SCREENING FOR BACTERIAL WILT TOLERANCE IN TOMATO (EGGPLANT AND PEPPER)

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1. **Description**

**The Disease**

Bacterial wilt is a major disease in a diverse range of crops including cucurbits (cucumber, pumpkin, bittergourd), potato, peanut, banana, pepper, eggplant and tomato. In all, hundreds of plant species in 44 families are affected by bacterial wilt. Its symptoms, as its name suggests, include plant wilting.

**Causative Agent**

The disease is caused by the bacterial pathogen *Ralstonia solanacearum* (previously known as *Pseudomonas solanacearum* and *Burkholderia solanacearum*) which is widely distributed throughout the world. It is more common in tropical, subtropical and warm temperate climates. In Thailand, *Ralstonia* is found throughout the country. Good quality soils with high nutrient content, neutral pH and high in organic matter are favourable for *Ralstonia*. Soils with high pH, high in calcium, carbonate and magnesium (calcareous soils) are not favourable for *Ralstonia*, but are also not favourable for *solanaceous* crops. As farmers continuously crop *solanaceous* species more and more, the incidence of *Ralstonia* is on the increase.

The extensive number of plants affected by *Ralstonia* includes not only important hosts, such as tomato, but also vegetable crops such as pumpkin, cucumber as well as a number of weeds. An extensive number of hosts from more than 50 families of weeds that have been reportedly affected by the bacterium. The importance of indigenous and introduced vegetation has been clearly established when potato and tomato crops have wilted in fields considered to have virgin soil; fields that were never previously planted with a *solanaceous* crop. There have also been many reports of members of the family Asteraceae, which includes ornamental (and weed) varieties of daisy and asters as well as herbs, which are hosts of Ralstonia. Other members of the same family have reportedly harboured strains of *Ralstonia*, some with no apparent symptoms. It is safe to say that many species of weed, ornamentals and herbs are hosts of *Ralstonia* and no weed or crop should be considered otherwise unless confirmed.

**Control**

Continuous cropping of susceptible crops only increases the incidence of the disease in the field and as such decreases crop yield. **There are no known methods of eradicating the disease from a field.** The only method of returning the field to a usable state is by one of three methods: fumigation; whereby the field is totally covered with plastic and the soil is fumigated with methyl bromide or by other chemical fumigants in a pellet form; addition of competitive soil borne fungi or bacteria either directly by inoculation or through the addition of manure; and crop rotation; non-host crops, such as corn, some cucurbit species such as wax gourd or leafy vegetables with high cyanide content in their leaves, such as mustard, are cultivated. In the case of leafy vegetables, the crop MUST be ploughed back into the soil to allow release of the cyanide in addition to the additive effect of solar radiation.
Races, Biovars, Phylotypes and Sequevars
There is some uncertainty in dividing *Ralstonia* isolates into races or groups. Traditionally isolates have been divided into races based on host range; Race 1 – potato, tobacco, diploid banana, peanut and olive; Race 2 – musaceous hosts including heliconia and triploid banana; Race 3 – potato; Race 4 – ginger; Race 5 – mulberry. However, this is clearly not a totally satisfactory nor complete method for isolate characterization as there have been some reports of Race 3 isolates also infecting solanaceous crops, particularly pepper. In 1964, *Ralstonia* isolates were divided into five biovars based on their ability to produce acid from three sugars. This offered the possibility of, for example, differentiating between tomato *Ralstonia* isolates based on their habitat (biovar 1 from normal fields, biovar 2 from rice paddy’s). This still doesn’t differentiate clear differences in host range of isolates infecting different solanaceous crops nor differences in virulence within crops. More recently DNA profiling has been used which divides *Ralstonia* isolates into four genetic groups (or phylotypes) and subsequently each phylotype into subgroups or sequevars. Currently there are four phylotypes of *Ralstonia* in tomato; Asia (Phylotype I), America (Phylotype II), Africa (Phylotype III) and Indonesia (Phylotype IV). While this may offer more information regarding geographical relatedness it still doesn’t offer much in differentiating host range or virulence as previously mentioned.

For our purposes, it is clear that the only useful way of differentiating between isolates is based on host range (pepper, tomato, eggplant, cucumber, bittergourd, pumpkin etc), use of differential hosts within crops (resistant, moderate resistance, susceptible) and colony colour (in determining whether the isolate is virulent).

Genetics
The genetics of resistance to bacterial wilt is, to say the least, complex. It has been the focus of research for more than 20 years and yet, in this time, the complete story of resistance remains elusive. While it has been confirmed for tomato that at least four loci are involved in resistance, Bw1, Bw3, Bw4, Bw5, which have been mapped to chromosomes 6, 10, 4 and 6 respectively, no reliable markers have been developed. Some markers are available for these loci, however positive results for these markers is not confirmation of resistance as other genes or factors appear also to play a role in resistance. While the chromosome maps of tomato, pepper and eggplant are similar, these loci have not be confirmed for pepper and eggplant. It is clear however, that the resistance in pepper and eggplant is less complex than in tomato and with the availability of double haploids in pepper and eggplant, breeding for bacterial wilt resistance in these crops less of a daunting experience. Further, it would appear, at least in tomato, that some of these loci are linked to fruit quality as lines with high tolerance to bacterial wilt tend to have poor fruit quality. Some consideration should therefore be given to the use of grafted tomato seedlings using bacterial wilt resistant rootstock (possible rootstock include tomato, pepper and eggplant). Grafted seedlings also offer the advantage of improved yields as well as flooding tolerance, a trait not found in many tomato lines.
Resistance
In tomato, resistance to *Ralstonia* involves direct resistance of the host but also compatibility or the relationship between the host plant and the isolate. There is evidence to suggest that the host also selects the isolates and vice versa. So, breeding for resistance needs careful planning with clear market targeting.

