Why do Some Plants of the Same Species Flower and Produce Seed while Others Don't?

A Study on Variables Contributing to Growing Malfunctions of Forages

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"I am but one member of a vast team made up of many organizations, officials, thousands of scientists, and millions of farmers—mostly small and humble—who for many years have been fighting a quiet, oftentimes losing war on the food production front." –Norman Borlaug

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Acknowledgements: I would first like to thank Dr. Jean Hanson for being my mentor. She has taught me so much about the importance of seed production and problem solving. Dr. Jean was extremely busy every day, but she always made sure I was doing what I was supposed to be and helped lead me with my project. If I ever needed help or assistance, she would be right there for me. I would walk into her office to ask her just a few quick questions, and I would walk out 45 minutes later knowing so much more than I intended! Even though Dr. Jean is technically retired, she's one of the hardest working people in the Forage Diversity building. Everyone always wants her attention and help, so all day she's running around assisting everyone. Dr. Jean has an unlimited knowledge of plants; it's truly remarkable. She could answer anyone's question about anything. I really appreciate all the help she gave me while I completed my project. She gave me so much insight and wisdom that I will carry with me for the rest of my life.

Another person I would l like to thank is Tigist Endashaw. She greeted me the night I came to ILRI. I would stop in her office after work almost every day to visit with her. Whenever I came, she would drop whatever it was she was doing and focus solely on me. Most times I would only stay for a half hour or so because I knew how busy she was. There was a couple times where I stayed for almost two hours just because she wanted me there to keep her company while she was still working. Currently, Tigist is doing the work of three people while still maintaining the work of her own job. On top of that, she files all of my paperwork, makes sure I'm doing okay and am enjoying my time here, drives me to church on Sundays, takes me out to tea occasionally, and calls me almost every night to ask about how my day was. She truly is a remarkable person and I couldn't have asked for a better person to look out for me here. Tigist really made my internship here memorable.

I would also like to thank the staff in the Forage Diversity Department at ILRI for all of the support they gave me during my time here. So many of them made me feel welcome and were always taking me places to see more of Addis Ababa and making my internship here all the more memorable. All of the staff members were so open to helping me out whenever I needed it, Temeselew especially. He took time out of his busy schedule to help me out in making many various solutions for germinating and staining. He was so patient with me when I asked to help him in making the solutions, but honestly didn't have much of an idea as to what I was doing. All of the staff members in the Forage Diversity Department made me feel a part of the ILRI family. I couldn't have asked for a better place for my internship.

Lastly, I would like to thank my family for all of the support they have given me through this whole process. I don't think I could have done it without them pushing me to be the best I can be. I couldn't ask for a better support group. They have always been there for me and periodically sent me emails throughout my internship asking how my project is going and if I've made any headway in it. To my sister, Emily, who secretly left notes in my suitcase to remind me how much support I have back at home and for the pictures she also hid in my suitcase to remember where I came from. They have always been there for me, and they really helped me realize how much of an impact this experience will have on me for the rest of my life.

Forage Seed Production: Before seeds can be put into the market, both the seeds and plants have to undergo many tests and observations. Without seed production, there would be no plants in the world. Seed production starts right at the beginning of a formed seed on a plant. From the seed pod or plant, the seed is taken to be germinated and grown as a little seedling. It is said to believe that good seed in good soil yields abundantly. With this in mind, it's important to take care of the plants as well as the soil they're growing in. The purpose of ILRI is to create better lives through livestock. In order to do this, the forage that livestock eat have to be high in nutrients as well as convenient and profitable to grow. ILRI works to improve food security and reduce poverty in developing countries through research for better and more sustainable use of livestock. What is the key to this? Successful seed production. ILRI's global mandate for livestock research is:

> *"to measurably and sustainably improve the livelihood of resource-poor livestock keepers, make animal products more affordable and accessible for the poor and conserve natural resources in developing countries through partnerships and alliances for innovative livestock research, training and information exchange"*

ILRI's personal mission is:

> *"to help reduce poverty, hunger and environmental degradation through livestock research to enhance productivity and sustainability of agricultural systems in the developing world"*

Seeds are the cheapest input in crop production and key to agricultural progress. Without seeds, there would be no agriculture. To demonstrate the importance of seed production, I was taken to fields in Addis Ababa, Zwai, Debre Zeit, and Soddo. These are ILRI's forage seed production sites. While there, I helped with planting, observed the different irrigation and planting systems, and interviewed various seed producers who grow forages through the FeedSeed program. Forages are very valuable to these seed producers because forages have dual purposes. For most of the seed producers, they sell the seeds to government agencies, NGOs, and various farmers and keep the plant to feed their livestock. I also had the opportunity to help in the greenhouse and plant seeds that had been germinated. In doing this, I was able to see all of the steps of seed production. I saw the beginning—planting germinated seeds—all the way to forages growing in the fields in the seed production sites.

Getting to Know the Plants

Plant that Doesn't Flower or Set Seed

Onobrychis arenaria:

Gathered Information: *Onobrychis arenaria* is a perennial herb. Its accession number is 5685, was planted on 28 May 2008, and its common names are sand sainfoin and sand esparsette. The stems on it are erect and branching, reaching a length of 30-90 centimeters high with rare hairs or glabrous. Leaves on *Onobrychis arenaria* are 10-30 millimeters long and 2-5 millimeters wide. One plant contains 6-15 pairs of elliptical or linear-lanceolate leaflets. The leaves cover the stems completely, running up the whole length of each stem. The inflorescences on *Onobrychis*

arenaria are long and narrow. On and inflorescence, there are both flowers and seed pods. The flowers are purple-pink in color and measure 8-10 centimeters in length. The seed pods are semi-pubescent, ovate, and measure 5 millimeters long on average, with each pod containing a single kidney-shaped seed. The seeds range from 4-6 millimeters in length. *Onobrychis arenaria* flowers in May-June and the seeds ripen in August-September. It gets cross-pollinated mainly by honey bees. Through research, I found that this plant needs to be abundantly visited by honeybees, bumbles, and solitary bees come pollination time.

Onobrychis arenaria grows on semi-dry grasslands, meadows, and grazed Magerrasen. It grows best in moderately dry to fresh, lime or gypsum-based rock, sand, and loess soils. It is an Eastern European-Asian species and native to the steppe regions of Eurasia. Its growing range extends west to France and south to Greece. As stated before, it's native to southern Europe and western Asia, on dry sunny slopes, embankments, at vineyard and orchard edges, and sandbanks. It needs to grow in a bright place with direct sunlight. *Onobrychis arenaria* is adapted to warm, temperate climates, and not adapted to a wide range of soil and environmental conditions, meaning that the areas it can grow in are very limited.

