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Chlorophyll fluorescence estimation of fodder galega (Galega orientalis Lam.) *in situ* and dependence on different leaf rank and cultivars

L. Baležentienė and D. Šiuliauskienė

Lithuanian University of Agriculture, Studentų 11, Akademija LT–53361, Kauno r., Lithuania; e–mail:ligita.balezentiene@lzuu.lt

Abstract. The fluorescence measurement *in situ* of various developmental levels of leaves and various cultivars of fodder galega (*Galega orientalis* Lam.) was carried out at the Research Station of the Lithuanian University of Agriculture. The object of the investigation was to evaluate the differences of fluorescence of galega cultivars *Vidmantai*, *Laukiai*, *Melsviai* and a breeding number *L04–4*. The estimation of fluorescence efficiency characterizes the intensity of photosynthesis indirectly and is related to biosynthesis. The yield of the synthesized biomass was also determined. The most intensive fluorescence yield value of the 2nd and the 3rd leaf ranks and plants was determined at the flowering stage *in situ*. The indices of fluorescence (fluorescence in steady–state light Ft and maximal fluorescence Fm, quantum yield of electron transport Y and electron transport rate ETR) were smaller in a lower leaf rank and in more old leaves of the galega cultivars tested. All quantities of the investigated fluorescence indices statistically significant (P = 0.95) decreased and reached the minimal value both of the older leaves (the 4th–the 6th leaf ranks) and of the youngest (the 1st rank) leaves in comparison with the peak values of the 2nd and the 3rd leaf ranks of all investigated cultivars. It was established that a strong and statistically significant parabolic curvature ($0.78145 \leq \eta \leq 0.97385$) of different leaf ranks depend on fluorescence indices Ft, Fm and Y/ETR with a leaf rank predomination by 60.3–86.0, 67.2–84.9 and 62.8–94.8% respectively.

Key words: chlorophyll fluorescence, fodder galega, leaf rank

INTRODUCTION

In recent years the use of chlorophyll fluorescence measuring as a pulse–amplitude modulated (PAM) fluorescence method is informative and attractive for assessing photosynthetic characteristics. The method was first applied in the 1980's because it is non–invasive, quantitative and provides information about photosynthetic efficiency (Schreiber, 1997; Ralf et al., 1998). This method can be applied practically *in situ* and has become an important method for determining the influence of various ecological factors, pollution or stress factors (salinity, carbon limitation etc.) on photosynthesis (Maxwell et al., 2000; Durako & Kunzelman, 2002) caused by the environment. Chlorophyll fluorescence characterizes photo activity of chlorophyll, reflects electron transport in photo system II (PS II) and demonstrates the efficiency of photosynthetic energy conversion, which has a fundamental importance for the atmosphere and for biomass production (Grabolle & Dau, 2005). Schreiber (1997);

many other authors offered warnings about the fundamental relationship between the quantum of fluorescence yield and photochemical energy conversion. However, PAM fluorescence cannot completely replace classical methods, especially when it is impossible to measure the net production or gas exchange balances (Beer & Ilan, 1998).

By PAM fluorescence fluorometry the leaf is subjected to a pulse of saturating light and two measurements are made: Ft–initial steady–state fluorescence of a light–acclimated tissue and Fm–the maximum fluorescence at a given photosynthetic photon flux density (PPFD). The essence of the fluorescence method lies in the application of the saturating light pulse due to this photochemical conversion in the photo system II starting with the quantum yield and non–irradiative energy dissipation. The initial steady–state Ft and the maximum Fm values of fluorescence are used to determine the photo system II efficiency, or quantum yield Y (Fm–Ft/Fm=ΔF/Fm) of electron transport through PS II at a given irradiance, when part of the reaction centres are closed (Genty et al., 1989; Beer & Björk, 2000). The Fm can change depending on various internal and external factors (Maxwell et al., 2000). Y is a sensitive indicator of photosynthetic stress (Ralf, 1999). Consequently, these fluorescence indicators can be used for plant ecophysiology studies and for characterizing new cultivars (Lang et al., 1996; Fracheboud et al., 1998). The calculation of absolute photosynthetic electron transport rate ETR (c x 0.5 x PAR x Y) depends on PPFD at the leaf surface and the absorbed PPFD by the leaf (Schreiber, 1997). It is assumed that 84% of the incident quanta (PPFD) are absorbed by terrestrial plant leaves and only 0.5 of this PPFD are distributed to PS II (Beer et al., 1998).

