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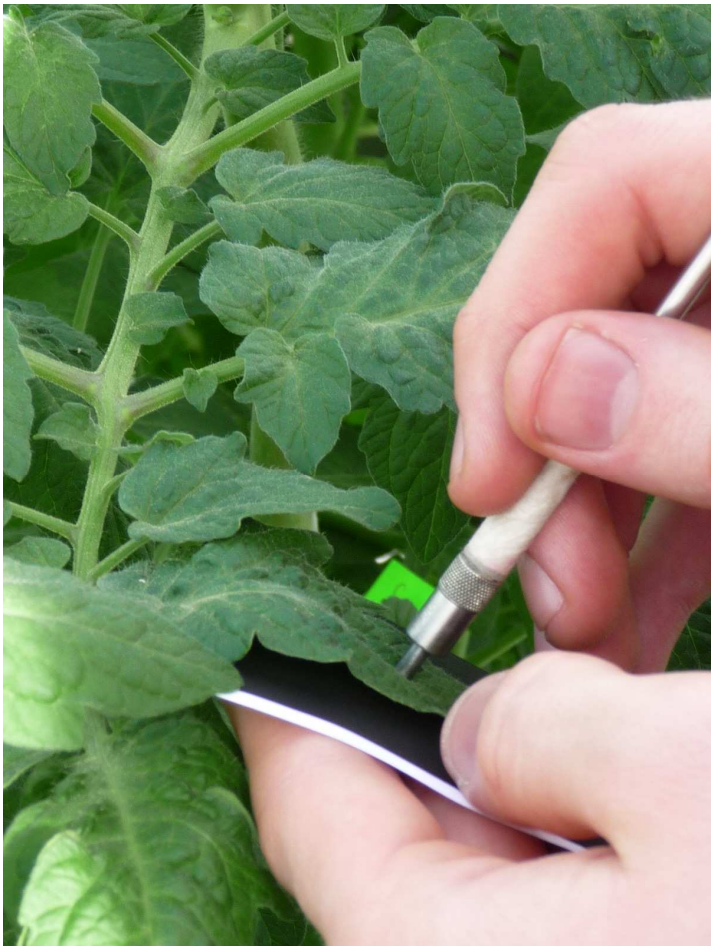

Fytal

The Application of Differential Pathlength Spectroscopy in Greenhouses

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Abstract

Measurements have been performed on plant leafs with (F)DPS ((Fluorescence) Differential Pathlength Spectroscopy). This is a technique that uses two glass fibers to detect the light emitted from one of these fibers. These two measured spectra are subtracted to obtain a very shallow measurement. The measurements were done with a black background on leafs of tomato plants that received different treatments being dry or wet and differently cut, the same was done for roses. Also chrysantheum leafs were measured infected with stunt and Verticillium. The spectra, which mainly showed the absorption, reflection and fluorescence of chlorophyll, that were found in these measurements were analyzed with Labview & PCA (Principal Component Analysis).
It has been shown that the intensity of the measurements is sensitive to the background which is why the measurements were preformed with a black background.
In the measurements a trend seems to appear in the first principal component. This component shows a significant difference between dry and wet conditions according to a rank-sum test. The difference even increased in measurements that were taken at a later point. There was also a visually apparent difference found between the measurements on the first row and the rest of the plants. This is because this row received more light. In later experiments differences were again found but with differing results for the infected chrysantheums big differences were seen. For the measurements of roses and tomato's the differences were never clear. The differences were proven to exist with several rank-sum tests but the measurements are not accurate enough to be of value to the farmers, especially fluorescence measurements vary much. It is advised to measure in a light region where the reflected signal is only dependent on the scattering and not on the scattering and the absorption.
A way to improve the data analyses is to create a fit of the measured spectra and extract all of the components. This way the cause of changes between measurements can be explained where with the PCA-method differences can be found but not explained.

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Introduction

In the Faculty of the Erasmus MC (Fluorescence) Differential Pathlength Spectroscopy[12] ((F)DPS) has been developed. This technique uses light from the visible spectrum (350 nm – 1000 nm) and sends it into the collection and delivery glass fiber. The light travels through the fiber and is emitted out of the fiber tip. The fiber tip is placed on biological tissue that will be measured. The light is reflected from the tissue and is captured by both the collecting and delivery glass fiber and the collecting glass fiber right next to it. By subtracting both corrected measured spectra resulting from both fibers from each other a very shallow measurement can be preformed.
(F)DPS is currently used to detect forms of cancer that grow superficially in biological tissue of people on the skin or in bronchial mucosa[3] for example.
This research looks at the applications of (F)DPS in agriculture and mainly agriculture in greenhouses. The question is if (F)DPS can accurately asses the state of a plant. In this research tomato plants, roses, calathea's and chrysanthemums will be investigated. Each plant will be given different environmental stresses. The roses and tomato plants will be cut differently and will be held under dry and normal conditions. The tomato plants will also have leaf cuttings taken which will be placed in water with a variable EC (Electrical Conductivity). The chrysanthemums are given different treatments. There are 10 different races of chrysanthemums investigated. Each race has a control batch with no anomalies a batch with the plant virus Stunt and a batch with the mold Verticilium. If a correlation is found between the measurements and the conditions of the plant in question (F)DPS technique can be used in agriculture to asses the welfare of plants and farmers can act accordingly.

1. Theory of plant tissue measurements

The theory of plant tissue measurements describes (F)DPS which is used to measure plant leaves and the theory of plant tissue measurements also describes the techniques used to analyze the data from the measurements with (F)DPS. The problems we can expect to encounter are investigated as well.

1.1 DPS

Differential pathlength spectroscopy[1] (DPS) is based on two glass fibers ,a delivery and collection fiber (dc-fiber) and a collection fiber (c-fiber) that have been placed next to each other (only separated by a thin layer of cladding). Light is sent through the dc-fiber and emitted from the fiber tip. The fiber tip is usually placed on a biological tissue. The light emitted from the fiber then reflects from the biological tissue into both fibers. The signal is measured by two spectrographs. By subtracting both corrected signals a very shallow measurement in the order of magnitude of 0,4 d_{fiber}[1] can be preformed on the tissue. As fiber diameter a value between 100 µm en 400 µm is usually chosen. To minimize the amount of reflection resulting from the change of refractive indices at the fiber tip the fiber tip is polished at an angle of 15 degrees.

To simplify the formulas the variables that are wavelength dependent are know written as follows:

$$f(\lambda) = \bar{f} \qquad [0]$$

For a graphic representation of the different reflections see fig. 1

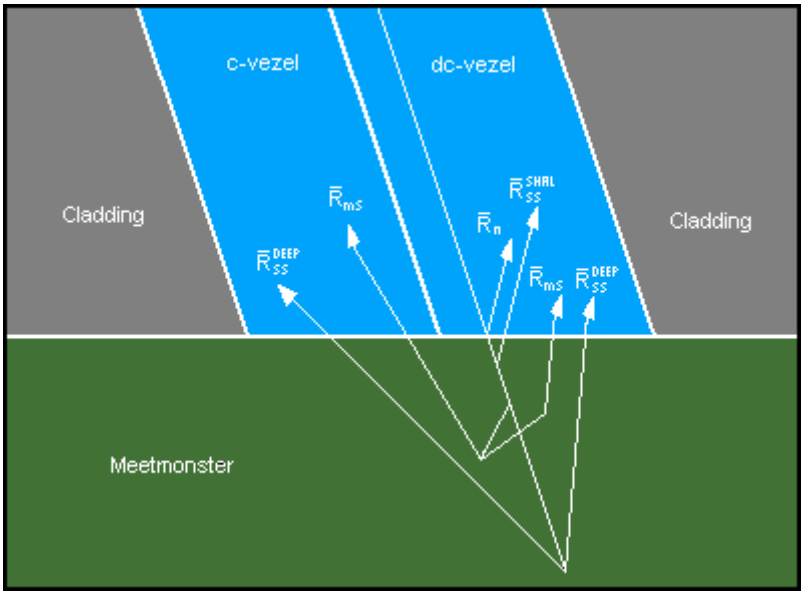

Fig. 1: Schematic of the DPS measurement with the fiber touching the sample

Figure 1 shows the different reflections. The photons can reflect because of the transition from fiber to sample. This effect can be measured and subtracted from the result. The photons also reflect in the sample. The photons from the shallow reflections mostly end up in the dc-fiber the further the photons travel from the fiber tip the greater the chance that the photons

are detected by both the c-fiber and eventually the same amount of photons are detected by both fibers and are therefore subtracted away. Only the photons that were scattered near the probe tip will be measured so a shallow measurement can be preformed.

In earlier research[1] a formula has been derived for the calculation of the difference in reflection:

$$\bar{R}^{\exp} = c\left(\frac{\bar{I} - \bar{I}_n}{\bar{I}_{WS2} - \bar{I}_{WS99}} - \frac{\bar{J}}{\bar{J}_{WS2} - \bar{J}_{WS99}}\right) = c(\bar{I}_{dc} - \bar{J}_c) \qquad [1]$$

Where:

$\bar{R}^{\exp}$ =	The calibrated DPS signal	[a.u.]
$\bar{I}_{WS99}$ =	The intensity on the dc-fiber during a measurement on black Spectralon (Labsphere SRS-99)	[a.u.]
$\bar{J}_{WS99}$ =	The intensity on the c-fiber during a measurement on black Spectralon (Labsphere SRS-99)	[a.u.]
$\bar{J}_{WS2}$ =	The intensity on the c-fiber during a measurement on white Spectralon (Labsphere SRS-02)	[a.u.]
$\bar{I}_{WS2}$ =	The intensity on the dc-fiber during a measurement on white Spectralon (Labsphere SRS-02)	[a.u.]
$\bar{I}_n$ =	The intensity on the dc-fiber caused by reflection on the fiber tip	[a.u.]
$\bar{I}$ =	The intensity measured on the dc-fiber	[a.u.]
$\bar{J}$ =	The intensity measured on the c-fiber	[a.u.]
$\bar{I}_{dc}$ =	The calibrated intensity on the dc-fiber	[a.u.]
$\bar{J}_c$ =	The calibrated intensity on the c-fiber	[a.u.]
c =	The calibration constant	

Formula one consists of units that all have arbitrary units this is because the used spectrographs can only measure counts?
With formula 1 the measurements can be calibrated for the reflections on the fiber tip and in the glass fiber itself. Formula 1 also calibrates for the transmission of the fibers.

The differential reflection signal is described[1] by:

$$\bar{R} = C_1 \bar{\mu}_s' \exp(-0{,}8 d_{fiber} \bar{\mu}_a) \qquad [2]$$

Where:

C_1 =	The proportional constant	[-]
$\bar{\mu}_a$ =	De absorption coefficient	[cm^{-1}]
d_{fiber} =	De fiber diameter	[m]
$\bar{\mu}_s'$ =	De scattering coefficient	[cm^{-1}]

1.1.1 FDPS

Fluorescence differential path-length spectroscopy[2] (FDPS) works according to the same principle as DPS but measures the fluorescence in stead of reflection. When performing FDPS the sample is excited with blue LED's at 365nm and 385nm. To calibrate the measurements a measurement is taken on Spectralon (Labsphere USFS-200-010).

From these:

$$\bar{I}^{F}_{sample} = \bar{\bar{F}}^{dc}_{sample} \cdot \bar{\bar{T}}_{dc} \qquad [3]$$

Where:

$\bar{I}^{F}_{sample}$	= The total fluorescence measured by the dc-fiber	[a.u.]
$\bar{\bar{F}}^{dc}_{sample}$	= The number of fluorescent photons collected by the dc-fiber	[a.u.]
$\bar{\bar{T}}_{dc}$	= The transmission through the dc-fiber	[-]

$$\bar{J}^{F}_{sample} = \bar{\bar{F}}^{c}_{sample} \cdot \bar{\bar{T}}_{c} \qquad [4]$$

Where:

$\bar{J}^{F}_{sample}$	= The total fluorescence measured with the c-fiber	[a.u.]
$\bar{\bar{F}}^{dc}_{sample}$	= The number of fluorescent photons collected by the c-fiber	[a.u.]
$\bar{\bar{T}}_{c}$	= The transmission through the c-fiber	[-]

With formula 3 & 4 the FDPS spectrum can be calculated:

$$\bar{\bar{R}}\bar{\bar{F}}^{\exp} = \bar{\bar{F}}^{dc}_{sample} - \bar{\bar{F}}^{c}_{sample} \qquad [5]$$

Where:

$\bar{\bar{R}}\bar{\bar{F}}^{\exp}$	= The FDPS signal	[a.u.]

1.2 Data Analysis

To analyse the measured spectra a number of statistical operations are used to determine if there is a correlation between the data and the plants.

1.2.1 PCA

Principal component analysis[4,5] (PCA) is a multivariate statistical analyses technique that was primarily used to compress data to a smaller set of variables called principle components (PC). It analyses the data by trying to find patterns where data points increase together.
PCA works as follows: First an average of the data is taken and subtracted so the data is now the deviation from the mean. Next the covariance matrix and the eigenvectors are calculated. The eigenvectors get arranged in order of magnitude so they form the PC's. The first principle component explains the most variance; the second component explains the most after the first PC has been subtracted and so on.
In this research PCA is used to analyze the spectra from the (F)DPS measurements. With PCA the data can be translated into PC's. Then it is investigated if the PC's can be correlated with the environmental stresses applied on the plants.

1.2.2 Rank-sum test

To prove there is a difference between to sets of data a Wilcoxon rank-sum test[6,7] is performed. This test is also known as the Wilcoxon–Mann–Whitney test and the Mann–Whitney U-test.
In this test the data is sorted from high to low then each data point gets a rank depending on how high it is in the sorted data. Then from the two different distributions the average of the ranks is determined. The difference in the two averages in the ranks tells how different the two distributions are. This difference is quantified in p. If A and B are perfectly the same p =1 (see Fig.2). But A and B are practically always shifted from each other. The question is whether they come from a different distribution (see Fig.3). In this case p gives the chance A and B come from different distributions.

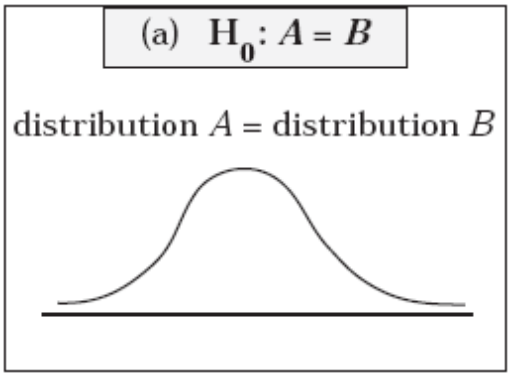

Fig. 2: distribution A and B overlapping

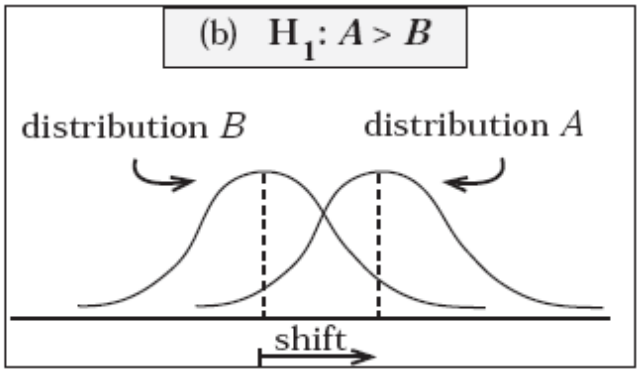

Fig. 3: distribution A and B shifted

So if $p = 0{,}03$ this corresponds to a chance of 3 % that A and B are from different distributions. However this test might not always be right since it's still an analyses technique without parameters. So a difference could be found in the noise of the measurements.

1.2.3 Correlation coefficients

To proof there is a linear correlation in the data between an x and y the correlation coefficients (CC) are calculated. This is a statistical analysis technique that calculates the slope of a set of data points and then by measuring the spread around this line the correlation is calculated. If the CC increases between the x and y the correlation improves (see Fig. 4).

