

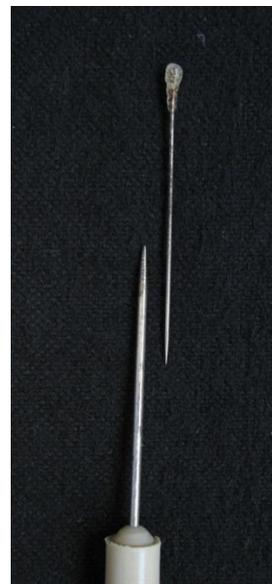
Illustrated Guide to Crossing Brachypodium

Garvin Lab Methods (2009)

The information and images assembled here are to be included in a manuscript describing methods and quantifying different variables associated with crossing Brachypodium. Due to broad interest in crossing Brachypodium, I am releasing this compilation of information that has been developed in my lab to allow Brachypodium researchers to establish successful Brachypodium crossing in their labs now, rather than wait for the publication. If you have any further questions, just contact me.

-Dave

Basic Tools for Crossing



Fine scissors (the finer the better), a fine needle, and a loupe. The loupe came from Daigger, their 5X magnification one is fine (10x is available but it is not better and is more cumbersome). To the right is a comparison of size between a dissection needle and the needle I use. I put a gob of cement on the end to make it easier to hold. The needle tip was dulled just a bit so that it will not punch through the palea during emasculation. Go to a craft store and get a variety selection of needles – try the thinnest one. Note – a stick pin is too large.



Loupe is worn on a headband (eg old PAG lanyard) in front of one eye. You will quickly get used to doing some manipulations looking through the other eye, and then when you need magnification you can look through the loupe. Wedge the bottom of loupe on cheek below eye to keep in place when using

I do my work in the mouth of a growth chamber. The good light really helps, and I do not have to take plants elsewhere. I prop the pot with plants being manipulated on a stack of larger pots, to a comfortable height

Floret Development

-Floret series from a single spikelet (basal floret at bottom) upward ,to help highlight some developmental points relevant to crossing. This should help you calibrate your eye to selection of properly staged florets. To the right are isolated paleas. Reference both images to get a feel for the stage of development.



Extremely immature and not useful yet. Tiny green anthers.

Very immature. Note columnar shape of stigma, tiny ovary size, and greenish anthers. Too young to be used.

Stigma just starting to get feathery, but in frontal view of intact floret it still looks pretty compact. Ovary is still small and subject to dislodging. Anthers still small and not useful. Still too young for use.

Stigma getting more feathery and ovary continues to grow. This is still perhaps a day early (note in frontal view that the stigma does not appear particularly feathery yet). We are working on some more precise quantification of anther stage and fertility, and will provide info once in hand.

Stigma very feathery. Note that this is getting obvious in the frontal view of intact floret. This is getting into the sweet spot for manipulations. Suitable for emasculating and use as female in crosses.

May be hard to see, but note that anthers are larger. This would probably be a good anther source for pollination, by methods described. Early in day you could try carefully emasculating and using as a female in crosses, as long as you determine that removed anthers have not dehisced

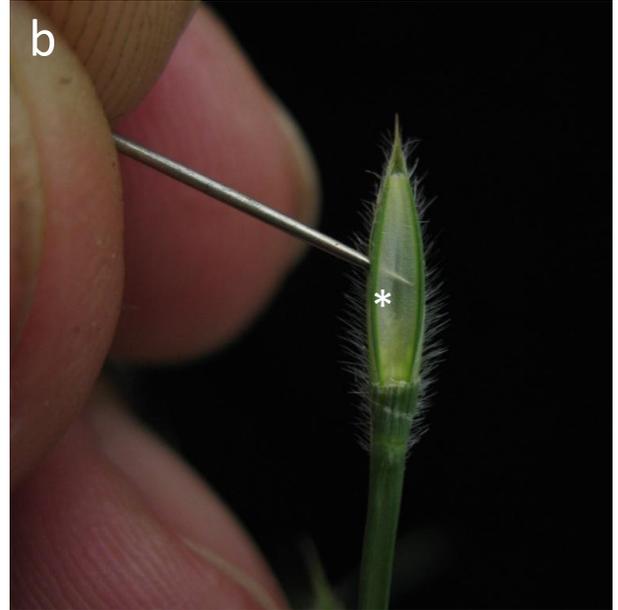
Anther dehiscence likely occurred the day before. Note in the frontal view that the anthers are pushed well up the floret, and old anthers take on a distinct yellow color. You can see shed pollen on the lemma, and the ovary is already swelling in response to successful fertilization