There are clear differences in host resistances between tomato varieties. Firstly, some isolates can invade the root system and cause wilt while others cannot. Secondly, some isolates can invade the root system but do not cause wilt. Some isolates will cause mild symptoms in some tomato lines and severe or no symptoms in others. Finally, there are differences in the rate of symptom development. The latter is of particular importance in breeding programs where the variety is destined for areas where the bacterial wilt disease pressure is low. Under these conditions the level of infection and rate of infection is lower than under hot conditions and the degree of resistance required by the breeder can be predetermined, allowing for higher quality fruit as well as a reduced breeding period. So, when planning the breeding program pay particular attention to the level of resistance required for the target market and communicate this to the plant pathologist. Selecting the correct screening program will pay dividends both in time and quality of the final product.

Farming practices impact on a tomato plant’s resistance to bacterial wilt. If the soils are favourable to nematodes (generally sandy soils), a variety’s resistance to bacterial wilt can break down. Similarly, poor farmer practices such as rough transplanting which damages the plant’s root system, lack of water, poor seedling potting mix, or other factors all of which contribute to transplant shock, also reduce a variety’s resistance. However, these farming practices should be considered separately when determining or planning a screening protocol for genetic resistance to a disease. First step is to breed for genetic resistance. Second step is to select for farming practices. However, there are some tricks to the programs. For example, with *Ralstonia* it is known that some resistances are based on preventing entry to the bacteria at the root level while another is at the cellular level in the phloem. In the latter case the bacteria can access the root system readily. While it appears that this is not a just a resistance mechanism in the plant, but in fact a combination of resistance and an interaction between the plant and the bacterium, it can be used to your advantage in a screening program. Assuming that in the farmer’s field that, due to poor practices, the root system or even the stem is damaged and *Ralstonia* infects the plant via these damaged areas. If you have conducted the screening program with the aim of cellular resistance at the phloem level, then the variety in question MAY (not saying definitely will) cope with the stress associate with poor farmer practices. So screening by either using a *Ralstonia* isolate that can readily invade the root system or by using leaf cutting with a standard *Ralstonia* isolate COULD allow you to select a bacterial wilt resistant variety that is also more tolerant to poor farmer practice. However, there is a trade off. Screening in this way you might also select against other promising varieties that have resistance to bacterial wilt under normal conditions with, perhaps, good fruit quality. So, the moral of the story is make sure you have a clear aim for your breeding program, carefully plan your screening program and openly discuss it.
The Screening Program
There are a number of phases to the bacterial wilt screening program. Firstly, collection and maintenance. Particular attention should be paid to the collection of a good representation of samples and characterizing them using the differential hosts, not just of tomato but also of pepper and eggplant. This is best done by leaf cutting. While it might be seem advantageous to screen a tomato variety with the an isolate collected in the variety’s geographical market, this is misleading for a number of reasons. Firstly, in the future the geographical market for that variety might change. Secondly, the farming practices employed by all farmers differ as too the soil conditions, weather conditions etc. It is difficult to translate regional or local conditions by screening with a locally obtained isolate when in one or two years time farming practices have selected more new isolates and the original isolate no longer reflects those now populating the soils of the region. Rather, breeding programs should select the level of resistance – high, moderate, or low. This reflects markets rather than regions and provides for more flexibility in breeding programs. Similarly, this also applies to breeding for markets in other countries within Asia. One exception could be Indonesia where the phylotype is, on current information, significantly different. Definitely, *Ralstonia* isolates in Indonesia are generally more pathogenic than in the rest of Asia. Again, the level of resistance should be selected and not the country. Careful planning in characterizing local isolates and comparing their virulence, based on standard differential hosts, should be done whilst in the breeding program planning stage. Selecting germplasm by screening with one isolate collected from one field in another country is negligent; it is ignoring the facts of the true nature of bacterial wilt resistance as you might in fact be screening for low level resistance when in fact you need high resistance. Finding out that you have selected poorly three to five years into the program has major financial consequences both in costs of the R&D program but more importantly, loss of potential sales and market share. Screening in Thailand using isolates collected in other countries could result in the release of an isolate that could adapt to local conditions and result in major disease outbreaks, destroying the local market that you have bred for. This is particularly the case for Indonesian isolates. It is also environmental damaging, least of all its illegal!!! Consequences to the company, both legally and in damage to its reputation (the latter being more important) could prove insurmountable.

The level of resistance should also be selected; low, moderate or high. If a moderate or low level of resistance is required additional considerations such as rate of infection and inoculating with multiple isolates may be necessary.

The speed at which the program needs to be conducted also needs consideration. For example; if the population in question needs to be transplanted and seed collected, then the results may need to be finalized before day 28. This also applies to multiple disease screening programs. If you needed to screen initially for bacterial wilt and then for TYLCV, you may need to complete the bacterial wilt program by day 21 to facilitate TYLCV screening. In planning the program these factors play a major role particularly for bacterial wilt where resistance to the mode of infection varies.

Finally, weather conditions during the screening program impact greatly on the results. For example; programs conducted during the winter period might result in an 80% death rate in the susceptible check. This would also be reflected in your screened population where 20% might in fact not be resistant. The same also holds for programs conducted in
very hot conditions. Remember, the optimum conditions for bacterial wilt infection are soil temperatures of around 30°C.