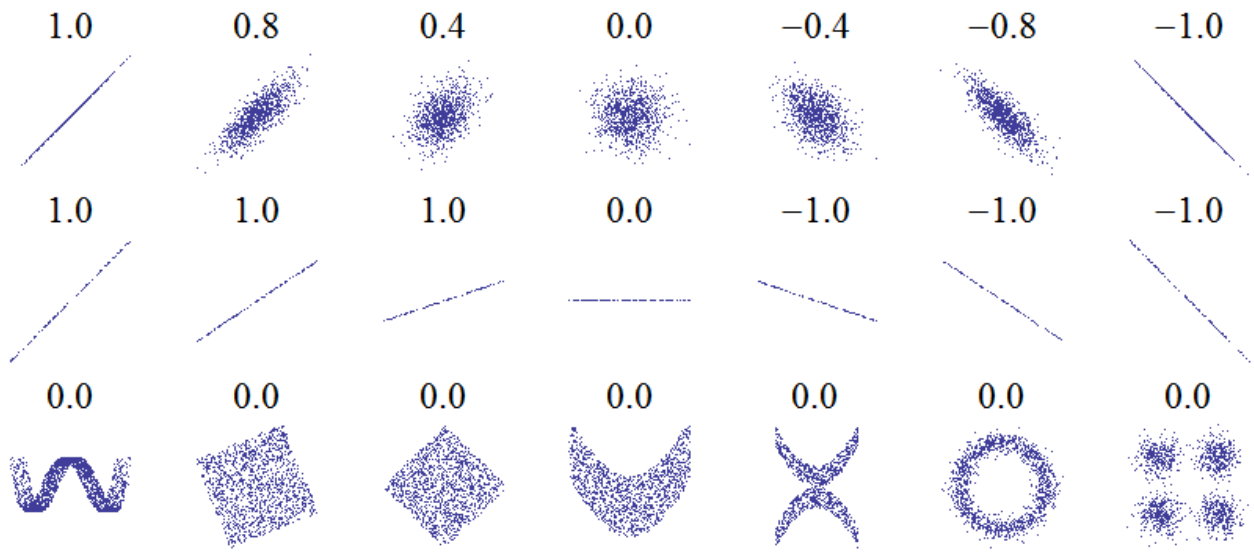

Fig. 4: Data distribution and CC values, the slope of the data doesn't matter only the deviation of the data around the line changes the CC also any non linear correlations as shown in row three don't matter either.

Normally a CC of −0.3 to −0.1 and 0.1 to 0.3 is considered a small correlation and −0.5 to −0.3 and 0.3 to 0.5 is considered a medium correlation and −1.0 to −0.5 and 0.5 to 1.0 is considered a large correlation. However this depends on what kind of experiment is performed some experiments require a much larger correlation to pass for example an measurement of a physical law should have an almost perfect correlation and a correlation of -0.9 or 0.9 is considered a low correlation. Therefore in this investigation we also calculate the p-value which is also calculated in the rank-sum test and gives the probability that the relation resulted from randomness.

1.3 Plant leafs

The investigation focuses on measurements on leafs of plants but before any measurements are done there is a need to understand more about leafs. A few properties of plant are explained here.

1.3.1 Leaf anatomy

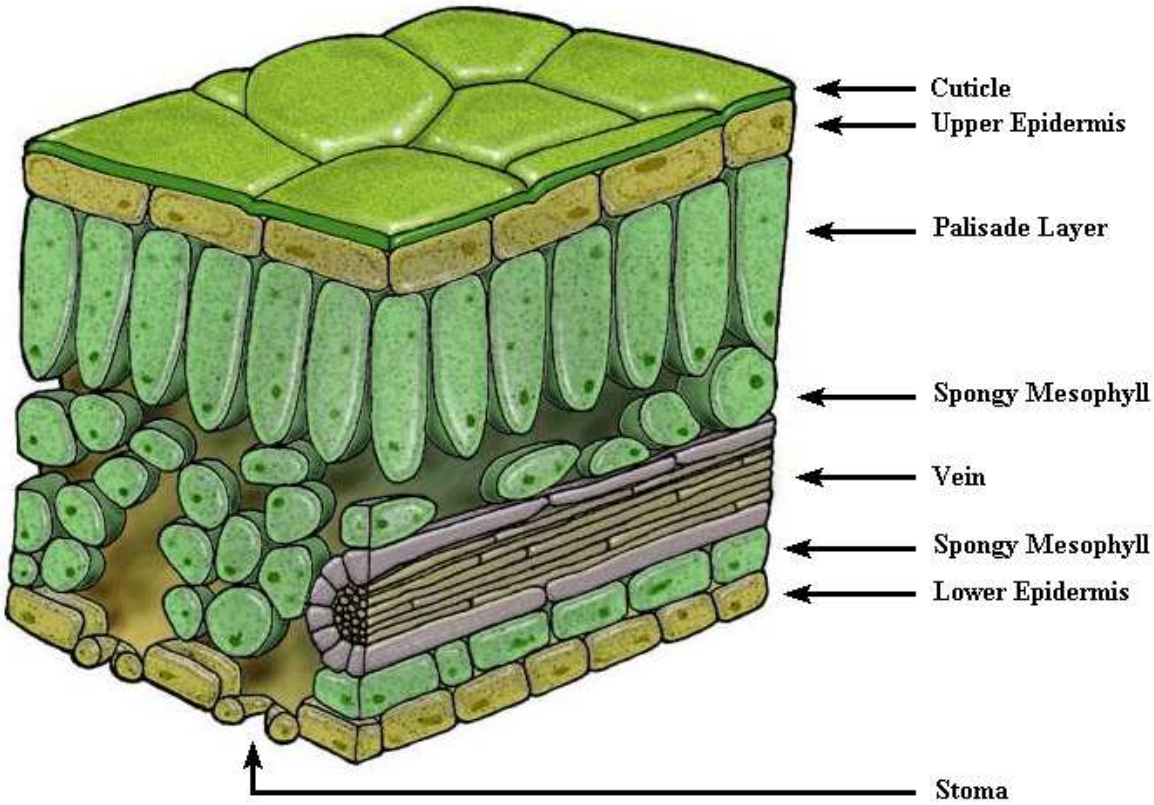

Fig. 5: cross section of a typical leaf [18]

As seen in Fig. 5 the leaf consists of many layer in this investigation the measurement is done trough the whole leaf. The top layers of the leaf are quite regularly distributed. The spongy mesophyll layer has gaps in between the cells the veins also run trough the spongy mesophyll layer. The leaf is protected by an epidermis on both sides of the leaf this is a layer of cells one cell thick that acts like the skin of the leaf.
The individual cells consist of a nucleus a vacuole and many other things but most important the cell has a cell wall that is stiff and keeps the leaf from wilting. The vacuole[21] in the cell stores water, it expands with more water and contracts when the water evaporates. If the plant contains more water then the leafs are firm but when the leafs dry out the vacuole no longer provides enough pressure on the cell wall and the leafs wilt. This is possibly measureable with DPS[1].

Leaf surface

During the initial theoretical investigation a few possible problems came to light that could interfere during measurements.

The first of these were trichomes, these are little protruding spines that grow on the surface of the leaf (see Fig.4). Trichomes are like little hairs on the plant leaf surface that prevent the plant from dehydrating to fast. How these trichomes effect the measurement is very difficult to determine. Especially since every trichome is different in size. For now nothing is done to negate the effect of the trichomes and it is assumed that the trichomes are too small to affect the measurements significantly.

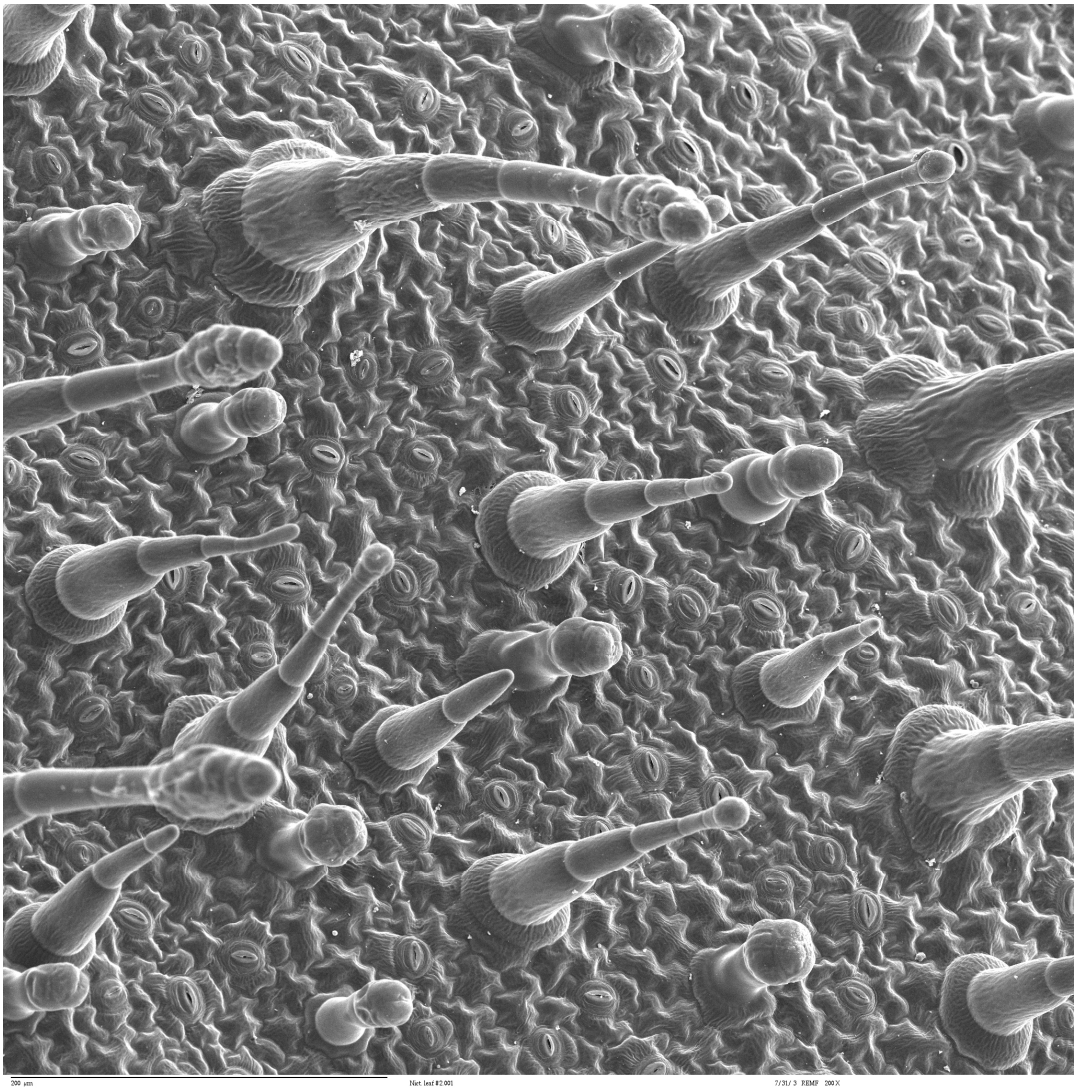

Fig. 6: Leaf epidermis under an electron microscope, visible protrusions: trichomen

There are also stomata present on the leaf surface this mouth like structure (see Fig. 6) controls the water flow in the plant. When the stomata open the water vapor passes trough the stomata. The resulting pressure difference sucks op more water from the surface keeping the plant hydrated.

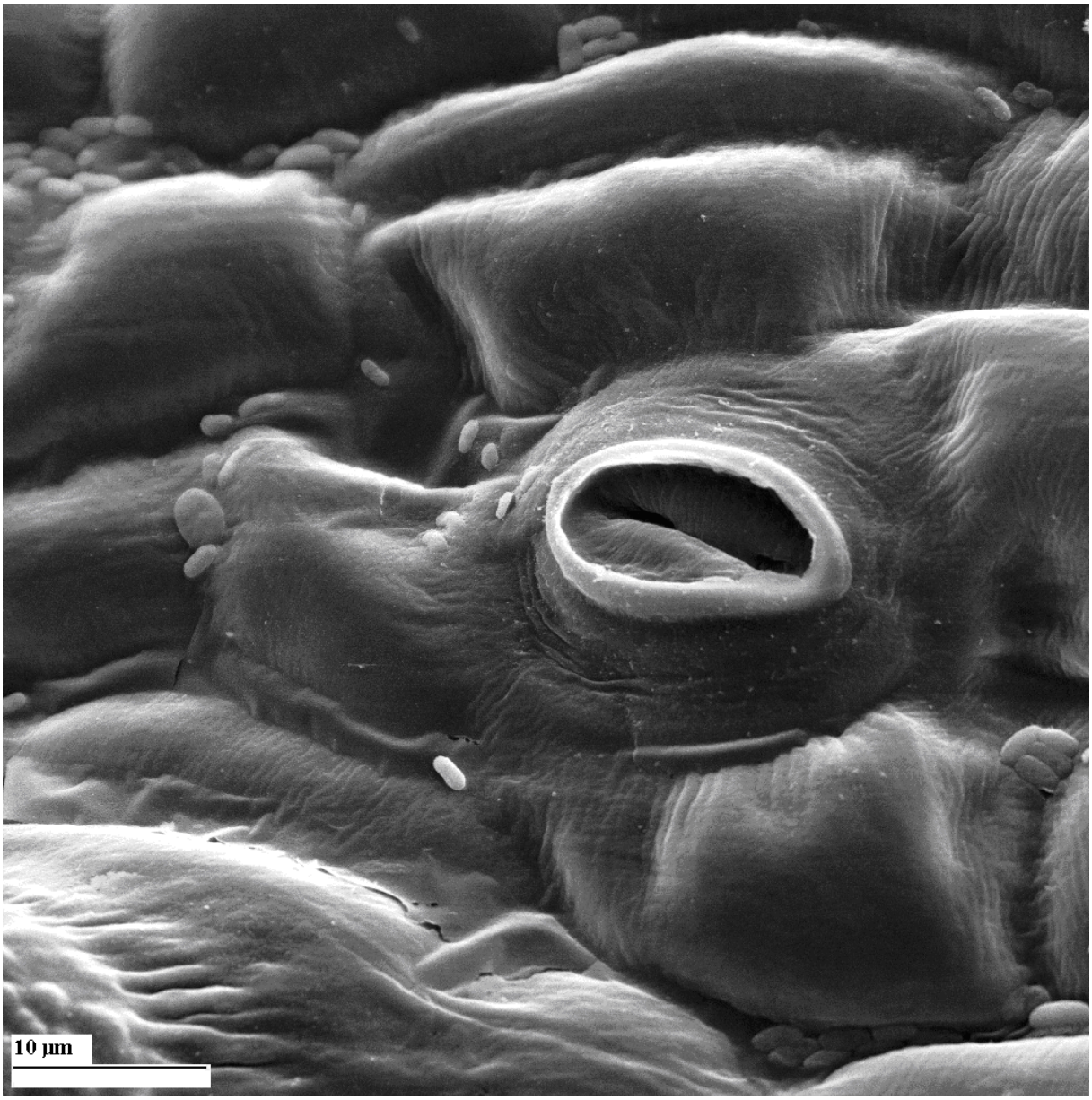

Fig. 7: Leaf epidermis under an electron microscope, visible opening: stomata

The plant surface also has a so-called cuticle. This is an oily water-resistant layer that protects the plant. This cuticle may stick on the fiber tip during the measurements and cause a deviation in the measurements. The cuticle thickness varies per plant and is for example very thick on tomato plants.

1.3.2 Plant nutrition

A plant needs water with minerals. The amount of minerals can be roughly determined by measuring the EC (Electrical Conductivity) measured in milliSiemens per centimeter. The EC can be changed by adding more salt or water. An EC of 0 means the water contains practically no minerals and the higher the EC becomes the more minerals the water contains. A low EC will result in a high water intake by the plant and a high EC will result in a low water intake but high nutrient intake by the plant[22].

1.3.3 Plant spectra

The spectra measured with the so-called "de Valk" FDPS device are caused by scattering and different absorbing and fluorescing substances. The scattering looks like the curve shown in Fig.8. The reflection spectrum is formed by the absorbers and this scattering. By subtracting the original scattering curve with the measured curve a measure for the absorption is obtained.

