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# Desktop Plant Stress Guide:

*The following pages represent a compilation of research done using chlorophyll fluorescence for plant stress detection, and measurement. It is organized by plant stress type, with important introductory notes listed first.*

**Edition 4.1 – 09/30/15**

**Note: Recent chloroplast migration work has created game changing research that should make researchers reconsider, dark adaptation times, the times to reach steady state photosynthesis under light adapted conditions, the types of actinic light sources that should be used for chlorophyll fluorescence measurements, and photosynthesis measurements (Cazzaniga 2013, Dall'Osta 2014). See page 8 for details, or the application note on chloroplast migration.**

This guide is intended as a starting point for research. Results may sometimes vary by species, plant type, or special interest.

Results were compiled from world wide published research, *independent of fluorometer brand name*. While chlorophyll fluorescence is sensitive to most types of plant stress, in some cases, this is not true. In those cases, quality special fluorescence assays are listed, or other quality alternative solutions are suggested.

The best tests for different types of plant stress are listed on the following pages. Tests are listed by plant stress type and in order, with the best tests listed first. For more information, contact Opti-Sciences at 603-883-4400, or **www.optisci.com**

# Table of Contents

## *A review of measuring protocols –value and limitations*


$F_V/F_M$ - value and limitations page 3
Y(II) or ΔF'/Fm' - value and limitations page 4
ETR – value and limitations page 5
OJIP - value and limitations page 6


## *Notes on measurement with the effects of chloroplast migration:*


Picking a leaf page 8
Dark Adaptation - How long is long enough? page 8
Y(II) – QuantumYield of PSII or ΔF'/F' – Light adapted measurement at steady state photosynthesis. page 10
ETR or *J* for gas exchange people page 11
Light curves page 11
Rapid light curves page 11
Meanings of terms, accuracy, repeatability and reliability when measuring chlorophyll fluorescence page 11


## *Cook book checklists for reliable chlorophyll fluorescence measurements*


Cook book checklist for $F_V/F_M$ page 13
Cook book checklist for OJIP page 15
Cook book checklist for Y(II) or $\Delta F/F_M$'and ETR page 16
Cook book checklist for quenching measurements such as NPQ page 19


## *Specific Plant Stress Types*


Drought Stress page 23

Light Stress page 26

Heat Stress page 28

Nutrient Stress page 30

Cold Stress page 34

Over-Wintering page 35

$CO_2$ Stress page 35

Air Pollution Stress page 36

Herbicide Stress page 36

Pesticide Stress page 39

Chemical Stress page 39

pH Stress page 41

Biotic Stress page 41

Herbivory (Animal) Stress page 43

Weed Stress page 43

Radiation Stress page 43



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# Measuring protocols

*$F_V/F_M$, Y(II), and ETR* are all very robust tests that have been shown to correlate well with carbon assimilation under many conditions. However, they do have some limitations in plant stress measurement. Some types of plant stress do not affect PSII especially in early phases. ***To compensate for these limitations, inventive researchers have found ways to solve some of these issues, by developing unique working assays. These improvements are listed under specific stress categories.***

**The strengths and limitations of each protocol are provided in a summary on the next pages. For information on measuring specific types of stress, go to the appropriate area in the table of contents.**

**$F_V/F_M$ – Dark-adapted test** - a measurement ratio that represents the maximum potential quantum efficiency of Photosystem II if all capable reaction centers were open. 0.79 to 0.83 (Maxwell K., Johnson G. N. 2000), (Kitajima and Butler,1975) is a range of the average approximate optimal value for most plant species with lowered values indicating plant stress. $F_V/F_M$ has a photochemical component and a non-photochemical component (Baker 2004). It offers the advantage that all samples can be compared in a known dark-adapted state (Baker 2004). Compare samples with a similar light history because it can take between 40 minutes to 60 hours for chronic photoinhibition to relax after several hours of bright sunlight. (Lichtenthaler 2004). *$F_V/F_M$ is a fast test that usually takes less than two seconds, but requires proper dark adaptation. This test was developed by Kitajima and Butler (1975)*

**$F_V/F_O$ –** This the same dark-adapted measurement normalize over $F_O$ instead of $F_M$. It is a more sensitive plant stress detector due to the fact that it is normalized over the minimum fluorescence measurement rather than the maximum fluorescence measurement as found in $F_V/F_M$. $F_V/F_O$ but it does not correlate with carbon assimilation.

*$F_V/F_M$ is a normalized parameter that tests whether or not plant stress affects PSII in a dark-adapted state. $F_V/F_M$ is the most used chlorophyll fluorescence measuring parameter in the world. "The majority of fluorescence measurements are now made using modulated fluorometers with the leaf poised in a known state." (Baker 2004)*

## *Limitations of $F_V/F_M$ and $F_V/F_O$*

1. Not sensitive to early or moderate water stress. (Bukhov & Carpentier 2004) (Zivcak 2008)
2. Not sensitive to early or moderate water stress in $C_4$ plants (da Silva J. A. & Arrabaca M.C. 2008)
3. $F_V/F_M$ is not sensitive to nitrogen stress until very low levels are reached. (Baker 2004) (Chlorophyll content meters are recommended for nitrogen and sulfur stress. They are sensitive to nitrogen and sulfur stress, but they are not as sensitive to most other types of plant stress as chlorophyll fluorescence measurement.)
4. $F_V/F_M$ is not sensitive to sulfur stress until starvation levels are reached. (Baker 2004)
5. Not sensitive to heat stress below 45°C centigrade in Oak. (Haldiman P, & Feller U. 2004)
6. Not sensitive to DCMU herbicide stress. (Nedbal & Whitmarsh 2004)
7. $F_V/F_M$ is sensitive to some types of herbicide stress types, and not others. (Nedbal & Whitmarsh 2004)
8. Not sensitive to nickel stress. (Joshi & Mohanty2004)
9. Not sensitive to zinc stress. (Joshi & Mohanty2004)
10. Not sensitive to NaCl stress in rice, but it is sensitive to NaCl stress in sorghum and chickpea. Result here seem vary from plant to plant. It seems to work with some $C_3$ plants, and some $C_4$

plants, but not other $C_3$ plants and other $C_4$ plants. See NaCl stress listed under chemical stress, in this guide, for more detailed information. (Moradi & Ismail 2007) (Netondo 2004) (Eyidogan 2007), (Moradi & Ismail 2007), (Netondo 2004) (Eyidogan 2007)

## Y(II) or $\Delta F/F_M$' or Effective Quantum Yield of PSII- **Light adapted test** –

a normalized measurement ratio that is an indication of the amount of energy used in photochemistry by PSII under steady-state photosynthetic lighting conditions. (Genty 1989), (Genty 1990), (Maxwell K., Johnson G. N. 2000), (Rascher 2000) It is affected by the closure of reaction centers and heat dissipation caused by non-photochemical quenching (Schreiber 2004). Compare samples with a similar light history because it can take between 40 minutes and 60 hours for chronic photoinhibition to relax after several hours of bright sunlight. (Lichtenthaler 2004). *Y(II) is a fast test that usually takes less than two seconds. This test was developed by Bernard Genty* (Genty 1989), (Genty 1990).

*Y(II) tests whether or not a plant stress affects PSII in a light adapted state at steady state photosynthesis. Y(II) also varies with light level or PAR (photosynthetically active radiation), as well as temperature. For this reason, it is crucial to use a PAR clip for field measurements of Y(II) and ETR without changing the leaf angle to the source of light. Y(II) has been shown to be a more sensitive test than $F_V/F_M$ for some types of plant stress. However, when comparing samples, light intensity and temperature must be controlled.*

### *Limitations of Y(II)*

1. Yield of PSII or Y(II) and ETR are sensitive to drought stress in $C_4$ plants. (da Silva J. A. & Arrabaca M.C. 2004), (J Cavender-Bares & Fakhri A. Bazzaz 2004 ) (Cerovic 1996)
2. Yield Y(II) in the *Burke assay,* is very sensitive to early drought stress *in* $C_3$ plants (Burke 2007), (Burke 2010) Drought stress can be detected within 24 hours or irrigation cessation. This is a special assay recommend for $C_3$ plants. The Burke assay is required for early $C_3$ plant drought stress measurement. Without it, Y(II) will only measure severe drought stress in $C_3$ plants, due primarily to photorespiration (Flexas 1999)
3. $F_S/F_O$ components of Yield Y(II) and $F_V/F_M$ ($F_S$ is a component of Y(II), and $F_O$ is a component of $F_V/F_M$). When they are combined in a ratio, they are sensitive to moderate drought stress in $C_3$ plants at near saturation light levels. This is adequate for grapes but not most other plants. (Correspondence with Flexas),(Flexas 1999), ( Flexas 2000), ( Flexas 2002) *Yield of PSII or Y(II) by itself is not sensitive to drought stress in $C_3$ plants until it is severe due to photorespiration* (Flexas 1999). ( Flexas 2000), ( Flexas 2002)
4. *Sensitive* to heat stress at 35°C and above in Oak, a $C_3$ plants (Haldiman P, & Feller U. 2004)
5. Y(II) is not sensitive to nitrogen stress until it is severe, unless the *Cheng (2001) assay* at high light levels is used. See nutrient stress for details and alternatives. (Chlorophyll content meters are recommended for nitrogen and sulfur stress. They are sensitive to nitrogen and sulfur stress, but they are not as sensitive to most other types of plant stress as chlorophyll fluorescence measurement.)
6. Y(II) is not sensitive to sulfur stress until starvation levels are reached. (Baker 2004)
7. Not sensitive to early or moderate $CO_2$ stress. (Siffel & Braunova 1999)
8. Not sensitive to NaCl stress in Rice, but it is sensitive to NaCl stress in sorghum and chickpea. Result here seem vary from plant to plant. It seems to work with some $C_3$ plants, and some $C_4$ plants, but not other $C_3$ plants and other $C_4$ plants. See NaCl stress listed under chemical stress in this guide for more detailed information. (Moradi & Ismail 2007) (Netondo 2004) (Eyidogan 2007)

**ETR – also known as *J* among gas exchange researchers, is relative electron transport rate.**
It is normally calculated according the following average formula:

ETR=Y(II) X PAR X 0.5 X 0.84.

To measure ETR requires the measurement of Y(II) and PAR at the leaf plane, and in the same directional orientation as the leaf. The use of a PAR Clip is highly recommended for this reason. On a sunny day, the leaf is usually at steady state photosynthesis at its current distance and angle to the sun or another light source. Changing the distance or the angle to the light source can cause the leaf to no longer be at steady state. Light intensity at the leaf varies inversely with the square of the distance. When using artificial light sources. a small change in distance can make a large difference. The 0.5 PSII to PSI reaction center ratio is an average value. The 0.84 leaf absorptance value is also an average value.

## *Limitations of ETR*

1. ETR is sensitive to the same types of plant stress as Y(II).
2. ETR should not be used to compare different samples unless leaf absorption is known or measured and the ratio of PSII to PSI reaction centers is known or it has been measured. (Baker 2008). 0.84 is an average leaf light absorption value. Since leaf light absorption can vary from 0.70 to 0.90 in healthy plants (Eichelman 2004) an error can be introduced unless leaf absorption ismeasured. Leaf absorptance changes with plant stress, leaf age, chlorophyll content, species (Eichelman 2004), and light level (Cazzaniga 2013), (Dall'Osta 2014)

   The average ratio of PSII reaction centers to PSI reaction centers is 0.50. The ratio of PSII reaction centers to PSI reaction centers varies from 0.4 in some $C_4$ plants to 0.6 in some $C_3$ plants (Edwards 1993, Laisk 1996,). the most used method for measuring the ratio of PSII to PSI, involves the use of spectral analysis of samples at $77^oK$ (Anderson 1999), (Zell 2010). This ratio varies by type of plant, $C_3$ or $C_4$, by plant species, by sun grown leaves vs. shade leaves, and in carbon deficient leaves.

   Others feel that it is all right to use ETR average values for many applications (Schreiber 2004). Certainly for more exacting work, absorption values and PSII ratio values for ETR can Be replaced by measured plant values. (Edwards 1993), (Laisk 1996), (Eichelman 2004) (Baker 2008). For more information on this subject, refer to the OSI application note on PAR measurement and ETR.

***For the reasons stated in the limitations above, it is more common to use Y(II) for plant stress measurement than ETR, since they are both just as sensitive to the same types of plant stress.***

*OJIP tests for plant stress in a dark-adapted state.*

It has been found that by using a high time resolution scale, as an actinic light source has been turned on, that the rise to maximum fluorescence from minimum fluorescence, has intermediate peaks and dips designated by the OJIP or OKJDIP nomenclature. Over the years, there have been multiple theories of what the rise, time scale, peaks and dips mean. (Vredenburg 2004, 2009, 2011, 2012), (Strasser 2004), (Zhu 2005 & 2012). The most widely held view, at this time, is presented by Zhu X-G, Govindgee, Baker N.E, deSturler E. Ort D.R, Long S.P. (2005) & Zhu X-G., Wang Y., Ort D.R. Long S.P. (2012). While there is more than one school of thought with different recommendations as to how this information can be used for plant stress testing, the Strasser protocol is the one most used for plant stress measurement. (Strasser 2004).

Like the other measuring protocols, the research shows that OJIP works better for some types of plant stress than it does for others. The research shows similar plant stress measuring sensitivities to $F_V/F_M$, and $F_V/F_O$. (See the plant stress type and the list of limitations below).

Measurements take a couple of seconds, and provide fluorescence data measured with high time resolution. Protocols, such as Strasser's, relate to how plant stress conditions affect different parts of the OJIP fluorescence rise. The Strasser Protocol uses a logarithmic time scale for the X graphing axis.

So what does the rise with steps mean? (Description taken from Zhu 2005 and 2012) (Strasser 2004)
In general, in a properly dark adapted samples, there are open reaction centers, and $QB^-$ nonreducing PSII reaction centers. The start or slope of the O-J rise is affected by the probability that excitation energy migration from a close core antenna- reaction center to an open core antenna – reaction center. Higher probability of excitation transfer delays the rise. O and $F_O$ are affected by the ratio of size of peripheral antenna to core antenna. If peripheral antenna size is larger, O and $F_O$ are lower. O and $F_O$ are also affected by the number $QB^-$ nonreducing PSII reaction centers. The greater the number of non-reducing reaction centers, the higher the value. $F_O$ is measured antenna fluorescence before the reduction of any $Q_A$. $F_O$ is measured in modulated fluorometers and estimated with linear regression analysis in continuous fluorometers. O is commonly the point measured at 40 μsec. after the actinic light has been turned on.

1. The O-J rise represents the photochemical reduction of pheophytin and $Q_A$. J at 2 ms, represents maximum values for $Q_AQ_B^-$ and $Q_A^-\ Q_B^-$. J becomes more defined as the dark adapted OEC (Oxygen evolution complex) ratio of $S_1$:$S_O$ moves from 1:0 to 0:1. The Dip after J becomes more defined with a higher $S_O$ value. Higher $S_O$ values provide a greater $P_{680}^+$ concentration that is a strong fluorescence quencher.
2. The J-I rise is affected by the photochemical reduction of $Q_B$. I at 30 ms, represents the first shoulder in the $Q_AQ_B^{2-}$ chemical reaction that end at P with a maximum for $Q_AQ_B^{2-}$. If properly dark adapted, it starts with the ratio of $Q_B$ : $Q_B^-$ = 1:0 and ends with the ratio at 0:1. The dark adapted ratio of $Q_B$ : $Q_B^-$ affects the slope and height of I.
3. P represents maximum values for $Q_AQ_B^{2-}$ and $P_QH_2$. The height and slope of the rise from I to P is affected by the rate constant for reoxidation of $PQH_2$ to PQ and the size of the PQ, plastoquinone pool.
4. $PQH_2$ is re-oxidized by the cytochrome $b_6f$ complex. The rise of chlorophyll fluorescence ends with reoxidation of $PQH_2$ to PQ by the cytochrome $b_6f$ complex.