So, in planning the program, take into account the weather conditions, the speed at which the program needs to be completed, the level of resistance or tolerance required by the market and, if necessary, farming practices. In interpreting the results, take into account the results of the resistant, intermediate and susceptible checks. Don’t be hasty in declaring the program a success. Repeating a program, just to be sure, reduces the mistake of introducing a new variety to market only to find it is not resistant!!! It is better to spend an extra 10,000 baht now than waste millions of baht years down the line.
2. Breeding Summary

Bacterial wilt is a major disease of tomato and is caused by the bacterium *Ralstonia solanacearum*. The host range of *R. solanacearum* is extensive and includes other solanaceous crops as well as cucurbits, banana and weeds. However, not all isolates of *R. solanacearum* infect tomato. Further, some isolates infect tomato but cause mild symptoms while others cause severe symptoms.

Resistance to *R. solanacearum* in tomato is very complex with at least five known genes. Generally speaking resistance appears to segregate with lower quality fruit. Varieties with high levels of resistance to *R. solanacearum* tend to be resistant to all tomato isolates of the bacterium. Similarly, varieties highly susceptible to *R. solanacearum* tend to be susceptible to all tomato isolates of the bacteria. However, the resistance of “moderately resistant” tomato varieties to *R. solanacearum* varies between bacterial isolates. Further, the level of resistance of all varieties is dependant on the weather conditions; hot wet conditions favour the development of the disease while under cooler, drier conditions even normally susceptible varieties show some tolerance to the bacteria. But this is not resistance, it only reflects the level or concentration of bacteria in the soil. This concentration (or amount) of bacteria in the soil is determined not only by the weather conditions but also soil type. Sandy soils or those with high pH or low nutrient content tend to be less favourable to the development of *R. solanacearum*, but at the same time are less favourable to tomato cultivation.

<table>
<thead>
<tr>
<th>Disease</th>
<th>Bacterial Wilt (<em>Ralstonia solanacearum</em>)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetics</td>
<td>Multiple genes. Status: recessive but not fully understood.</td>
</tr>
<tr>
<td>Source of resistance</td>
<td>Various</td>
</tr>
<tr>
<td>Resistance required in parents</td>
<td>High resistance: tolerance required in both parents.</td>
</tr>
<tr>
<td>Screening program</td>
<td>Available</td>
</tr>
<tr>
<td>Programs</td>
<td>Seedling inoculation by leaf cutting.</td>
</tr>
<tr>
<td>Markers</td>
<td>Some available¹</td>
</tr>
<tr>
<td>Double Haploids</td>
<td>None available</td>
</tr>
</tbody>
</table>

1. Since the original writing of this protocol more reliable markers have become available. As of writing this protocol, none were available, and the mapping of the resistance traits had not been published.
So, in planning a breeding program the important things to note:

1. If you need a high level of resistance, both parents need to have high levels of resistance to the bacteria.
2. The genetics of resistance is complex involving more than 5 genes.
3. Screen for the desired level of resistance (low, moderate or high) under optimal conditions for bacterial infection, not for an Asian geographical area or market (perhaps with the exception of Indonesia). Isolates in given geographical areas will vary over time depending on what crops are grown in those areas.
4. Screening in Thailand using isolates from other countries is a major farming risk and could cause problems for other breeding programs. Apart from that, it is also illegal and if publicly know would severely damage the good reputation of Chia Tai.
5. Include all controls (S-, MS- and R-) whether you are screening in the screenhouse or in the field.
6. Always rate the resistance level of your lines based on the resistance level of the controls included in the trial.
7. If the results are unclear, repeat the trial rather than “hoping everything will be OK”.

Table 2. Summary of screening methods. Standard method is “Cut Leaf”.

<table>
<thead>
<tr>
<th>Screening method</th>
<th>Date of inoculation (age of seedling)</th>
<th>Result (age of seedling)</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Drenching</td>
<td>Day 14</td>
<td>Day 35</td>
<td>Consistent result. Reflects true field resistance</td>
</tr>
<tr>
<td>Root damage and drenching</td>
<td>Day 14</td>
<td>Day 35</td>
<td>Generally consistent.</td>
</tr>
<tr>
<td>Root dipping</td>
<td>Day 14</td>
<td>Day 28</td>
<td>Stresses the plant. Inconsistent results.</td>
</tr>
<tr>
<td>Cut leaf</td>
<td>4 – 5 leaf stage (2 cotyledon, 2 – 3 leaves) Normally day 21 in summer.</td>
<td>Day 28</td>
<td>Very quick and results obtained within 7 days. Some lines that may be resistant in the field under normal conditions may be susceptible by leaf cutting.</td>
</tr>
</tbody>
</table>

**Optimum screening location (screenhouse)**
- North and north-eastern Thailand (screenhouse or fixed outside location)

**Optimum period of year for program**
- All year round in north and north-eastern Thailand
- The central plains of Thailand, November to March is best but it can also be done all year round

Screening Protocol for Bacterial Wilt Tolerance in Tomato (Eggplant and Pepper)
**Isolates**
Consult your “Isolate’s Book”
(Re-isolate and characterise every 9 months)

**Controls**

**Resistant Check:** Permata (EW Indonesia – F1)
Hawaii 7998 (AVRDC)

**Intermediate Check:**
Moderate Resistant: Tawan (F1, Thailand)
Moderate Susceptible: Donna (CT – F1)

**Susceptible Check:** L390 (AVRDC), Mission, Sida
3. Collection storage and maintenance

a. Isolation of *Ralstonia solanacearum* from field samples

**Identifying symptoms in the field**

A. Wilting is first observed on the uppermost shoot and youngest leaves. This is best observed during the hottest part of the day and is a result of damage to the phloem by the invading *Ralstonia*. The plant still appears green and otherwise healthy.

B. Vascular browning of the bundle sheath (discolouration).

*Note: Some soil borne fungal pathogens, such as Fusarium and Sclerotium, and nematodes may also cause vascular discolouration.*

C. In some tomato cultivars, dark brown or black strips along the stem (also referred to as brown water soaking) and hollowed stems (pith necrosis) can be observed.

