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Simultaneous responses of photosystem II and soluble proteins of rapeseed to cold acclimation

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Abstract: This experiment was conducted to assess the quantitative and qualitative changes in soluble proteins as well as some chlorophyll fluorescence parameters in the leaves of a winter canola (*Brassica napus* L., cv. Licord) under continuous low temperature. Over the experiment, seedlings were initially grown at 15/10 °C (d/n). At fourth fully expanded leafy stage (day 30), a part of the plants were transferred to 4/2°C for 4 weeks. Plants were sampled for protein extraction from leaves in which chlorophyll fluorescence parameters (F_o, F_v, F_m, F_v/F_o, F_m/F_o, F_v/F_m, F_o', F_V', F_m' and some other calculated) were also measured. The results showed a clear increase in soluble proteins quantity caused by cold treatment. The enhancements appeared abruptly following the cold exposure to 4°C and lasted. The electrophoretic protein patterns showed changes in the intensity of some polypeptides, besides, induction a new probable protein weighing 47-kW in response to cold treatment. Cold-triggered reduction in maximum quantum yield of PSII (F_v/F_m) was connected especially with drastic decreasing F_v and F_m. Interestingly, high quantitative amounts of soluble proteins along with induction of the new probable polypeptide induced at cold temperature, were attributed to low deduction of maximum quantum yield of PSII. Additionally, more imperative chlorophyll fluorescence parameters changed e.g. qP, NPQ, qL, Y(II) or ϕ_{PSII} etc at light. Nowadays, radar charts or spider plots are the most sophisticated multivariate statistical tools representing physiological responses of plants to abiotic stress conditions or even morphophysiological studies of plants. In rapeseed many researches performed by applying the radar charts for low temperature stresses and interpreted their effects more advancely than common statistical tools. We observed a good representation of the chl fluorescence parameters fluctuations using radar plots. Overall, cold-induced soluble proteins accumulated after longer cold-acclimation, can contribute in photosynthetic apparatus protection against low-temperature damages.

Key words: Canola; Chlorophyll fluorescence; Cold acclimation; Protein.

Introduction

Oil seeds are the second global food resources among which *Brassica napus* L. is the third annual oil seed in the world (1-4). Oilseed rapes are the world's third most important source of vegetable oils after palm and soybean (5-9). Low temperatures are important environmental factors limiting plant distribution, survival, and crop yields worldwide. Frost resistance is seemingly the main factor influencing winter survival of winter cereals and also winter oilseed rape (10). It is concluded that exposing the plants to low, nonfreezing temperatures; called cold acclimation, could increase the tolerance of temperate plant species to freezing (11-13). In other words, development and the degree of hardening achieved by rapeseed plants, are essentially key factors in their ability of tolerate very low temperatures. Nonetheless, the unhardened rapeseed plants could survive at -4°C temperature, while a fully-hardened spring-type ones is able to survive much lower temperatures (-10° to -12°C). The hardened winter rapeseed can survive short periods of exposure to temperatures between 15° and -20°C.

Many biophysical, biochemical and molecular changes occur during the cold acclimation process, which enable the survival of plants at below freezing temperatures (14). A strong correlation between cold acclimation and freezing/frost tolerance in both winter and spring types rapes has been noticed (15, 16). Where, it is suggested that acclimation of two-week old rapeseed seedlings for 7 days at 4 °C followed by frost treatment at −4 °C for 16 h, is preferred for assessment of frost tolerance of rapeseed seedlings (15). Cold acclimation of plants also infer higher growth capacity and better freezing (frost) and high-light tolerance and higher potential for production of forage mass after freezing (17, 18). Speculating the mechanisms underlying cold acclimation, could lead to selection strategies needed for germplasm enhancement for cold tolerance (19). Cold acclimation is a complex process that involves changes in lipid membrane composition, increased total soluble proteins and soluble sugars, amino acids, and organic acids, accumulation of osmolytes and protective proteins, increased concentration of other cryoprotectants such as sugars and proline (13, 14, 20), active oxygen species detoxification, several genes induction (21-23). Obviously, exposure to low, nonfreezing temperatures dramatically induces genetic, morphological, and physiological and cellular membranes changes in plants, which result in the development of cold hardiness and the acquisition of freezing tolerance (24-28). Incidences of early or late frosts during the growing

season have serious detrimental effects on canola yield and seed quality (23).

Numerous physiological changes in the leaves of plant occur in response to low temperatures (14, 29-32). Changes in protein phosphorylation have been observed within minutes (33). Altered gene expression, including changes in both mRNA accumulation, alteration of lipid composition in plasma membranes and leaf lipid compositions, possible increase the ratio of lipids to proteins in membranes, accumulation of protecting compounds, such as carbohydrates, free amino acid or other osmolytes and induction of new gene activity (14, 29, 31, 34), enzyme activity (30) and cold-regulated (COR), dehydration-responsive, and ice recrystallization inhibition (IRI) genes, and down-regulation of photosynthesis and respiration-related genes (19), was observed within a few hours (35). Moreover, biochemical and molecular analyses have demonstrated differential gene expression and the accumulation of specific proteins during the induction of freezing tolerance (29, 30, 36, 37). A number of plant proteins encoded in response to low-temperature have been reported in *B. napus* (14, 38, 39).

Rubisco (ribulose-1, 5-bisphosphate (RuBP) carboxylase/oxygenase) enzyme plays a pivotal role in CO_2 assimilation via carboxylase activity in all photosynthetic organisms. Rubisco, the major photosynthetic enzyme, is sensitive to the variation in environmental temperature (40, 41); small subunit of Rubisco (SSU) is suppressed more relative to large subunit of Rubisco (LSU) under low temperature stress. Similarly, low-temperature treatment involves the suppression of SSU synthesis in etiolated *B. napus* seedlings (42), and suppression of both SSU and LSU; SSU being more sensitive in non-tolerant rice (43, 44). Although less studies have been made on Rubisco modifications, but its substantial role e.g. through "introduction of 'improved' Rubisco into transgenic plants" or "Rubisco protein engineering" should be improved as described earlier (45).

Chlorophyll fluorescence (CF) is a very sensitive mean, allowing the sensation of changes in general bioenergetic status of plants' photosynthetic apparatus (46, 47). It provides a rapid, efficient, easy and non-invasive tool for detecting functional changes of photosynthetic apparatus under abiotic or biotic stresses (48, 49). In fact, there are complexes for chlorophyll proteins included in thylakoid membrane, by which, fluctuations of chlorophyll fluorescence could be sensitive indicators to monitor the membrane fluidity changes, stability and organization which are induced by stresses like heating (50) and chilling or freezing. Mishra *et al*. (51) showed and stated the potential of - chlorophyll (chl) fluorescence emission, to reveal and screen plant [cold] tolerance of cold acclimated plants when examining *Arabidopsis thaliana* accessions. There have been many fluorescence parameters defined in the literature (52). Inevitably, low temperatures decrease dark-or light adapted chlorophyll fluorescence-related parameters. The techniques based on measurement of chlorophyll fluorescence induction, belong recently to fundamentals of plant stress research (53). Low temperature can causes the reduction of quantum yield of photosystem II (PSII) ($F_v/_{Fm}$: maximum photochemical quantum efficiency (yield) of PSII photochemistry with all reaction centers open) in many plants, e.g. *Hordeum vulgare* (18), *A. thaliana* (54), *Lolium multiflorum* (55), *Avena sativa* (56), and *B. napus* (10, 16, 23, 52, 57), which could be widely used along with maximal variable fluorescence level of dark-adapted ($F_{v=}F_{m-}F_o$) and maximum fluorescence of dark-adpated ($F_{m=}$ABS/CSM) as reliable characteristics for evaluating and screening of the genotypic differences by environmental stresses like cold and chilling stress tolerance. As reported, decrease in some chlorophyll fluorescence parameters and especially F_v/F_m was parallel with the increasing amounts of the cold-induced polypeptides (58). Various studies indicated that photosynthetic apparatus (chloroplasts) as well as mitochondria are the primary sites of unfavorable effects of low temperature. Under low temperatures, cold-induced-proteins which directly or indirectly (by modifications of carbohydrates and lipids metabolism) protect chloroplasts and mitochondria from low-temperature damages (59, 60). Typically, commercial instrumentation for measuring variable chlorophyll "a" fluorescence follows one of the following two approaches (61):

