SCREENING FOR FUSARIUM WILT RESISTANCE IN TOMATO

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1. Description

The Disease
Fusarium wilt in tomato is a major disease of tomato. It was first described by G.E. Massee in England in 1895. It is of worldwide importance where at least 32 countries had reported the disease. It is particularly severe in countries with temperate and tropical climates. The disease nearly destroyed tomato production in many countries and it has only been the development and use of resistant cultivars that has reduced this threat.

Causative Agent
Fusarium oxysporum f. sp. *lycopersici* (Sacc.) (Fol) causes Fusarium wilt in tomato. The fungus penetrates though the roots and proliferates in the vascular tissue, impeding water transport, causing spectacular wilting, leaf-yellowing and rapid death of the plant. There are three races of Fol, Races 1, 2 and 3. Races 1 and 2 are found throughout most tomato growing regions while Race 3 has only been reported in parts of USA and Australia.

Resistance
Resistance to race 2 confers resistance to race 1, but not vise-versa (see Table 1). It has yet to be absolutely confirmed whether resistance to Race 3 confers resistance to Races 1 and 2. It is know that resistance to races 1 and 2 does not confer resistance to Race 3. Resistance to Races 1 and 2 is referred to as "semi-dominant", meaning that in its heterozygous state (I_1i or I_2i) resistance is not 100%. Furthermore, both heterozygous and homozygous states for Fol Race 1 confers some tolerance to Race 2, significantly above that would be found in susceptible cultivars. Generally homozygous for Fol-2 (I_2I_2) confers approximately 100% resistance to both Races 1 and 2. Because of this "semi-dominant" resistance and tolerance conferred by Race 1 resistance against Race 2 isolates, there is often confusion over the interpretation of the results. This underscores the need to follow the standard protocol exactly and include all the controls indicated. Variations in the onset of the infection between winter and summer is significant, often up to 10 days.

Genetics
Resistances to all races of Fol have been identified in wild tomato species and incorporated into the cultivated tomato *Lycopersicon esculentum*. The interaction between Fol and tomato is race-cultivar specific. Race-specific disease resistance in plants is determined by **single dominant or semi-dominant resistance (R) genes** that is involved in the recognition of the products of avirulence (Avr) genes from pathogens and the activation of plant defence responses. R-genes conferring resistance to Fol race 1 have been identified and mapped to chromosomes 11 and 7. The I-2 gene, conferring resistance to Fol race 2, is a member of the coiled coil (CC)-nucleotide-binding site (NBS)-leucine-rich repeat (LRR) class of resistance genes. I-2 lies within a cluster of seven similar genes on the long arm of chromosome 11.

Table 1. Typical resistances in tomato when inoculated with *Fusarium oxysporum* f. sp. *lycopersici* Race 2. Period of inoculation, warm nights (22°C) with warm to hot days (30°C plus) in Chiang Mai.

Variety (source)	Resistance state	% Plants with leaf yellowing	% Plants with vascular browning (≥10 cm)	Gene	Number of days to symptoms
Peto 94 (Peto seed)	Resistant	0%	40%	I_2I_2	21 days
Delta F1 (EWS)	Resistant (moderate)	15%	60%	I_1I_2	21 days
	Susceptible	75%	80%	I_1I_1	21 days
Bonny Best	Susceptible	90%	100%	ii	21 days
AVRDC lines					
Fantastic	Susceptible	90%	100%	ii	21 days
UC82-L	Resistant			I_1I_1	
MH-1	Resistant			I_2I_2	
I3R-1 (BL783)	Resistant			I_2I_2 I_3I_3	

2. Breeding summary

In breeding for resistance, breeding programs should select for Race 2 resistance (and Race 3 if the market requires). Varieties resistant to Race 2 are also resistant to Race 1. Although the resistance is single-gene dominant, under high disease pressure some plants heterozygous for the resistance will develop symptoms (can be up to 40% in the field). However, this is rare.

Breeding objectives:

1. Breed for resistance to Race 2.
2. Resistance is dominant.
3. Symptom development in the screenhouse (and field) is slow (up to 28 days after inoculation).
4. Symptom development is quickest under cool to warm conditions.
5. Always include the recommended controls in both field, screenhouse and laboratory based screening programs.
6. **Only parents or inbred lines need to be screened (dominant gene).**

Table 1. Summary of Fusarium Wilt in tomato.

Disease		Fusarium Wilt (*Fusarium oxysporum* f. sp. *lycopersici*)
Genetics		Dominant genes (total number not confirmed). Race 2 resistance confers resistance to Race 1.
Source of resistance		Various
Resistance required in parents		Resistance obtained with heterozygous state. For highest level of resistance, homozygous resistance required in both parents.
Screening program	**Available**	Yes.
	Programs	Seedling inoculation by drenching.
	Marker*	Available. Differentiates no-gene, heterozygous and homozygous states.
Double Haploids		None available.

*** Screening by using the marker is recommended – it is much quicker and more accurate. *Only parents* need to be screened.**

Table 2. Summary of screening methods.

Screening method	Date of inoculation (age of seedling)	Result (age of seedling)	Comment
Lab based - PCR	Extract DNA from any plant material	Results within two days.	The PCR-based protocol is the protocol of choice. It is quick, can be done all year round, cheaper and very accurate.
Drenching*	Day 14	Day 35	Consistent result. Reflects true field resistance but slow (28 days).
Root damage and drenching*	Day 14	Day 35	Generally consistent.
Root dipping	Day 14	Day 28	Stresses the plant. Inconsistent results.
Cut leaf			Doesn't work.

* From December to February (winter) programs in northern Thailand can be done in plastic houses. During other months they should always be done in temperature controlled rooms. In the central plains, programs should always be done in temperature controlled rooms. Optimum temperatures are between 15 and 25 degrees C.

3. Screening Protocol – PCR based

Protocol for *I-2 gene* detection (resistance to Fol) in tomato by PCR

Step 1: Confirm presence or absence of the I-2 gene by multiplex PCR

Step 2: Confirm whether the lines are homozygous or heterozygous using a CAPS marker

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<u>Step 1:</u> Confirm presence or absence of the I-2 gene

1. Tomato leaf DNA extraction

Extract tomato leaf (any stage) DNA using a modified NaOH lysis procedure.

1.1 Label each plant.

1.2 Cut a small "disc" of tomato leaf tissue using a 1.5 ml eppendorf tube.

1.3 Place the leaf disc into a 96 well plate or 1.5 ml eppendorf tube as a pre-designated.

1.4 Add 50 μl of 0.5 M NaOH into each well *(0.5 M NaOH: 2 g NaOH per 100 ml of dH_2O)*.

1.5 Grind leaf tissue to a liquid consistency using a pestle. Optionally, a pestle in a small bench-top hand drill can be used.

1.6 After each sample is ground, wash the pestle in a 0.1% chlorine (final) solution and rinse three times with R/O.

1.7 Precipitate the leaf extracts by centrifuging in a microtitre plate benchtop centrifuge at 200 rpm, 4°C for 10 minutes or if using eppendorf tubes, at maximum speed (~13,500 rpm) in a microfuge for 2 minutes.

