Sukhwinder Kaur and Lynn Epstein, 24 May 2020. LE update 19Nov20

# RNA Isolation from Celery Crowns for eventual TagSeq

## I. Exp. 2: Challenger-U and FOA R2 inoculated and harvested at 21dpi. Also, Exp. 3 (2019) 21 dpi: U & Foa R2 Exp. 4 (2020) U-R2-R3- & R4, 7, 14 & 21 dpi

FOA Race 2 culture-**Inoculum preparation:**
FOA Race 2 cultures were grown on PDA with 100ug/ml chloramphenicol (8 plates-100 mm) for 22 days at RT (23-24C) under lights. Conidia (mostly microconidia) were harvested and passed through two layers of sterilized cheese cloth to remove any mycelia. 0.1% WA was prepared the day before infestation. On the day of infestation, a conidial suspension (maintained on ice) was prepared in 0.1% water agar and adjusted to final 7.6 X $10^6$ conidia/ml.

**PRODUCTION OF SEEDLINGS & INFESTATION**

1. Coated Challenger seed was obtained from Syngenta, Woodland, CA
2. About 150 seeds (25-30seeds per plate) were plated in 100mm Petri dishes on a single layer of (sterilized twice on different days at dry cycle) moist white germination paper, moistened with 3 or 5 ml sdi water. Note that germination paper should be maintained as moist with no free-floating water in the Petri dish.
3. The Petri dishes with seeds were incubated in the dark at 23-24C for five days and then transferred to lights at 23-24C for 9 days. After 14 days of total incubation, these seedlings were transplanted into 72 cell trays in sterilized seeding soil. Note that this is soil that is designated for seeding flats; it is not the perlite GH mix used for transplanting and FOA screening in infested soil.
4. All the trays were kept in the GH with temperature around 70-75F in liners and watered from the bottom of the trays. For the first two weeks, trays were covered with clear plastic tray covers until true leaves grew. Then the seedlings were sprinkler-irrigated 3X/day for 3 min, and later for 5 min, for 6 weeks until infestation/transplanting.
5. "Eight weeks old" seedlings (including time for germination) were gently removed from the trays, washed to remove soil and gently pat-dried with paper towels to remove excess water. The seedlings were inoculated by dipping in 150 ml of a suspension of 7.6 X $10^6$ conidia/ml 0.1% water agar prepared as indicated above, for 10 min in batches of 5 seedlings at a time and transplanted into sterilized greenhouse soil (perlite:GH 3:1). Uninoculated controls were dipped for 10 min in 0.1% water agar only. There were 2 treatments---mock inoculated and FOA R2 inoculated, each with 5 plants in each of 4 replicates. The trial can be considered a completely randomized design. Although

seedlings were handled gently, some wounding occurred when removing roots from the plug.

**HARVEST. Mortars and pestles were autoclaved.**

6. After 21 days post-inoculation, the seedlings were transferred to 186 Hutchison for harvest..In a bucket, soil was removed and crowns were washed thoroughly with tap water. The 1-3 roots on the crown were removed and each crown (ca. 1 cm in length) was cut longitudinally in half from 5 plants/rep with a razor blade. One-half of each of the crowns was transferred to pre-cooled aluminium foil, wrapped and immediately flash frozen in liquid N2; these crowns were stored in case extraction from a 2nd determination was required. For the other half of each of crown that was for the next-day RNA extraction, the crowns were crushed with a pre-cooled pestle while keeping everything on ice and transferred to pre cooled aluminium foil,wrapped and immediately FLASH FROZEN IN LIQUID NITROGEN .
7. All samples were stored in a -70C FREEZER. RNA was extracted next day.

ONLY CHALLENGER WAS PROCESSED FOR RNA EXTRACTION.

**RNA extraction. IMP-Always use low retention filter tips. Clean all the pipets with RNase away solution. Do not inhale RNase away but let the soln. dry completely. Clean the surface with RNase away soln.**
**Heat all the glassware to be used at 170C for two h.**
**Soak pestles and mortars and spatulas etc. in 1N NaOH and 0.1% EDTA soln overnight and then rinse it thoroughly with DEPC treated NF water.**

**RNA Extraction Protocol:**

1. For each sample, aliquot 1.4 ml of extraction buffer into a sterile 2 ml centrifuge tube and warm to 65 ℃. Just before adding the sample, add 20μL of β-mercaptoethanol.
2. Add ca 5X by weight–(0.5g) sterilized sand (pre-baked at 232C for 2h) and PVPP (0.02 g) to a cold mortar and pestle. Chill w/ liquid N2.
3. Just before you're ready to add the ground tissue, add 20μL of β-mercaptoethanol to the extraction buffer.
4. The five half-crowns (ca. 100 mg) were ground in liquid N2, with the mortar and pestle.
5. Transfer the ground tissue to the tube (T-1) with warm (65 ℃) buffer. Vortex vigorously for 1 min and then incubate at 65 ℃ for 10 min to lyse cells completely.
6. Centrifuge at 15,700 g for 5 min and transfer the supernatant into a new sterile tube (T-2).
7. Add an equal volume (900-1000 μL) of chloroform: isoamyl alcohol (24:1), vortex vigorously for 1 min and centrifuge at 15,700 g for 10 min at room temperature.