A. continuous excitation systems: these fluorimeters, are ones planned to measure the Kautsky induction or fast fluorescence induction (62). For this, measurement of chlorophyll fluorescence is performed after sample dark adaptation usually by covering samples via leaf clips for 15-30 minutes. Usually, the instruments used to measure this chlorophyll fluorescence are called "plant stress meters" and are typically portable, light and programmable to analyze measured signals (63), which provides an affordable & convenient method of rapidly screening samples using 1 second measurements of the fast chlorophyll fluorescence kinetics. Most of the measured and calculated fluorescence parameters are as follow: F_o, F_m, F_s, F_o', maximum fluorescence from light-adapted leaf, PSII centers closed ($F_{m'}$), F_v/F_o, F_v/F_m, time (in ms) to reach maximal fluorescence F_m (t_{Fm}), area, $F_{v'}/F_{m'}$, coefficient of photochemical fluorescence quenching, ranging from zero (upon application of a saturation pulse) to 1 (qP), coefficient of non-photochemical quenching of variable fluorescence, ranging from 0 (in the dark-acclimated state) to 1 or $1-[F_m-F_o']/[F_m-F_o]$ so-called qN, non-photochemical quenching coefficient or $[F_m-F_m']/[F_m-F_o]$ so-called qNP, non-photochemical fluorescence quenching or $(F_m-F_m')/F_m'$) so-called NPQ, coefficient of photochemical quenching, based on the lake model (connected PS II units) or $qP*F_o'/F_t$ so-called qL, quantum yield of non-regulated energy dissipation in PSII or $1/(1+NPQ+qL(F_m/F_o-1))$ so-called Φ_{NO}, quantum yield of regulated energy dissipation in PSII or $1-\phi_{PSII}-\phi_{No}$ so-called Φ_{NPQ}-K, Φ_{fD} and Φ_{NPQ-G} (63) at 50 µs (O step), 100 µs, 300 µs (K step), 2 ms (J step), 30 ms (I step) and maximal fluorescence intensity, $F_P = F_m$ (at time denoted as t_{Fm}) (64, 65), raising to a curve called OJIP curve. Derived chlorophyll fluorescence parameters e.g. those derived from JIP-test are categorized as follow modified after Strasser *et al*. (65): 1) extracted parameters from the recorded fluorescence transient OJIP like: continuously recorded fluorescence or $F_m'-qP(F_m'-F_O')$ so-called F_t, F_{50}µs or F_{20}µs, t_{Fm}, F_P and area…. 2) fluorescence parameters derived from the extracted data like: F_o nearly equals F_{50}µs or F_{20}µs, F_m, F_V, relative variable fluorescence at time *t* or V_t … 3) specific energy fluxes like: φ_{PO}=TRo/ABS=F_v/F_m, φ_{RO} …

4) phenomenological fluxes like: ABS/CS=F_o, F_m=ABS/CSM, JIP-test parameter: trapped energy flux per cross section (at *t*=0). A phenomenological flux or activity orF_v/F_m*F_o or =(F_v/F_m)F_o so-called TRo/CS=φ_{PO}.(ABS/CS) … 5) performance indexes like: PI_{total}, PI_{ABS}.

B. *pulse modulated* systems or saturation pulse methods: It enables the so-called quenching analysis using modulated fluorescence and saturation pulses. In this type of measurement, measuring light is switched on and off (modulated pulse) at high frequency. Basic chlorophyll fluorescence parameters are derived from this fluorescence kinetics. The most known instruments applying to measure and analyze this kind of measurements are e.g. PAM2000/2001, PAM2500 (Walz Heinz GmbH, Effeltrich, Germany). When samples are dark-acclimated, four characteristic levels related to fluorescence yield are recognized in a plot with logarithmic time scale: F_o, F_K, F_J, F_I, and F_m called OKJIP or OJIP curve, where O term stands for $F_{50\mu s}$, K term for $F_{300\mu s}$, J for F_{2ms}, I for F_{30ms} and P for M or maximal fluorescence (F_m). The OJIP approach or JIP-test can be applied to monitor the effect of various abiotic and biotic stresses and photosynthetic mutations affecting the structure and function of the photosynthetic apparatus (65). JIP-test analysis is a very powerful tool in basic and applied studies, because it is very rapid (hundred samples may be analyzed in a working day), and inexpensive, and provides a large quantity of information. Rapacz *et al*. (66), stated the OJIP test as a reliable indicator of winter hardiness and freezing tolerance even under fluctuating winter environments.

Spider plot or radar chart presentation are designed to plot the relative values (relative to the corresponding value of the control, which thus become equal to unity) of selected expressions, such as the specific fluxes. As a multiparametric description of structure and function of each photosynthetic sample, presented by an octagonal line, provides a direct visualization of the behavior of a sample facilitating the comparison of plant material as well as the classification of the effect of different environmental stressors on it in terms of the modifications it undergoes to adapt to new conditions (65). The spider plot easily identifies deviation (in positive or negative way) from a typical shape, and represents some kind of 'fingerprint' of each stress or physiological status. It enables also to recognize the more sensitive or more resistant genotypes (53). Unfortunately we found not any research reported the effects of low temperatures on canola which has been examined using the radar plots for analyzing the chlorophyll fluorescence parameters, but for other abiotic stresses on canola or low temperatures in other plants, the reports have been made. In this case, the role of radar plots or spider charts has been applied to survey the effect of salt stress treatments on rapeseed (67) , the responses of 8 canola cultivars for physiological (chlorophyll fluorescence) and productivity traits under normal conditions (68), the effect of flooding stress in the greenhouse in some rapeseed cultivars for JIP-test parameters (69).

The aim of the present study was to examine the effect of low-temperature treatment (4ºC) on the changes of leaf soluble polypeptides, chlorophyll fluorescence parameters viz, F_o, F_v, F_m, F_v/F_m, F_v/F_o, ratio of F_m by F_o or F_m/F_o and other more and their relationships in the winter rapeseed (*Brassica napus* Licord) cultivar.