Hypothesis: I believe that the factor contributing to the lack of flowers is because it's not growing in direct sunlight. It's in the greenhouse, receiving a good deal of heat from the sun shining on it, but making the plant receive indirect sunlight. Also, it needs to grow in warm, temperate climates. Addis Ababa, Ethiopia is a tropical environment. There is a significant difference between temperate and tropical zones. Temperate zones have warm summers and cool winters with year-round rain or snow. They typically have four seasons every year. Tropical zones have a climate that is hot all year, but has a distinct wet season and dry season during the year. *Onobrychis arenaria* is a perennial plant, so during the winter season, it goes into a dormant period. While growing in the greenhouse in Addis Ababa, Ethiopia, it doesn't receive this cold, dormant period. In order for it to grow, it has to receive this condition. This will be discussed in the testing section under Change of Environment.

Plants that do Flower, but Don't Set Seed

Canavalia sp. located in Zwai:

Background: The species I selected was *Canavalia sp.* Its accession number is 1251, and was planted on 19 January 2004. The species of it is unknown. It's located in a plot in Zwai. I have to ask whoever is going to the field that week to bring back some samples of the development stage I need to observe. This slows down my research sometimes because I can only do one step of the process at a time. On the week of June 9th, Dr. Jean brought me back my first samples of *Canavalia sp.* so I could look at it under the microscope, observe if there was any pollen, the amount of ovules, if there was any pollen on the stigma, where the stigma is in relation to the anthers, and many other observations.




Gathered Information: Dr. Jean handed me a sheet regarding all of the information known about the *Canavalia sp.* growing in Zwai. While looking at this information, I found that its country of collection was Tanzania and the district and area of collection was Mwanza. The exact site of collection was the Beach of Sanana Island in Lake Victoria near Mwanza. The altitude of this area is 1,080 meters. Average annual rainfall is 1,002 millimeters. I also found the average temperature in this area is 23°C. I then compared this environment to Addis Ababa, Ethiopia. Because Zwai is near Addis Ababa, I used there as a reference to environment. The altitude of Addis Ababa is 2,300 meters. Average annual rainfall is 1,165 millimeters. The average temperature in Addis Ababa is 16.34°C. When just looking at these environmental factors, there is some discrepancy between the two areas.

The higher the altitude, the more rainfall, the cooler the temperatures, and the less amount of sunlight will occur. These factors are very apparent when comparing the environment of Mwanza to Addis Ababa. This leads to one of my ideas as to why *Canavalia sp.* might not be producing seeds. With such a difference with environments of where it was grown previously compared to where it's grown now, there is a huge difference with altitude and rainfall. When looking at the temperature, there is also a gap between 23°C and 16.34°C, but that slight difference shouldn't affect the production of seeds. The spread of this *Canavalia sp.* is 400 centimeters. Also, its growth zone is tropical and it's a vine legume. While it was growing in Mwanza, there were few seeds, and out of those, only a moderate amount was ripe.

Hypothesis: #1: One of my hypotheses as to why this *Canavalia sp.* won't seed is because of its growing environment. I think this because there is another *Canavalia sp.* with the same accession

number growing in Soddo, and it produced seeds in 2009. Also, on the information sheet about our plant species, it states that it produced a few seeds before that as well. Ever since it has been growing in Zwai, no seeds have been produced. This makes me believe it's an environmental factor affecting the plant's reproduction.

#2: My second hypothesis is the flowers don't produce any pollen. If a flower doesn't produce any pollen, then seeds won't be produced.

#3: After dissecting the flower and realizing that some of the flowers do contain pollen, the pollen needed to be germinated and stained. Pollen that germinates in sucrose solution grows a pollen tube, therefore fertilizing the ovules in the ovary, and creating seeds. Stained, or viable, pollen will turn a fluorescent color when observed under the UV blue light under the microscope lens. This hypothesis will also be discussed later in the testing sections under Germination of Pollen and Aniline Blue Fluorochrome Staining.

***Canavalia sp.* located in Soddo:**

Background: On Friday, June 20th, a worker on ILRI traveled to Soddo and gathered samples of a species of *Canavalia* that won't produce seeds. Its accession number is 12761, so it's not the same species as my other *Canavalia* that I'm studying because the accession number for that species is 1251. Also, you can tell right away it's not the same species because the flowers are a completely different dark purple, whereas 1251's petal color is lavender. It is a perennial legume with a tropical growth zone. The plant height of it is 150 centimeters with a plant spread of 350 centimeters.

Gathered Information: Dr. Jean handed me a sheet containing all of the information known about the *Canavalia sp.* growing in Soddo. While looking at this information, I found that its country of collection was Kenya and the district and area of collection was Kwale. The exact site of collection was the Mombasa town near Beach Hotel. The altitude of this area is 50 meters. Average annual rainfall is 1,100 millimeters. I also found the average temperature in this area is 27°C. I then compared this environment to Soddo, Ethiopia. The altitude of Soddo ranges from 1,600-2,100 meters. Average annual rainfall is 1,125 millimeters, and average temperature is 14.3°C. When just looking at these environmental factors, there are some differences between the two areas. The altitude between Mombasa and Soddo is very large. Altitude has a large impact on plants and organisms, so this probably affects the growing of the *Canavalia sp.* along with the difference in average temperatures. Also, during its growing period in Mombasa, there were many seeds, but very few ripe seeds. This shows that when produced, the seeds don't mature; rarely do they ripen.

Hypothesis #1: My first hypothesis as to why the *Canavalia sp.* won't produce seed is because of its growing environment. As stated above, the altitude difference between the two areas is very large. The higher the altitude, more rain, less sunshine, and cooler temperatures are present. Mombasa has an elevation of about 50 meters, which is a huge difference from Soddo's 1,600-2,100 meter elevation. I would try altering the growing environment of this *Canavalia sp.*, giving it more sunshine and a warmer temperature.

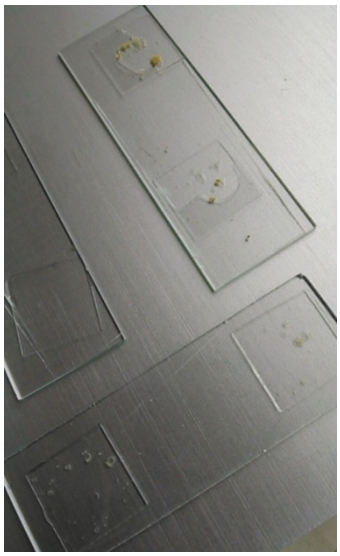
#2: Another one of my hypotheses is the flowers don't produce any pollen. Seeds won't be produced if no pollen is present on the flowers.

