Anthracnose Screening Protocol 2021

Outline

1. Sample collection
2. Fungal growth and Inoculum
3. Differential series growth
4. Disease rating
5. Further Readings
   1. There are references that are recommended you read prior to performing this protocol
6. References
7. Sample Collection

Anthracnose samples can be collected using brown paper bags or zip lock bags. Be sure to label each bag with the location of the plot collected, date and fungi collected. These samples will then need to be taken to the lab same day and fungal colonies will be grown on agar. If this is not done on the same day, bring a cooler with ice in it to store your samples and immediately transfer your samples to a freezer after you are done in the field.

Symptoms of anthracnose can be observed in Fig. 1 and Fig.2. Further details on anthracnose symptoms and identification can be found at [PP-1233 Anthracnose of Dry Beans (ndsu.edu)](https://library.ndsu.edu/ir/bitstream/handle/10365/5423/pp1233.pdf?sequence=1&isAllowed=y#:~:text=Anthracnose%2C%20caused%20by%20the%20fungus,at%20any%20stage%20of%20development.).

1. A close-up of a leaf

   Description automatically generated with medium confidence (b) A close up of a plant

   Description automatically generated with medium confidence

Photograph (a) provided by James Kelly, Michigan State University (3)

Photograph (b) provided by the Government of Manitoba, licensed under the OpenMB Information and Data Use License (Manitoba.ca/OpenMB)

**Fig. 1:** Examples of anthracnose infections in common dry beans (a) diseased leaves characterized by dark brown/black lines across leaf veins and midribs (b) dark sunken lesions appearing on bean pods

1. Fungal Growth and Inoculum

You will need to prepare either Potato Dextrose Agar (PDA) or Mathur Agar to grow the fungal samples on. The following procedure will explain how to prepare the agar and plate the fungus: [Fungi\_InoculumPrep.pdf (msu.edu)](https://www.canr.msu.edu/beanbreeding/_pdf/Fungi_InoculumPrep.pdf)

You can find the following information from the procedure above:

* Media preparation (PDA)
* Media preparation Mathur Agar
* Plating the fungus
* Inoculum and inoculation preparation: 100mL spore suspension
* Media preparation Bean Pod Agar Media
* Inoculum preparation
* Fungal re-isolation
* Long-term storage

Before plating your fungus on the media, you will need to sterilize the infected samples. Surface-sterilize your infected bean tissue samples (the pieces of stems and pods or whole seeds) by submerging them for 1 min in a solution of 10% household bleach (5.25% NaOCl) followed by 3 rinses in sterile distilled water. Allow the tissue to hydrate on moist, sterile filter paper for 24h at room temperature. Then take a tissue surrounding a single lesion and excise and crush it. Use an inoculating loop to streak the extract onto your agar (Halvorson et al., 2016).