Materials and Methods

Plant material and growth conditions

Seeds of winter *B. napus* cv. Licord (2n = 4x = 38), a France originated cold tolerant cultivar, were germinated in 14×13 (cm) plastic pots in a field-soil/sand/clay mixture (5:2:2, v/v) under controlled conditions at 15/10ºC (day/night) with a 16/8 (day/night) photoperiod and photosynthetic photon flux density (PPFD; Philips AGRO sodium light source) of about 170 µmol m^{-2} s^{-1}. At the fully expanded 4 leafy stage (plants with about 30 -day old), seedlings were divided into two groups. Control plants remained in the same conditions and plants for low-temperature treatment (cold acclimation) were transferred to 4/2ºC (d/n) with the same photoperiod and PPFD for 4 weeks at growth chamber. Intrinsically, the sampling times for low temperature treatment were after 30 (before transfer to 4°C), 37 (w 1), 44 (w 2), 51 (3rd w), 58 (4th w in cold) days. At the same sampling times and daytime, samples were also taken from the controls. Growth under low temperature acclimating conditions was slower; therefore, cold-acclimated plants reached the same developmental stage as non-acclimated plants at a different chronological age.

Chlorophyll fluorescence measurement

The chlorophyll fluorescence of leaves was measured (from day 30 namely a day before, up till day 58 namely 4 w of cold acclimatization) at the ambient temperature of plant growth using a portable chlorophyll fluorimeter (PAM2500, Walz Heinz GmbH, Effeltrich, Germany, 2008), as measured by researchers (53, 70-72) and described in details by Gray *et al*. (28) and Öquist and Wass (73), on the middle section of the youngest fully expanded leaves, using a saturated light pulse method (6000 μ molm^{-2}s^{-1}) for 0.8 s after a dark treatment (20 min) using dark clips, which is, as Brestic and Zivcak (53) emphasized, recently the most frequently used and generally accepted chlorophyll fluorescence technique. As pointed out by Brestic and Zivcak (53), while PEA plant efficient analyzer, Hansatech GB) device is used instead, the data are recorded with maximum efficiency 100 kHz, hence, the first point is $F_{10\mu s}$, but the reliable data starts from 30 µs. In this method, a leaf must be dark adapted for at least from 15 to 30 min prior to the measurement. Among the various parameters which were calculated according to OJIP-test, our focus was on the parameters that were significantly affected by cold stress. The basal or minimal fluorescence (F_o) in darkness (PSII centers open), is measured by a weak modulating light beam (ML). Then the application of a saturating pulse (SP) raises the fluorescence to a maximum value (F_m). Maximum quantum yield of PSII Photochemistry, F_v/F_m=1-(F_o/F_m), in some cases called also φ_{PO} is the most frequently used parameter, which is markedly reduced for stressed and/or damaged plants. The F_v denotes the variable fluorescence. Actinic light (red light) was then used to measure the steady-state chlorophyll fluorescence (F_s). The protocol namely simple dark-to-light slow fluorescence induction as Brestic and Zivcak (53) denoted, is usually realized with fixed time between flashes, usually about 5 min.

It is time sufficient for reaching initial steady state, but usually not for opening stomata in the light-adapted state. Then, F_m' was measured by applying a saturating pulse, while F_o' was measured by switching on the actinic light for 2 s after the saturating pulse and applying far-red light. CF parameters then defined, e.g. NPQ was defined as $F_m/F_m'-1$; actual quantum efficiency of PSII (PSII) was defined as $(F_m'-F_s)/F_m'$; photochemical quenching (qP) was defined as $1-(F_s-F_o')/(F_m'-F_o')$ (49, 74) and so on.

Protein extraction and determination

Samples were collected, leaves were cut, weighed, wrapped in aluminum foil and stored in liquid nitrogen until later use. Total soluble proteins were extracted from the leaves by a fresh buffer including: 50 mM Tris-HCl (pH 7.5), 0.04% 2-mercaptoethanol (v/v), 2 mM EDTA (3 µl per 1 mg leaf fresh weight) using ice-cold mortar and pestle. Then, the extract was centrifuged for 21 min at 11,000 rpm at 4ºC. The pellets discarded and the protein concentration was determined in the supernatant spectrometrically at 595 nm by means of dye-binding method (75).

Bioling solubility

In order to extract protein for electrophoresis, sample buffer contained 62.5 mM Tris-HCl (pH 6.8), 5% 2-mercaptoethanol (v/v) , 6% (w/v)SDS, 1% (v/v) glycerol , and 0.01% Bromophenol blue (w/v) was prepared. An aliqut of the supernatant (in a 1:5 ratio of supernatant to sample buffer) was subjected to 100 ºC heat treatment for 4 min. The boiled sample was centrifuged at 6000 rpm for 20 min to remove insoluble proteins.

Electrophoresis

After samples collection, equal amounts of total proteins (45 µg) were separated on a 15% SDS-PAGE gel. Gels were then stained overnight with Coomassie blue (0.1% Coomassie blue in 40% methanol and 10% acetic acid) and then gels were de-stained and scanned in the plant breeding and biotechnology laboratory, Shahrekord Universitt, Iran, during 2011 yr.

Data analysis

Experimental design was a factorial on the basis of completely randomized design (CRD) with 3 replications. Two temperature regimes (control 15/10ºC; cold 4/2ºC), and 5 sampling times (from day 30 to day 58 from cultivation) were evaluated. Statistical including ANOVA and post-ANOVA analyses, were conducted using the software STATISTIX (Analytical Software PO Box 12185, Tallahassee FL 32317, USA; courtesy of Gerard Nimis, analytical aoftware support) to determine the significance between temperature treatments, and further Fisher's LSD test at the 0.05 probability level was carried out to create homogeneity groups of the means ($P<0.05$). The multivariate radar chart approach were then applied to more sophistically reveal and interpret the changes occurred to physiological, chl fluorescence, parameters under cold.

Results and Discussion

Quantitative analysis of total soluble proteins

Low-temperature stress resulted in increasing total soluble proteins content in cold-treated winter canola seedlings (from 4.09 to 5.33 mg g^{-1} fresh weight or FW), whereas protein level was stable leading to a minor increase in control plants. The higher rate of the increase was observed during the end of the low-temperature regime (3– 4 w under cold). According to the ANOVA results, there were significant ($P<0.01$) differences between control and cold treatments of temperature, between weeks of cold exposure, and also for the interaction of both these variables (Table 1).

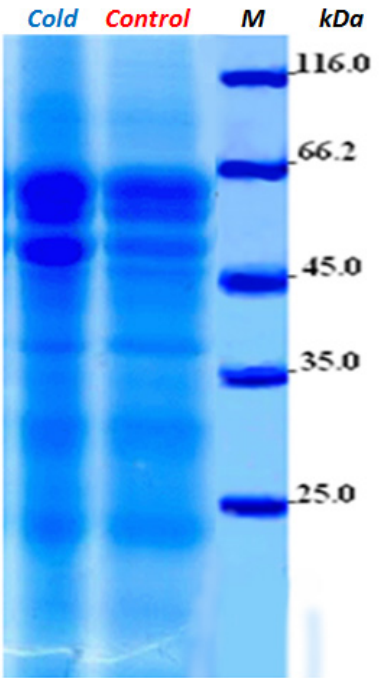

Figure 1. Cold-induction of a probable new polypeptide with 47 kDa MW on experimental day 44 (14 d at 4 ºC). *M* denotes the sized marker.

Table 1. mean squares for some of the important chlorophyll fluoresence parameters and protein amount in the experiemnt.

