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A Method of Determining the Susceptibility of a Plant to a Bacterial Disease

Hyuliya Letifova Kalkanova[1]*

[1]*Research in Floriculture, Brivresearch Centre, Netherlands*

Abstract

A method of determining the susceptibility of a plant to a bacterial disease, said method comprising the step of infecting plant material of the plant with bacteria of a bacterial species specific for a plant disease. To do this, the method comprises the steps of obtaining a leaf sample from the plant, infecting the leaf sample obtained from the plant with the bacteria of the bacterial species specific for the plant disease, maintaining the leaf sample alive, and determining bacterial growth by detecting a biopolymer produced by the bacteria. Powered by TCPDF (www.tcpdf.org).

Introduction

The present invention relates to a method of determining the susceptibility of a plant to a bacterial disease, said method comprising the step of infecting plant material of the plant with bacteria of a bacterial species specific for a plant disease. Plant diseases may have various causes, such as malnutrition, viral infection or bacterial infection. With respect to the latter, causative (etiological) bacteria may infect a plant by invading it via the stomata, the hydratodes or an injury (damage) and multiply there. A plant carrying the bacteria has reduced commercial value or is even a loss. It may not be allowed to export the plant. If it is desired to create plants that are resistant to the bacterial disease, a plant that is relatively susceptible to the bacterial infection is obviously not suitable as starting material. If a plant, or plant material used for testing, develops symptoms of the disease, it is obviously infected. However, if the symptoms do not develop, then the plant may be a carrier or it may be resistant. In the former case, the plant is tolerant but still not fit for export and/or breeding and/or propagation. The object of the present invention is to provide a method capable of grading a plant with respect to its capability to resist bacterial infection.

To this end, a method according to the preamble is characterized in that the method comprises the steps of obtaining a leaf sample from the plant, infecting the leaf sample obtained from the plant with the bacteria of the bacterial species specific for the plant disease, maintaining the leaf sample alive and, determining bacterial growth by detecting a biopolymer produced by the bacteria.

Instead of relying on the visual appearance such as a change in morphological change or color of the plant material, bacterial growth itself is determined. If there is no bacterial growth, the plant is fully resistant. If there is bacterial growth despite the absence of a change in visual appearance, the plant is tolerant.

Bacterial growth can be determined using any suitable method, such as fluorescence in case of genetically modified bacteria. More fluorescence indicates more bacteria. Bacteria causing the plant disease can be

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***Corresponding author:** Dr. Hyuliya Letifova Kalkanova, Research in Floriculture, Brivresearch Centre, Netherlands. Tel: +310616574637; Email: hlkalkanova@gmail.com

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made fluorescent by introducing a gene for a fluorescent protein such as GFP using routine procedures. To this end, the bacterial species may be genetically engineered by introducing a plasmid carrying the gene for the fluorescent protein. In general, there will also be a gene coding for an antibiotic resistance, to easily select successfully engineered bacteria.

For routine measurements for a particular combination of plant species and pathogen, there is not necessarily a need for the use of a control (blank) because the typical signal level representative of the biopolymer detected will be known.

The duration of the incubation of the plant sample is in general many days, such as one to four weeks. To reduce the risk that the leaf sample perishes due to attack by a different bacterial species or by fungi, the leaf sample is preferably sterile before inoculation with the bacterial species. This is conveniently achieved if the plants to be tested are tissue-cultured plants, which are grown under sterile conditions. After inoculation, the inoculated leaves are preferably shielded from contamination by other infectious agents.

According to a favourable embodiment, a control is used chosen from i) a further leaf sample of a resistant plant, ii) a further leaf sample of a further plant, said plant and the further plant both being identical clones, wherein the control is not infected with the bacteria specific the plant disease. This allows for a more accurate determination of whether growth has occurred. Because cloned plants are identical, their genetically determined resistance properties will also be the same.

