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Photosynthetic capacity of canola (*Brassica napus* L.) plants as affected by glycinebetaine under salt stress

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Summary

Salinity causes reduction in growth and severe losses of crop productivity by affecting various biochemical and physiological processes including photosynthesis. Plants sense and adapt to salt stress by modulating different physiological processes including accumulation of osmoprotectants. Glycinebetaine (GB) is an important osmoprotectant and found to have ameliorative effects on the growth of plants by altering ion homeostasis, photosynthetic and antioxidant capacity. In this study, it was attempted to reveal how GB improves photosynthetic activity and salt tolerance of two canola cultivars which differ in degree of salt tolerance. Two cultivars (Dunkled and Cyclone) of canola (*Brassica napus* L.) were grown under non-saline or saline (150 mM NaCl) conditions. Glycinebetaine (100 mM) was foliarly applied to both non-stressed and salt stressed plants of both canola cultivars at the vegetative growth stage. Salt stress reduced growth of both canola cultivars, however, cv. Dunkled was superior to cv. Cyclone. Foliar application of GB improved leaf relative water contents (RWC), osmotic potential and proline accumulation in salt stressed plants. The chlorophyll fluorescence transient (CFT) remained unchanged at O and J phase while at I and P phase it was affected by salt stress in both cultivars that was ameliorated by GB application. The positive values of K band after 1000 µs under salt stress revealed the reduced efficiency of oxygen evolving complex (OEC). The GB application enhanced electron transport chain and decreased heat dissipation under salt stress. This effect was more in cultivar Cyclone as compared to Dunkled. Furthermore, a considerable proteomic variation was noted in canola cultivars after application of GB under both saline and non-saline condition. The results suggested that exogenous foliar application of GB ameliorated the adverse effects of salt stress on both cultivars of canola by osmotic adjustment, growth improvement, increased light absorption by reaction centers, efficient energy trapping and enhanced electron transport chain in both cultivars of canola.

Introduction

Abiotic stresses like salinity, drought, high and low temperature severely affect the crop productivity which strains more on food insecurity (Ashraf and Harris, 2005; Ashraf et al., 2008). It was reported by many researchers that crop yield losses due to salinity and drought is 80 % along with actual losses. High salinity causes both ionic and osmotic stresses (Zhang et al., 2009; Ahuja et al., 2010) by modifying the plant cell plasma membrane, lipid and protein composition, and ultimately impairing optimal growth and development (Fujii and Zhu, 2009; Rodríguez-Milla and Salinas, 2009). Salt induced growth and yield reduction are associated with many factors i.e reduced photosynthetic metabolism, leaf chlorophyll content and photosynthetic capacity (Athar et al., 2009; Athar et al., 2015), diversion of energy in the processes of osmotic adjustment and ion exclusion and nutritional imbalance. Salt induced reduction in photosynthesis is associated with the partial stomatal closure and/or the non-stomatal limitation which is involved in the dark enzymatic processes of CO_2 assimilation e.g. the decrease in Rubisco activity and content, or Pi-regeneration capacity (Ashraf and Harris, 2013).

Plant salt tolerance is correlated with accumulation of osmoprotectants in chloroplasts such as glycinebetaine and proline (Ashraf and Harris, 2004). Glycinebtaine (GB), a quaternary ammonium compound, stabilizes the PSII structure and maintains its activity under saline conditions (Chen and Murata, 2011). It also maintains the association of Rubisco activase with Rubisco and inhibits the production of ROS under stress conditions (Murata et al., 2007). Moreover, GB protects the membrane from high concentrations of Na^+ and Cl^- (Hanson and Bendixen, 1995). Some crops are accumulating GB in high quantities like sugar beet, some accumulate it in moderate amounts and some crops are non accumulator of GB such as canola. So, for a non accumulator crop, exogenous foliar application of this osmoprotectant acts as an alternate shotgun approach to ameliorate growth reduction under salt stress (Mäkelä et al., 1996). Exogenous foliar application of GB improves growth, survival and tolerance in a wide variety of GB accumulator and non accumulator plants. In our earlier studies, it is reported that exogenous foliar application of GB ameliorated the salt induced adverse effects in canola (Athar et al., 2009) by improving the photosynthetic capacity (Athar et al., 2015). It has been reported that salt stress enhances the photoinhibition of PSII by damaging the D1 protein (Chen and Murata, 2011). Chlorophyll a fluorescence measurement has been one of the most used techniques for providing rapid insights in the ability of a plant to tolerate environmental stresses such as salt stress (Kalaji and Guo, 2008; Kalaji et al., 2011). In addition, it also facilitates the understanding to what extent salt stress has affected the structural and functional stability of the photosynthetic apparatus (Baker, 2008; Stirbet and Govindjee, 2011). Of various chlorophyll fluorescence techniques, the chlorophyll a fast fluorescence transient measures the photochemical performance of PS-II, reflecting the time course of photochemistry. Based on the theory of energy flow in thylakoid membranes, Strasser et al. (1995) have developed the JIP test which has been found to be very sensitive to stress induced changes in PSII even before visible symptoms appear on the leaves. Keeping in view the above mentioned reports, the present study aimed to assess how exogenous foliar application of GB ameliorates the adverse effect of salt stress on photosynthetic capacity in two cultivars of canola.