1.8 Transfer 5 μl of plant extract to a 96 well plate (or eppendorf tube) containing 245 μl of 0.1 M Tris-HCl, pH 8.0 (1:50 dilution). Mix by pipetting (eppendorf tube by vortexing).

1.9 Centrifuge at 200 rpm, 4°C for 10 minutes or if using eppendorf tubes, at maximum speed (~13,500 rpm) in a microfuge for 2 minutes.

1.10 Store at –20°C or 4°C overnight.

NB. In all cases, a positive control (**<u>known</u>** positive control for *I-2 gene*) and negative control (**<u>known</u>** negative control for the *I-2 gene*) **<u>MUST</u>** be included. In both cases these should be extracted at the same time as the samples. Controls can be leaf discs stored at -20°C.

<u>Controls</u>: Negative – Fantastic (ii), Bonny Best (ii), UC82-L (Gene I_1I_1)

Heterozygous – Delta (I_1I_2) (East-West Seed)

Homozygous - MH-1 or 13R-1(I_2I_2)

2. PCR

Primers*

Primer TFusF1: 5' CTG AAA CTC TCC GTA TTT C 3'
Primer TFusrr1: 5' CGA AGAGTG ATT GGA GAT 3'
Primer TFusrr2^{T}: 5' CCT GGA TGA ACA GCT GAG 3'

^{T} – Can be used instead of TFusrr1, but TFusrr21 is better.

The internal control Ve2 primers;

Primer Ve2F: 5' ATT TGC TGC CCC TAC TAT GTA TCC 3'
Primer Ve2R: 5' TGA ATT GTA AGT TGT TGG AGG TCC 3'

Preparation of PCR master mix: as follows:

Reagents	10 μl Reaction	Master mix (100 reactions) of 10 μl each
10 mM dNTPs	0.5	50
10X Buffer	1.0	100
50 mM $MgCl_2$	0.25	25
TFusF1 primer (10 ρmol/ul)	0.25	25
TFusrr1 primer (10 ρmol/ul)	0.25	25
Ve2F primer (10 ρmole/ul)	0.05	5
Ve2R primer (10 ρmole/ul)	0.05	5
dH_2O	7.05	705
Taq DNA Polymerase** (5 U/μl)	0.1	10
Total	9.5	950
DNA template	0.5	-
Total volume	10	-

- Pipette 9 μl of PCR master mix to PCR tubes.
- Add 0.5 μl of DNA template.

NB: Don't forget the PCR Negative control

* Choaa A. El Mohtar et.al., 2007. *Marker-Assisted Selection of Tomato Genotypes with the I-2 Gene for Resistance to Fusarium oxysporum f. sp. Lycopersici Race2.* Plant Disease 91: 758-762.

** Check the stock *Taq* DNA polymerase concentration first

PCR reaction:

1 cycle:	94°C for 3 min
35 cycles:	94°C for 1 min
	57°C for 1 min
	72°C for 2 min
1 cycle:	72°C for 10 minutes

Amplified DNA of the specific *I-2* genotypes should be approximately 600 bp.
Amplified DNA of the internal control should be approximately 800 bp.

Agarose Gel Electrophoresis

Electrophoresis DNA in 1% agarose gels at 100 volt for approximately 40 minutes (this will vary depending on the electrophoresis unit used).

- Prepare 1% agarose gel in 0.5X TBE buffer *(TBE 10X: to 800 ml dH_2O add 108g Tris, 55g boric acid, 9.3 g EDTA and make up to 1 litre)* and dissolve by heating in a microwave.
- Cool the molten agarose to 50 – 60°C, pour into the mould, insert the comb and allow the gel to set.
- Submerge the gel in 0.5X TBE buffer and remove the comb.
- To each 10 μl sample add 2 μl 6X gel loading buffer (GLB)
  6X GLB:
  12.5 mg bromophenol blue
  12.5 mg xylene cyanol
  1.875 ml 80% glycerol
  1.5 ml EDTA
  1.665 ml dH_2O
- Load the DNA approximately 14 μl into the wells. Include the appropriate molecular weight markers as well as the negative and positive controls.
- Run the gel at 100 volts for approximately 40 minutes until the bromophenol blue dye front is within 1 cm of the end of the gel.

Visualisation of DNA using SYBR-Gold or Ethidium Bromide

- Submerge the gel and stain in SYBR-Gold (1:10,000 dilution – normally 5 μl stock in 50 ml 0.5X TBE buffer) or ethidium bromide solution (10 mg/ml) for 20 minutes.
- Rinse the gel in dH_2O.
- Visualize the amplified DNA fragments under ultraviolet-illumination
- Verify results against DNA molecular weight and positive, negative control.

2. Analysis of DNA fragments pattern

- Verify results against DNA molecular weight markers (MW) and the positive and negative controls. This indicates the absence or presence of the loci (Figure 1).

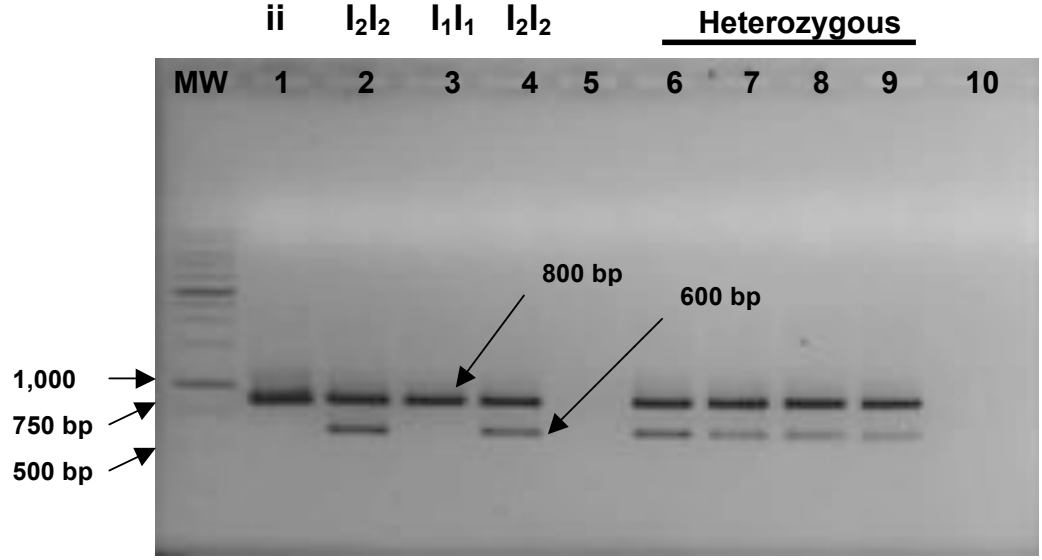


Figure 1. Agarose gel electrophoresis of PCR amplified DNA associated *I-2 gene* detection in tomato. Controls include homozygous (resistant Race 2 (I_2I_2) lines), lanes 2 and 4, negative (no *I-2 gene*) lanes 1 and 3, and heterozygous (I_1I_2) lanes 6-9. Two DNA patterns are possible:

- **Negative result**: A single band of approximately 800 bp indicates an absence of the *I-2* gene. Lanes 1 and 3

- **Homozygous and heterozygous**: Two bands of approximately 600 and 800 bp indicate the homozygous and heterozygous. Lanes 2, 4, 6, 7, 8 and 9.

