Desktop Plant Stress Guide:

Edition 6.2 – 06/07/22

Note: Sam Wilson and Alexander V. Ruban (2020) show evidence that while chloroplast migration is a real thing, it does not show itself in chlorophyll fluorescence measurement. Therefore, at higher actinic light levels q_Z should replace q_M for quenching measurements. q_Z is a slower reacting photoprotective mechanism that involves zeaxanthin. It takes between 10 minutes and 30 minutes to adjust or relax. **(Nilkens 2010)**

Note: *Light stress*- Over the last decade Alexander Ruban & Erick Murchie and collaborators have developed and researched an alternative method of measuring plant photoprotective mechanisms and photodamage that was independent of time-based quenching relaxation techniques. As a result, there is no longer an overlap of plant photoprotective mechanism times, and photodamage, making the results less ambiguous (Ruban A.V, Murchie E.H, 2012) (Ruban 2017)**.** For more information, see the quenching section on pNPQ, qP_d & $q_I.$

Note: Early drought stress measurement in C_3 plants -Developed by German researchers, the Templer protocol worked for *early drought stress* measurements in C_3 plants. It uses F_V/F_M at temperatures above 26ºC or 78.8ºF successfully. See the section on drought stress for more information (Templer 2017).

Note: *Waterlogging stress* or flooding stress in soybean, a C_3 plant – Researchers found that F_V/F_M, OJIP, and the ratio fluorescence chlorophyll content measuring parameter F_{735}/F_{700} (Kim 2018) worked for waterlogging stress measurement. *Waterlogging stress* in Scots Pine seedlings and the ratio fluorescence chlorophyll content measuring parameter F_{735}/F_{700} (Repo 2016).

Note: *Heat stress* in orchard grass, a C_3 plant. The ratio fluorescence chlorophyll content measuring parameter, F_{735}/F_{700}, was successful in measuring heat stress at 35ºC compared to samples at 20ºC. However, this may be a special case as chlorophyll content increased with heat stress. (Jones G.B. 2017)

Note: Zinc deficit stress is measurable with chlorophyll content measurement – See the section on nutrient stress (Kazemi M. 2013) (Derakhshani Z. 2011)**.** Hyperaccumulation of Zinc and Cadmium **(Martos 2016)**

Note: It will become apparent, after reviewing this plant stress guide, that the combination of a chlorophyll fluorometer and a chlorophyll content meter provide an affordable measuring capability for almost all types of plant stress. Measurements for both types of field portable instruments are fast, allowing for measurement of larger plant populations. A chlorophyll content meter provides better solutions for some nutrient plant stress types, and chlorophyll fluorometers provide better results for most other types of plant stress. Gas exchange measurements work well for all types of plant stress measurement. However, they are more expensive and measurements take longer.

This guide was intended as a starting point for research. Results may sometimes vary by species, plant type, or special interest. **Full publication references are in the separate PDF file sent with this document.**

Results were compiled from worldwide 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, alternative solutions are suggested. The best tests for different types of plant stress were listed on the following pages. **Tests were listed by plant stress type, with the best tests listed first.** For more information, contact Opti-Sciences at 603-883-4400, or **www.optisci.com**

Table of Contents

Specific Plant Stress Types

> Complete references are in a separate companion document in alphabetical order

A review of measuring protocols –value and limitations

Notes for measuring

Cook book checklists for reliable chlorophyll fluorescence measurements

Drought Stress:

Important Notes: $\Delta F/F_M$' or Y(II), and ETR are effective tests for water stress in C4 plants.

In C3 plants, the Templer protocol (Templer 2017) using F_V/F_M at 26ºC, or higher, works for early drought stress detection one day after the ending of watering. The only other method that reports good results for <u>moderate water stress is Fs/Fo. It only works for moderate water stress found in plants like grapes and trees. It is not adequate for most other crops according to Flexas (from email on his work).</u> In C3 plants, photorespiration is thought to be the reason for less than adequate results in regard to early water stress testing for most crop plants (Flexas 2002). (Flexas 1999) and (Flexas 2000) provide a good review of the limitations of standard chlorophyll fluorescence techniques for water stress measurement. The standard tests: <u>F_V/F_M, and Y(II), will only work for *severe drought* stress measurement in C3 plants due to photorespiration (Flexas 1999, 2000).</u> F_V/F_M can be used for severe water stress after more than 7 days without water and <u>it will work for measuring trees but it should not be used for crops.</u> (da Silva J. A. & Arrabaca M.C. 2008).

In addition, samples that are subject to drought stress display <u>heterogeneous fluorescence</u> from one place on a leaf to another. For more information on this topic see Baker (2008). 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 C3 plants

gs, E, and A from gas exchange measurements – gs, or stomatal conductance, is the most sensitive measuring parameter for measuring drought stress in most C3 plants**.** Both E or transpiration rate, and A or carbon assimilation rate are also sensitive drought stress measuring parameters**.** For drought plant stress measurement, Infrared-gas-exchange instrument or IRGA will work nicely. *This will detect early drought stress in all kinds of crops and other plants.* **Higher priced solution that takes a few minutes.**

gs from a porometer – Stomatal conductance from a porometer in C3 and C4 plants is **medium priced solution for drought stress measurement, that takes a less than 1 minute.** (Zhaoquan G. 2017) porometers are preferred for measuring isohydric plants at midday (plants that close their stomata as water potential decreases).

Ψ water potential from pressure bombs, leaf psychrometers, – **destructive tests that are inexpensive.** The use of water potential measurement is preferrable for midday measurement of anisohydric plants (plants that keep their stomata open at photosynthetic rates high, for longer periods, even with decreasing leaf water potential). However, water potential, Ψ, **works well for all types of higher plants if the measurement occurs pre-dawn** (Harb J. B., Keller M., 2018). Soil water potential measurements also work - **soil matric sensors, soil tensiometers** (Li X., 2020)

Soil moisture sensors – There are systems for continuous monitoring of soil moister and for sampling.

F_V/F_M measurements <u>combined with ambient temperatures at or above 26ºC or 78.8º F</u>
Researchers from the Max Planck Institute, Julius Kuhn Institute, Friedrich-Alexander University, Saatzucht Josef Breun GmbH, Saaten-Union Biotec GmbH and Heinrich-Heine University have measured

early drought stress when combined with moderate heat stress in drought resistant Mediterranean barley genotypes and elite German breeding lines. *Since it is common that both types of plant stress are found together in field crops, this research opens up new opportunities for drought stress research using an affordable alternative to gas exchange in C_3 plants. After raising the temperature from 20^oC to 26^oC, Fv/Fm was abe to measure drought stress one day after watering was stopped.* (Templer 2017) **Fast inexpensive solution**

Fs/Fo & Fs - Fo is from the predawn dark-adapted Fv/Fm test, and Fs is from the steady state light adapted Y(II) fluorescence test at a specific time of day. **This test is sensitive to moderate water stress and it is adequate for work with grapes and trees but it is not sensitive enough for most other C_3 plant crops**. Fs was normalized over predawn Fo. It is more sensitive to C_3 plant water stress than Y(II). Tested in C_3, C_4, and CAM plants. Fs/Fo allows comparison between samples. Ambient actinic light was used at levels between 800 to 1250 μmols. A monitor fluorometer will measure Fo predawn and Fs at different times of day. Flexas found that the method could be used to monitor grape vines and activate optimal watering times. No saturation flash is necessary. (Flexas 2002). **Medium priced solution.**

Note: After conversations with John Burke, the Burke assay is no longer on the list of best C_3 plant drought stress tests. He seemed to have reservations on the test. For more information, contact John Burke directly. The Burke assay is still recommended for C_4 plants.

Best Tests C4 Plants

ΔF/F$_M$' 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). **Medium priced solution**

ETR/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.* **Higher priced solution**.

Porometers, water potential instruments and soil moisture instruments will also work for C4 drought stress measurement.

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. **Higher priced solution.**

Fs/Fo Light adapted test can also be used for water stress in steady state, Samples must be dark adapted to obtain Fo in Fv/Fm, and then samples must be brought to steady state photosynthesis to measure Fs. 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) **Medium priced solution.**

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)
Medium priced solution.