According to a favourable embodiment, before the step of infecting the leaf sample, a sterilisation step is performed. The plant may be sterilised as a whole, but preferably the leaf sample is sterilised. Sterilisation is conveniently done using a fluid, in particular a sterilising gas or liquid such as aqueous hypochlorite solution. This helps to make sure the correct bacterium is detected and/or increase the likelihood that the plant material is maintained alive for the duration of the method.

According to an important embodiment, the plant from which the leaf sample is obtained is kept alive. If the plant from which the plant material is obtained is one of many genetically identical plants (for example obtained by cloning), then the plant from which the plant material was obtained may be sacrificed. For many other applications, in particular breeding or propagation, keeping the plant from which the plant material was derived alive is advantageous to achieve such a goal. The measure that

the plant is kept alive can be done in any manner, and it suffices if at least a cell of the plant or tissue sample of the plant is kept alive from which a full plant can be grown. The least susceptible plants are preferably used for breeding.

According to a favourable embodiment, the leaf sample is comminuted and subjected to a polynucleic acid amplification, the biopolymer detected being an amplified polynucleic acid specific for the bacteria used for infection.

The polynucleic acid may be DNA or RNA. The term «specific for the bacteria» does not imply that the stretch of polynucleic acid amplified is natural, endogenous DNA or RNA. The bacterial species may have been genetically engineered, and the amplified stretch may be a marker, such as specific for an antibiotic resistance gene. This saves the trouble of having to perform sequencing the bacterial species used for infection.

According to a favourable embodiment, the amplification is performed using PCR.This method lends itself well to automation. It is also possible to monitor the progress of the PCR reaction, so if bacterial growth is detected unequivocally, the amplification may be stopped which saves time.

According to a favourable embodiment, the number of amplification cycles is used as a measure for the bacterial growth. Thus, if there is bacterial growth, the amplification can be terminated earlier, saving time.

According to a favourable embodiment, the plant is an orchid species. This is an important application area. Acidovorax is an important cause of losses in the orchid business and an important specific application area.

According to a favourable embodiment, the plant sample is maintained in a container with at least one of i) the stomata and ii) a superficial cut in the leaf sample material upward facing.

This helps to keep the bacteria after infecting the leaf sample on its surface and avoids that they are transferred to the container reducing the chance that the leaf sample is successfully invaded by the bacterial species. Hence, they are more likely to infect the leaf sample. Most plant leaves have stomata on both sides of the leaves, orchids being an exception. According to a favourable embodiment, the leaf sample is subjected to a stress condition. This stress condition makes the leaf sample more susceptible to the infection by the bacteria, making it easier and/or quicker to determine the susceptibility of the plant to infection.

The stress condition is preferably not a lack of humidity for the plant material. The stress condition is for example a higher or lower temperature that deviates more than 2˚C from the average temperature at which the plant from which the leaf sample is obtained was grown, preferably at least 5˚C.

Finally according to a favourable embodiment, the plant is an orchid species and the leaf sample is shielded from light while said leaf sample is maintained alive.

This has been found to reduce the time necessary to determine the susceptibility. Preferably at least 50% of the light is blocked, preferably at least 90%, more preferably at least 96%. According to a favourable embodiment, the determination of bacterial growth is performed for the leaf sample at least one more time after at least one further day of incubation of the leaf sample.

This helps to improve the reliability of the method in case a leaf sample just happened to be infected more slowly. The earlier leaf sample basically functions as a control helping to determine more accurately the extent to which bacterial growth has occurred. Preferably the leaf sample comprises

at least two leaves or leave sections. Preferably, the further incubation is performed for three days, more preferably for a week. According to a favourable embodiment, for at least one day after contacting the leaf sample with the bacteria, the relative air humidity is at least 90%.