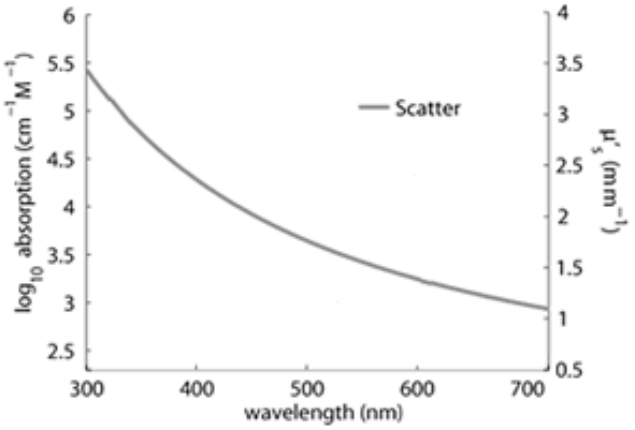

Fig. 8: The effect of scattering without absorbers as a function of wavelength

Some research on substances that are common in plants has been done. Most of the research has gone into chlorophyll[13] which is the most dominant substance in the plant spectra. This is because chlorophyll is one of the most important substances required for photosynthesis. There are mainly two different types of chlorophyll called chlorophyll a and chlorophyll b. Which have quite different absorption and fluorescence spectra[10] (see Fig. 14).
There are more substances like Anthocyanin[14] (absorbs $\lambda = 550\,\text{nm}$), Carotenoïds[15] (several substances with different absorption lines), Phycocyanin[16] (absorbs $\lambda = 620\,\text{nm}$; fluoresces $\lambda = 650\,\text{nm}$), NADH[17] ($\lambda = 260\,\text{nm}$ & $\lambda = 340$) and NAD^+ ($\lambda = 260\,\text{nm}$) present in plants but these are less dominant in the spectra.

1.3.4 Infections

If DPS can measure certain conditions of a plant then it might also possible to detect certain infections.

Verticillium Mold

Verticillium[19] (see Fig. 9) is a type of mold that can infect a number of plants. Nearly all non woody plants are similarly affected. The lower and older leaves often turn yellow and later wilt and wither. The symptoms gradually progress to the upper parts of the plant. Diseased plants are often stunted and, if infected early, generally die prematurely.
For example, outward symptoms on tomato may not develop until the plants are bearing heavily or are under drought stress. Yellow blotches then can develop on older leaves and the veins within the yellowed areas show a brown discoloration. Light, chocolate brown dead areas later develop in these blotches.
Since one of the symptoms is a discoloration a measurable difference is expected between the infected and healthy plants.

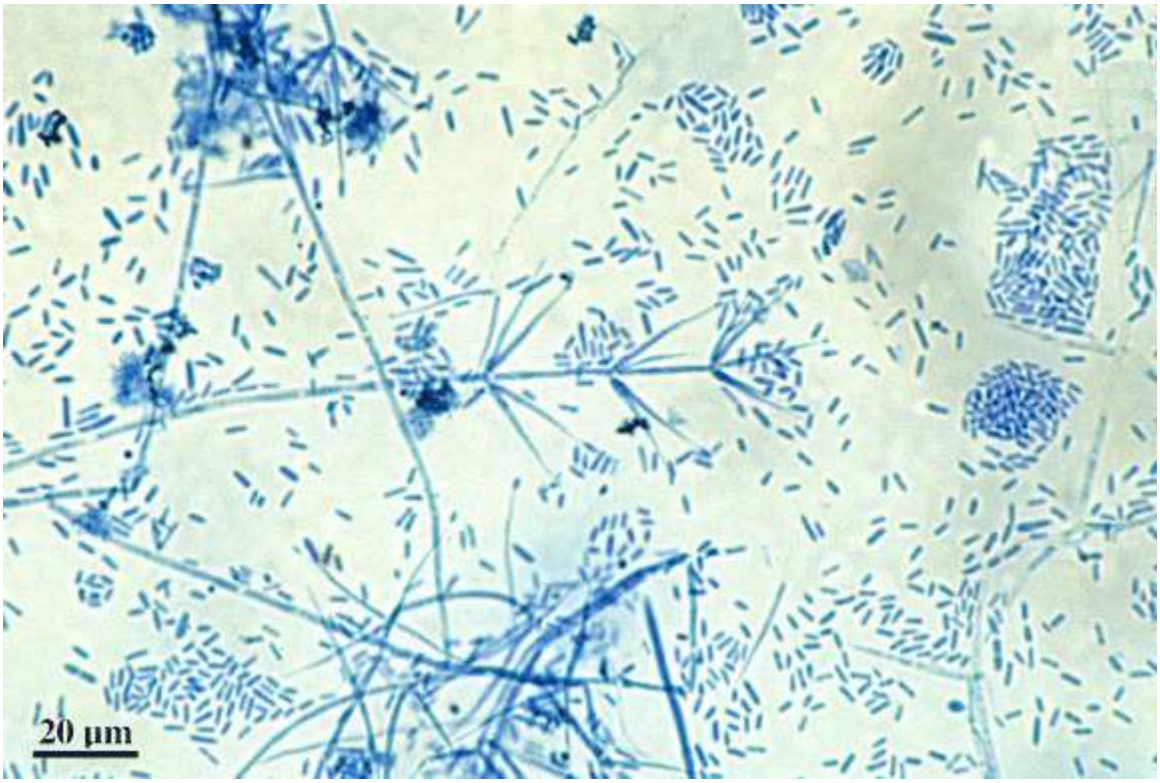

Fig. 9: Verticillium mold under a microscope

Stunt Viroid

Stunt[20] is a viroid that can infect chrysanthemums. The stunt virus is very hard to detect in plants and the virus is very contagious. An infection with stunt results in inferior blooms opening 7-10 days earlier than those on uninfected plants. Also affected plants may be half the height of uninfected plants.

2. The Methodology

In this chapter the measurement method is described and explained. The experimental setup and the protocol are described. After that the types of measurements are explained. Finally the procedure used to process the data is described.

2.1 Experimental setup

The experimental setup "De Valk" is mobile and is for this reason used to do the measurements in the greenhouse. Schematically the experimental setup looks as follows:

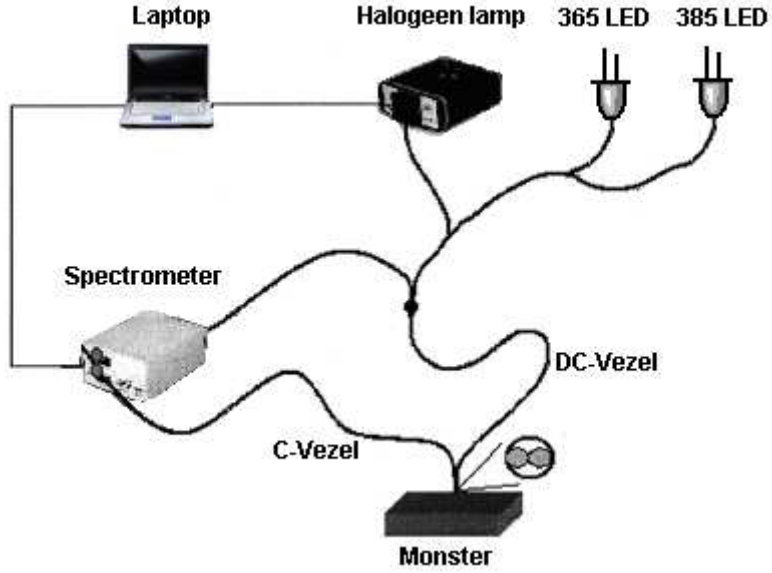

Fig. 10: A schematic representation of the experimental setup that was used to measure plants

De setup consists of:

- "De Valk" containing:
  - A Halogen lamp
  - Two blue LED's (365nm/ 385nm)
  - Two spectrometers
- A number of glass fibers
- A glass fiber link(4:1)
- A laptop

The following items were also used

- The "Calibration box" with Spectralon (Labsphere SRS-02, Labsphere USFS-200-010, Labsphere SRS-99)
- A black container where in a calibration with water can be preformed
- A black background made from black foam
- A notebook (for notes and the log)
- A bottle with 70% alcohol to clean the fiber tip

Light from a halogen lamp in combination with the light of two LED's are guided into a glass fiber (dc-fiber). The light is guided trough the fiber and is emitted at the end (fiber tip) where it is reflected in the c-fiber and dc-fiber and absorbed by the sample in question. The light collected by both fibers is collected by two separate spectrometers. The spectrometers are controlled by a labview program on the laptop and the program stores the measured data.

2.2 Procedure

First of all the setup is calibrated. This is done with a “calibration box”. In this box are three reflection standards from Spectralon (Labsphere SRS-02, Labsphere USFS-200-010, Labsphere SRS-99), one for a black measurement, one for a white measurement and one for a fluorescence measurement. With these measurements the transmission thru the fibers can be determined. To measure the reflection on the fiber tip another measurement is done in a black container filled with water (which approaches the refractive index of the sample).
After calibration the setup is ready to perform measurements. When doing these measurements its very important that the spectrometers aren’t overloaded with photons. This can be done by changing the integration time of the measurement. This is done separately for the reflection and the fluorescence measurements. When needed the amount of samples taken are increased to decrease the noise in the signal. Unfortunately the amount of samples can’t be too big because the individual measurements would take too long.
In the greenhouse 10 to 16 measurements where done per leaf. The tip touches the cuticle (see chapter 1.3.1) every time a measurement is preformed. This causes the tip to get contaminated after a few measurements (especially on tomato plants) so the tip is cleaned with 70 % alcohol. The leaf isn’t cleaned because chlorophyll dissolves in alcohol and that results in the alcohol destroying the leaf and therefore affecting the measurements. A log is kept that keeps track of the properties of the measured plant and the measurement number. So the data can be analyzed later on.

2.3 Measurements on leafs

Before the measurements in the greenhouse measurements have been done on plants in the laboratory to get an idea what the spectra of the measurements are going to look like. see Fig.12 & Fig.13 for an example of a typical reflection and fluorescence measurement.

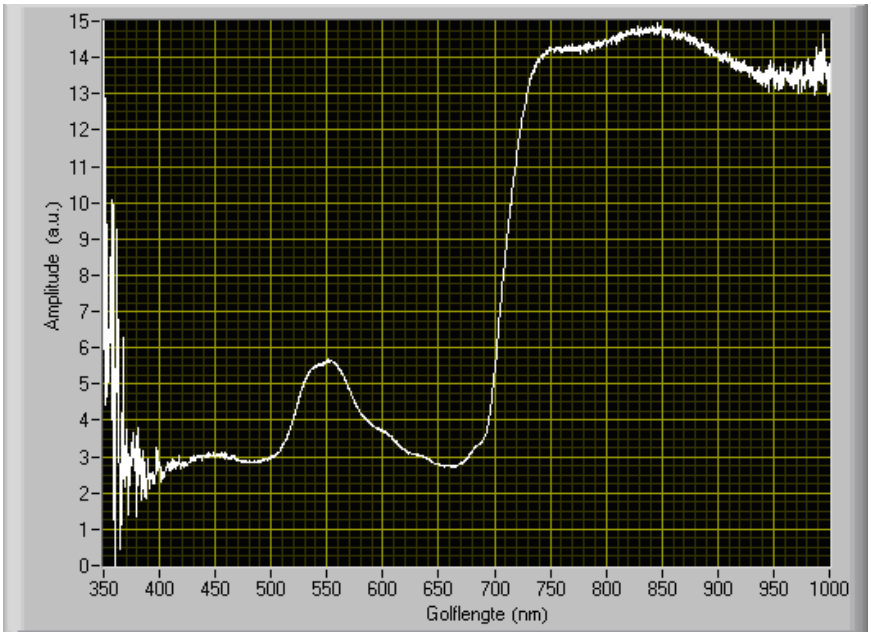

Fig. 12: Example of a reflection spectrum

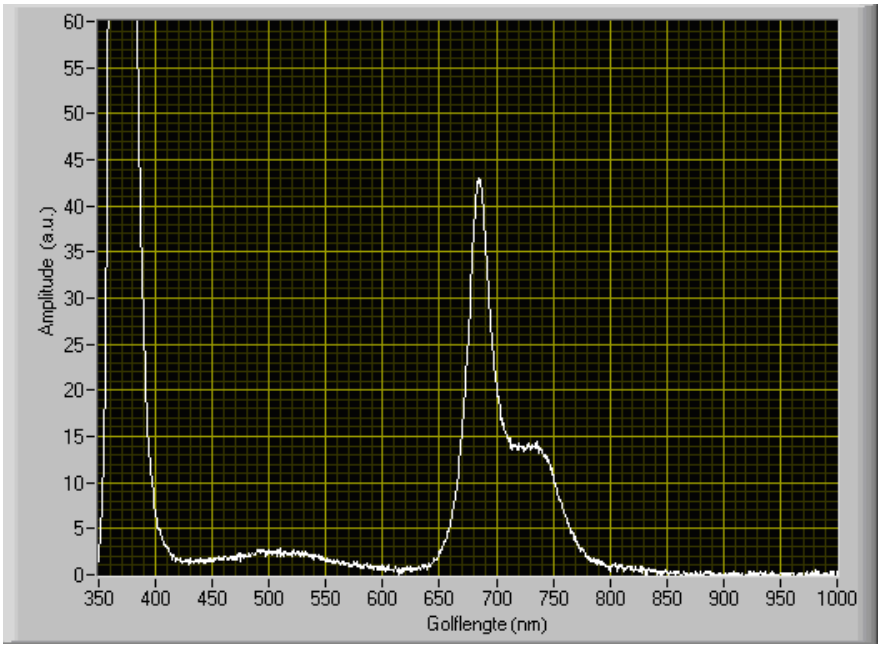

Fig. 13: Example of a fluorescence spectrum with at 370 nm the excitation wavelength and right between 650 nm and 800 nm the fluorescence of chlorophyll

The fluorescence of chlorophyll (see Fig.13) appears to be dominant in the fluorescence spectrum. The reflection spectrum shows that the absorption is quite high. This absorption is mainly caused by chlorophyll. In the high and low end of the visible spectrum of light the noise in the reflection measurements is high. This is because the halogen lamp doesn't emit much light in this wavelength, the spectrographs are less sensitive to this wavelength and the fibers absorb more light in lower wavelengths. If this is a problem the number of samples per measurements can be increased. If the number of samples can't be increased the noise can be decreased by using a brighter lamp (or extra LED's) and a better spectrograph.

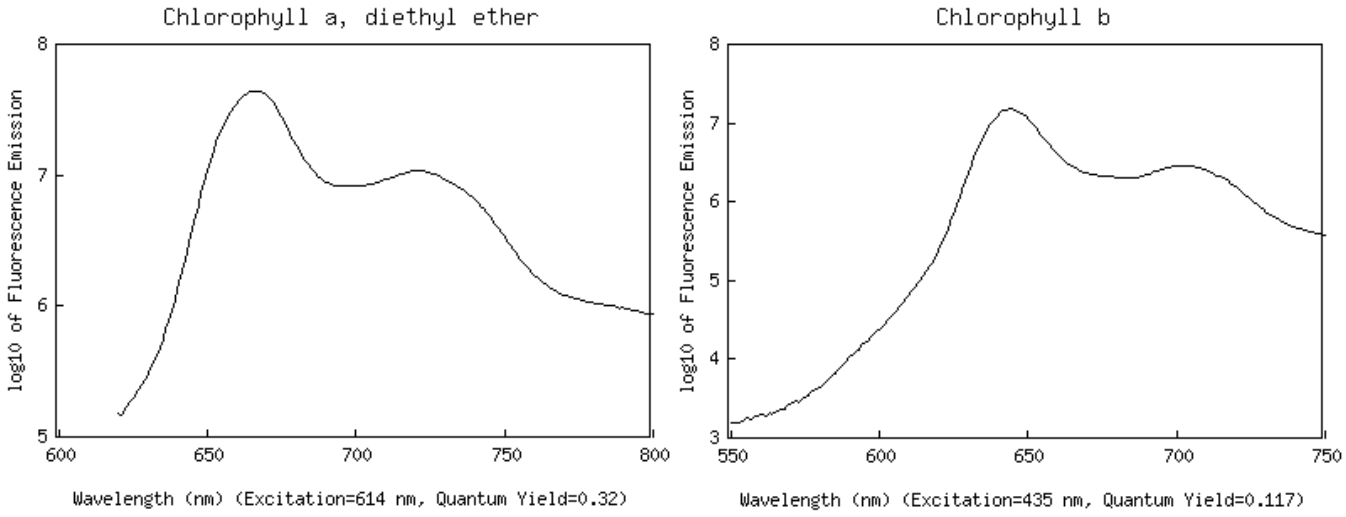

Fig.14: Fluorescence spectrum of chlorophyll a en b in diethyl ether [10]

2.3.1 Variation in one point of a leaf

To register the effect of noise and other unforeseen variables the variation in one point of a leaf is measured several times while nothing but time chances. This is done by balancing the fiber tip on a leaf, so the pressure on the leaf won't vary. About 20 measurements are taken on

every single spot. From these measurements certain parameters are calculated using labview (see chapter 2.5.1). Then the parameters are plotted as a function of time and the standard deviation is calculated in the measurements. From these deviations the maximum is taken to account for most of the variance in the measurements.