NPQ – Slow test, increases with moderate to late water stress. This is a dark adapter test.
(Cavender-Bares J. & Fakhri A. Bazzaz 2004) **Medium priced solution works for trees but not crops.**

Non-Sensitive to Early or Moderate Drought Stress:

F_V/F_M - Fast dark-adapted test is <u>not</u> sensitive to early or moderate water stress in **C3 plants**, 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 <u>7 days</u>. 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 **<u>not</u>** sensitive to early or moderate water stress in **C4 plants,** only severe stress. (da Silva J. A. & Arrabaca M.C. 2008). It can be used for severe water stress after about <u>7 days</u>.
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 C4 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 <u>7 days</u> (Thack 2007). <u>It is not adequate for crops</u>.

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

Waterlogging or Flood Stress:

F_V/F_M, OJIP & Chlorophyll Content using Ratio Fluorescence - F_{735}/F_{700} – All tests are fast tests and both F_V/F_M and OJIP require dark adaption. F_{735}/F_{700} does not require dark adaption. Tests were conducted on soybean, a C3 plant (Kim 2018) **Includes a range of pricing solutions.**

F_{735}/F_{700} - Ratio Fluorescence – Waterlogging stress of Scots Pine seedlings, a C3 plant (Repo 2016) **Inexpensive solution**.

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), (Nilkens 2010) & (Dall'Osta 2014). (Ruban 2017)

Fast tests:

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. **Inexpensive solution**.

$\Delta F/F_M'$ or **Y(II)** For Quantum yield of PSII 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. **Medium priced solution**

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. **Inexpensive solution.**

Longer tests:

Ruban & Murchie photoprotective NPQ protocol – Over the years various plant mechanisms have been discovered including a rapid reacting photoprotective mechanism called q_E, state transitions thought to help plants survive under low light conditions, a slower reacting photoprotective mechanism called q_Z, acute photoinhibition and chronic photoinhibition or photodamage. One of the biggest problems with measurements of these types is that they use quenching relaxation times to estimate the mechanisms success in different plants. However, the relaxation of the mechanisms overlap in time, and so measurements are ambiguous. Over the last several years, Alexander Ruban and his Erick Murchie developed a method for measuring plant photoprotective mechanisms that are not time based and do not overlap with photodamage. The protocol provides the parameters **pNPQ** or photoprotective NPQ, the highest light level without photodamage, the light level where 50% of all leaves have no photodamage, the optimal plant growing light intensity, a discrete measurement of the intensity where photodamage begins and the photodamage level for various intense light values. qP_d is value that allows a measurement of when photodamage begins, q_I is also available to measure photodamage. Instead of the time-based quenching relaxation method, it is more like a light curve using 5-minute steps at low light levels and goes to the highest actinic light levels. Furthermore, it measures F_O' and compares values of F_O' "calculation" to determine when photodamage occurs.

Quenching and Quenching Relaxation Test – Another test to study photo-protection mechanisms involves quenching relaxation measurements. They include the ΔpH of the thylakoid lumen, the xanthophyll cycle, 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 ΔpH of the thylakoid lumen, and the xanthophyll cycle (Muller, Niyogi 2001), q_T is a parameter for measuring state transitions where they exist, q_Z is parameter for a longer adapting and relaxing plant photoprotective mechanism that involves zeaxanthin. It takes from 10 minutes to 30 minutes to fully adjust (Nilkens 2010). Finally, q_I is a measure of photoinhibition & photodamage. 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), (& Klughammer 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) and for **q_Z** see Nilkens (2010). 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), and for q_M see (Cazzaniga S. 2013**). Medium priced solution.**

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. Steady state has a different meaning for gas exchange researchers. Generally, they mean after q_E reaches steady state between 4 -7 minutes at a given actinic light level (**Conversations with Tracy Lawson University of Essex)**. 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.

Longer 30-minute adjustments are also a valid approach to light curves. (Muller, Niyogi 2001), (Kramer 2004), (Hendrickson 2004). **Medium priced solution.**

Automated fluorometer routines are programmed for desired light intensities, step time duration, the number of saturation pulses per step and the number of steps. 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)

RLC -Rapid Light Curves – designed to measure a plant's response to <u>**changing light conditions**</u>.
For under canopy work, partly cloudy conditions, windy conditions and for aquatic plants, Rapid light Curves (RLCs) have advantages. It is a longer quasi- dark-adapted, or momentary dark-adapted 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 steps 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 useful 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, occurs at "I_M". Minimum saturation intensity is called "I_K", and initial slope of the RLC curve is α or alpha). Rapid light curves are available on the OS1p, the OS5p+ the PSP32 automated monitor fluorometer and the iFL. Light saturation rate, rapid light curve measurements, highly correlate with the concentration and maximum activity of Rubisco (Macintyre 1997), (Macintyre 1996).

Steady state photosynthetic rate measurements 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 dependent, and results change depending on the time of day (Rasher 2000). Rapid Light Curves that use 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 provide relevant information on the saturation characteristics of electron transport (Schreiber 2004). Momentary dark adaptation for 5 to 10 seconds is a recommendation by Ralph (2005). For a review of RLCs 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. **Medium priced solution.**

Heat Stress:

In C_3 plants, heat is an oxidative stress, and as a result, measuring sensitivity can be delayed depending on the measuring protocol that one uses. According to Haldiman (2004), the light adapted test **Y(II) will detect heat stress at 35ºC** and higher. **F_V/F_M will only detect heat stress at 45°C and higher**. **Gas Exchange has been shown to detect heat stress at 30ºC or higher** (Haldiman 2004). 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. NPQ will detect heat stress in Oak leaves at 35°C and higher.

Fast tests:

$\Delta F/F_M$' or **Y(II)** Y(II) is a light adapted fast test that takes about two seconds. Y(II) is the most sensitive chlorophyll fluorescence test for measuring heat stress quickly. As stated above, **it will detect heat stress at about 35°C or higher** (Haldiman P, & Feller U. 2004), (Dascaliuc A., Ralea t., Cuza P., 2007). It is important to use a PAR clip when measuring Y(II) because values vary with actinic light level as well, and PAR clips measure PAR at leaf level and angle as well as leaf temperature. **(Medium priced solution)**

A – Gas-exchange carbon assimilation will detect heat stress at **30ºC** and above (Haldiman 2004). **(Expensive solution that requires a gas-exchange instrument and time for the leaf to climatize to the measuring chamber)**

F_{735}/F_{700} - ratio chlorophyll fluorescence in the CCM-300 chlorophyll content meter, will detect heat stress in orchard grass a C_3 plant at about 35°C. *However, this may be a special case as chlorophyll content increased with heat stress* (Jones G.B. 2017). **(Inexpensive solution)**

F_V/F_M – **Is not sensitive to moderate heat stress below 45º 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 44º C and above. **The test is not sensitive to heat stress below 44ºC.**

Longer tests:

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. Quenching Tests – **Moderate heat stress above 35ºC**, NPQ and q_P in Oak - Q. pubescens (Haldiman P, & Feller U. 2004) These are long, time-consuming tests suited to a small number of plants. More expensive fluorometers are a requirement. 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, 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°C while Y(II) and NPQ detect heat stress at 35°C. (Haldiman P, & Feller U. 2004) These are longer, tests suited to a smaller number of plants. **This is the most expensive type of instrumentation for measuring plant stress but it provides the most complete answer.**

Other quenching parameters include qN, qP, (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 q_N 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, Schreiber (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, tests suited to a small number of plants. More expensive fluorometers are required.

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 nutrient 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 sulfur stress, and the most highly used methods involve chlorophyll content meters. They are available using two different methods. One type uses a light absorption technique 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 & SPAD *These are chlorophyll meter parameters, 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 (Torres-Dorante 2015) Maize under dry conditions (Mashego 2012), Rice (Saberioon 2014), Soybean (Van Heerden 2007), Potato (Lazarević 2014), Vineyard wine grapes (D'Attilio 2014), 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 cannot 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) (Buschmann 2007). Gitelson provides a formula for **direct readout in chlorophyll content** in mg^{-2} m^{-2}. *References exist for both difficult to measure samples and easier to measure samples*:17-day old rice seedlings (Kan 2015), maize (Butts 2017), nursery fertilizer status (Clark 2017).

Other Tests – Nitrogen stress

K Step – Fast dark-adapted test that is sensitive at <u>severe levels</u> to <u>nitrogen</u> 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 <u>severe levels,</u> but not sulfur deficiency. (Baker and Rosenqvist 2004) A more expensive fluorometer is required.

$\Delta F/F_M$' Y(II) - Fast light adapted test that can also be used for <u>nitrogen</u> stress at steady state. <u>Nitrogen stress must be severe to detect nitrogen stress without high actinic light.</u> (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).