8. Transfer the upper phase to a new tube (T-3) and add an equal volume (700-800 µL) of chloroform. Vortex vigorously for 1 min and centrifuge at 15,700 g for 10 min at room temperature.
9. Transfer the upper phase to a new tube (T-4) and add an equal volume (500-600 µL) of 4 M LiCl.
10. Precipitate RNA at -20°C for 2 hours and then leave at 4°C overnight.
11. Centrifuge at 15,700 g for 15 min at 4°C and remove supernatant.
12. Wash the pellet gently with 1.0 ml of ice cold 75% freshly prepared ethyl alcohol, centrifuge at 15,700 g for 5 min at 4°C and remove the supernatant.
13. Wash the pellet gently again with 1.0 ml of ice-cold 75% ethyl alcohol. Centrifuge at 15,700 g for 5 min at 4°C and remove the supernatant.
14. Centrifuge again at 5,900 g for 5 min, remove the remaining ethanol and air-dry the resultant pellet.
15. Re-suspended the pellet in 200-300 µL NF water (but not DEPC-treated)
16. Do the Qubit and Nanodrop quantification. RNA was quantified using the Invitrogen Qubit RNA BR Assay Kit Cat# Q10210 on a Qubit® 2.0 fluorometer (Invitrogen, Life Technologies, Carlsbad, CA)
17. At this step RNA quantity should be 80-90 ng/ul Qubit and Nanodrop quantity is 97-250 ng/ul, 260/280 ratio is >2.0 (2.08-2.14) and 260/230 ratio is also >2.0 (2.2-2.9)

**Ambion Turbo DNase-free kit: (200ul RNA)**

1. Add 0.1 volume 10x Turbo DNase buffer. Vortex buffer at least for 30 sec before use (Best to do this in a 1.5 mL low binding centrifuge tube).
2. Add 5 µL TURBO DNase and mix gently.
3. Incubate at 37 °C for 30 min, gently mixing by flicking the tube every 10 min.
4. Add 0.1 volume re-suspended DNase Inactivation reagent (vortex 30 sec before use), again for 30 sec and incubate at room temperature for 2 min, vortexing every 30 seconds.
5. Centrifuge at 10,000g for 2 min and transfer supernatant to a new tube being careful not to transfer the inactivation buffer at the bottom of the tube.
6. Approx.150-170 ul RNA should be recovered.

Check quantity and quality of RNA by Qubit (70-90 ng/ul) and Nanodrop (ND) (90-150ng/ul, 260/280 and 260/230 ratio >2.

UCD Genome Center requires further cleaning with columns
I followed **RNA clean & concentrator Tm5 cat# R1015 from Zymo research kit**.
80-120ul of high quality RNA was recovered after this step.
Again do ND and Qubit quantification.

At this step, RNA should have an indicated concentration by Qubit of 85-100 ng/ul and by Nanodrop of 140-150ng/ul. The Nanodrop quality indicators, 260/280 and 260/230 ratios should be >2.

Submit RNA to Genome Center for TagSeq lib.

Extraction Buffer:

| | Stock Solution | 50 mL Working Solution |
|---|---|---|
| 3% CTAB, | | 1.5 g |
| 100 mM Tris-HCl pH 8.0, | 1 M | 5 mL |
| 1.4 M NaCl, | 5 M | 14 mL |
| 20 mM EDTA | 0.5 M | 2 mL |
| 5% PVP | | 2.5 g |
| RNase free water | | 29 mL |
| β-mercaptoethanol – added just before use | | 10 µL / 700 µL |

All solutions need to be RNase-free. Make up 50 ml – heat to 65C to dissolve CTAB and PVP. Add β-mercaptoethanol only to volume required for preps.
Other solutions needed: chloroform:isoamyl alcohol, chloroform, 4M LiCl, 75% EtOH.

1.