Notes:

1. The rise of fluorescence intensity in healthy plants, usually results in two intermediate peaks and a third maximum peak. The J peak is followed by D, or a dip, the I peak is next, and the P peak is the maximum fluorescence value. Under some types of moderate to severe plant stress, other peaks have also been found (Strasser 2004). Sometimes a "K step or peak" appears at 300 μsec. It only occurs only at high light levels, when there is severe nitrogen, iron, or sulfur deficiency. (Strasser 2004) (Vredenberg 2004).
2. The time to P or $F_M$ ($P=F_M$) is variable.
3. The decline in fluorescence intensity after the P step, or S, M, and T phases, are affected by the initiation of photosynthesis with photochemical quenching, nonphotochemical quenching or photoprotective mechanisms such as change in the Δph of the thylakoid lumen and the xanthophyll cycle. State transitions and chloroplast migration relaxation also eventually impact these later fluorescence values. If adequate dark adaptation is not uses to allow these mechanisms to relax errors will occur. See the section on dark adaptation for more details.
4. Since OJIP values change with actinic light intensity (Vredenberg 2004), it is important to always use the same light intensity for Strasser protocol plant stress measurements. It is common to use 3000 μmols or 3500 μmols for the Strasser OJIP protocol. Calibration of the light source is recommended. Some instruments do this automatically.
5. Different OJIP protocols have been developed, by different schools, for measuring plant stress. Some develop measuring parameters from the characteristics of the various peak intensities, and the slope of the fluorescence rise. Some have use the timing of the fluorescence peaks.
6. The Strasser school, has developed a series of parameters to help describe plant function, and try to improve plant stress detection, and measurement. $PI_{ABS}$ or performance index, VJ, and other parameters, are used to measure plant stress. (Strasser 2004).

## *Limitations of OJDIP and OJIP*

1. OKJIP is not sensitive to heat stress until 44°C is reached. (Strasser 2004)
2. $PI_{ABS}$, or Performance index, is not sensitive to drought stress until *seven days have passed*, on many of the plants tested, but it is slightly more sensitive than $F_V/F_M$ (Thack 2007). It is not as sensitive as $F_S/F_O$ or the Burke assay for $C_3$ plants and it is not as sensitive as Y(II) for $C_4$ plants. Other solutions are listed under drought stress.
3. $PI_{ABS}$ is derived from several OJIP parameters including $V_J$, the initial slope of the fluorescence rise - $M_O$, $F_V/F_M$, $F_V/F_O$ The rise from J to P, and the rise from O to J. (Strasser 2004)

$$PI_{ABS} = ( V_J / M_O) (F_V/F_M) ( F_V/F_O) ((F_M-F_J)/ (F_J-F_O))$$

4. $PI_{ABS}$ is a primarily a stress detection tool, and does not correlate with carbon assimilation.
5. The peaks of OKJDIP are light intensity dependent (Vredenburg 2011). For that reason, it is important To use the same actinic light intensity for all measurements. Much of Strasser's early work Recommended 3,000 μmols. Today, 3,500 μmols is recommended. Both of these values are available on the OS30p+ and the OS5p+. Other intensities may be used, however the same intensity should always be used when comparing the plant stress of different samples.

## Notes for stress measuring *with the effects of chloroplast migration*:

**1**. It is Common to use the youngest fully mature leaf blade for diagnosis of deficiencies in plants (Reuter and Robinson 1997)

2. Dark adaptation is a technique used in some chlorophyll fluorescence measurements to fix a common known reference point relative to various measurements (Maxwell and Johnson 2000). Deciding where to put that reference is based on an understanding plant mechanisms that can affect measurements, and what one wants to measure. Recommended times can vary by chlorophyll fluorescence test type, and environmental conditions.

Due to new research on chloroplast migration, dark adaptation times of at least 20 to 35 minutes are required for plant stress measurement of terrestrial plants. Anecdotal evidence indicates that some research reviewers require the equivalent of "overnight" dark adaptation or pre-dawn values. While the time used is dependent on what is being measured, different journals may have their own ideas as to what constitutes reliable dark adaptation. See the section below for an in-depth discussion.

To obtain reliable modulated $F_V/F_M$ or OJIP test values, decisions need to be made for control and test measurements. The plant mechanisms listed below will lower $F_M$, and possibly raise $F_O$, changing OJIP and $F_V/F_M$ measurements downward like other types of plant stress. One must decide which mechanisms are of concern for specific types of plant stress measurement and dark-adapt accordingly.

$F_V/F_M$ is affected by both photochemical and non-photochemical factors. If a leaf is dark adapted and measured, then subjected to very high light levels for a long period of time, then dark adapted and re-measured, the first measurement will be higher then the second measurement. The decline in $F_V/F_M$ measurement may be due to a decrease in reaction centers capable of photochemistry or un-reversed non-photochemical quenching. (Baker N.R., Oxborough K. 2004) . It can take between forty minutes and sixty hours for photoinhibition to relax or repair (Lichtenthaler 2004). Therefore, light history should be similar between samples to be compared.

Papageorgiou reports that results may vary greatly depending on how long dark adaptation is done. A few minutes of dark adaptation is enough to re-oxidize the plastoquinone pool and the $CaMn_4OxCly$ cluster, while longer periods deplete respiratory substrates through respiration in cyanobacteria and chlororespiration in higher plants and algae. Longer times will also deplete ATP pools, and trans-membrane ion concentration gradients. Dark adaptation also shifts higher plants and algae toward state 1 conditions and cyanobacteria to state 2 conditions (where they exist). (Papageorgiou G.C. Tismmilli-Michael M. Stamatakis K. 2007). Under high actinic light, or near saturating light conditions, cell chloroplasts migrate from the tops of plant cells to the sides of plant cells, increasing leaf light transmission and decreasing leaf light absorptance. This process significantly affects both light and dark-adapted fluorescence measurements. During dark adaptation, chloroplasts migrate back to the tops of cells. This process takes between 20 minutes to 35 minutes (Cazzaniga S. 2013) (Dall'Osta 2014). See the application note on $q_M$ chloroplast migration at [www.optisci.com](www.optisci.com) for more information.

Full activation of Rubisco takes between three and four minutes in vascular plants as well as photoplankton. Deactivation of Rubisco in the dark, takes between 12 -18 minutes in vascular plants and from 9 minutes to 28 minutes in some photoplankton. The longer deactivation is thought to offer an advantage for species subjected to erratic bright light for maximum utilization of light (MacIntyre 1997).

Rapid acting photo-protective mechanisms activated by exposure to variable light intensities (designated in the parameters $q_E$ and Y(NPQ) are controlled by the xanthophyll cycle and thylakoid lumen $\Delta$ph. They

relax in a several seconds to few minutes during dark adaptation. (Muller, Niyogi 2001),(Kramer D. M., Johnson G., Kiirats O., Edwards G. (2004). According to Lichtenthaler (1999), this time is 4-6 minutes. Baker (2008) indicates that the adjustment and relaxation times can be longer, up to 7 minutes, in field plants.

The affects of state transitions on chlorophyll fluorescence have recently been shown to be more complex than previously thought (Where they exist). Classical state transition theory saw state transitions as a low light survival mechanism that allowed light balance between Photosystem II and Photosystem I. $F_M$' or maximum fluorescence under light adapted conditions would decrease over a fifteen to twenty minute time frame, and then relax during dark adaptation over a fifteen to twenty minute time frame. State I – State 2 transition quenching relaxation (called $q_T$) was considered to be most significant at lower light levels in terrestrial plants and could represent more than 60% of quenching at low light levels. *It was also thought that at high light levels it represents about 6% of total quenching.* (Lichtenthaler H. Burkart S 1999). Recent evidence shows that the fluorescence change thought to be the result of state transitions and acute photoinhibition is in fact caused by chloroplast migration at least at higher light levels and near saturating light levels in land plants. Chloroplast migration takes between 20 minutes and 35 minutes to adjust and to relax. (Cazzaniga S. 2013), (Dall'Osta 2014) $q_M$ has been shown to be as much as 30% of NPQ under high light conditions (Cazzaniga S. 2013), (Dall'Osta 2014). (See the application note on $q_M$ and $q_T$ for more information.)

In the past, it was thought that the effects of acute photo-inhibition, caused by exposure to high light intensities for an hour or two, could be reversed with 20 to 30 minutes of dark adaption (Theile, Krause & Winter 1998). However, recent evidence indicates quenching relaxations in the dark, for these time periods, are likely to be caused by chloroplast migration $q_M$ instead of $q_I$ photoinhibition. (Cazzaniga S. 2013), (Dall'Osta 2014). Times for relaxation should extend from 20 to 35 minutes with this in mind.

Chronic Photoinhibition $q_I$, is the result of exposing leaves or plants to high light conditions for several hours. After sunny summer days, there is almost always some residual photoinhibition built into all chlorophyll fluorescence measurements. That is alright as long as samples are compared to other samples with a similar light history. As stated earlier, it can take between forty minutes and sixty hours for photoinhibition to relax or repair. Samples that have been exposed to a few over cast days do not have the same light history as samples after a sunny day (Lichtenthaler H 2004). If one wants to measure photoinhibition, consider partially shielding samples from photoinhibitory light conditions for at least 60 hours to eliminate any and all photo-inhibition. Then dark adapt for at least 35 minutes and make a measurement. (many experts like overnight dark adaption). The $F_V/F_M$ measurement, used in quenching measurements, then becomes a more reliable reference for measuring photoinhibion after a long exposure to high light conditions. The OS5p+ has a dark adaptation timer. Actinic light will be turned on after the time has run out. It can be set for up to 20 hours.

**Quenching and quenching relaxation measurements:**

When making longer quenching and quenching relaxation parameter measurements related to photo-inhibition and photo-damage mechanisms that are common in chronic high light stress, high heat stress, cold stress and over wintering stress, one should understand that it could take days for full relaxation or repair of the non-photochemical quenching parameter, $q_I$ to pre-stress conditions. Reversal or relaxation of chronic photo-inhibition caused by several hours of high light exposure starts to relax at about 40 minutes and may take up to 60 hours to fully relax under dark adaptation (Lichtenthaler H. & Babani F. (2004) (Theile, Krause & Winter 1998).

To get an accurate control value for $F_M$ and $F_O$, under chronic photo-inhibition conditions, (Maximum fluorescence, and minimum fluorescence components of non-photochemical quenching parameters) *it is common to dark adapt for a full night using pre-dawn values for field plants and plants that have been*

*exposed to photoinhibitory light levels for extended periods of time.* In some cases, it may make sense to dark-adapt for longer periods of time. (Maxwell and Johnson 2000). Under laboratory conditions, times of 20 minutes to 35 minutes may be adequate if photoinhibiton is not an issue ( Research reviewers may have ideas of their own and require the equivalent of overnight dark adaptation or predawn dark adaptation). It is understood that in plants with a recent *high light history* that there will likely be some residual photoinhibition built into all dark-adapted measurements. This is alright as long as the light history of measured samples has been built in to the experimental design. Unless light stress is the focus of the experiment, it is important to compare samples with similar light history. In addition, quenching measurements of different samples should not be compared unless the $F_V/F_M$ values of the samples are identical. This is necessary because $F_V/F_M$ is the yard stick used to gauge other quenching parameters (Baker 2008) (See the quenching application note for more details.)

In Aquatic Plants Gorbunov (2001) is a good source for corals, and Consalvey (2004) is a good source for Algae. For information regarding dark adaption for rapid light curves Ralph (2005) and Rascher (2000) are good sources.

The use of far-red pre-illumination that is available on some fluorometers is designed to rapidly re-oxidize PSII by activating PSI. While this can be valuable in fieldwork (Maxwell and Johnson 2000), it does not affect the relaxation of other non-photo-chemical quenching mechanisms (Consalvey 2004).

Dark adaptation can be accomplished by using dark adaptation leaf clips or cuvettes. Some researchers use hundreds of inexpensive clips to make measurements on larger population quickly. Shrouds, darkened rooms, and darkened growth chambers may also be used.

Since light output usually decreases as a light source heats up, it is important to ensure that the actinic light intensity is maintained through out the quenching test. If not, then the plant will likely not be at steady state photosynthesis for the test, and the actual PAR level, at the leaf, will be lower that one wants. Some systems provide a PAR sensor, and a feed-back loop to constantly maintain the actinic PAR level at the leaf throughout the quenching test. If that is not available, then using an external actinic PAR light source may be the best option. Wait until the actinic light source heats up, and the actinic PAR level at the leave has reached a relatively stable level, before exposing the leaf sample to the light source. Measure PAR with a PAR clip, or a separate PAR sensor, if that is the only thing available. Remember, light intensity varies inversely with the square of the distance from the source, and so small distances can make a significant difference if the source is not the sun. A good way to make measurements is to dark adapt use a dark shroud over the PAR clip, or the PAR clip may be used in a darkened room.

*It is common to use overnight pre-dawn dark adaptation for quenching measurements. If one considering shorter dark adaptation times, it makes sense to check with target journal research reviewers because some have strong feelings on the subject.*

**3. Y(II) – or $\Delta F/F_M$' Quantum yield of PSII measurements** only take a couple of seconds, however, reliable quantum yield measurements, Y(II), must be taken at steady-state photosynthesis. While leaves at the top of plants, that are exposed to ambient sun light, on a clear day, are considered to be at steady state photosynthesis, changes in lighting due to clouds, shading and wind can disrupt steady state conditions. Steady state is an equilibrium condition reached after a several minutes of exposure to ambient solar or artificial radiation. If light levels change, it can take up to thirty-five minutes for the plant to adjust to the new steady state (Cazziniga 2013). While Maxwell and Johnson (2000) tested 22 different species of British plants, and found that steady state occurred in fifteen to twenty minutes in the plants measured, new evidence about chloroplast migration at higher actinic light levels takes between 20 to 35 minutes to fully adjust (Cazziniga 2013)( Dall'Osta 2014) to steady state. Measurements taken under variable lighting conditions may not provide reliable yield results (Rascher 2000). *For the reasons listed above, it is*

*important not to change the orientation of the leaf relative to the actinic light source when taking a PAR (photosynthetically active radiation) or a Y(II) measurement.*
No dark adaptation is required.

Y(II) measurements can vary significantly with light irradiation variation (light level) and temperature variation. If these two parameters are not controlled, or measured, it is possible to misinterpret results. For example, in a case where an investigator is measuring salt stress, the Y(II) measurement from one plant may be higher than on a second plant where irradiation is lower. One might consider this lowering of Y(II) to be the result of stress, when the only difference may be light irradiation level.

*For field measurements and laboratory measurements, where irradiation and temperature can change, not only because of distance to the light source, but also due to leaf angle change , it is recommended that a PAR Clip be used to measure irradiation and leaf temperature along with Y(II). Light intensity varies inversely with the square of the distance from the light source. The PAR sensor will detect small or no differences in irradiation as the PAR sensor is moved closer or further from the sun, in field measurements, because the sun is so far away. However, it can make a big difference in the lab, or in growth chambers.* (For more information on Y(II) measurements, and the factors that affect yield measurement, contact Opti-Sciences for the yield application note or the PAR measurement application note). Changing the leaf angle to a light source can make a big difference in steady state condition.

When studying under canopy samples, one may want to shroud the leaf, and use an internal artificial actinic light source for making Y(II) measurement at different light levels. Make sure that steady state is reached before the measurement. When in doubt, use pre illumination of at least 35 minutes to reach steady state photosynthesis. Rapid light curves are designed to be used under variable light conditions and they should also be considered for under canopy measurement.

## 4. ETR - Relative Electron Transport Rate

**ETR is a calculated parameter that includes Y(II) and the light irradiated on the leaf. Four electrons must be transported for every $CO_2$ molecule assimilated or $O_2$ molecule evolved. (Schreiber 2004)**
Electron Transport Rate - ETR = (Y(II)) x (PAR µmols) x (0.84 leaf absorptance) x (0.5 PSII/PSI ratio)

(Y(II) is the quantum yield of PSII) X (PAR is Photosynthetically Active Radiation measured between 400nm and 700nm in µmols quanta $m^{-2}$ $s^{-1}$.) X (0.84 is the average leaf absorption) X (0.5 is the average ratio of PSII reaction centers to PSI reaction centers). Average plant values are used in the standard equation. 0.84 is an average value for many species of plants (Bjorkman and Demming, 1987). Research has shown that the leaf absorption coefficient can vary between 0.7 and 0.9 in healthy plants (Eichelman H. 2004) and it can vary with plant stress, leaf age, chlorophyll content, species (Eichelman 2004), *and light level* (Cazzaniga 2013) (Dall'Osta 2014).