Other Nutrients

<u>Boron</u>
CCI – Chlorophyll content meter was sensitive to early Boron stress in most safflower cultivars
(Day S., Çıkılı Y., Aasim M., 2017)
$\Delta F/F_M$' 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.
<u>Calcium</u>
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
<u>Chlorine</u>
$\Delta F/F_M$' 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).
<u>Cobalt</u>
$\Delta F/F_M$' or Y(II) - <u>Cobalt.</u> (Joshi & Mohanty2004) (Tripathy 1983) An intermediate priced fluorometer is required.
<u>Copper</u>
$\Delta F/F_M$' or Y(II) - <u>Copper.</u> 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 <u>copper</u> deficit.
(Adams, Norvell, Philpot & Peverly 2000), (Kriedemann 1985) A more expensive fluorometer is required.
<u>Iron</u>
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 <u>iron</u> deficiency in soybean & maize
(Jiang, Gao, & Zou 2006) An inexpensive priced fluorometer is required.
<u>Magnesium</u>
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.
<u>Manganese</u>
F_V/F_O - A fast dark-adapted test very sensitive to <u>Manganese</u> deficiency.
(Adams, Norvell, Philpot & Peverly 2000), (Kriedemann 1985) (Hannam 1985) an inexpensive fluorometer is required
<u>Molybdenum</u>
CCI – Chlorophyll content meter (Biscaro 2009) Measures the effects of adding molybdenum and nitrogen uptake.
<u>Nickel</u>
ETR - <u>Nickel</u>. 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
Fv/FM – Has been shown to be sensitive to <u>phosphorus</u> stress (Stark, Niemyska, Bogdan & Tawlbeh 2000)
PIABS - PIABS is sensitive to <u>phosphorus stress</u> in Sorghum (Ripley, Redfernand, Dames 2004)
Potassium
Yield of PSII or Y(II), NPO, and qP - 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
Fv/FM - was found to detect only <u>starvation levels of sulfur</u> stress in Chlamydomonas
(Antal T., Volgusheva A., Kukarskikh G., Krendelva T., Tusov V., Rubin A. 2005) (Baker 2008)
Zinc
Chlorophyll content measurement – will detect a zinc deficit. (Kazemi M. 2013) (Derakhshani Z. 2011)
Fs in Yield of PSII or Y(II) - <u>Zinc -</u> Fv/FM is <u>not</u> a good indicator of zinc stress. (Joshi & Mohanty2004) (Tripathy & Mohanty 1980) (Krupa 1993)
F₇₃₅/F₇₀₀ chlorophyll content measurement - Hyperaccumulation of Zinc and Cadmium (Martos 2016).

Important Nutrient tests limitations:

Combining the use of chlorophyll fluorescence and chlorophyll content measurement makes sense for affordable nutrient plant stress measurement. This is due to the difficulties in measuring some types of nutrient plant stress such as nitrogen, sulfur, molybdenum, and zinc stress with chlorophyll fluorescence.
Fv/FM - 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 <u>not</u> a good test for **zinc** (Joshi & Mohanty2004). It is also <u>not</u> a good test for **nickel.** (Joshi & Mohanty2004)
ΔF/FM' 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 <u>chlorophyll content meters work well for both Nitrogen and Sulfur stress</u>. (Yara fertilizer management guide on line)
qP - Slow modulated test is sensitive to Sulfur deficiency <u>at starvation levels</u>.
(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.
Fs in ΔF/FM' or Y(II) - <u>Zinc -</u> Fv/FM is <u>not</u> a good indicator of zinc stress. (Joshi & Mohanty2004) (Tripathy & Mohanty 1980) (Krupa 1993)

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** (Buschmann 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 ΔF'/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 ΔpH 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 ΔF'/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)

F$_{735}$/F$_{700}$ – Chlorophyll content measurement of soybean in *elevated CO$_2$* (Jin 2017)

Non-sensitive CO$_2$ Stress tests

ΔF/F$_M$' or Y(II) - Fast light adapted test that is <u>not</u> 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).

Air Pollution Stress

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

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 F_O to F_M 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 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 – *Glycosate* an EPSPs 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 – *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)
ΔF/F_M' 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* <u>*are listed under nutrient stress*</u>.
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 F_V/F_O (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)

Fv/F$_M$ - *Cadmium*. (Baker and Rosenqvist 2004), (Popovic et al., 2003).

F$_{735}$/F$_{700}$ – *Cadmium* chlorophyll content measurement hyperaccumulation of Cadmium and Zinc (Martos 2016).

ΔF/F$_M$' or Y(II) - *Cobalt*. (Joshi & Mohanty2004) (Tripathy 1983)

ΔF/F$_M$' or Y(II) - *Copper*. Sensitive test (Joshi & Mohanty2004) (Lanaras 1993)

Fv/F$_M$ - *Copper* (Baker and Rosenquist 2004), (Popovic et al., 2003)

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

Fv/F$_M$ - *Lead* (Joshi & Mohanty2004), (Parys 1998) (Romanowska 1998)

Fv/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*. Fv/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)*. qN is a very sensitive indicator of salt stress in <u>Rice</u>. <u>Fv/F$_M$ and yield were not sensitive</u> to salt stress in Rice (Moradi & Ismail 2007)

q$_N$, q$_P$, Fv/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)

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

Fv/F$_M$ – *NaCl (Salt)* Fv/F$_M$ was sensitive to salt stress in chickpea seedlings (Eyidogan 2007)

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

ΔF/F$_M$' or Y(II) – *NaCl (Salt)* Y(II) was sensitive to salt stress in chickpea seedlings (Eyidogan 2007)

ΔF/F$_M$' or Y(II) – *NaCl (Salt)* <u>not sensitive</u> 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)

ΔF/F$_M$' 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)

Fv/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)

Fs in Y(II) - *Zinc* - Fv/Fm is <u>not</u> a good indicator of zinc stress (Joshi & Mohanty2004) (Tripathy & Mohanty 1980). Fs 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).

pH Stress

Fv/F$_M$ – Fv/Fm was found to detect severe acid rain stress at a pH of 1.8 or below.
(Velikova, Yordanov 1996)

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 <u>crown rust on oat leaves</u> (Sholes & Rolfe 1996)

N**PQ -** This is a longer dark-adapted measurement for <u>tobacco mosaic virus on tobacco</u> (Osmond 1998), (Lohaus 2000)

F$_V$/F$_M$ - Fast dark-adapted test can be used for <u>Bean rust</u> (Peterson & Aylor 1995)

ΔF/F$_M$' or Y(II)- Fast light adapted test used for <u>cedar fungus</u> (Ning 1995)

F$_M$ -F$_S$ /F$_M$ - This is a longer dark-adapted test that requires several minutes to reach steady state photosynthesis. <u>tobacco mosaic virus on tobacco</u> (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 <u>chickpea leaves fungus</u> (Esfield 1995) (Weiss 1998)

F$_V$/F$_M$ - Fast dark-adapted test can be used for biotic stress <u>lemons infected by Penicllium digitatum</u> (Nebal 2000)

F$_O$/F$_V$ - Fast dark-adapted test can be used for biotic stress <u>Brassica Blackspot by destruxins</u> (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 <u>Maize rust resistance.</u> (Duraes 2001)

F$_V$/F$_M$ - Fast dark-adapted test can be used for biotic stress. <u>Maize rust resistance.</u> (Duraes 2001)

Herbivory – (Animal Stress):

ΔF/F$_M$' 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 – F$_{735}$/F$_{700}$ chlorophyll content measurement (Butts 2017).

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)

Wind Stress

Various OJIP parameters are sensitive to wind stress Results can vary by species. In one species of tree, Fagus sylvatica or European beach, photosynthetic performance increased. In another species, Fraxinus excelsior or common ash, photosynthetic performance decreased, and in a third species, Abies alba, or silver fir, there was very little change due to wind. (A.J. Clark, W. Landolt, J.B. Bucher, R.J. Strasser 2000)

Wind during measurement should not bother dark adapted measurements, like Fv/Fm, Rapid light curves (Rascher 2000) and OJIP.

Measuring protocols

Fv/FM, 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 for measuring some plant stress types. Results can also vary by whether the plant is a C_3 plant, a C_4 plant or a CAM plant. Some types of plant stress, in C_3 plants, delay fluorescence measurement response until the stress is more severe due to photorespiration.