After your fungus has been plated, it is ready to be re-plated in 5 days. You will need to identify fungal colonies in a microscopic examination of the colonies on agar. This pathogen produces brownish white, uniform, circular, fluffy growth in the center with concentric rigs and appressed growth at periphery grown on PDA. Refer to the following study done by G. Rajesha and S. G. Mantur for further morphological descriptions of Colletotrichum lindemuthianum: [20153038003.pdf (cabdirect.org)](https://www.cabdirect.org/cabdirect/FullTextPDF/2015/20153038003.pdf). Establish pure cultures of the pathogen by using single-spore isolation, refer to the following link for further guidance on single spore isolation: [A rapid approach for isolating a single fungal spore from rice blast diseased leaves (sciencedirectassets.com)](https://pdf.sciencedirectassets.com/282494/1-s2.0-S2095311919X00064/1-s2.0-S2095311919625815/main.pdf?X-Amz-Security-Token=IQoJb3JpZ2luX2VjECoaCXVzLWVhc3QtMSJHMEUCIQDHHX5fgF%2BOykNtIaIOF2kDoq5LzN53XyN43V8aWwr5mwIgNU74nPORVU5P4qzO%2BOg2eyUqm6MtlUP7sNFiaBxabisq%2BgMIYxAEGgwwNTkwMDM1NDY4NjUiDGX8s91k%2F85YRC1LsSrXA3N2JqBfYkY7PuWseYiasHTtrsYj6Bv6LLxnjHMbGGv4ULycpeO5xUsrHiig130DhdOpOp9E%2B0DRCG7w6u0GqD50m1MglY44kP%2Bsqogd1Ok8mU1SdQrZv8lEXjeTicR13RrIe7AxhVIBC1VS%2BQ2SV1%2B7vDbOo2hVZ1zJ%2F8G4Bq0079mMmgftxT8YOmnpctDltZdTfNIH0m2KIA5ZRCsNT7OGsJBTDrQHlp2p%2F0yBYRBIvpJY2Vxx%2FMdo%2FBNKHuTXNvsSnisJiygdXzDSC%2FN13vigC75y0wa9nvOip5g0hQv9vAggXMN9rxLmAyeRkHGLVuVo0UaAk6NWRlFs2NvXOd3R8%2B8aYdZ03B4UPjjLaxiFRRFMEuI3ulHnnYhc7koAA2AiosDmVgTqBV5m6yhPuKa1Ai%2B1z5gcK%2FYO0AJoO1gyS%2FWvnoGru%2Fi6C%2Bw%2FL%2Fo3nNvXK8qQN05YHYQ9XwbUzjdbqu5p4y37LpdSKwSJ8p8cC5lXXyaPSG7iYrUElHgi3NadgMJQw22Lf0Ct3XWVoBJGgdrazr16fcfNLBtOjEFBxXb1KRN%2BcJZd16sjZMc65%2BOtoDJWFpfkyAgAWSbGKLNjjqYKhOjJvvVW%2BKAH5XnmjpNTGFh%2FwTCsq5%2BJBjqlATAmqgU4wujhLLMKVvFToSb6nUsyGQr5A%2F12VsV9Gi5eHo5AzAb8RJG5gY4oH7xRtoVmCJTbQA1IMLhmcyLsVNGwe5xTaKEcpd88BV2v91FuKoFk2GSYW5rgJz59gX6%2FTaa6miRv6JKq1VSOtp%2BrpO1RefBXNVok0ob%2FaTpCxgrsR2RZrPbBcojcEAefgADncnPiHkP8IV4WwOO8TEFGyRYy7q9P7Q%3D%3D&X-Amz-Algorithm=AWS4-HMAC-SHA256&X-Amz-Date=20210826T192421Z&X-Amz-SignedHeaders=host&X-Amz-Expires=300&X-Amz-Credential=ASIAQ3PHCVTYT56LJ2V4%2F20210826%2Fus-east-1%2Fs3%2Faws4_request&X-Amz-Signature=feddd2a93385410dc5a9b82ba3e56518184158e1cefd4c3d99404d91ca2cfbd9&hash=efef46d53bf7b1d6882ff696a92d3b4b1ed674ab542e41f197bc76a1cecb9df8&host=68042c943591013ac2b2430a89b270f6af2c76d8dfd086a07176afe7c76c2c61&pii=S2095311919625815&tid=spdf-711ee8ad-979a-43f1-a5bb-9ece18127e0b&sid=20eeb84f6c1d46434f3b0f28f6075dc24632gxrqa&type=client) (FEI, 2019). Additionally, this video can aid in showing you how to transfer fungal cultures once identified: [Forest Pathology - transferring fungal cultures - YouTube](https://www.youtube.com/watch?v=Yv_QBWAN7hQ&ab_channel=D.Benson). 10 days after the second re-plating, the fungus is ready to be turned into an inoculum. Please refer back to the procedure Fungi Inoculum Prep above on how to prepare your inoculum.

Next, you will need to measure and adjust the spore concentration with a hemocytometer. If it is your first time using a hemocytometer, this video may help on how to set it up: [Loading a Hemacytometer for Manual Cell Count - YouTube](https://www.youtube.com/watch?v=5EGD39ln5LM&t=141s&ab_channel=ARUPLaboratories). Clean the hemocytometer prior to use and the cover glass with 70% ethanol. Then dry it with sterilized lens paper. Then mix the spore solution well and add 10 µl of the spore solution to each side of the hemocytometer using the appropriate pipette. Carefully position the cover-glass over the sample. Place the hemocytometer under the microscope under 10X objective to locate the grid (Fig. 3). Count the number of spores (Fig. 2) in all 4 corners (red) and the center square (blue). If the spore falls on the left or bottom line do not count it. If the spore falls on the right or top line, count it. Calculate the total number of spores. The spores per mL of suspension is equivalent to the average spore count per large square x . Dilute the spore solution to the desired concentration (spores/mL) using the formula C1V1= C2V2 where the desired final concentration (C2) is conidia .