Material and method

Seeds of two canola (*Brassica napus* L) cultivars (cv. Dunkled and cv. Cyclone) differing in salinity tolerance were obtained from Ayub Agriculture Research Institute, Faisalabad, Pakistan. The experiment was conducted at the Botanic Gardens of Bahauddin Zakariya University Multan, Pakistan, with 10/14 light/dark period at 800-1000 mmol m^{-2} s^{-1} PPFD, a day/night temperature cycle of 26/15 °C and 60±5 % relative humidity. Before experimentation, 500 seeds

of each canola (*Brassica napus* L.) cultivar were surface sterilized in 5 % sodium hypochlorite for 5 minutes. Seeds were sown in 32 plastic pots (28 cm diameter and 32 cm depth), each of which filled with 10 kg thoroughly washed river sand. Plastic pots have drainage holes covered with a piece of muslin cloth at the bottom. All pots were irrigated with 2 L of full strength Hoagland's nutrient solution. The seeds were allowed to emerge for one week and then thinned to 8 plants per pot initially. After a period of further one week, plants were thinned to 5 plants per pot of uniform size and placed equidistantly. Four weeks after the start of the experiment, pots were irrigated with full strength Hoagland's nutrient solution containing 0 or 120 mM NaCl. Salinity level was developed by the addition of NaCl stepwise in aliquots of 40 mM every day until the appropriate treatment was attained. Varying concentrations of GB [0 or 100 mM in 0.1 % Tween-20 solution] (~5 ml per plant and ~1.5 L for whole sub-experiment) applied as a foliar spray to plants of both canola cultivars growing in non-saline or saline conditions. The control (0 mM GB) plants were sprayed with the same volume of 0.1 % Tween-20 solution. The pH value of the sprayed solution was maintained at 6.5 to ensure the maximum penetration into the leaf tissue and to avoid the leaf injury. Plants were sprayed in the evening to avoid drying of solution on leaves and allowing maximum GB solution penetration into the leaf tissue. Hoagland's nutrient solution containing (0 or 120 mM NaCl) was replaced every week to replenish nutrients. However, treatment solution was applied in excess to each pot so as to flush through all the salts previously present in the sand and to ensure the desired salt level. After four weeks of GB treatment (2 months old plants), plants were harvested, washed with distilled water, blotted dry and separated into shoots and roots, and data for fresh biomass recorded. However, canola plants growing in 120 mM NaCl solution were washed in cold 140 meq/L $LiNO_3$ solution, isotonic with the corresponding salt treatment. However, 1 mM $Ca(NO_3)_2$ $4H_2O$ was added in $LiNO_3$ solution to maintain membrane integrity and to avoid marginal changes in ion content during washing. These plants were then oven-dried at 65 ºC for 72 h and dry biomass recorded. However, before harvest, relative water content (RWC), leaf proline, chlorophyll contents, and chlorophyll fluorescence parameters were measured.

Leaf relative water contents (RWC): A fully developed and young leaf from each plant was taken and fresh weight of each leaf recorded. All leaf samples were dipped in distilled water for six hours and measured the turgid weight of each sample leaf. Then all the sample leaves were dried in oven at 70 °C to measure dry weights.
RWC= [L fresh wt. – L dry wt.]/[L turgid wt. – L dry wt.]*100

Leaf osmotic potential: Third leaf from top was taken from each cultivar and was frozen at -20 °C for seven days, after which time the frozen leaf material was thawed and the cell sap was extracted with the help of a disposable syringe. The extracted sap was directly used for the determination of osmotic potential using an osmometer (Wescor 5500, USA).

Chlorophyll content: A fully developed and young leaf from each plant (usually third leaf from top) was used to measure chlorophyll contents using a portable chlorophyll meter (Minolta SPAD-502, Japan).