*Note: Pseudomonas corrugata also produces pith necrosis in tomato. The two bacteria can be distinguished by *P. corrugata*’s ability to cause rot in onion.*
Figure 1. The typical wilt symptoms of bacterial wilt can often be easily seen at a distance in a field (A). They are easily distinguished from those of *Fusarium* in that the leaves of bacterial wilt infected plants remain green, or in the case of older plants, appear dead. Generally, no leaf yellowing is observed. Closer observation of the plant reveals vascular browning (B), brown water soaking (C), pith necrosis (C) and ooze (D).

Sample collection in the field

1. At least 5 wilted plants should be randomly collected from each field and more than one location (farmer) should be sampled. Cut the stem at approximately 10 cm above the collar level and take the portion with roots attached.

2. If you are not returning to the lab within 24 hours, check for bacterial streaming (bacterial ooze).

3. Store plant samples accordingly:

   3.1 If the samples will be returned to the laboratory within 24 hours, store the cut stem samples in plastic bags in an ice box with ice. The plastic bag should be sealed and marked with a sample number, details of which must be recorded in your field notebook. Details should include the date and time collected, plant variety, sample symptoms, weather conditions on collection and since the crop was planted, conditions of the soil (heavy, light, sandy, clay, wet, dry) and conditions of the other plants/crops growing nearby (e.g. wilt symptoms, variety, others crops including pepper and eggplant which may or may not also be infected). Details are important as they are indicative of the virulence and host range of the isolate(s).

   3.2 If the samples will not be returned to the laboratory within the next 24 hours, the samples should be prepared accordingly. Carefully wash the...
stem samples with clean water to remove the soil. Finally wash the stem samples with 70% ethanol and allow to dry. Using a clean knife or cutter (must have been wiped with 70% ethanol and dried), first remove the top 2 cm of the stem (probably dry now) and cut the stem into 2 – 3 cm lengths. Place the cut stem sections into plastic tube (normally 50 or 15 ml falcon tubes, if the stem sections are thin, use microfuge tubes). Place the tubes into sealable plastic bags (best to use zip lock plastic bags) and store the sample on ice. Each tube should be labeled with a sample number and details recorded as mentioned above.

**Processing samples in the lab**

**Isolation of *Ralstonia***

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

2. Rinse with ddH$_2$O and air dry with clean paper towel.

**NB:** ddH$_2$O refers to double distilled water which 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 air-dry on clean paper towel.

4. Using a sterilized sharp knife or blade (dip in 95% ethanol and flame and let it cool before using) remove both ends of the stem (Figure 1) and dispose of them in the waste. Cut the remaining stem into 2 – 3 cm in lengths.

5. Spilt the stem sections vertically using a sterilized knife or blade and place them into sterile glass test tubes containing approximately 2 - 3 ml of sterile ddH$_2$O water. The milky suspension (bacterial ooze) should be streaming from the vascular vessels. Presence of vascular streaming should not be used as an absolute guide to the presence of *Ralstonia solanacearum*.

6. Allow the samples to stand for 5 - 10 minutes at room temperature (RT). During this period, the water should become more turbid. The level of turbidity will vary depending on the degree of infection. Often, “bacterial ooze” will be observed in a young freshly collected tomato sample within a few minutes after cutting. If this is the case, proceed immediately to step 7.2.
Figure 2. Ooze Test.

1. Using a sterilized sharp knife or blade (in the lab, dip in 95% ethanol, flame and let it cool before using, in the field use a clean knife or cutter wiped with 75% ethanol) remove both ends of the sample.
2. Using the remaining sample, cut 2 – 3 cm pieces and place them into test tubes containing approximately 2 - 3 ml of sterile water. The stem section should be immersed just below the top of stem. In the field, the cut stems can be placed in a glass of clean water.
3. Allow the stems to stand for approximately 10-15 min. Ooze should be apparent.
Using the laminar flow, flame a loop to red-hot and cool it in air for few seconds or by touching a TZC plate (Kelman’s tetrazolium chloride medium is used as the isolated medium). Isolation of Ralstonia can either be from bacterial ooze collected using the ooze test (7.1) or directly from cut stems (7.2).

7.1 Using a **bacterial ooze suspension** (released from the stem), streak one loop-full of the ooze solution on TZC (as shown on Figure 4) in order to get single colonies.

7.2 Using a **cut stem with white, creamy, sticky bacterial ooze**, streak a loop-full of the ooze on TZC (as shown on Figure 4).

8. Incubate the plates at 30 - 32°C for 48 hours (2 days). While plant pathogenic bacteria can grow on the media within 24 hours, they generally require more than 24 hours to adapt to the medium and form their distinctive colonies.

9. On TZC, *R. solanacearum* produces slimy, white colonies with pink or red centers (Figure 3). There are variations in the color and size of the centers among the population of the bacteria even though they are all *R. solanacearum*. Variations in the size, colour and shape tend to indicate whether the colonies are pathogenic or non-pathogenic. Colonies that are neither slimy, white, have pink centers or regular shaped, tend to be non-pathogenic (Figure 3).

![Figure 3](image)

**Figure 3.** Typical pathogenic *R. solanacearum* colonies: irregular shaped, slimy, white colonies with pink or red centers.
10. Select three to five single colonies, which are fluidal (slime), round or irregular in shape, and pink to light red centers with white edges. Mark the position of the selected colonies on petri dish with a pen or any marker available in each lab. **Small or large colonies that are regular, round, smooth edged with dark red centers ARE NOT pathogenic.**

11. Streak each colony individually on fresh TZC according to the patterns shown in Figure 5 (top) and incubate the bacteria at 30 - 32°C for 48 hours. Multiple colonies can be streaked on sectioned plates to save on time and plates (Figure 5).