Screening for Fusarium (Races 1 and 2) Resistance in Tomato

3. Summary

- Tomato lines that have **DO NOT contain the *I-2-gene*** are **"Fusarium Race 2 susceptible"**.

- Tomato lines that contain the *I-2* gene (600 bp) are **"Fusarium Race 2 resistant".**

- This **"Step 1"** multiplex PCR method does not allow the differentiation of homozygous from heterozygous resistant genotypes.

- This multiplex PCR method does not allow for the identification of Fusarium Race 1 resistant lines.

Trouble Shooting

1. The concentration of *I-2 gene* specific band (600 bp) should be 4 – 5 fold higher than that of the Ve2 (800 bp) internal control.

2. If it is believed that both parents are homozygous for the *I-2 gene* but the progeny is either heterozygous or are segregating for homozygous and heterozygous, then the original assumption of both parents being homozygous is incorrect.

3. On occasions you will observe a faint or absent 600 bp band associated with the *I-2 gene*. Firstly check the appearance of Ve2 (800 bp) internal control band. If the band associated with Ve2 is of equal or lower concentration, or is absent, the result is in question. The concentration of *I-2 gene* specific band (600 bp) should be 4 – 5 fold higher than that of the Ve2 (800 bp) internal control. The Ve2 band is the control to ensure that the quality and quantity of DNA was satisfactory for PCR, avoiding false positive results.

4. How do I determine whether the lines are Race 1 resistant? Apply the "Screening for resistance by inoculation" protocol.

5. How do I determine whether the lines are heterozygous or homozygous? Apply "Step 2" of the PCR protocol.

<u>Step 2:</u> Confirm whether the lines are homozygous or heterozygous using a CAPS marker.

1. Tomato leaf DNA extraction

Extract tomato leaf (any stage) DNA using a modified NaOH lysis procedure.

1.1 Label each plant.

1.2 Cut a small "disc" of tomato leaf tissue using a 1.5 ml eppendorf tube.

1.3 Place the leaf disc into a 96 well plate or 1.5 ml eppendorf tube as a pre-designated.

1.4 Add 50 μl of 0.5 M NaOH into each well *(0.5 M NaOH: 2 g NaOH per 100 ml of dH_2O)*.

1.5 Grind leaf tissue to a liquid consistency using a pestle. Optionally, a pestle in a small bench-top hand drill can be used.

1.6 After each sample is ground, wash the pestle in a 0.1% chlorine (final) solution and rinse three times with R/O.

1.7 Precipitate the leaf extracts by centrifuging in a microtitre plate benchtop centrifuge at 200 rpm, 4°C for 10 minutes or if using eppendorf tubes, at maximum speed (~13,500 rpm) in a microfuge for 2 minutes.

1.8 Transfer 5 μl of plant extract to a 96 well plate (or eppendorf tube) containing 245 μl of 0.1 M Tris-HCl, pH 8.0 (1:50 dilution). Mix by pipetting (eppendorf tube by vortexing).

1.9 Centrifuge at 200 rpm, 4°C for 10 minutes or if using eppendorf tubes, at maximum speed (~13,500 rpm) in a microfuge for 2 minutes.

1.10 Store at –20°C or 4°C overnight.

NB. In all cases, a positive control (**<u>known</u>** positive control for *I-2 gene*) and negative control (**<u>known</u>** negative control for the *I-2 gene*) **<u>MUST</u>** be included. In both cases these should be extracted at the same time as the samples. Controls can be leaf discs stored at -20°C.

<u>Controls</u>: Negative – Fantastic (ii), Bonny Best (ii), UC82-L (Gene I_1I_1)

Heterozygous – Delta (I_1I_2) (East-West Seed)

Homozygous - MH-1 or 13R-1(I_2I_2)

3. **PCR**

Primers*

Primer TAOF: 5’ GGG CTC CTA ATC CGT GCT TCA 3’
Primer TAOR: 5’ GGT GGA GGA TCG GGT TTG TTT C 3’

Preparation of PCR master mix:

Reagents	10 μl Reaction	Master mix (100 reactions) of 10 μl each
10 mM dNTPs	0.5	50
10X Buffer	1.0	100
50 mM $MgCl_2$	0.25	25
TAOF primer (10 ρmol/ul)	0.25	25
TAOR primer (10 ρmol/ul)	0.25	25
dH_2O	7.15	715
Taq DNA Polymerase** (5 U/μl)	0.1	10
Total	9.0	100
DNA template	0.5	-
Total volume	10	-

- Pipette 9 μl of PCR master mix to PCR tubes.
- Add 0.5 μl of DNA template.

NB: Don’t forget the PCR Negative control

* M. Staniaszek et al., 2007. *A CAPS marker $TAO1_{902}$ diagnostic for the I-2 gene conferring resistance to Fusarium oxysporum f. sp. Lycopersici race 2 in tomato*. Plant Breeding 126, 331-333.

** Check the stock *Taq* DNA polymerase concentration first

PCR reaction:

1 cycle:	94°C for 3 min
40 cycles:	94°C for 15 sec
	62°C for 20 sec
	72°C for 30 sec
1 cycle:	72°C for 10 minutes

Amplified DNA of the specific *I-2* genotypes should be approximately 902 bp.

4. Restriction enzyme digestion of PCR amplified products

Digest PCR products with *RsaI* restriction enzyme.
RsaI restriction enzyme size**:**

GT⇩AC
CA⇧TG

Preparation of restriction master mix: as follow:

Reagents***	Single reaction (ul)	Master Mix (50 Reactions) of 12 ul each
10X Buffer I (NEB)	1.2	60
dH2O	0.8	40
RasI (10U/ul)	0.2	10
PCR amplified DNA	10	
Total	12.0	110

- Pipette 10 ul of PCR product to eppendorf tubes containing 2 ul of 'master mix'
- Gently vortex and spot spin
- Incubate at 37C for 3 hours
- Add 2 ul of 6X gel loading buffer
- Run gel. Alternatively digests can be stored at 4°C

*** check manufacturer's specifications of *RsaI*

Agarose Gel Electrophoresis

Electrophoresis DNA in 1% agarose gels at 100 volt for approximately 40 minutes (this will vary depending on the electrophoresis unit used).

- Prepare 1% agarose gel in 0.5X TBE buffer *(TBE 10X: to 800 ml dH_2O add 108g Tris, 55g boric acid, 9.3 g EDTA and make up to 1 litre)* and dissolve by heating in a microwave.
- Cool the molten agarose to 50 – 60°C, pour into the mould, insert the comb and allow the gel to set.
- Submerge the gel in 0.5X TBE buffer and remove the comb.
- To each 10 μl sample add 2 μl 6X gel loading buffer (GLB)

  6X GLB:
  12.5 mg bromophenol blue
  12.5 mg xylene cyanol
  1.875 ml 80% glycerol
  1.5 ml EDTA
  1.665 ml dH_2O
- Load the DNA approximately 14 μl into the wells. Include the appropriate molecular weight markers as well as the negative and positive controls.
- Run the gel at 100 volts for approximately 40 minutes until the bromophenol blue dye front is within 1 cm of the end of the gel.