Chlorophyll fluorescence: The data for chlorophyll fluorescence were recorded following nomenclature by Tsimilli-Michael and Strasser (2008), Stirbet and Govindjee (2011) and the literature available on the websites of manufacturers of chlorophyll fluorescence meters. Fast chlorophyll a fluorescence transients were recorded on third leaf by using portable fluorescence meter, FluorPen FP 100 (Photon System International, Czech Republic). Before taking measurements, leaves of each genotype/cultivars were dark adapted for 30 minutes by wrapping the aluminium foil to the leaf surface to ensure complete oxidation of photosynthetic electron transport chain or PSII with open reaction centers (RCs). The fluorescence emission was induced in a 4 mm diameter area by exposing to a saturating actinic light at the intensity of 3000 μmol m^{-2} s^{-1}. The fast chlorophyll fluorescence kinetics was measured from 10 μs to 2 s and the settings of FluorPen was as: Fo (initial fluorescence) as O (20 μs) when all the reaction centers are open, L (150 μs), K (300 μs), J (2000 μs) and I (30000 μs) are the intermediate and P (500000 μs) as the maximum fluorescence (Fm). The original (without normalization) chlorophyll a fluorescence transients of different treatments were plotted. For detailed analysis of the whole digitized fluorescence kinetics, different normalizations, calculation of kinetic differences or ratios as well as time-derivatives were undertaken. The original OJIP transients were double normalized between the two fluorescence extreme O (Fo) and P (Fm) phases and the variable fluorescence between OP expressed as V_{OP} ($V_{OP} = (F_t\text{-}Fo)/(F_m\text{-}Fo)$ was determined. Similarly, chlorophyll a fluorescence transients were double normalized between Fo (30 ms) and F_K (300 ms) expressed as V_{OK} [$V_{OK} = (Ft\text{-}F_O)/(F_K\text{-}F_O)$] to reveal the possibility of fluorescence rise at an early step at about 300 ms. Further the chlorophyll a fluorescence transients were double normalized between F_O and F_J expressed as V_{OJ} [$V_{OJ} = (F_t\text{-}F_O)/(F_J\text{-}Fo)$]. The O-I phase was evaluated by double normalization of fluorescence transients between Fo and F_I expressed as V_{OI} (<1) [$V_{OI} = (Ft\text{-}F_O)/(F_I\text{-}F_O)$]. The I-P phase was evaluated following two approaches: (i) normalization of fluorescence transients V_{OI} between the time range of 30-300 ms expressed as V_{OI} or PI (>1) [$V_{OI} = (Ft\text{-}F_O)/(F_I\text{-}F_O)$] and (ii) transient normalisation to the time range of 30-200 ms expressed as V_{IP} [$V_{IP} = (Ft\text{-}FI)/(F_M\text{-}F_I)$]. Moreover, differences in transients with respect to a reference were calculated as L-band ($\Delta V_{OK} = V_{OK\ (salt\ stress)} - V_{OK(control)}$) and K-band ($\Delta V_{OJ} = V_{OJ\ (salt\ stressed)} - V_{OJ(control)}$). Similarly, ΔV_{OI} and ΔV_{IP} were calculated for the detailed analysis of chlorophyll fluorescence kinetics. Other phenomenological and biophysical parameters were also calculated using JIP-test as described by Tsimilli-Michael and Strasser (2008) that give information about the structural and functional state of PS II.

Protein Extraction: Fresh leaves (0.5 g) were ground in 4 mL of cooled phosphate buffer (50 mM, pH 7.8). The homogenous mixture was centrifuged at 15000 g at 4 °C for 20 minutes and the supernatant used for protein analysis. Sodium azide was added to avoid any type of growth in protein sample.

Sodium Dodocyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE): One dimensional gel electrophoresis (E-VS10-SYS, Germany) was performed on 12 % polyacrylamide to visualize the protein samples according to the standard procedure (Laemmli, 1970). After electrophoresis, gel was stained with Coomassie brilliant blue R-250 dye for approx. one hour. The corresponding gel was visualized after de-staining process and the sample protein bands were compared with the bands of the protein marker (Invitrogen, 69079-3).

Statistical analysis of data: The data obtained from all parameters are described as mean value ± SE. By using the statistical software COSTAT, data were subjected to three way analysis of variance (ANOVA). The mean values were compared with least significance difference (LSD) test following Snedecor and Cochran (1989).

Result

A marked inhibitory (P<0.001) effect of salt stress was observed on fresh and dry weights of shoots and roots of both canola cultivars. Cultivar Dunkled had significantly (P<0.001) higher shoot fresh and dry weights than those of cv. Cyclone under both normal and saline conditions (Fig. 1a; 1b). However, cultivars did not differ in root fresh and dry weights. GB application caused a significant (P<0.001) increase in shoot fresh and dry weights of both cultivars. Foliar application of GB significantly increased (P<0.001) root fresh and dry weights of both cultivars under normal conditions, whereas under saline condition GB application slightly improved root fresh weight of cv. Dunkled only (Fig. 1c; 1d).