Research has also shown that the fraction of light that is absorbed and used by PSII reaction centers varies from at least .40 to .60 (Laisk and Loreto,1996). The average ratio of PSII reaction centers to PSI reaction centers is 0.50. This ratio varies by type of plant, $C_3$ or $C_4$, by plant species, by sun grown leaves vs. shade leaves, and in carbon deficient leaves. The most used method for measuring the ratio of PSII to PSI, involves the use of spectral analysis of samples at $77^oK$ (Anderson 1999), (Zell 2010).

Even if the default average values are used, some hold that ETR can provide useful relative comparative information between different samples and the same sample under different conditions (Schreiber 2004). ETR should not be used to compare different samples unless leaf absorption is known or measured and the ratio of PSII to PSI reaction centers is known or it has been measured. (Baker 2008)

Baker (2008) says that when measuring ETR, it is important to also measure leaf absorbance with an integrating sphere. Absorbance can change significantly with plant stress, leaf age, chlorophyll content, species (Eichelman 2004), *and light level* (Cazzaniga 2013). Baker says that the ETR values of different samples should only be compared if leaf absorbance is known. *For this reason, some researchers prefer Y(II) measurements to ETR for stress measurement because the additional variables do not have to be considered. The iFL integrated gas exchange – chlorophyll fluorometer and Y(II) meter now measure leaf absorptance, providing a solid estimate of integrating sphere measurements.*

PAR sensor location error according to Rascher (2000). When artificial light sources are used, Rascher found that the location the PAR sensor relative to the leaf surface could cause an error of up to 10%. This error is insignificant if sun light is used, due to the much greater distance from the light source. Rascher used an independent PAR sensor, and measured the irradiation intensity at the leaf plane. He then made corrections due to PAR Clip sensor location, by comparing the differences between the PAR clip values, and the leaf plane values. *This correction may not be needed for most relative comparison ETR applications, however it may be required for more exacting work when necessary.*

*Do not change the angle of the leaf when measuring. The amount of radiation falling on the leaf can change dramatically and the leaf will not be at steady state photosynthesis, introducing multiple errors.*

**5. Light Curves** By plotting ETR vs. PAR, potential ETR rates, photosynthetic capacity, and ETR rate limitations, at given light intensities, can be determined. (U. Schreiber 2004). Plants are allowed to reach steady state photosynthesis, at each light level, before measurement. Until recently, it was thought that it took between fifteen to twenty minutes a specific light level, to reach steady state photosynthesis, according to Maxwell and Johnson (2000). However, recent research has found that at high actinic light levels chloroplast migration occurs and substantially affects chlorophyll fluorescence measurements. Chloroplast migration takes between 20 to 35 minutes to fully adjust to steady state. Note: Four electrons must be transported for every $CO_2$ molecule assimilated or $O_2$ molecule evolved. In this case, steady state photosynthesis must be reached at each light level before measurement. This means that the actinic light source should be on for at least between twenty and thirty five minutes. For more information, contact Opti-Sciences for the light curve application note, and the one regarding $q_M$ or chloroplast migration. Leaf absorption measurement can improve ETR value reliability.

**6. Rapid Light Curves** Rapid Light Curves are a good solution in a variable light environment such as under canopy work, and working with aquatic plants. The reason is that most chlorophyll fluorescence measuring parameters require steady state light adapted conditions or known dark-adapted conditions to be reliable. The exceptions are the "NO" lake model quenching parameter, and the rapid light curve parameters, $ETR_{MAX}$, $I_K$, $I_M$ and $\alpha$ . Light saturation rate, as measured by rapid light curves, highly correlates with the concentration and maximum activity of Rubico (Macintyre 1997), (Macintyre 1996). *Measured steady state photosynthetic rates overestimate actual photosynthetic rates in a variable light environment* (Macintyre 1997). RLC measuring results are light history dependant, with different results at different times of the day. Different researchers recommend different dark adaptation times. They vary from momentary 5-10 seconds (Ralph 2005), to longer times (Rascher 2000). For more information, contact Opti-Sciences for the Rapid light curve application note.

**7. Understanding measurement accuracy, repeatability, and reliability.**

**Accuracy** is the ability to hit the bull's eye.

In many types of measurements, accuracy is determined by calibrating to a measuring a standard that is traceable to the National Institute of Standards and Technology (NIST). With such measurements, tolerances are always involved. **Repeatability** is the ability to achieve the same measurement again and again to a certain tolerance level. A **Reliable** measurement is one that is accurate and repeatable**.**

In the case of Chlorophyll fluorescence, Accuracy is achieved by following the guidelines that have been determined by research as they relate to instrument use and plant mechanisms. Check lists are provided below for each of the common measuring protocols.

Repeatabilty can be also be optimized by following these guidelines related to instrument use and plant mechanisms.

In plant stress measurement, reliability is a measurement that is accurate and repeatable.

**Normalized Ratios -$F_V/F_M$, and Y(II) or $\Delta F/F_M'$**

$F_V/F_M$ and Y(II) are normalized ratios that do not use a traceable standard. Instead, their accuracy is determined by properly using the measuring instrument, and following the lessons learned about plant physiology, by several great researchers. For most species, the optimal $F_V/F_M$ reading for stress free plants is in the range of 0.79 to 0.84 (Maxwell and Johnson 2004). To achieve reliable measuring results, refer to the cookbook checklists below. They highlight the important measurement variables involved in each measuring protocol.

# Cookbook checklists

**To ensure reliable measurements, follow the "cookbook checklists" on the following pages as a recipe for success.**

## First –$F_V/F_M$

$F_V/F_M$ allows comparison of samples in a known dark-adapted state.

***To get an accurate measurement, one has to follow tested guidelines.***

1. **Dark-adapt properly** knowing the plant's light history. It takes only a few minutes for the xanthophyll cycle and the Δ ph of the thylakoid lumen to return to a dark-adapted state. ( State transitions (where they exist), however, take between fifteen to twenty minutes (Ruban 2009) (Lichtenthaler 1999) . These times can vary somewhat in field plants, and can take slightly longer. Deactivation of Rubisco in the dark, takes between 12 -18 minutes in vascular plants and from 9 minutes to 28 minutes in some photoplankton (MacIntyre 1997). In plants that have a high actinic light history, chloroplast migration occurs and relaxes during a twenty to *thirty five minute* window (Cazzaniga S. 2013). In addition, field plants and other plants that have been exposed to photoinhibition conditions for a number of hours, will retain a certain amount of NPQ for up to 60 hours (Lichtenthaler 2004). This means that even if dark adaptation is overnight, there will almost always be some residual NPQ built into most summer field sun grown leaf measurements of $F_V/F_M$ after a sunny day. This is all right as long as one is comparing samples with a similar light history. But it will cause problems when samples have a different light history unless one is measuring "light stress" and comparing results. It is common for researchers to choose dark adaptation times anywhere from twenty minutes to thirty five minutes, or overnight, using pre-dawn values. If the leaf has been exposed to high light conditions, *thirty-five minutes would be a safe dark adaptation time* to account for chloroplast migrations (Cazzaniga S. 2013) (Dall'Osta 2014). Shorter times may be used to study the effects of plant protective mechanisms. For more information contact OSI for the Dark adaptation application note. (These guidelines are different for quenching measurements and for Rapid Light Curves.) . *Some Journal research reviewers have their own ideas regarding reliable dark adaptation times. If you plan to publish, it may make sense to check with reviewer. The equivalent of overnight dark adaptation is acceptable for all known reviewers.*

2. **Modulation light intensity setting** $F_V/F_M = (F_M-F_O)/F_M$. Minimum fluorescence , is a measurement of a fully oxidized photo system II before any $Q_A$, or quinone "A" has been reduced. It is a dark adapted value, measured by exposing the leaf antennae to a very low intensity modulated light. The intensity must be set high enough to properly allow minimum detection of chlorophyll fluorescence, but not high enough to drive photochemical reduction of any $Q_A$, the primary quinone in the light reaction. If it is set too high, it will drive photochemical reduction of any $Q_A$ and provide an $F_O$ value that is too high. *When setting the modulating light intensity, the Ft value or fluorescence signal should not rise over a 30 second period* when a leaf is exposed to the modulated light. If it does, the intensity must be lowered. *OSI now offers an automated modulated light set up routine for its OS5p+,OS1p,OS30p₊ , iFL , Y(II) meter, and $F_V/F_M$ meter chlorophyll fluorometers.*
3. **Shade leaves vs. Sun leaves**. – The $F_V/F_M$ ratio will be slightly higher on sun leaves than on shade leaves (Lichtenthaler 2004).
4. **$F_V/F_M$ will be higher with a white saturation pulse than a red saturation pulse.** Some fluorometers use a red saturation pulse. This is not an issue for comparative measurements of plant stress with similar instruments, but values measured on a fluorometer with a white saturation pulse should not be directly compared to measurements of a fluorometer with a red saturation pulse. There is evidence to show that systems with a red saturation pulse correlate but measure consistently slightly lower than systems with white light saturation lights. (Cessna 2010)
5. **Maximum $F_V/F_M$ values vary with species**. The average maximum $F_V/F_M$ value is between 0.79 - 0.83 (Maxwell and Johnson 2000).
6. **Field plants should only be compared to field plants and green house plants should be compared to green houseplants** due to light history. (Lichtenthaler 2004)
7. **It is common to use the youngest fully mature leaf blade for diagnosis of deficiencies in plants** (Reuter and Robinson 1997)
8. **The duration of the saturation pulse** should be between 0.5 seconds and 1.5 seconds for higher plants, and 25 to 50 milliseconds for Phytoplankton and cyanobacteria. (Schreiber 1995). Times outside these ranges increase the error in $F_V/F_M$ measurements. Shorter durations prevent complete saturation of PSII regardless of the light intensity. Longer durations create a form of saturation pulse NPQ that rounds the tail end of the pulse maximum value, and reduces the average maximum saturation pulse value. A figure of 0.8 is sometimes set at the factory for some fluorometers, because it works for all higher plants tested. Some fluorometers have automated routines that ensure the correct saturation pulse duration. *This is not as important in the latest chlorophyll fluorometers from Opti-Sciences. They now use an eight point 25 millisecond rolling average to determine $F_M$ at its highest point, regardless of saturation pulse duration, as long as the saturation pulse is wide enough to saturate the sample. Therefore, saturation pulse NPQ will not affect measurements with modern Opti-Sciences chlorophyll fluorometers. It is available on the OS5p+,OS1p,OS30p₊ , iFL , Y(II) meter, and $F_V/F_M$ meter chlorophyll fluorometers.*
9. **Saturation pulse intensity.** Dark adapted leaves saturate easily with lower saturation pulse intensities. It may take a few hundred µmols to saturate shade leaves and sun leaves will saturate below 1,500 µmols. Lower values may not fully saturate PSII, and provide an error. Higher values always work with dark adapted samples. (Ralph 2005) (Requirements are different for Y(II).) Very intense saturation pulses will only damaged dark adapted plants if they are too frequent at the same location. Research has shown that once per hour is safe in the dark (Albert Porcar-Castell 2008).
10. **Some $F_V/F_M$ fluorometers have the ability to pre-illuminate dark adapted leaves with far-red light.** When this feature is used for five to ten seconds before an $F_V/F_M$ measurement takes place, it activates PSI, and ensures that all electrons have been drained from PSII before the measurement of $F_O$. While this feature ensures that PSII is completely re-oxidized, it does not relax the xanthophyll cycle, state transitions, chloroplast migration, and photoinhibition. *Time is still required in a darkened environment to relax all forms of NPQ and to obtain a reliable $F_V/F_M$ measurement.* (Maxwell and Johnson 2000)
11. **Part of the minimum fluorescence, $F_O$, and maximum fluorescence, $F_M$, in $F_V/F_M = (F_M-F_O)/F_M$ contains Photosystem I fluorescence** as well as PSII fluorescence. With $F_V/F_M$, one is trying to

measure the maximum variable fluorescence of PSII in a dark-adapted state. PSI fluorescence is not variable, but the low fluorescent signal from PSI does overlap with PSII. This produces an error. In $C_3$ plants, about 30% of $F_O$ fluorescence is due to PSI, and in $C_4$ plants about 50% of $F_O$ fluorescence is due to PSI fluorescence. PSI produces about 6% of the fluorescence found in $F_M$ in $C_3$ plants, and about 12% of Fm in $C_4$ plants (Pfundle 1998). This not a problem when comparing $F_V/F_M$ measurements for plant stress because PSI fluorescence does not change. It remains constant.

16. **Chlorophyll fluorescence heterogeneity** is measurement variation over the surface of a single leaf. While for most applications, it is not of concern, it can create problems when measuring some types of plant stress and under certain conditions. According to Baker (2008), plants under **drought stress**, **cold stress**, **$CO_2$ stress, or biotic stress** show significant patchy chlorophyll fluorescence heterogeneity. Fv/Fm does not work for drought stress until it is severe, but it may be used for cold stress. This means that if measurements are taken with a standard chlorophyll fluorometer, on different parts of the same leaf, there may be significant variation. The problem may be overcome by developing a sampling pattern, and making multiple measurements on a single leaf, and averaging the results. See the Opti-Sciences application note on chlorophyll fluorescence heterogeneity for more information. (Correspondence with Claus Buschmann). The iFL averages chlorophyll fluorescence measurements over the large chamber area, eliminating heterogeneity as an issue
17. **Light history –** Compare samples with similar light history only. Field plants and other plants that have been exposed to photoinhibition conditions for a several hours, can retain a certain amount of NPQ for up to 60 hours (Lichtenthaler 2004). This means that even if dark adaptation is overnight, there will almost always be some residual NPQ built into most summer field measurements of $F_V/F_M$ plants that have been exposed to photoinhibitory light conditions for several hours. Measurements should not be compared to plants that have been exposed to overcast conditions for this reason unless some form of light stress is the focus of the experiment. If photoinhibition measurement is the focus, it may make sense to partially shade the samples from photoinhibitory conditions for at least 60 hours.

***The best experiments are ones that take these issues into account.*** PSI fluorescence is involved in all measurements. It does not vary with light level or plant stress. (Schreiber 2004). With this in mind, comparing samples with similar light histories allows comparison of many types of plant stress. The Plant Stress guide provided by Opti-Sciences references papers that deal with specific types of plant stress and limitations of different chlorophyll fluorescence parameters for measuring plant stress.

***There are fluorescence solutions and assays available that are sensitive to most types of plant stress. $F_V/F_M$ is not as sensitive as Y(II) for many types of plant stress. However, It does have the advantage that all samples, with similar light histories, can all be dark adapted to the same known state. Light level does not need to be controlled.***

$F_V/F_M$ is not a sensitive test for drought stress, heat stress, nitrogen stress, nickel stress, sulfur stress, zinc stress, some herbicides and salt stress in some types of plants (Opti-Sciences Plant Stress Guide 2014). It can be used effectively in most other types of plant stress. *For specific research results on specific types of plant stress, see the specific type of plant stress of interest.*

# Cookbook checklist OJIP

## OJIP - has an additional requirement when compared tor $F_V/F_M$

“Strasser OJIP” is the OJIP protocol most used for measuring plant stress. Measurements require a fixed and calibrated actinic light intensity to get reliable measurements. It was found by Vredenburg (2011) that some of the OJIP peaks and timing for the peaks change at different light intensities. Early Strasser work

was done at 3,000 µmols. Later Strasser work was done at 3,500 µmols. (The OS30p+ automatically calibrates it's red actinic light source to 3,500 µmoles when the instrument is turned on.

Follow the $F_V/F_M$ checklist for all other OJIP requirements for reliable measurement.

## Cookbook checklist before making light adapted Y(II) or $\Delta F/F_M$' measurements.

**Y(II) or $\Delta F/F_M$'** is the quantum yield of PSII. It is a normalized measurement ratio that is an indication of the amount of energy used in photochemistry by PSII under steady-state photosynthetic lighting conditions (Genty 1989), (Maxwell K., Johnson G. N. 2000). Y(II) is affected by closure of reaction centers and heat dissipation caused by non-photochemical quenching. (Schreiber 2004) Photochemistry, heat dissipation, and chlorophyll fluorescence compete for light energy absorbed by the leaf. First reported by Bernard Genty in 1989, this light adapted test became possible with the advent of modulated fluorometers. It is the most versatile plant stress measuring parameter, because it has been shown to detect more types of plant stress, earlier, than any other chlorophyll fluorescence protocol. Measurement requires built in IR filtering.