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 normalized measurement ratio that represents the maximum potential quantum efficiency of Photosystem II if all capable reaction centers were open. (Maxwell K., Johnson G. N. 2000), (Kitajima and Butler,1975) The range of the optimal value for most plant species is from 0.79 to 0.83, with lower values indicating plant stress. Fv/FM has a photochemical component and a non-photochemical component (Baker 2004**). It offers the advantage that one can compare all samples in the same, known, dark-adapted state** (Baker 2004). It is important to compare samples with a similar light history because while photoinhibition starts to relax or repair after 40 minutes in the dark, it can take between 30 to 60 hours for chronic photoinhibition to relax or repair after several hours of bright sunlight. (Lichtenthaler 2004). *Fv/FM is a fast test that usually takes less a few seconds but requires proper dark adaptation. This test was developed by Kitajima and Butler (1975) Values correlate in a linear fashion with gas exchange carbon assimilation in C4 plants and in a curvilinear fashion with C3 plants.* ***Measuring with dark adaption clips allows fast measurement of large plant populations.***

F_V/F_O – This the same dark-adapted measurement normalized 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 Fv/FM. However, Fv/FO but it does not correlate with gas-exchange carbon assimilation like Fv/FM does.

Fv/FM is a normalized parameter that tests whether or not plant stress affects PSII in a dark-adapted state. Fv/FM 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. Templer (2017) reports that Fv/FM is sensitive to drought stress in C_3 plants, one day after watering, if the temperature is above 26ºC.
2. Not sensitive to early or moderate water stress, only sensitive severe drought stress associated with trees not acceptable for crops or grapes. (Bukhov & Carpentier 2004) (Zivcak 2008)
3. Not sensitive to early or moderate water stress in C_4 plants (da Silva J. A. & Arrabaca M.C. 2008)
4. Fv/FM is not sensitive to nitrogen stress until it reaches severe levels. (Baker 2004)
   (Chlorophyll content meters are recommended for nitrogen and sulfur stress. They are sensitive to nitrogen stress, sulfur stress, and other types of nutrient stress but they are not as sensitive to most other types of plant stress as chlorophyll fluorescence measurement.)
5. Fv/FM is not sensitive to sulfur stress until starvation levels are reached. (Baker 2004)
6. Not sensitive to heat stress below 45ºC centigrade in Oak, a C_3 plant. (Haldiman P, & Feller U. 2004)
7. Not sensitive to DCMU herbicide stress. (Nedbal & Whitmarsh 2004)
8. Fv/FM is sensitive to some types of herbicide stress types, and not others. (Nedbal & Whitmarsh 2004)
9. Not sensitive to nickel stress. (Joshi & Mohanty2004)
10. Not sensitive to zinc stress. (Joshi & Mohanty2004)
11. 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 ∆F/F$_M$' or φ$_{PSII}$ - 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). Since Y(II) values vary with light intensity, it is necessary to compare values at the same actinic light level unless light stress is the focus. Furthermore, compare samples with a similar light history because while photoinhibition starts to relax or repair after 40 minutes in the dark, it can take between 30 to 60 hours for chronic photoinhibition to relax or repair after several hours of bright sunlight. (Lichtenthaler 2004). *Y(II) is a fast test that usually takes a few seconds. This test was developed by Bernard Genty* (Genty 1989), (Genty 1990).

If light levels change, it can take up to thirty minutes for the plant to adjust to the new steady state (Nilkens 2010). 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, Y(II) measurements taken under variable lighting conditions may not provide reliable yield results (Rascher 2000).

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, use a PAR Clip 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 no differences in irradiation as the PAR sensor is moved closer or further from the sun, in field measurements, because the sun is 93 million miles away and any changes in distance are small by comparison. *However, small changes in distance, from a light source, in a lab, can significantly change actinic light intensity.* Wind and changing the leaf angle to a light source can also make a big difference in steady state condition.

When studying under canopy samples, where illumination can change, 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 exists before measurement. When in doubt, use pre-illumination of at least 30 minutes to reach steady state photosynthesis. **Rapid light curves were designed to be used under variable light conditions and they should also be considered for under canopy measurement.**

Limitations of Y(II)

1. Yield of PSII or Y(II) and ETR are sensitive to early drought stress in C$_4$ plants.
   (da Silva J. A. & Arrabaca M.C. 2004), (J Cavender-Bares & Fakhri A. Bazzaz 2004)
   (Cerovic 1996)
2. F$_S$/F$_O$ components of Y(II) and 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 and trees but not most other crops. (F$_S$ is a component of Y(II), and F$_O$ is a component of F$_V$/F$_M$)
   (Correspondence with Flexas), (Flexas 1999), (Flexas 2000), (Flexas 2002)
3. *Y(II) by itself is not sensitive to drought stress in C$_3$ plants until it is fairly 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. **(Chlorophyll content meters are recommended for nitrogen and sulfur stress and management.** 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) **Chlorophyll content meters are recommended for sulfur stress and management**

7. <u>Not</u> 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 – 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 or Electron transport rate = Yield of PSII x PAR x ratio of PSII to PSI reaction centers x leaf absorptance.

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. Therefore, while making measurements under sun light, the distance from the light source is insignificant, because a few feet are insignificant compared to the 93 million miles from the sun to the plant. When using artificial light sources; however, 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.

(Y(II) is the quantum yield of PSII) X (PAR is Photosynthetically Active Radiation measured between 400nm and 700nm in μmols $m^{-2}\,s^{-1}$.) 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*) (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°K (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. *For this reason, some researchers prefer Y(II) measurements to ETR for stress measurement because the additional variables do not have to be considered.*

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.

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) one can introduce an error unless one measures leaf absorption. 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, one can replace absorption value and PSII ratio value estimates for 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 or OKJDIP

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

OJIP protocols use a high time resolution scale of the rise of chlorophyll fluorescence to maximum fluorescence value from a dark-adapted minimum fluorescence, using an actinic light source. Its rise has intermediate peaks and dips that the OJIP or OKJDIP nomenclature designates. 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). There is more than one school of thought, on how to use the OJIP rise for plant stress testing. However, *the Strasser protocol has be 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 and limitations with OJIP as one finds with F_V/F_M, and F_V/F_O. (See the plant stress type and the list of limitations below).

Measurements take a few seconds and provide high time resolution fluorescence data. 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 sample, 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 open core antenna to a 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 the peripheral antenna size is larger, O and F_O are lower. The number QB- nonreducing PSII reaction centers (damaged reaction centers) also affects O and F_O. The greater the number of non-reducing reaction centers, the higher the value. F_O is a measurement of antenna fluorescence before the reduction of any Q_A. Modulated fluorometers measure F_O while continuous OJIP fluorometers estimate F_O with linear regression analysis. O is commonly the measurement point at 40 µsec. after turning on the actinic light.

1. The O-J rise represents the photochemical reduction of pheophytin and Q_A. J at 2 ms, represents maximum values for $Q_A Q_B$- 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 $Q_A Q_{B2}$- chemical reaction that end at P with a maximum for $Q_A Q_{B2}$-. 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 $Q_A Q_{B2}$- and $P_Q H_2$. 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 $b_6 f$ complex. The rise of chlorophyll fluorescence ends with reoxidation of PQH_2 to PQ by the cytochrome $b_6 f$ 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 at high light levels, when there is severe nitrogen, iron, or sulfur deficiency. (Strasser 2004) (Vredenberg 2004). **Chlorophyll content meters are much more sensitive to both nitrogen and sulfur plant stress than OJIP or K step measurements.**
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 relaxation the slower q_Z photoprotection mechanism relaxation. If dark adaptation is not adequate errors will occur. See the section on dark adaptation for more details.
4. **Since <u>OJIP values change with actinic light intensity</u> (Vredenberg 2004), it is important to always use the same actinic light intensity for Strasser protocol plant stress measurements. It is common to use 3,000 µmols m^{-2} s^{-1} or 3,500 µmols m^{-2} s^{-1} for the Strasser OJIP protocol. <u>Strasser's later work was done at 3,500 µmoles m^{-2} s^{-1}</u> . Results are actinic light intensity dependent. Calibration of the light source is recommendation. Some instruments like the OS3p+ 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 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. Strasser uses PI_{ABS} or performance index, VJ, and other parameters, to measure plant stress. (Strasser 2004). The OS30p+ and OS5p+ use the Strasser OJIP protocol and calibrated actinic light sources.