Salt stress reduced the leaf osmotic potential (more negative) in both canola cultivars and exogenous foliar application of GB cause a further decrease in leaf osmotic potential of both canola cultivars. In addition, impact of salt stress and GB application on leaf osmotic potential of both cultivars was the same (Fig. 2a). Relative water content (RWC) decreased in salt stressed plants of both canola cultivars. Cultivar Dunkled had greater RWC values than cv. Cyclone under normal conditions, while the reverse was true under saline conditions (Fig. 2b). Exogenous application of GB reduced RWC in non-stressed plants of cv. Dunkled and salt stressed plants of cv. Cyclone only. Chlorophyll content measured as SPAD units decreased due to imposition of salt stress in cv. Dunkled only. Exogenous foliar application of GB did not change the chlorophyll contents of cv. Dunkled under both non-saline and saline conditions. However, application of GB reduced the chlorophyll contents of cv. Cyclone under saline conditions (Fig. 2c).

Chlorophyll a fluorescence transients of dark adapted leaves plotted on logarithmic time scale clearly showed differential impact of salt stress and exogenous application of glycinebtaine on O-J-I-P phases of two canola cultivars (Fig. 3). The fluorescence rise at O-J and J-I phases was significantly decreased in cv. Dunkled due to salt stress, whereas in cv. Cyclone such a decrease in fluorescence was found at O-J phase only. Foliar application of GB did not change amplitude of fluorescence at O-J phases of both canola cultivars (Fig. 3). However, exogenous GB application compensated the loss of fluorescence at O-J phase by increasing fluorescence level at J-I and I-P phases in salt stressed plants of both canola cultivars. In addition, in non-stressed plants of cv. Cyclone it remained almost unaffected (Fig. 3a; 3b). Since changes in the OJIP shape are translated to changes of the structural and functional parameters of PSII, chlorophyll a fluorescence transients were expressed as Ft/Fo so that differences concerning the Fo values would not interfere with the other differences as well as it decreased heterogeneity among replicates (Fig. 3c; 3d). Chlorophyll a fluorescence transients normalized by Fo (Ft/Fo) showed typical O, J, I and P steps. Moreover, it also showed that salt stress and application of GB increased Fv in both canola cultivars which are almost equivalent to increase in primary photochemistry (j_{Po}). To avoid any interference and heterogeneity due to different Fo and Fm values, all fluorescence transients were doubly normalised and presented as relative variable fluorescence, which are plotted on log time scale (Fig. 3e; 3f). Transients showed that exogenous application of GB caused a dip in fluorescence at J-I phase in salt stressed plants of both cultivars.

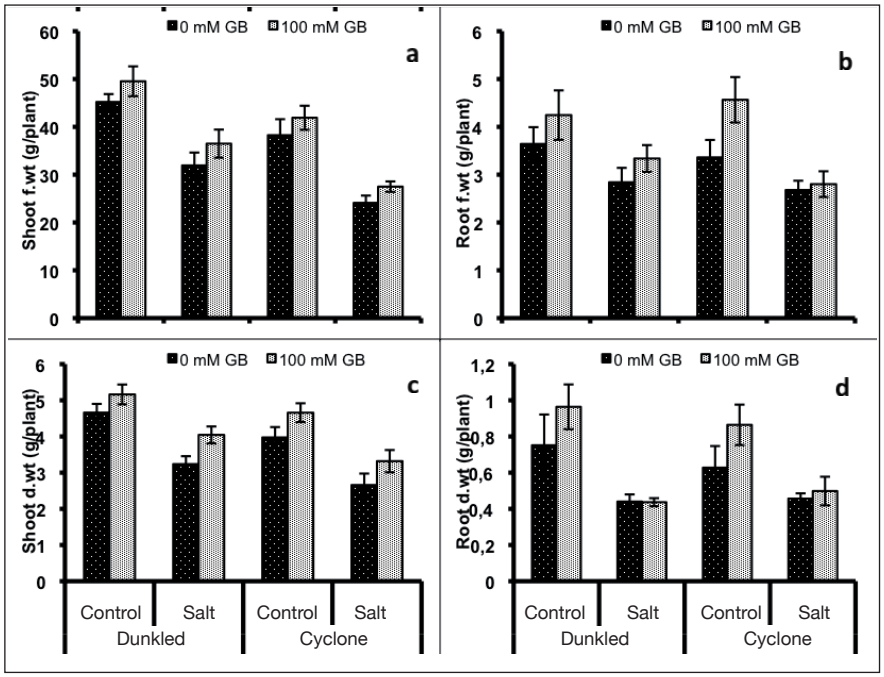

Fig. 1: Shoot and root fresh and dry weight of two cultivars of canola (*Brassica napus* L.) when three week old plants were subjected to foliar application of glycinebetaine and salt stress for further two weeks.