**PAR** is photosynthetically active radiation. Radiation on the leaf is measured between the wavelengths of 400nm to 700 nm. PAR sensors and thermisters for measuring temperature are calibrated to other instruments that are traceable to the NIST. It is recommended that recalibration should occur every two years. Most modern sensors are solid state, so drift is minimal. **Y(II) is sensitive to most types of plant stress.** We have listed some important notes below.

**Checklist before making Y(II) measurements:**
$F_M$' = maximum fluorescence in a light adapted environment at steady state photosynthesis. $F_S$' = the fluorescence signal in a light adapted environment at steady state photosynthesis.
Y(II) is = ($F_M$' – $F_S$') / $F_M$' = $\Delta F/F_M$'

1. **Leaves must be at steady state photosynthesis.** Above canopy leaves on a clear day, in the field, are considered to be at steady state photosynthesis. (Maxwell and Johnson 2000). *In the past*, it was thought that this process took between 15 to 20 minutes on a sunny day. (Maxwell and Johnson 2000). However, it can take from twenty to thirty five minutes at higher actinic light levels (Cazziniga 2013) (Dall'Osta 2014). New evidence requires that chloroplast migration time be included in the time to reach steady state photosynthesis. At higher actinic light levels, chloroplasts migrate from the top of cells in the dark, and at lower light levels, to the sides of cells at high light levels. This process takes between *20 to 35 minutes*. The fluorescence changes previously thought to be caused by state transitions, and by "acute" photoinhibition, are actually caused by chloroplast migration. It is a mechanism that increased leaf transmittance and decreases leaf absorptance. Chloroplast migration can account for up to 30% of non-photochemical quenching at higher light levels. Chloroplast migration changes both $F_S$ and $F_M$'. (Cazzaniga S. 2013) (Dall'Osta 2014).
2. **It is dangerous to make Y(II) measurements on below canopy leaves in the field**. The shade from higher leaves and wind can interrupt a plant's adjustment to steady state under ambient conditions. The xanthophylls cycle, and Δph of the thylakoid lumen adjust in several seconds to several minutes. It can take longer in field plants, up to seven minutes. (Baker 2008) (Lichtenthaler 1999). State Transitions take between fifteen and twenty minutes to completely adjust (where they exist). It has been found that state transitions were a big factor at lower light intensities where they existed, but they were not as much of a factor at high light intensities. Chloroplast migrations take between 20 and 35 minutes at high actinic light levels. Rapid light curves and $F_V/F_M$ may be better solutions for below canopy work where appropriate. Rapid light curves are designed for measuring the affects of rapid light changes. *The alternative, is to use an internal fluorometer actinic light source, under a shroud, expose the leaf*

*sample to light for up to thirty five minutes, to reach steady state, and then make a measurement.* If necessary, use a shroud, a tripod, and a PAR clip. Use the internal actinic illuminator to pre-illuminate samples to steady state photosynthesis to get reliable Y(II) measurements.

3. **Y(II) values vary with light level and with temperature**. The higher the light level, the lower the YII) value. When measuring Y(II) in the field, it is extremely important to measure leaf irradiation or light level, at the leaf level, and at the same angle of as the leaf orientation. Y(II) also varies with leaf temperature. Comparing Y(II) values taken at different light levels, and different temperature levels, can introduce significant errors, unless it is the change, at different light levels and heat levels, that is of interest. This is commonly done with a PAR Clip. (Genty 1989), (Genty 1990) *PAR clips are essential for most field and laboratory Y(II) applications.* Light intensity varies with the square of the distance from the light source. In the field, small changes in distance make little difference because the sun is so far away. In the laboratory or in growth chambers, small distance changes can make a significant difference in PAR light reaching the leaf surface.
4. **Shade leaves vs. Sun leaves**. – The Y(II) ratio will be higher on Sun leaves than on shade leaves for the same light irradiation level (Lichtenthaler 2004). Light level will affect each differently.
5. **Field plants should only be compared to field plants** and green houseplants should be compared to green houseplants due to light history. (Lichtenthaler 2004)
6. **Leaf orientation.** When making a Y(II) measurement, it is important not to change the orientation of the leaf. The leaf is at steady state photosynthesis in its current orientation. Changing the orientation changes the amount of light falling on the leaf, and the leaf will no longer be at steady state photosynthesis.
7. **It is common to use the youngest fully mature leaf blade for diagnosis of deficiencies in plants** (Reuter and Robinson 1997)
8. **The duration of the saturation pulse** should be between 0.5 seconds and 1.5 seconds for higher plants, and 25 to 50 milliseconds for algae and cyanobacteria (Schreiber 1995). Times outside these ranges increase the error in Y(II) measurements. Shorter durations prevent complete saturation of PSII regardless of the light intensity (Roseqvist & van Kooten 2006). Longer durations create a form of saturation pulse NPQ that rounds the top trailing edge of the pulse maximum value, and reduces the average maximum saturation pulse value (Roseqvist & van Kooten 2006). Some fluorometer allow adjustment of this parameter, and others are preset at the factory at either. 0.8 seconds, or 1.0 seconds for higher plants.. *This is not as important in the latest chlorophyll fluorometers from Opti-Sciences. They now use an eight point rolling average to determine $F_M$' at its highest point, regardless of saturation pulse duration, as long as the saturation pulse is wide enough to saturate the sample. The eight point rolling average prevents saturation pulse NPQ from being a problem. This feature exists on the OS5p, the OS5P+, the OS1p, the iFL the OS30p+, the Y(II) meter & the $F_V/F_M$ meter*
9. **Saturation pulse intensity**. Saturation pulse intensity is more of an issue with Y(II) than with $F_V/F_M$. When dark adapting, shade leaves will saturate at a few hundred μmols, and sun leaves will usually saturate below 1,500μmols (Ralph 2004). However, a problem has been found when measuring Y(II) at high light levels. It has been discovered that at high actinic, or sun light levels, leaves resist the complete closure of all PSII reaction centers that is expected when using the most intense saturation pulse. Even with a 20,000 μmol saturation pulse, some reaction centers remain open. As a result up to a 41% error was found in Y(II) measurements using standard square saturation flash techniques at high actinic light levels (Loriaux 2006) (Loriaux 2013) when compared to gas exchange measurements. To correct for this issue, a method was developed using a multiple phased single saturation flash was used. The fluorescence intensity output was measured for each phase. The initial maximum saturation flash of 7,000 μmols for 0.3 seconds was made and then, a 20% down ramp in light intensity was created at a rate of 0.01 mol photons $m^{-2}s^{-2}$. Finally, a second 0.3 second flash at 7000 μmols was used to detect any saturation pulse NPQ. The measured fluorescence results were then subjected to east squares linear regression using PAR values of PAR/10,000. The Y axis intercept represented a fluorescence value with an infinitely intense saturation flash. The Loriaux 2013 paper was co-Authored by Bernard Genty, the creator of Y(II). The Loriaux 2013 method is an included

option on the OS5p+, the iFL, the OS1p and the Y(II) meter chlorophyll fluorometers. One can still use the standard square topped flash if desired.

10. **PSI fluorescence -** Part of the fluorescence signal contains PSI fluorescence as well as PSII fluorescence. With Y(II), one is trying to measure variable fluorescence of PSII in a light adapted state. PSI fluorescence is not variable, but the low fluorescent signal from PSI does overlap with PSII. This produces a small error but it is not a problem for comparing similar samples, because PSI fluorescence does not change with light intensity, temperature, or plant stress. (Baker, Oxborough 2004)
11. **"Super-saturating flash" error** is produced by using a very intense saturation light source that is longer that 2ms, causing multiple turnovers of primary PSII receptor $Q_A$ and the reduction of plasotoquinone to plastoquinol. This raises $F_M$' (or $F_{MS}$') and can cause an overestimate of Y(II) by less than 10% (Baker and Oxborough 2004), (Schreiber 2004). Use of a super-saturation flash is by far the most common method of measuring yield of PSII in higher plants. *As long as one is interested in plant stress and not exact correlation to $CO_2$ carbon assimilation, this is not an issue.*
12. **Cold stress can produce a non-linear correlation** with $CO_2$ assimilation. Electron transport of PSII in cold stressed corn far exceeds the requirements for $CO_2$ assimilation by more than three to one, indicating that under these conditions, other electron sinks are at work. The ratio of ETR (a product of Y(II), PAR, leaf absorption ratio, and PSII absorption ratio) to $CO_2$ assimilation, under cold stress, can be diagnostic for cold stress. (Fryer M. J., Andrews J.R., Oxborough K., Blowers D. A., Baker N.E. 1998)
13. **The ratio of ETR to $CO_2$ assimilation can be diagnostic for drought stress in $C_3$ plants**. $C_3$ plants exhibit strong electron transport rates for early and moderate levels of water stress even when $CO_2$ assimilation has decreased due to water stress. This indicates that there are other electron sinks for electron transport. (Ohashi 2006). *This problem of early water stress measurement and detection may be overcome by using a special assay discussed in Burke 2007 and Burke 2010. A discussion of the Burke assay can be found at* www.optisci.com. Request the water stress application notes. The iFL is ideal for comparing ETR to $CO_2$ assimilation.
14. **Mangrove leaves growing in the tropics.** Here again electron transport rate is more that three times that of $CO_2$ assimilation. It is believed that this is mostly due to reactive oxygen species as an electron sink. (Baker Oxborough 2004), (Cheeseman 1997)
15. While linear correlation occurs between Y(II) and ETR with $CO_2$ assimilation in $C_4$ plants and curvilinear correlation between Y(II) and ETR with $CO_2$ assimilation in $C_3$ plants, (Genty 1989), (Genty 1990), (Baker Oxborough 2004), *exact* correlation between fluorescence ETR and gas exchange carbon assimilation is not possible due to the fact that most fluorescence comes from only the upper most layers of the leaf, while gas exchange measurements measure lower layers as well (Schreiber 2004).
16. **Chlorophyll fluorescence heterogeneity** is measurement variation over the surface of a single leaf. While for most applications, it is not of concern, it can create problems when measuring some types of plant stress and under certain conditions. According to Baker (2008), plants under **drought stress**, **cold stress,** and **$CO_2$ stress** show significant patchy chlorophyll fluorescence heterogeneity. This means that if measurements are taken with a standard chlorophyll fluorometer, on different parts of the same leaf, there may be significant variation. The problem may be overcome by developing a sampling pattern, making multiple measurements on a single leaf and averaging the results. Gas exchange systems with integrated fluorometers, such as the iFL-LCpro-SD, measure over a large area and eliminate this issue as a problem. See the Opti-Sciences application note on chlorophyll fluorescence heterogeneity for more information.
17. **Light history** - Since chronic photoinhibition takes up sixty hours to relax, there can be un-relaxed photoinhibition built into all light adapted measurements made on samples that have a "high" light history from the previous day or two. It is also likely that a few overcast days will allow complete relaxation of photoinhibition. For this reason, it is important to take this variable into account when comparing samples measured on different days and under different conditions. Light history should be considered when designing experiments for reliable results.

**18. Actinic light spectrum**. It was recently found that under natural field conditions, chloroplast migrations occured at higher actinic light levels in $C_3$ plants (Cazzaniga 2013) and $C_4$ plants (Maai 2011). *Furthermore, it was found that this light avoidance mechanism significantly affected chlorophyll fluorescence measurements.* Using wild and mutant Arabidopsis plants, the chloroplast migration, was responsible for up to ***30% of total NPQ*** (non-photochemical quenching) at high actinic light levels. This mechanism was found to be *regulated by intense white and intense blue actinic light. Chloroplast migration did not respond significantly to intense red actinic light.* As a result, **using a white actinic light source or a combination of red LEDs and blue LEDs that provide a *intense blue actinic light*, will prevent measuring errors due to chloroplast migration.** Sunlight provides an intense blue spectrum. (Cazzaniga 2013) (Dall'Osta 2014) The OS1p, the OS5p, the OS5p+ and the iFL have white actinic light sources with intense blue spectrums.

**Y(II) vs. $F_V/F_M$ -**Y(II) is a more versatile measuring parameter than $F_V/F_M$, that is proven to measure more types of plant stress at more sensitive levels. It does require comparison to samples used as a standard, at the same PAR light level, and temperature. $F_V/F_M$ offers the advantage that samples can be compared after being dark-adapted to the same known state. However, $F_V/F_M$, (the dark adapted test) is not sensitive to drought stress until about 7 days have passed without water (Bukhov & Carpentier 2004), heat stress (Haldiman P, & Feller U. 2004), nitrogen stress (Baker 2004), sulfur stress (Baker 2004), nickel stress below 45°C (Joshi & Mohanty2004), zinc stress (Joshi & Mohanty2004), some types of chemical stress, and some types of herbicide stress. For more information about specific types of plant stress, *go to the table of contents.* Both are fast tests. A PAR Clip is a highly recommended accessory for the measurement of Y(II) as Y(II) varies with PAR light level and temperature.

## Cookbook checklist before making NPQ and other quenching measurements.

*Quenching measurement parameters, such as NPQ, are the least understood, and most often misused parameters that are available with advanced chlorophyll fluorometers. This Check list is designed to improve the understanding of proper quenching protocol usage.*

There are a few quenching protocols to choose from: The Kramer lake model, Hendrickson lake model, puddle model, and quenching relaxation protocols. For an in depth discussion of the differences, and advantages of each, please request the *Opti-Sciences Quenching application note* at www.optisci.com.

**To get reliable measurements, one should follow tested guidelines.**

**1. Dark-adapt properly knowing the plant's light history.** It takes only a few minutes for the xanthophyll cycle and the Δph of the thylakoid lumen to return to a dark-adapted state. State transitions, however, take between fifteen to twenty minutes. These times can vary somewhat in field plants and can take slightly longer (Baker 2004). Under high actinic light conditions, it takes chloroplast migration from 20 to 35 minutes to relax to a dark adapted state. Chloroplast migrations will significantly affect fluorescence measurements if measurements are made before they are fully relaxed. (Cazzaniga S. 2013) (Dall'Osta). In addition, field plants and other plants that have been exposed to photoinhibition conditions for a number of hours, will retain a certain amount of NPQ for up to 30 to 60 hours (Lichtenthaler 2004). This means that even if dark adaptation is overnight, there will almost always be some residual NPQ built into summer field measurements of $F_V/F_M$, and other displayed quenching parameters. For this reason, it is important to only compare samples with a similar light history. ***When doing quenching measurements on field plants, it is common for researchers to use pre-dawn or overnight dark adaptation times*** (Maxwell & Johnson 2000). *(For more information, see dark*

*adaptation application note.) If photoinhibition is your focus, Then you may want to partially shade samples from photoinhibitory light conditions for at least 60 hours to get a more reliable $F_V/F_M$ and a more reliable $q_I$ measurement. Before choosing a shorter dark adaptation time for lab work or growth chamber work, check with a reviewer from a target publication. They have strong feelings on the subject. However, overnight or pre-dawn values are generally accepted .*

