on each side of the main root from 2 to 4.5 cm. Since you sprayed the plates with GR24, most weed seed on the plate, whether near or far from the sorghum root, ought to have received adequate germination stimulant. Reimage the rectangle you marked on Day 3, the “near-host-root”, and snap another image of the new “far-from-root” 2×2.5 cm area for each plate. Again, keep the plates oriented in the same way (kernel to the right) as you did on Day 3 and keep track of each image file name with your “near and far” images of each plate.

By comparing these areas, in terms of *Striga* germination rate particularly, you can measure any inhibition that a sorghum root might have on *Striga*. Germination counts are made on your Day 3 and Day 5 images by dividing the number of germinated *Striga* seeds in the image by the total number of seeds and express as a percentage to determine the germination rate. You may also want to determine the percent germinated *Striga* near the root with haustoria at both Days 3 and 5. To do this, count the number of germinated *Striga* with haustoria. Only those *Striga* radicles that are

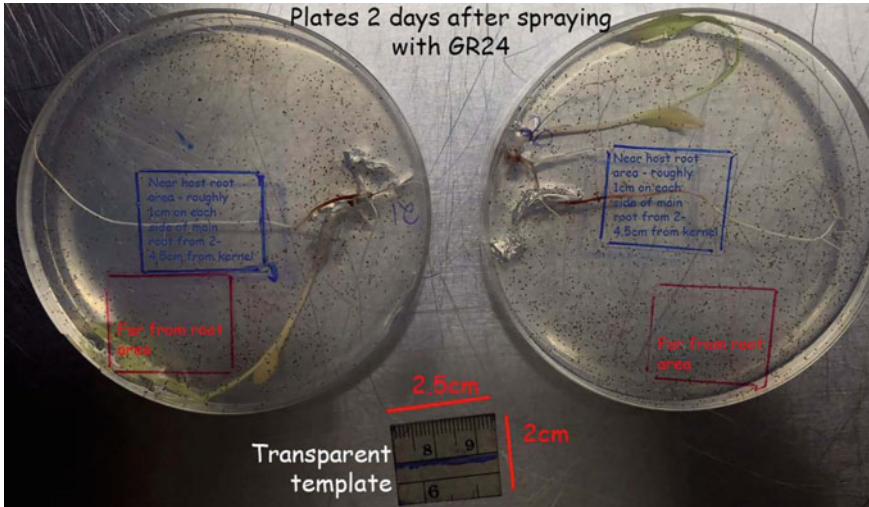


Fig. 9 Agar gel assay plates marked for “Near & Far” measures. After reading the germination distance (MGD) at Day 3 and imaging the area 2–4.5 cm from the kernel for observations and germination counts (blue rectangles), plates are sprayed with 35–40 μL of 10^{-5} M GR24 (final conc. = 10^{-8} M) to germinate remaining *Striga* seed on plate that was not stimulated by the host root and returned to the incubator for two more days. At Day 5, before reimaging the “near” area (blue rectangle), a second (red) rectangle is drawn on the plate as far from the main root as possible tracing the same 2×2.5 cm template cut from a transparent ruler (bottom). Both areas are then imaged and germination rates within each compared for any indication of germination inhibition

wider at their apical end relative to their basal width are counted as having haustoria. Divide this count by the number of *germinated* weed seeds in the area (Fig. 10).

To determine if there is any germination inhibition near the host root, use the two images for each plate taken after GR24 treatment, the Day 5 images. Count the number of germinated seeds and total seeds in each rectangle to determine the germination rates in each place. Then divide the germination rate calculated for the “near-host-root” area by the germination rate of the “far-from-root” area to obtain the “germination index” (GI). To avoid effects of peculiarities on certain plates where root-free areas are hard to find, average the far-from-host germination counts for all plates in a set (all plates from all entries tested in that set) and use this value as the germination count by which to divide each near-host-root rates to determine the GI. Average these germination indices for all plates observed for a given accession (Fig. 11). Example images from which these counts are taken are shown in Figs. 12 and 13. A GI wildly different than one could indicate some inhibition of the sorghum accession on *Striga* germination. Most sorghum does show some germination inhibition by this measure, that is, GI in the range of 0.7–1.0. Sorghum accessions that consistently show a $\text{GI} \leq 0.65$ in this assay, especially with multiple *Striga* seed sources, we consider to have a germination inhibition mechanism of *Striga* resistance. The impact of this trait on overall resistance and its chemical nature remains to