7. *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 in C$_3$ plants until at least *seven days have passed*, on many of the plants tested, but it is slightly more sensitive than Fv/F$_M$ (Thack 2007). Other solutions are listed under drought stress. This is adequate for trees but not most crops or grapes.
3. PI$_{ABS}$ is derived from several OJIP parameters including V$_J$, the initial slope of the fluorescence rise - M$_O$, Fv/F$_M$, Fv/F$_O$, the rise from J to P, and the rise from O to J. (Strasser 2004)
   PI$_{ABS}$ = (V$_J$ / M$_O$) (Fv/F$_M$) (Fv/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 <u>light intensity dependent</u>** (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 m^{-2} s^{-1}. **In his later work he used 3,500 μmols m^{-2} s^{-1}** Both of these calibrated actinic light 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.
8. OJIP is not sensitive to most types of nutrient stress such as nitrogen and sulfur stress as would be expected from Fv/F$_M$ research (Arrobas, M 2016). **Chlorophyll content meters are recommended for nitrogen and sulfur stress and management.**

Quenching and quenching relaxation measurements:

It can take days for chronic photoinhibition to repair to pre-stress conditions (30 to 60 hours to fully relax under dark adaptation) (Lichtenthaler H. & Babani F. (2004) (Theile, Krause & Winter 1998). This is common in chronic high light stress, high heat stress, cold stress and over wintering stress. (Lichtenthaler H. & Babani F. (2004) (Theile, Krause & Winter 1998).

As a result, it may make sense to partially shade leaves from photoinhibitory actinic light levels for up to 60 hours. and then dark-adapt for the equivalent of overnight to get an accurate "control" value for F$_M$ and F$_O$, to eliminate chronic photo-inhibition conditions, (Maxwell and Johnson 2000). *It is common to dark adapt for a full night using pre-dawn Fv/F$_M$ values for field plants and plants that have been exposed to photoinhibitory light levels for extended periods of time.* One understands that in plants with a recent *high light history* likely have residual photoinhibition built into all dark-adaption measurements under these conditions. This is alright as long as the light history of measured samples has been built in to the experimental design. If light stress is the focus of the experiment partially shading the plants for at least 60 hours is a better solution. In addition, quenching measurements of different samples should not be compared unless the Fv/F$_M$ values of the samples were identical. This is necessary because Fv/F$_M$ is the yard stick to gauge other quenching parameters (Baker 2008) (See the quenching application note for more details.)

The use of far-red pre-illumination that is available on some fluorometers 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). Far-red pre-illumination is necessary for Kramer Lake model quenching, puddle model quenching and Ruban & Murchie's photoprotective NPQ protocols. Far red light is not necessary for the Hendrickson Lake Model protocol.

The laws of physics make lamp light output drop as a light source heats up. To prevent this, Opti-Sciences uses closed loop lighting systems with a PAR sensor to maintain a constant programable instrument actinic light level and to prevent this issue. This stable light source capability is currently available with the OS5p+, the OS1p, the iFL, the Y(II) meter and the PSP32 systems. If it is not available, 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 than one wants. 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 <u>quenching </u>measurements.

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. *However, most gas exchange researchers define steady state conditions as the time it takes q_E*, the fast-reacting photoprotective mechanism, to adjust. This means that steps can be from 4-7 minutes at each light level. It is common to start low and work to higher light levels to prevent photoinhibition.

Until recently, fluorescence researchers thought that it took between fifteen to twenty minutes, at specific light level, to reach steady state photosynthesis, according to Maxwell and Johnson (2000). However, more recent research finds that, at high actinic light levels, a longer term adjusting photoprotective mechanism, known as q_Z, that involves zeaxanthin, adjusts and relaxes in from 10 minutes to 30 minutes. This means that the actinic light source should be on for at least between 20 and 30 minutes. *Both approaches have value. However, the 4 to 7 minute per step is the most common approach.* Leaf absorption measurement can improve ETR value reliability.

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 conditions or known dark-adapted conditions to be reliable. The exceptions are the "Y(NO)" lake model quenching parameter, and the rapid light curve parameters, ETR_{MAX}, I_K, I_M and α. Light saturation rate, as measured by rapid light curves, highly correlates with the concentration and maximum activity of Rubico (Macintyre 1997), (Macintyre 1996). *Steady state photosynthetic rates overestimate actual photosynthetic rates in a variable light environment* (Macintyre 1997). RLC measuring results are light history dependent, with different results at different times of the day. Different researchers recommend different dark adaptation times and light step periods. Dark adaption times vary from momentary 5-10 seconds (Ralph 2005), to longer times (Rascher 2000). Light step periods vary from 10 seconds to 20 seconds (Rasher 2000). **For best curve-fitting results, one should select at least six actinic light steps that are below leaf light saturation and two actinic steps above leaf light saturation.** For more information, contact Opti-Sciences for the Rapid light curve application note.

Notes for stress measuring

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

2. There are three types of photosynthetic metabolic pathways in higher plants for carbon fixation. They are C_3, C_4, and CAM. In addition, there are some hybrid C_3-C_4 species. From a plant stress measuring perspective, this is important factor. The reason is that C_3 plants, under "oxidative plant stress", "normally" do not show changes in chlorophyll fluorescence until the oxidative plant stress is much more severe than other types of plant stress. Drought stress heat stress and cold stress are oxidative types of plant stress. This happens in C_3 plants because rubisco will not only combine with CO_2 but it will also combine with various forms of oxygen. Fortunately, there are protocols and methods to solve most of these problem. See the Templer protocol under drought stress for C_3 plants. C_3-C_4 plant hybrids, should be treated like C_3 plants for plant stress measuring purposes due to possible delays in the response of chlorophyll fluorescence. About 85% of land plants are C_3 plants, including most food crops. C_4 plants are mostly grasses. However, they also include some plants that evolve from grasses like maize and sorghum and sugarcane. CAM plants include cactus and orchids. Oxidative plant stress is not an issue for C_4 and CAM plants.

3. For most types of nutrient plant stress, a chlorophyll content meter works much better than chlorophyll fluorescence. Chlorophyll fluorescence will only detect nitrogen and sulfur stress at severe or starvation levels. In addition, chlorophyll content measurements work better for molybdenum, iron and zinc plant stress.

4. 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 has been based on an understanding of plant mechanisms. that have affected measurements. It also depends on what one wants to measure. Recommended times can vary by chlorophyll fluorescence test type, and environmental conditions.

It is important to note that the decision on how long to dark adapt can depend on either the latest science or tradition for F_V/F_M measurements. While there are countless papers that use 20-to-30-minute dark adaption times for F_V/F_M measurements, most quenching protocols all use the equivalent of overnight dark adaption and the equivalent of pre-dawn F_V/F_M measurement for quenching's reference measurement. Some old school researchers and reviewers only believe in overnight predawn dark adaption for all types of dark adaption protocols. We recommend checking with potential research reviewers before starting experimental design.

While chloroplast migration is a real thing and takes between 20 to 35 minutes to occur, chlorophyll fluorescence measurements do not reflect or adjust to chloroplast migration **(Sam Wilson and Alexander V. Ruban 2020)**. With that in mind, and the results of the Nilkens' group, we find that q_Z, a slow reacting photoprotective plant mechanism, involving zeaxanthin, takes from 10 minutes to 30 minutes to relax in the dark **(Nilkens 2010)**. And at lower actinic light levels, state transitions take from 15 minutes to 20 minutes to relax in the dark. Therefore, dark adaptation times of at least 20 to 30 minutes are a requirement for terrestrial plant stress measurement. Anecdotal evidence indicates that some research reviewers require the equivalent of "overnight" dark adaptation or pre-dawn values. Different journals may have their own ideas as to what constitutes reliable dark adaptation. See the section below for an in-depth discussion.

F_V/F_M is affected by both photochemical and non-photochemical factors. If a leaf was 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 than 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). Photoinhibition and photodamage starts to relax or repair at about 40 minutes in the dark. However, it can take between 30 hours and 60 hours for photoinhibition to relax or repair in the dark. (Lichtenthaler 2004). Therefore, one should only compare samples with a similar light history unless one is studying light history.

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 state transitions exist). (Papageorgiou G.C. Tismmilli-Michael M. Stamatakis K. 2007). Under high actinic light, or near saturating light conditions, a slower adjusting plant photoprotective mechanism, q_Z, that involves zeaxanthin, takes between 10 to 30 minutes to adjust and relax (Nilkens 2010).