2. **For quenching measurements, samples that are compared, must have the same $F_V/F_M$ values.** Quenching measurements of different samples with different $F_V/F_M$ values should not be compared (Baker 2008). $F_V/F_M$ is used as the measuring standard for non-photochemical quenching measurements, and if the measuring standard is different, the quenching values are meaningless. Comparing values from samples with different $F_V/F_M$ values is like measuring items with a ruler that has dimensions that change.
3. **Modulation light intensity setting** $F_V/F_M$ is $(F_M-F_O)/F_M$. $F_O$, or minimum fluorescence is a dark-adapted value made by exposing the leaf antennae to a very low intensity modulated measuring light, that is not set high enough to drive photosynthesis or chemically reduce $Q_A$ (Zhu 2005), but set high enough to make a measurement. The modulation light intensity must be set correctly for best accuracy and repeatability. If it is set too high, it will drive photosynthesis and provide an $F_O$ value that is too high. The modulated light allows the measurement of pre-photosynthetic antennae fluorescence. Maximum fluorescence is measured when exposing a leaf to a saturation flash with light intense enough to close all PSII reaction centers. The OS1p, the OS5p+, the iFL, and the OS30p+ all have automatic modulated light set up routines for ease of use. One can still use the manual method as well. This is done by placing the leaf in the leaf clip, PAR clip or leaf chamber and exposing the leaf to the modulated light. If the Ft value on the screen rises over a 10 to 20 second period it is set too high. If it is set too low, a too low message will appear on the screen.
4. **Leaves must be at steady state photosynthesis for most quenching measurement parameters.** Until recently it was thought that this process took between fifteen and twenty minutes at a lower and medium light levels (Maxwell and Johnson 2000) to reach steady state. However, recently it was discovered that chloroplast migration was responsible for the intermediate chlorophyll fluorescence change up to twenty to thirty five minutes, replacing state transitions and acute photoinhibition as the two sources of chlorophyll fluorescence change during these time scales. As a result actinic light levels should be on for at least 35 minutes to reach steady state photosynthesis. Chloroplast migrations will significantly affect fluorescence measurements at high actinic light levels if measurements are made before they are fully adjusted. (Cazziniga 2013) (Dall'Osta 2014). For example, if there are 18 saturation pulses spaced 2 minutes apart, the leaf will be exposed to the actinic light for 36 minutes after dark adaptation. Since an internal fluorometer artificial light source is normally used, the test allows one to compare Below canopy leaves as long as the $F_V/F_M$ values are the same. According to Klughammer (2008), the only non-photochemical parameter that does not have to be taken at steady state photosynthesis is Y(NO) from Hendrickson or Kramer.
5. **Use a fluorometer with a stable actinic light output.** Depending on the brand and type of fluorometer, the intensity output of the actinic light can change over time. When an actinic light is on, it can heat the fluorometer and cause a lowering of the light output. The intensity of the actinic LED light source output changes as the heat from the LED changes the LED temperature. More advanced systems have ways to ensure a steady actinic light level, either by using a stable light source or monitoring light output with a PAR clip to maintain a constant light level. If light intensity changes significantly over a 20 -35 minute actinic illumination period, *the sample is no longer at steady state photosynthesis. The OS1p, the OS5p+ and the iFL use a PAR sensor to measure light irradiated onto the leaf surface and correct any changes in intensity. Corrections are made at least every 0.1 seconds or faster. All units have a stable actinic light output for the most reliable measurements.*
6. **Y(II) values and quenching values vary with light level, leaf angle to the light source and with temperature.** The higher the light level, the higher the NPQ value. When measuring NPQ in the field or the lab, it is extremely important to measure PAR leaf irradiation at the leaf, and leaf temperature. Light varies inversely with the square of the distance from the light source, and varies significantly with leaf angle to the source. Comparing Y(II) and quenching values taken at different light levels, different

angles to the light source and different temperature levels, introduces a significant error, unless it is the change that is of interest. This is commonly done with a PAR Clip, a tripod, and a shroud over the sample for quenching measurements.

7. **Shade leaves vs. Sun leaves**. – The Y(II) ratio will be higher on Sun leaves than on shade leaves (Lichtenthaler 2004) for the same light intensity.
8. **Field plants should only be compared to field plants,** and green houseplants should be compared to green houseplants due to light history. (Lichtenthaler 2004)
9. **Leaf orientation is not important because an artificial actinic light source is used.**
10. **It is common to use the youngest fully mature leaf blade for diagnosis of deficiencies in plants (Reuter and Robinson 1997).**
11. **The duration of the saturation pulse** should be between 0.5 seconds and 1.5 seconds for higher plants, and 25 to 50 milliseconds for algae and cyanobacteria (Schreiber 1995). Times outside these ranges increase the error in Y(II) measurements. Shorter durations prevent complete saturation of PSII regardless of the light intensity (Roseqvist & van Kooten 2006). Longer durations create a form of saturation pulse NPQ that rounds the top trailing edge of the pulse maximum value, and reduces the average maximum saturation pulse value (Roseqvist & van Kooten 2006). Some fluorometer allow adjustment of this parameter, and others are preset at the factory at either. 0.8 seconds, or 1.0 seconds for higher plants.. *This is not as important in the latest chlorophyll fluorometers from Opti-Sciences. They now use an eight point rolling average to determine $F_M$' at its highest point, regardless of saturation pulse duration, as long as the saturation pulse is wide enough to saturate the sample. The eight point rolling average prevents saturation pulse NPQ from being a problem. This feature exists on the OS5p, the OS5P+, the OS1p, the iFL the OS30p+, the Y(II) meter & the $F_V/F_M$ meter*
12. **Saturation pulse intensity**. Saturation pulse intensity is more of an issue with Y(II) than with $F_V/F_M$. When dark adapting, shade leaves will saturate at a few hundred µmols, and sun leaves will usually saturate below 1,500µmols (Ralph 2004). However, a problem has been found when measuring Y(II) at high light levels. It has been discovered that at high actinic, or sun light levels, leaves resist the complete closure of all PSII reaction centers that is expected when using the most intense saturation pulse. Even with a 20,000 µmol saturation pulse, some reaction centers remain open. As a result up to a 41% error was found in Y(II) measurements using standard square saturation flash techniques at high actinic light levels (Loriaux 2006) (Loriaux 2013) when compared to gas exchange measurements. To correct for this issue, a method was developed using a multiple phased single saturation flash was used. The fluorescence intensity output was measured for each phase. The initial maximum saturation flash of 7,000 µmols for 0.3 seconds was made and then, a 20% down ramp in light intensity was created at a rate of 0.01 mol photons $m^{-2}s^{-2}$. Finally, a second 0.3 second flash at 7000 µmols was used to detect any saturation pulse NPQ. The measured fluorescence results were then subjected to east squares linear regression using PAR values of PAR/10,000. The Y axis intercept represented a fluorescence value with an infinitely intense saturation flash. The Loriaux 2013 paper was co-Authored by Bernard Genty, the creator of Y(II). The Loriaux 2013 method is an included option on the OS5p+, the iFL, the OS1p and the Y(II) meter chlorophyll fluorometers. One can still use the standard square topped flash if desired.
13. **The time between saturation pulses is important.** Rosenqvist and van Kooten (2006) state that between one to two minutes is required for complete relaxation of saturation pulse NPQ. If saturation pulses are not separated by this distance range, then an error caused by saturation pulse NPQ will result. Furthermore, It will accumulate with each saturation pulse. When in doubt, space saturation pulses two minutes apart or more. *We have found that when the actinic light is off it can take longer that two minutes* for saturation pulse NPQ to fully dissipate as seen during quenching relaxation measurements. If one sees the bottom of the fluorescence graph start to rise, it is either due to the modulated light intensity or a build up of saturation pulse NPQ after longer relaxation tests. In this case, we find that spacing the saturation flashes 3 to 4 minutes apart during the relaxation phase of the test works very well.

14. **Overlap of PSI fluorescence** -Part of the minimum fluorescence, the $F_O$ parameter, in $F_V/F_M$ $((F_M - F_O)/F_M)$, contains PSI fluorescence as well as PSII fluorescence. With $F_V/F_M$, one is trying to measure the maximum variable fluorescence of PSII in a dark-adapted state. PSI fluorescence is not variable, but the low fluorescent signal from PSI does overlap with PSII. This produces an error. In $C_3$ plants, about 30% of FO fluorescence is due to PSI, and in $C_4$ plants about 50% of Fo fluorescence is due to PSI fluorescence. PSI produces about 6% of the fluorescence found in Fm in $C_3$ plants, and about 12% in $C_4$ plants. (Pfundle 1998). This not a problem when comparing quenching measurements for plant stress because, PSI fluorescence does not change with light level or plant stress.
15. **PAR** is photosynthetically active radiation. Radiation on the leaf is measured Between the wavelengths of 400nm to 700 nm. PAR sensors and thermisters for measuring temperature are calibrated to other instruments that are traceable to the NIST. Since Y(II) and quenching parameters change with light and temperature, as well as plant stress levels, there are advantages to using a shrouded leaf and PAR Clip when making quenching measurements. In addition, it is important that the actinic light level does not change over the length of the measurement, because it will cause an error in quenching measurement results. This can be done with a stable internal light source, or a system that allows a PAR Clip that provides measurement feedback to maintain a constant light level required for steady state photosynthesis during the quenching measurement.
16. **Far-red pre-illumination**. Some fluorometers have the ability to pre-illuminate dark-adapted leaves with far-red light. When this feature is used for 5 to 10 seconds before an $F_V/F_M$ measurement takes place. It activates PSI, and ensures that all electrons have been drained from PSII before the measurement of $F_O$. While this feature ensures that PSII is completely re-oxidized, it does not relax the xanthophyll cycle, state transitions, chloroplast migration or photoinhibition. Time is still required in a darkened environment to relax all forms of NPQ and to obtain reliable quenching values.
17. **Far-red illumination.** This is usually used in the post actinic light mode to allow measurement of $F_O$' a parameter that reflects quenched $F_O$. This value is used in Kramer lake model parameters, and puddle model $q_N$ and $q_P$. It is not used in Hendrickson simplified lake model parameters, or in NPQ.
19. **Light history** - Since chronic photoinhibition starts to relax at forty minutes and takes from thirty to sixty hours to relax (Lichtenthaler 2004), there can be un-relaxed photoinhibition built into all light adapted and dark-adapted measurements made on samples that have a “high” light history from the previous day or two. It is also likely that a few overcast days will allow complete relaxation of photoinhibition. For this reason, it is important to take this variable into account when comparing samples measured on different days and under different conditions. Light history should be considered when designing experiments for reliable results.

**The best experiments are ones that take these issues into account**. PSI fluorescence is involved in all measurements. It does not vary with light level or plant stress (Schreiber 2004). With this in mind, comparing samples with similar light histories allows comparison of many types of plant stress.
The Plant Stress Guide provides referenced papers that deal with specific types of plant stress and limitations of different chlorophyll fluorescence parameters for measuring plant stress.

# Drought Stress:

**Important Notes: Yield of PSII or Y(II), and ETR are effective tests for water stress in $C_4$ plants. In $C_3$ plants, the only method that reports good results for early water stress is the special Burke assay (2007, 2010) listed below. Fs/Fo only works for moderate water stress found in plants like grapes. It is not adequate for most other plants according to Flexas. In $C_3$ plants, photorespiration is thought to be the reason for less than adequate results in regard to early water stress testing. (Flexas 2000). Flexas 1999 and Flexas 2000 provide a good review of the limitations of standard chlorophyll fluorescence techniques for water stress measurement. The standard tests: $F_V/F_M$, and Y(II), will only work for *severe drought* stress measurement in $C_3$ plants due to photorespiration (Flexas 1999, 2000). $F_V/F_M$ can be used for severe water stress after about 7 days without water. It should not be used for crops.** (da Silva J. A. & Arrabaca M.C. 2008).

**In addition, samples that are subject to drought stress display heterogeneous fluorescence from one place on a leaf to another. For more information on this topic see Baker (2008). *The Burke assays do not seem to be affected by this fact.* If the Burke assay is not being used for $C_4$ plants, then it is important to deal with this issue. To overcome this issue, it is recommended that measurements be made at multiple locations on the same leaf, and results may be averaged. Integrated fluorometer – gas exchange systems overcome this issue by averaging the fluorescence reading over the same area as gas exchange measurements. Imaging fluorescence displays the heterogeneity. Using several measurements on the same leaf, at different locations on the same leaf, with a non-imaging fluorometer provides a higher measurement resolution of the heterogeneity. See the Opti-Sciences application note on fluorescence heterogeneity for more information on the subject.**

## Best Tests $C_3$ plants

**Y(II) in the Burke Special assay- (Designed for large and small plant populations).** This is a light adapted test that can be used for ***very early water stress***. This test was designed for small or large populations of plants. At dawn, leaf disc samples are collected with a leaf punch, and kept moist in a tissue culture tray. They are transferred to a larger tray and covered with glad wrap. The samples are measured using Y(II) and then placed in an oven heated to 40°C for one half hour. The tray is removed from the oven, allowed to cool for thirty minutes, and reach steady state photosynthesis under room level lighting. Y(II) measurements are then made of each sample. It has been found that the non-water stressed leaves will measure lower than water stressed leaves even when irrigation had ended 24 hours earlier. This assay works because leaves that are under water stress retain sugars manufactured at night and so they are less susceptible to heat stress. It works for $C_3$ plants and $C_4$ plants. (Burke 2010), (Burke 2007) *For this test to work properly, either samples must be taken at or before sunrise, or whole plant dark adaptation by artificial means is required, before sample collection. When artificially dark adaptation is used, night length dark adaptation times should be used. Samples must always be kept wet. Momentary dark adaption is used before measurement. The newer 2010 paper procedure is the one to follow for best results regardless of whether the plants are $C_3$ or $C_4$ plants.*

**$F_S/F_O$ & $F_S$ -** $F_O$ is from the dark-adapted $F_V/F_M$ test, and FS is from the steady state Y(II) fluorescence

test. This test is sensitive to moderate water stress and it is adequate for work with grapes but it is not sensitive enough for most other $C_3$ plant crops. Fs, a component of (YII) is not a normalize ratio but is has been found to be more sensitive to $C_3$ plant water stress than Y(II). Tested in $C_3$, $C_4$, and CAM plants. $F_S/F_O$ is normalized ratio using $F_S$ that allows comparison between samples. $F_O$ is a predawn value of $F_O$. Actinic light is used at saturating levels between 800 to 1250 μmls. (Flexas 2002), (Flexas 2000), (Flexas 1999).

## Best Tests  $C_4$ Plants

**Yield or Y(II) -** Fast light adapted test can also be used for water stress in **$C_4$ plants.** Good correlation between Y(II) and gas exchange measurements (da Silva J. A. & Arrabaca M.C. 2004).

**ETR/A or *J*/A** - Fast light adapted steady state fluorescence test. In $C_4$ Plants. The ratio of ETR to carbon assimilation, ETR/A, is known to be consistent in $C_4$ plants. **This is not true in $C_3$ plants**. *ETR requires a PAR Clip.* (J Cavender-Bares & Fakhri A. Bazzaz 2004 ) (Cerovic 1996). ETR/A requires a combined integrated fluorometer - gas exchange system. *When comparing ETR or J values on different leaves, leaf absorption should be measured and entered into the formula for ETR.*

**Y(II) in the Burke Special assay-** This is a light adapted test that can be used for ***very early water stress***. This test was designed for small or large populations of plants. At dawn, leaf disc samples are collected with a leaf punch and kept moist in a tissue culture tray. They are transferred to a larger tray and covered with glad wrap. The samples are measured using Y(II) and then placed in an oven heated to 40°C for one half hour. The tray is removed from the oven and allowed to cool and reach steady state photosynthesis under room level lighting. Y(II) measurements are then made of each sample. It has been found that the non-water stressed leaves will measure lower than water stressed leaves even when irrigation had ended 24 hours earlier. This assay works because leaves that are under water stress retain sugars manufactured at night and so they are less susceptible to heat stress. It works for $C_3$ plants and $C_4$ plants. (Burke 2010), (Burke 2007)

## Other Tests

**Combined Y(II) and Carbon Assimilation (A) –**The combination of gas exchange and fluorescence is a powerful tool to use for water stress, as it shows how water stress affects different parts of light and dark reaction. The combined use of the two types of instruments has been found to be very useful for specific types of plant stress measurements, such as water stress, heat stress and cold stress. In these types of plant stress, the results of electron transport, as measured with a fluorometer, can show significant differences from carbon assimilation measurements, from gas exchange measurements.

**$F_S/F_O$** Light adapted test can also be used for water stress in steady state, Samples must be dark adapted to obtain $F_O$ in $F_V/F_M$, and then samples must be brought to steady state photosynthesis to measure $F_S$. It is not as sensitive to water stress as the Burke assay but it may be used for plants like grapes (in $C_3$ plants). (Flexas 1999)

**Light curve–** Slow test that helps identify water as the cause of stress.
This is a longer light adapted test. $F_S$ has been found to decrease as light intensity increases. (Flexas 2000)

**NPQ –** Slow test, increases with moderate to late water stress. This is a dark adapter test. (Cavender-Bares J. & Fakhri A. Bazzaz 2004 )

-Continued -What tests are not sensitive to drought stress.