2.3.2 Influence of the background

To measure the influence of different backgrounds on the measurements the background must be changed while the fiber tip remains in the same position. To achieve this, a leaf is attached to the fiber tip with some tape. Now the fiber tip with the leaf attached to it can be placed on different backgrounds. The pressure on the tip may vary but this is kept at a minimum by handling the probe with care. Measurements are taken on a black (Spectralon Labsphere SRS-99), a gray (Spectralon Labsphere SRS-50) and a white ((Spectralon Labsphere SRS-02) background. These backgrounds are made from reflection standards Spectralon that behave like Lambert reflectors.

Fig 15: Reflection standard Spectralon Labsphere SRS-02

2.3.3 Placement on the plant

The measurements can vary depending on the placement of the probe. The variations may depend on the leaf chosen whether it is on top of the plant or closer to the ground. Measurements can vary as a function of the placement of the probe on a leaf. The measurements can be very different if the measurement is preformed near the base or at the top of the leaf. On each leaf 40 measurements or less, when the leaf isn't big enough, are taken to average out the variation of the surface of one leaf. So the variation can be analyzed as a function of the position of the leaf on the plant.

2.4 Measuring in the greenhouse

To make accurate measurements the plants are best held in accurate conditions. This is why most measurements are done in the greenhouse.

2.4.1 Tomato plants

The project "Fytal in action" is located in a greenhouse located in Delfgauw. In the greenhouse there are 6 rows of tomato plants. The treatments of the tomato plants consist of two different water treatments (wet or dry) and three different cutting treatments (3 leafs and 6 fruits or 3 leafs and 3 fruits or 1,5 leaf and 6 fruits), see Fig. A1 Attachment 1. On every leaf 15 randomly placed measurements are done. Every measured leaf is found on the first branch above the first set of flowers and on the top of the branch.

2.4.2 Roses

In the same greenhouse in Delfgauw roses are also grown. These roses are also given two different water treatments and three different cutting treatments. In this case roses were selected with a flower that was just opening up. The leaf measured was the top leaf on the first

branch with 5 leaves and every leaf was measured 12 times in the length and 8 times in the width with equal increments from bottom to top and side to side.
A grid measurement was also done on two leafs, one leaf from a rose that was held dry and a leaf from a rose that was given sufficient water.

2.4.3 Chrysantheums

With help from Deliflor 10 different races of chrysantheums were measured.
All these races where given three different treatments: no viroids or mold, Infected with stunt viroid or infected with Verticillium (mold).
On each of these thirty possible combinations two plants were measured. On each plant one leaf is measured. Where every measurement consists of 9 measurements in the length and 6 in the width of the leaf with equal increments from bottom to top and side to side.

2.5 Data processing

2.5.1 Labview

To control the experimental setup labview is used. In this program the integration time and the number of samples per measurement are controlled and the calibration measurements are preformed.
For the data processing labview is used first. The first part of the data processing with labview is using the raw calibration data from the previous labview program to correct for the transmission and reflections in the fibers. Next another labview program (see Fig. A2 attachment 2) is used to combine the calibrated spectra in one file and to analyze the fluorescence and reflection spectra from the measurements:

In the fluorescence spectra the following parameters are calculated:

- The amplitude of the two maxima at 685nm and 735nm
- The properties of the fits (fits are done with 3 Gaussians) thru the spectra

In the reflection spectra the following parameters are calculated:

- The fit of the scattering
- The minimum at 660nm en the maximum 550nm
- A measure for the absorption calculated by taking the difference between the scattering and the reflection (Surface between the fit of the scattering and the reflection spectra, as explained in 1.4)
- The absorption at the excitation wavelength
- The intensity on 850nm

Next it is investigated whether one or more of the parameters or a ratio between the parameters can be correlated to the conditions of the plant.

2.5.2 Matlab

In Matlab a program was written (see attachment 3) with which PCA can be preformed on the measurements. Matlab was used because in the statistics toolbox from matlab the operant “princomp” can easily do a PCA on any data set. First the program reads the data and removes faulty measurements. Then PCA is performed on this clean data. The data is further analyzed with a rank-sum test and correlation coefficients if the data can’t be visually separated to see if the data shifts.
With another program (see attachment 3) in matlab a graphical representation of a leaf is be made so the course of the data becomes visible.

3. Results of the (F)DPS measurements

The results are organized by the type of plant. Measurements were taken on roses, tomato plants, calathea's, chestnut tree leafs and chrysanthemums. The measurements are described chronologically were findings in earlier measurements are adapted in later measurements.

3.1 Measurements on two roses

A number of measurements were done on two roses to get an idea of the measurements.

3.1.1 Variation in one point of a leaf

In the laboratory measurements were taken on the leaf of a rose. For every set of measurements the placement of the probe on the leaf wasn't changed. The maximum deviations are derived from the measurements (See table A1 Attachment 4)

Table 1: Maximal standard deviation on the processed data from the reflection spectra

Average at 850 nm	2 %
The difference between the scattering and reflection (Surface between the scattering fit and the spectra)	2 %
Maximum at 550 nm	3 %
Minimum at 662 nm	4 %

The measurement shows a descending trend in the intensity of the fluorescence (See Fig. 19), this is because a measurement is done with an intense blue light, this breaks down the chlorophyl. It does seem that the chlorophyll content recovers over time.

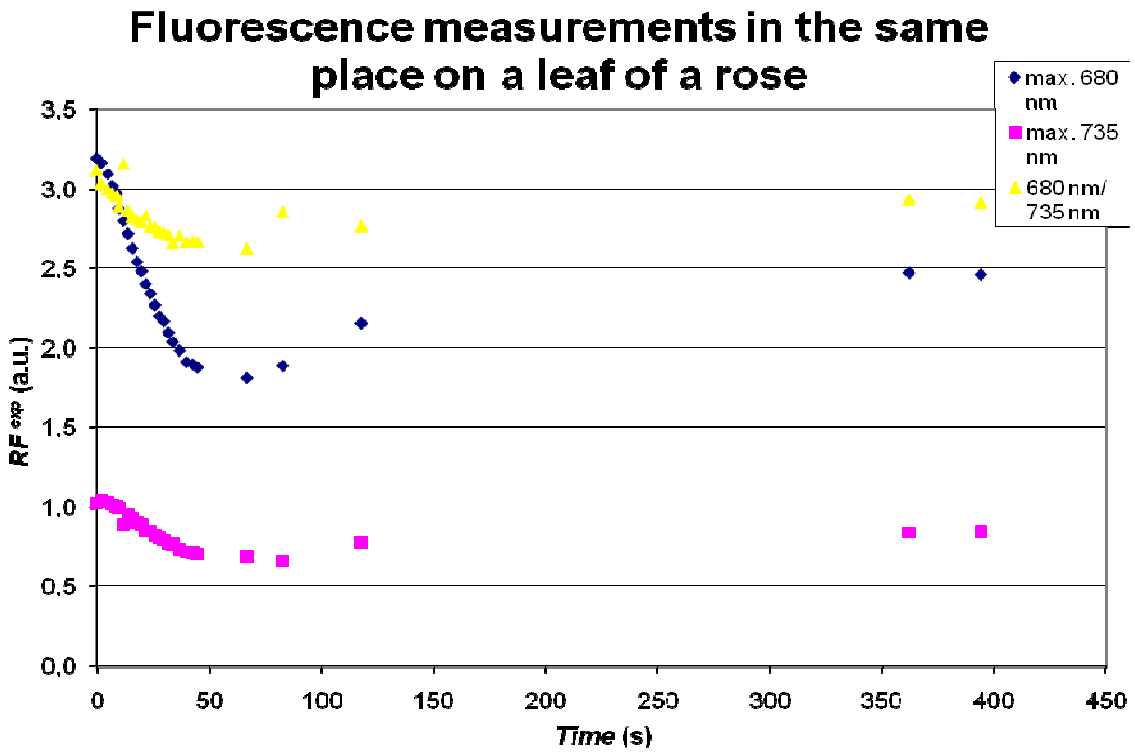

Fig. 19: Fluorescence measurements on the same place on a leaf of a rose

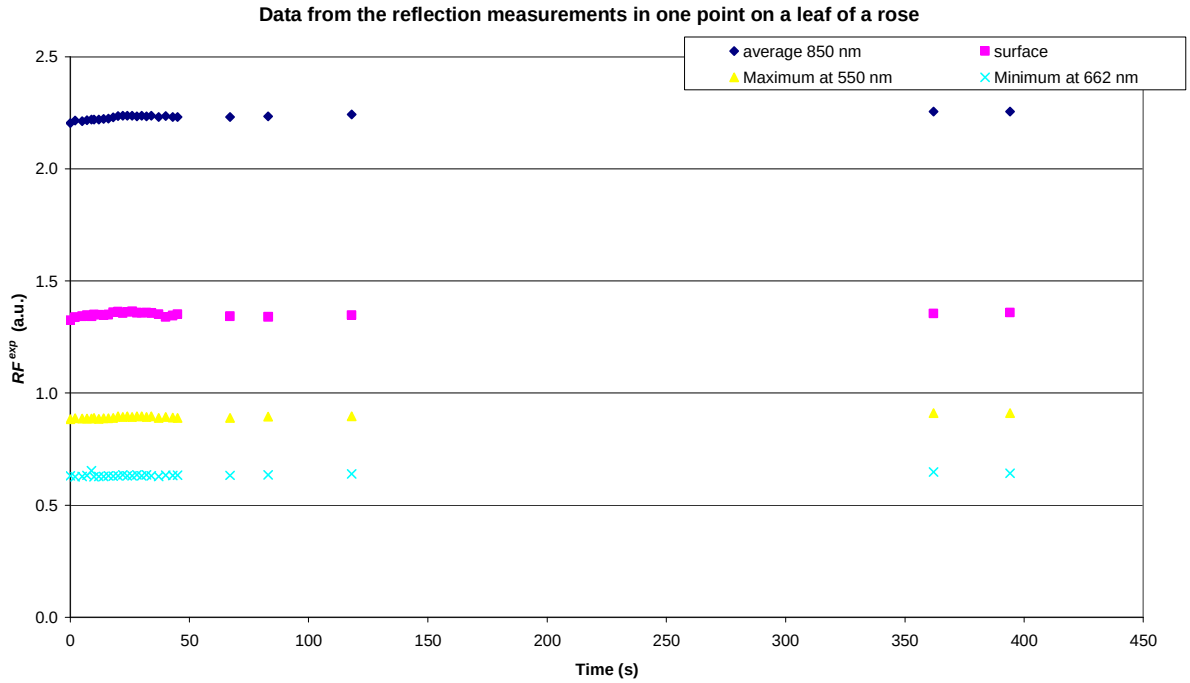

Fig.20: Reflection measurements on the same place on a leaf of a rose

The reflection measurements don't change depending on the measurement (See Fig.20). Even after waiting a few minutes no significant change is detected. The small difference can be explained by noise caused by the limited amount of photons collected by the spectrographs.

3.1.2 Influence of the background

In earlier measurements it seemed that the measurements were influenced by the background. To investigate this effect measurements are taken on three different backgrounds: a black (Spectralon Labsphere SRS-99), a gray (Spectralon Labsphere SRS-50) and a white ((Spectralon Labsphere SRS-02) background. The measurements are taken as described in 2.3.2 . Every measurement consists of a series of measurements on one point of a leaf with alternating backgrounds.

The measurement numbers in Fig. 21-24 each represent a different leaf. In figure 21 the measured intensity at 850 nm is plotted for different leafs and backgrounds. The effect of the background becomes apparent as the intensity increases with a lighter background. The same effect is encountered in figure 22.

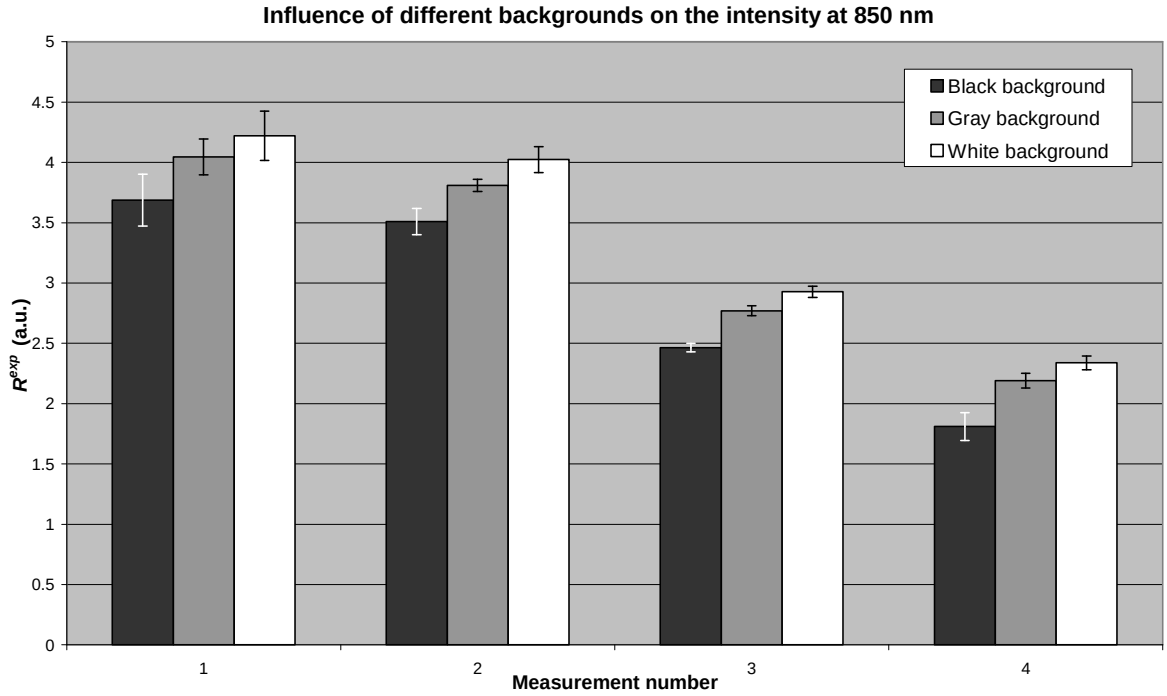

Fig. 21: The influence of different backgrounds on the measured intensity at 850 nm in the reflection spectra.

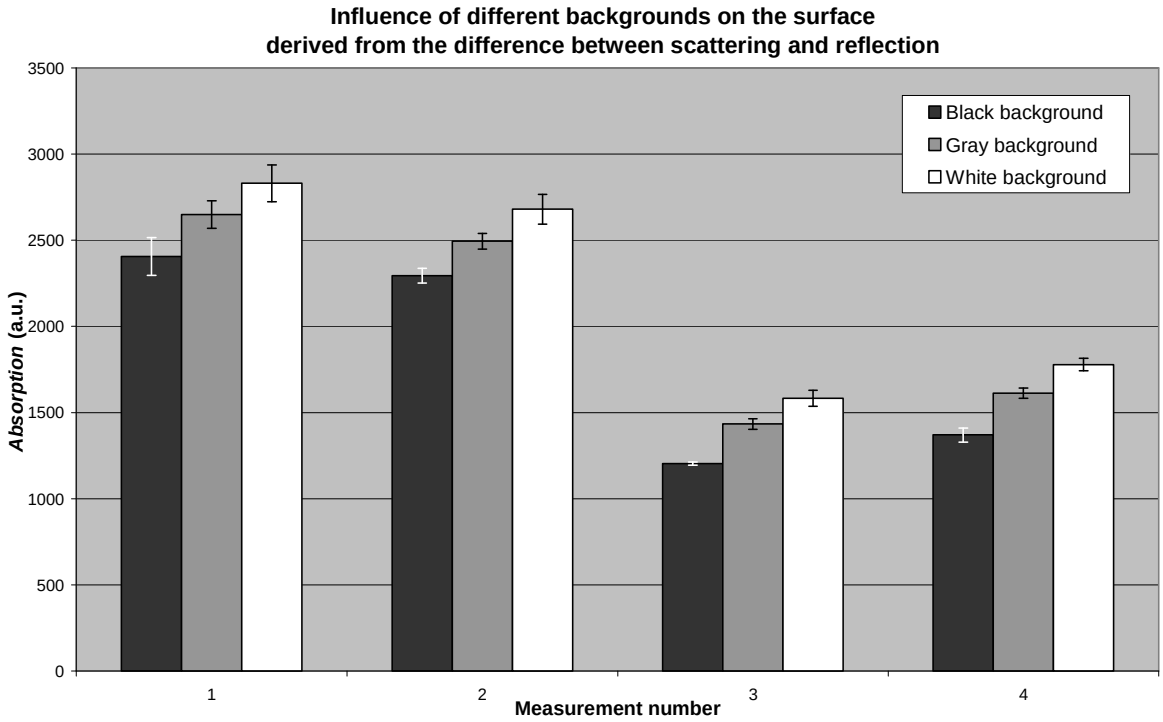

Fig. 22: The influence of different backgrounds on the measured surface in the reflection spectra.