**Tip:** The following video may be helpful but be mindful of the differences within this protocol and the video, as the video is for Angular Leaf Spot (ALS): [Kirkhouse Trust - Isolation of a fungal pathogen and producing inoculum - YouTube](https://www.youtube.com/watch?v=F92GEhJaubc&ab_channel=KirkhouseTrust)

A close-up of a circuit board

Description automatically generated with medium confidence

Photograph provided by Fred Brooks, University of Hawaii at Manoa, Bugwood.org

**Fig. 2:** Bean anthracnose (Colletotrichum Lindemuthianum)

A picture containing text, shoji, crossword puzzle, building

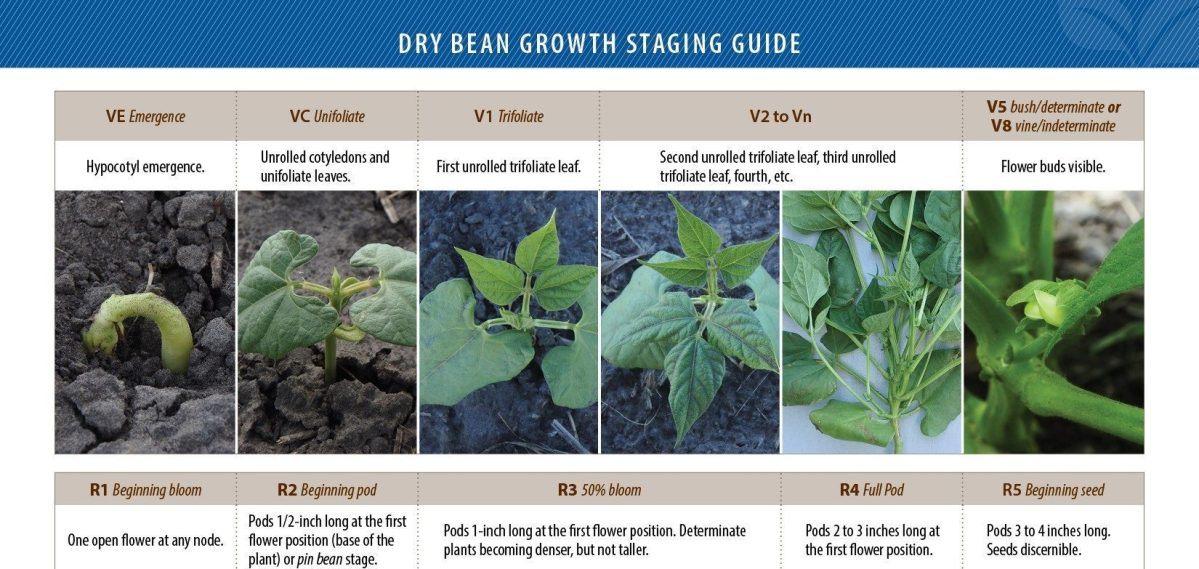
Description automatically generated

**Fig. 3:** Hemocytometer counting chamber

1. Differential Series Growth

While you are growing your fungus, you will need to grow the differential series in the greenhouse. It is recommended that you grow three replicates per cultivar, arranged in a completely randomized design. It is also recommended that a known susceptible cultivar is also grown, such that you expect this cultivar to show symptoms of disease. ‘Othello’ has been used previously as it is known to be susceptible to race 73.

Once the plants have reached the adequate growth stage, it is time to inoculate. Inoculate when the unifoliate leaves are fully expanded (~9-10 days after seeding) (Fig. 4). Spraying the plants with the conidia/mL suspension until run-off of the upper and lower sides of the leaves and onto the stems (Conner et al., 2020).



Photograph provided by Manitoba Pulse Soybean Growers

**Fig. 4:** Dry Bean Growth Staging Guide depicting unifoliate leaves under the VC growth stage

Then place the plants in a humidity chamber for 5 days at 20°C under 14 h fluorescent light. After 5 days return them to the greenhouse and 10 days after inoculation, evaluate the plants.

1. Disease Rating

Ten days after inoculation, disease symptoms will have appeared, and you can evaluate disease ratings to determine which race(s) of anthracnose were present in your sample(s). Disease reaction is based on a 1-9 severity scale.

|  |  |  |
| --- | --- | --- |
| **Disease rating** | **Resistant/Susceptible** | **Description** |
| 1-3 | Resistant (R) | No visible disease symptoms or only a few, very small lesions, primarily on the leaf veins |
| 4-9 | Susceptible (S) | Numerous large lesions or sunken cankers on the abaxial sides of the leaves of hypocotyls |

(Kelly, J.D., 2010)

Race designations were then determined. Do this by adding up the binary numbers associated with differentials that were determined to be susceptible and create a table.