# Non-Sensitive to early or moderate Drought Stress:

**$F_V/F_M$ -** Fast dark-adapted test is not sensitive to early or moderate water stress, only severe stress (Bukhov & Carpentier 2004) (Zivcak M., Brestic M, Olsovska K. Slamka P. 2008) In some species $F_V/F_M$ is more sensitive to water stress than in other species. (Deng X. Hu Z., Wang H., Wen X., Kuang T. 2003) It can be used for severe plant stress where drought lasts about 7 days. This may be adequate for long-term drought in forestry applications, however, is not adequate for crops.

**$F_V/F_M$ -** Fast dark-adapted test is **not** sensitive to early or moderate water stress in **$C_4$ plants,** only severe stress. (da Silva J. A. & Arrabaca M.C. 2008). It can be used for severe water stress after about 7 days. It is not adequate for crops.

**$PI_{ABS}$** - Fast dark-adapted test for *detecting* water *stress after seven days* after cessation of irrigation on wheat using OKJIP protocol. It is not as sensitive as Y(II), ETR, *J*/A or the Burke assay in $C_4$ plants. This is a normalized OJIP parameter for comparing data between samples. The test correlates well with $CO_2$ gas exchange data during water stress measurements. (Zivcak M., Brestic M, Olsovska K. Slamka P. 2008) (Thach 2007) , but it only works for severe drought stress after about 7 days (Thack 2007). It is not adequate for crops.

**K Step -** Fast dark-adapted test for water stress using OJIP protocol **(See $PI_{ABS}$)** (Strasser 2004). Works only for severe drought stress.

**$F_V/F_M$ - Leaf treated with high light irradiation and polyethylene glycol to induce water stress**.
20 mm leaf plugs are collected and treated with polyethylene glycol PEG at 6000 mol weight at various concentrations to induce water stress and exposed to 1500 to 1800 µmols for two hours before dark adaption . (Nair D. B., Alam B., Jacob J. 2005).

# Light Stress:

**While light stress can be measured effectively by most fluorescence protocols, it is common to study light stress using more elaborate chlorophyll fluorometers that allow longer quenching and quenching relaxation protocols.**

**To understand the effects of light stress on plants, the following papers provide a good start:** (Lichtenthaler 1999, 2004), (Muller, Niyogi 2001), (Kramer 2004),(Cazzaniga S.2013) & (Dall'Osta 2014).

## Best Tests

**Quenching and Quenching Relaxation Test – Best test** to study photo-protection mechanisms Including the △ph of the thylakoid lumen, and the xanthophyll cycle, as well as state transitions (where they exist), chloroplast migration, and photo-inhibition are quenching relaxation tests. Measuring parameters have been developed for each mechanism. $q_E$ represents the fast acting photoprotective mechanisms that involve △ph of the thylakoid lumen, and the xanthophyll cycle (Muller, Niyogi 2001), $q_T$ is a parameter for measuring state transitions where they exist, $q_M$ is parameter for measuring chloroplast migration, a mechanism that affects chlorophyll fluorescence more at high actinic light levels (Cazzaniga S.2013) (Dall'Osta 2014). $q_Z$ is a measuring parameter for an unknown mechanism thought to be related to the xanthophyll cycle. In many cases, $q_Z$ is probably $q_M$ (Cazzaniga S.2013). Chloroplast migration also exists in monocots (Maai 2011). Finally $q_I$ is a measure of photoinhibition. Other quenching parameters have been developed as well to allow measurement of the effects of light stress They include: the Kramer lake model quenching protocol, the Hendrickson lake model quenching protocol that allows resurrection of NPQ from the puddle model of antennae –reaction center interaction to the newer lake model.. Kramer parameters include: Y(II), $q_L$, Y(NPQ), Y(NO). (Kramer 2004), Hendrickson parameters include: Y(II), Y(NPQ), Y(NO) and NPQ (Hendrickson 2004), (& Kluhammer and Schreiber 2008). Lake model parameters that include $q_E$, $q_T$, and $q_I$ see (Ahn, Avenson 2008) For standardized definitions see (van Kooten O., & Snel J.F. 1990). For lake model parameters see Kramer (2004), Hendrickson (2004) and Ahn, Avenson (2008) NPQ , $q_E$, $q_T$, $q_I$ (Muller, Niyogi 2001) for $q_Z$ see (Nilkens 2010) and for $q_M$ see (Cazzaniga S.2013). *This is a longer dark-adapted test.*

***For definitions* of quenching parameters** $q_E$, $q_T$, $q_I$, with NPQ see (Muller P., Xiao-Ping L, Niyogi K. 2001). For $q_E$, $q_T$, $q_I$ with $q_N$ see (Lichtenthaler 1999) For lake model definitions Y(II), $q_L$, Y(NPQ), Y(NO) see Kramer D. M., Johnson G., Kiirats O., Edwards G. (2004). For simplified lake model parameters that include NPQ, see Hendrickson (2004), and Klughammer, Schreiber (2008). For division of lake model parameters into $q_E$, $q_T$, and $q_I$ see Ahn, Avenson (2008). For standardized puddle model quenching definitions see (van Kooten O., & Snel J.F. 1990). For $q_Z$ see (Nilkens 2010), and for $q_M$ see (Cazzaniga S.2013).

**For Quantum yield of PSII Y(II) or (△F/Fm') correction** in high light conditions see Earl (2004) and (Loriaux, 2006 & 2013) It has been found that under high actinic light conditions, a correction of quantum yield of PSII value is necessary to restore the correlation of ETR with Carbon assimilation measurements. Without this correction, it is not possible to close or completely chemically reduce all PSII reaction centers, a requirement for reliable Y(II) and ETR measurement. The methods are discussed in the papers and poster listed here.

***Light Response Curves*** This is a longer test (usually dark-adapted and then a light adapted test) where actinic light levels are increased or decreased after steady state photosynthesis has been reached and measured. These are curves that show the results of light level on Y(II) and Electron

Transport Rate. The effects of light level increases and decreases can be studied easily. (Muller, Niyogi 2001), (Kramer 2004), (Hendrickson 2004).Automated fluorometer routines are programmed for desired light intensities, step time duration, the number of saturation pulses per step and the number of steps. With the addition of the Cazzaniga 2013 paper on chloroplast migration, the length of time to reach steady state chlorophyll fluorescence is 20 to 35 minutes for each step.

**Yield of PSII or Y(II) -** Fast light adapted test can also be used for light stress in steady state sensitive to light stress. (Cavender-Bares & Bazzaz 2004)

**$F_V/F_M$ -** Fast dark-adapted test can be used to detect light stress. (Adams & Demming-Adams 2004) $F_V/F_M$ correlates to carbon assimilation.

# Other Tests –light stress

## For under canopy work and aquatic plants

**Rapid light Curves (RLC)–** A longer dark-adapted, or momentary dark adapter test, that usual take takes less than five minutes, but may take longer. *Steady state photosynthesis is not reached.* Data from several measurements at different times of day are recommended by some, for reliable results (Rasher 2000). An internal fluorometer actinic illuminator is used to step light up, or down to determine ETR response at different times of day. This provides a diurnal light history of the sample, it also allows investigation of the saturation characteristics of plants and correlates well to Rubisco activity under variable light conditions *(Macintyre 1997), (Macintyre 1996)*. Rapid light curves are used for aquatic plants, and under canopy plants, where light is constantly variable, and other methods of testing can be difficult. (Ralph 2005)

The parameters $ETR_{MAX}$, or optimal ETR, the intensity where $ETR_{MAX}$ occurs $I_m$, minimum saturation intensity $I_k$, and initial slope of the RLC curve $\alpha$ are made available in the OS5p+. *Light saturation rate as measured by rapid light curves highly correlates with the concentration and maximum activity of Rubico (Macintyre 1997), (Macintyre 1996). Measured steady state photosynthetic rates overestimate actual photosynthetic rates in a variable light environment (Macintyre 1997).*

Different researchers use different dark adaptation times, different step durations, different numbers of steps, and they step in different directions, up and down. They are light history dependant, and results change depending on the time of day that they are taken (Rasher 2000). Rapid Light Curves that uses 10 second steps have been found to have an unacceptably high level of error in benthic diatoms and longer steps are recommended (Perkins R.G, Mouget J-L, Lefebvre S., Lavaud J. 2006) The ability to saturate all reaction centers can be dependent on light history and method (Perkins R.G, Mouget J-L, Lefebvre S., Lavaud J. 2006). Rapid light curves are believed to provide relevant information on the saturation characteristics of electron transport (Schreiber 2004). Momentary dark adaptation for 5 to 10 seconds is covered by Ralph (2005). RLC as a way to measure full activation of Rubisco in a variable light environment see (MacIntyre 1997) contact Opti-Sciences for the RLC application note.

**$PI_{ABS}$** - Fast dark-adapted test sensitive to light stress using OKJIP protocol (Thach 2007). This parameter is a light stress detector but values do not correlate to gas exchange well.

# Heat Stress:

**The traditional method for measuring heat stress involves quenching measurements and non-photochemical quenching parameters such as NPQ. Chlorophyll fluorometers that perform this test are more expensive than basic systems. According to Haldiman (2004), NPQ will detect heat stress in Oak leaves at 35$^{o}$C and higher. He also found that Y(II) will also detect heat stress at 35$^{o}$C and higher. $F_V/F_M$ will only detect heat stress at 45$^{o}$C and higher. Gas Exchange has been shown to detect heat stress at 30$^{o}$C or higher (Haldiman 2004).**

## Best Tests

**Y(II) is a light adapted fast test that takes about two seconds. NPQ is a test that takes about twenty to thirty five minutes and overnight dark adaptation. $F_V/F_O$ increase in the dark is a long test.**

**Yield of PSII or (YII)** **-** Fast light adapted sensitive test for Moderate heat stress above **35$^{o}$C** in Oak – *Q. pubescens* (Haldiman P, & Feller U. 2004), ( Dascaliuc A., Ralea t., Cuza P., (2007) This is a two second test that can be used for small or large populations of plants.

**Quenching Tests –** Moderate heat stress above **35$^{o}$C**, **NPQ** and **$q_P$** in Oak - *Q. pubescens* (Haldiman P, & Feller U. 2004) These are long, time-consuming tests suited to a small numbers of plants. More expensive fluorometers are required.

**NPQ** is sensitive to study moderate heat stress in Spinach plants. (Tang Y., Wen X., Lu Q., Yang Z., Cheng Z., & Lu C. 2007). This is a long, time consuming test suited to a small number of plants. More expensive fluorometers are required.

**Combined Y(II) or ETR and Carbon Assimilation (A) –**The combination $CO_2$ of gas exchange and chlorophyll fluorescence instrumentation is a powerful tool to use for heat stress, as it shows how heat stress affects different parts of the light and dark reactions. The combined use of the two types of instruments has been found to be very useful for specific types of plant stress measurements, such as water stress, heat stress and cold stress. In these types of plant stress, the results of electron transport, as measured with a fluorometer, can show significant differences from carbon assimilation measurements, in gas exchange measurements. Gas exchange detects heat stress at 30$^{o}$C while Y(II) and NPQ detect heat stress at 35$^{o}$C. (Haldiman P, & Feller U. 2004) These are longer, time consuming tests suited to a smaller number of plants. This is the most expensive type of instrumentation for measuring plant stress.

Other quenching parameters include **$q_N$, $q_P$,** (Schreiber U. 2004) *For definitions of quenching parameters* $q_E$, $q_T$, $q_I$, with NPQ see (Muller P., Xiao-Ping L, Niyogi K. 2001). For $q_E$, $q_T$, $q_I$ with qN see (Lichtenthaler 1999) For lake model definitions $q_L$, Y(NPQ), Y(NO) see (Kramer D. M., Johnson G., Kiirats O., Edwards G. 2004). For simplified lake model parameters that include NPQ, see Hendrickson (2004), and Klughammer, Shreiber (2008). For division of lake model parameters into $q_E$, $q_T$, and $q_I$ see Ahn, Avenson (2008). For standardized quenching definitions see (van Kooten O., & Snel J.F. 1990). For standardized puddle model quenching definitions see (van Kooten O., & Snel J.F. 1990) These are longer, time consuming tests suited to a small number of plants. More expensive fluorometers are required.

## Other Tests – Heat stress

**$F_V/F_M$ –** *Is not sensitive to moderate heat stress below* ***45° C.***
(Haldiman P, & Feller U. 2004) (Schreiber U. 2004), (Baker and Rosenqvist 2004) (Crafts-Brander and Law 2000).

**$PI_{ABS}$ -** Fast dark-adapted test sensitive to heat stress using OJIP protocol. This is a normalized parameter for comparing different samples.(Strasser 2004) results reported at **44° C** and above. The test is not sensitive to heat stress below 44°C

**K Step -** Fast dark adapted test sensitive to heat stress using OJIP protocol sensitive (Strasser 2004) **(see $PI_{ABS}$ above)**

# Nutrient Stress:

**Using standard types chlorophyll fluorescence measurement for some types of nutrient stress works well, *however, non-standard methods are required for other types of nutient stress measurement including nitrogen and sulfur stress.* There is a special assay available by Cheng (2001) that incorporates high light levels to measure nitrogen stress listed below. However, the most cost effective tools for nitrogen and sulfer stress, and the most highly used methods involve chlorophyll content meters. They are available using two different methods. One type uses a light absorption techniques at two different light wavelengths. The second uses ratio fluorescence detection at two different wavelengths. Ratio fluorescence has added the advantages that it works well not only with larger leaves but also with very small samples, conifers, grasses, Arabidopsis, stems, or even cactus, because the measuring aperture does not need to be filled to get a reliable measurement. Ratio fluorescence also provides a larger reliable measuring range, especially at higher chlorophyll content levels, and direct read out in chlorophyll content level in mg$^{-2}$ m$^{-2}$ (Gitelson 1999). Gas exchange provides excellent results at a much higher price. For references and details, see the papers sited below.**

## Best Tests

### *Nitrogen*

**CCI or SPAD** *These are absorbance – transmittance indices, not fluorescent parameters.* These instruments transmit light at two different wavelengths through leaves. One is in the red range that is very sensitive to chlorophyll content, and the other is in the far-red range. The far-red wavelength is not sensitive to chlorophyll content, but it is affected by leaf thickness and refractive index. The ratio of the two numbers provides CCI and SPAD. This type of instrument has been heavily used for nitrogen stress measurement, and nitrogen management protocols. Maize (Wang 2008), Maize under dry conditions (Mashego 2012), Maize (Bukan 2011), Maize and Wheat (Francis 1999), Maize (Shapiro C., Schepers J., Francis D., Shanahan J., 2006), Rice (Koontz 2011), Maple trees (van den Berg 2004), Asian Pear (GHASEMI 2011), Artichoke (Rodrigo 2011), Compares CCI and SPAD (Knighton 2005).

*This is the most cost effective way to measure nitrogen stress at usable levels.* Nitrogen stress and sulfur stress can not be distinguished. For this reason, it is common to add sulfur before the study of the effects of nitrogen stress. This is the most used, and most cost effective way to measure nitrogen stress.

**Ratio Fluorescence - $F_{735}/F_{700}$.** Various fluorescence ratios have been tried, but the $F_{735}/F_{700}$ fluorescence ratio provides the best correlation to chlorophyll content results and the largest measuring range. CCI or SPAD work well for standard samples but they have problems with small leaves like immature crop plants, conifers, turf grasses, Arabidopsis, CAM plants such as cactus, or moss on rocks. The ratio fluorescence test offers an affordable solution for difficult samples. It has the advantage that the measuring aperture does not need to be covered for reliable measurement This ratio also has the advantage that it offers more than twice the chlorophyll content measuring range of absorption style chlorophyll content meters, 41 mg m$^2$ to 675 mg m$^2$ (Gitelson 1999) (Buschman 2007). Gitelson provides a formula for **direct readout in chlorophyll content** in mg m$^2$.