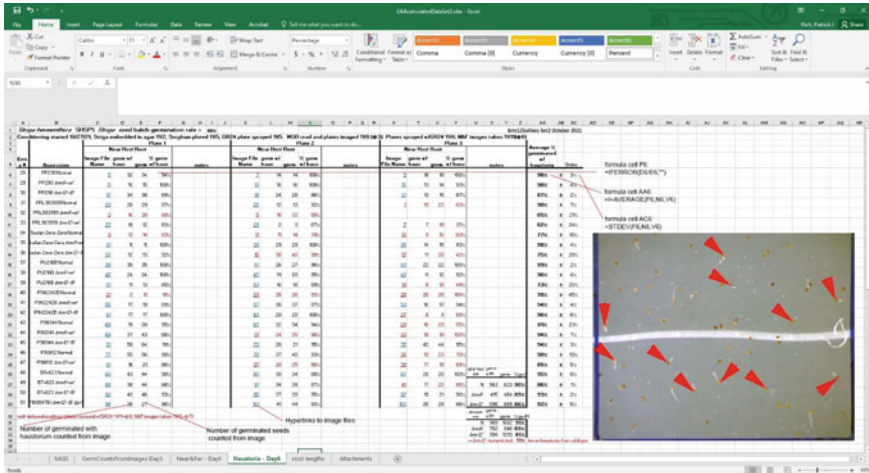


Fig. 10 Example data sheet for recording Day 5 proportion of germinated *Striga* forming haustoria. Red arrows on example image indicate germinated *Striga* with haustoria

be determined. It is, however, clearly distinct from low *Striga* germination stimulant activity.

You may also note some other allelopathic influences of certain accessions on surrounding *Striga* in the Day 5 “near” images. Do haustoria form in germinated *Striga* at similar rates? Do these haustoria look unusual? Do the *Striga* come out of their seed coats? Are *Striga* radicals unusually short or opaque near the sorghum