# Stock solutions

**0.5M EDTA**
Add 74.45g EDTA disodium salt dihydrate to 300mL dd$H_2O$
pH to 8.0 with NaOH
Volume to 400mL

**1M Tri-Sodium Citrate**
Dissolve 51.612g Trisodium Citrate in dd$H_2O$ to 200mL

## Exp. 1. Tall Utah 52-70 R Improved

**Celery uninfested and FOA R2, R3 and R4 infected sample collection from GH**
Notes
GH 46
We need (ca. 100 mg wet weight per replicate; we're pooling to make the reps more uniform). For the Tall Utah, I would pool, say 6 crowns per each of 5 **replicates**, i.e., would need a minimum of 120 uniform (max 160) Tall Utah. **Need conducive temp for disease -27C=80.6F**. Collect all at same time point, just before any browning of the crown, at **21 dpi** (need to have uniform infection, although pooling should reduce that problem)

1. Tall Utah-race 2
2. Tall Utah-race 3
3. Tall Utah-race 4
4. Tall Utah-mock inoc
5. Young fungal hyphae –race 2. Note- would have to be grown in liquid. Maybe 3 day old, at a time of active hyphal growth. Same temp as **GH-27C**. Fungal hyphae could be say a pool of 3 plates.
6. Young fungal hyphae –race 3
7. Young fungal hyphae –race 4

TU-52-70R imp from Harris Moran (2014 seed) was seeded on 12/28/2017 in three 72 cell trays in double steam sterilized seeding soil. (Note: Tall Utah-52-70 R Improved (TU) seeds can also be obtained from Burpee Seed Company). Uncoated seeds were surface disinfected with 5% commercial bleach (0.307% Sodium hypochloride)) for 2 min rinsed once with sdi water and pat dried in folds of sterilized paper towels.
Germination noticed on 1/8/2018.
4 trmts with 6 plants in each of 5 reps=120, INOCULATION DATE 2/22/2018-8 WEEKS OLD TRANSPLANTS
Prepare Inoculum of Race2, Race3, Race4,-ON PDA PLATES-2/6/2018
Two 500 ml flasks of each of three races enough to inoculate 40 plants, Total 6 flasks, Soak 4 cups of millet grains overnight ON 2/8/2018, TRANSFER MILLET GRAINS TO 6 FLASKS-500 ML CAPACITY-100ML MARKING, AUTOCLAVE ON 2/9/2018
Inoculate with Race 2, 3, 4 two flasks each on 2/13/2018, Incubate under lights at 25-27C.
Double steam sterilized soil for 120 tubes-30 each for U-R2-R3-R4
Labels 30 each for GH46-TU-U-1-1-DATE (6) GH46-TU-U-5-1(6)-DATE
GH46-TU-R2 -1-1-DATE, GH46-TU-R2 -5-6-DATE, SAME FOR R3 AND R4
Harvested at 21 dpi-3/15/2018.
For GH bring autoclaved pestles mortar, 50.0 ml tubes, scrappers, ice, ice chest RNALater solution.
USE separate buckets for washing U, FOA R2, R3 and R4 infected plants.
Cool mortar and pestle, RNAlater solution keep on ice.
Harvest the plant, remove all soil and wash, remove all roots and cut crown piece put in cooled mortar do all the 6plants, the crush these in RNA later solution keeping everything cold and quickly transfer in in 50 ml tube. Add more cold RNA later solution to submerge all the tissue.
Transfer in ice chest.
All samples harvested and in RNAlater soln, brought to lab and stored at 4C fridge until RNA extracted (approx. within a week).

# RNALater Solution

| **Component** | **1500mL** | **750mL** | **400mL** |
|---|---|---|---|
| dd$H_2O$ | 935mL | 467.5mL | 249.33mL |
| Ammonium sulfate | 700g | 350g | 186.67g |
| 1M Sodium Citrate | 25mL | 12.5mL | 6.67mL |
| 0.5 M EDTA | 40mL | 20mL | 10.67mL |

In 100g batches, dissolve Ammonium Sulfate in dd$H_2O$, while stirring on low heat.
Add stock solutions.

Cool to RT and adjust to pH 5.2 using concentrated $H_2SO_4$ (about 14 drops/L)
Filter to remove any particulates.
Store at RT

**Use:**
Chill to 4˚C before use.
Add 5-10 volumes to fresh sample,
Store O/N at 4˚C

Stable at 4˚C for 1 week
Stable at -20 to -80 ˚C indefinitely
(Remove excess supernatant before freezing, samples can be thawed at RT)

Blot excess solution away prior to RNA extraction

**Notes:**
If precipitation occurs heat to 37˚C and agitate
**Keep away from bleach!**
Do not rinse samples; RNAse inactivation is reversible
Optional for dense liquid samples - Add equal volume ice-cold PBS before spinning down.

**References:**
Patent:
http://www.google.com/patents/US8178296
Gist:
https://gist.github.com/brantfaircloth/0229c3ae545f4bcfe4de
Step by step:
https://www.protocols.io/view/RNAlater-Recipe-c56y9d