Emasculation

-start by clipping spikelet back to the one floret to be worked with (I virtually always use the basal one). Clip this off as close as possible (see images below) to make it easy to see reproductive organs. I often do emasculations in the morning in prep for crosses in the afternoon. If the emasculated florets gape, tie them gently shut until used (see pollination method for image). Before I do any emasculations, I make sure that there are going to be potential florets available to serve as pollen donors later in day.



Place fine needle sideways at top of palea and lemma and push back a bit against lemma to get inside floret



Slide needle into floret, at open crease (*) ease needle between folds of palea and slide down and under anther



Gently slide anther up crease with needle. Repeat on other side. You will probably find that one anther will be easier than the other to remove based on your handedness. Try rotating the pot 180° and then bend over the culm to gain access to other anther

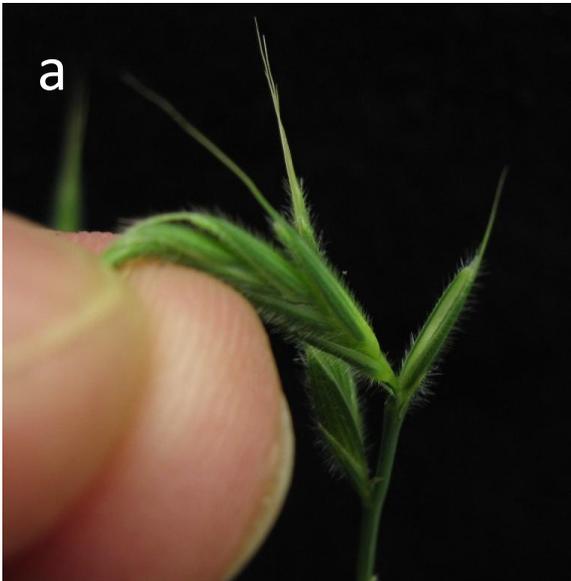


This shows anthers teased up near top of palea folds. Just tease them all the way out. You could probably clip off the floret below the anthers to remove them as well; I don't do this because I want the floret to close back up completely once I am done emasculating

With good lighting, I can easily do this by naked eye. Once a floret is chosen and the spikelet trimmed, it takes me about 30 seconds to emasculate. If enough plants are available, I can identify and emasculate 30 or so an hour. You may want to use the loupe to assess the anthers removed to be sure that they are not too mature and thus may have dropped mature pollen.

Pollen Recovery - Method 1

-I personally use the loupe for manipulations e-f. I do my pollen recovery and pollination steps in the afternoon btwn 2 and 4 pm typically.



Look through florets for a potential pollen donor. Just bend spikelets back to be able to look at anthers through palea, as shown



View of promising floret through palea –the anthers are pushed up a bit and are larger than earlier developmental stages (trust me, with practice this is pretty easy to spot)



Clip back floret just above anthers – this will encourage anthers that are close to dehiscence to move closer to stage of use. You can encourage this along by gently breathing right on the floret after clipping back