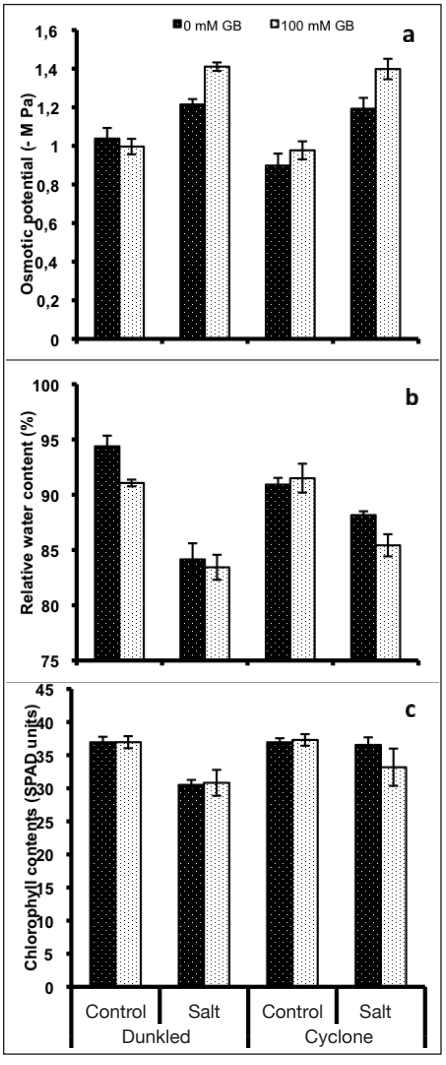

Fig. 2: Osmotic potential, relative water content and chlorophyll content of two cultivars of canola (*Brassica napus* L.) when three week old plants were subjected to foliar application of glycinebetaine and salt stress for further two weeks.

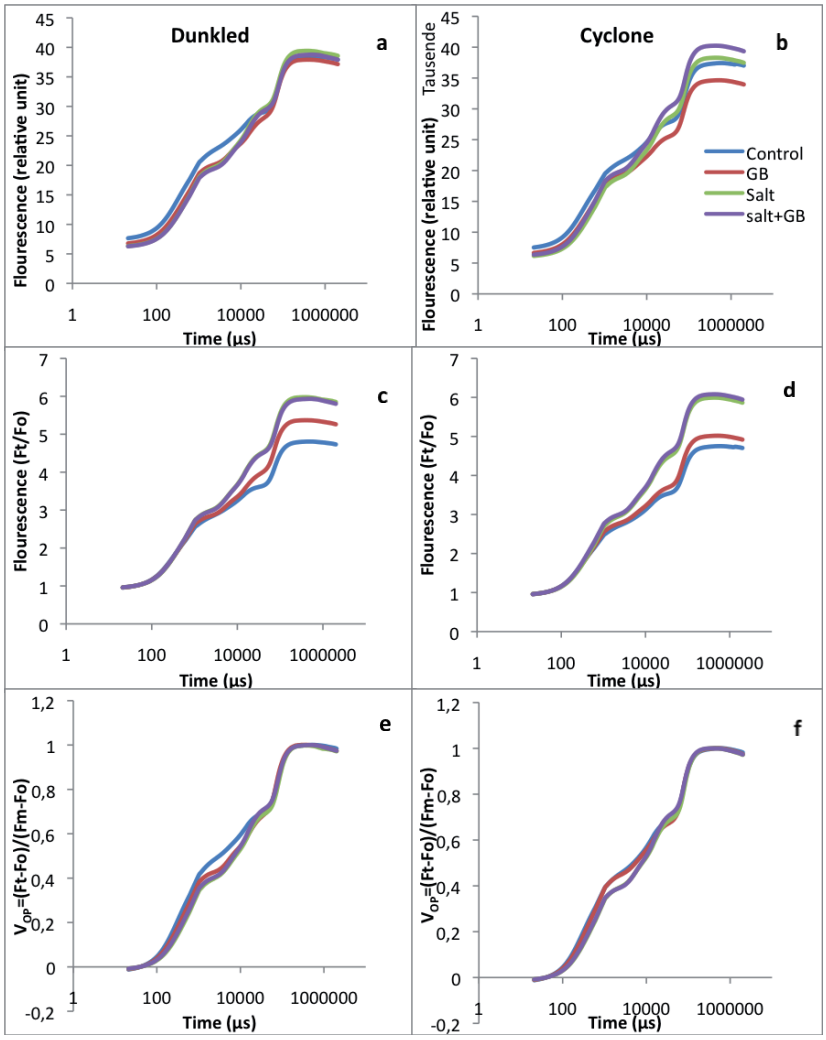

Fig. 3: Chlorophyll flourescence with and without normalization of two cultivars of canola (*Brassica napus* L.) when three week old plants were subjected to foliar application of glycinebetaine and salt stress for further two weeks.