SV	df	F_v/F_m	F_v'/F_m'	F_v	F_v'	F_m	F_m' …	…qP	NPQ	TR_o/CS	Y(NO)	Y(NPQ)	Prot
Temp	1	425.63**	627.06**	3876.03**	2940.3	1280.53*	1526.53**	3845.72**	5596.72**	64.8458*	16.8827ns	1295.93**	4538.7**
Sampling times(D)	4	32.33**	86.36**	364.95**	447.88	23.2ns	502.87*	736.24**	3317.32**	25.4429ns	91.1521**	165.63**	2030.67**
Temp×D	4	23.47**	66.74**	474.28**	260.88	84.37ns	362.87*	488.46**	1789.65**	11.2891ns	72.0563*	236.01**	1892.7**
Error	20	4.93	1.01	26.67	43.3	206.73	124.6	13.17	116.61	13.9411	18.2749	13.49	54.2
CV(%)		2.98	1.66	3.5	10.83	7.38	11.56	10.7	10.4	10.07	11.27	9.06	1.72

ns ($P>0.05$), *($P<0.01$), **($P<0.01$); "Temp" denotes temperature treatment as control and cold. Days consisted of 0, 7, 14, 21and 28 (4 weeks). In this table, F_v' is the maximal variable fluorescence level from light-adapted leaves, namely $F_v'=F_m' - F_o'$ and Y(NO) is quantum yield of non-photochemical energy conversion in PSII other than that caused by down-regulation of the light-harvesting function or simply as F_t/F_m and also Y(NPQ): quantum yield of non-photochemical energy conversion in PSII due to down-regulation of the light-harvesting function; F_t/F_m'-F_t/F_m.

Electrophoretic banding patterns of leaf total soluble proteins

Low temperature was appeared to be associated with lower abundance of LSU intensity in seedlings of winter canola (Fig. 1) following 4 d exposure to cold temperature. At the 2nd w of cold treatment, the intensity of LSU and SSU bands were increased. Low-temperature caused remarkable changes in soluble protein patterns of the winter cultivar. At the 8th d (2-3 w) of cold acclimation, the accumulation of 14.1, 30.2 and 36.5 kDa polypeptides were increased compared to the control. Moreover, the presence of a probable new cold-induced band of 47 kDa at the 2nd w of cold exposure expressed, and lasted until the 4th w of cold treatment. When cold-treated plants were returned back to the control conditions, the intensity of the bands which has been increased under cold stress, decreased and returned back to its original intensity (data not shown). Overall, these results highlighted that, low-temperature changed the intensity of pre-existing bands by increasing, decreasing, disappearance some of pre-existing polypeptides and even induction of one or more new polypeptides.

In this context, Boothe *et al*. (76) reported that under a 4ºC cold regime, the quantity of BN28 polypeptide and relative transcripts increased, and a decline in protein levels observed soon after returning plants to control temperatures (22/14ºC), and little or no protein could be detected after 7 d of de-acclimation**.** Thus, as a result, the cold tolerant genotypes of canola have the capability to accumulate higher amounts of soluble proteins in agreement with electrophoresis results (as considered e.g. by Boothe *et al*., (76) ; Trischuk *et al*., (14)) when compared both with the control. The full investigation of almost all of the polypeptides affected by cold acclimation or low-temperatures stresses should be further studied via 2D electrophoreses as assessed in canola by Meza-Basso *et al*. (42), and Trischuk *et al*. (14) who pointed out that, these preliminary results cannot be simply interpreted as a difference in the rate of degradation of storage proteins, but rather suggest that some specific polypeptides might be preferentially synthesized at chilling temperature 4ºC, which should be assayed further via radioactive labeling and western blotting techniques as performed by Trischuk *et al*. (14). They reported that in winter wheat and both spring and winter canola, accumulation of a prominent 47-kDa dehydrin protein occurred in response to cold acclimation which decreased after 14 d to non-acclimated levels. In our experiments the probable new expressed 47-kD protein confirmed the results retained above. Dehydrins belong to LEA (late embryogenesis abundant) group of proteins which accumulate in plants in response to low temperature or other dehydrative cellular processes and have been found in a large variety of species (77).

The present study was conducted to study the effect of a moderate-duration low-temperature treatment (4°C) on the fluctuations and of 1D electrophoretic patterns of soluble proteins and most of the chlorophyll fluorescence parameters in winter canola (*B. napus* cv. *Licord*). The results demonstrated that during low-temperature treatment, leaf soluble proteins continued to be synthesized and accumulated, thereafter following removing the cold treatment, turned back to the original amount. This demonstrates mainly that changes in proteins (including Rubisco subunits) induction or lowering is in response to cold treatments. As shown, temperature regime, and different sampling times had significant effects on quantitative amounts of leaf soluble proteins. Generally, in the non-tolerant species like spring canola, synthesis of Rubisco massages and polypeptides are suppressed, with SSU further are suppressed than LSU.

At low temperatures, changes in soluble polypeptides like Rubisco subunits accumulation and activity were connected not only with stressful effects of low temperature but also with the photosynthetic acclimation to cold. As a result, at low temperatures the structural kinetic properties of this photosynthetic enzyme are altered, leading to its increased stability and activity at low temperature, where low temperatures leading to a higher Rubisco amounts than the higher temperatures. Electrophoresis studies showed that, on experimental day 30 (out of cold) some polypeptides weighing 13.5, 14 (SSU), 14.1, 30.2, 36.5, 55 (LSU) and 55.9 kDa were observed. Thus, the existence of such polypeptides during cold conditions could not inevitably be solely a reason for cold-induced tolerance, but their density increase could be remarkable, as they play a presumable role in plant growth and development. Researchers have shown that in canola cultivar, "*Jet neuf*", a 48 h exposuring to a zero temperaturecold, in the 2D electrophoresis pattern the concentrations of 14 polypeptides increased, of 7 polypeptides decreased (even invisible), the intensity (thickness) of Rubisco small subunit (SSU) declined (42). In this subject, it has been reported that LSU and SSU can function as instruments to studying photosynthesis apparatus response to low-temperatures, where they provide information about gene products and expression regulation in such cases. Similarly, low-temperature treatment has resulted in suppressed SSU synthesis in etiolated *B. napus* seedlings (42) and suppression of both SSU and LSU, with SSU being more sensitive in non-tolerant rice (44). Rubisco subunits, especially SSU, at the beginning of cold stress responded approximately earlier than other polypeptides, their concentrations decreased, and started again to increase10 d later. This relates to chloroplast response to low-temperatures. In the present study, overall increase in soluble proteins accumulation in leaves may be related to chloroplast response to low-temperatures, because at the time in which increased accumulation of soluble proteins was observed an ambient increase in F_m was also visible. Generally, chloroplasts and mitochondria are the organelles which are affected mainly by low-temperatures. According to Pessarakli (78), mitochondria have been recognized to be the main cellular compartment which are affected by chilling treatment. The similar results were obtained in the research of Routaboul *et al*. (54) on *Arabidopsis* which showed that at the beginning of cold treatment, SSU activity and concentration decreased which was followed by similar changes concerning LSU. Later on, the concentrations of leaf soluble proteins and the photosynthetic polypeptides, reincrease. This is associated with plant recovery in cold, which is connected with the action of cold-inducible genes (59, 60). Recently, Jurczyk *et al*. (79) examined the effects of pre-hardening (15 °C) and cold acclimation (4 °C) temperatures on some of the

photosynthetic traits such as Rubisco (Ribulose-1,5-bis-phosphate carboxylase/oxygenase) enzyme activity and the expression of two variants of its activase gene in *Lolium perenne*, and showed that led to enhancement in its activity under these low temperature regimes in freezing tolerant genotypes of *Lolium perenne*. Unusually, they pointed that Rubisco activity increased in the Lp5 freezing-tolerant *L. perenne* genotype during the low temperature treatment relative to the control. They showed that Rubisco activity was more in the experimental than in the control plants for all sampling times. As mentioned before, Rubisco modifications through Rubisco protein engineering could amend physiological processes in plants. Improving Rubisco performance should be done e.g. by improvement of the activity of its ancillary protein *i.e.* "Rubisco activase" enzyme, and modulation of the synthesis and degrading its inhibitory sugar phosphate ligand(s) (80).