This helps to keep the bacteria viable so as to give them ample opportunity to infect the leaf sample. Preferably, the humidity is maintained using mmobilized water as a source of water vapour, for example as a gel such as an agar agar gel. This reduces the risk of contamination. It is preferred that the relative humid conditions are maintained for at least one week, more preferably at least 10 days from the day of inoculation, as this helps the plant material to fend the bacteria off. The relative humidity is preferably at least 95%, more preferably at least 98% en even more preferably at least >99% [1-3].

The invention will now be illustrated with reference to the example section below.

Materials and Methods

Culturing bacteria

For the method according to the invention for detecting the susceptibility to a bacterial disease, a pathogenic strain has to be used. A suitable pathogenic strain is Acidovorax cattleyae, ATCC Number: 33619, causative agent of a bacterial disease for Phalaenopsis cultivars (orchid species). The Acidovorax cattleyae used for the experiments was be grown in a petri-dish on agar-agar medium, prepared using per 500 ml water and,

- D-sorbitol-2 gram
- L-pyroglutamic acid–0.5 gram
- K_2HPO_4-3 gram
- Na_2HPO_4-3 gram
- $MgSO_4.7H_2O$-3 gram
- Tween 8-10 ml
- Victoria Blue-40 mg
- Bromothymol blue-15 mg
- Agar-15 g

The pH was adjusted to 7.4 before autoclaving at 121˚C for 20-30 mins. Before the agar solidified, the following antibiotics were added:

- Ampicillin (50 mg/ml)-3 ml
- Vancomycin (12.5 mg/ml)-2 ml

After inoculation, the petri-dish was sealed with Parafilm and 35 incubated for seven days at 28˚C.

A single colony was picked and grown in an Erlenmeyer flask on 20 ml liquid medium having the same composition but without agar until it reached the log-phase.

To determine the bacterial concentration for a given optical density, five ten-fold dilutions were made with sterilized tap water and plated in duplicate on Petri-dishes (100 µl per dish). Also, the turbidity was determined for all bacterial suspensions using a portable ISO turbidity measuring device (HI 98713, HANNA Instruments, Nieuwekerk a/d IJssel, Nederland). The Petri-dishes were incubated for one week at 28˚C. The following correlation between optical density and bacterial concentration was obtained (Table 1).

Infection of a plant sample

Plant material: Two different phalaenopsis varieties were used in de experiment. There were about 180 clones made for each cultivar, to check the reproducibility and reliability of the method according to the invention. Clones are genetically identical and are made by in-vitro micropropagation from a Phalaenopsis flower spike (stem carrying a flower). Clones were maintained at 28˚C and about 2000 lux light intensity on a standard non-liquid growth medium and transferred each 12-14 weeks to fresh medium.

Each week the young plant leaves were cut from the plant itself and were inoculated with bacteria suspension.

Sterilisation of cut leaves: As leaf explants are collected from the plants growing under 10 natural environment, surface sterilization of explant was performed to prevent microbial contamination. This was done as follows:

1. Wash the leaves with 1% SavlonTM approximately for 10 minutes.

2. Rinse with sterile distilled water (SDW) two times.

3. Dip the explants in 70% ethanol for 30 seconds and wash with SDW two times.

4. Soak the explants in 0.1% sodium hypochlorite for 3 minutes followed by several rinses of SDW.

Inoculation methods: Three inoculation methods were compared for the two phalaenopsis varieties.

In the first inoculation method, the two youngest actively growing leaves were cut from the plant and dipped in a bacterial suspension and both leaves were together stored in sterile petri-dish by room temperature without light contact between leaves samples and environment. The petri-dish was shielded from light using a black plastic bag.

In de second inoculation method, the two youngest actively growing leaves were cut from the plant, then each leaf was provided with a small cut (ca 5 mm) on its top surface. Then both leaves were dipped in a bacterial suspension and were together stored in sterile petri-dish by room temperature without light contact between leaf

Table 1: The correlation between optical density and bacterial concentration.

Optical Density	Bacteria Numbers (CFU) per 0.1 µl
0.46	1348
0.44	1299
0.22	131
0.20	106
0.18	22
0.15	18
0.13	3

samples and environment.