Full activation of Rubisco takes between three and four minutes in vascular plants as well as in phytoplankton. Deactivation of Rubisco, in the dark, takes between 12 -18 minutes in vascular plants and from 9 minutes to 28 minutes in some phytoplankton. 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 mechanism q_E involves the xanthophyll cycle and changes in the thylakoid lumen Δph. They relax in the range of several seconds to a 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 effects 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 actinic light survival mechanism that allowed light balance between Photosystem II and Photosystem I. F_M' or maximum fluorescence under light adaption conditions, decreases over a 15 to 20 -minute time frame, and then relax during dark adaptation over a 15 to 20 - 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). More recent evidence shows that the fluorescence change thought to be the result of state transitions is in fact caused by a slower reacting photoprotective mechanism at higher light levels. q_Z, a zeaxanthin-based mechanism, has been shown to adjust and relax over a 10-to-30-minute time scale (Nilkens 2010). While chloroplast migration also occurs and relaxes over about a 20-minute to 30-minute time frame, at near saturating light conditions in land plants, evidence now exists that chloroplast migration does not affect chlorophyll fluorescence measurements (Sam Wilson and Alexander Ruban 2020). 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). More recently, Ruban suggested that these various mechanisms, acute photoinhibition, q_Z, and state-transitions overlapped in time and as a result, time-based measurements of these mechanisms were ambiguous at best (Ruban 2017), (Ruban A.V, Murchie E.H, 2012) (Wilson & Ruban 2020). As a result, he developed a method to completely separate photoprotective NPQ and photodamage mechanisms that was not time based.

Chronic Photoinhibition q_I, is the result of exposing leaves or plants to high light conditions for hours. After sunny summer days, there is almost always some residual photoinhibition built into all chlorophyll fluorescence measurements. That is alright as long as one compares samples to other samples with a similar light history. As stated earlier, chronic photoinhibition starts to relax or repair after 40 minutes in the dark. Furthermore, it takes between 30 to 60 hours for photoinhibition to completely relax or repair. As a result, 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 photoinhibition. Then dark adapt the equivalent of overnight for quenching measurements. That way, the F_V/F_M measurement, in quenching measurements, becomes a more reliable reference for measuring photoinhibition after a long exposure to high light conditions.

5. 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.

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.

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

Reliable measurements are ones that are both 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 0.83 (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

The biggest advantage of F_V/F_M is that it allows measurement of samples in the same known dark adaption state. F_V/F_M is a normalized ratio that does not use a traceable standard. Instead, it's accuracy is determined by properly using the 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). Lower values indicate plant stress.

To get a reliable 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. This fast-acting mechanism is "q_E". It can take up to 4 minutes with indoor and green house plants, and it can take up to 7 minutes in field plants (Baker 2008). At Higher actinic intensities, a longer term photoprotection mechanism, "q_Z" for zeaxanthin, takes from 10 minutes to 30 minutes to fully adjust or relax in the dark (Nilkens 2010) In lower plants there is evidence that state transitions occur. (State transitions 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 phytoplankton (MacIntyre 1997). In addition, field plants and other

plants that have been exposed to photoinhibition conditions for a number of hours, will take from 30 hours up to 60 hours to repair (Lichtenthaler 2004). Photoinhibition starts to repair and relax after about 40 minutes in the dark (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 Fv/Fm. This is all right if one is measuring "light stress" and comparing results, but when measuring other types of plant stress, light history should be taken into account when comparing samples. It is common for researchers to choose dark adaptation times anywhere from twenty minutes to overnight, using pre-dawn values. 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.) If possible, testing should be done to find the time required to reach a **stable steady dark-adapted state.** If not, then 30 minutes is safe for F_V/F_M measurements with a similar light history.

2. Modulation light intensity setting Fv/Fm = (Fm-Fo)/Fm. Minimum fluorescence, F_O, is a "pre-photosynthetic" dark adaption value. For measurement, one exposes the leaf antennae to a very low intensity modulated light. The intensity must be set properly to allow detection, but not high enough to drive photosynthesis. If it is set too high, it will drive photosynthesis and provide an Fo value that is too high When setting the modulating light intensity, place a dark clip on a leaf and close the shutter. Wait for five minutes, place the fluorometer probe into the dark clip and open the shutter with the fluorometer on and open to modulated light adjustment. The "Ft" value or fluorescence signal should not rise over a 30 second period when on a leaf, under these circumstances. If it does, lower the modulation light intensity and check again. If it does not detect a signal, then the intensity of the modulation light must increase. It is a good idea to test one of your samples that is either under stress or has lower chlorophyll content otherwise it may only work on healthy, high chlorophyll content leaves and on the leaves under stress. *OSI now offers an automated modulated light set up routine or modulated light set up aids on all of its newer chlorophyll fluorometers.*

3. Shade leaves vs. Sun leaves. – The Fv/Fm ratio will be slightly higher on sun leaves than on shade leaves (Lichtenthaler 2004).

4. Fv/Fm 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 lower than systems with white light saturation lights. (Cessna 2010)

5. Maximum Fv/Fm values vary with species. The average maximum Fv/Fm value is between 0.79 - 0.84 (Maxwell and Johnson 2000).

6. Compare samples with a similar light history. Field plants should only be compared to field plants and green house plants should be compared to green houseplants. Due to the fact that it can take from 30 up to 60 hours for chronic photoinhibition to relax or repair in the dark, photoinhibition can be involved in some measurements more than others. (Lichtenthaler 2004) Results after a sunny day in the summer may be different that measurements on the same plant after a few days of overcast, again because it takes a long time for photoinhibition to relax or repair.

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 Fv/Fm 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. All of Opti-Sciences newer chlorophyll fluorometers provides a rolling 25 ms. 8-point measuring average to determine the highest F_M value and prevent saturation pulse NPQ

from being a source of error. This ensures a reliable value, even if the saturation pulse width or duration is too long.

9. Saturation pulse intensity. Dark adapted leaves saturate easily with lower saturation pulse intensities. It may take a few hundred μmols m^{-2} s^{-1} to saturate shade leaves and sun leaves will saturate around 1,500 μmols m^{-2} s^{-1}. 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 light adaption, Y(II) values.)

10. Some Fv/Fm fluorometers have the ability to pre-illuminate dark adapted leaves with far-red light. In the dark, the fluorometer exposes the leaf to far red light for five to seven seconds before an Fv/Fm measurement. When this takes place, it activates PSI, and ensures that all electrons have been drained from PSII before the measurement of Fo. While this feature ensures that PSII is completely re-oxidized, it does not relax the xanthophyll cycle, state transitions, or photoinhibition. *Time is still required in a darkened environment to relax all forms of NPQ and to obtain a reliable Fv/Fm measurement.* (Maxwell and Johnson 2000). Far red light is not a requirement for reliable Fv/Fm measurements. For best results, measure all samples the same way, either with far red light or without far red light.

11. Fluorescence heterogeneity presents itself as different Fv/Fm measurements on different parts of the leaf. It has been found to occur under cold stress conditions, with biotic stress, and under water stress conditions. By using multiple measurements and a sampling plan, heterogeneity can be over come (Buschmann C. in correspondence by e-mail 2008). Imaging fluorescence will also solve the issue.

12. Part of the minimum fluorescence, the Fo parameter, in Fv/Fm (Fm – Fo)/Fm), contains PSI fluorescence as well as PSII fluorescence. This is not a problem for plant stress measurement because PSI fluorescence values do not change. However, it is important to understand that it exists. 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. 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% of Fm in C$_4$ plants (Pfundle 1998).

It is important to use the best method for measuring plant stress. In some cases, F$_V$/F$_M$ provides the best results, in some cases the light adaption Y(II) does, and in some cases, chlorophyll content is the best choice.

F$_V$/F$_M$ is not a sensitive test in C$_4$ plants for nitrogen stress, nickel stress, sulfur stress, & zinc stress. Use a Chlorophyll content meter instead.

F$_V$/F$_M$ is not a sensitive test in C$_3$ plants heat stress (Y(II) will detect heat stress at 35°C and F$_V$/F$_M$ will not detect heat stress until 45°C), nitrogen stress, nickel stress, sulfur stress, zinc stress, some herbicides, salt stress in some types of plants and drought stress below 26°C. Y(II) will detect drought stress in C$_3$ plants after about 7 days after watering. That works for trees but not most crops. (Opti-Sciences Plant Stress Guide 2010) For nutrient stress, use a chlorophyll content meter. Fv/Fm is effective for most other types of plant stress. *For specific research results on specific types of plant stress, see the Plant Stress Guide offered by Opti-Sciences Inc.*

F$_V$/F$_M$ is not a sensitive test in CAM plants for nitrogen stress, nickel stress, sulfur stress, & zinc stress. Use a Chlorophyll content meter instead.