Currently, the kinetics of the parameters of chlorophyll fluorescence induction are finding application in new spheres of research and are creating possibilities in plant breeding as a characteristic of photosynthetic activity and bio-productivity of cultivars.

The aim of this research was to determine differences of fluorescence parameters of galega cultivars and various ranks of leaves.

MATERIALS AND METHODS

The galega cultivars were sown in twice–replicated plots at the Research Station of the Lithuanian University of Agriculture. The galega plots consisted of 6 $m^{2.}$ The trials were set on a sandy moraine humic horizon of Calcary–Epihypogleyic Luvisol, LVg–p–w–cc. Three fodder galega (*Galega orientalis* L.) cultivars Vidmantai, Laukiai, Melsviai (included in National Varieties List, 2001; 2002) and breeding number L04–4 were tested for fluorescence parameters in situ. The Vidmantai was used as a control cultivar. The productivity measurements of the cultivars' green mass GM, dry matter DM, seed yield, mass of 1000 seed, number of seeds per pod and plant height were taken.

The chlorophyll fluorescence was measured *in situ* at the flowering stage of galega in 4 replications. The steady–state fluorescence yield (Ft) in the light and maximum fluorescence yield (Fm) during the light flash were recorded and used to determine the quantum yield of electron transport (Y). Pulse–amplitude modulated fluorescence was measured by chlorophyll fluorometer diving–PAM–200. The yield of electron transport has been used as a sensitive indicator of the photosynthetic ability of cultivars. Irradiance during fluorescence measurement runs 850 μmol m^{-2} s^{-1} PAR

energy with time resolution of 10 μ s^{-1} in red (650 nm) light region. The quantum yield of electron transport was estimated according to the equation: Y = (Fm–Ft) / Fm (Schreiber, 1997; Genty at al., 1989). The electron transport rate illustrates the absorbed electron quanta that is distributed to photo system II and was determined using the equation: ETR = c x 0.5 x PAR x Y μmol m^{-2} s^{-1}; c–part of absorbed PPFD by leaf (Genty et al., 1989). The photosynthetic responses (Ft, Fm, Y, ETR) were examined with respect to the age of the leaf, from the youngest and fully developed leaves to the oldest (1st–6th leaf ranks). The level of statistical confidence, multivariate test of homogeneity, Levene's and Box M of variances/co- variances, stochastic interactions between the initial Ft, maximal Fm fluorescence and estimated Y and ETR data were calculated by the methods of variance and regression analysis using the statistical package STATISTICA of Stat Soft for Windows standards. This integrated system of data analysis and management includes a complete collection of classical methods, which allows effectively collect data tables on a local disc with a distant data depot.

RESULTS AND DISCUSSION

The biometrics characteristic of the examined cultivars illustrates the main agro–biologic features of galega cultivars (Table1).

Table 1. Biometric parameters of galega cultivars.

	Cultivar				Mean	Standard deviation	Variation coefficient %
	Vidmantai	*L04–4*	*Laukiai*	*Melsviai*			
Green mass t ha^{-1}	51.6	52.7	58.2	60.4	55.7	3.68	6.6
Dry matter t ha^{-1}	12.1	12.2	13.2	13.9	12.9	0.75	5.8
Seed yield g m^{-2}	13.8	12.0	18.0	8.5	13.1	3.42	26.2
Mass of 1000 seed g	8.1	7.6	6.9	7.0	7.4	0.48	6.5
Height cm	127.3	128.0	119.0	137.0	127.8	6.37	5.0
Seed per pod un.	4.0	4.0	7.5	5.0	5.1	1.43	27.9

Melsviai, with the highest value of DM – 13.9 t ha^{-1}, due to the tallest (137 cm) stems, was determined to be the most productive cultivar; Laukiai was the shortest – 119.0 cm height.