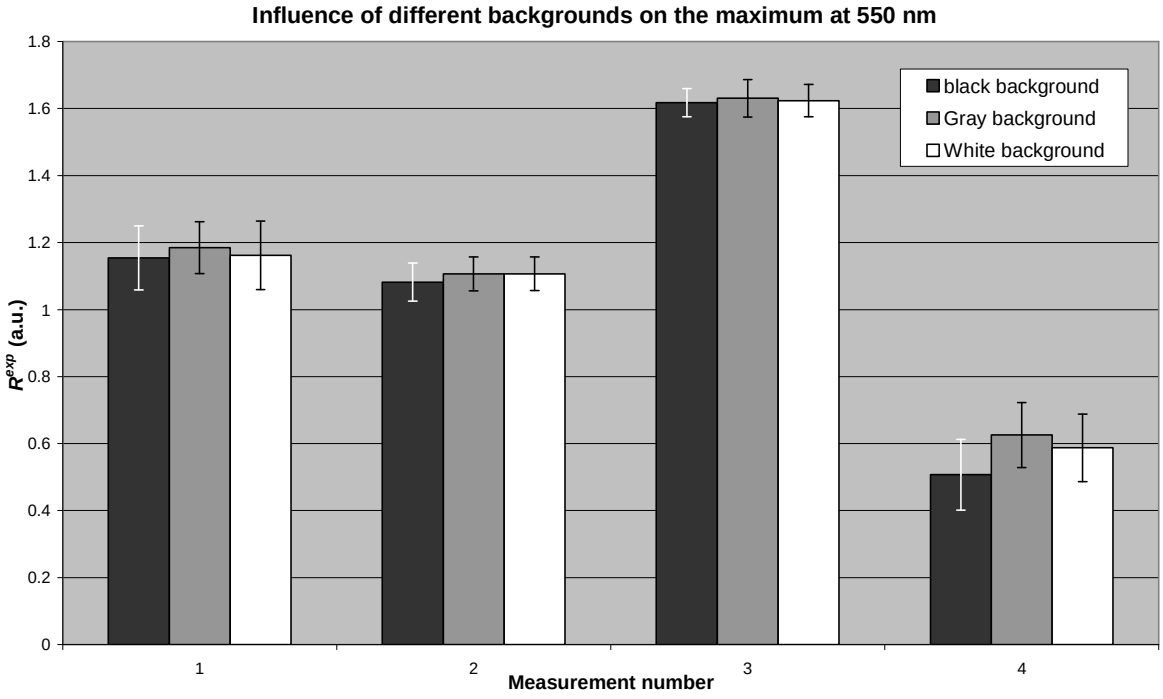

Fig. 23: The influence of different backgrounds on the measured maximum at 550 nm in the reflection spectra.

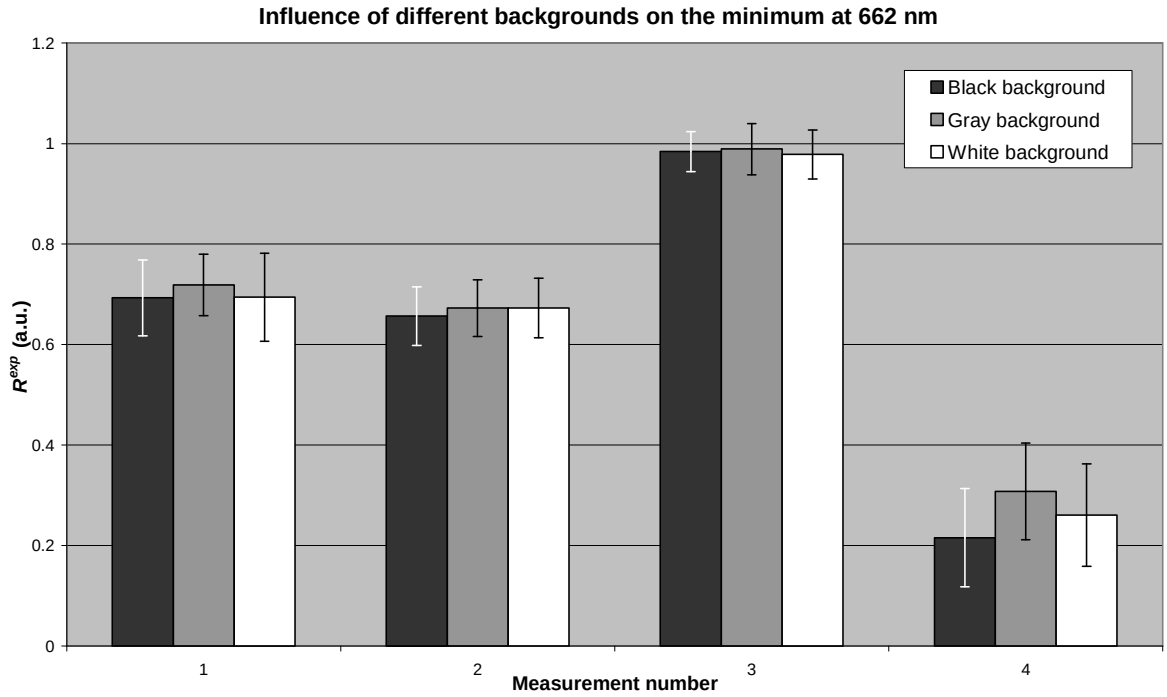

Fig. 24: The influence of different backgrounds on the measured minimum at 662 nm in the reflection spectra.

In figure 23 & 34 the values of the intensities at the maximum at 550 nm and the minimum at 662 nm are shown. The signal doesn't differ significantly because of the different backgrounds.
Concluding: the background influences some of the measured aspects of the spectra, so in the next measurements it is chosen to consistently use a black background to eliminate this effect.

3.1.3 Placement on the plant

Twenty-two leafs of a rose are measured to see how the measurements vary. The difference between scattering and reflection are calculated because this is a good measure for the variance in the measurements.

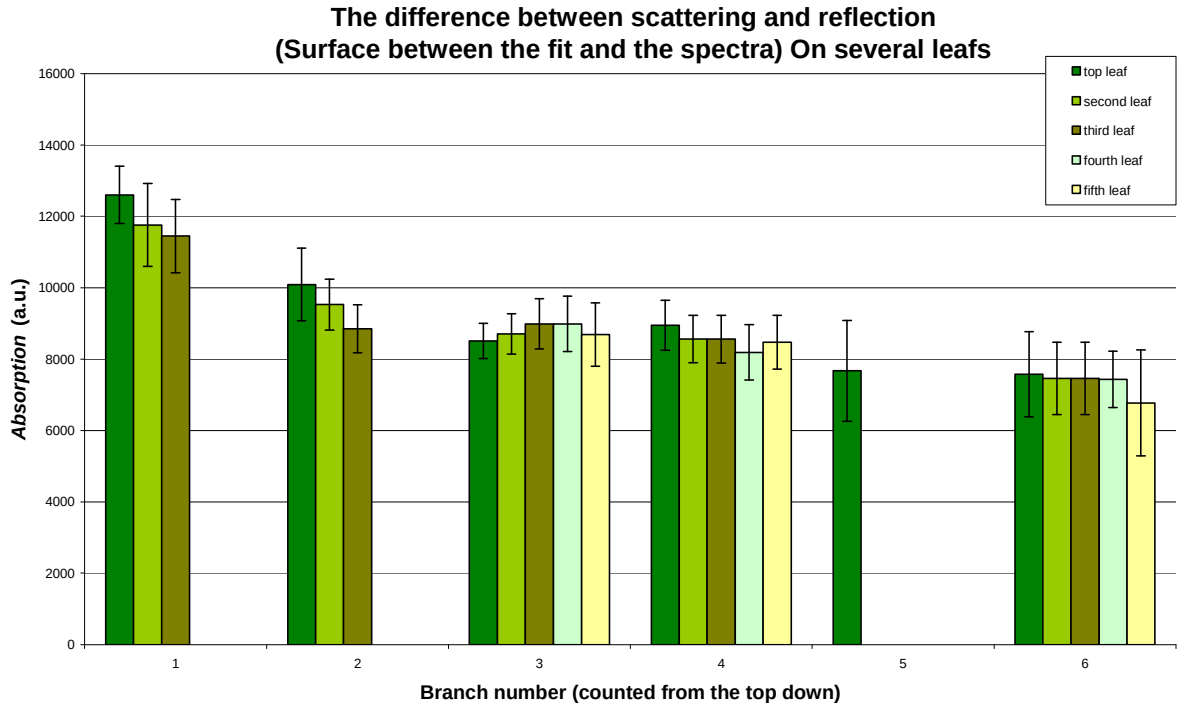

Fig. 25: The influence of the leaf orientation on the plant on the total absorption

In figure 25 every bar represents an average of the calculated surface between the scattering and reflection from several measurements on one leaf. Different sets of leafs have been measured on different branches and have been plotted accordingly in figure 25.
Visually no significant changes in the spectra were detected during the measurements. When the measurements are processed into Figure 25 it becomes apparent that this isn´t the case most of the time. Especially the absorption in the upper leafs deviates from the rest of the measurements. A hypothesis of the cause would be the fact that the top leafs haven't fully grown yet and that this results in a higher absorption. Even in the lower regions of the plant some shifts are encountered so in future measurements the place of the leaf is carefully chosen taking extra care of choosing fully grown leafs.
For more data see table A2 Attachment 5.

3.3 Measurements on tomato plants

The First measurements in a greenhouse were taken on leafs of tomato plants.
The tomato plants were measured three separate times. The main analysis of the data was performed by PCA. The PC's are plotted in graphs to give a representation of the data. If the points in a graph separate and two different clusters are formed then the data differs from each other which would prove that DPS is a measurement technique capable of measuring these different conditions.
Measurements were done on 17-03-2009 & 07-04-2009 & 28-04-2009

The Labview-data (See Attachment 8) didn't show a clear difference between dry and wet plants. The PCA data looks like the following:

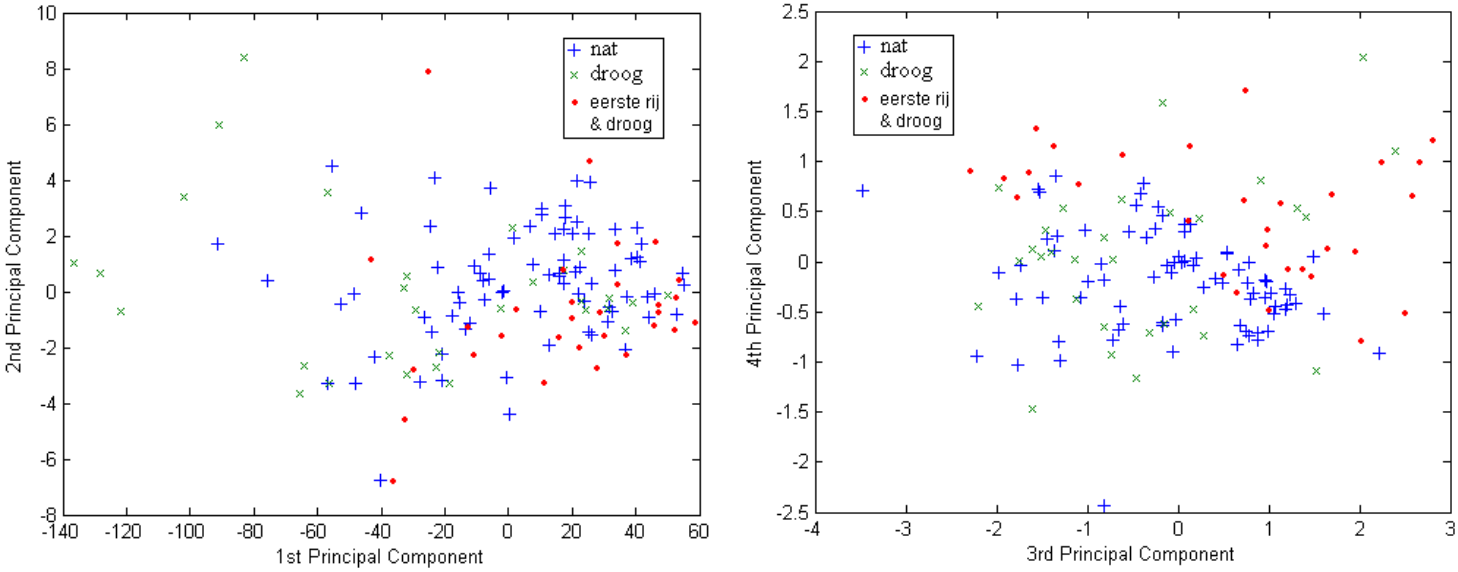

Fig. 29: PCA of the fluorescence spectra of tomato plant leafs measured on 17-03-2009

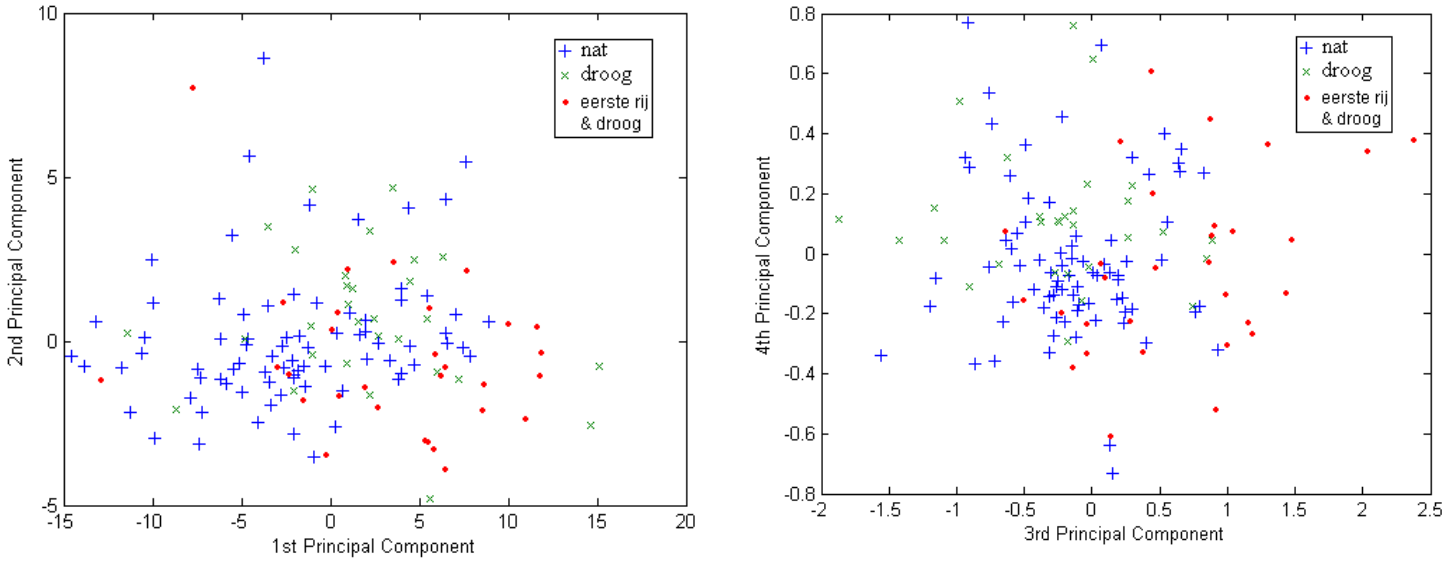

Fig. 30: PCA of the reflection spectra of tomato plant leafs measured on 17-03-2009

Fig 29 & 30 show the PCA data graphically. There is no clear difference between the measurements. Only the reflection data of the 3rd PC of the first row of plants (dry) clearly differ from the rest of the data points. The first row receives more light because of its placement in the greenhouse. Which is probably the difference measured.