In the third inoculation method, two youngest actively growing leaves were cut from the plant and dipped in bacterial suspension then they were stored in a sterile petri-dish with a moist sterile filter. The same storing conditions from the previous methods were used. Sterile filters are made from cotton make up pads. They were sterilized at 121ºC for about 20 min in a glass pot. Then they were dipped in sterile tap water and squeezed out of an excess moisture and placed on the bottom of each petri-dish. Both leaves were then placed on the filter. Petri-dishes were then stored by the same storing conditions as the previous methods. The objective of the filters in the petri-dish was a increase of the air humidity, high air humidity improves bacterial growth.

Determining bacterial growth using pcr

The comparison of inoculation methods began one week after inoculation.

Samples were used for each method and Phalaenopsis cultivar. Each time one leaf was taken out a petri-dish and used in a PCR. Cq values were determined for each sample. Quantification cycle (Cq) is the metric used for analyzing qPCR results. The Cq value represents the number of cycles needed to reach a set threshold fluorescence signal level. The exact level used for this threshold should be chosen so that it captures data during the exponential phase and is the same for all samples analyzed in a run. In practice, to determine Cq values background fluorescence levels are subtracted from the raw data. The background value is typically based on the relatively stable fluorescence level of the first few cycles. Then, a fluorescence threshold value is chosen either manually or using an instrument-specific algorithm. The data analysis searches data curves for each sample and interpolates a Cq value that represents where that sample crossed the threshold. Thus, the specific Cq obtained is a relative value. It is relative to the starting template copy number, but it is also specific for the instrument and reagents used, the efficiency of the PCR amplification, the efficiency of cleavage or hybridization of the fluorogenic probe, and the sensitivity of detection. A lower Cq correlates with a higher amount of starting template and a higher Cq value correlates to a lower amount of starting template.

In the beginning of the experiment all samples were inoculated with the same amount of bacteria (100 ul with an OD of about 0.22). Each week the samples were taken and the CQ value of the sample was analyzed. If the CQ value of the second sample decreased in comparison with the first sample the there was a bacteria growth. If the bacteria growth did't do any damage to the sample surface and there were no symptoms observed then is the plant sample (plant cultivar) tolerant to the disease. If the bacterial growth does any damage and there were symptoms observed then the sample (plant cultivar) is susceptible to the disease. If the CQ value didn't change, there was no decrease nor an increase, in comparison to the first sample that means there was no bacteria growth. If there was no bacteria growth on the sample (plant cultivar) end there were no symptoms observed then the sample (plant cultivar) is deemed resistant to the disease. Resistance is the ability of the plant to inhibit the growth of the pathogen. Suitable primers for the detection of a bacterial disease can be determined using commercially available software, such as IDT Biotools

Oligo Analyzer 3.0. For Acidovorax cattleyae, ATCC Number: 33619 suitable primers are 5'-caagtcctca tggcccttat ag-3' and 5'-acggttaggc tacctacttc t-3' (forward and reverse primer respectively).

These can be obtained from Integrated DNA Technologies Coralville, Iowa, USA).

Results

Three inoculation methods were compared for two phalaenopsis varieties.

A. In the first inoculation method, the two youngest actively growing leaves were cut from the plant and dipped in a bacterial suspension and both leaves were together stored in sterile petri-dish by room temperature without light contact between leaves samples and environment.

1. Leaves were on the 24th of april 2014 inoculated with bacterial suspension and the negative control samples were inoculated with sterile tap water.

2. Two weeks later leaves were observed and samples were used in PCR. Symptoms (small water-soaked spots are the first symptoms caused by Acidovorax cattleyae. These spots enlarge rapidly under humid conditions and turn brown to black, often surrounded by a yellow halo) were mildly visible on leaves of cultivar 2, but were not yet visible on leaves of cultivar 1. PCR Cq value results were for the negative control sample for both cultivars 0, for cultivar 2 Cq = 18.8, for cultivar 1 Cq = 19.2. Both Cq values indicate the presence of bacteria on each of the inoculated samples.