In order to validate the observed differences, a semi-quantitative evaluation was performed using the differences of suitably normalised transients exhibited by stressed and non-stressed plants and expressed as OP, L and K bands. L and K bands are usually hidden among the O-J-I-P steps of the original transients. The difference kinetics of DV_{OP} (Fig. 4a) of GB applied plants of both cultivars under non-saline or saline conditions showed a negative bands in the O-I region (and more in J-I region, photo-electrochemical quenching) of the OJIP transient, which indicates that processes from exciton trapping to PQ reduction are faster than in respective control plants. However, negative band of DV_{OP} of GB treated plants of cv. Dunkled was more negative than thin in GB treated plants of cyclone under both non-saline and saline conditions. Moreover, amplitude of difference kinetics of DV_{OP} in GB treated plants of cv. Cyclone at O-I region was greater than that in salt stressed plants of cv. Cyclone, whereas in cv. Dunkled such difference in amplitudes is not observed (Fig. 4a). To reveal changes in chlorophyll fluorescence at each step, difference kinetics at L and K steps were also calculated and presented as ΔV_{OK} (L band, energetic connectivity among PSII units) and ΔV_{OJ} (K band, antenna size or OEC activity), respectively (Fig. 4b; 4c). Negative amplitude of L-band of GB treated plants of both cultivars under saline or non-saline condition indicated GB treated plants had greater energetic connectivity among PSII units than in control plants. However, amplitude of L band in cv. Cyclone applied with 100 mM GB was higher than in plants of cv. Dunkled applied with 100 mM GB under non-saline conditions (Fig. 5b), whereas under saline conditions cultivars did not differ. Difference kinetics as ΔV_{OJ} (K band) revealed that a negative band appeared at 300 µs in GB treated plants of both canola cultivars under both non-saline and saline conditions (Fig. 4c). Cultivar difference in amplitude of negative K-band due to foliar application of GB is only visible under non-saline condition, where cv. Dunkled presents lower amplitude of the L band.

Changes in I-P phase have been measured by two normalization methods, which is associated with the electric trans-thylakoid potential generated by the proton pump fuelled by Cyclic Electron Transport (CET) in PSI (processes related with the electron flow from reduced PQ (PQH2) to PSI end electron acceptors i.e. Fd and NADP) (Strasser et al., 2010; Vredenberg, 2011). Exogenous application of GB increased the amplitude of I-P curve in both canola cultivars under non-saline conditions, whereas under saline conditions amplitude is lower in both canola cultivars (Fig. 5a; 5b). This suggests that GB increased the pool of electron carriers of PSI end to be reduced from electrons coming from PQ in both canola cultivars. Moreover, I-P phase measured as $V_{IP} = [(Ft\text{-}F_I)/(Fm\text{-}F_I)]$ showed that (Fig. 6c; 6d) salt stress reduced the rate constant of cv. Dunkled and exogenous application of GB improved the rate constant in cv. Dunkled. However, such changes in rate constant due to GB application are