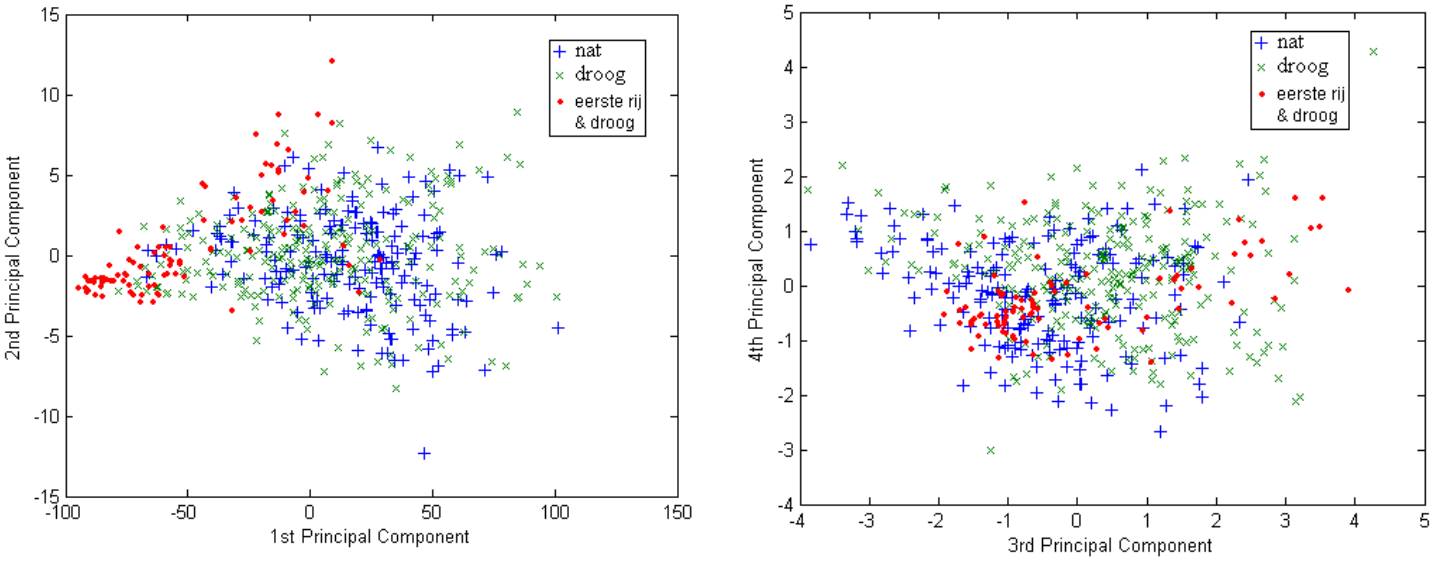

Fig. 31: PCA of the fluorescence spectra of tomato plant leafs measured on 07-04-2009

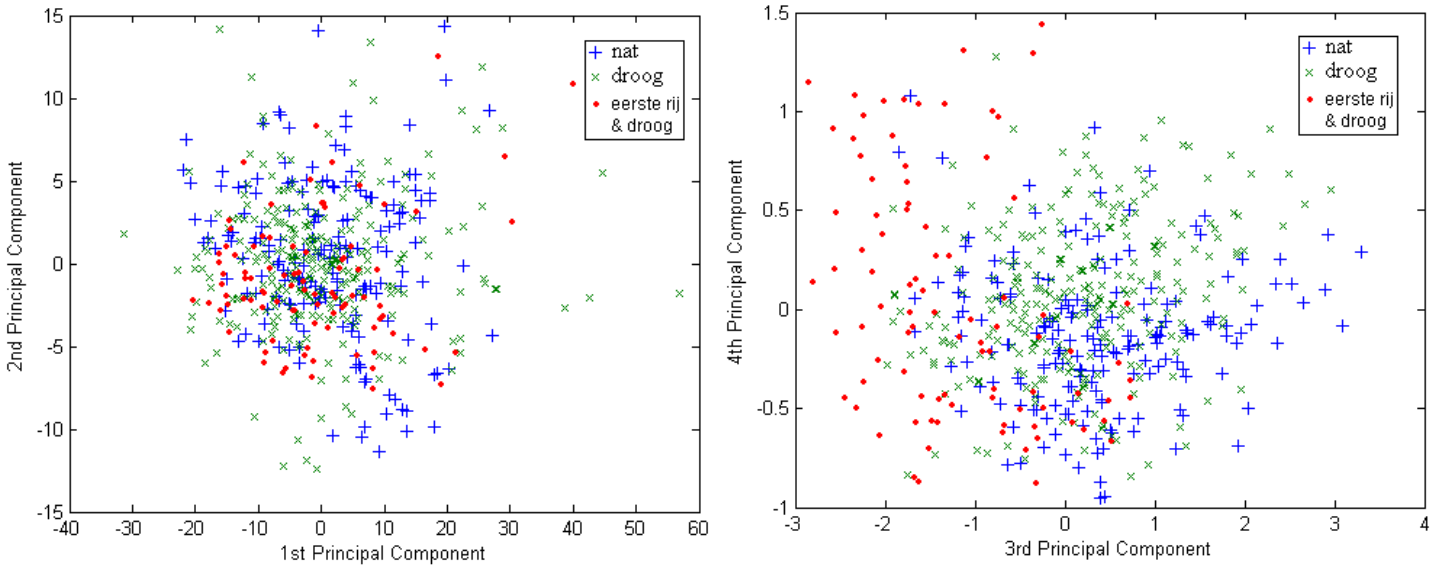

Fig. 32: PCA of the reflection spectra of tomato plant leafs measured on 07-04-2009

The second time in the greenhouse another series of measurements was done. The spectra were analyzed with PCA and plotted in figure 31 and 32. No clear separation between dry and wet tomato plants is visible in the graphs. There is however a clear separation between the first row and the other rows just as seen in the first measurements. The difference is now also apparent in the first PC of the fluorescence.

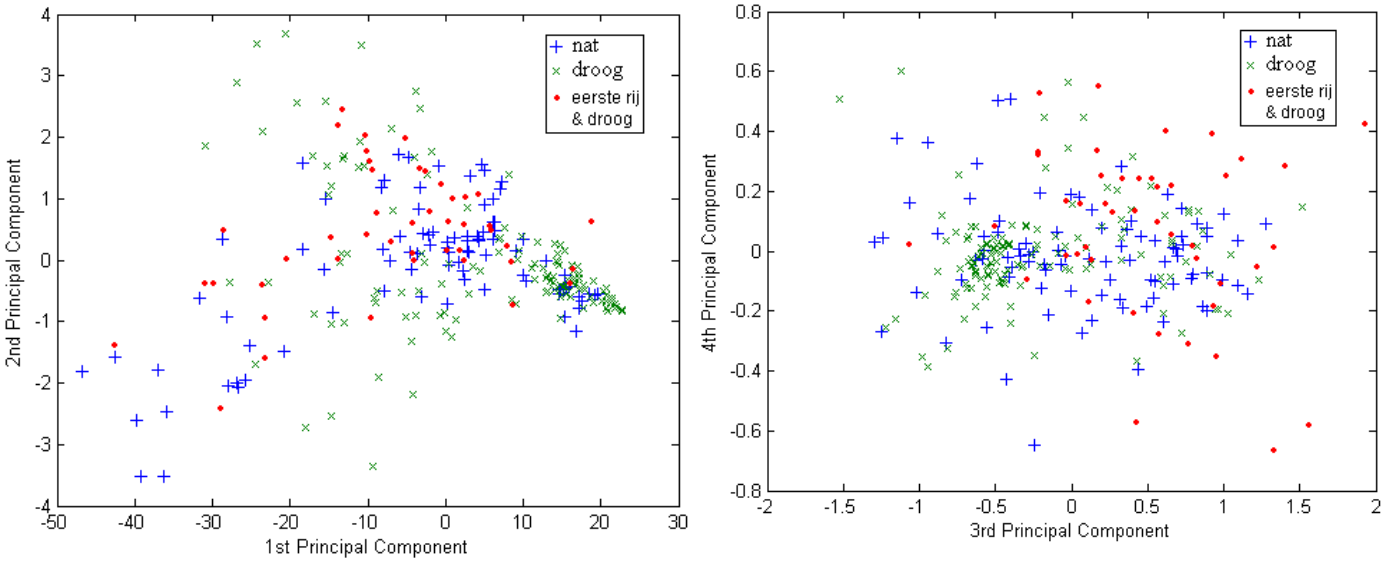

Fig. 33: PCA of the fluorescence spectra of tomato plant leafs measured on 28-04-2009

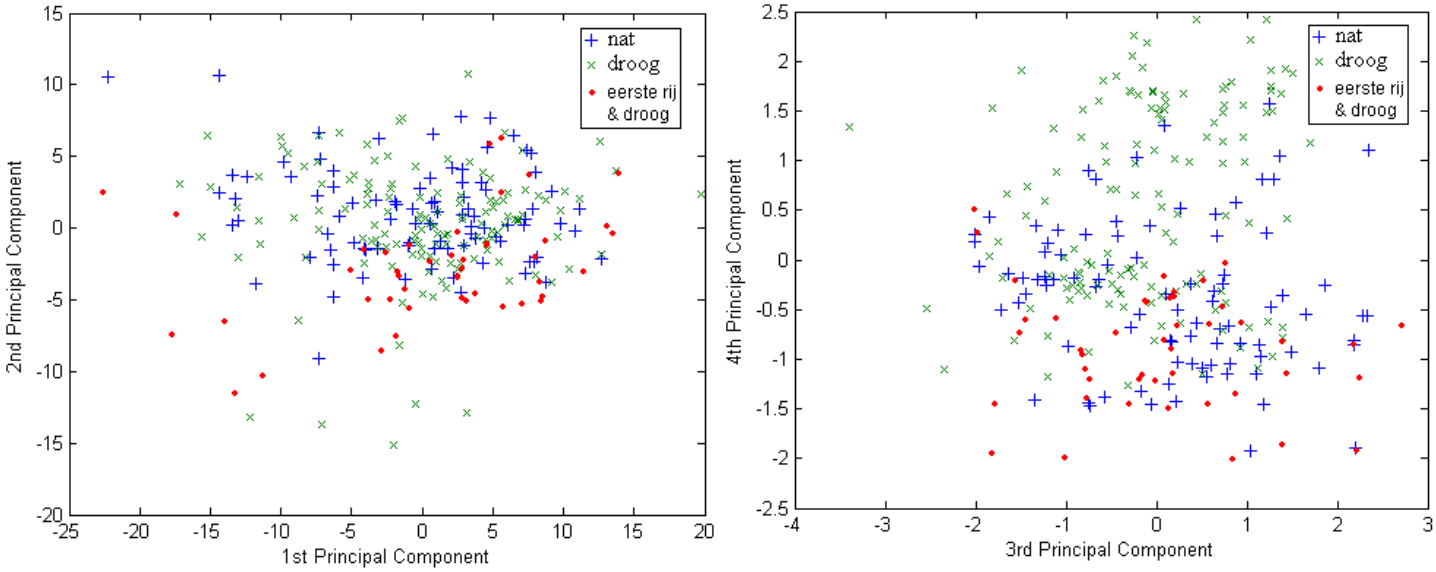

Fig. 34: PCA of the reflection spectra of tomato plant leafs measured on 28-04-2009

The third time in the greenhouse another series of measurements was done. The spectra were analyzed with PCA and plotted in figure 33 and 34. In the third data set the difference between the first row and the rest of the data isn't significant any more. This is possibly because the weather was much cloudier and the plants didn't receive very much light and so the light wasn't very different between the first row and the other rows.
For the first time a difference is visible between the dry and wet tomato plants in the 4th PC.

A Rank-sum test was performed on the PCA data (See table A3 & A4 in attachment 7 for more insight in the data):

Table 3: PCA on fluorescence spectra with rank-sum test data

	17-03-2009		07-04-2009		28-04-2009	
Fluores-cence	Percent explained (%)	p	Percent explained (%)	p	Percent explained (%)	p
1st PC	99.45	0.004	99.2	$8 \cdot 10^{-4}$	99.2	$6 \cdot 10^{-6}$
2nd PC	0.35	0.2	0.56	0,1	0.53	0.01
3rd PC	0.095	0.1	0.12	$2 \cdot 10^{-11}$	0.18	$1 \cdot 10^{-8}$
4th PC	0.028	0.1	0.054	$8 \cdot 10^{-5}$	0.016	0.2

Table 4: PCA on reflection spectra with rank-sum test data

Reflection	17-03-2009		07-04-2009		28-04-2009	
	Percent explained (%)	p	Percent explained (%)	p	Percent explained (%)	p
1st PC	87.1	0.001	86.3	0.05	72.8	0.5
2nd PC	11.0	0.05	12.4	0.4	23.7	0.06
3rd PC	1.05	0.6	0.85	0.08	1.6	0.2
4th PC	0.17	0.002	0.13	$7 \cdot 10^{-8}$	1.5	$2 \cdot 10^{-17}$

A few interesting observations were made in the with the Rank-sum test analyzed PCA data (see table 3 & 4):

1. The difference in the first PC (the amount of fluorescence) of the fluorescence spectrum increased in time.
2. There is a shift between wet and dry measured plants in the third PC in the fluorescence spectrum but this difference decreases in the last measurement although it remains substantial.
3. The first three PC's in the reflection spectra don't show any significant difference between the wet and the dry plants. The first PC seems to separate in the first measurement but fails to do the same in the following two.
4. The fourth PC in the reflection spectra however gives a definite separation of the wet and dry plants. Which increases after every measurement as expected.

Now that there is a trend visible in the principal components what do the principal components look like and are they the same? To look at this the first four PC's are shown in Fig. 35 trough 40. The first two PC's of the fluorescence spectra and the first three PC's of reflection spectra are the same. However higher PC's are different and are harder to compare so even though a large difference was shown in the 4th PC this PC changes and it is not certain whether this PC explains the same variance every time. The same holds true for the 3rd PC of the fluorescence spectra.