#3: It was discovered that some of the flowers do contain pollen after dissecting flower samples. Now, my hypothesis is that the pollen doesn't germinate or isn't viable. In order to figure this out, I needed to germinate and stain the pollen. When pollen germinates in sucrose solution, it grows a pollen tube, which then fertilizes the ovules in the ovary, thus creating seeds. Pollen that stains will turn a fluorescent color when observed under the UV blue light under the microscope lens. If the pollen fluoresces, it means it's viable. This hypothesis will also be discussed later in the testing section under germinating/staining.

Tests Performed/Results on *Onobrychis arenaria*

Change of Environment: Because my hypothesis as to why it won't flower is due to its growing and environmental conditions, I decided to see if that was truly the issue. I felt the *Onobrychis arenaria* should be placed in an incubator for 4-6 weeks to try to imitate the winter season it requires. The incubators haven't been used for two years, so it took a few weeks to fix and get them working again. When they were ready to use, Dr. Jean thought it would be better to try vernalization on a different plant first. The Forage Diversity Genebank only has one *Onobrychis arenaria* plant in the greenhouse and has a very limited amount of seeds of it. During my internship, I wasn't able to try vernalization, the dormant and winter period, on the *Onobrychis arenaria*, so there aren't any results from this test.

Tests Performed/Results on *Canavalia sp.* located in Zwai



Germination of Pollen: The sucrose solution to see the germination of pollen was created on June 10th. I germinated the pollen grains of the *Canavalia sp.* on June 18th. For the germination process, I placed one drop of the solution onto the right side of the slide. I then held the opened flower over the drop of sucrose solution and used the forceps to knock the pollen off the anthers and into the sucrose solution. After that, I followed the same procedure on the left side of the slide. I repeated this process a second time on another slide. It takes about four hours for pollen to germinate in this solution. While it germinates, it has to be placed in an incubator at a constant temperature of 30°C, the ideal temperature for germination to occur. After the four hours, I was unable to find any pollen tubes growing out of the pollen grains. The morning of June 19th, I decided to observe the pollen grains again. Some pollen tends to take longer germinating. There still weren't any pollen tubes growing, so I concluded that the pollen on this species of *Canavalia* doesn't germinate.

Even though this *Canavalia* won't germinate, it could still be viable. This means that the pollen could pollinate the flower and result in seed pods producing. To test out the viability of the pollen I had to create a stain. I created the staining solution on June 19th. The Genebank had all of the chemicals needed for the stain except Malachite Green, with which was replaced with Brilliant Green. The process for staining pollen is similar to germinating it. When a pollen grain is viable, it will turn green, and if it's not viable, it will not change.

Aniline Blue Fluorochrome Staining: On July 3rd, I started the process of testing the viability of the pollen. This solution would make the viable pollen fluorescent. There are five main steps to the Aniline Blue Fluorochrome Staining. These steps are located in Appendix 6. In order to observe the pistils under the microscope, they had to be placed on a slide with a cover slip on top to flatten it out. The pistils are very large though, so I had to press down on the cover slip in order to flatten the cover slip down so it lied flat on the slide. I did that, but I still wasn't able to see the ovules inside the pistil, so I had to flatten down the cover slip even more! I used a heavy microscope plate so my fingerprints didn't get onto the cover slip. I heard a small "pop!" and immediately picked up the microscope plate to see what had happened! Underneath, I found a large yellow glob near the stigma. I had no idea what it was because looking at the pistil under the microscope before, I didn't see anything. Now, this light yellow glob appeared that popped right out of the pistil!

I decided to observe this under the microscope to find out what it was! Under the UV light, the huge clump was a large fluorescing mass! It didn't look like the pollen I've been observing though, so I wasn't sure if it was pollen or not. I thought I saw a few grains, but they were extremely small, so maybe it wasn't pollen at all and just a different structure that fluoresced. When Dr. Jean looked at it, she said it looked to be pollen but was difficult to tell and wasn't for certain either. After a little bit more time looking at it, she declared it wasn't pollen. After looking at it, we found a few oval-beanlike figures that contained a nucleus and other structures in cells. Out of the thousands of oval-beanlike figures, only a few were found to be developed and containing a nucleus, meaning they're underdeveloped. Dr. Jean also had Yeshe look through the microscope to see what she thought this huge mass was, but she had no idea either. For something to compare to, Yeshe and I dissected a few flower samples I had taken of the *Canavalia sp.* in Zwai to see what the pollen looked like. I dissected 10 different flowers and didn't find pollen on any of the anthers. This means that all of those are sterile. If all 10 of those flowers are sterile, that means many more flowers must also be sterile.

Hand Pollination: On June 30th, I traveled to Zwai to help with gathering samples for DNA molecular testing. Because it's flowering, but not producing seed, I'm thinking maybe it's not pollinating correctly. To find this out, I decided to hand pollinate it while there. I used the pollen off one of the flowers on an inflorescence and pollenated two flowers on a different inflorescence. While doing some research about *Canavalia sp.*, I found a list that included many different *Canavalia's* and for the ones it listed, they were known for out-crossing pollination. Because of this, there is a large chance that my *Canavalia sp.* of study also outcrosses. There are multiple plants of each species planted in each plot in Zwai. While hand pollinating, I had to make sure that I used flowers from different plants. If I didn't, the pollination might not work.

Because there are two different *Canavalia sp.* accessions being grown in Zwai, I thought that maybe my *Canavalia* of study would seed if I hand pollinated it with the other *Canavalia sp.* The plants have different accession numbers, but since no one knows the species of either, there is still a slight chance that they could be the same species. I decided to try hand pollinating my issue *Canavalia sp.* using the pollen from the *Canavalia sp.* that is growing perfectly. The plots

they're grown in are a distance away from each other, so there was a chance that I lost some pollen on the way to my one of study, but when I did the pollination, I still saw a large amount of pollen attached on the paintbrush and falling off onto the anthers. I performed the same procedure on a few of these flowers as I did before.

After every flower I pollinated, I placed a tag around it so that it was known which flowers were pollinated. This will make is convenient and easier to observe in the future. On the tags I pollinated using the same accession number plant, I wrote down: "Hand pollinated, pollinated with same accession 1251, 30 June 2014." For the tags that I pollinated using the different accession of *Canavalia sp.*, I wrote down: "Hand pollinated, pollinated with different accession: 15592, block 873, 30 June 2014."

I was able to travel to Zwai two weeks after my hand pollination to see if any seed pods had been produced. If not, I was going to gather some samples from the plot and examine them further in the lab. When I reached the plot, I spotted two seed pods near the area I had hand pollinated! I'm pretty sure these were from the two flowers I had pollinated because even though the tags fell off, I found them under some leaves very close by. I then taught Tekla, a manager at Zwai, how I hand pollinated the flowers so he can continue that process and produce more seeds.