3. One week later leaves were again observed, and samples were again taken. Symptoms were further developed on cultivar 2, but were not yet visible on cultivar 1. PCR Cq value results were for the negative control sample for both cultivars 0, for cultivar 2 Cq = 16.1, for cultivar 1 Cq = 17.5. Both Cq values indicate the presence and growth of bacteria on each of the inoculated cultivar samples in comparison with the first round of Cq value for the samples a week ago, that in combination with visible symptoms on cultivar 2 in combination with increased bacteria growth suggest that this cultivar is susceptible, but the absence of symptoms on cultivar 1 in combination with increased bacteria growth suggest a presence of tolerance level to the bacteria.

B. In the second inoculation method, the two youngest actively growing leaves were cut from the plant, then each leaf was provided with a small cut (ca 0.5 cm) on its top surface. Then both leaves were dipped in a bacterial suspension and were together stored in sterile petri-dish by room temperature without light contact between leaf samples and environment.

1. 210 leaves were cut from the plants. Leaves were on the 18th of April 2014 inoculated with bacterial suspension and the negative control samples were inoculated with sterile tap water.

2. Two weeks later leaves were observed and samples

were used in PCR. Symptoms were mildly visible on cultivar 2, but were not yet visible on cultivar 1. PCR Cq value results were for the negative control sample for both cultivars 0, for cultivar 2 Cq = 19.3, for cultivar 1 Cq = 19.6. Both Cq values indicate the presence of bacteria on each of the inoculated samples.

3. One week later leaves were again observed, samples were again taken. Symptoms were further developed on cultivar 2, but were not yet visible on cultivar 1. PCR Cq value results were for the negative control sample for both cultivars 0, for cultivar 2 Cq = 17.5, for cultivar 1 Cq = 17.6. Both Cq values indicate the presence and growth of bacteria on each of the inoculated cultivar samples in comparison with the first round of Cq value for the samples a week ago, that in combination with visible symptoms on cultivar 2 in combination with increased bacteria growth suggest that this cultivar is susceptible, but the the absence of symptoms on cultivar 1 in combination with increased bacteria growth suggest a presence of tolerance level to the bacteria.

4. One week later leaves were again observed, samples were again taken. Symptoms were further developed on cultivar 2, but were not yet visible on cultivar 1.

PCR Cq value results were for the negative control sample for both cultivars 0, for cultivar 2 Cq =15.9, for cultivar 1 Cq =16.4. Both Cq values indicate the presence and growth of bacteria on each of the inoculated cultivar samples in comparison with the second round of Cq value for the samples a week ago, that in combination with visible symptoms on cultivar 2 in combination with increased bacteria growth suggest that this cultivar is susceptible, but the absence of symptoms on cultivar 1 in combination with increased bacteria growth suggest a presence of tolerance level to the bacteria.

X. In the third inoculation method, two youngest actively growing leaves were cut from the plant and dipped in bacterial suspension. Then they were stored in a sterile petri-dish with a moist sterile filter. The same storing conditions from the previous methods were used. Sterile filters are made from cotton make up pads. They were sterilized at 121˚C for about 20 min in a glass pot. Then they were dipped in sterile tap water and squeezed out of an excess moisture and placed on the bottom of each petri-dish. Both leaves were then placed on the filter. Petri-dishes were then stored by the same storing conditions as the previous methods. The objective of the filters in the petri-dish was a increase of the air humidity, high air humidity improving bacterial growth.

1. Leaves were on the 12th of June 2014 inoculated with bacterial suspension and the negative control samples were inoculated with sterile tap water.