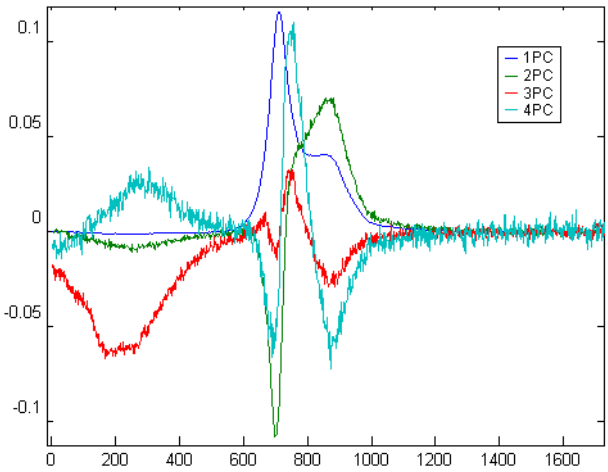

Fig 35: Fluorescence spectra extracted with PCA, tomato's 17-03

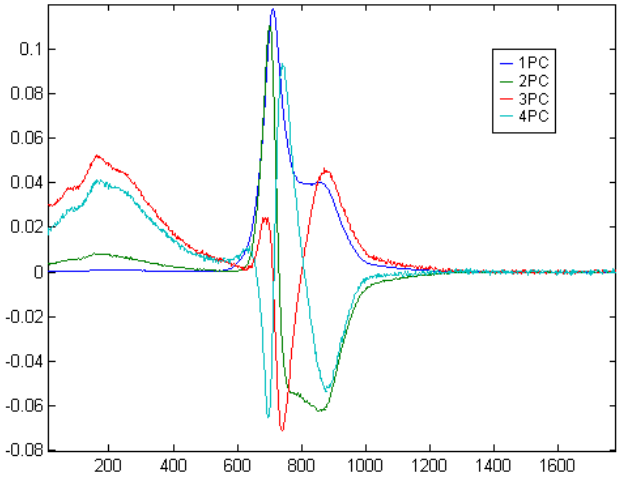

Fig 36: Fluorescence spectra extracted with PCA, tomato's 07-04

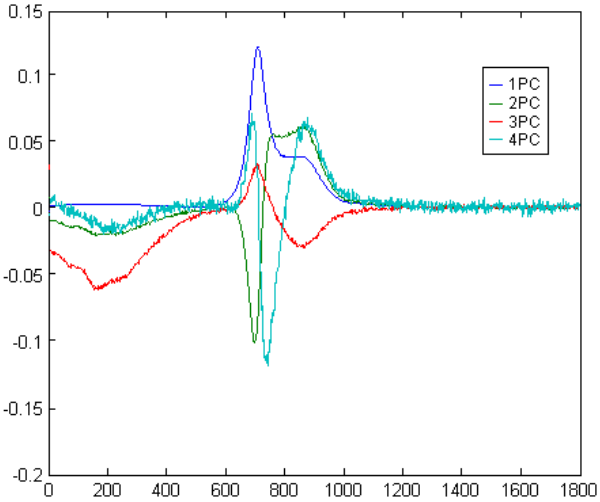

Fig 37: Fluorescence spectra extracted with PCA, tomato's 28-04

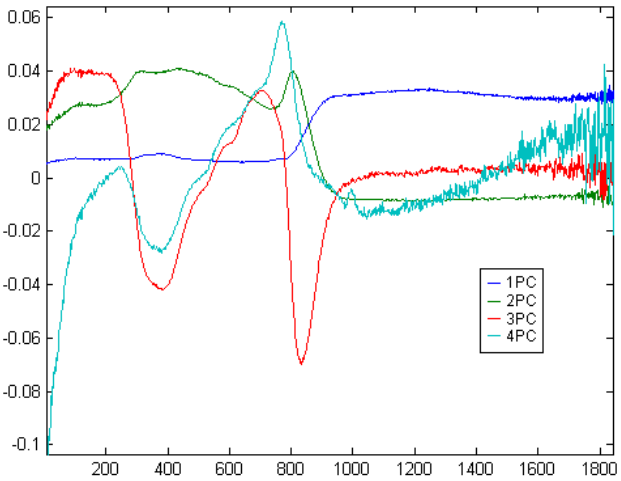

Fig 38: Reflection spectra extracted with PCA, tomato's 17-03

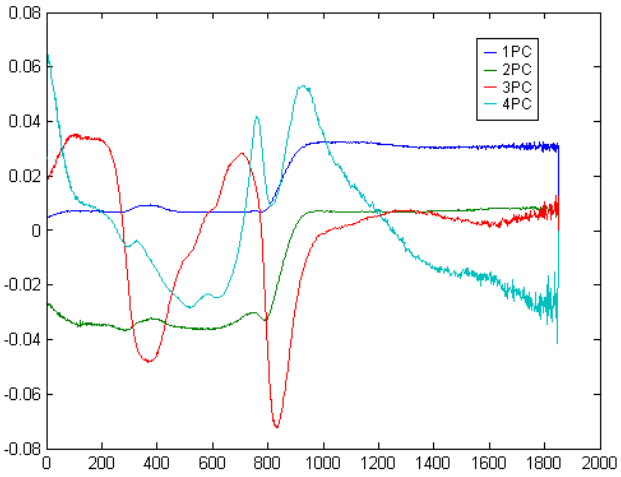

Fig 39: Reflection spectra extracted with PCA, tomato's 07-04

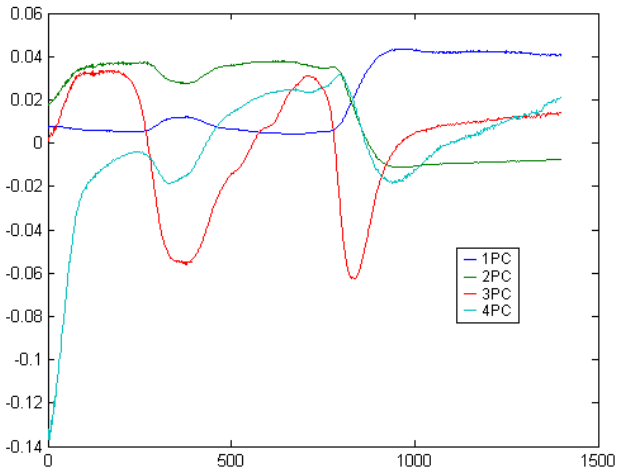

Fig 40: Reflection spectra extracted with PCA, tomato's 28-04

3.4 Chrysanthemums

After the tomato plants chrysanthemums have been measured. The chrysanthemums are divided in ten races with three different treatments. The first of the three different treatments is a reference which means the plants are grown normally. The plants that are given the second treatment are infected with a viroid called Stunt and the plants that are given the third treatment are infected with a mold called Verticilium. The question in the measurements is if a difference can be found between the reference and the infected plants.

3.3.1 In the greenhouse

In the greenhouse one leaf was measured per plant and two plants were measured per race and treatment. The measurements on the leaf consisted of nine measurements from bottom to top and six measurements from side to side of the leaf. Every plant of the ten races either had no infection, were infected with the mold verticilium or infected with the stunt viroid.
PCA was performed on this data too Fig. 41 shows the 3rd and 4th PC data from the fluorescence spectra.

1 + 2 x

3 · 4 *

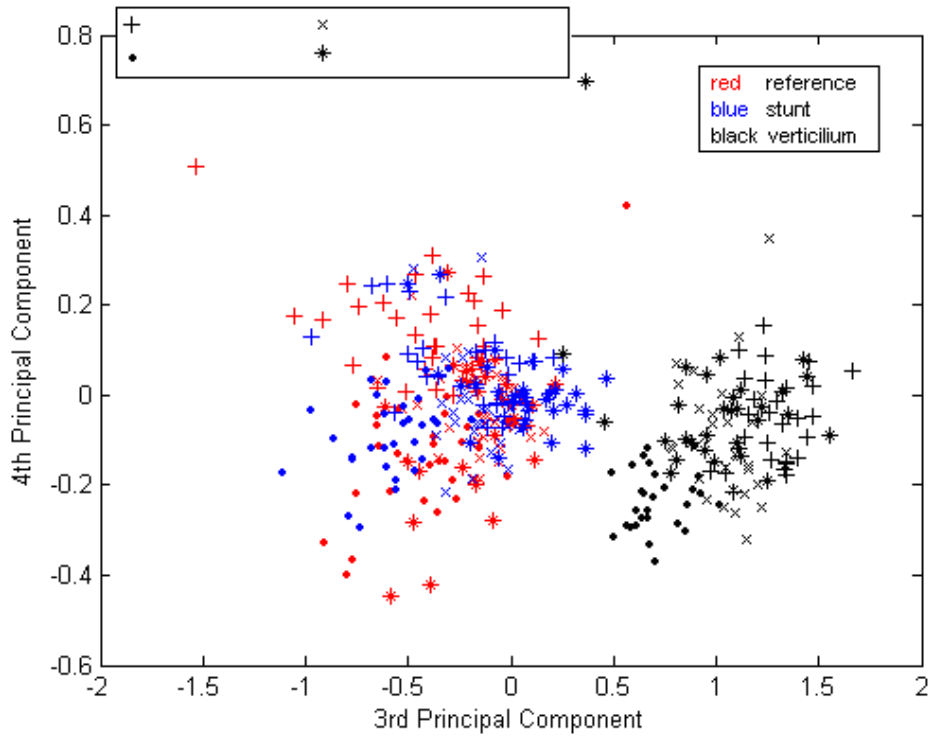

Fig. 41: Graphical representation of the 3rd and 4th PC of the fluorescence spectra of chrysanthemum leafs

There was a clear difference between the reference and the chrysanthemums infected with Verticilium. Especially the first five measured races showed a big difference, see table 5. This corresponds to how susceptible the plants are to the mold.
Differences were found in other components but the difference didn't seem to correlate just as well with the susceptibility and the differences measured were also smaller although significant differences were found between the plants.

Table 5: The p value's found from the difference between the Verticilum and reference plants for the different races combined with the visually determined susceptibility

Race name	visual susceptibility	p (%) 3e PC Fluorescence	p (%) 4th PC Reflectance
1	neutral	$4 \cdot 10^{-11}$	$5 \cdot 10^{-11}$
2	susceptible	$3 \cdot 10^{-11}$	$3 \cdot 10^{-11}$
3	susceptible	$5 \cdot 10^{-11}$	$4 \cdot 10^{-11}$
4	susceptible	$3 \cdot 10^{-11}$	$3 \cdot 10^{-11}$
5	susceptible	$2 \cdot 10^{-10}$	$1 \cdot 10^{-4}$
6	resistant	$4 \cdot 10^{-5}$	$4 \cdot 10^{-2}$
7	neutral	$1 \cdot 10^{-6}$	1
8	susceptible	$1 \cdot 10^{-6}$	$6 \cdot 10^{-1}$
9	resistant	$8 \cdot 10^{-7}$	$2 \cdot 10^{-9}$
10	neutral	$7 \cdot 10^{-2}$	$3 \cdot 10^{-7}$

The visual susceptibility is determined by the farmer looking at the plant and determining how affected the plant is in comparison to other healthy plants. A farmer will look at the number of branches, height, leaf size and leaf color. The p values are calculated with a rank-sum test.
For the stunt viroid a difference was found in the 4th PC. When the Visually established susceptibility is placed next to the rank sum test values they seem to correlate. But the same data doesn't correlate to the measured susceptibility. So the measured data is the effect of the stunt viroid and not the stunt viroid self.

Table 6: The p value's found from the difference between the Stunt and reference plants with a measured and visually determined susceptibility rating

	Measured	Visual	Stunt (viroid)
Race name	*Susceptibility*	*Susceptibility*	p (%) 4th PC Reflectance
1	neutral	Very susceptible	$5 \cdot 10^{-11}$
2	susceptible	Very susceptible	$7 \cdot 10^{-9}$
3	Very susceptible	Very susceptible	$5 \cdot 10^{-6}$
4	resistant	resistant	$5 \cdot 10^{-3}$
5	neutral	susceptible	$5 \cdot 10^{-8}$
6	very susceptible	susceptible	$1 \cdot 10^{-8}$
7	resistant	neutral	$2 \cdot 10^{-1}$
8	susceptible	very susceptible	$1 \cdot 10^{-3}$
9	neutral	very susceptible	$6 \cdot 10^{-10}$
10	susceptible	resistant	$3 \cdot 10^{-3}$

No clear differences were found in other PC's between the reference and affected plants that also correlated to the susceptibility data (See Attachement 8 Table 12).
However, big differences where found when comparing al the races together See table 7

Table 7: The p-values of the data from the six most significant PC's

PC	Reflection Verticilium	Fluorescence Verticilium	Reflection Stunt	Fluorescence stunt
1st	$1\cdot10^{-19}$	0.07	$1\cdot10^{-35}$	$3\cdot10^{-5}$
2nd	$8\cdot10^{-14}$	0.01	$2\cdot10^{-5}$	$8\cdot10^{-5}$
3rd	$9\cdot10^{-4}$	$1\cdot10^{-7}$	$6\cdot10^{-11}$	0
4th	0	0.1	$4\cdot10^{-6}$	$1\cdot10^{-8}$
5th	0.5	$2\cdot10^{-30}$	$6\cdot10^{-20}$	$2\cdot10^{-26}$
6th	0	$3\cdot10^{-4}$	0	$2\cdot10^{-5}$

According to the rank-sum test there is a big separation in the 6th PC of the reflection spectra for both the stunt and the Verticilium. The spectra are shown in Fig. 42 & 43.

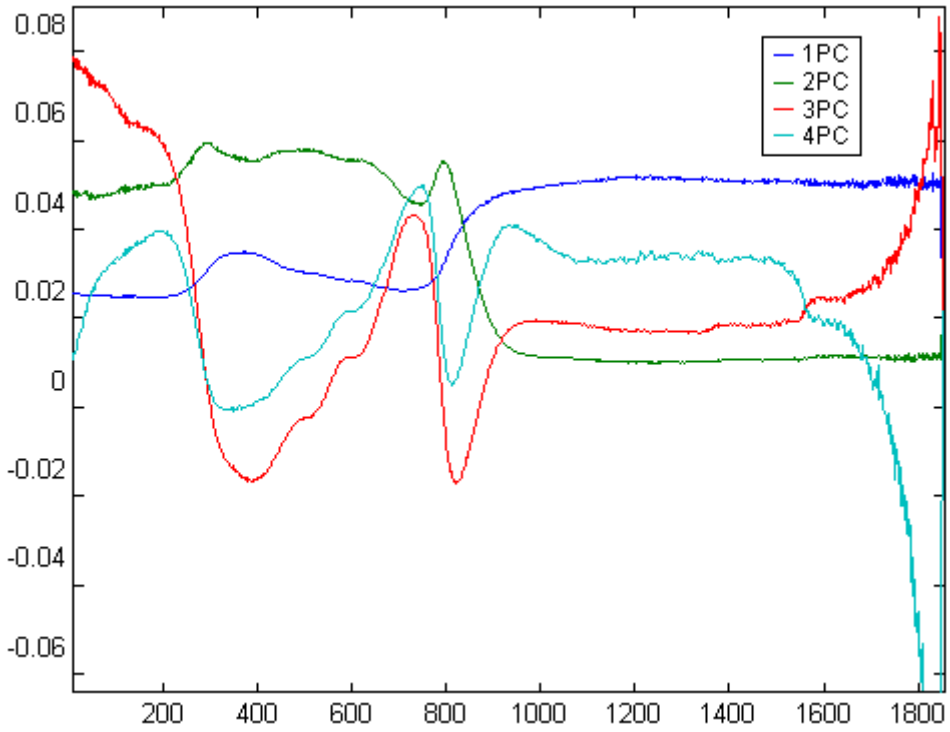

Fig 42: Reflection Components 1 trough 4

The fourth component looks like the fourth component measured with the tomato's looks like (the third measurement) the spectrum looks like that of chlorophyll a or b but it could also be a change in the spectra. Which immediately shows the weakness of PCA. That is that differences can be found but they're almost impossible to explain.
The same holds true for the other PC's (see Fig. 43) where differences were found. It could be that the measurement only show a difference because of a difference in sunlight the plants received or because of the order the plants were measured.

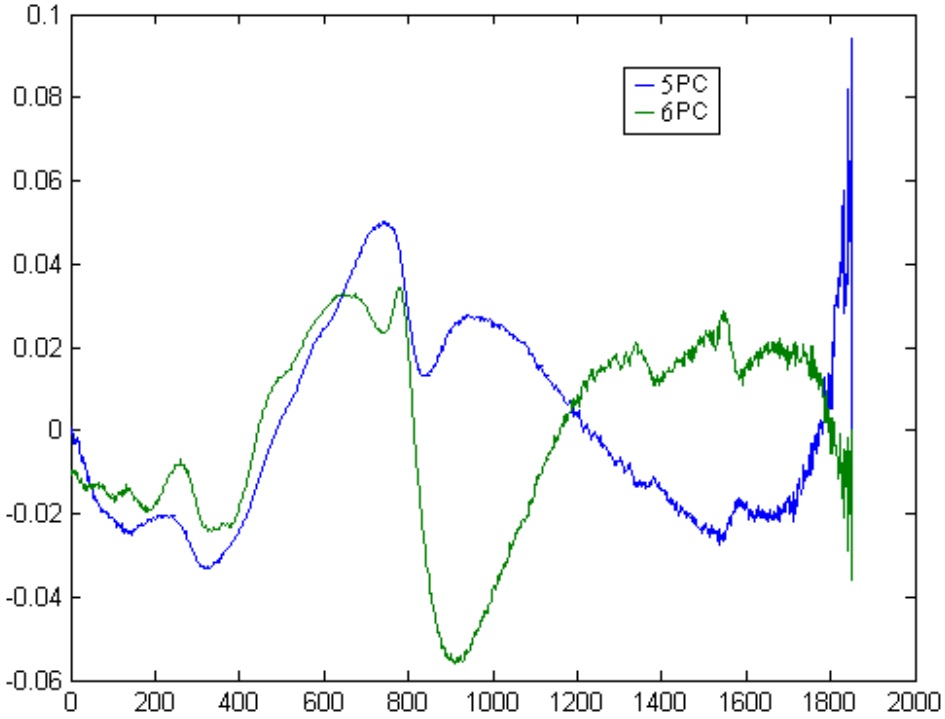

Fig 43: Reflection Components 5&6

More research should be done into the spectra of plants so different parameters can be extracted and the differences found can be related to the infections a plant might have. It is clear however that (F)DPS can measure differences in the state of plants. However the accuracy with which it differs isn't very high. With these infections the plants react much more. This means the farmer can find the disease much faster but the (F)DPS device can to.

3.5 Roses

In the same greenhouse were the tomato plants are grown roses are also grown. The roses are also under different water related stresses and are cut differently. One leaf per rose is measured. Two roses of every unique treatment are measured. This data was again processed with PCA. Fig 44 & 45 show the most interesting data.