12. The colonies morphology appeared on the medium should be homogeneous which indicate purity of the bacteria. If more than one type of the colony is observed, repeat step 10 on a fresh TZC plate. The bacterium from each sample is designated as an “isolate”.

**Figure 4.** Typical non-pathogenic *R. solanacearum* colonies: regular shaped, defined red or dark red centers with no white borders or slim.
Figure 5. Streak plate technique.

1. Flame the loop to red hot and cool on a TZC plate.
2. Using the loop, touch the ooze, colonies, or dip the loop in the bacterial suspension and streak on the media surface as in pattern A.
3. Re-flame the loop, cool and streak with pattern B.
4. Repeat step 3, and streak with pattern C.
5. Label the plate accordingly – date, media, isolate.
Isolate Characterization

In order to confirm the pathogenicity of each isolate, it is necessary to inoculate the differential hosts. While the isolate may have had the characteristic “pathogenic” shape and colour on a TZC plate, it may not be pathogenic. Use the pathogenicity protocol in section 3b. below and use the control checks for tomato. Control checks for eggplant and pepper should also be included to determine the host range (NB. For 2008, don’t inoculate other species as we are still in the development stages.)

After the isolate has been characterized store the bacterium in 20% glycerol (see method below) and record the isolate details in the isolate record book.

b. Pathogenicity protocol

A pathogenicity test is required for all bacterial strains isolated from wilted plant samples to ensure the bacteria is a causal agent of wilting and has maintained its virulence. The test should be done as soon as the bacterium is purified and prior to storage.

Preparation of Inoculum

A. If the bacterium was cultured on TZC:

1. Use a loop (flame to red hot and cool it down prior use) or a scalpel and pick a 48 hour single colony (4 - 5 mm in diameter) and put it into a test tube contains 5 ml of sterile ddH₂O (pH 7.0).
2. Repeat for at least three single colonies (select at random).
3. Mix the bacteria very well by using a vortex.
4. Concentration of the inoculum prepared from single colony will be approximately $10^6 – 10^8$ CFU per ml.

B. If the bacterium had been stored in glycerol:

1. Using a sterilized loop, scratch the iced surface of the bacterial suspension from the tube and streak a fresh TZC plate and incubate at 30 - 32°C for 48 hours.
2. Use a loop (flame to red hot and cool it down prior use) or a scalpel and pick a 48 hour single colony (4 - 5 mm in diameter) and put it into a test tube contains 5 ml of sterile ddH₂O (pH 7.0).
3. Mix the bacteria very well by using a vortex.
4. Concentration of the inoculum prepared from single colony will be approximately $10^6 – 10^8$ CFU per ml.
Inoculation

There are various inoculation techniques available (stem puncture, soil drenching, leaf clipping, root dipping etc.). However, the aim of this test is to determine pathogenicity of the bacterium in the host plants. The technique should be fast and reliable, therefore, leaf cutting is preferred, or at least this modified leaf cutting technique. Three colonies of each strain need to be tested separately and three plants should be used per colony. Therefore, for each colony three susceptible checks (L390), three intermediate checks (L180) and three resistant checks (H7996) each being 21 days old (days after sowing), should be used.

[Note: Some researchers just use a single plant and only the susceptible check. This is not advised as errors in inoculating do occur which could result in an incorrect result. It is also strongly advised that the checks for other hosts (eggplant, pepper, cucumber, bittergourd and pumpkin).

1. Using a sharp pair of scissors, cut off the three lowest leaves at their base.
2. Dip a sterile toothpick in the bacterial suspension (described above).
3. Ensuring that the tip has a drop of suspension at its end, inoculate each freshly cut stem in turn. Each drop should be approximately 10 µl of bacterial suspension.
4. Ensure that one un-inoculated plant of each control is maintained.
5. Maintain the plants in the screenhouse normally.
6. Wilting should occur 2 - 5 days after inoculation.
7. Record the result at day 3 and day 5.

c. Long-term storage for *Ralstonia solanacearum*

Storage from colonies on TZA or NA plates (Preferred method)

*Why preferred? There has been some debate over the use of phosphate buffered based media and its affect on growing Ralstonia to maintain its pathogenicity. In fact, even for inoculations some researchers will not use NBY. But I haven’t seen any direct evidence that it causes problems.*

1. Streak plate the bacteria on fresh TZC medium.
2. Incubate the culture at 32°C for 48 hours. The colonies should be approximately 3 - 4 mm in diameter. On TZC, the colonies are irregular round with red pink centers and slimy appearance (this is an indicator of pathogenicity). All colonies must be homogeneous.
3. Prepare a cryovial or similar screw capped eppendorf tube (1.5 or 2 ml) containing 375 µl of sterile 80% glycerol and 400 µl sterile ddH₂O.
4. In the laminar flow, transfer three loop-fulls of the bacterium, by randomly scraping colonies from the TZC media, into cryovials containing the prepared glycerol solution. **Make three copies of each isolate.**

5. Vortex until the suspension is completely mixed.

6. Label the tube with the appropriate isolate code and date. Store at –20°C (better at -80°C but we don’t have one).

7. Check the survival efficiency of the isolate every 9 - 12 months by streaking the culture on a TZC plate. Ensure that the colonies are fluidal, irregular in shape with small pink centers and white edges (this is an indicator of the pathogenicity).

8. Re-isolate each isolate by inoculating on the susceptible host and place under long-term storage.

**Storage from an overnight liquid culture, NBY (Should only be used if no plate culture is available)**

1. Inoculate by streak plate pathogenic isolates of *Ralstonia solanacearum* on TZC medium.

2. Grow overnight at 30°C until the colony is approximately 2 –3 mm in diameter. Ensure that the shape of the colony is irregular and pink in colony with a white ooze appearance (this is an indicator of the pathogenicity).