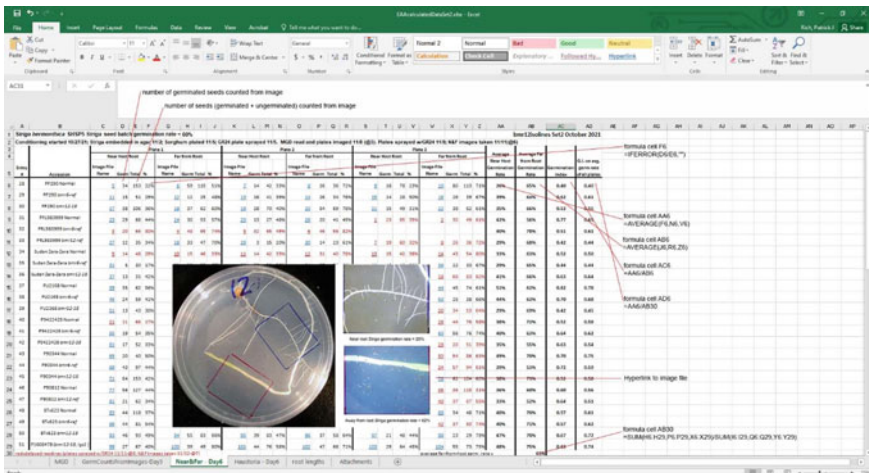


Fig. 11 Example data sheet for measuring germination inhibition

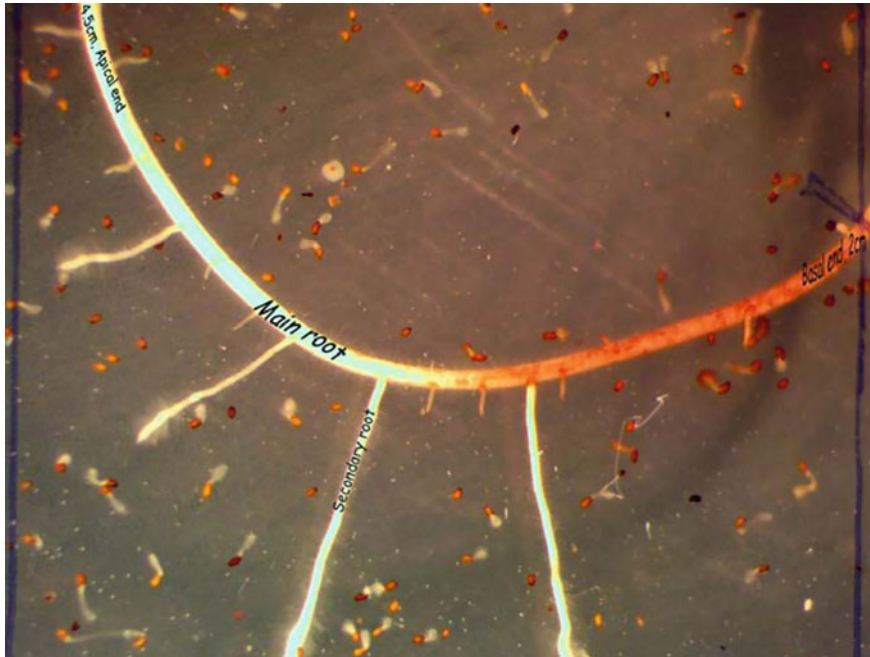


Fig. 12 Image of “near-host-root” area. Image taken at Day 5 (2 days after GR24 treatment) covering an area of 2×2.5 cm. This includes *Striga* within approximately 1 cm of the host main root over a length of 2 cm (right) to 4.5 cm (left). Note that most germinated *Striga* in this area have haustoria. Toward the basal end (right) an orange colored exudate can be seen being absorbed by surrounding *Striga*

root? Do you notice colored exudates (yellow, orange or red) bleeding from the sorghum root and being absorbed by the surrounding *Striga* (Fig. 12)?

You may also want to count the number of lateral branches on the sorghum root, their lengths, etc. as an indicator of rooting habit. These kind of measurements are easier if you can get a digital image (or images) of the entire plate. If the microscope you have won't quite zoom out that far, a smart phone camera using the “square” setting is a good way to image the entire plate. Even better if you can compare two images of the same root, perhaps at Day 3 and Day 5, to get an idea about root growth rate over that specific time. Software that comes with some microscopes or root analysis software like ImageJ[®] (available for free from the United States National Institutes of Health at <https://imagej.nih.gov/ij/>) can assist with such measures.

Even more from the agar plates. If you use deeper (25 mm) plates with thicker agar layers (60 mL) you should be able to keep the plates for observing any *Striga* attachments on the sorghum seedlings. Leaving the plates stacked only two or three high on a lab bench under fluorescent lights can keep the sorghum seedlings alive and growing for three weeks. As long as the plate remains free from mold or bacterial contamination and the sorghum shoot remains green, plates remain useful. Wiping

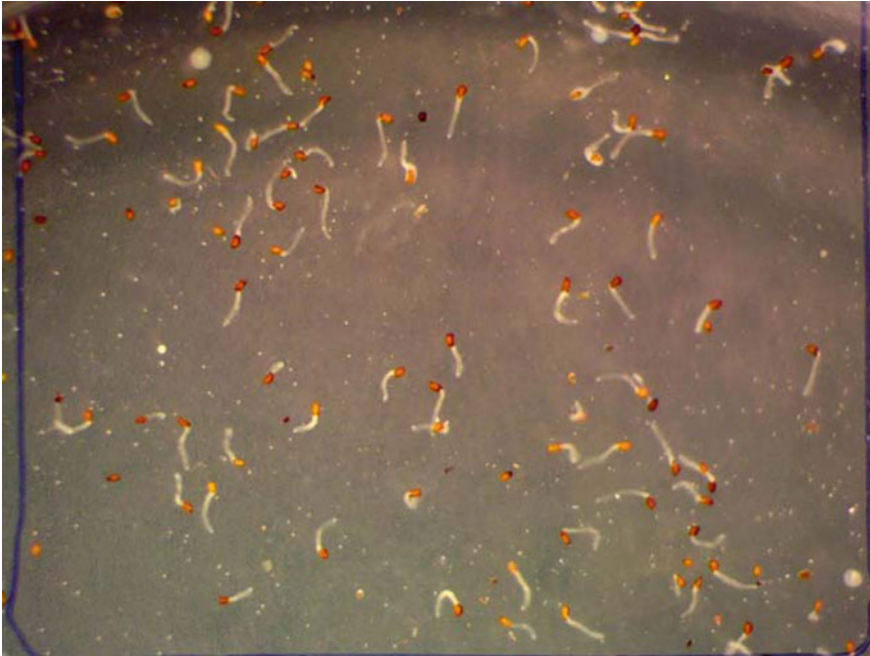


Fig. 13 Image of “far-from-root” area from the same plate. Image taken at Day 5 (2 days after GR24 treatment) from the same plate covering a 2×2.5 cm area away from the sorghum root. Note germinated *Striga* here lack haustoria since they are not near enough to the host to receive the haustorial initiation signal