Visualisation of DNA using SYBR-Gold or Ethidium Bromide

- Submerge the gel and stain in SYBR-Gold (1:10,000 dilution – normally 5 μl stock in 50 ml 0.5X TBE buffer) or ethidium bromide solution (10 mg/ml) for 20 minutes.
- Rinse the gel in dH_2O.
- Visualize the amplified DNA fragments under ultraviolet-illumination
- Verify results against DNA molecular weight and positive, negative control.

4. Analysis of DNA fragments pattern

- Verify results against DNA molecular weight markers (MW) and the positive and negative controls. This indicates the absence or presence of the loci (Figure 1).

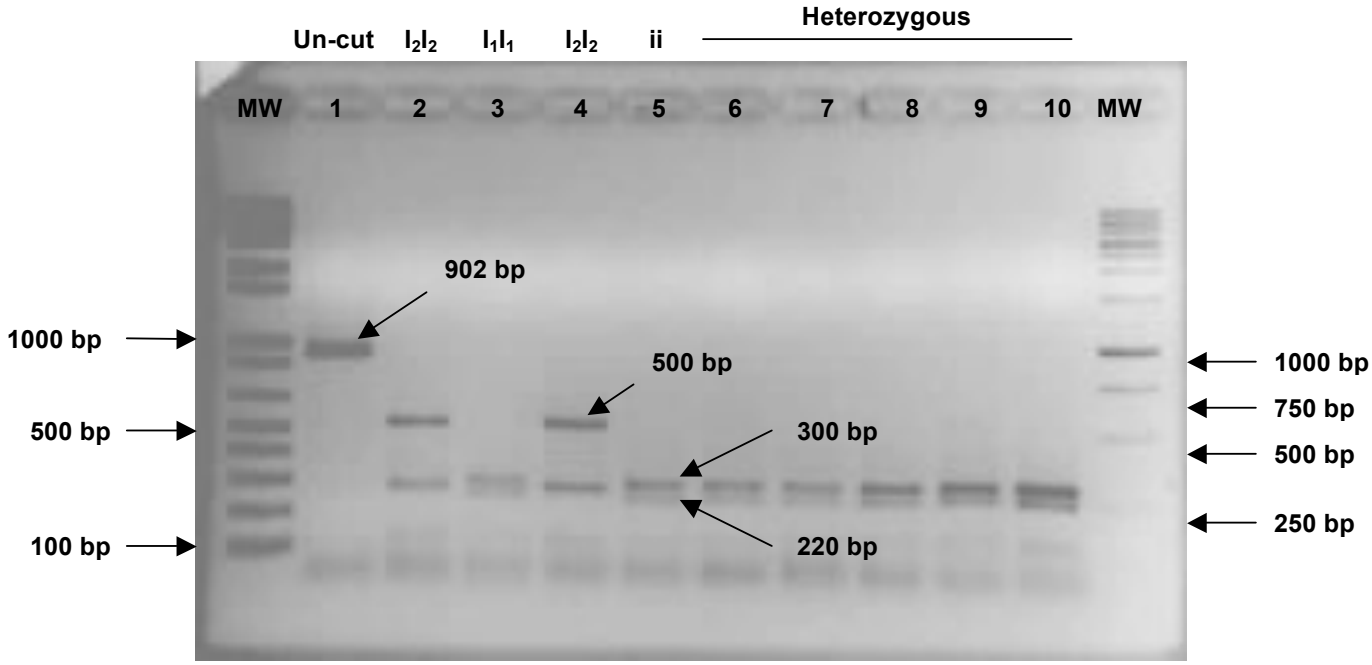


Figure 1. Agarose gel electrophoresis of PCR amplified DNA associated *I-2 gene* detection in tomato. Controls include homozygous (resistant Race 2 (I_2I_2) lines), lanes 2 and 4, negative (no *I-2 gene*) lanes 3 and 5, and heterozygous (I_1I_2) lanes 6-10. Lane 1 is the un-cut amplified DNA of approximately 902 bp. Two DNA patterns are possible:

- **Homozygous**: Two bands of approximately 500 bp and 300 bp indicate the homozygous. Lanes 2, and 4.

- **Heterozygous and negative result**: Two bands of approximately 300 bp and 220 bp indicating the heterozygous state or the absence of the *I-2* gene. Lanes 3, 5, 6, 7, 8, 9 and 10

5. Summary

- Tomato lines that have **DO NOT contain the *I-2-gene*** are **"Fusarium Race 2 susceptible"**.

- Tomato lines that contain the *I-2* gene, both homozygous and heterozygous, are **"Fusarium Race 2 resistant".**

- **In Step 1 you have two different banding patterns.** The homozygous pattern, two bands of 500 and 300 bp, and the heterozygous/susceptible state with bands of 300 and 220 bp. Note that the result for the heterozygous and susceptible states is the same in Step 1.

- **Step 2** is only for differentiating the homozygous from the heterozygous resistant state.

- This CAPS PCR method does not allow for the identification of Fusarium Race 1 resistant lines.

Trouble Shooting

4. Screening protocol - Inoculation

a. Inoculation of tomato lines in the screenhouse with *Fusarium oxysporum* f. sp. *Lycopersici*

Before you start!!!

This screening will reflect tomato plants grown under field conditions whilst being able to control the variables often associated with field conditions, which usually do not affect the pathogenicity of bacterial wilt. It is essential that all plants be in a similar condition – age, height, vigor etc.

Seedling preparation

Tomato seeds should be sown in plastic trays containing a peat moss based potting mix. For Chia Tai a white peat mix (Pinstrup) is used. **Twenty-five seeds of each line should be sown** as at least twenty plants are required per line.

At 12 days after sowing (das) transplant the seedlings to 2 inch plastic pots containing a 1:1 ratio of potting mix and sand. Seedlings should have the first true leaves, however, this depends on the individual lines and weather conditions.

Watering of seedlings

Watering of seedlings should be as required but consistent, normally twice per day being once in the morning and then in the afternoon. The morning watering should contain 50 mg/l soluble fertilizer (21-21-21) while the afternoon watering should be just water. The soil should be thoroughly wet with watering draining from the base of the pot. Over-watering should be avoided.

On the day of inoculation, tomato plantlets **MUST NOT BE WATERED** in the morning and inoculations should, where possible, be done late in the afternoon. **If the seedlings do need watering in the morning then the weather is really too hot for the Fusarium program!!** This is not always possible especially when a large number of plantlets are to be inoculated. However, after inoculation the plants **MUST NOT BE WATERED UNTIL THE FOLLOWING MORNING.**

Experimental design

The inoculations should be divided into three replications, each replication being five plants. Five plants should be left un-inoculated. This is a total of twenty plants. This also includes the controls.