Laukiai had the greatest reproductive value (18 g m^{-2} of seed and 7.5 seed per pod in average). The variation coefficients of dry matter yield and mass of 1000 seeds of the tested cultivars were the least: 5.8 and 6.5% respectively.

The high variance coefficients of seed yield (26.2%) and seed per pod (27.9%) showed the greatest differences among the tested cultivars.

There is one basic assumption that must be satisfied before the analysis of variance will be used: applying of variables variance equation which was verified by the test of homogeneity of variances/co-variances presented in Levene's and Box M test in ANOVA/MANOVA (Table 2). Levene's test for the homogeneity of variances amounts was performed by one–way ANOVA on the absolute deviation scores (from the respective cell means). Box M is a multivariate test of the homogeneity of variances and co-variances for multiple dependent variables or covariates.

Table 2. *Levene's* and *Box M* test of homogeneity of variances.

ANOVA: Single Factor–Degrees of freedom for all *F*'s: 5,18					
Variable	*F*	*P*–level	Variable	*F*	*P*–level
Ft *L04–4*	1.424	0.263	Y *L04–4*	1.365	0.283
Ft *Laukiai*	1.356	0.287	Y *Laukiai*	0.664	0.655
Ft *Vidmantai*	1.111	0.396	Y *Vidmantai*	2.484	0.070
Ft *Melsviai*	1.123	0.390	Y *Melsviai*	1.437	0.259
Fm *L04–4*	1.241	0.331	ERT *L04–4*	1.365	0.283
Fm *Laukiai*	1.001	0.445	ERT *Laukiai*	0.664	0.655
Fm *Vidmantai*	1.722	0.181	ERT *Vidmantai*	2.484	0.070
Fm *Melsviai*	1.497	0.240	ERT *Melsviai*	1.437	0.259
ANOVA: Two Factors–Degrees of freedom for all *F*'s: 23,72					
Variable	Box M	*P*–level	Variable	Box M	*P*–level
Ft	38.955	0.059	Y	32.541	0.188
Fm	24.980	0.515	ETR	32.541	0.188

The results of computing confirmed that the probability of the type I error *P* was bigger than the selected acceptable in a practice level of significance $\alpha = 0.05$, therefore there was no hypothesis H0 about the equality of variable variance that could not be rejected. Consequently, factual values of *F* test and the probability of the type I error were equable, using the same constant coefficients calculation of Y and ETR.

According to the multivariate test of the homogeneity of variance and covariance for multiple dependent variables and two–way variance analysis, *F*–ratio at probability $P = 0.95$ confirms the total of investigated factors of fluorescence (Ft, Fm, Y and ETR) depending on cultivar and leaf rank (Table 3). The calculated *F*–ratio values (for Ft – $F = 57.131$; $F = 474.316$; $F = 25.244$; for Fm – $F = 105.227$; $F = 1350.154$; $F = 46.453$; for Y and ETR – $F = 3.859$; $F = 158.469$; $F = 10.978$; $P < 0.0000$) are bigger than *F*–ratio values with 3 and 72, 5 and 72, 15 and 72 freedom degrees at $\alpha = 0.05$ level of critical value and by 95% guarantee that both factors (a cultivar and a leaf rank) and their interaction have statistically significant influence on measured PS II fluorescence indices Ft, Fm, Y and ETR (Table 3).

Table 3. Statistics of *ANOVA*: two–factors dependence of fluorescence parameters (Ft, Fm, Y and ETR) on a cultivar and a leaf rank.