Checklist OJIP

OJIP - has an additional requirement when compared to Fv/Fм

Follow the Fv/Fм checklist for all other OJIP requirements for reliable measurement. "Strasser OJIP" is the OJIP protocol most used for measuring plant stress. Measurements require 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 changed at different light intensities.* Early Strasser work was done at 3,000 µmols m^{-2} s^{-1}. **Later Strasser work was done at 3,500 µmols m^{-2} s^{-1}.** Both calibration intensities are available on the OS30p+ (The OS30p+ automatically calibrates its red actinic light source when the instrument turns on. Follow the Fv/Fм checklist for all other OJIP requirements for reliable measurement.

Checklist before making light adapted Y(II) measurements.

Accuracy is the ability to hit the mark. In many types of measurements, accuracy is determined by calibrating to a measurement standard traceable to the National Institute of Standards and Technology (NIST). With such measurements, tolerances are always involved.

Y(II) or ΔF/Fм' measurements are normalized ratios, and it does not have a traceable standard. Instead, proper instrument usage as it relates to plant physiology determines accuracy. Here, the standard has been developed by the research of experts in the field.

Repeatability is the ability to achieve the same measurement, within a specified tolerance level.

A **Reliable** measurement is one that is accurate and repeatable.

Chlorophyll fluorometers primarily measure normalized ratios that relate relative measurements of variable chlorophyll fluorescence found in Photosystem II. These measurements are capable of measuring many types of plant stress. This application note provides a cookbook style checklist of issues that must be considered in order to get reliable Y(II) measurements.

Y(II) or Δ F/Fm' 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). The closure of reaction centers, heat dissipation through photoprotective nonphotochemical quenching and nonphotochemical heat dissipation, not due to regulation, affect Y(II) measurements. (Schreiber 2004). Photochemistry, heat dissipation, and chlorophyll fluorescence are competitive processes that compete for energy. Conditions that favor greater photochemistry cause lower chlorophyll fluorescence and heat dissipation. The relationship between Y(II) and photochemistry is linear in C$_4$ plants, and curvilinear in C$_3$ plant. Because the plant enzyme, rubisco, will bond with various forms oxygen as well as with CO$_2$, under photorespiratory conditions, photorespiration, in C$_3$ plants, may limit some types plant stress measurement sensitivity in C$_3$ plants. Photorespiratory stresses include drought stress, heat stress and cold stress.

First reported by Bernard Genty in 1989, this light adapted test became possible with the advent of modulated chlorophyll fluorometers. It is the most versatile plant stress measuring parameter, because it has been shown to detect more types of plant stress, earlier, that any other chlorophyll fluorescence method. The disadvantage is that, unlike Fv/Fм, values vary with ambient actinic light intensity.

Cookbook checklist before making Y(II) measurements.
F_M' is maximum fluorescence, under actinic light, at steady state photosynthesis, using a saturation pulse for measurement. F_S' is the steady-state fluorescence signal under actinic light.
Y(II) is = $(F_M' - F_S') / F_M'$ or $\Delta F/F_M'$

1. Leaves must be at steady state photosynthesis. Under lower and medium light levels this takes between fifteen and thirty minutes at a given light level. Above canopy leaves on a clear day, in the field, are considered to be at steady state photosynthesis. (Nilkens 2010) (Maxwell and Johnson 2000). P 77. It takes about 2 to 7 minutes for the fast photoprotective mechanism, q_E to adjust in the light (Baker 2004) and it takes from 10 minutes to 30 minutes for the slow reacting photoprotective zeaxanthin, q_Z, to adjust in the light. As a result, it can take up to 30 minutes for a plant to reach steady state photosynthesis. At near saturating light conditions, chloroplast migration also occurs in plant cells. However, recent research (Wilson S. Ruban A. 2020) shows that chloroplast migration does not affect the fluorescence signal. Wind will also affect steady state conditions.

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. Steady state conditions are a requirement for reliable measurement of Y(II). The xanthophylls cycle, and Δph of the thylakoid lumen (q_E) adjust in about 4 to 7 minutes in field plants. (Lichtenthaler 2004) (Baker 2004). State Transitions take between 15 and 20 minutes to completely adjust. While state transitions are a significant factor at lower actinic light intensities, in most plants, they are not a factor at high light intensities. At higher light intensities q_Z takes between 10 -30 minutes to adjust (Nilkens 2010).

Under canopy, "Rapid Light Curves" and F_V/F_M may be better solutions. Rapid light curves were designed for measurement of plants under quickly changing light conditions. The alternative is to use an internal fluorometer actinic light source, under a shroud, expose the sample to the actinic light for up to 30 minutes, to reach steady state, and then make a measurement.

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 and leaf temperature. Comparing Y(II) values taken at different light levels and different temperature levels introduce a significant error, unless it is the change, at different light levels and heat levels, that is of interest. A PAR Clip allows measurement of PAR and leaf temperature near or at the leaf level. (Genty 1989), (Genty 1990)

4. Shade leaves vs. Sun leaves. – The Y(II) ratio will be higher on sun leaves than on shade leaves (Lichtenthaler 2004).

5. 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)

6. Leaf orientation. When making a Y(II) measurement, with or without a PAR Clip, **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 Phytoplankton and cyanobacteria. Times outside these ranges can increase the error in Y(II) measurements with most chlorophyll fluorometers. Shorter durations, than the times above, prevent complete saturation of PSII, regardless of the light intensity (Rosenqvist & van Kooten 2006). 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 (Rosenqvist & van Kooten 2006). However, The OS1p, the OS5p, the Y(II) meter in the Plant Stress Kit, the PSP32 monitor fluorometer and the OS5p+ have an algorithm that uses a 25 millisecond 8-point rolling average capability to detect the

correct F_M' value as long as the saturation flash is long enough. This prevents saturation pulse NPQ from being a problem.

It can take from 60 seconds to 120 seconds for saturation pulse NPQ to fully dissipate, and so it is best to wait for at least two minutes between measurements at the same location. (Rosenqvist & van Kooten 2006).
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 m^{-2} s^{-1}, and sun leaves will usually saturate below 1,500 µmols m^{-2} s^{-1}. Indoor plants saturate at much lower light intensities. However, a problem exists when measuring Y(II) on plants that are under high actinic light levels for a several hours. These leaves resisted the complete closure of all PSII reaction centers or chemical reduction of the reaction centers, even with the highest saturation light intensity. Even with a 7,000 µmols m^{-2} s^{-1} saturation pulse, some reaction centers remain open. And since closing all PSII reaction centers is a requirement for reliable Y(II) and ETR measurement, a solution is necessary. Up to a 22% error was found in Y(II) measurements and a 41% error in ETR measurements using standard square topped flash under those conditions.

The solution was, a single, multiple phased saturation flash. The name of the method is F_M' correction. The evidence shows that the method provides the ETR one expects when comparing results to gas exchange methods. This option is on all Opti-Sciences instruments for light adaption measurements, including the Y(II) meter, OS1p, OS5p, OS5p+, iFL and PSP32 instruments. For more on this method, ask for the application note on F_M' correction or read the development papers. (Earl 2004) (Loriaux S.D., R.A Burns, Welles J.M., McDermitt D.K. Genty B. 2006) (Markgraf, T. and Berry J. 1990). Opti-Sciences uses the protocol in Loriaux 2013.
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 adaption 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 exists when 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' 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 Y(II) 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 in C_4 plants. Chlorophyll fluorescence electron transport of PSII in corn, a C_4 plant, under cold stress, far exceeds the requirements for CO_2 assimilation by more than three to one. This indicates that under these conditions, other electron sinks are at work. The ratio of ETR 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). Corn is a C_4 plant and photorespiration is not an issue.
13. The ratio of ETR to CO_2 assimilation can be diagnostic for water 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 2005).
14. Mangrove leaves growing in the tropics. Here again electron transport rate is more than three times that of CO_2 assimilation. It was believed that this was mostly due to reactive oxygen species as an electron sink. (Baker Oxborough 2004), (Cheeseman 1997)
15. Linear correlation occurs between Y(II) and ETR verses CO_2 assimilation in C_4 plants (Genty 1989). Curvilinear correlation occurs between Y(II) and ETR verses CO_2 assimilation in C_3 plants, (Genty 1989), (Genty 1990), (Baker Oxborough 2004). E*xact* correlation between Y(II) & ETR with gas exchange carbon assimilation is not possible due to the fact that fluorescence comes from only the upper most layers of the

leaf while gas exchange measurements measure the whole leaf (Schreiber 2004).

16. Chlorophyll fluorescence Heterogeneity – Chlorophyll fluorescence can vary from one part of a leaf to another and become patchy under certain circumstance. *Under drought stress, cold stress, or CO_2 stress it is best to take multiple measurements on the same leaf and average the values* (Baker 2008).