Chlorophyll fluorescence results

In current research, cold acclimation temperature affected significantly the value of dark-or light adapted CF parameters viz, maximum quantum yield of PSII (F_v/F_m), maximum primary yield of photochemistry of PSII (F_v/F_o); variable (F_v), maximum (F_m) quantum yield of PSII and especially of trapped energy flux per cross section viz TR_0/CS and other CF parameters compared to the control (Fig. 2), confirming earlier reports (66, 67, 70, 81, 82). When plants were transferred back to the control circumstances, CF parameters and even protein density e.g. F_v/F_m recovered to their initial values. The observed changes in F_v/F_m were revealed in the first days of cold treatment as a result of increaseing in the initial fluorescence (F_o=ABS/CS), but decrease of variable (F_v) and maximal (F_m=ABS/CSm) chlorophyll fluorescence parameters, whereas the further increase of F_v/F_m resulted mainly from increasing F_m value which was statistically significant previously than the recovery of F_v/F_m. On the other hand, F_o values remained higher than the control since the end of the cold treatment (Fig. 2). The pair-wise association between chl parameters revealed some of the weak correlation coefficients, which were not significant (P>0.05) at control, were significantly higher and stronger under cold (data not shown), confirming earlier reports (83).

Generally and as anticipated, the chlorophyll fluorescence parameters: F_o, Y(NO), Y(NPQ), TR_o/CS, t_{Fm} (time needed to reach F_m), F_o/F_m, F_o'/F_m', 1-qP, NPQ, qN, qNP (non-photochemical quenching coefficient), dissipated energy flux per cross section (at t=0). A phe-

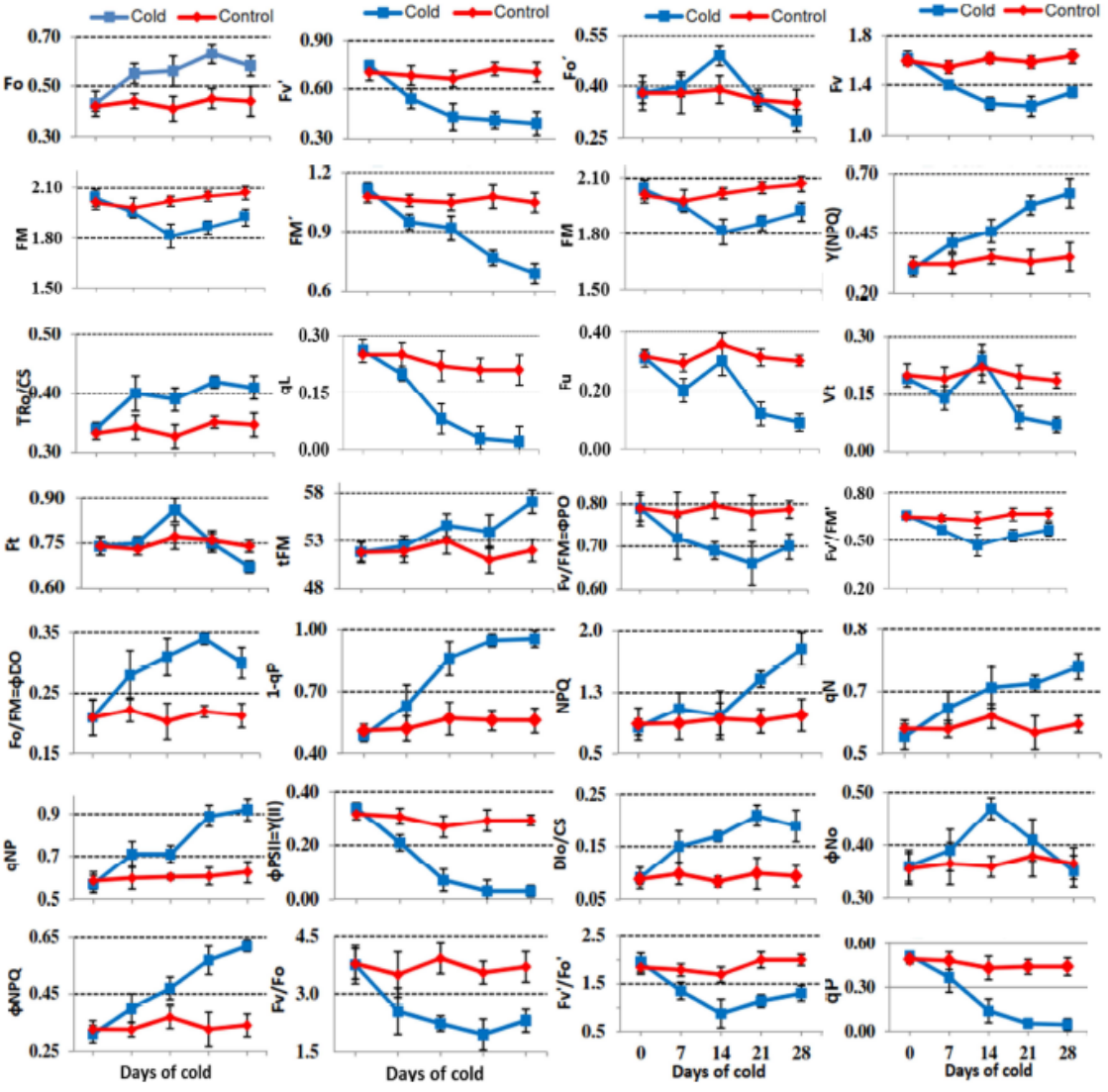

Figure 2. The changes of chlorophyll fluorescence parameters at dark-or light adapted conditions of Licord canola genotype in the cold (4/2 ±2) compared with control (15/10 ±2 d/n). It is also shown here, the changes of quantum yield of PSII (Fv/Fm) ratio in dark and light addaptation along with the changes of total soluble proteins (prot) of leaf under cold conditions.

nomenological flux or activity; F_o-TR0/CS so-called DI_o/CS and ϕ_{NPQ} (quantum yield of regulated energy dissipation in PSII) increased as compared to the control under cold acclimation treatment. In contrast, other CF parameters i.e.: F_o', F_v, F_v', F_m, F_m', coefficient of photochemical quenching based on the lake model with connected PSII units or simply as $qP*F_o'/F_t$ so-called qL, F_t (the continuously recorded fluorescence), Fu (variable fluorescence at time *t*), V_t (relative variable fluorescence at time t), F_v/F_m and F_v'/F_m' (maximum photochemical quantum efficiency (yield) of PSII photochemistry with all reaction centers open from dark/light-adapted leaves, respectively), qP (coefficient of photochemical fluorescence quenching), Φ_{PSII} (effective quantum yield (efficiency) of PSII photochemistry), Φ_{NO} (quantum yield of non-regulated energy dissipation in PSII), F_v/F_o (maximum primary yield of photochemistry of PSI), F_v'/F_o', F_v/F_m(=φ_{PO}=TRo/ABS =1-F_o/F_m) decreased compared with the control.