Tests Performed/Results on *Canavalia sp.* located in Soddo

Germination/Staining of Pollen: While the pollen was still fairly fresh, I decided to germinate it. Pollen dries out within 24 hours, but since the flowers were placed in the refrigerator as soon as possible, there was a chance the pollen still was okay to use. I used the sucrose solution that was made on June 10th to germinate the pollen. I placed one drop of the sucrose solution on the right side of the slide, then used the forceps to knock the pollen off the anthers and into the solution. I repeated this on the left side of the slide. I then used a different flower containing pollen and repeated this process. This way I would have more data to look at. I didn't check to see if the pollen had germinated yet because I used the staining solution after that so that the germinated pollen would stain. This will help me see the germinated pollen easier as well as see if the pollen is viable or not. While looking through the microscope, no germinated pollen was found, and only a few appeared to be stained.

Conclusion: *Onobrychis arenaria* is a perennial legume typically grown in temperate zones. This means every year, it needs to go through vernalization, or a dormant period. For plants in temperate zones, this period occurs during winter. "It is usually essential for an organism to undergo periods of inactivity which may require the formation of special protective structures,

and these periods are represented by the several states and degrees of dormancy. The development of dormancy has therefore been a major factor in evolution which has allowed the synchronization of life processes among the members of a population, and between their successive developmental stages and seasons. It may be concluded that dormancy is a state in which, even though normally favourable conditions of warm temperatures, adequate water and aeration are supplied, growth and development do not take place until a special set of conditions has been experienced." (Villiers) Because of this, I believe this is the reason the *Onobrychis arenaria* plants hasn't flowered in the time it's been planted in the greenhouse in 2008.

Through much research and many tests, there was finally a result for the *Canavalia sp.* located in Zwai. The Aniline Blue Fluorochrome Staining test was what really aroused questions. There was a large fluorescing mass that popped out of the pistil which appeared to look like underdeveloped pollen. After looking for pollen in other flower samples to compare the fluorescing mass to, I was unable to find any. Because of this, I can say that most likely, the majority of the flowers on this plant are sterile, thus preventing it from producing seeds. The staining was performed after I had already hand pollinated four flowers in the plot in Zwai. Two weeks after the pollination though, two seed pods were found growing on one of the inflorescences I had hand pollinated. The seed pods had formed from the pollination of the same accession number. Dr. Jean has had numerous people do years and years of research about the *Canavalia* genus, trying to figure out what the problem was and how to make them produce seeds. They've been working on figuring out a solution for many years now, and I finally uncovered it. The seeds inside each pod are very large, so there are only a couple of seeds in each. This means that many pods will have to be produced in order to gather a large amount of seeds to start growing for production. From now on, Tekla will continue to hand pollinate the *Canavalia sp.* using the same process I used to try to replicate what I did and produce more seed pods.

My recommendation on what to do for the *Canavalia sp.* located in Soddo is to try hand pollinating the flowers. When I germinated and stained the pollen, it looked similar to the pollen from the *Canavalia sp.* located in Zwai. Because of this, the same issue with their reproduction is likely to be occurring. This is why I believe that hand pollinating it will be the solution. Hand pollination will be more successful if done early afternoon because then the flowers have had some time to dry off from the night, but not too hot to dry the pollen out. Also, then the pollen isn't wet from the morning dew.

I am at a lack of words to describe how much of an impact my time at ILRI has had on my life. It has taught me to be an independent person, as well as hardworking. Every single task I performed was extremely tedious and required patience. Not one job was completed in the period of just one day. Each and every task contained multiple steps, and each step took a long time to prepare and carry out. Dr. Jean was talking to me one day about the importance of science and how much work it requires and I'll never forget what she said:

> *"Science is a lot of intricate work. You have to be very meticulous when doing anything. If you don't have the patience or dedication to carry out all the tasks required, then don't come into the science field. There's a lot to it and it's the basis for everything; if you don't put forth all of the effort, your end product won't be accurate, and that data isn't*

reliable. Don't make that mistake. Work hard, complete all the steps thoroughly, and do everything yourself; don't tell others to do it for you. You'll get the best results if you do things yourself with some help."

There are so many things I learned here; I learned not just science, but about myself and the culture and nature of another country and its people. Having the two different *Canavalia sp.* grow in Zwai and Soddo, I was able to travel to both field sites in order to visit them and observe the growing environments of both plants. The field sites are very different from each other when it comes to irrigation, soil types, and how and where plants are planted. On another occasion of traveling, I interviewed nine seed producers that are growing forages through the FeedSeed program. On the questionnaire I created, my questions ranged from their highest education level to their seed yield to how they got into seed production. Hearing each of their stories was truly inspiring. All of their stories were so different, and yet, I could find a connection to all of them. For most of the seed producers, seed production was their way of making it through. They had no choice. On average, they grew crops on less than 8 acres of land. This was how they were trying to make their way through life. I couldn't believe this. These interviews with the seeds producers really helped to open my eyes to the way of life here, and for that, I could never thank them enough for sharing their stories with me.

References

"Addis Ababa." *Wikipedia*. Wikimedia Foundation, n.d. Web. 27 June 2014. <http://en.wikipedia.org/wiki/Addis_Ababa#Climate>.

"Aphids." *Aphid Control Using Beneficial Insects and Natural Insecticides*. 2014. Web. 4 June 2014. <http://www.arbico-organics.com/category/pest-solver-guide-aphids>.

"Aphids, Scales and Mites On Home Garden and Landscape Plants." *G7274 Aphids, Scales and Mites on Home Garden and Landscape Plants*. University of Missouri Extension, 1 Mar. 2014. Web. 4 June 2014. <http://extension.missouri.edu/p/g7274>.

"Aphids." The Regents of the University of California, 25 Apr. 2014. Web. 4 June 2014. <http://ucipm.ucdavis.edu/PMG/PESTNOTES/pn7404.html>.

"AZ Master Gardener Manual: Environmental Factors." *AZ Master Gardener Manual: Environmental Factors*. N.p., n.d. Web. 27 June 2014. <http://ag.arizona.edu/pubs/garden/mg/botany/environmental.html>.

" Canavalia pubescens." *Native Plants Hawaii*. N.p., n.d. Web. 27 June 2014. http://nativeplants.hawaii.edu/plant/view/Canavalia_pubescens#environmentalinformation>.

"Canavalia sp., Magic Bean, Kaattuthambattan, Beach Bean, Seaside Bean, Jackbean -."*rare plants for home and garden*. N.p., n.d. Web. 27 June 2014. <http://toptropicals.com/catalog/uid/canavalia_sp.htm>.

"Canavalia." *Wikipedia*. Wikimedia Foundation, 6 Mar. 2014. Web. 27 June 2014.

<http://en.wikipedia.org/wiki/Canavalia>.