3. In the laminar flow, transfer a loop-full of the bacteria to 10 ml of sterile NBY in a 125 ml flask (or into a test tube or flask with a diameter of at least 20 mm).

4. Incubate the culture overnight (normally 16 to 18 hours) by shaking at 120 rpm and 32°C.

5. The following morning check the solution. The culture should be cloudy; concentration between $10^{10}$ and $10^{12}$ CFU/ml.

6. Prepare a cryovial or similar screw capped eppendorf tube (1.5 or 2 ml) containing 375 µl of sterile 80% glycerol. **Make three copies of each isolate.**

7. To this add 625 µl of the NBY bacterial suspension (the final glycerol concentration is 30%). Vortex until the suspension is completely mixed.

8. Label the tube with the appropriate isolate code and date. Store at –20°C.

9. Check the survival efficiency of the isolate every 9 - 12 months by streaking the culture on a TZC plate. Ensure that the colonies are fluidal, irregular in shape with small pink centers and white edges (this is an indicator of the pathogenicity).
10. Re-isolate each isolate by inoculating on the susceptible host and place under long-term storage.

**d. Propagation of *Ralstonia solanacearum* for inoculation**

**Using NBY liquid medium (Preferred)**

For multiplication

1. Inoculate by streak plate pathogenic isolates of *Ralstonia solanacearum* on TZC medium.

2. Grow overnight at 30°C until the colony is approximately 2 –3 mm in diameter. Ensure that the shape of the colony is irregular and pink in colony with a white ooze appearance (this is an indicator of the pathogenicity).

3. In the laminar flow, transfer a loop-full of the bacteria to 10 ml of sterile NBY in a 125 ml flask (or into a test tube or flask with a diameter of at least 20 mm).

4. Incubate the culture overnight (normally 16 to 18 hours) by shaking at 120 rpm and 32°C.

5. The following morning, check the solution. The culture should be cloudy; concentration between $10^{10}$ and $10^{12}$ CFU/ml.

6. Prepare a sterile cryovial or similar sterile screw capped eppendorf tube (1.5 or 2 ml) containing 375 µl of sterile 80% glycerol. **Make three copies of each isolate.**

7. To this add 625 µl of the NBY bacterial suspension (the final glycerol concentration is 30%). Vortex until the suspension is completely mixed.

8. Label the tube with the appropriate isolate code and date. Store at –20°C.

9. Record entry in the isolate book.

10. Check the survival efficiency of the isolate every 9 - 12 months by streaking the culture on a TZC plate. Ensure that the colonies are fluidal, irregular in shape with small pink centres and white edges (this is an indicator of the pathogenicity).

11. Re-isolate each isolate by inoculating on the susceptible host and place under long-term storage.
For inoculation

1. Inoculate by streak plate pathogenic isolates of *Ralstonia solanacearum* on TZC medium.
2. Grow overnight at 30°C until the colony is approximately 2–3 mm in diameter. Ensure that the shape of the colony is irregular and pink in colony with a white ooze appearance (this is an indicator of the pathogenicity).
3. In the laminar flow, transfer a loop-full of the bacteria to 10 ml of sterile NBY in a 125 ml flask (or into a test tube or flask with a diameter of at least 20 mm).
4. Incubate the culture overnight (normally 16 to 18 hours) by shaking at 120 rpm and 32°C.
5. The following morning check the solution. The culture should be cloudy; concentration between $10^{10}$ and $10^{12}$ CFU/ml.
6. Prepare the spectrophotometer by “zeroing” it (setting the instrument to zero). To 700 µl of ddH$_2$O, add 300 µl NBY. Pipette the solution into the cuvette, mix and insert into the spectrophotometer. Set the reading to zero.
7. Check the bacterial concentration using spectrophotometer and determine absorbance (OD) at 600 nm.
   a. **Spectrophotometer:** To 700 µl of ddH$_2$O, add 300 µl of culture. Mix thoroughly. Pipette the solution into the cuvette, mix and insert into the spectrophotometer. Record the reading.
   b. **ELISA plate reader:** To 1540 µl of ddH$_2$O, add 660 µl of culture. Mix thoroughly. Pipette 400 µl of the solution into five randomly located wells on the ELISA plate. Insert into the plate reader. Record the reading.
8. Dilute the bacterial suspension to OD = 0.2 (approximately $2 \times 10^6$ CFU/ml) using ddH$_2$O. **(NB. There seems to be some discrepancy over the concentration and OD. AVRDC use OD$_{600}$ = 0.2, believing that it is $2 \times 10^6$ CFU/ml. The important thing here is to use the OD$_{600}$ which gives the result we need and for tomato this is OD$_{600}$ = 0.2.) If a lower level of resistance is required, a lower OD$_{600}$ could be used but should always be evaluated in relation to the controls.**

   **Further note:** When using the ELISA plate reader, record the readings using 400 µl aliquots. Using less, such as 100 µl, gives a lower absorbance and as such the calculated concentration is higher.
9. The culture is now ready for inoculation.
Using NBY agar (NOT preferred)

For multiplication

1. Subculture the bacteria from –20° or –80° C by streaking on TZC plate and incubate the culture at 30 - 32° C for 48 hr.

2. Select three single colonies, which are fluidal and have red pink centers, by using a loop touch all three colonies and re-streak on NBY agar with single streak. Incubation overnight at 30 - 32 ° C.

3. To each plate, add 1 ml of sterile ddH2O. Flame a glass rod soaked with 95% ethanol. Allow the glass rod to cool before spreading the colonies. Transfer the solution to a clean tube using aseptic techniques.

4. Transfer 100 µl bacterial suspension to fresh NBY agar and spread using a sterile glass rod. Incubate overnight at 30 - 32 ° C.