2. One week later leaves were observed and samples were used in PCR. Symptoms were heavily visible on cultivar 2, but were not yet visible on cultivar 1. PCR Cq value results were for the negative control sample for both cultivars 0, for cultivar 2 Cq = 17.3, for cultivar 1 Cq = 18.4. Both Cq values indicate the presence of bacteria on each of the inoculated samples.

3. One week later leaves were again observed, samples were again taken. Symptoms were further developed on cultivar 2 and resulted in complete susceptibility of it, but no symptoms appeared on cultivar 1. PCR Cq value results were for the negative control sample for both cultivars 0, for cultivar 2 Cq = 16.3, for cultivar 1 Cq = 19.6. Cq value of the second cultivar indicate the presence and growth of bacteria, although the Cq value of the first cultivar shows an increase. That suggests a presence, but not growth of the bacteria. The second cultivar shows increase in symptoms and decrease in Cq value, the first one shows a increase in Cq value and no symptoms of disease at all. That shows a susceptibility of the second cultivar and resistance of the first cultivar to the bacterial disease. This experiment showed that maintaining the leaf sample in good health by an elevated relative humidity is important to allow the leaf sample to fend of the infection.

D. Comparison of the inoculation methods and the reached results.

1. All methods were found to suitable for inoculation

2. The same results were reached with the first two methods (cultivar 2 susceptible; cultivar 1 tolerant), only the third one showed a resistance of cultivar 1 and susceptibility of cultivar 2.

3. All methods can be used for determining the degree of susceptibility of a cultivar. The third showed the difference between resistance and tolerance levels a cultivar.

4. The speed of the three methods varies. With method 1 the symptoms were fully visible after 4 weeks, with method 2 symptom developing took 2-4 weeks, and by method three symptoms appeared after one-two weeks. For a given inoculation method used, the method according to the invention may provide a gain in time for pre-selection purposes because susceptible plants can be detected in an earlier stage.

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Claims

1. A method of determining the susceptibility of a plant to a bacterial disease, said method comprising the step of infecting plant material of the plant with bacteria of a bacterial species specific for a plant disease, characterized in that the method comprises the steps of,

- Obtaining a leaf sample from the plant,
- Infecting the leaf sample obtained from the plant with the bacteria of the bacterial species specific for the plant disease,
- Maintaining the leaf sample alive, and
- Determining bacterial growth by detecting a biopolymer produced by the bacteria.

2. The method according to claim 1, wherein a control is used chosen from i) a further leaf sample of a resistant plant, ii) a further leaf sample of a further plant, said plant and the further plant both being identical clones, wherein the control is not infected with the bacteria specific the plant disease.

3. The method according to claim 1 or 2, wherein before the step of infecting the leaf sample, a sterilisation step is performed.

4. The method according to any of the preceding claims, wherein the plant from which the leaf sample is obtained is kept alive.

5. The method according to any of the preceding claims, wherein the leaf sample is comminuted and subjected to a polynucleic acid amplification, the biopolymer detected being an amplified polynucleic acid specific for the bacteria used for infection.

6. The method according to claim 5, wherein the amplification is performed using

PCR.

7. The method according to any of the claims 5 or 6, wherein the number of amplification cycles is used as a measure for the bacterial growth.

8. The method according to any of the preceding claims, wherein the plant is an orchid species.

9. The method according to any of the preceding claims, wherein the plant sample is maintained in a container with at least one of i) the stomata and ii) a superficial cut in the leaf sample material upward facing.

10. The method according to any of the preceding claims, wherein the leaf sample is subjected to a stress condition.

11. The method according to claim 10, wherein the plant is an orchid species and the leaf sample is shielded from light while said leaf sample is maintained alive.

12. The method according to any of the preceding claims, wherein the determination of bacterial growth is performed for the leaf sample at least one more time after at least one further day of incubation of the leaf sample.

13. The method according to any of the preceding claims, wherein for at least one day after contacting the leaf sample with the bacteria, the relative air humidity is at least 90%.

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