out condensation inside the lid of the plate where the sorghum shoot touches also helps to keep them going. If, after 10 days from when you planted the sorghum in the agar, the sorghum shoot is green and the agar layer hasn't dried out (still comes to at least the half full mark in the bottom of the plate), you can check them under the microscope for attachments. With the magnification set in the mid-range, follow the main root and its branches to look for *Striga* that have attached. Circle and mark each with a unique number on the bottom of the plate and take a picture of each at higher magnification. The position of each on the root system (whether they are on the main root or a branch, for instance) can be recorded from a whole plate image taken with a smart phone at each observation day. If you can get two or three images over time of particular attached *Striga* on a live host, you can have a measure of its ability to support early *Striga* growth. Imaging individual attachments marked at 10 days and reimaging at 15 and 20 days (or there about) after planting the sorghum allows one to get an idea of whether and how well *Striga* grow on a particular sorghum accession. Simple data collected from tracked individual attachments as “successful” if the parasite grows over the course of observation or “unsuccessful” if an attached *Striga* does not grow allows one to compare sorghum accessions both in terms of the average number of attached parasites per seedling and percentage of those that

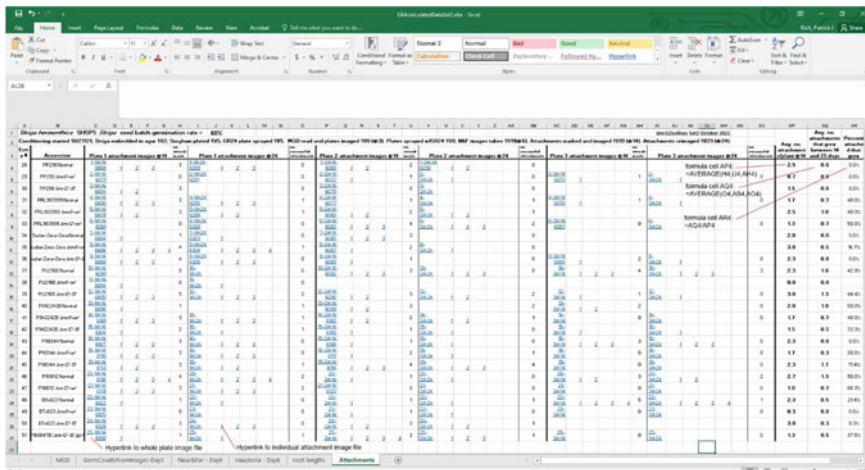


Fig. 14 Example data sheet for keeping track of *Striga* attachments and quantifying post-attachment resistance in sorghum in agar plates

succeed (Fig. 14). Such measures can indicate differences in their compatibilities as *Striga* hosts. With the proper checks, the extended agar assay in 25 mm plates can be used as an initial screen for post-attachment *Striga* resistance.

It is very important that you keep track of the fitness of the sorghum on the plate with each set of images and make sure the agar hasn't dried out if you go to the trouble to follow the growth of individual parasites over time (Fig. 15). Whole plate images are useful for this purpose. All *Striga*, even on a susceptible host, is going to look sick and die if its host is dying or dead. Don't bother looking at plates with a sorghum whose shoot is brown or on which the agar layer is drying up. Also your record keeping needs to be such that you know which image corresponds to which *Striga* on which plate so that you can put together a string of images at the end of the observation period (perhaps 3 images over 2 weeks, Fig. 16). This is, of course, laborious and probably not worth it if your attachment frequency is low. Attachment frequency tends to be higher in thicker agar layers (60 mL) in deep (25 mm) plates. Resistance at this early post-attachment stage, generally called incompatibility, may be observed if you have several good plates of a given accession. If a sorghum accession consistently shows fewer attachments than a susceptible check in the same set, this may indicate some post-germination, pre-attachment resistance. If the *Striga* attaching to plants of one accession don't grow, turn purple or grows at a slower rate than those on a susceptible check, you might be observing incompatible resistance (Fig. 17).