Susceptible check:	Bonny Best (ii), Fantastic (ii)
Moderate check:	Delta F1 - EWS (I_1I_2)
Resistant check:	UC82-L – AVRDC (I_1I_1), MH-1 (I_2I_2), 13R-1 (BL783) – AVRDC (I_2I_2 I_3I_3)

Materials required

1. Beaker containing bacterial suspension;
1. Cup for pouring the suspension;
2. Marker pens;
3. Wash bottle containing 70% ethanol for swabbing;
4. Paper towels, plastic tags, gloves, large washing tub for washing the used containers overnight with 1% final sodium hypochlorite;
5. Fan to keep cool;
6. Yellow tags labelled with the plant line, date of inoculation and bacterial isolate. White tags labelled with the plant line and sowing day should already be in the pots (Figure 3).

1. Positive control -15 plantlets of the Fusarium wilt susceptible line "??"
2. Negative control – 15 plantlets of the Fusarium wilt tolerant line "Peto 94"
3. Non-inoculated "??" control – 5 plantlets
4. Non-inoculated "Peto 94" control – 5 plantlets
5. Inoculated sample - 30 plantlets of each line being screened
6. Non-inoculated sample – 5 plantlets of each line being screened

Inoculation (Drenching)

1. Check that all pots are correctly labeled with a white tag indicating the line and the date the seed was sown (e.g. No.12, 22/7/44) (Figure 1).

2. Check all plants and make sure that all are approximately the same height and condition.

3. **DO NOT DAMAGE THE ROOTS** of the seedlings.

4. Add 30 ml of suspension culture (this amount should be 10% of the total volume of the pot. If a 3 - inch pot, being 300 ml, then add 30 ml of culture. If inoculation at 36 cells tray add 5ml of spore solution)

5. Check that all pots are correctly labeled with a white tag indicating the line and the date the seed was sown (e.g. #No. 12, 22/7/44) and a yellow tag with the plant line, date of inoculation and Fusarium isolate.

6. Record details in the "Screening request form" and in your workbook.

7. Ensure that the plants receive their next scheduled watering (next day if inoculated in the afternoon or at no sooner than 4 hours later if inoculated in the morning).

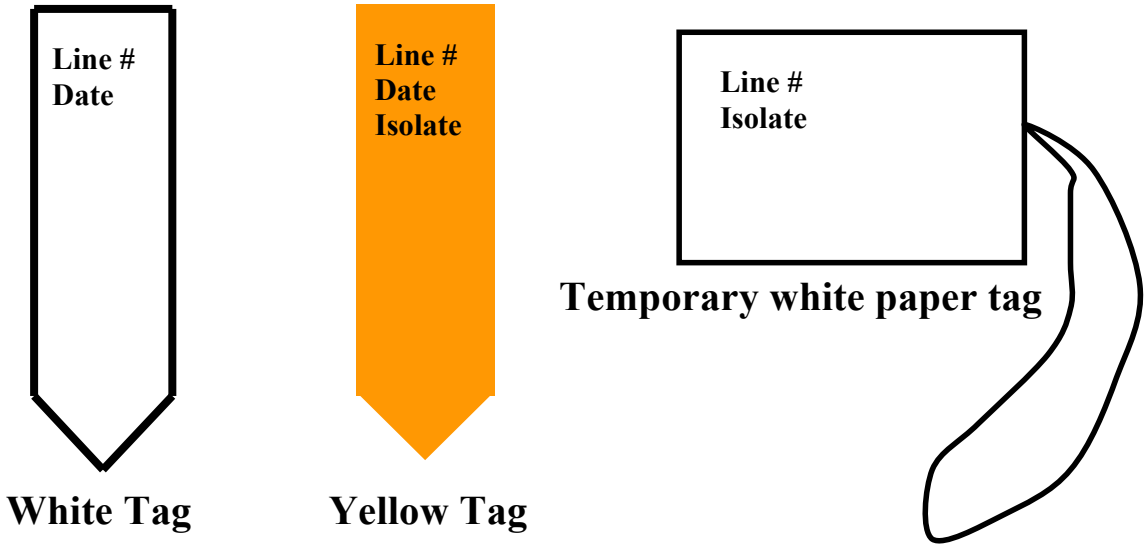


Figure 1. Labelling of tags.

1. White tags should be labelled with the line number and the date of sowing
2. Yellow tags should be labelled with the line number, date of inoculation and the Fusarium isolate number.
3. Temporary white paper tags with string should be labelled with the line number and the Fusarium isolate number.

b. Evaluation of tomato lines inoculated with *Fusarium oxysporum* f. sp. *lycopersici*

1. Check all seedlings after 24 hours. If symptoms of wilt, ensure that seedlings have been watered. Continue to monitor every 24 hours.

2. Evaluate seedlings at intervals of 1, 2, 3 and 4 weeks. Results should be recorded as the percentage of wilting of each plant and the rating of each line with wilt symptoms (see disease severity rating and evaluation sheet).

Disease severity rating

Rating	Description
0	Healthy
1	10% of leaves have yellowing
2	Leaves are yellowing and wilting of young leaves
3	Most leaves are yellowing and leaves are wilting
4	Dead

NB. For the fusarium wilt program there is no need to use formulas as the genetics is very clear cut; being single-gene dominant.

A selection of plants, resistant, intermediate and susceptible, should be analysed for stem browning. Remove the plant from the soil, cut it length-wise down the stem to the very based. Measure the height of the browning – the susceptible lines should have significantly more stem browning (higher up the stem) than the resistant or intermediate lines. The resistant lines will still have some stem browning (approximately 10 cm) but this will vary from line to line.

Stage 0

Stage 1

Stage 2

Stage 3

Stage 4

Figure 2. Disease severity rating.

c. *Fusarium oxysporum* f. sp. *lycopersici* isolates and inoculation controls

Controls:
Performance of controls under glasshouse conditions inoculated by soil drenching and root damage-drenching. Variation between hot and cool season inoculation vary by 40%.

***NB. Glasshouse conditions at Kanchanaburi exceed 40°C and as such are not suitable for Fusarium wilt programs. Programs should be done in the temperature controlled room or done in the months of December to February.**

****During the months of March to October programs in Chiang Mai should also be done in the temperature controlled room.**

5. Disposal

Disposal of tomato lines and soil inoculated with *Fusarium oxysporum* f. sp. *lycopersici*

1. At the completion of the inoculation, remove seedlings and soil from the pots and dispose of the plant material into the pathogen waste container for autoclaving.

   As fusarium is a produces spores, all material should be autoclaved twice. After the initial autoclave, the material MUST be allowed to cool to below 40°C to allow spores that survive the autoclaving to germinate. Then the material should be autoclaved a second time.

2. The used plastic bags and pots should be thoroughly washed and then soaked overnight in 10 mg/l chlorine final (approximately 0.2 ml of 5% Clorox per litre of water).

3. After overnight soaking, the bags should be removed, drained, rinsed with tap water and dried.

6. Collection storage and maintenance

a. Isolation of *Fusarium* from field samples

Identifying symptoms in the field

Sample collection in the field

1. It is only necessary to collect stem samples – best if they are approximately 10 cm in length and are from the collar region (the base of the plant immediately above the soil).

2. If the sample is not to be taken directly to the laboratory, store in a plastic bag and maintain on ice. The plastic bag should be marked with a sample number and details recorded in a notebook. These details should include the date, time collected, plant variety, sample symptoms, weather conditions, conditions of the soil (heavy, light, sandy, clay, wet, dry) and conditions of the other plants growing nearby (e.g. all with wilt symptoms, some tomato and others including pepper and eggplant which are also infected with bacteria or fungi).