Measurements of chlorophyll fluorescence at low temperature reveals large effects, showing the initial reduction of many of CF parameters like F_m (=ABS/CSm) which reflects an increased density of inactive reaction centers in response to stresses indicating that active reaction centers could convert into inactive one, and make reduction of the efficiency of trapping and a decline in PSII activity (84), and also reducing maximum quantum yield of PS II (F_v/F_m or φ_{PO}) and other chlorophyll fluorescence-related parameters except for e.g. t_{Fm} and further increase in this parameter indicating photosynthetic acclimation to cold. As mentioned by Brestic and Zivcak (53), F_v/F_m or $\Phi_{PO,}$ is the most common frequently used parameter, applied often defined as the indicator of photo-inhibition or other kind of impairs caused to the complexes of PSII. This ratio is also in steady state for many different species of plants when measured under non-stressed conditions, equivalent to 0.832 (85). For stressed and/or damaged plants, F_v/F_m is markedly reduced. In other words, in healthy leaves, the F_v/F_m value is always close to 0.8, independent of the studied plant species. Lower amounts of F_v/F_m exhibits that a part of PSII reaction centers are impaired(injured), called photo- inhibition, which is frequently observed in plants under stress conditions. As Maxwell and Johnson (49) cited, changes in F_v/F_m and F_o are accepted and widely used as reliable diagnostic indicators of photoinhibition. Generally, cold acclimation of plants, increase the frost tolerance in leaves, and subsequently deducing the susceptibility to photosynthesis photoinhibition under chilling temperatures. Cold-acclimation of plants for long-terms, exhibits slightly decreased quantum yield and lowered F_v/F_m ratio. Hypothetically, this kind of mechanism prohibits destruction of the photosynthetic apparatus.

In this experiment, t_{Fm} (time to reach the maximum state of F_m) increased from 51.80 to 57.12 in 4 w of cold treatment compared to control. Accordingly, Kalaji *et al*. (86) reported that t_{Fm} or Tf(max) of barley genotypes of Syria grown 1 day under low light or PAR stress increased for cv. *A. Aswad* genotype to about 140% and decreased for cv. *A. Abiad* genotype to about 86% of control. It could be as a mean to show the sample stress which causes the reaching of F_m much earlier than expected, as mentioned by Kalaji and Guo (61). According to Brestic and Zivcak (53), t_{Fm} is an important factor which is the time when the maximum fluorescence is reached. As Kalaji *et al*. (86) pointed out, t_{Fm} could also help to identify early light stress effects on the photosynthetic machinery of barley [and certainly, other crop plants].

The decrease especially in F_v/F_m under cold stress is due to the effect of low hazardous temperatures on PSII and electron transport of photosynthesis. This is related to destabilization of thylakoid membrane which leads to reduction of electron transfer in PSII (87). In other words, the optimal (F_v/F_m), along with the effective ($\Delta F/F_m'$) quantum yield of PSII, reflect the number of active PSII reaction centers. The F_v/F_m has a widespread utilizations in order to detecting stress-induced perturbations in the photosynthetic apparatus, since declining the F_v/F_m can be a result of the development of processes called slowly relaxing quenching, and photodamage to PSII reaction centers, reducing the maximum quantum yield of PSII i.e. F_v/F_m (52, 88).

In this experiment, the decrease in F_v/F_m was accompanied by decreasing $F_{v,}$ F_m, trapped energy flux per cross section (TR_0/CS), F_m/F_o, φ_{Po} and more other CF parameters as mentioned earlier (Fig. 1), which confirms the results reported by Aroca *et al*. (89) and Rapacz *et al*. (57). Also the conclusion given there is applicable to the present results: the reason of the decrease in primary photosynthetic photochemistry in cold is probably connected with membrane destruction and destabilization under low temperature. The recovery effects on primary photochemistry observed in the present experiment was connected mainly with the increase in F_m. It is also suggested that cold tolerant barley genotypes altered photosynthetic capacity during cold acclimation (48).

Rapacz, individually collaboratively, made sequential studies on oilseed rapeseed plant (or even on wheat and barley crops) especially the study of low temperatures stress including freezing, chilling, cold acclimation and de-acclimation ranging from 1999 to 2016 years (10, 16, 57, 66, 90-92) performed studies on the effects of freezing and non-freezing (chilling and vernalization) temperatures on European winter rapeseed "*Brassica napus* var. oleifera cultivars during the Last 19 years. In an experiment, they reported a high association between vernalization requirement with frost resistance and field survival. Moreover, Rapacz *et al*. (57) in a study on rapeseed showed that during 10 weeks of low temperature, the content of soluble sugars increased permanently in the leaves of spring and winter rapeseed. The reported a sigmoid decrease and increase in chlorophyll fluorescence parameters i.e. F_m, F_o, F_v, F_v/F_m, qN and Φ_{PSII}, although, the increase of F_m, and decrease of qN, also occurred during 70 days of cold (+5/+2 ºC) days. In the case of chlorophyll a fluorescence, Rapacz *et al*. (18) showed that the amounts of F_o declined in winter barley during 14 d of cold acclimation at +2 ºC. In which, F_v/F_m or maximum photochemical quantum yield of PSII decreased from 0.82, at the beginning of cold (d zero) to 0.77 (2 w in cold). Moreover, qP decreased from 0.81 to 0.614 (in average) and NPQ from 0.89 to 1.03 increased.

In this research, cold acclimation caused increase/decrease of Φ_{NO} and increase of Φ_{NPQ} parameters, called

as quantum yield of regulated energy dissipation in PSII (Φ_{NPQ}), and quantum yield of non-regulated energy dissipation in PSII (Φ_{NO}) (Fig. 1) which suggested by Kramer *et al*. (93). It is assumed that $\Phi_{PSII} + \Phi_{NPQ} + \Phi_{NO} = 1$. While Φ_{NPQ} reflects the quantum yield for dissipation by down-regulation (active, organized process), FNO is the yield of other non-photochemical losses. The reduction of Φ_{NPQ}, Φ_{PSII}, NPQ, qN, qP and qL and increase of Φ_{NO} is occurred under different concentrations (40 to 160 µM) of Artemisinin as reported by Hussain and Reigosa (94) while studying the responses of Arabidopsis plant to Artemisinin-induced stress for 7 days concluding that Artemisinin has a phytotoxic impact including inhibition of non-photochemical fluorescence and heat energy dissipation of on excitation energy fluxes Φ_{NPQ}, NPQ and Φ_{NO}, though non-photochemical quenching (NPQ) is the most efficient photoprotective response in plants (94).

Photochemical quenching of variable chlorophyll fluorescence, qP or qL shows the photochemical capacity at actual mode of PSII in light-adapted state, which is connected with the photochemical energy conversion by charge separation in reaction centers of PSII. We obtained both declining so the decreasing qP ranging from 0.49 to 0.44 in control and from 0.51 to 0.05 in cold regime (Fig. 1). It quantifies the actual fraction of PSII reaction centers being in the open state, namely with reoxidised QA (93, 95). The qP, also makes translation of light quantum energy into chemical energy process, reflecting photosynthetic efficiency and the light use situation of plant (96, 97). As Mathobo *et al*. (97) referred, the decrease of qP may be influenced by increasing the proportion of closed PSII centers and also, the increase of qN may be influenced by major portion of absorbed light energy being used by plants through photosynthesis. In this subject de Souza *et al*. (81) reported a declining trend for qP and qL for maize plants under drought stress.