The agar assay is not ideal for measuring these post-germination resistance mechanisms because the sorghum seedling is so confined and attachment frequency tends to be lower in agar than on other media like that described in Chapter "Phenotyping for Resistance to *Striga Asiatica* in Rice and Maize Mutant Populations In Madagascar". With several sets of good plates with multiple *Striga* attachments and careful



Fig. 15 If given proper light and humidity and a thick enough layer of agar, it's possible to keep plates for three weeks or even longer. Even in this set of standard depth (15 mm) plates, young *Striga* can be seen growing on 20-day-old sorghum seedlings. The sorghum eventually succumbs to the cramped conditions and lack of nutrients

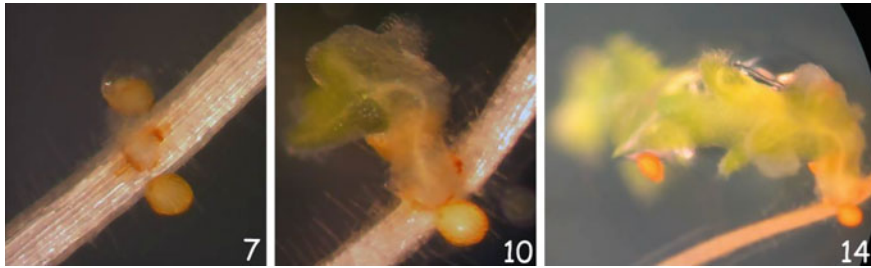


Fig. 16 Images of the same *Striga* attached to the root of a susceptible sorghum over time in agar. Images taken at 7, 10 and 14 days after planting the sorghum seedling in agar embedded with conditioned *Striga* seed. Plate was treated with GR24 after reading MGD in the Agar Gel Assay. In this case, images at the latter days had to be taken at lower magnifications than the original image to capture the larger *Striga* shoot

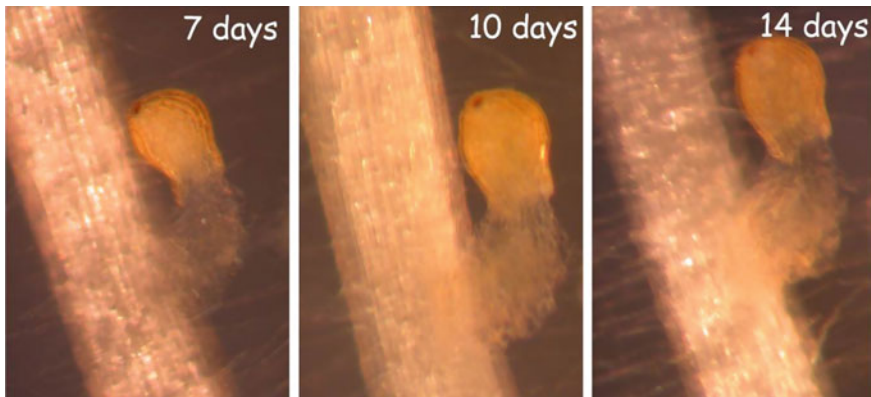


Fig. 17 Images of the same *Striga* attached to the root of a resistant sorghum over time in agar. Images taken at 7, 10 and 14 days after planting the sorghum seedling in agar embedded with conditioned *Striga* seed. Plate was treated with GR24 after reading MGD in the Agar Gel Assay. In contrast to the series of images collected on the susceptible sorghum, this attached *Striga* does not grow

observations, such reactions can be indicated from the same plates used to determine if pre-attachment *Striga* resistance mechanisms are at play. Post-attachment *Striga* resistance can then be confirmed in other co-culture media.

Conclusions

The most important information (and what the assay was originally designed for) one gets from the agar gel assay is the MGD, which is an established measure of *Striga* germination stimulant activity (Hess et al. 1992). As long as one has the proper

amount of *Striga* seed embedded in the agar, and the germinability of that seed is adequate (> 30%, as measured on the blank plates), one can determine whether a sorghum accession has the resistance mechanism of low *Striga* germination stimulant activity. To date, the only *Striga* resistance gene identified in sorghum is *LGS1* which controls germination stimulant activity (Gobena et al. 2017). Recessive alleles at this locus changes the type of strigolactone exuded by sorghum roots. Mutagenesis could result in new *lgs1* alleles which could be confirmed in mutant lines by chemical analysis of root exudates (This chapter) and sequencing of PCR amplicons targeting *LGS1*. More useful would be gained resistance expressed as germination inhibition, lower haustorial inducing capacity or at the post-germination stages of the *Striga*-host interaction. Then *lgs1* could be introgressed into these mutant lines through marker assisted backcrossing to offer more durable *Striga* resistance through pyramiding. The extended agar assay may detect such novel resistance mechanisms and ultimately, with further investigation, lead to gene identification underpinning the gained *Striga* resistance.

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