17. Light history – Photoinhibition starts to relax and repair after 40 minutes in the dark. And since it takes chronic photoinhibition between 30 to 60 hours to relax and repair, one should not compare samples with different recent light histories unless light history is your focus. There will be some residual photoinhibition after a bright summer day and there may be no residual photoinhibition after a few overcast days (Lichtenthaler 2004).

18. PAR is "photosynthetically active radiation". PAR measurements are between the wavelengths of 400nm to 700 nm. PAR sensors, for measuring light intensity at the leaf, and thermistors, for measuring leaf temperature go through calibration to other instruments that are traceable to the NIST. We recommend that recalibration should occur every two years. Most modern sensors are solid state, so drift is minimal.

19. Y(II) is more sensitive to some types of plant stress, for example: Y(II) will detect heat stress at 35ºC while F_V/F_M will not detect heat stress until 45ºC. We have listed some important notes below. For more information request the Opti-Sciences Plant Stress Guide and quantum photosynthetic yield application note at www.optisci.com

1. 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. Y(II), *by itself, is only sensitive to severe drought stress* in C_3 plants. (Flexis 2002)
3. Y(II) is Sensitive to heat stress at or above 35ºC in Oak, a C_3 plant. (Haldiman P, & Feller U. 2004)
4. Y(II) is not sensitive to nitrogen or sulfur stress until starvation levels are reached. (Baker 2004)
5. Y(II) is not sensitive to early or moderate CO_2 stress. (Siffel & Braunova 1999)
6. Y(II) is not sensitive to NaCl stress in rice a C_3 plant, but it is sensitive to NaCl stress in sorghum a C_4 plant and chickpea a C_3 plant. (Moradi & Ismail 2007) (Netondo 2004) (Eyidogan 2007)

F_V/F_M is not as sensitive as Y(II) to heat stress, nickel stress, zinc stress, some types of chemical stress, and some types of herbicide stress.

Cookbook checklist for making NPQ and other quenching measurements.

Over the last twenty years there have been many changes in our understanding of chlorophyll fluorescence quenching measurements. The development of two lake model quenching protocols, the development of quenching relaxation protocols and Ruban & Murchie's photoprotective NPQ protocol that is not a time-based relaxation protocol. This Check list was designed to improve the understanding of proper quenching protocol usage. 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 at lower light levels. These times can vary somewhat in field plants and can take slightly longer (Baker 2004). Under high actinic light conditions, a slower reacting photoprotective mechanism, q_Z, that uses zeaxanthin, adjusts and relaxes in a 10-minute to 30-minute time frame (Nilkens 2010). In addition, field plants and other plants that are under high actinic light, for a number of hours, are subject to acute and chronic photoinhibition conditions. As a result, plants will retain a certain amount of photoinhibition and photodamage for multiple days. It takes up to 30 to 60 hours (Lichtenthaler 2004) for photoinhibition to relax and repair. 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 the 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. **In order to compare quenching values, samples <u>must have the same F_V/F_M values.</u>** Don't compare quenching measurements of different samples with different F_V/F_M values (Baker 2008). The initial F_V/F_M value is the measuring standard for all succeeding 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 $(F_M-F_O)/F_M$ and F_O, or minimum fluorescence, is a dark-adaption value. The modulated light allows the measurement of pre-photosynthetic antennae fluorescence. The chlorophyll fluorometer measures F_O by exposing the leaf antennae to a very low intensity modulating measuring light, that is not set high enough to drive photosynthesis or chemically reduce Q_A but set high enough to make a measurement (Zhu 2005). 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. Maximum fluorescence occurs 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 PSP32 all have automatic modulated light set up routines for ease of use. One can still use the manual method as well. To do this, one places the leaf in the leaf clip, PAR clip or leaf chamber and dark adapt for about 5 minutes. Then expose the sample to the instrument modulated light. If the Ft value (momentary fluorescence readout 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. If the value stays steady, it is set correctly.

4. **Leaves must be at steady state photosynthesis for most quenching measurement parameters. After dark adaption and F_V/F_M measurement, the instrument actinic light turns on at a set PAR value until the sample reaches steady state photosynthesis.** Until recently it was thought that this process took between fifteen and twenty minutes at a lower light levels (Maxwell and Johnson 2000) to reach steady state. However, at higher light levels a longer, slower reacting photoprotective mechanism also exists. It is "q_Z" for zeaxanthin and this mechanism takes from 10 minutes to 30 minute to adjust and relax. As a result, steady state occurs after q_Z adjusts (Nilkens 2010). 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 Lake model protocols. Chloroplast migration occurs, but chlorophyll fluorescence does not detect its effects (Wilson S., & Alexander Ruban A. 2020)

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 this causes 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 to 30-minute actinic illumination period, *the sample is no longer at steady state photosynthesis. The OS1p, the OS5p+ the iFL and the PSP32 systems use a PAR sensor to measure light irradiation at the leaf surface and maintain a constant actinic light intensity over time. Corrections occur 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. Make measurements is with a PAR Clip, a tripod, and a shroud over the sample for dark adaption and illumination by the instrument actinic light source during 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). To compare samples, the Fv/Fm values must be the same on different samples (Baker 2004).

9. **Leaf orientation is not important because one uses an artificial actinic light under a shroud.**

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 (Rosenqvist & 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 (Rosenqvist & van Kooten 2006. *Saturation pulse NPQ is not an issue in the latest chlorophyll fluorometers from Opti-Sciences. They now use a rolling eight-point 25 ms. average to detect 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, and the PSP32 systems It is also available on instruments that do not do quenching measurements.*

12. **Saturation pulse intensity**. Saturation pulse intensity is more of an issue with Y(II) than with Fv/F_M. When dark adapting, shade leaves will saturate at a few hundred µmols, and sun leaves will usually saturate below 1,500 µmols m^{-2} s^{-1} (Ralph 2004). However, there is a problem when measuring Y(II) at on leaves that are under near saturating light conditions for several hours. Under these conditions, leaves resist the complete closure of all PSII reaction centers, a requirement for reliable measurement. Even with a 20,000 µmols m^{-2} s^{-1} saturation pulse, some reaction centers remain open. As a result, up to a 22% error is possible in Y(II) measurements using standard square saturation flash techniques and up to a 41% error in ETR values (Loriaux 2006) (Loriaux 2013) when comparing to gas exchange measurements. To correct for this issue, a method was developed using a multiple phased single saturation flash. When using this F_M' correction method, fluorescence ETR value behave as one expects when comparing gas exchange results. The Loriaux 2013 paper was co-Authored by Bernard Genty, the creator of Y(II). The Loriaux 2013 method is available on all Opti-Science instruments capable of Y(II) measurements including the OS5p+, the iFL, the OS1p the PSP32 and the Y(II) meter chlorophyll fluorometers. One can still use the standard square topped flash as well. For more details on F_M' correction get the F_M' correction application note, or read Loraiux 2013.

13. **The time between saturation pulses is important.** Rosenqvist and van Kooten (2006) state that between one to two minutes is a requirement 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 than 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 buildup 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. **O**verlap **of PSI fluorescence** -Part of the minimum fluorescence, the F_O parameter, in Fv/F_M (($F_M - F_O$)/F_M), contains PSI fluorescence as well as PSII fluorescence. With Fv/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% 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". PAR measurements are between the wavelengths of 400nm to 700 nm. PAR sensors, for measuring light intensity at the leaf, and thermistors, for measuring leaf temperature, go through calibration to other instruments that are traceable to the NIST. We recommend that recalibration should occur every two years. Most modern sensors are solid state, so drift is minimal.

16. **Far-red pre-illumination**. Some fluorometers have the ability to pre-illuminate dark-adapted leaves with far-red light. One uses this feature to illuminate the leaf for 5 to 7 seconds before an F$_V$/F$_M$ measurement takes place. It activates PSI and ensures that all electrons drain 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, q$_Z$ or photoinhibition. Time in the dark is still requirement to relax all forms of NPQ and to obtain reliable quenching values.

17. **Far-red illumination.** One also uses far red light after the actinic light turns off in the post actinic light mode to allow measurement of F$_O$' a parameter that reflects quenched F$_O$. This value is part of the Kramer lake model parameters, puddle model q$_N$ and q$_P$ and in Ruban & Murchie's photoprotective NPQ protocol. Hendrickson simplified lake model parameters do not use F$_O$'.

Complete publication references are in a separate companion PDF file and are in alphabetical order.