Continued on next page -

# Other Tests – Nitrogen stress

**Yield of PSII or Y(II)** ***at high light levels*** **-** Fast light adapted test that can also be used for nitrogen stress at steady state for $C_3$ plants. Various nitrogen levels can be distinguished better using high light levels (Cheng 2001).This is a special assay that requires measuring Y(II) at high light levels to make nitrogen stress measurements at usable levels. It improves the resolution for nitrogen stress measurements to usable levels**.**

**K Step –** Fast dark adapted test that is sensitive at severe levels to nitrogen deficiency in soybean & maize (Strasser 2004), (Baker 2008) An OJIP fluorometer is required and the actinic light intensity should be at 3,000 µmols or 3,500 µmols because the K step changes with light level (Vredenburg 2011)

**$q_P$ -** Slow modulated test that shows some nitrogen deficiency at severe levels, but not sulfur deficiency. (Baker and Rosenqvist 2004) A more expensive fluorometer is required.
**Yield Y(II) -** Fast light adapted test that can also be used for nitrogen stress at steady state. Nitrogen stress must be severe to detect nitrogen stress without high actinic light. (Cavender-Bares and Bazzaz 2004) (Baker and Rosenqvist 2004) High light levels are needed in combination with yield to measure nitrogen stress at usable levels. An intermediate priced fluorometer is required (Cheng 2001).

## *Boron*

**Yield of PSII or Y(II) and ETR** – Fast Light adapted test sensitive to Boron deficiency in sunflowers (Kastori R., Plesnicar M., Pankovic D., Sakac Z., 1995) An intermediate priced fluorometer is required.

## *Calcium*

**$F_V/F_M$ –** Was found to detect $C_a$ stress in tomato plants ( Shmidts-Eiberger, Haefs, Noga) and apple trees (Shmidts-Eiberger, Haefs, Noga 2002). An inexpensive fluorometer is required

## *Chlorine*

**Yield of PSII or Y(II) & ETR , $F_V/F_M$** are all sensitive test for Chlorine stress in watermelon (Zhang, Wang, Huang, Xing, Lin Wang 2010) An intermediate priced fluorometer is required.

**CCI –** Chlorophyll content meter (Cayanan 2008) (Cayanan 2009).

## *Cobalt*

**Yield of PSII or Y(II) -** Cobalt. (Joshi & Mohanty2004)(Tripathy 1983) An intermediate priced fluorometer is required.

## *Copper*

**Yield of PSII or Y(II) -** Copper. Sensitive test (Joshi & Mohanty2004) (Lanaras 1993) An intermediate priced fluorometer is required.

**$F_O/F_{5min}$ –** A slow dark adapted test that is sensitive to copper deficit.
(Adams, Norvell, Philpot & Peverly 2000), (Kriedemann 1985) A more expensive fluorometer is required.

***Iron***

**CCI –** Chlorophyll content meter used to detect chlorosis due to sulfur and iron deficiency. (Christianson 2012)

**K Step –** Fast dark adapted test that is sensitive iron deficiency in soybean & maize (Jiang, Gao, & Zou 2006) An inexpensive priced fluorometer is required.

**Yield of PSII or Y(II)** – variation of 6% with a loss of up to 70% of chlorophyll. When chlorophyll loss exceeds 70%, changes in $F_V/F_M$ are dramatic. Sugar beets (Beta vulgaris L.) (Morales F., Abadia A., Abadia J. 1991) An intermediate priced fluorometer is required.

***Magnesium***

**$PI_{ABS}$ – $PI_{ABS}$ has been shown to be sensitive to Mg deficiency**
(Hermans C, Johnson GN, Strasser RJ, Verbruggen N, 2004) An intermediate priced fluorometer is required.

***Manganese***

**$F_V/F_O$ -** A fast dark adapted test very sensitive to Manganese deficiency.
(Adams, Norvell, Philpot & Peverly 2000), (Kriedemann 1985) (Hannam 1985) an inexpensive fluorometer is required

***Molybdenum***

**CCI** – Chlorophyll content meter (Biscaro 2009) Measures the effects of adding molybdenum and nitrogen uptake.

***Nickel***

**ETR -** Nickel. This also means that Y(II) is sensitive to Nickel stress. $F_V/F_M$ is not a good indicator of Nickel stress. (Joshi & Mohanty2004), (Tripathy 1981) An intermediate priced fluorometer is required.

***Phosphorus***

**$F_V/F_M$ –** Has been shown to be sensitive to phosphorus stress (Stark, Niemyska, Bogdan & Tawlbeh 2000)

**$PI_{ABS}$ -** $PI_{ABS}$ is sensitive to phosphorus stress in Sorghum (Ripley, Redfernand, Dames 2004)

***Potassium***

**Yield of PSII or Y(II), NPO, and $q_P$ -** were effective in detecting K deficiency in rice plants. Experiments with K deficiency in reference to photoprotection mechanisms. (Weng, Zhen, Xu, Sun 2008)

***Sulfur***

**CCI or SPAD** in leaf absorption chlorophyll content meters. *These are not fluorescent parameters* that measure greenness of a leaf and leaf optical density. They are used in chlorophyll content meters for fertilizer and nitrogen management programs. Readings for sulfur stress and nitrogen stress are indistinguishable. (Yara fertilizer management guide on line). Fluorescence is not a good indicator of sulfur stress. (Baker and Rosenqvist 2004) (Christensen 2012) ***This is a cost effective way to measure sulfur stress.***

**$F_V/F_M$ -** was found to detect only starvation levels of sulfur stress in Chlamydomonas (Antal T. , Volgusheva A., Kukarskikh G., Krendelva T., Tusov V., Rubin A. 2005) (Baker 2008)

***Zinc***

**$F_S$ in Yield of PSII or Y(II) -** Zinc **-** $F_V/F_M$ is not a good indicator of zinc stress. (Joshi & Mohanty2004) (Tripathy & Mohanty 1980) (Krupa 1993)

# Important Nutrient tests limitations:

**$F_V/F_M$ -** Fast dark adapted test that is only sensitive to nitrogen content only *at very low levels, and Sulfur at starvation levels.* (Baker and Rosenqvist 2004). It is also not a good test for Zinc (Joshi & Mohanty2004). It is also not a good test for nickel. (Joshi & Mohanty2004)

**Yield of PSII or Y(II)-** Fast light adapted test is sensitive to Sulfur deficiency only *at starvation levels* (Baker and Rosenqvist 2004). It can be used for nitrogen stress at high light levels (Cheng 2006). However, absorption chlorophyll content meters work well for both Nitrogen and Sulfur stress. (Yara fertilizer management guide on line)

**$q_P$ -** Slow modulated test is sensitive to Sulfur deficiency at starvation levels . (Baker and Rosenqvist 2004)

Gas exchange will work well for all types of nutrient plant stress, but tests are slow, making them suitable only for small populations. They are also the most expensive instruments.

# Cold Stress: **All tests below are important in Cold stress studies.**

**Important Notes: Cold stress provides unexpected results when using chlorophyll fluorescence. ETR measurements are three times higher than expected under cold stress (see the ETR/ $CO_2$ Assimilation test for more details).**

**In addition, samples that are subject to cold stress display *heterogeneous fluorescence* from one place on a leaf to another. For more information on this topic, see Baker (2008). To overcome this issue, it is recommended that measurements be made at multiple locations on the same leaf, and results may be averaged. Integrated fluorometer –gas exchange systems overcome this issue by averaging the fluorescence reading over the same large area as gas exchange measurements. Imaging fluorescence displays the heterogeneity. However, using a few measurements, at different locations on the same leaf, with a non-imaging fluorometer provides a higher measurement resolution of the heterogeneity. The results can be averaged for a reliable result (Bushmann 2008). See the Opti-Sciences application note on fluorescence heterogeneity for more information on the subject.**

## Recommended Tests

**ETR/ $CO_2$ Assimilation or *J* /A -** The ratio of ETR in PSII to $CO_2$ assimilation changes in cold stress indicating other electron sinks in cold stress. Under cold stress conditions, ETR is about three times higher than predicted by carbon assimilation measurements.(Fryer M. J., Andrews J.R., Oxborough K., Blowers D. A., Baker N.E. 1998) *This test uses a combination fluorometer and $CO_2$ - $H_2O$ gas exchange system. It has the added advantage that it is measuring fluorescence over the entire leaf chamber area, eliminating the heterogeneous fluorescence issue.*

**Y(II) or $\Delta F'/F_M'$ - Yield of PSII -** Fast light adapted sensitive test can also be used for moderate cold stress in steady state. (Oquist and Huner 1991), (Ball 1994), (Krause 1994), (Adams1994), (Adams1995), (Ball 1995).

**$F_V/F_M$ -** Fast dark-adapted test can be used for moderate cold stress. (Oquit and Huner 1991), (Ball 1994), (Krause 1994), (Adams 1994, 1995), (Ball 1995).

**Light Curves /Stepped Actinic Test –** Light response curves and the effects of light level increases and decreases with cold stress can be studied easily. This is a longer light adapted test. (Oquist and Huner 1991), (Ball 1994), (Krause 1994), (Adams1994, 1995), (Ball 1995).

**ETR -** This is a short or long light adapted test related to yield and PAR or light level. A PAR clip is required. (Oquist and Huner 1991), (Ball 1994), (Krause 1994), (Adams 1994, 1995), (Ball 1995).

**Quenching and Quenching Relaxation Test –** Test to study relaxation kinetics after exposure to light and chilling temperatures. Studies of the ∆ph of the thylakoid lumen, xanthophyll cycle, and photo-inhibition with NPQ, $q_N$, $q_P$, $q_L$, $q_E$, $q_T$, $q_I$, Y(NPQ), Y(NO). This is a longer dark adapted test. (Cavender-Bares J., Bazzaz F., 2004)

# Over-Wintering Stress

## Recommended Tests

**$Y(II)$ or $\Delta F'/F_M'$ - Yield of PSII -** Fast light adapted sensitive test can also be used for moderate cold stress in steady state. (Adams & Demming- Adams 2004) (Oquist and Huner 1991), (Ball 1994), (Krause 1994), (Adams 1994,1995), (Ball 1995).

**$F_V/F_M$ -** Fast dark-adapted test can be used for moderate cold stress. (Adams & Demming- Adams 2004), (Oquist and Huner 1991), (Ball 1994), (Krause 1994), (Adams 1994,1995), (Ball 1995).

**Quenching and Quenching Relaxation Test –** Test to study relaxation kinetics after exposure to light and over-wintering plants. Studies of qI mechanisms become possible as well as the ∆ph of the thylakoid lumen, xanthophyll cycle, and photo-inhibition with NPQ, $q_N$, $q_P$, $q_L$, $q_E$, $q_T$, $q_I$, Y(NPQ), Y(NO). This is a longer dark adapted test. (Adams & Demming- Adams 2004) (Cavender-Bares J., Bazzaz F.,2004)

**Light Curves /Stepped Actinic Test –** The effects of light level increases and decreases with cold stress can be studied easily. This is a longer light adapted test. (Adams & Demming- Adams 2004), (Oquist and Huner 1991), (Ball 1994), (Krause 1994), (Adams 1994, 1995), (Ball 1995).

# $CO_2$ Stress:

## Best Tests

**$CO_2$ stress causes *heterogeneous fluorescence* across the leaf, for this reason, a larger part of the leaf should be characterized with multiple measurements at multiple locations on the same leaf and averaged (Baker 2008), (Buschmann – email correspondence). Integrated chlorophyll fluorescence measurement and gas exchange measurement offer the best way to measure $CO_2$ stress. Chlorophyll fluorescence is averaged over the same large area as gas exchange measurements, eliminating the issue of patchy fluorescence response. Early on, Y(II) values actually increase while carbon assimilation decreases**(Siffel & Braunova 1999)**. Furthermore, $A/C_i$ curves or $A/C_C$ curves can be used to characterize leaves at different $CO_2$ levels. $A/C_C$ curves require an integrated system.**

**$A/C_i$** curves using a gas-exchange instruments with micro-environmental control are a good way to measure $CO_2$ stress (Sellin 2013).

**$F_V/F_M$ -** Fast dark-adapted test is sensitive to early $CO_2$ stress. (Siffel & Braunova 1999)

**$PI_{ABS}$ -** Fast dark adapted test sensitive to $CO_2$ stress using OJIP protocol. (Strasser 2004)

**$q_P$ -** A longer slow light or dark adapted test that has been used in compound stress situations related to water and light stress with $CO_2$ stress (Bukov & Carpentier 2004), (Cornic 1989), (Brestic 1995)

## Non-sensitive $CO_2$ Stress tests

**Yield of PSII or Y(II) -** Fast light adapted test that is not sensitive to $CO_2$ stress initially and has been show to actually increase early on. It will decline after a period of time. While it is not valuable to detect $CO_2$ stress, it may be valuable to identify it in conjunction with $F_V/F_M$, and NPQ.
(Siffel & Braunova 1999)

**NPQ -** This is a longer dark adapted measurement. It has been shown there is no quenching in the total absence of $CO_2$. (Siffel & Braunova 1999).

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# Air Pollution Stress

**$F_V/F_M$ -** Fast dark-adapted test is sensitive to ozone stress. (Mikkelsen 1994)
(Calatayud, Pomares, and Barreno 2006)

**Yield or Y(II) -** Fast light adapted test can also be used for ozone stress in steady state.
(Calatayud, Pomares, and Barreno 2006) (Carrasco-Rodriguez J. and del Valle-Tascon S., 2001)

**$q_P$ -** Slow test. Ozone stress showed a lower $q_P$ (Calatayud, Pomares, and Barreno 2006)
(Carrasco-Rodriguez J. and del Valle-Tascon S., 2001)

**NPQ** – Slow test, ozone stress showed an increase in NPQ stress. This is a dark adapter test.
(Calatayud, Pomares, and Barreno 2006) (Carrasco-Rodriguez J. and del Valle-Tascon S., 2001)

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# Herbicide Stress:

### Different herbicides work in various ways. Some parameters are successful with certain types of herbicide stress and not for others.

**For example: $F_V/F_M$ is not sensitive to DCMU stress but VJ is sensitive to DCMU stress.**

**Herbicides are listed in alphabetical order and the test used to identify stress is listed on the left.**

**$F_V/F_M$ , & NPQ -** *Atrazine* , a PSII inhibitor. Both tests were sensitive to atrazine use in some different genotypes of sweet corn. (Kopsell 2010)

**$V_J$ –OJIP –** *Atrazine* , a PSII inhibitor, by observing the transition from Fo to Fm in the OJIP test, a rise in Fo and a rise in J provide a sensitive test for stress. (Hiraki, van Rensen, Vredenberg, & Wakabayashi 2003) (Percival 2005)

**Yield of PSII & NPQ -***Basta* (AgrEbo) is composed of 18.5 % *Glufosinate-ammonium* <Ammonium -DL-homoalanine-4-YL-(methyl)phosphinate> Yield and NPQ are sensitive tests for Basta herbicide stress. (Takayama K. , Konishi A., and Omasa K.. 2003)

**$V_J$ –** *Bentazone*, a PSII inhibitor, $V_J$ (or $F_{VJ}$) is the fluorescence rise from O to J in the OJIP

test, provides a sensitive test for stress. (Christiansen, Teicher and Streibig 2003)

**$V_J$ – OJIP -** *DCMU* has little effect on Fv/Fm (Nedbal & Whitmarsh 2004). However by observing the transition from Fo to Fm in the OJIP test, a rise in Fo and a rise in J provide a sensitive test for stress. (Hiraki, van Rensen, Vredenberg, & Wakabayashi 2003), (Percival 2005)

**NPQ -** *DCMU*. A longer dark adapted test will provide stress information on DCMU. (Nedbal & Whitmarsh 2004)

**NPQ –** *DDT*. A sensitive test for DDT that is also dependent on zeaxanthin quantity in leaves. If there is little or no zeaxanthin production, NPQ can detect DDT stress. If zeaxanthin has been produced, NPQ is not affected by DDT. (Bilger & Bjorkman 1994)

**$V_J$ –OJIP –** *Diuron* by observing the transition from Fo to Fm in the OJIP test, a rise in Fo and a rise In J provide a sensitive test for stress. (Hiraki, van Rensen, Vredenberg, & Wakabayashi 2003) (Percival 2005)

**$V_J$ –** *Fluorochloridone* a PDS inhibitor, $V_J$ (or $Fv_J$) is the fluorescence rise from O to J in the OJIP Test, provides a sensitive test for stress. (Christiansen, Teicher and Streibig 2003)

**$V_J$ –** *Glycosate* an EPSPs inhibitor, $V_J$ (or $Fv_J$) is the fluorescence rise from O to J in the OJIP test, provides a sensitive test for stress. (Christiansen, Teicher and Streibig 2003)

**$V_J$ –OJIP –** *TU-1178* by observing the transition from Fo to Fm in the OJIP test, a rise in Fo and a rise in I provide a sensitive test for stress. (Hiraki, van Rensen, Vredenberg, & Wakabayashi 2003)

**$V_J$ –OJIP –** *TU-1282* by observing the transition from Fo to Fm in the OJIP test, a rise in Fo and a rise in I provide a sensitive test for stress. (Hiraki, van Rensen, Vredenberg, & Wakabayashi 2003)

# Herbicide effects on Arabidopsis at standard dose:

In $F_V/F_M$ & $F_V/F_O$, $F_O$ is minimum fluorescence measured with very low intensity modulated light of dark adapted sample before any $Q_A$ is reduced by a saturation flash.