3. It is also important to record whether the plant appears to be infected with other pathogens e.g. bacteria with "ooze" emanating from the stem section – this is a clear indicator of bacterial wilt.

Processing samples in the lab

1. Remove all dirt and other non-plant material and wash the stem with tap water.

2. Rinse with ddH_2O and dry with clean paper towel.

NB: ddH_2O refers to double distilled water that is the quality that the reverse osmosis (RO) unit produces. It is not sterile.

3. Surface sterilize the stem sections by wiping with 70% ethanol. Allow the stem sections to dry on clean, dry paper towel.

4. Transfer to the laminar flow and cut into 2 cm long sections using a sterile knife or scalpel blade (flamed with 95% ethanol and cooled).

5. Dip in chlorine solution 0.1% (1% of 10% stock) for 10 seconds.

6. Wash in sterile ddH_2O three times.

7. Dry with tissue paper – commercial tissue paper is good enough (no need to autoclave it).

8. Cut pieces length-wise into half.

9. Place sections onto PDA (containing 2 ml of filter sterile citric acid stock solution per 150 ml PDA). Each plate should contain 4 sections.

10. Incubate the plates (inverted) at room temperature until mycelium develop (about 3-4 days).

11. Touch a single mycelium and transfer to a fresh PDA plate (containing 0.4 ml filter sterile citric acid stock solution per 150 ml PDA).

12. Incubate for 7 days at 28^{O}C room. At this stage the plate should be covered with fungi mycelium.

b. Long-term storage of *Fusarium oxysporium* isolates

Storage in skim milk and silica gel (Preferred method)

1. Prepare approximately 3.45 g of sterile silica (without pH indicator) in a glass tube (sterilize about 2 hours at 180 ^{0}C (the silica beads should be approximately 2 – 3 cm high in the tube)).

2. In the laminar flow and using a PDA plate from *a. Isolation of Fusarium from field samples*, step 15; add 1 ml of the sterile 7% skim milk solution (powdered skim milk – 7 g/100ml distilled water) and spread over the plate using a glass spreader.

3. Transfer the mycelium-skim milk solution to the glass tube containing the silica beads.

4. Thoroughly shake and vortex the tube.

5. Immediately cool on ice for 5 minutes.

6. Remove from the ice and again vortex the solution. Ensure that the solution has coated the beads (usually 75% of the beads are covered).

7. Return to the ice for 30 minutes.

8. Store at 4°C.

After a period of one month storage, transfer the tube to the laminar flow. Using a flamed and cooled forceps, transfer one silica bead from the tube to a PDA plate (containing 0.4 ml citric acid solution). Incubate at room temperature for approximately 2 days. Examine the mycelium under to microscope to confirm the isolate of *Fusarium*.

c. Propagation of *Fusarium oxysporium* for inoculation

Preparation of spore solution

1. Transfer a long-term tube containing fusarium coated silica beads to the laminar flow. Using a flamed and cooled forcep, transfer two silica beads from the tube to a PDA plate (containing 0.4 ml filter sterile citric acid stock solution per 150 ml PDA).

2. Incubate for 4 - 7 days at 28^{O}C room. At this stage the plate should be covered with fungi mycelium.

3. To each plate add 5 ml of ddH_2O (doesn't have to be sterile ddH_2O).

4. Spread the ddH_2O over the plate with a glass rod.

5. Transfer the solution to a beaker.

6. Repeat another 2 times.

7. Filter the solution through cheesecloth into a fresh beaker.

8. Check the concentration of the spores using a haemacytometer (see Appendix Using the Levy Haemacytometer).

9. Dilute the spores to a final concentration of 7.5×10^6 spore per ml using ddH_2O.

Appendix

Culture Media

Potato Dextrose Agar (PDA)

Variations in screening program

Reduced time-frame needed for screening program

Why?

Often there is a need to undertake multiple screening programs on the one line (such as in a F1 population). In this case to reduce the cost of the program (avoiding multiple transplanting into larger pots saving on time and seedling mix) and/or the lines also need to be transplanted into the field, the seedling roots can be damaged and then immersed in *Fusarium* inoculum before replanting in pots. This has a two fold effect:

1. It damages the root system allowing quicker infection of the plant via the damaged roots by the fungi, and

2. It also increases the stress on the plant as the normal plant "defences" have been compromised by the damage in addition to the physical damage to the roots which the plant also tries to repair.

Table 2. Summary of screening methods. Standard method is "Root damage and drenching".

Screening method	Date of inoculation (age of seedling)	Result (age of seedling)	Comment
Drenching*	Day 14	Day 35	Consistent result. Reflects true field resistance but slow (28 days).
Root damage and drenching*	Day 14	Day 35	Generally consistent.
Root dipping	Day 14	Day 28	Stresses the plant. Inconsistent results.
Cut leaf			Doesn't work.

* Programs can only be done in Chiang Mai, or in the temperature controlled room at Kanchanaburi. From March to November programs in Chiang Mai should be done in the temperature controlled room due to the extreme heat.

Program Variation

In the screenhouse (taking up at point #9).

8. Check that all pots are correctly labeled with a white tag indicating the line and the date the seed was sown (e.g. No.12, 22/7/44) (Figure 1).

9. Check all plants and make sure that all are approximately the same height and condition.

10. Remove the seedlings from the trays and remove the soil by brushing the then washing the excess off using water.

11. Grouping the seedlings in their lines (ie. 15 per group), cut the bottom tips of the roots off (**DAMAGING THE ROOTS)** and immediately place the seedlings in the beaker of suspension culture. Period of inoculation should be 30 minutes (no more, no less).

12. Repot the seedlings immediately. Add approximately 30 ml of water (this amount should be 10% of the total volume of the pot. If a 3 - inch pot, being 300 ml, then add 30 ml of water. If inoculation at 36 cells tray add 5ml of water). This ensures that the plants do not dehydrate.

13. Check that all pots are correctly labeled with a white tag indicating the line and the date the seed was sown (e.g. #No. 12, 22/7/44) and an orange tag with the plant line, date of inoculation and Fusarium isolate.

14. Record details in the "Screening request form" and in your workbook.

15. Ensure that the plants receive their next scheduled watering (next day if inoculated in the afternoon or at no sooner than 4 hours later if inoculated in the morning).