Effect	*df* Effect	*F*	*P*–level	Effect	*df* Effect	*F*	*P*–level
	Ft (*df* Error–72)				Fm (*df* Error–72)		
Cultivar	3	57.131	5.23E–19	Cultivar	3	105.227	2.96E–26
Rank	5	474.316	0.00E+00	Rank	5	1350.154	0.00E+00
Interaction	15	25.244	9.31E–23	Interaction	15	46.453	0.00E+00
	Y (*df* Error–72)				ETR (*df* Error–72)		
Cultivar	3	3.859	1.28E–02	Cultivator	3	3.859	1.28E–02
Rank	5	158.469	0.00E+00	Rank	5	158.469	0.00E+00
Interaction	15	10.978	3.36E–13	Interaction	15	10.978	3.36E–13

Table 4. Cultivars, leaf ranks and their interaction on Fm, Y and ETR.

Ft					
Rank	Cultivar F (15.72) = 25.244; $P < 0.000$				Average F (5.71) = 474.316; $P < 0.000$
	L04–4	*Laukiai*	*Vidmantai*	*Melsviai*	
1	0.349	0.342	0.367	0.365	0.356
2	0.516	0.417	0.476	0.494	0.476
3	0.570	0.395	0.563	0.546	0.518
4	0.365	0.386	0.443	0.410	0.401
5	0.346	0.315	0.368	0.315	0.336
6	0.288	0.317	0.307	0.234	0.286
Average F (3.72) = 57.131; $P < 0.000$	0.405	0.362	0.421	0.394	0.396

Fm					
Rank	Cultivar F(15.72) = 46.453; $P < 0.000$				Average F (5.71) = 1350.154; $P < 0.000$
	L04–4	*Laukiai*	*Vidmantai*	*Melsviai*	
1	0.470	0.459	0.481	0.492	0.475
2	0.760	0.634	0.831	0.743	0.742
3	0.859	0.613	0.927	0.845	0.811
4	0.533	0.546	0.604	0.588	0.568
5	0.489	0.423	0.461	0.416	0.448
6	0.356	0.417	0.373	0.269	0.354
Average F (3.72) = 105.227; $P < 0.000$	0.578	0.515	0.613	0.559	0.566

Y					
Rank	Cultivar F(15.72) = 10.978; $P < 0.000$				Average F (5.72) = 158.469; $P < 0.000$
	L04–4	*Laukiai*	*Vidmantai*	*Melsviai*	
1	0.258	0.256	0.235	0.258	0.252
2	0.321	0.342	0.427	0.334	0.356
3	0.336	0.355	0.393	0.353	0.359
4	0.317	0.294	0.267	0.303	0.295
5	0.293	0.257	0.203	0.242	0.249
6	0.192	0.240	0.174	0.132	0.185
Average F (3.72) = 3.859; $P < 0.000$	0.286	0.290	0.283	0.270	0.283

ETR					
Rank	Cultivar F(15.72) = 10.978; $P < 0.000$				Average F (5.72) = 158.469; $P < 0.000$
	L04–4	*Laukiai*	*Vidmantai*	*Melsviai*	
1	93.2	92.6	85.0	93.1	91.0
2	115.8	123.4	154.1	120.8	128.5
3	121.5	128.2	141.9	127.6	129.8
4	114.6	106.0	96.4	109.5	106.6
5	105.8	92.7	73.2	87.4	89.8
6	69.5	86.8	63.0	47.7	66.7
Average F (3.72) = 3.859; $P < 0.000$	103.4	104.9	102.3	97.7	102.1

According to the applied Fisher LSD and Tukey HSD tests statistically significant differences ($P < \alpha$) were found (Table 4).

The least Ft mean value among the youngest leaves (the 1st leaf rank) was 0.356 (0.342 of Laukiai). The Ft tended to increase with leaf age and reached the peak at the 3rd leaf rank–0.518 mean value (0.570 of L04–4). Subsequent increasing of leaf age determined the decreasing of Ft mean value from 0.401 (4th leaf rank) to 0.286 (the 6th leaf rank).

