Collaboration for Plant Pathogen Strain Identification

Guidelines for the Identification of Races of *Fusarium oxysporum* f. sp. *niveum* using Differential Watermelon Lines

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Host: Watermelon (*Citrullus lanatus*)
Pathogen: *Fusarium oxysporum* f. sp. *niveum*

Background

Fusarium wilt of watermelon is caused by *Fusarium oxysporum* f. sp. *niveum* (E. F. Sm.) W. C. Snyder & H. N. Han. This soilborne fungal pathogen was first described in the United States in Georgia and South Carolina in 1894 and only causes disease in watermelon (Smith 1894). Today, the disease is found worldwide in most watermelon growing areas (Egel and Martyn 2013). Primary infection occurs in the roots and movement of field soil on tools and equipment contributes to disease spread within and between fields. The formation of thick-walled chlamydospores enables pathogen survival in the soil for up to 15 – 20 years, which makes disease management difficult. Management can be accomplished with the use of resistant cultivars and by grafting onto resistant squash, bottlegourd, or citron melon 'Carolina Strongback' rootstocks (Keinath and Hassell 2014, Keinath et al. 2019). However, Fon can still be highly destructive and significantly limit production if more virulent isolates or new races appear. Disease symptoms will vary depending on the age of the plant at infection, environmental conditions, aggressiveness, and density of the pathogen population. Symptoms can also vary depending on host genotype (Kleczewski and Egel 2011). Damping off may occur in young seedlings, while in older plants an initial overall graying of foliage is followed by chlorosis, wilt, and necrosis. Wilting of individual runners can also be seen in older plants (**Fig. 1**). Wilt occurs more rapidly when plants are stressed for water and at fruit set. When stems of affected plants are cut lengthwise the characteristic necrosis of the vascular tissue can be seen (**Fig. 2**) (Kleczewski and Egel 2011, Martyn 1996).

Figure 1. Wilting of individual runners that can be seen in older plants (Purdue University)

Figure 2. Characteristic necrosis of the vascular tissue (Purdue University)

Races and resistance

Races of *Fusarium oxysporum* f. sp. *niveum* are defined based on their level of pathogenicity on differential hosts as opposed to defined resistance genes (**Table 1**; Cirulli 1972, Martyn and Netzer 1991, Zhou et al. 2010). While resistance to Fon: 1 has been shown to be inherited by a single dominate gene pattern the gene is not yet characterized (Netzer and Weintall 1980). The susceptible and resistant responses of Black Diamond, Charleston Grey, and Calhoun Grey are based on the percent wilt of inoculated seedlings, where ≥33% wilt is rated as a susceptible response, and ≤33% wilt is rated as a resistant response (Martyn and Bruton, 1989).

Table 1. Classification of races of *Fusarium oxysporum* f. sp. *niveum* according to pathogenicity on differential host cultivars of *Citrullus lanatus* (adapted from Martyn and Netzer 1991)

Differential Hosts	Fon: 0	Fon: 1	Fon: 2
Black Diamond	S	S	S
Charleston Grey	R	S	S
Calhoun Grey	R	R	S
PI 296341 FR	R	R	R

S = Susceptible, R = Resistant

Today, races 1 and 2 are widespread, so prevention and management of this disease is critical (Engle and Martyn 2013, Martyn 2014, Keinath et al. 2020). There are now multiple reports of race 3 in the US which overcomes the resistance in PI 296341-FR (Amaradasa et al. 2018, Petkar 2019, Zhou et al. 2010). However, distribution is limited, and we were unable to validate a reference isolate. While race 0 can be problematic for some heirloom watermelon varieties, it is of little economic importance in commercial watermelon production areas (Engle and Martyn 2013, Keinath et al. 2020). Given the limited economic impact of Fon: 0, this race, and the differential cultivar Charleston Grey were dropped as a reference material offered by CPPSI (**Table 2)**.

Table 2. Expected responses of the *Citrullus lanatus* differential hosts and isolates of Fon races available as CPPSI reference materials

Differential Hosts	Fon: 1	Fon: 2
Black Diamond	S	S
Calhoun Grey*	R	S
PI 296341 FR**	R	R

S = Susceptible, R = Resistant

*The CPPSI source of Calhoun Grey seed exhibited variable response in resistance to Fon: 1. This is the current CPPSI source available via the USDA-GRIN system. If using this seed for your breeding program it is recommended to screen and select for resistant plants in subsequent populations. A new seed source with a consistent resistance response to Fon: 1 has been acquired. This seed is available upon request, domestically, please contact the CPPSI Director as listed below.