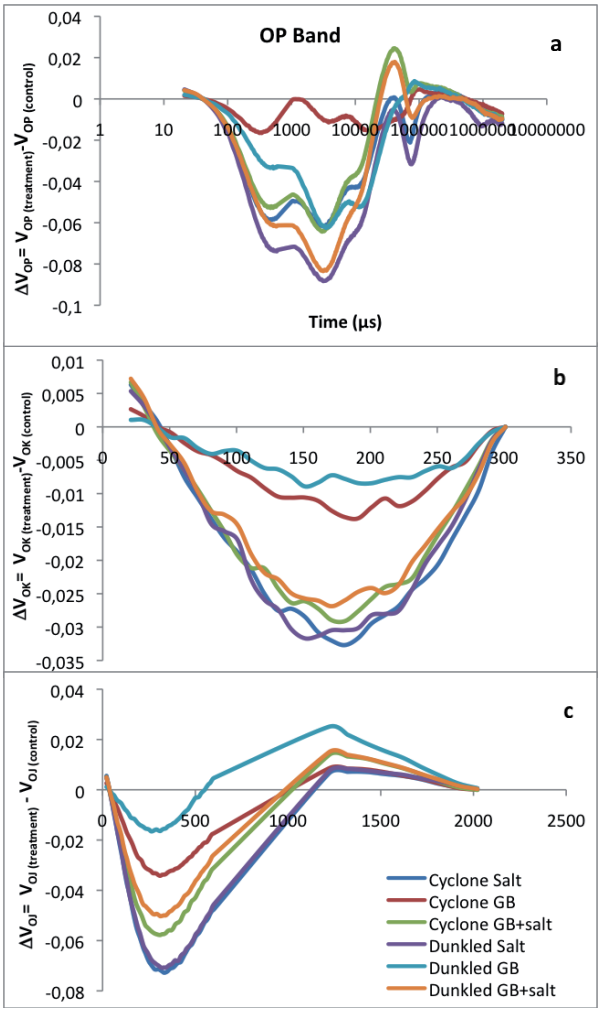

Fig. 4: ΔVOP, ΔVOK, ΔVOJ of two cultivars of canola (*Brassica napus* L.) when three week old plants were subjected to foliar application of glycinebetaine and salt stress for further two weeks.

not observed in cv. Cyclone (Fig. 5c; 5d).

Salt stress induced changes in biophysical parameters derived from the chlorophyll fluorescence curve of both cultivars of canola are presented as radar plot (Fig. 6a; 6b). All data of these parameters were normalized to the reference control and each variable at the reference control was standardized by giving a numerical value of 100. Salt stress decreased the variable fluorescence at step J (V_J) of both canola cultivars. Exogenous foliar application of GB did not affect V_J of both canola cultivars. However, salt stress and GB application did not change the V_I (relative variable fluorescence at step I) in both cultivars (Fig. 6a; 6b; Tab. 1). Among ratios of chlorophyll fluorescence, F_V/F_M (maximum primary yield of photochemistry of PSII) remained unaffected due to salt stress and GB application in both canola cultivars, F_V/F_O and F_M/F_O more sensitive parameters for photosynthetic capacity reduced due to salt stress in both cultivars of canola and exogenous application of GB improved both these parameters in both canola cultivars (Fig. 6a; 6b; Tab. 1). However, such improvement in F_V/F_O and F_M/F_O due to GB application was more in cv. Dunkled under non-saline conditions, whereas the same was true for cv. Cyclone under saline conditions. Salt stress reduced the rate of primary photochemistry (Mo) of both canola cultivars. However, exogenous application of GB slightly improved in cv. Dunkled only (Fig 6a; 6b). Similarly, total PQ pool (Area), multiple turnover of PQ reduction and oxidation (Sm) and number of Q_A^- redox turnover until F_M (N) reduced in cv. Cyclone only and GB application did not change these biophysical parameters (Fig. 6a; 6b; Tab. 2). The derived specific energy fluxes such as absorbance flux per active reaction center (ABS/RC), trapped energy flux per reaction center (TR_o/RC), electron transport flux (further than Q_A^-) per reaction center (ET_o/RC) and dissipation energy flux per reaction center (DI_o/RC) reduced due to salt stress in both canola cultivars and foliar application of GB did not affect these JIP test parameters in both cultivars (Fig. 6a; 6b; Tab. 4).

The relationship between log PI_{ABS} and relative electron transport yield can be considered as plant's ability to transform light energy into chemical energy (NADH) which is subsequently directed into metabolic reactions in the biochemical process of photosynthesis (Hermans et al., 2003). In this respect our result suggests that the plants that were sprayed with GB under salinity stress have higher ability to convert light energy into chemical energy which can be used further to reduce CO_2 to carbohydrate. The higher ability to convert light energy into chemical energy is observed in cultivar Cyclone (Fig. 7).

Comparative protein expression of two canola cultivars was performed using one-dimensional SDS-PAGE (Fig. 8), which shows that 22, 56 and 100 kDa water soluble protein expression increased salt stressed and exogenously applied plants of cv Dunkled, whereas in Cyclone expression of 22 and 56 kDa proteins was low.