We here show that cold acclimation caused decline the other CF derived parameters such as effective photochemical quantum yield of PSII or F_v'/F_m*qP which equals $(F_m'-F_t)/F_m'$ or simply as Y(II) or Φ_{PSII}", other than that of qL and qP (and increase the trend of qN, NPQ and Y(NPQ) (Fig. 1) confirmed by de Souza *et al*. (81). The parameters; Y(II), qP and qL are associated with photochemical and qN, NPQ and Y(NPQ) with non-photochemical quenching) and Y(II) + Y(NPQ) + Y(NO) = 1. Here, cold acclimation changed qP and Φ_{PSII} or Y(II) declining gradually, and Y(NO) increased (from 0.36 to 0.48) and Y(NPQ) increased (from 0.30 to 0.62) respectively. The sharp declining of the photochemical quenching-related parameters [qL, qP and Y(II)], and proportional increase of non-photochemical quenching-related ones [qN, NPQ and Y(NPQ)], suggest that maize plants which submitted to drought stress, maintained their capacity to regulate dissipation of light energy, in spite of the stressful condition (81). Additionally, the efficiency of PSII (Φ_{PSII}) represents the proportion of electrons excited from the chlorophyll which are used during photochemical reduction of NADP+ (98). In this area, according to Perks *et al*. (99) the more rapid decline occurred in Φ_{PSII} parameter, reflects a decrease in net assimilation rate and variations in the proportion of open PSII reaction centers (qP) ra-

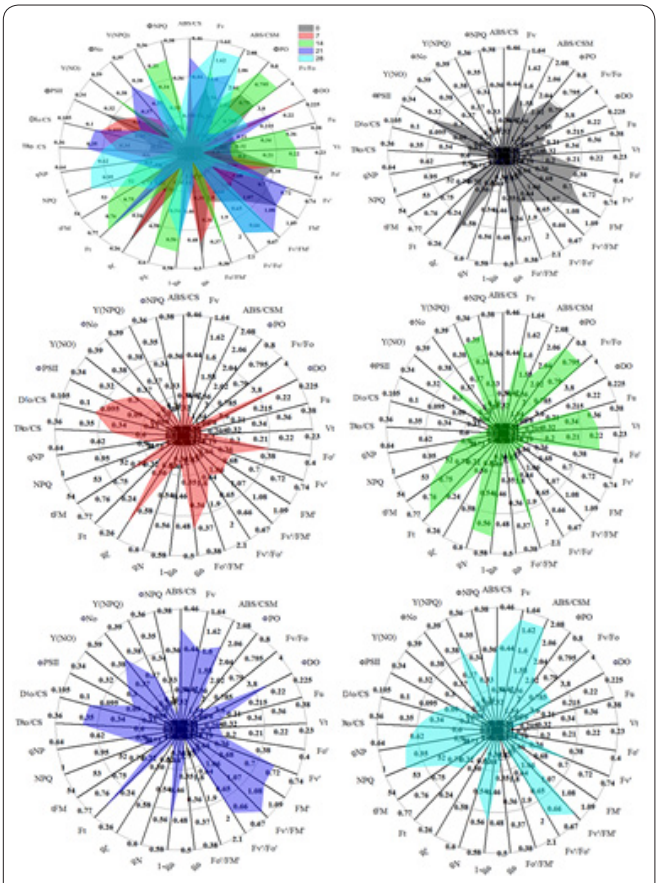

Figure 3. Radar/spider plot/charts indicating the changes in chl a fluorescence parameters (some of the OJIP test parameters) at control conditions till 4 weeks (0, 7, 14, 21, 28 days) after the onset of measurements.

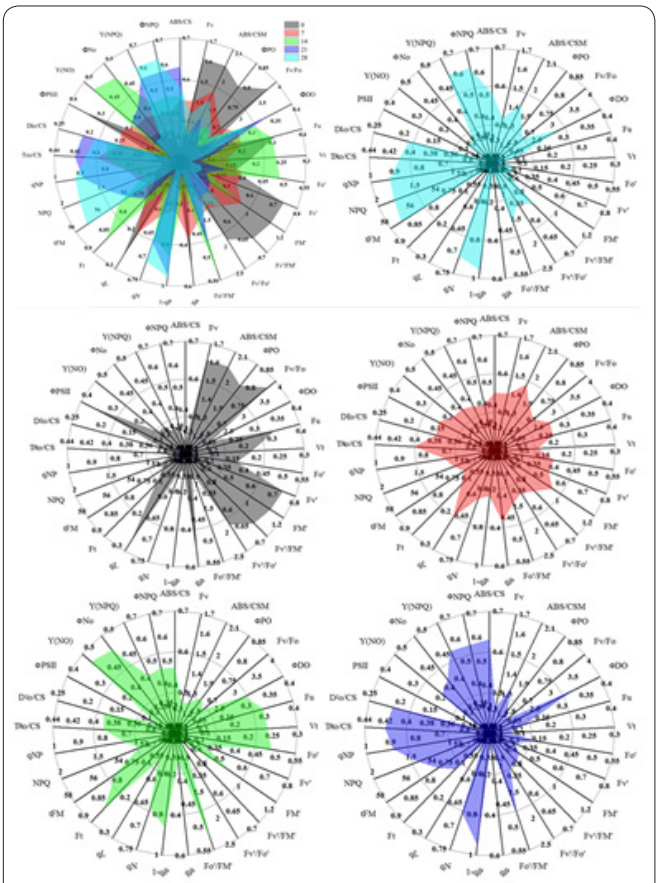

Figure 4. Radar/spider plot/chart displaying the changes in chl a fluorescence derived parameters (some of the OJIP test parameters) at cold acclimation conditions till 4 weeks (0, 7, 14, 21, 28 days) after the onset of measurements.

ther than a decline in the photochemical efficiency of open reaction centers under steady state, light adapted, where they suggest that fluctuations made to Φ_{PSII} parameter, could be useful as a predictor of plant vitality and post-planting establishment, especially for those subjected to cold storage.

Another ratio which should be noticed in stress studies is F_v/F_o or maximum primary yield of photochemistry of PSII, or the efficiency of electron donation to PSII reaction center and the rate of photosynthetic quantum conversion at PSII reaction center, and according to Li *et al*. (100) to provide an estimation of leaf photosynthetic capacity. In this experiment it started to decline a day before onset of cold (3.76) till 3th w of cold (1.94) and then increased at the last week of cold treatment (2.31) compared to the control (Fig. 1). Li *et al*. (100) reported a declining pattern of this parameter from 3.23 to 1.85 in drought affected plants compared to controls (3.76 to 3.96). A decrease in F_v/F_o, irrespective of whether it arises from F_v or F_o, is an indicator of structural alterations in PSII (101), and could be a tool to estimate leaf photosynthetic capacity. Moustakas *et al*. (102) while studying the effects of aluminum on the photosynthetic apparatus of different cereals concluded that, exposure of some of wheat and cereal cultivars to aluminum stress, caused injury to the thylakoid structure.