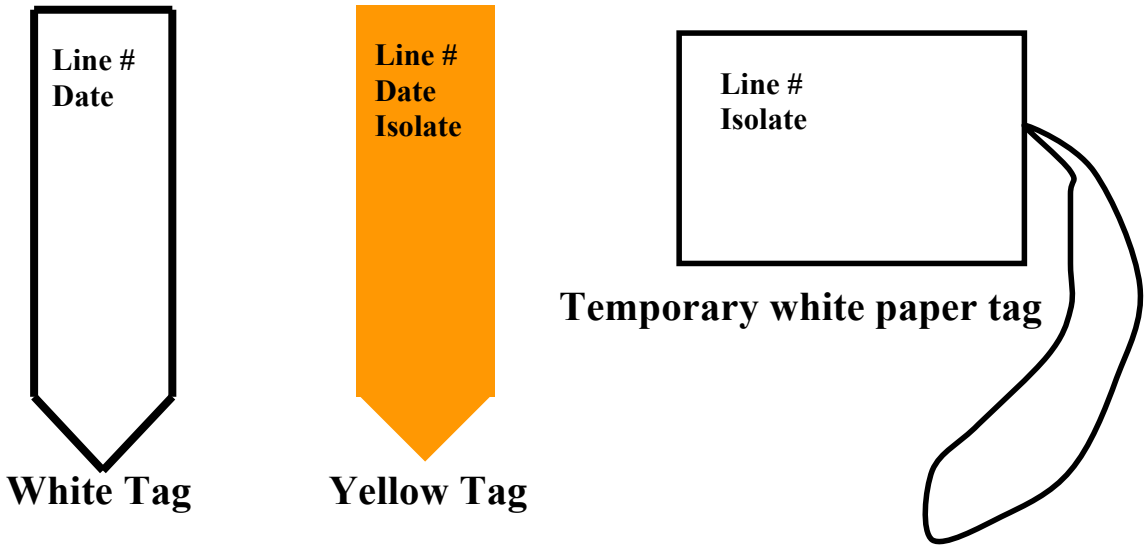
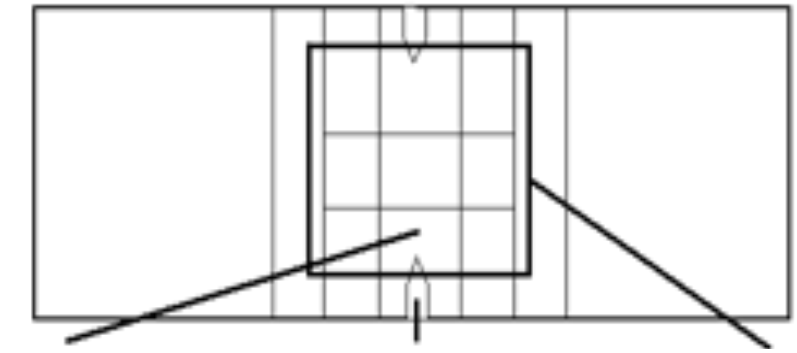


Figure 1. Labelling of tags.

1. White tags should be labelled with the line number and the date of sowing
2. Yellow tags should be labelled with the line number, date of inoculation and the Fusarium isolate number.
3. Temporary white paper tags with string should be labelled with the line number and the Fusarium isolate number.

USE OF THE LEVY HAEMACYTOMETER

The haemacytometer you will be using (Figure 1) is a Levy ultra plane counting chamber. It is used for counting the number of particles in a very small but known volume of liquid. It consists of a thick microscope slide with two microscopic grids at its centre. The grids are flanked by two vertical channels on each side and separated by a central horizontal channel. Each grid has a small tapered channel leading into it. A cover slip is provided that is large enough to cover both grids. These things are EXPENSIVE, handle with care!



One grid (D) **Add liquid here** **Coverslip**

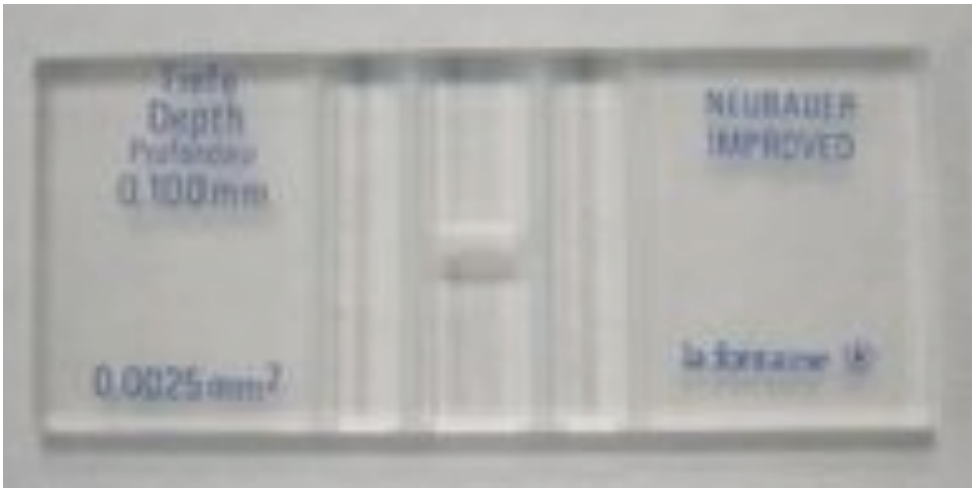


Figure 1. Levy haemacytometer

The two grids on the haemacytometer consist of a series of vertical and horizontal intersecting parallel lines engraved in the glass (Figure 2).