Chourykaew, Boonsanong, Chumpol Khunwasi, Thaweesakdi Boonkerd, and Tosak Seelanan. "FloralVisitors and Fruit Set in Afgekia sericea Craib (Fabaceae)." *The Natural History Journal of Chulalongkorn University* 4: 31-44. Web. 27 June 2014.

Cranshaw, W.S., and D.C. Sclar . "Spider Mites." *Spider Mites*. Colorado State University Extension, 1 Jan. 2014. Web. 4 June 2014. <http://www.ext.colostate.edu/pubs/insect/05507.html>.

Drees, B. M. , and John Jackman. "Spider mite." *Spider mite*. Golf Publishing Company, 1 Jan. 1999. Web. 4 June 2014. <http://texasinsects.tamu.edu/cimg371.html>.

"Ethiopian Treasures." *Ethiopian Climate and Seasons*. Web. 27 June 2014. <http://www.ethiopiantreasures.co.uk/pages/climate.htm>.

Franklin-Tong, V. E.. "Signaling and the Modulation of Pollen Tube Growth." *The Plant Cell Online* 11: 727-738. *The Plant Cell*. Web. 27 June 2014.

"ILDIS LegumeWeb." - *ILDIS LegumeWeb*. N.p., n.d. Web. 27 June 2014. <http://www.ildis.org/LegumeWeb?version~10.01&LegumeWeb&tno~6203&genus~Onobrychis&species~arenaria>.

"International Livestock Research Institute." *Home*. N.p., n.d. Web. 8 July 2014. <http://www.ilri.org/>.

Jabłoński, Bolesław, and Zbigniew Kołtowski. "Nectar Secretion and Honey Potential of Honey Plants Growing Under Poland's Conditions: Part XIII." *Journal of Apicultural Science* 46: 25-30. Web. 27 June 2014.

Kemp, Roger. *Cell division and heredity*. New York: St. Martin's Press, 1970. Print.

Lawrence, William John Cooper. *Plant breeding,*. New York: St. Martin Press, 1968. Print.

Lebbie, S. H. B.. "Introduction to the workshop." *session1*. N.p., n.d. Web. 3 July 2014. <http://ilri.org/InfoServ/Webpub/fulldocs/AnGenResCD/docs/SouthAfrican_AARNET/ILRI.htm#P81_7311>.

Lewis, Dan. *Sexual incompatibility in plants*. London: E. Arnold, 1979. Print.

"Norman Borlaug." BrainyQuote.com. Xplore Inc, 2014. 10 July 2014. http://www.brainyquote.com/quotes/authors/n/norman_borlaug.html

">>Onobrychis arenaria ssp. arenaria Onobrychis tanaitica Spreng.." >>Onobrychis arenaria ssp. arenaria, Onobrychis tanaitica Spreng.. N.p., n.d. Web. 27 June 2014. <http://www.gardening.eu/arc/plants/Masts/Onobrychis-tanaitica-Spreng/47521/>.

"Onobrychis arenaria subsp. arenaria information from NPGS/GRIN." *Onobrychis arenaria* subsp. arenaria information from NPGS/GRIN. N.p., n.d. Web. 27 June 2014. <http://www.ars-grin.gov/cgi-bin/npgs/html/taxon.pl?311973>.

"Plant Care Articles." *EasyBloom*. N.p., n.d. Web. 27 June 2014. <http://www.easybloom.com/plantlibrary/care/why-wont-my-plant-flower>.

Piper, C. V.. "The American Species of Canavalia and Wenderothia." : 555-588. Web. 27 June 2014.

"Relatives." *AgroAtlas*. N.p., n.d. Web. 27 June 2014. <http://www.agroatlas.ru/en/content/related/Onobrychis_arenaria/>.

"RESPONSE OF SWORD BEAN (CANAVALIA SP.) TO DIFFERENT INTENSITIES OF LIGHT STRESS." *RESPONSE OF SWORD BEAN (CANAVALIA SP.) TO DIFFERENT INTENSITIES OF LIGHT STRESS*. N.p., n.d. Web. 27 June 2014. <http://www.actahort.org/books/752/752_90.htm>.

"Sainfoin - Frequently Asked Questions." . Alberta: Agriculture and Rural Development, 14 Aug. 2013. Web. 2 July 2014. <http://www1.agric.gov.ab.ca/$department/deptdocs.nsf/all/faq14389>."Sand-Esparsette."

"Scientific name." *Factsheet*. N.p., n.d. Web. 27 June 2014. <http://www.tropicalforages.info/key/Forages/Media/Html/Canavalia_brasiliensis.htm>.

Sinnott, Edmund W.. *Principles of genetics*. 5th ed. New York: McGraw-Hill, 1958. Print.

Villiers, Trevor Angus. *Dormancy and the survival of plants*. London: Edward Arnold, 1975. Print.

Vörösváry, G., L. Holly, and L. Horváth. "CONSERVATION PRIORITIES FOR CROP WILD RELATIVES IN HUNGARY." . Institution for Agrobotany, n.d. Web. 8 July 2014. <http://www.pgrforum.org/Documents/Conference_posters/Vorosvary_et_al.pdf>.Web. 27 June 2014. <http://cropgenebank.sgrp.cgiar.org/images/file/forage_legumes/breeding%20systems%20table.pdf

Web. 27 June 2014. <http://www.envirothon.org/files/2014/Pollinators_.pdf>.

Wikipedia. Wikimedia Foundation, 22 June 2014. Web. 27 June 2014. <http://de.wikipedia.org/wiki/Sand-Esparsette>.

"1251 ETH013." *Accession profile: 1251*. N.p., n.d. Web. 27 June 2014. <https://www.genesys-pgr.org/acn/id/2870838>.

Appendix 1—Compiled Plant Information

PAGE: 2 ILRI GERMPLASM DATA

Passport data:

Accession number: 5685
Genus and species: Onobrychis arenaria

Longevity: Perennial
Plant type: Legume
Screening type: Experimental
Growth zone: Temperate

Other accession numbers: IFON-180,IG-109442,PI-312962

PAGE: 1 ILRI GERMPLASM DATA

Passport data:

Accession number: 1251
Genus and species: Canavalia sp.

Collector's name: Solomon Mengistu,Maynard Lugenja
Collecting institute: ILCA/TALIRO/WIIAD
Date of collection: 16/08/87
Collector's number: SMT-275B
Country of collection: Tanzania
District of collection: Mwanza
Area of collection: Mwanza
Exact site of collection: Beach of Sanane Island in Lake Victoria near Mwanza
Altitude: 1080m
Annual rainfall: 1002mm
Temperature: 23°C
Soil colour: Brown
Soil drainage: Freely draining
Soil texture: Sandy loam
Habitat: At the foot of a granite rocky hill Island.
Associated legumesAeschynomene elaphroxylon Sesbania sesban
Associated grasses:Pennisetum polystachium Hyparrhenia filipendula
Flowers: Very few
Seeds: Few
Ripe seeds: Moderate
Leafiness: Many
Plant spread: 400cm
Plant morphology: Vine
Plant type: Legume
Screening type: Experimental
Growth zone: Tropical
Plant density: Moderate
Relative abundance: Moderate
Comments: Markedly prostrate invading the huge granite rocks.