According to the Fisher LSD test all the means of Ft of investigated cultivars indicate statistically significant differences, but the Tukey HSD test did not determine the statistically significant Ft difference between L04–4 and Melsviai ($P = 0.07969$).
The maximum Fm value – 0.927 is determined in the 3rd leaf rank of Vidmantai as well as the peak Fm mean value (0.811) between cultivars. The Fm value increases with age from the 1st (0.459–0.492) up to the 3rd (0.613–0.927) leaf rank due to the cultivar characteristic size of the forming leaflet. The Fm value of the 1st leaves rank was less than that of the 4th (0.568 mean value) and lower (0.448 and 0.354) ranks. Fm value decreases with a leaf's age starting from the 4th leaf rank. The least Fm value (mean value 0.354) is determined in the 6th leaf rank (0.269–0.417).

The most liberal posterior distribution Fisher LSD and conservative Tukey HSD tests have confirmed statistically the significance of the influence of the examined cultivars on all Fm means. Statistically significant influence of leaf ranks on Ft and Fm mean values confirm the tests applied to both.

Y mean value determines the smaller of the young, not-fully developed leaves of the 1st rank and the old leaves of the 5th– and 6th ranks in comparison with the middle rank (the 2nd–the 4th) leaves. Y mean value of this fluorescence factor of the youngest, 1st rank leaves (0.252) was higher than that of the 5th leaf rank (0.249) of tested cultivars. The peak Y value (0.342 and 0.427) was found in leaves of the 2nd rank of Laukiai and Vidmantai and of the 3rd rank (0.355 and 0.393) of Laukiai and Vidmantai. The least mean value (0.185) was determined in the 6th leaf rank.

ETR variation tends to follow the same indices as the other fluorescence indices: Ft, Fm and Y. The maximum values of ETR occurred in 115.8–154.1 or 128.5–129.8 mean value of the 2nd–the 3rd leaf ranks. The minimal ETR value 47.7; 63.0 and 69.5 determined of the 6th leaf rank of Melsviai, Vidmantai and L04–4 respectively.

According to the Fisher test only two statistically not significant Y and ETR differences were determined among the1st and the 5th ($P = 0.66472$), the 2nd and the 3rd ($P = 0.64863$) ranks. The Tukey HSD test confirmed these differences in Y and ETR with a probability $P = 0.99800$ and $P = 0.99743$ respectively.

The significance of Y and ETR varies between cultivars. According to the Tukey HSD test statistically significant differences of the Y and ETR means were determined between Laukiai and Melsviai ($P = 0.01011$), but after the Fisher LSD test statistically significant differences have been determined between Melsviai and the other cultivars: L04–4 ($P = 0.01319$), Vidmantai ($P = 0.04364$) and Laukiai ($P = 0.00189$).

The applied regression analysis of the quantitative parameters of fluorescence of different cultivars is specified as strong and statistically significant ($P = 0.95$) parabolic curvature dependence ($0.78145 \leq \eta \leq 0.97385$). The second–degree parabola regression model has been chosen ($\bar{y}_x = b_0 + b_1 x + b_2 x^2$) to describe the estimated function between a leaf rank and Ft, Fm, Y and ETR (Table 5).

Table 5. Statistics of regression analysis of leaf rank dependence on cultivars fluorescence parameters.