**PI 296341 FR is reported resistant to Fon: 1 and Fon: 2. However both traits segregate independently in this cultivar and one or the other can be lost during seed increases if subsequent generations are not selected carefully (Egel and Martyn 2013, Martyn and Netzer 1991). In the presence of a suspected race 3, both PI 296341 FR and Calhoun Grey are susceptible.

New sources of resistance to Fon: 2 have been released by the US Vegetable Lab, designated USVL246-FR2 and USVL252-FR2 (Wechter et al. 2016). These lines were developed from PI 482246 and PI 482252, respectively.

Preparation of host plants and inoculum:
Methods for inoculation of watermelon seedlings vary. Most protocols use either spore suspensions or a mycelium-agar slurry as inoculum. Other sources of method variation include the age of the seedlings at inoculation, the methodology for dipping roots into the inoculum solution, and the environmental conditions under which the seedlings are maintained after inoculation. The following is an inoculation procedure that has been demonstrated to give consistent results for this work.

Seeds are sown in a commercial potting mixture or vermiculite and grown in a greenhouse at 25-30ºC with a 16 hour photoperiod to germinate. Before planting PI 296341 FR, nick or slice the seed coat at the wider cotyledon end – make sure to make a tiny cut through the seed coat. This will promote imbibing and facilitate more consistent germination and emergence.

At the same time, inoculum is started by transferring the pathogen to ~10 plates ½ PDA grown at 25°C in an incubator or 10 flasks of cultured in potato dextrose broth (300 mL for a 1L flask) and placed in a rotary shaker at 25°C and set fast enough to keep the culture aerated (**Fig. 3**). Czapek media may also be used instead of PDA.

Figure 3. Flask of inoculum in liquid culture on a rotary shaker at 25°C (Sakata)

Inoculation, incubation and evaluation for resistance and susceptibility:
Seedlings are inoculated when the first trifoliate leaf has fully expanded (~10 to 14 days after sowing). The inoculum is prepared by straining the culture through cheesecloth (**Fig. 4**) (or by scrapping ½ PDA plates and straining though cheesecloth) and collecting the spore suspension. Depending on the virulence of the isolate used, the spore suspension is adjusted to ~100ml of 1 X 10^6 spores/ml to 2 X 10^6 spores/ml dilution.

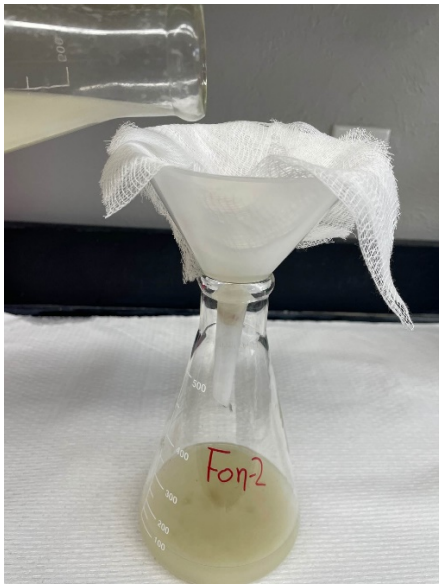

Figure 4. Strain inoculum culture through cheese cloth (Sakata).

Figure 5. Removal of seedlings from the potting soil (Webb, USDA).

For inoculation, remove seedlings from their containers (**Fig. 5**) and wash the roots with water to remove as much potting soil as possible. Some protocols suggest to trim roots prior to inoculation but we have found it is not necessary. The washed roots are then submerged in 100ml of inoculum for 5 minutes gently swirling the mixture every few minutes (**Fig. 6**). A mock inoculation (**Fig. 7**) is strongly recommended to rule out the impact of the root dip and transplanting processes.

Figure 6. Root dip inoculation (Sakata).

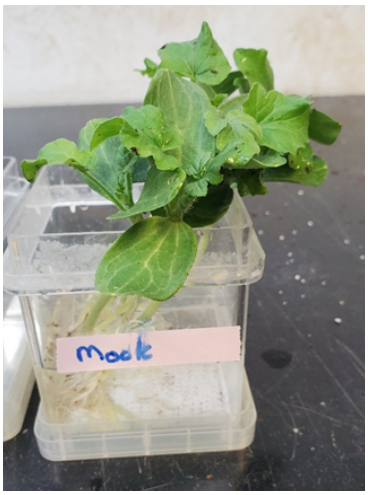

Figure 7. Root dip mock inoculation (Webb, USDA).