PAGE: 4 ILRI GERMPLASM DATA

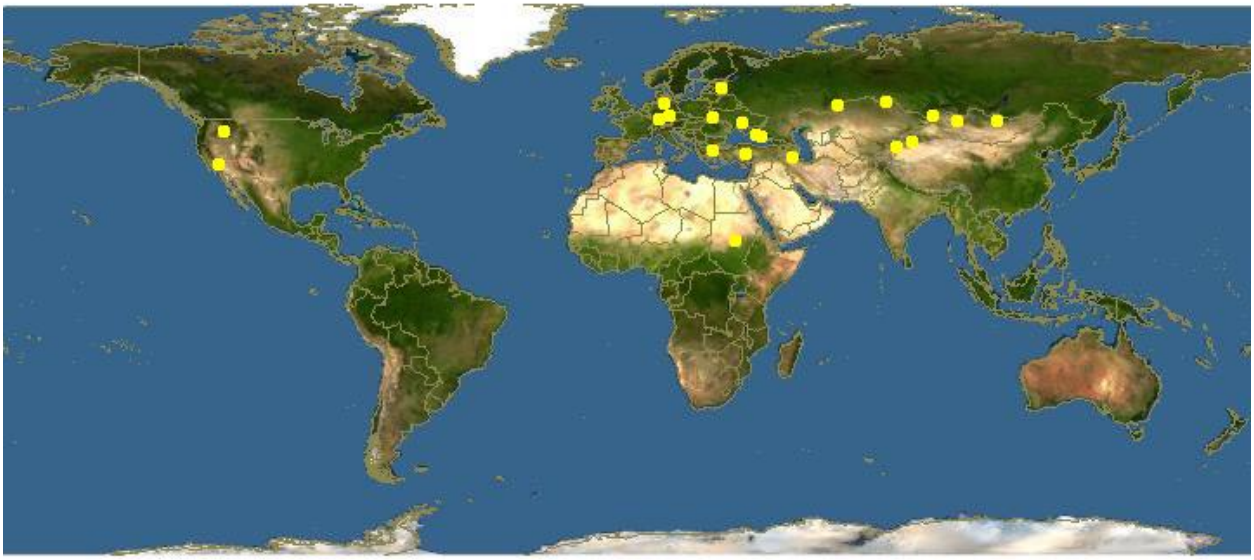
Passport data:

Accession number: 12761
Genus and species: Canavalia sp.

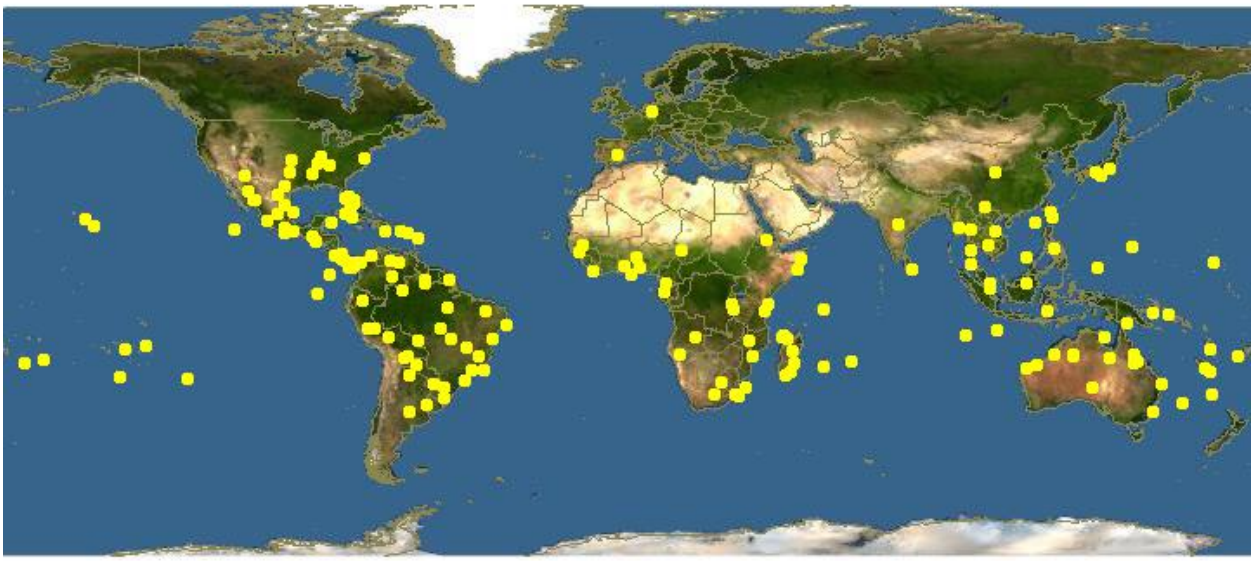
Collector's name: Solomon Mengistu,Keller-Grein G.
Collecting institute: ILCA/CIAT
Date of collection: 31/08/84
Collector's number: K-080
Country of collection: Kenya
State of collection: Coast
District of collection: Kwale
Area of collection: Mombasa
Exact site of collection: Mombasa town near Beach Hotel
Map reference: Series Y-503 sheet SB-37-3 Edition 3-SK 1971
Latitude: 04°02'S
Longitude: 039°13'E
Altitude: 5m
Annual rainfall: 1100mm
Temperature: 27°C
Slope: 0%
Parent rock: Lagoonal
Soil name: Arenosol
Soil colour: Brown
Soil drainage: Moderate
Soil texture: Sandy loam
Habitat: Coastal bushland
Associated legumesTephrosia Alysicarpus sp.
Associated grasses:Panicum sp. Digitaria sp.
Seeds: Many
Ripe seeds: Very Few
Leafiness: Moderate
Plant height: 150cm
Plant spread: 350cm
Plant morphology: Vine
Longevity: Perennial
Plant type: Legume
Screening type: Experimental
Growth zone: Tropical
Plant density: Very few
Relative abundance: Uncommon
Comments:
Other accession numbers: CIAT-19219

Appendix 2—Distribution Maps of Plants

Onobrychis arenaria



Canavalia sp.