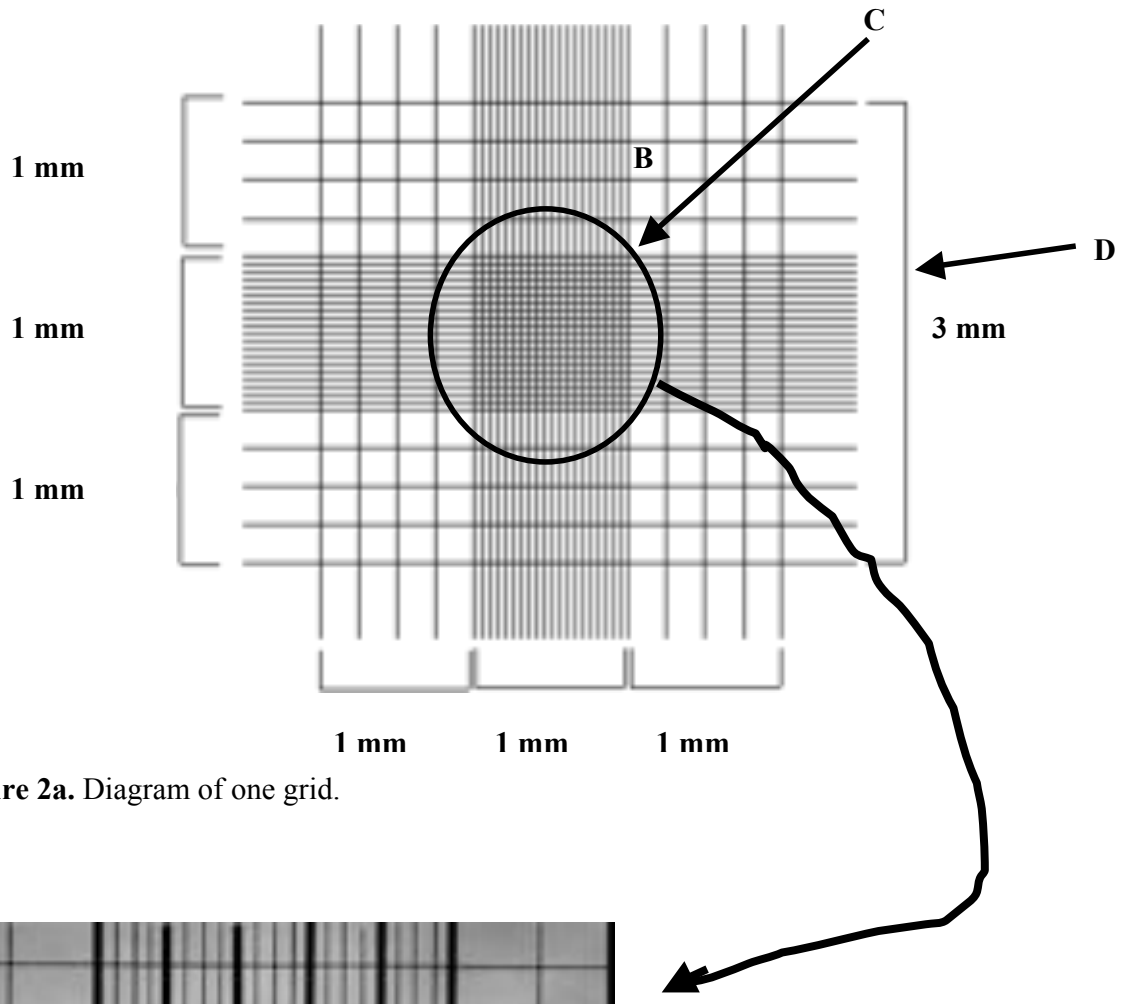


Figure 2a. Diagram of one grid.

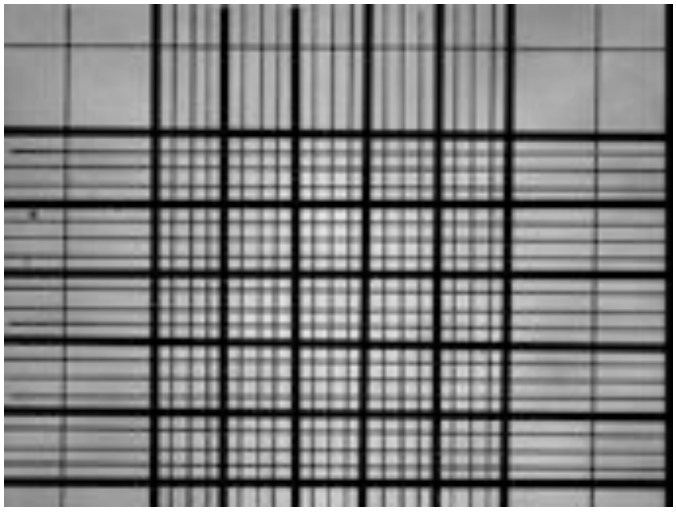


Figure 2b. Diagram of the central part of the grid (C). Note that the 0.04 mm^2 squares (A) are each divided into 16 smaller squares. At the upper left and right are two of the 0.0625 mm^2 corner squares (B).

The largest square is 3 mm on each side (9 mm^2). At each of the four corners of this large square are squares 1 mm2 ruled into 16 smaller squares, each 0.0625 mm^2. The central square millimetre is divided in 25 (not 16) squares, each 0.04 mm^2, each of

which is subdivided into 16 smaller squares 0.0025 mm^2 (= 1/400 mm^2). In summary you have available to you five squares of different sizes:

1. 9 mm^2 - the large single square (3 mm X 3 mm) (D)
2. 1 mm^2 - the four corner and central squares (1 mm X 1 mm) ©
3. 0.0625 mm^2 - the 4 x 4 subdivisions of the corner squares (0.25 mm X 0.25 mm) (B)
4. 0.04 mm^2 - the 5 x 5 subdivisions of the central square (0.2 mm X 0.2 mm) (A)
5. 0.0025 mm^2 - the smallest subdivisions of the central square (0.05 mm X 0.05 mm)

The haemacytometer measures volume and therefore the depth of the grid is as important as its area. It is designed so that when you add fluid exactly the same amount remains in the grid and the excess is channelled away (within reason!). The depth from grid to coverslip is exactly 0.1 mm.

To calculate the total volume within any square you multiply the area of the square times 0.1. For example the smallest squares contain 0.005 x 0.005 x 0.1 = 0.0000025 mm^3 (= 2.5 x 10^{-6} mm^3). Calculating this in cm^3 (= cc's or ml's) multiply 0.0005 x 0.0005 x 0.01 to get 2.5 x 10^{-9} ml. The volumes of the five square sizes are thus:

1. 9.0 x 10^{-4} ml
2. 1.0 x 10^{-4} ml
3. 6.25 x 10^{-6} ml
4. 4.0 x 10^{-6} ml
5. 2.5 x 10^{-9} ml

CALCULATING CELL NUMBER USING THE HAEMACYTOMETER

A. Filling the chamber

1. Place the cover slip over the central part of the haemacytometer;

2. Mix the cell suspension well before starting and then proceed as quickly as possible to avoid any settling;

3. Using a capillary tube or a pipette with a fine tip take up some of the cell suspension and introduce it very slowly to the tear-shaped channel at the edge of one of the grids so that it runs in under the coverslip. Do this carefully to avoid any overflow into the side channels;

4. Do not disturb the coverslip and allow the haemacytometer about 3 minutes to settle before moving it to the stage of the microscope.

B. Counting

1. Using low power, locate the various regions of the haemacytometer as discussed above.
2. Using the 40 x objective determine which of the 5 square sizes contain a number of cells convenient for counting. This number will usually be somewhere between 20 and 50.
3. Count the number of cells in each of 5 different squares of the same size.
4. Calculate the number of cells/ml based on the volume of the square used for doing the count. Be sure to divide the result by 5 since you did the counts in five squares not one.

Example

1. After examining the grids the 0.25 mm x 0.25 mm squares had the most comfortable number of spores to count.
2. You counted five squares of 0.04 mm^2 - the 5 x 5 subdivisions of the central square (0.2 mm x 0.2 mm) – square 1: 24; square 2: 34; square 3: 32; square 4: 23; and square 5: 37.
3. This is an **average** of 30 spores per square.
4. The volume is $4x10^{-6}$ ml.
5. This is a total of 30 spores per $4x10^{-6}$ ml or 30 x $1/4x10^{-6}$ = $7.5x10^{6}$ spores per ml.
6. Now you need a concentration of $5x10^{6}$ so you would dilute the solution by 50% - to 500 ml of spore solution add 250 ml of ddH_2O.

Summary:

Square size used: 0.25 mm x 0.25 mm

Number of squares counted: 5

Average number of spores counted per square: 30

Volume of each square: (0.25x0.25x0.1) mm^3

Convert to cubic centimetres or ml's: divide by 1000 (1 cm^3 = 1 ml = 1000 mm^3)

Total: $7x10^{6}$ spores per ml

Fusarium Screening Data Recording Sheet

Date seeds sown: **Date of inoculation:** **Date of record:** **Isolate used:**

A. CONTROLS

	Line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	AV	Comments
Suscentible																		
Tolerant																		

B. TEST

Line	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Screening for Fusarium (Races 1 and 2) Resistance in Tomato

Fusarium Screening Data Recording Sheet

Date seeds sown: **Date of inoculation:** **Date of record:** **Isolate used:**

B. TEST (continued)

Line	21	22	23	24	25	26	27	28	29	30	AV	Comments