Use the table below as an example:

|  |  |  |  |
| --- | --- | --- | --- |
| Differential Cultivar | Binary no. | Mean Disease Rating (1-9) | Phenotypic Reaction (R/S) |
| Michelite | 1 |  |  |
| MDRK | 2 |  |  |
| Perry Marrow | 4 |  |  |
| Cornell | 8 |  |  |
| Widusa | 16 |  |  |
| Kaboon | 32 |  |  |
| Mexico 222 | 64 |  |  |
| PI | 128 |  |  |
| To | 256 |  |  |
| Tu | 512 |  |  |
| AB 136 | 1024 |  |  |
| G 2333 | 2048 |  |  |

For example, differentials Michelite(1), Cornell (8), and Mexico 222 (64) are susceptible for race 73. By adding their binary numbers you get the race designation 73.

1. Further Reading
2. Conner, R. L., Boland, G. J., Gillard, C. L., Chen, Y., Shan, X., McLaren, D. L., Hou, A., Penner, W. C., Melzer, M. S., Balasubramanian, P., Hwang, S.-F., & McRae, K. B. (2020). Identification of Anthracnose races in Manitoba and Ontario from 2005 to 2015 and their reactions on Ontario dry bean cultivars. *Canadian Journal of Plant Science*, *100*(1), 40–55. https://doi.org/10.1139/cjps-2019-0003
   1. [Identification of anthracnose races in Manitoba and Ontario from 2005 to 2015 and their reactions on Ontario dry bean cultivars (cdnsciencepub.com)](https://cdnsciencepub.com/doi/full/10.1139/cjps-2019-0003)
3. Halvorson, J. M., Lamppa, R. S., Markell, S. G., & Pasche, J. S. (n.d.). *Characterization of COLLETOTRICHUM lindemuthianum Races Infecting dry EDIBLE Bean in North Dakota*. Taylor & Francis. https://www.tandfonline.com/doi/full/10.1080/07060661.2015.1137081?scroll=top&needAccess=true.
   1. [Characterization of Colletotrichum lindemuthianum races infecting dry edible bean in North Dakota: Canadian Journal of Plant Pathology: Vol 38, No 1 (tandfonline.com)](https://www.tandfonline.com/doi/abs/10.1080/07060661.2015.1137081?journalCode=tcjp20)
4. Kelly, J. D. *Anthracnose*, Http://Arsftfbean.uprm.edu/Bic/Wp-Content/Uploads/2018/04/Anthracnose.pdf, 2010.
   1. [Anthracnose (uprm.edu)](http://arsftfbean.uprm.edu/bic/wp-content/uploads/2018/04/Anthracnose.pdf)
5. References
6. ARUPlabs, director. *Loading a Hemacytometer for Manual Cell Count*. *YouTube*, ARUP Laboratories, 31 Mar. 2016, [www.youtube.com/watch?v=5EGD39ln5LM&t=141s&ab\_channel=ARUPLaboratories](http://www.youtube.com/watch?v=5EGD39ln5LM&t=141s&ab_channel=ARUPLaboratories)
7. del Rio, L., & Bradley, C. (2002, July). *Anthracnose of dry beans*. https://library.ndsu.edu/ir/bitstream/handle/10365/5423/pp1233.pdf?sequence=1&isAllowed=y#:~:text=Anthracnose%2C%20caused%20by%20the%20fungus,at%20any%20stage%20of%20development.
8. “Forest Pathology - Transferring Fungal Cultures.” *YouTube*, YouTube, 6 Aug. 2014, www.youtube.com/watch?v=Yv\_QBWAN7hQ&ab\_channel=D.Benson.
9. FEI, L.-wang, LU, W.-bo, XU, X.-zhou, YAN, F.-cheng, ZHANG, L.-wei, LIU, J.-tao, BAI, Y.-jun, LI, Z.-yu, ZHAO, W.-sheng, YANG, J., & PENG, Y.-liang. (2019). A rapid approach for isolating a single fungal spore from rice blast diseased leaves. *Journal of Integrative Agriculture*, *18*(6), 1415–1418. https://doi.org/10.1016/s2095-3119(19)62581-5
10. Kirkhouse1, director. *Kirkhouse Trust - Isolation of a Fungal Pathogen and Producing Inoculum*. *YouTube*, YouTube, 12 July 2012, www.youtube.com/watch?v=F92GEhJaubc&ab\_channel=KirkhouseTrust.
11. Poudel, Bikash. “Counting Fungal Spores Using A Hemocytometer.” *Sustainable Agriculture*, 18 June 2015, agristudentbikash.wordpress.com/2015/06/18/counting-fungal-spores-using-a-haemocytometer/.
12. Rajesha, G., and S. G. Mantur. “Studies on Morphological and Cultural Characters of Colletotrichum Lindemuthianum Inciting Anthracnose of Dolichos Bean.” *Journal of Mycopathological Research*, vol. 52, no. 1, 28 Apr. 2014, pp. 121–124., doi:20153038003.