Appendix 3—Field Map of Soddo and Zwai

Soddo Top Field

Row	31	30	29	28	27	26	25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
24	622	621	620	619	618	617	616			614																					
23	612	611	610	609	608	607	606					2001																			
22	602	601	600	599	598	597	596		593			2002																			
21		592	591	590	589	588	587					2003																			
20		583	582	581	580	579	578					2004							240												
19		574	573	572	571	570	569		566			2005							238	239											
18	565	564	563	562	561	560	559					2006							230	231	232										
17	555	554	553	552	551	550	549		536			2007							218	219	220	221	222								
16	545	544	543	542	541	540	539		537			2008							204	205	206	207	208	209							
15	535	534	533	532	531	530	529					2009							189	190	191	192	193	194	195	196					
14	525	524	523	522	521	520	519		506			2010							173	174	175	176	177	178	179	180	181				
13	515	514	513	512	511	510	509		506			2011							155	156	157	158	159	160	161	162	163	164	165	166	
12	505	504	503	502	501	500	499					2012							136	137	138	139	140	141	142	143	144	145	146	147	
11	495	494	493	492	491	490	489	488				2013							116	117	118	119	120	121	122	123	124	125	126	127	128
10	485	484	483	482	481	480	479		476			2014							97	98	99	100	101	102	103	104	105	106	107	108	109
9	475	474	473	472	471	470	469		467			2015							77	78	79	80	81	82	83	84	85	86			
8	465	464	463	462	461	460	459					2016							59	60	61	62	63	64	65	66					
7	455	454	453	452	451	450	449		446			2017							43	44	45	46	47								
6	445	444	443	442	441	440	439					2018							29	30	31										
5	435	434	433	432	431	430	429					2019							16												
4	425	424	423	422	421	420	419		416			2020																			
3	415	414	413	412	411	410	409																								
2	408	407	406	405																											
1	404	403																													
Col																															

Canavalia sp. Accession #12761

Canavalia sp. Accession #1251

Zwai

Row	40	39	38	37	36	35	34	33	32	31	30	29	28	27	26	25	24	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
31	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002			
30	965	945	922	899	876	853	829	805	781	757	733	708	683	658	633	608	582	556	530	504	477	450	423	396	374	352	330	308	286	264	242	220	198	173	152	124	94	64		
29	964	944	921	898	875	852	828	804	780	756	732	707	682	657	632	607	581	555	529	503	476	449	422	395	373	351	329	307	285	263	241	219	197	172	151	123	93	63		
28	963	943	920	897	874	851	827	803	779	755	731	706	681	656	631	606	580	554	528	502	475	448	421	394	372	350	328	306	284	262	240	218	196	171	150	122	92	62		
27	962	942	919	896	873	850	826	802	778	754	730	705	680	655	630	605	579	553	527	501	474	447	420	393	371	349	327	305	283	261	239	217	195	170	149	121	91	61		
26	961	941	918	895	872	849	825	801	777	753	729	704	679	654	629	604	578	552	526	500	473	446	419	392	370	348	326	304	282	260	238	216	194	169	148	120	90	60		
25	960	940	917	894	871	848	824	800	776	752	728	703	678	653	628	603	577	551	525	499	472	445	418	391	369	347	325	303	281	259	237	215	193	168	147	119	89	59	34	
24	959	939	916	893	870	847	823	799	775	751	727	702	677	652	627	602	576	550	524	498	471	444	417	390	368	346	324	302	280	258	236	214	192	167	146	118	88	58	33	
23	958	938	915	892	869	846	822	798	774	750	726	701	676	651	626	601	575	549	523	497	470	443	416	389	367	345	323	301	279	257	235	213	191	166	145	117	87	57	32	
22	957	937	914	891	868	845	821	797	773	749	725	700	675	650	625	600	574	548	522	496	469	442	415	388	366	344	322	300	278	256	234	212	190	165	144	116	86	56	31	
21	956	936	913	890	867	844	820	796	772	748	724	699	674	649	624	599	573	547	521	495	468	441	414	387	365	343	321	299	277	255	233	211	189	164	143	115	85	55	30	
20	955	935	912	889	866	843	819	795	771	747	723	698	673	648	623	598	572	546	520	494	467	440	413	386	364	342	320	298	276	254	232	210	188	163	142	114	84	54	29	
19	954	934	911	888	865	842	818	794	770	746	722	697	672	647	622	597	571	545	519	493	466	439	412	385	363	341	319	297	275	253	231	209	187	162	141	113	83	53	28	
18	953	933	910	887	864	841	817	793	769	745	721	696	671	646	621	596	570	544	518	492	465	438	411	384	362	340	318	296	274	252	230	208	186	161	140	112	82	52	27	11
17	952	932	909	886	863	840	816	792	768	744	720	695	670	645	620	595	569	543	517	491	464	437	410	383	361	339	317	295	273	251	229	207	185	160	139	111	81	51	26	10
16	951	931	908	885	862	839	815	791	767	743	719	694	669	644	619	594	568	542	516	490	463	436	409	382	360	338	316	294	272	250	228	206	184	159	138	110	80	50	25	9
15	950	930	907	884	861	838	814	790	766	742	718	693	668	643	618	593	567	541	515	489	462	435	408	381	359	337	315	293	271	249	227	205	183	158	137	109	79	49	24	8
14	949	929	906	883	860	837	813	789	765	741	717	692	667	642	617	592	566	540	514	488	461	434	407	380	358	336	314	292	270	248	226	204	182	157	136	108	78	48	23	7
13	948	928	905	882	859	836	812	788	764	740	716	691	666	641	616	591	565	539	513	487	460	433	406	379	357	335	313	291	269	247	225	203	181	156	135	107	77	47	22	6
12	947	927	904	881	858	835	811	787	763	739	715	690	665	640	615	590	564	538	512	486	459	432	405	378	356	334	312	290	268	246	224	202	180	155	134	106	76	46	21	5
11	946	926	903	880	857	834	810	786	762	738	714	689	664	639	614	589	563	537	511	485	458	431	404	377	355	333	311	289	267	245	223	201	179	154	133	105	75	45	20	4
10	1004	925	902	879	856	833	809	785	761	737	713	688	663	638	613	588	562	536	510	484	457	430	403	376	354	332	310	288	266	244	222	200	178	153	132	104	74	44	19	3
9	1003	924	901	878	855	832	808	784	760	736	712	687	662	637	612	587	561	535	509	483	456	429	402												131	103	73	43	18	2
8		923	900	877	854	831	807	783	759	735	711	686	661	636	611	586	560	534	508	482	455	428	401												130	102	72	42	17	1
7						830	806	782	758	734	710	685	660	635	610	585	559	533	507	481	454	427	400												129	101	71	41	16		
6											709	684	659	634	609	584	558	532	506	480	453	426	399												128	100	70	40	15		
5																583	557	531	505	479	452	425	398												127	99	69	39	14		
4																				478	451	424	397												126	98	68	38	13		
3																																			125	97	67	37	12		
2																																				96	66	36		
1																																				95	65	35		
Col																																								