For inoculation

1. To each spread-plate of bacteria, add 5 ml of sterile ddH2O. Flame a glass rod soaked with 95% ethanol. Allow the glass rod to cool before scrapping the colonies. Transfer the bacterial suspension to a clean beaker.

2. Check the bacterial concentration using a spectrophotometer by determining absorbance (OD) at 600 nm. Dilute the bacterial suspension to OD_{600} 0.2 (approximately 2x10⁶ CFU/ml) using ddH₂O. (NB. There seems to be some discrepancy over the concentration and OD. For E.coli, an OD_{600} of 0.2 is 2x10⁶ CFU/ml. AVRDC use OD_{600} = 0.2, believing that it is 2x10⁶ CFU/ml. The important thing here is to use the OD_{600} which gives the result we need and for tomato this is OD_{600} = 0.2.)

3. The culture is now ready for inoculation.
Preparation

Prepare proposal after discussions with breeder

Copy proposal to database

Sow seeds (including controls inoculating)

Prepare inoculum

Inoculate TZC plate

Select colony and inoculate broth culture

Check concentration

Prepare inoculum (maintain on ice)

Inoculate plants (5 leaf stage)

Next day inspect plants for signs of stress (wilt). Ensure that plants are watered.

Collect data and discuss with breeder

Prepare report; publish and copy to breeder

Evaluation and report

Screening Flowchart
4. Screening protocol

Inoculation of tomato lines in the screenhouse with *Ralstonia solanacearum*

**Seedling preparation**
Tomato seeds should be sown in plastic trays or two-inch plastic pots 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.

If the seedlings need to be transplanted from trays to two-inch plastic pots, this should be done 12 days after sowing (das). Seedlings should have the first true leaves however, this depends on the individual lines and weather conditions.

Before inoculation, transfer all plants to the bacterial wilt screening area. (NB. Later in 2008 we may change to using a potting mix containing a 1:1 ratio of Pinstrup and sand. This works well from experience and also reduces the cost of the program.)

**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 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 BE WATERED** 30 minutes to 1 hour before inoculation in addition to any normal watering in the morning. Inoculations should be done, where possible, in the afternoon. Sometimes, when large numbers of plants are to be inoculated, the program **SHOULD** be done over more than one day. Having large numbers of people inoculating the plants only increases the potential problems. At the completion of the inoculations, the plants **MUST NOT BE WATERED UNTIL THE FOLLOWING MORNING OR FOR AT LEAST FOUR HOURS.**

**Experimental design**
The inoculations should be divided into three replications, each replication being at five plants. **Five plants should the “technique” control - the two cotyledons and one true leaf should be cut but the scissors MUST be free of inoculum (swab with 75% alcohol and dry before cutting)**. This is a total of twenty plants. This also includes the controls.

**Controls**
See breeding summary on page 9.
**Materials required**

1. Beaker containing bacterial suspension;
2. Cup for pouring the suspension;
3. Marker pens;
4. Wash bottle containing 70% ethanol for swabbing;
5. Paper towels, plastic tags, gloves, large washing tub for washing the used containers overnight with 1% final sodium hypochlorite;
6. Fan to keep cool;
7. Red 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).

**Inoculation (Leaf clipping)**

Inoculation is done at the five-leaf stage – two cotyledons, three true leaves (approximately 21 DAS). The inoculation should be planned on the stage of the plant and not the period as the period will vary from season to season and from line to line.

1. The bacterial suspension should be 2x10^6 CFU (OD\textsubscript{600} 0.2).

2. Check that all pots are correctly labelled with a white tag indicating the line and the date the seed was sown (eg. “Line” 16-12-01 indicates by day-month-year);

3. Check all plants and make sure that all are approximately the same height and conditions without damping off or other soil-borne diseases. All plants should have five leaves.

4. Using a sharp pair of scissors. Dip the scissors into the bacterial suspension gently mixing it. Now cut the lowest three leaves of the tomato plant (include one true leaf).

5. Repeat for each individual plant. **REMEMBER, THE SCISSORS NEED TO BE DIPPED IN THE INOCULUM, AND STIRRED, BEFORE EACH LEAF IS CUT.**

6. Replace the inoculum after each bench row (approximately 300 plants).

7. Check that all pots are correctly labelled with a red tag indicating the line and the date the seeds was sown and a red tag with the date of inoculation and bacterial isolate.

8. Record details in the “Screening Request Form” and in your workbook.

**NB: The next morning, ensure that the plants receive their scheduled watering.**

**If symptoms of wilt, ensure that seedlings have been watered.**
Figure 3. Labelling of tags.

1. White tags (or silver coloured plastic tape) should be labelled with the line number and the date of sowing.
2. Red tags should be labelled with the line number, date of inoculation and the bacterial isolate number.
3. Temporary white paper tags with string should be labelled with the line number and the bacterial isolate number. These should only be used if no plastic tags are available.
5. Evaluation
a. Evaluation of tomato lines inoculated with *Ralstonia solanacearum*

1. Check all seedlings after 24 hours. If symptoms of wilt, ensure that seedlings have been watered. **Wilting due to bacteria does not occur within 24 hours!!!**

2. Check the seedlings for symptoms of wilt daily. Record any lines that wilt.

3. Once the susceptible checks have wilted and started to day (usually seven days after inoculation) record data on all lines (see evaluation sheet). Results should be recorded as:

   Disease scale according to the rating system of Winstead and Kelman.
   