In other parameters listed below $F_O$ is fluorescence at 40µs , $F_P$ = P, $F_I$ = J at 2ms, in the OJIP protocol

**$F_V/F_M$, 1-($F_O/F_P$), 1-($F_I/F_P$) –** *2,4D* in the phenoxy group, is a synthetic auxin herbicide. These parameters were sensitive to 2,4D use after 48 hours. Baker uses Fi instead of J as his designation but they are the same. (Baker and Rosenqvist 2004)

**$F_V/F_M$, 1-($F_O/F_P$), 1-($F_I/F_P$) –** *Asulam*. These parameters were sensitive to Asulam use after 6 hours. Baker uses Fi instead of J as his designation but they are the same. (Baker and Rosenqvist 2004)

**$F_V/F_M$, 1-($F_O/F_P$), 1-($F_I/F_P$) –** *Bifenox*. These parameters were sensitive to Bifenox use after 48 hours. Baker uses Fi instead of J as his designation but they are the same. (Baker and Rosenqvist 2004)

**$F_V/F_M$, 1-($F_O/F_P$), 1-($F_I/F_P$) –** *Diclofop-methyl* . These parameters were sensitive to Diclofop-methyl use after 6 hours. Baker uses Fi instead of J as his designation but they are the same. (Baker and Rosenqvist 2004)

**$F_V/F_M$, 1-($F_O/F_P$), 1-($F_I/F_P$) –** *Glycosate*. These parameters were sensitive to Glycosate use after 6 hours. Baker uses Fi instead of J as his designation but they are the same. (Baker and Rosenqvist 2004)

**$F_V/F_M$, 1-($F_O/F_P$), 1-($F_I/F_P$) –** *Imazapyr* . These parameters were sensitive to Imazapyr use after 6 hours. Baker uses Fi instead of J as his designation but they are the same. (Baker and Rosenqvist 2004)

$F_O$ is minimum fluorescence, $F_O$ is fluorescence at 40µs , $F_P$ = P, $F_I$ = J at 2ms, in the OJIP protocol

# Pesticide Stress:

**Different pesticides work in various ways. Some parameters are successful with certain types of pesticide stress and not for others.**

**Copper based Algicides and Fungicides –** are main sources of Cu stress in plants, see Copper stress under Chemical Stress.

**Mercury based Organo-mercury fungicides –** A main source of Mg stress in plants, see Mercury stress under Chemical Stress.

**$PI_{ABS}$ , $F_VF_M$ –** Lindane. Sensitive test on cyanobacteria Anabaena (Bueno, Fillat, Strasser, Rodriguez, Marina, Smienk. Moreno, Barja 2004)

**Yield of PSII or Y(II) –** Trimax stress on Cotton Germ M.,
(Gonias E. D. Oosterhuis D.M., Bibi A.C. & Brown R.S. 2003)

# Chemical Stress:

**While some types of chemical stress can be measured by various parameters including $F_V/F_M$, some require specific parameters for measurement.**

In $F_V/F_M$ & $F_V/F_O$, $F_O$ is minimum fluorescence measured with very low intensity modulated light of dark adapted sample before any $Q_A$ is reduced by a saturation flash.

In other parameters listed below $F_O$ is fluorescence at 40µs , $F_P$ = P, $F_I$ = J at 2ms, in the OJIP protocol

**Listed by chemical.** *Nitrogen, boron, calcium, chlorine, cobalt, copper, iron, magnesium, manganese, molybdenum, nickel, phosphorus, potassium, sulfur, and zinc* *are listed under nutrient stress*.

**$F_V/F_O$** - *Aluminum* (Joshi & Mohanty2004), (Pereira 2000)(Baker and Rosenqvist 2004)

**$(F_P – F_I)/F_I$** - *Aluminum* (Baker and Rosenqvist 2004)

**$V_J$** - *Aluminum* Fi is = J in OJIP $V_J$ = Fi-Fo/ Fm-Fo (Joshi & Mohanty2004), (Moustakas 1993, 1995, 1997)

**$F_V/F_M$** - *Aluminum* (Joshi & Mohanty2004), (Moustakas 1996)
Not as sensitive as Fv/Fo (Baker and Rosenqvist 2004).

**$q_P$ , & $q_N$** - *Aluminum* (Joshi & Mohanty2004), (Moustakas 1996)

**$q_N$** - *Cadmium*. qN is more sensitive to Cadmium concentration than Fv/Fm.
(Joshi & Mohanty 2004) (Krupa 1993) Skorzynska and Baszynski 1997)

**$F_V/F_M$** - *Cadmium*. (Baker and Rosenqvist 2004), (Popovic et al., 2003).

**Yield of PSII or Y(II)** - *Cobalt*. (Joshi & Mohanty2004)(Tripathy 1983)

**Yield of PSII or Y(II)** - *Copper*. Sensitive test (Joshi & Mohanty2004) (Lanaras 1993)

**$F_V/F_M$** - *Copper* (Baker and Rosenquist 2004), (Popovic et al., 2003)

**Rfd** - *Copper*. Sensitive test (Joshi & Mohanty2004))

**$F_V/F_M$** - *Lead* (Joshi & Mohanty2004), (Parys 1998) (Romanowska 1998)

**$F_V/F_M$** - *Mercury* (Baker and Rosenqvist 2004), (Joshi & Mohanty2004), (Popovic et al., 2003)

**$q_N$** - *Mercury* (Joshi & Mohanty2004), (Lee 1995), (Xylander 1998)

**J & I in OJIP** -*Mercury* (Joshi & Mohanty2004), (Haldimann P., and Tsimilli-Michael M.2002)

**ETR** - *Nickel*. $F_V/F_M$ is not a good indicator of Nickel stress. (Joshi & Mohanty2004),
(Tripathy 1981)

*NaCl (Salt) – NaCl measurement success appears to show variable results by plant type, $C_3$ or $C_4$, and in some cases, by species.*

**$q_N$** – *NaCl (Salt)*. $q_N$ is a very sensitive indicator of salt stress in *Rice*. $F_V/F_M$ and yield were not sensitive to salt stress in Rice (Moradi & Ismail 2007)

**$q_N$, $q_P$, $F_V/F_M$, Y(II), & ETR** - *NaCl (Salt)* All parameters were sensitive to salt stress in Cereal Sorghum a $C_4$ plants (Moradi & Ismail 2007) (Netondo 2004)

**$F_V/F_M$** - *NaCl (Salt)* $F_V/F_M$ was sensitive to salt stress in the red mangrove, *Rhizophora mangle* L. (Biber 2006)

**$F_V/F_M$** – *NaCl (Salt)* $F_V/F_M$ was sensitive to salt stress in chickpea seedlings (Eyidogan 2007)

**Fv/Fm** - *NaCl (Salt)* not sensitive to salt stress in Rice (Moradi & Ismail 2007)

**Yield or Y(II)** – *NaCl (Salt)* Y(II) was sensitive to salt stress in chickpea seedlings (Eyidogan 2007)

**Yield or Y(II)**– *NaCl (Salt)* not sensitive to salt stress in Rice (Moradi & Ismail 2007)

**CCI or chlorophyll content index with a chlorophyll meter** - *NaCl (Salt)* was sensitive to salt stress in cotton a $C_3$ plant (Higbie 2010)

**Yield** or Y(II)– *Perchlorate* Y(II) is a very sensitive test for perchlorate stress in the aquatic plant, Alternanthera philoxeroides (Xie YF, Cai XL, Liu WL, Deng W 2009)

**$F_V/F_M$, NPQ, ETR** - *Perchlorate* These parameters will also detect perchlorate stress at different levels in the aquatic plant, Alternanthera philoxeroides (Xie YF, Cai XL, Liu WL, Deng W 2009)

**Spad /CCI –** *Perchlorate* is a sensitive test for perchlorate stress in the aquatic plant, Alternanthera philoxeroides (Xie YF, Cai XL, Liu WL, Deng W 2009)

**$F_S$ in Y(II) -** *Zinc* - Fv/Fm is not a good indicator of zinc stress(Joshi & Mohanty2004) (Tripathy & Mohanty 1980). $F_S$ is the steady state fluorescence level at a specific light intensity without the saturation flash information $F_M$. It takes between 20 minutes and 35 minutes to reach steady state photosynthesis at a specific light level (Cazzaniga 2013).

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# Ph Stress

**$F_V/F_M$ –** Fv/Fm was found to detect severe acid rain stress at a ph of 1.8 or below. (Velikova, Yordanov 1996)

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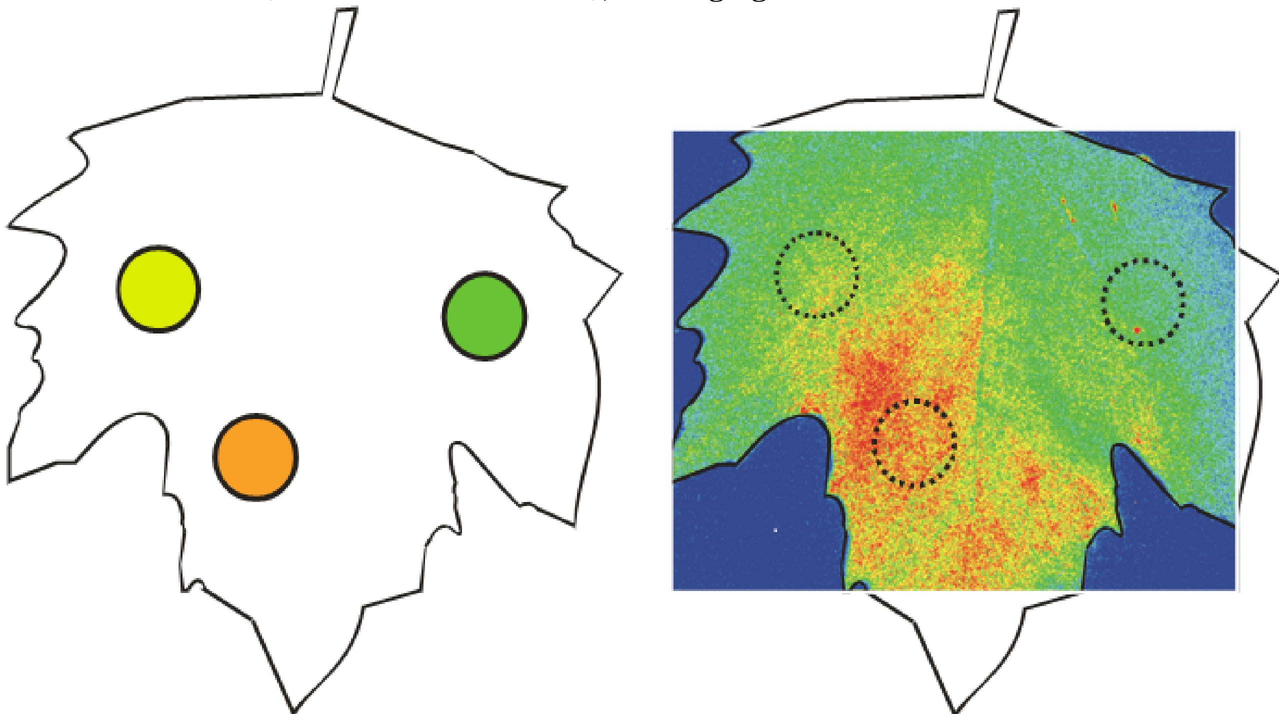
# Biotic Stress: The fluorescence parameter best suited to the type of infection is dependent on the type of Infection (Nedbal & Whitmarsh 2004) Therefore it is important to have versatile capability

**The tests listed in this category are not listed in order of sensitivity or effectiveness. While many references below involve fluorescence imaging, spot measurement can also be used for study.**

**Due to early site-specific infections, multiple point measurements on the same leaf in different areas are recommended. (Claus Buschmann 2008), or imaging fluorescence is recommended.**



**The picture above is from a Claus Buschmann email showing how a non imaging fluorometer may be used for biotic stress measurement. Non-imaging fluorometers provide higher resolution, but imaging system allow visualization of the entire leaf.**

**NPQ -** This is a longer dark adapted measurement for crown rust on oat leaves (Sholes & Rolfe 1996)

**NPQ -** This is a longer dark adapted measurement for tobacco mosaic virus on tobacco (Osmond 1998), (Lohaus 2000)

**$F_V/F_M$ -** Fast dark-adapted test can be used for Bean rust (Peterson & Aylor 1995)

**Yield or Y(II)-** Fast light adapted test used for cedar fungus (Ning 1995)

**$F_M$ -$F_S$ /$F_M$ -** This is a longer dark-adapted test that requires several minutes to reach steady state photosynthesis. tobacco mosaic virus on tobacco (Osmond 1990). $F_M$ is dark adapted and $F_S$ is the light adapted value at steady state photosynthesis.

**$F_V/F_M$ -** Fast dark-adapted test can be used for biotic stress chickpea leaves fungus (Esfield 1995) (Weiss 1998)

**$F_V/F_M$ -** Fast dark-adapted test can be used for biotic stress lemons infected by Penicllium digitatum (Nebal 2000)

**$F_O/F_V$ -** Fast dark-adapted test can be used for biotic stress Brassica Blackspot by destruxins (Buchwaldt & Green 1992)

**NPQ -** This is a longer dark adapted measurement recommended for virus infection in higher plants and algae. (Balachadran & Hurry 1997)

**$F_V/F_M$ -** Fast dark-adapted test can be used for biotic stress recommended for virus infection in higher plants and algae. (Balachadran & Hurry 1997)

**$F_V$ / $F_O$ -** Fast dark-adapted test can be used for biotic stress Maize rust resistance. (Duraes 2001)

**$F_V/F_M$ -** Fast dark-adapted test can be used for biotic stress. Maize rust resistance. (Duraes 2001)

# Herbivory – (Animal Stress):

**Yield of PSII or Y(II) –** Fast light adapted sensitive test for Arthropod damage showing greater damage than the size of the hole indicates stress. (Aldea, Hamilton, Resti, Zangerl, Berenbaum, Frank and Deluca 2006), (Zangerl 2002)

**$F_V/F_M$ -** Fast dark adapted test can be used to test for damage caused by insect larval foot hooks. (Hall, MacGregor, Nijsse, and Bown 2004)

# Weed Stress

**Maize –** chlorophyll content measurement (Tollenaar M., Dibo A.A 1994), (Tollenaar M. 1994) (Tollenaar M. 1997)

**Maize –** using transcriptome analysis for weed stress. (Moriles J. 2012)

**Rice** - weed stress measured by high performance liquid chromatography (HPLC) for phenolic compounds (Hea 2012)

# Radiation Stress

**γ (gamma radiation stress)** – on buckwheat - $F_V/F_M$, $F_V/F_O$,Y(II), ETR, photochemical quenching, and non-photochemical quenching are all sensitive to gamma radiation detection due to an increase in $q_I$ or photoinhibition. (JIA C.F 2008)

**Cosmic radiation during space flight** – on Chlamdomonas reinhardtii. $F_V/F_M$ and OJIP $V_t$ showed that some mutants health after space flight performed better than others. (Masci S. 2011)

**UVA and UVB sensitivity –** on red algae – $F_V/F_M$ was a sensitive test for measuring exposure of red algae to UBA and UVB radiation (DRING M.J. 1996)

**X-ray exposure** – Both $F_V/F_M$ and Y(II) are sensitive measurements for measuring X-ray exposure in plants. Kurimoto (2010)

**Neutron radiation** – Exposure of plants to neutron radiation can be measured with $F_V/F_M$ & Y(II) (Rea1 G 2008)