Canavalia sp. Accession #1251

Canavalia brasiliensis Accession #15592

Canavalia ensiformis Accession #14997

Canavalia ensiformis Accession #14881

Canavalia ensiformis Accession #14210

Canavalia ensiformis Accession #14336

Canavalia ensiformis Accession #15013

stores

offices

screen

Appendix 5—Recipe and Procedure for Germination

3.4.1 Germination of pollen grains in sucrose

Sucrose concentrations 5%, 10%, 15%, 20%, 25%, and 30% were tested. Each concentration was made by dissolving the sugar in calcium phosphate buffer (0.015M and pH 5.9) containing Boric acid (0.01%). From 3 randomly selected trees, 3 flower buds of different size (17-24 mm) were picked and the size of each bud measured. Then each bud was opened using a fine forceps and few pollen grains were picked and placed on 2 drops of each sucrose concentration on a microscopic glass slide. The pollen grains were uniformly dispersed in the drops and incubated in an incubator at 30 C and 90% RH for 3 hours. The number of germinating pollen grains were counted using an Olympus microscope under X100 magnification and 20 field of view for each concentration. A pollen grain was scored as germinated if an entire tube produced is greater than the grain diameter (Roberts *et al.*, 1982).

Appendix 6—Recipes and Procedures for Staining

Bedinger Lab Tomato: Pollen Staining Protocols

(September 22, 2010)

Emasculation

1. First, emasculate -1 stage tomato flowers (closed to slightly separating pale yellow petals. This is the stage of flower right before Bud Break, before any pollen is released from the anthers. The idea is that you are emasculating a flower that if left alone would open after 24hrs) by removing the anthers.
2. Mark the flowers by labeling with the female accession #, what it will be use for, and the date of pollination. Wait 24hrs.
3. Pollination is performed the next day by touching the stigma onto a surface (e.g. a 1.5 ml microfuge tube lid, or flower anthers) covered with pollen. Note: Pollen can be collected various ways, we mimic the vibration of bumble bee by using a hand-held tooth polisher to collect the pollen in a microfuge tube.
4. Pollinations are allowed to progress for 24 hours, unless specified otherwise (pollen tubes tend to be about 40% of the way down the style at 6 hours in a compatible/congruent pollination). Field flowers should be protected from pollinators by covering the inflorescence.

Fixing Pistils

1. After the desired amount of time post pollination, the remaining sepals, and petals are removed from around the pistil.

2. Using a scalpel or razor blade the pistil is cut at the base of the ovary just above the pedicel.

3. The excised pistil is placed in a 1.5 ml microfuge tube containing 0.5 ml (enough to completely submerge pistils) 3:1 95% EtOH: glacial acetic acid and left overnight or indefinitely.

Softening Pistils

1. Fix is removed by piptetting, and pistils are then submerged in 0.5 ml 5 M NaOH softening solution for 24 hours. Note: the tissue becomes extremely fragile and can be easily damaged after this stage. Also, the pistils tend to float right after adding NaOH so it good to gently shake the tube after a couple of hours and get the pistil to sink to the bottom.

Staining Pistils

1. After 24 hours the 5 M NaOH is carefully removed by pipetting (taking care not to disturb the pistil) and the pistil is gently washed 3-5 times with 0.5 ml ddH20 each time.

2. After the last wash the ddH2O is removed and replaced with 0.2 ml 0.001mg/ml ABF (Aniline Blue Fluorochrome) in 0.1M K2HPO4 pH 10 buffer.

a. Aniline Blue Fluorochrome was obtained from Biosupplies Australia (http://www.biosupplies.com.au). We prep a stock solution of 0.1mg/ml in ddH2O from the dry ABF they send. This is stored at +4 C. A further dilution of the stock solution to make a working stock solution is done using a 1:20 dilution of the stock solution into 0.1M K2HPO4. (In the past we have also used a 100X solution works best for us but you should try different amount that best work for your system).

3. Once the stain is added to the tubes, samples are immediately placed in the dark and allowed to sit for about 24hrs for best staining.

Mounting/Viewing Pistils

1. After incubation in ABF for 24 hours, pistils are removed and placed in a drop of 50% Glycerin on a microscope slide and covered with a cover slip.

2. View with a standard fluorescence microscope capable of exciting with a UV light source and DAPI emission filters to view the fluorescent signal from the tissue.

3. After viewing is complete, take nail polish and seal the edge of each cover slip. This stores the slide material so that we may return to it later.

www.irbtomato.org/Aniline_Blue_Staining_Protocol.pdf

A simplified method for differential staining of aborted and non-aborted pollen grains

Ross Peterson,[1] Janet P Slovin,[2] Changbin Chen[1]

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Stain solution

The final stain solution we used was prepared by adding the following constituents in the order given below and stored in the dark.
10 mL 95% alcohol
1 mL Malachite green (1% solution in 95% alcohol)
50 mL Distilled water
25 mL Glycerol
5 mL Acid fuchsin (1% solution in water)
0.5 mL Orange G (1% solution in water)
4 mL Glacial acetic acid
Add distilled water (4.5 mL) to a total of 100 mL.

Staining

Following at least two hours of fixation, the bud can be placed on a microscope slide and the fixative's liquid was thoroughly and carefully dried from the plant material with absorbent paper. Proper safety gloves should be worn to avoid the risk of chloroform from being absorbed through the skin. Apply 2-4 drops of the stain solution before the sample completely dries. If flower buds have been collected instead of free anthers, the buds should be dissected to release the anthers and pollen. Under a dissecting microscope the leftover plant debris can be carefully removed. To save stain solution, samples can be dissected prior to putting individual anthers into stain. Some anthers, such as those of Magnolias, are too large to be viewed intact and must be dissected further.
Once the sample is in the stain, slowly heat the slide over an alcohol burner in a fume hood until the stain solution is near boiling (~30 seconds). A more moderate rate of heating allows better penetration of the dye into the cellulose and protoplasm of the pollen. Extremely high temperatures resulting in smoking or bubbling of the stain can burn the dye and the sample. Heating can be adjusted by briefly moving the slide in and out of the flame. To ensure stain has been completely absorbed into the pollen grains, 10 to 15 minutes should be allowed for some species such as *Lonicera tatarica*, *Ginkgo biloba*, *Pinus resinosa* and *Rhododendron mucronulatum*.

Imaging

Place a cover-slip over the sample and apply even pressure on the cover-slip to ensure that all plant components converge to one plane. The cover-slip can be sealed using nail polish or wax. Slides were examined using a Leitz microscope (Ernst Leitz Wetzlar GmbH, Germany). Micrographs were taken using a Spot Insight digital camera (Diagnostic instruments, Inc. Sterling Heights, MI, USA) and edited with Adobe Photoshop CS2 (Adobe Systems Inc. CA, USA).