Cultivar	Indicators	b_0	b_1	b_2	Variance explained %
		Ft			
L04–4	Estimate	0.27136	0.14795	–0.02530	61.1
	t test (21)	4.42075	3.68400	–4.50520	
	P–level	2.38E–04	1.38E–03	1.94E–04	
Laukiai	Estimate	0.31184	0.05806	–0.01010	60.3
	t test (21)	12.11386	3.44753	–4.28758	
	P–level	6.12E–11	2.41E–03	3.27E–04	
Vidmantai	Estimate	0.24609	0.16541	–0.02666	80.1
	t test (21)	6.81309	6.99961	–8.06709	
	P–level	9.76E–07	6.54E–07	7.20E–08	
Melsviai	Estimate	0.25400	0.16693	–0.02929	86.0
	t test (21)	6.73579	6.76645	–8.48861	
	P–level	1.15E–06	1.08E–06	3.14E–08	
		Fm			
L04–4	Estimate	0.30112	0.28678	–0.04793	69.2
	t test (21)	3.23646	4.71135	–5.63095	
	P–level	3.95E–03	1.19E–04	1.37E–05	
Laukiai	Estimate	0.38727	0.13831	–0.02347	67.2
	t test (21)	7.89122	4.30774	–5.22683	
	P–level	1.02E–07	3.11E–04	3.52E–05	
Vidmantai	Estimate	0.28430	0.33774	–0.05629	70.7
	t test (21)	2.71311	4.92651	–5.87100	
	P–level	1.30E–02	7.13E–05	7.92E–06	
Melsviai	Estimate	0.27980	0.31858	–0.05512	84.9
	t test (21)	3.87570	6.74524	–8.34457	
	P–level	8.74E–04	1.13E–06	4.16E–08	
		Y and ETR			
L04–4	Estimate	0.16668	0.10967	–0.01742	82.4
	t test (21)	60.21331	39.61687	–6.29472	
	P–level	7.81776	7.86215	–8.93284	
Laukiai	Estimate	1.19E–07	1.09E–07	1.34E–08	78.0
	t test (21)	0.18044	0.09640	–0.01497	
	P–level	65.18364	34.82405	–5.40716	
Vidmantai	Estimate	8.89231	7.26150	–8.06247	62.8
	t test (21)	1.45E–08	3.75E–07	7.27E–08	
	P–level	0.21640	0.10429	–0.01930	
Melsviai	Estimate	78.17551	37.67343	–6.97159	94.8
	t test (21)	4.11947	3.03443	–4.01537	
	P–level	4.88E–04	6.30E–03	6.26E–04	
	Estimate	0.15704	0.12941	–0.02239	
	t test (21)	56.72919	46.74993	–8.08840	
	P–level	9.67437	12.18621	–15.07656	
	Estimate	3.45E–09	5.48E–11	9.75E–13	