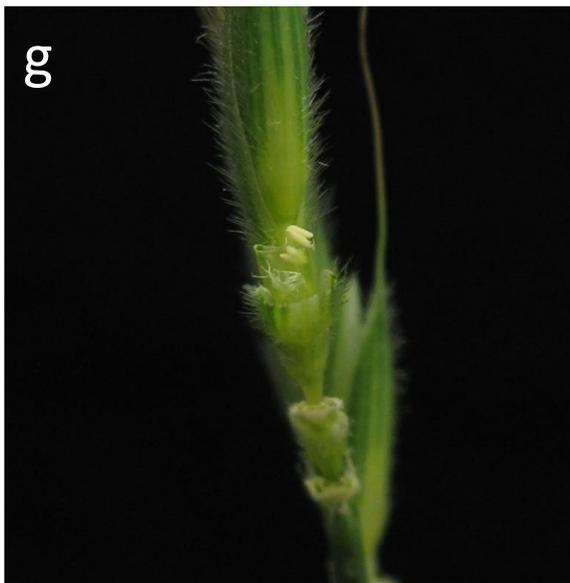
After about 15 minutes (plus/minus 10 min. or so) the anthers that will be useful will swell a bit more, but more importantly they will start to change position in the floret. Sometimes they move directly upward and poke out of floret. Or they may just shift inward as shown here (also see method 2, pane a, for image)



If anthers do not pop up cleanly, just take your needle and stick it through the lemma and into the ovary. Then lift slowly upward. This will squeeze the anthers up further and make them more accessible



The vertical slit opened by the needle in e is evident. Now, gently tease the anthers a bit further out by putting the needle under each and lifting. Note the anther appearance – creamy white, and very turgid. Often when you do this you will see a bit of pollen float away. This is a good sign



Another image of teased out anthers in good shape for use. You can see that the valve on one of the anthers has opened. If shedding pollen here, use directly for pollination (see that method). If not you can tease anthers on to your hand. The heat of the hand helps to encourage dehiscence. You can wiggle with needle a bit to encourage pollen release, in prep for pollination.



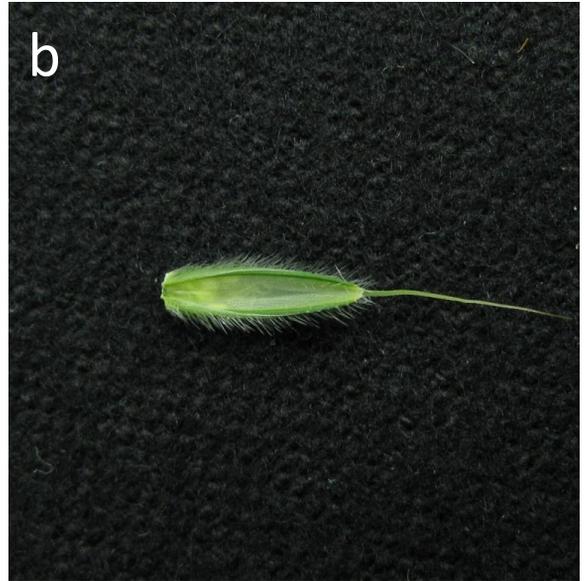
The good thing about a pollen plant is that you can chase new anthers up the length of the spikelet over time. Once you get good anthers from a floret on a given spikelet, remove the remnant of that floret. Day after day, the next floret in line is a good bet as a possible pollen donor. Spikelets so used are shown here. The trimmed spikelets serve as quick landmarks for anther hunting.

Pollen Recovery – Method 2

-I personally use loupe for manipulations c-d



Look for anthers that are turgid and look ready to dehisce. Good light will make it easy to spot these. Those shown here look good – see how full they appear, and they have also begun to move in the floret and shift upward



Clip off the floret near the top of the ovary (avoid disturbing anthers) and transfer it to palm or other working surface