The fraction F_o/F_m or φ_{DO} (F_o'/F_m' at light) called quantum yield baseline, in this experiment increased from 0.21 to 0.34. It is pointed out that, the higher F_o/F_m value shows that the initial rate of reduction of the plastoquinone (Qa) is higher than the rate of plastoquinone reoxidation by b (Q_b) and the activity of photosystem I (PSI) when plants were exposed to higher [e.g.] concentrations of NaCl (98). Moreover, Rohácek (103) points to the increase relation F_o/F_m as stress indicative suggesting normal values as standard, observed between 0.14 and 0.20.

The component qN represents the coefficient of non-photochemical quenching, which increased from 0.54 to 0.71 under cold stress. In the literatures (104) pointed out that chl *a* fluorescence is influenced by other factors termed collectively as non-photochemical quenching (qN) which comprises multiple components, including e.g. redox-state of some components of the photosynthetic electron transport chain etc. As proved by Gray *et al*. (28), some chl fluorescence parameters such as: F_m, F_m', initial, basal or minimal fluorescence yield or $F_{50\mu s}$ and $F_{20\mu s}$ from dark-adapted leaves (PSII centers open), where F_o=ABS/CS=F_m-F_v or simply F_o, F_o', F_s, F_s', F_v, F_v', F_v/F_m, qN, qP, and 1-qP are useful for screening cold acclimation and freezing tolerance in winter rye. Additionally, Guo-li and Zhen-fei (105) and de Souza *et al*. (81) reported a declining quantum yield of photosynthesis (qP), and F_v/F_m maximal quantum yield of PSII and increasing qN at 5 days of chilling stress 8 °C compared to 28 °C.

TR_o/CS or trapped (or phenomenological) energy flux per cross section and DI_o/CS or dissipated energy flux per cross section are JIP-test parameters, which increased in this experiment in cold treatment advance (passing through time). They get changed under abiotic or biotic stresses, increase or decrease, related to the type of stress. In this context, Gururani *et al*. (106) pointed out that phenomenological energy flux above (TRo/CS) along with other two such parameters, was reduced in zoysiagrass lines when the plants were subjected to cold stress. Li *et al*. (71) studied these two JIP parameters under photoinhibition in wheat under the effects of cold on photoinhibition of wheat plants. They reported that, in parallel with decreasing photosynthesis efficiency, the energy trapping flux and some other photosynthesis characteristics also repressed in response to photoinhibition. In their experiment, TR_o/CS decreased and DI_o/CS increased along with elevating high-light treatment (hour).

Chlorophyll fluorescence parameter V_t or relative variable fluorescence at time *t* in this experiment, declined from 0.24 to 0.07. Accordingly, to better visualize the effect of salt stress on the transient dynamics, drawn of the curves could be made as relative variable fluorescence, $V_t = (F_t - F_o)/(F_m - F_o)$ (107, 108), as a tool to measure the fraction of the PSII primary quinone electron acceptor in reduced state. Moreover, V_t is directly proportional to the number of closed RCs, and is linearly related to the rate at which centers are being closed (107). The figures 3 and 4 show the changes of chlorophyll fluorescence parameters; measured at control and cold acclimation (4/2 °C) conditions, respectively, of the leaves of winter canola *Licord* variety in this experiment.

As mentioned before, there is not any report, which studied the changes of the chl fluorescence parameters of rapeseed using radar plot under low temperature conditions. These changes are represented well here. So one can get enough results with a bit attention to such plots more appropriately than any other graphical or statistical method. They presented the deep changes of chl fluoresence parameters under cold compared with control conditions.

It is confirmed that higher photosynthetic rate during cold acclimation is essential for the expression of freezing tolerance in cold-tolerant plants due to affording energy for cold acclimation (57). This may be an effect of both increasing stability of membranes as well as increasing photosynthetic capacity which can be confirmed by the increase in Rubisco subunits accumulation. The interesting observation is that the short increase in F_m during cold acclimation is accompanied by the increase of cold-inducible soluble proteins. Up to now the protective effects of many cold-inducible proteins to photosynthetic apparatus and cold-responsive plasma membrane proteins were suggested or proved (58-60, 109-111). For instance, Dumont *et al.* (109) suggest that the better adaptation of pea (*Pisum sativum* L.) to chilling might be the result of the existence of a highly content of proteins involved in photosynthesis and in mechanisms for defense. It is well known that membrane lipid unsaturation plays a role in low-temperature damage to plants (34). Polyunsaturated lipids can stabilize and protect the photosynthetic machinery and complexes from low/high temperatures damages (112).

Throughout cold acclimation, several physiological changes occur, including alteration of lipid composition in plasma membranes and chloroplast envelopes (32, 113), accumulation of protectants like carbohydrates, free amino acids or other osmolytes, and induction of new genes activity. Cold acclimation primarily stabilizes the membranes against freeze injury (114). Moreo-

ver, enhancement of the levels of unsaturated fatty acids and thereby a drop in transition temperature, is the results and consequences of cold acclimation (115, 116). Artus *et al*. (58) showed that COR15a protein is accumulated under cold stress in chloroplasts of *Arabidopsis* and protect chloroplasts against low-temperature. As a result, Arabidopsis plants constructively accumulated this protein showed lower reduction in F_v/F_m after transfer to cold than wild-type plants. This study suggest that cold-induced genes as COR15a of *Arabidopsis* and also BN19, BN26, and BN115 of canola may be some homologues which produce proteins with same properties (58). In this context, it is interpreted that the main function of COR15a protein in *Arabidopsis thaliana* seems to be in vivo stabilization of thylakoid membranes against freezing-induced damage, which was demonstrated in transgenic plants overexpressing this gene (18, 58). It is reported that not only low temperatures but drought-induced polypeptides are also concomitant with fewer declines in F_v/F_m ratio, so they could serve as a marker for drought tolerance during the course of clonal selection (117).

Besides the protective role, some proteins involved in fatty acids metabolism may be connected with the protection of chloroplasts against damages. Interestingly, chloroplasts are the synthetic location of fatty acids in plants and eukaryotic algae. In this subject, It was shown that trienoic fatty acids are necessary for biogenesis and maintenance of chloroplasts at long-term exposure to low-temperatures (54). Whereas, Browse *et al*. (118), indicated that the trienoic fatty acids are important in protecting the photosynthetic machinery in Arabidopsis and cyanobacteria against photoinhibition at low temperatures. Besides the induction and increasing trienoic fatty acids in leaves of higher plants under cold acclimation, hexadecatrienoic (16:3) and linolenic (18:3) acids are assumed to protect the plants against chilling-related damage (119). In total, fatty acid composition of plant cells, especially of chloroplasts, is altered by environmental parameters which may, however, protect the plant against abiotic damages. Similarly, Scotti-Campos *et al*. (120) highlighted the protective role of phospholipid composition of chloroplast membranes of *coffea spp*. plants under gradual cold exposure to sustaining photosynthesis activity under cold temperatures.

Our results may indicate that further molecular studies of quantitatively increased cold-inducible proteins as their isolation and sequencing can be a basis for the identification of the gene(s) which can be useful in genetic transformation for obtaining oilseed rape cultivars maintained higher photosynthetic efficiency in cold. This in turn may be useful not only in breeding of freezing tolerant plants (22, 121-123) but also plants more suitable for cultivation in the regions of often temperature decrease occurred during the whole vegetation period. As suggested by that transformation of spring canola with five genes from bromegrass and fall rye associated with frost tolerance has produced breeding lines with some amelioration in cold tolerance (124).

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