After inoculation, the seedlings are immediately transplanted into new ‘cone-tainers’ containing pre-moistened potting mix, open trays and trays with cells are also used depending on inter-lab protocol. Inoculated plants are then allowed to recuperate in a cool, dark, humid environment overnight (**Fig. 8**).

Figure 8. Seedlings are transplanted into potting mix right after inoculation (Webb, USDA).

The day after inoculation the plants are moved to a growth room or greenhouse, and maintained for three weeks at 23-26°C with 16 hours of light/day. The soil should be kept moist, but not saturated. The seedlings will typically regain turgor after inoculation. The susceptible plants will start to wilt 5 to 7 days after inoculation. Three weeks after inoculation the results should be clear, with resistant plants remaining asymptomatic (**Fig. 9**), and susceptible plants developing symptoms including wilt, stunting, vascular discoloration, and/or complete death (**Fig. 10**).

Figure 9. Resistant response of PI 296341 FR no visible symptoms 3 weeks after inoculation (Webb, USDA)

Figure 10. Susceptible seedlings are dead or dying 3 weeks after inoculation (Webb, USDA)

Differentiation of races 1 and 2 is made by the pattern of responses in these hosts to each tested race. Charleston Gray was dropped as a differential host due to the lack of a clear susceptible response to race 1 (**Fig. 11**) and race 2 (**Fig. 12**).

Figure 11. Race 1 inoculation of L to R Black Diamond, Charleston Gray, and Calhoun Gray (R) (BASF)

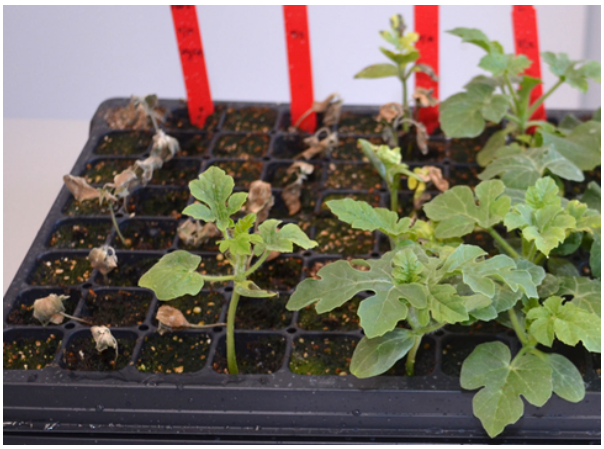

Figure 12. Race 2 inoculation of L to R, Black Diamond, Charleston Gray, Calhoun Gray and PI 296341 FR (BASF)

Ordering seeds of watermelon differential lines:
Seeds of each of the differential lines listed in Table 2 can be ordered from the USDA GRIN (Germplasm Resources Information Network: https://www.ars-grin.gov/). You may search the USDA GRIN database without logging in but cannot order seeds until you create an account and log in to the database.

Type in '**CPPSI***' in the search window to display all material deposited by CPPSI. Select the differential hosts to order. Twenty seeds of each of the differential lines can be ordered at no charge. The USDA National Plant Germplasm System in which the GRIN database is housed may not always have adequate seed of all the differentials listed to provide a full set of differentials.

Ordering isolates of *Fusarium oxysporum* f. sp. *niveum*:
Reference isolates of races 1 and 2 of *Fusarium oxysporum* f. sp. *niveum* can also be ordered from the National Center for Genetic Resources Preservation (NLGRP) via the online GRIN system. Follow the same GRIN access instructions for ordering seeds. ***Proof of valid permit for plant pathogens is required to receive isolates.***

For questions regarding ordering and permits for these reference races, contact:
Amy Gurza (amy.gurza@usda.gov) and/or **Andy Hagan** (andy.hagan@usda.gov)
National Lab for Genetic Resources Preservation Unit
1111 South Mason St.
Fort Collins, CO 80521

If you have difficulties ordering seeds or pathogen isolates, contact the CPPSI Director, **Kelley Clark** at kjclark@ucdavis.edu for assistance.

Feedback

Inquiries on how to participate and support CPPSI, provide feedback on new races identified, views on the inoculation protocols, differential hosts, or any related matter is welcomed. Please contact: Kelley Clark at kjclark@ucdavis.edu.

Liability waiver

The CPPSI Collaboration for Plant Pathogen Strain Identification, USDA NPGS/GRIN, APS, ASTA, and all other associated members and participating organizations or companies have done their best to provide information that is up-to-date and published in refereed journals and, therefore, no liability for the use of this information is accepted. The inoculation protocol described in this document has been demonstrated to be effective at identifying races of Fon and resistance in the above watermelon cultivars.

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