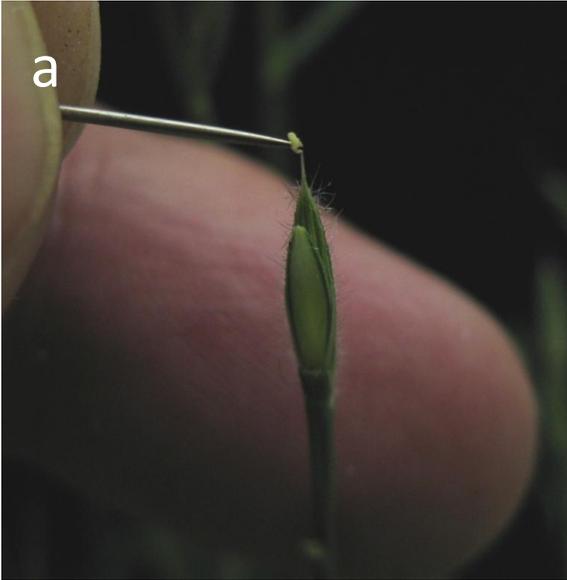
Use needle to separate the palea (which will retain the anthers) and the lemma



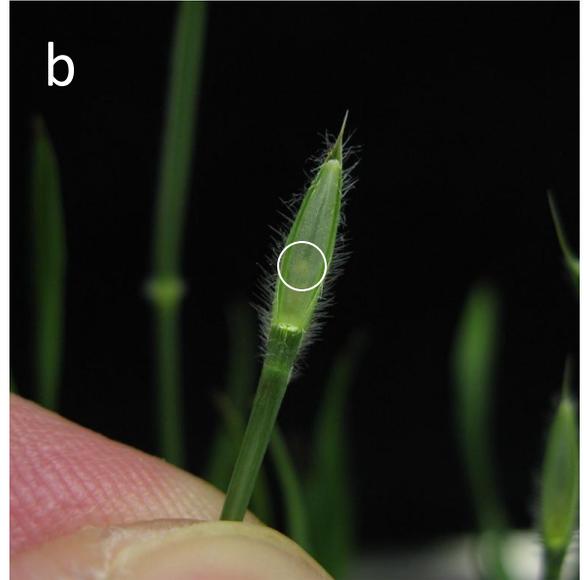
Use needle to tease anthers out of the bottom of the floret. Here you can see pollen being released from anthers due to being disturbed by removal

Pollination

-I use loupe for these manipulations



Regardless of which pollen recovery method used, to pollinate just skewer an anther shedding pollen with your needle. Agitate it a bit if needed to ensure that pollen is being released (you can easily see pollen using loupe). Then stick anther into female floret that has been opened a bit (see emasculatation method, pane a)



Once the anther is in the floret, push it down with the needle to the stigma - I do not bury it all the way down, but rather to the middle of the stigma "cloud" (anther circled above). Move the needle around the stigma a bit as well, if it has pollen on it. Then close the floret, tap by gentle flicking 4-5 times (to dislodge pollen onto the stigma)



I tie floret gently shut. It may help reduce dessication damage or accidental cross-pollination



Note: anthers from the same floret will not necessarily dehisce at the same rate. These two were taken out of the same floret and placed in my palm. The pane at left is at start. The pane at right shows how the upper anther is swelling in the lead-up to dehiscence far faster. The lower anther may not end up being used. Once removed like this, I typically do not like to wait more than 5 minutes for an anther to swell and dehisce (sometimes helped by a bit of agitation with needle). Perhaps with enough time the lower anther will be useful, but if it looks slow I will instead look for a different anther from a separate floret that is further along/a better candidate. Or wait until another day

Notes

These methods are for Bd21 plants grown in 20 hr days at 20°C

Time of day is a factor, as noted in the methods

Different lines show differences in developmental appearance, so these methods may need to be calibrated for your genotypes

I prefer pollen recovery method 1, because anthers that ultimately pop up are essentially always good to use

You can use more anthers for a given pollination if they are abundant

I do emasculations on the same day that we pollinate, but am testing whether emasculations can be done one or more days in advance

You can clip back spikelets to expose target florets for use in future emasculations at least a few days in advance. Florets prepped in advance may develop a bit faster

There are many steps here that can be tweaked to your liking. For instance, others have used a scope for all of these manipulations because they are comfortable doing so or do not have good up-close vision

If you have any questions or comments – please feel free to contact me at David.Garvin@ars.usda.gov , or garvi007@umn.edu