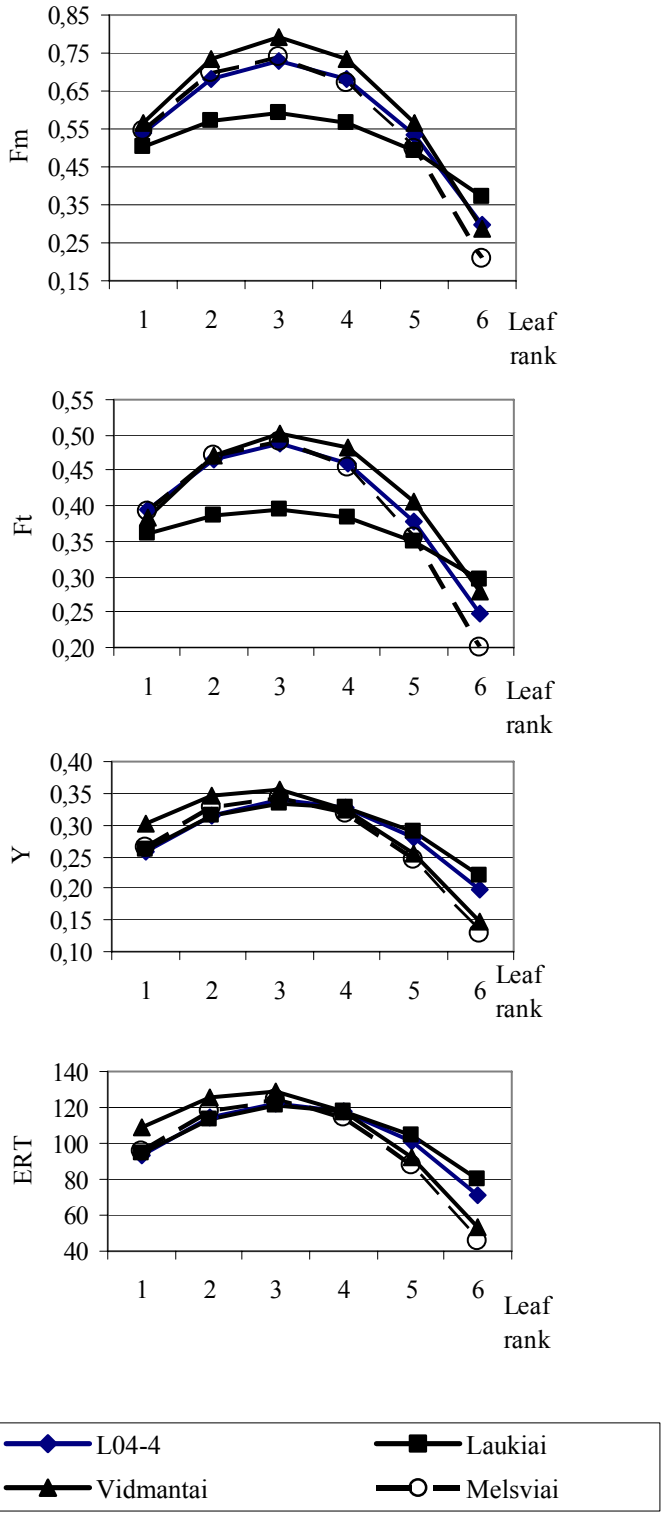

Fig. 1. Ft, Fm, Y and ETR dependence on leaf rank (2005 02 07).

The leaf rank predetermined Ft by 60.3–86.0%, Fm by 67.2–84.9% and Y and ETR–by 62.5–94.8%.

STATISTICA module of Nonlinear estimation /User specified regression has been applied to compute the selected statistical characteristics. The point estimation of the chosen module parameters b0, b1, b2 is being computed by the means of Quasi–Newton method, using a loss function: L = (OBS–PRED) **2).

The intensity of leaf rank influence on fluorescence indices of the investigated cultivars varied. The greatest influence of a leaf rank on Ft, Fm, Y and ETR was found in Melsviai. The least influence of leaf rank on Ft and Fm was recorded in Laukiai; on Y and ETR, in Vidmantai. The obtained dependencies can be statistically significant (Student test t and $P < 0.05$) as described by the second–degree parabola equations (Fig. 1).

Accordingly in these equations, the values of dependent variables – Ft, Fm, Y and ETR expand with the increasing of the independent variable–a leaf rank up to the 3rd. The older leaves (4th–6th rank) have begun to wither, necrotic lesions have emerged, metabolic and PAM fluorescence processes are being reduced. Therefore the lower leaf ranks (4th–6th) started to become a negative factor and values of PAM fluorescence indices decreased.

CONCLUSIONS

The cultivars examined have different agro-biological potential. The highest yield of GM (60.4 t ha^{-1}) and DM (13.9 and 13.2 t ha^{-1}) was determined in Melsviai and Laukiai, seed yield (18.0 g m^{-2}) – of Laukiai. The significant variation between cultivars and leaf ranks was observed for chlorophyll fluorescence parameters *in situ*. Steady–state (Ft) and maximum (Fm) fluorescence yield, quantum yield (Y) and electron transport rate (ETR) analysis indicate that their mean values significantly ($P = 0.95$) increased with an increasing leaf age up to the 3rd leaf rank and began to decrease from the 4th leaf rank according to the second degree parabolic curvature dependence ($0.78145 \leq \eta \leq 0.97385$).

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