   0 = no symptoms (Healthy)
   1 = one leaf wilted
   2 = two to three leaves wilted
   3 = four or more leaves wilted
   4 = whole plant wilting and dying
   5 = plant dead

   **The formula below can be used as a guide but for the moment we are not including it in reports – maybe in the future!!**

   Then calculate the bacterial wilt intensity using the following formula:

   \[ I = \left[ \frac{\Sigma (n_i \times v_i)}{(V \times N)} \right] \times 100 \]

   Where  \( I = \) wilt intensity (%); \( n_i = \) number of plants with respective disease rating; \( v_i = \) disease rating (0, 1, 2, 3, or 4 ); \( V = \) the highest disease rating (4); and \( N = \) the number of plants observed. \( \Sigma \) is the sum of. For an example calculation, see appendix.
### b. *Ralstonia solanacearum* isolates and inoculation controls

**Controls:**
Performance of controls under glasshouse conditions inoculated by soil drenching and leaf cutting. Variation between hot and cool season inoculation vary by 20% when using soil inoculation.

*NB. Glasshouse conditions in the central plains exceed 40°C for more than 4 hours and as such are not suitable for soil drenching.*

Controls – Thailand (Chiang Mai)

<table>
<thead>
<tr>
<th>Rating</th>
<th>Line</th>
<th>Soil Drench</th>
<th>Cut leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>% Healthy</td>
<td>% Healthy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hot Season</td>
<td>Cool Season</td>
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<tr>
<td>S</td>
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</tr>
<tr>
<td>MS</td>
<td>Tested line</td>
<td>40%</td>
<td>60%</td>
</tr>
<tr>
<td>MR</td>
<td>Tested line</td>
<td>60%</td>
<td>80%</td>
</tr>
<tr>
<td>R</td>
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<td>100%</td>
</tr>
<tr>
<td>HR</td>
<td>Permata</td>
<td>95%</td>
<td>100%</td>
</tr>
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</table>

Controls – Thailand (central plains)*

<table>
<thead>
<tr>
<th>Rating</th>
<th>Line</th>
<th>Soil Drench</th>
<th>Cut leaf</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>% Healthy</td>
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<tr>
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<td>HR</td>
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<td>105%</td>
<td>100%</td>
</tr>
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</table>
6. **Disposal**

**Disposal of tomato lines and soil inoculated with *Ralstonia solanacearum***

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.

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.
Appendix

Culture Media

80% glycerol

Glycerol is used as cryogen to preserve living cells. Therefore the quality of the glycerol, in terms of purity, is a crucial importance. Only use analytical grade (AR) glycerol. This is usually available either as 100% or 80%. Prepare the stock solution in smaller aliquots (20 - 50 ml) first before autoclaving.

Autoclave at 120° C for 15 minutes.

Nutrient Broth Yeast Extract (NBY) (per litre)

Beef extract 3.0 g
Peptone 5.0 g
Yeast extract 2.0 g
K₂HPO₄ 2.0 g
KH₂PO₄ 0.5 g
Glucose 15 g

After autoclaving, add 1.0 ml filter sterile 1M MgSO₄.7H₂O

TZC medium

Peptone 10 g
Casein hydrolysate 1.0 g
Glucose 5 g
Agar 15 g

Add to 900 ml dH₂O and make up to 1000ml. Autoclave at 120° C for 15 minutes.

After autoclaving, add 5 ml of 1% 2,3,5-triphenyltetrazolium chloride (TTC) to 1 litre (final concentration of TTC is 0.005%)

NB. This should not be stored and reheated. If you are using small numbers of plates, aliquot sterile TZC into single use amounts (1.5 ml eppendorf tubes). From storage, warm TZC in your hand or warm water and add the required amount (750 µl per 150 ml of TZC).

1% 2,3,5-triphenyltetrazolium chloride (TTC)

Prepare 100 ml of 1% TTC (white powder in a dark container, stored at 4°C) in ddH₂O. Sterilize by using a 0.2 µ filter. (DO NOT AUTOCLAVE). Store the solution at 4°C in sterile 1.5 ml tubes wrapped with aluminum foil to protect it from UV light.

Screening Protocol for Bacterial Wilt Tolerance in Tomato (Eggplant and Pepper)
### Evaluation Example

Table 1. Scoring of inoculation.

<table>
<thead>
<tr>
<th>Line</th>
<th>Rep 1</th>
<th>Rep 2</th>
<th>Rep 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-check</td>
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<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>MS-check</td>
<td>3</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>R-check</td>
<td>5</td>
<td>4</td>
<td>3</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2. Calculation of disease rating. Rating is always based on the result of the S-, MS- and R-checks.

<table>
<thead>
<tr>
<th>Line</th>
<th>Formula ( I = \frac{\sum(n_i x v_i)}{(V x N)} )</th>
<th>Result</th>
<th>Rating based on check</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-check</td>
<td>([51 + 42] x 100)</td>
<td>9300</td>
<td>(+5100) S</td>
</tr>
<tr>
<td>MS-check</td>
<td>([29 + 9] x 100)</td>
<td>3800</td>
<td>(2351 – 5100) MS</td>
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<tr>
<td>R-check</td>
<td>([3 + 3] x 100)</td>
<td>900</td>
<td>(0 – 2350) R</td>
</tr>
</tbody>
</table>

Example: Line 7
\[\text{[Sum } ((0 x 0)+(1 x 0)+(2 x 3)+(3 x 4)+(4 x 8)) + (4 x 8)] \times 100 = [50 + 32] \times 100 = 8200\]
# Bacteria Screening Data Recording Sheet

<table>
<thead>
<tr>
<th>Date of Recording:</th>
<th>Crop:</th>
<th>Date seed sown:</th>
<th>Date of inoculation:</th>
<th>Isolate used:</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Line</th>
<th>Rep 1</th>
<th>Rep 2</th>
<th>Rep 3</th>
<th>Total</th>
<th>Comments</th>
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<tbody>
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<td>1</td>
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Screening Protocol for Bacterial Wilt Tolerance in Tomato (Eggplant and Pepper)

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