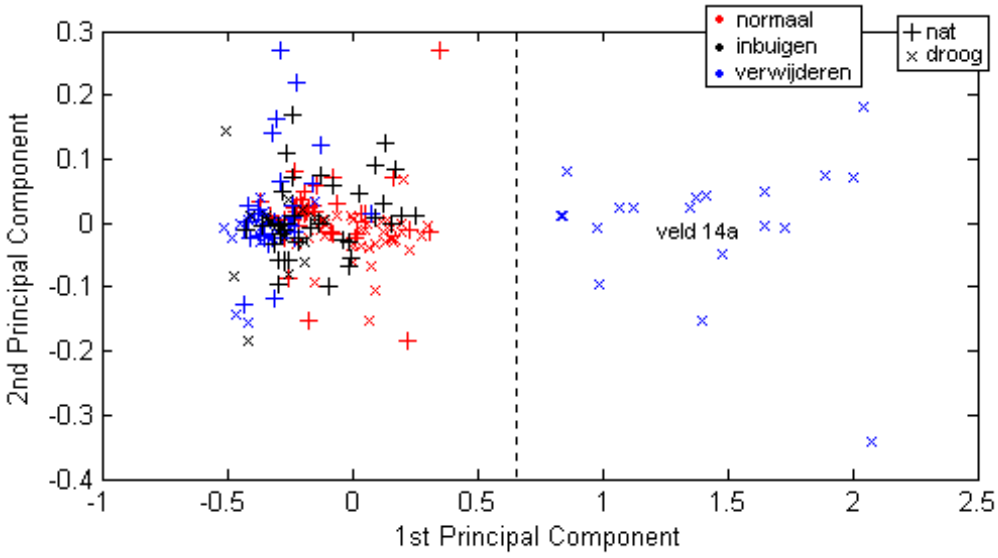

Fig. 44: The data from the PCA of the fluorescence spectra

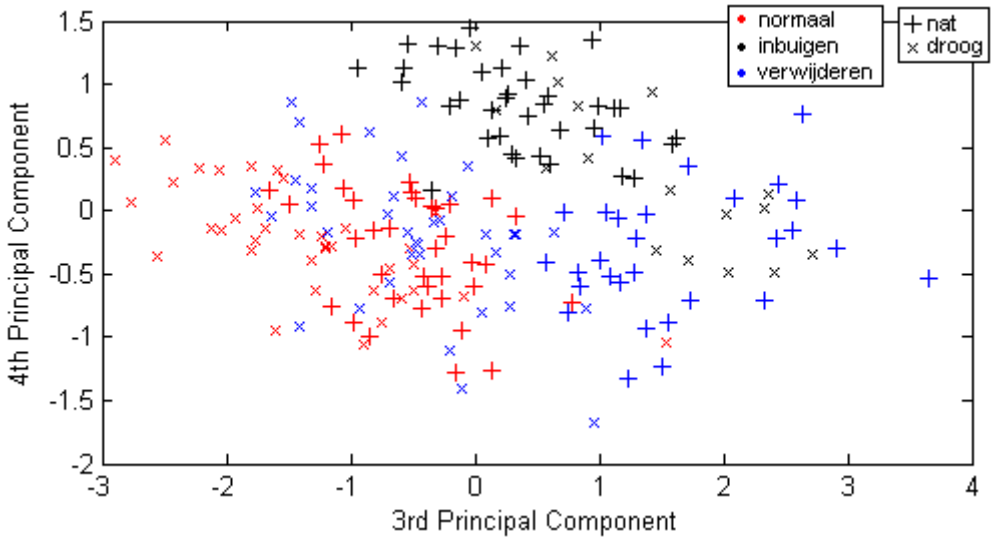

Fig. 45: The data from the PCA of the reflection spectra

There are clear differences in the first PC of the fluorescence spectra and the 3rd PC of the reflection spectra. The difference in the treatment of the ground shoot is most apparent in both the first PC of the fluorescence spectra and the 3rd PC of the reflection spectra.

4. Discussion & Recommendations

The measurements influence the measurement itself with the light it emits. This affects the chlorophyll so a lot of measurements on one point were avoided. Since a probe of 400 μm is used the measurements are done trough the whole leaf and the background affects the measurements so a black background was used. The first measurements in the greenhouse were done on tomato plant leafs. These leafs have an oily layer (cuticle) on the surface which sticks to the probe and has to be cleaned of with a cloth and 70% alcohol.
In the Tomato's a definite difference is found in many different principal components but in the beginning when the difference is the smallest the significance of the difference is never better than a p of 0.001 . Chrysanthemums were measured next. These were infected with a stunt viroid and a verticilium mold. Significant differences were found for both plants but only the data from the verticilium seemed to correspond to the determined susceptibility of the plants. Perhaps by measuring on the stem of a chrysanthemum the difference from caused by stunt can be shown[20]. The roses that were measured in the greenhouse showed a reaction to how they were cut. A clear difference wasn't found between the roses that were held dry and wet. A grid measurement was also performed on roses that were held wet and dry. A difference was found in the variation of the fluorescence of chlorophyll, the plant under water stress shows less variation. Lastly tomato plants were measured for a second time this time correlations were found in a number of principal components and in the slope of the reflection. But no clear difference was found in both the fluorescence and reflection spectra.
A lot of measurements have been performed and it can be said with certainty that the reaction of plants on different environmental stresses can be measured. However the differences measured are not very large and only appear clearly when the plants are wilting. This is not interesting in the agricultural business since the farmers can already see the changes. On top of that the differences originate from statistical analyses.
The principal components calculated vary every time measurements are done. Because of this and the unknown cause of the principal components the measurements and different found are hard to correlate to changes in the leaf. The solution for this would be to create a model for the measured spectra with this model a the spectra can be fitted and the different parameters can be extracted just as in blood hemoglobin and oxy hemoglobin are extracted[3]. With this the found differences can be explained.

Literature

1. Arjen Amelink and Henricus J.C.M. Sterenborg, “Measurement of the local optical properties turbid media by differential path-length spectroscopy” Applied Optics Vol. 43, No. 15, 3048-3054 (2004).
2. Arjen Amelink, Bastiaan Kruijt, Dominic J. Robinson and Henricus J.C.M. Sterenborg, “Quantitative fluorescence spectroscopy in turbid media using fluorescence differential path length spectroscopy” Journal of Biomedical Optics 13(5) 054051, (2008)
3. Martin P. L. Bard, Arjen Amelink, Vincent Noordhoek Hegt, Wilfried J. Graveland, Henricus J. C. M. Sterenborg, Henk C. Hoogsteden and Joachim G. J. V. Aerts, “Measurement of hypoxia-related parameters in bronchial mucosa by use of optical spectroscopy” American journal of respiratory and critical care medicine, Vol. 171 1179-1184 (2005)
4. http://en.wikipedia.org/wiki/Principal_components_analysis#cite_note-8
5. http://www.cs.otago.ac.nz/cosc453/student_tutorials/principal_components.pdf
6. http://en.wikipedia.org/wiki/Mann-Whitney_U_test
7. http://www.stat.auckland.ac.nz/~wild/ChanceEnc/Ch10.wilcoxon.pdf
8. Tzu H. Chow, Khay M. Tan, Beng K. Ng, Sirajudeen G. Razul, Tet F. Chia, Wee T. Poh and Chia M. Tay “Diagnosis of virus infection in orchid plants with high-resolution optical coherence tomography” J. Biomed. Opt., Vol. 14, 014006 (2009)
9. J. L. Baltzer and S. C. Thomas, “Leaf optical responses to light and soil nutrient availability in temperate deciduous trees” American Journal of Botany 92(2), 214-223 (2005)
10. Oregon Medical Laser Center (http://omlc.ogi.edu/spectra/ PhotochemCAD/)
11. K. Kitajima, K. P. Hogan “Increases of chlorophyll a/b ratios during accliation of tropical woody seedlings to nitrogen limitation and high light” Plant, Cell and Environment Vol. 26, 857-865 (2003)
12. Anatoly A. Gitelson, Mark N. Merzlyak and Olga B. Chivkunova “Optical properties and non-destructive estimation of anthocyanin content in plant leaves” Photochemistry and Photobiology, Vol. 74(1), 38-45 (2001)
13. http://en.wikipedia.org/wiki/Chlorophyll
14. http://en.wikipedia.org/wiki/Anthocyanin
15. http://en.wikipedia.org/wiki/Carotenoids
16. http://en.wikipedia.org/wiki/Phycocyanin
17. http://en.wikipedia.org/wiki/NADH
18. http://www.digitalfrog.com/resources/archives/leaf.jpg
19. http://en.wikipedia.org/wiki/Verticillium_wilt - cite_ref-3Department of Crop Sciences, University of Illinois at Urbana-Champaign: "Report on Plant Diseases", 1997 (http://web.aces.uiuc.edu/vista/pdf_pubs/VERTWILT.PDF)
20. Laurie T. Morelli, Paul E. Nelson and R. K. Horst, “Histopathology of the chrysanthemum cultivar bonnie jean infected with chrysanthemum stunt viroid” The American Phytopathological Society Vol. 77, No. 5, 655-660 (1987)
21. E. Heuvelink, “Productie in een fabriekje (de cel) vergt vergaande mate van organisatie” Onder glas, No. 4, 14-15 (April 2007)
22. E. Heuvelink & T. Kierkels, “Verschillende EC tijdens dag en nacht kan gunstig uitpakken: optimale EC afhankelijk van omstandigheden”, Onder glas, No. 10, 21-23 (Oktober 2007)

Explanation of abbreviations

DPS	Differential Path length Spectroscopy
FDPS	Fluorescence Differential Path length Spectroscopy
PC	Principal Component
PCA	Principal Component Analysis
a.u.	Arbitrary units
EC	Electrical Conductivity
CC	Correlation Coefficient

Attachments

1. Treatment schematic

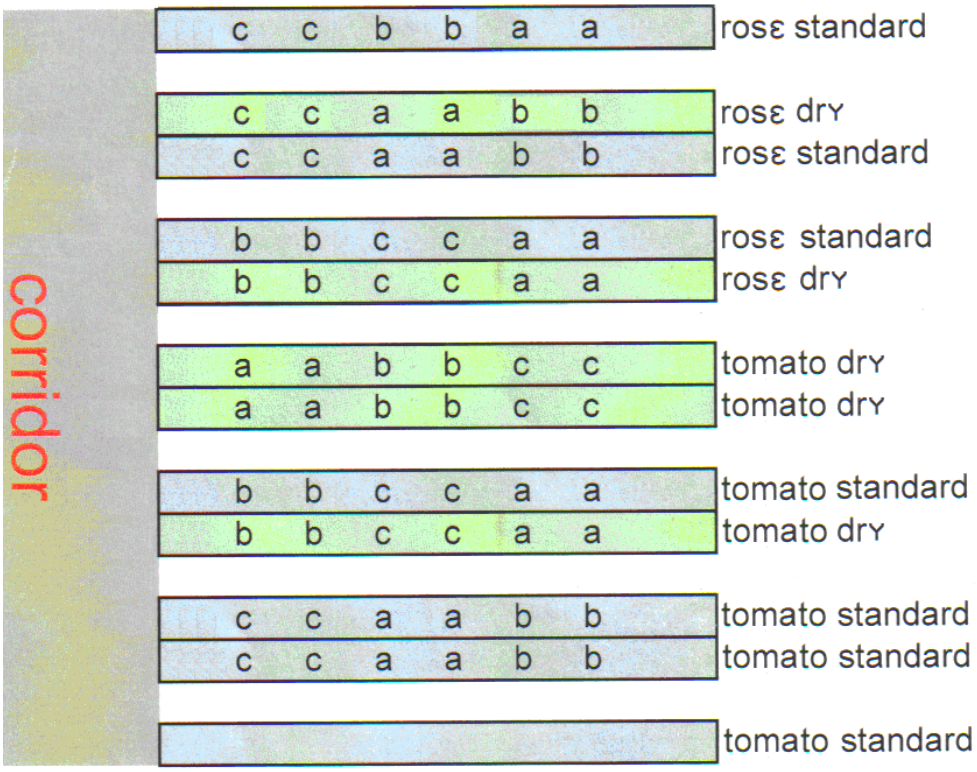

Fig. A1: Schematic of the tomato treatments in the greenhouse

With tomato *a = 3 leafs and 6 fruits (normal)*
b = 3 leafs and 3 fruits
c = 1.5 leafs and 6 fruits

With rose *a = normal*
b = an extra ground shoot bent
c = a ground shoot removed

2. Labview front panel

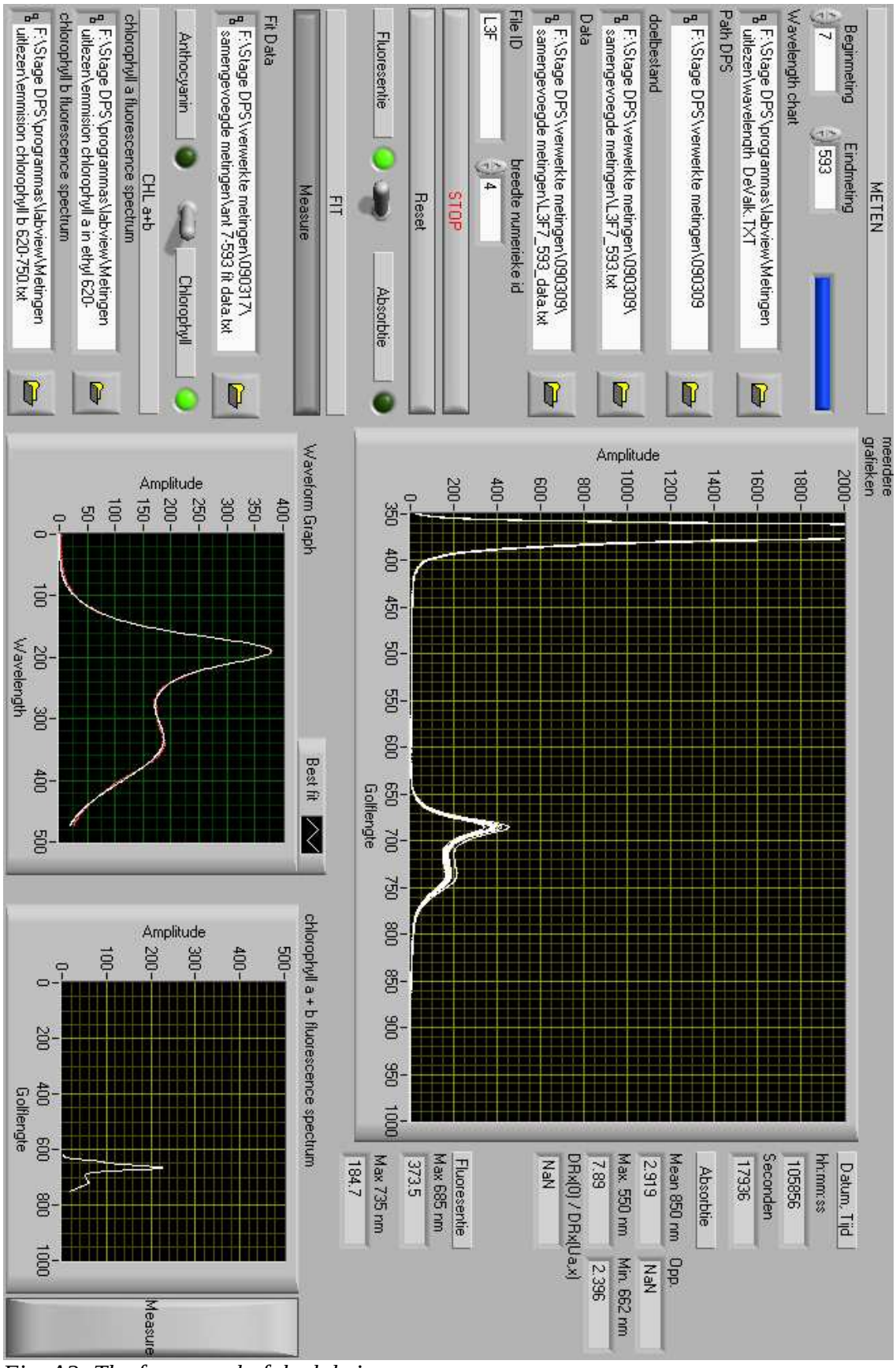

Fig. A2: The frontpanel of the labview program