Discussion

To overcome salt-induced reduction in growth of canola, exogenous application of glycinebetaine (GB) is considered to be an alternative strategy (Athar et al., 2009; Abbas et al., 2010) and this has been verified in the present study. These inhibitory effects of salt stress on canola cultivars were mainly due to salt induced osmotic stress and toxic effects of Na^+ accumulation (data not shown) along with reduced photosynthetic rate as has been found earlier (Ulfat et al., 2007; Athar et al., 2015). Salt stress and GB application decreased the leaf osmotic potential, representing an important factor which enhances the salt tolerance. Similar results were reported by Raza et al. (2006) that foliar application of GB reduced the osmotic potential in salt stressed plants of wheat which favor the osmotic adjustment. The main function of foliar applied GB is to promote photosynthetic capacity in canola cultivars. However, this improving effect of GB on photosynthetic capacity is cultivar specific and due to the cultivar's genetic potential (Yang and Lu, 2005; Chen and Murata, 2008), but its impact on the exact site of the photosynthetic machinery is still not clear. In a semi-quantitative evaluation, impact of salt stress and exogenous application of GB on different sites of photosynthetic machinery of salt tolerant and sensitive canola cultivars, whole transients of OJIP, suitably normalized transients and differences of normalized transients of stressed and non-stressed plants are measured. Whole transients data from the present study suggest that salt stress reduces the rate of primary photochemistry (fluorescence at O-J phase changes) and photo-electrochemical quenching (J-I phase changes) in both canola cultivars and exogenous GB application improved photosynthetic activity by compensating rate of reduction at PSI end electron acceptors in salt tolerant cultivar Dunkled (Fluorescence changes at I-P phase). These results are further supported by Fo normalized transient and transients of relative variable fluorescence between Fo and Fm (Fig. 3).

To further confirm the results, whole difference kinetics and difference kinetics at each step was performed (Fig. 4). In L-band, the low values of florescence under saline conditions show that energetic connectivity loss to some degree is because of reaching to the verge of salinity acclimation. In K-band, no positive peak till 1000 s µs shows the ability of both cultivars of canola to resist the imbalance in number of electrons at donor and acceptor side of photosystem II

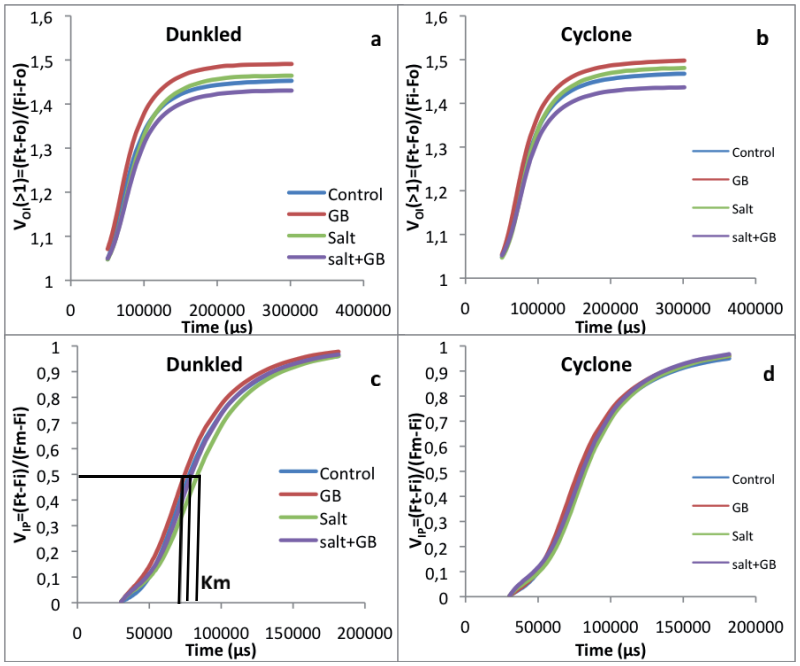

Fig. 5: VIP and VOI of two cultivars of canola (*Brassica napus* L.) when three week old plants were subjected to foliar application of glycinebetaine and salt stress for further two weeks.

which is induced by salinity. However, an increased K-band from 1000-2000 µs under saline condition reveals that the performance of OEC (oxygen evolving complex) reduced due to imbalance in e^- flow from the oxygen evolving complex to the reaction centers and in the direction of acceptor side of PS II. However, an attenuated K-band from 1000-2000 µs under GB foliar spray in saline condition suggesting the ability of both cultivars of canola to resist the imbalance in number of electrons at donor and acceptor sides of PSII. O-I part indicates the kinetic properties for oxidation/reduction of PQ pool. The negative ΔV_{OI} peaks recorded under salinity stress indicate that salinity-stressed plants of both cultivars of canola had the ability to maintain the PQ reduction rate and GB does not ameliorate this process under salinity stress to greater extent. The I-P phase which is the last phase of fluorescence transient indicates the change in electrons currents from the PQH_2 to electron acceptor of PS I. Increase in positive values of I-P phase indicates higher pool of the PS I's electron acceptor. A decrease in I-P phase on salt treatment and again rise under GB foliar spray might be a part of acclimation process of both cultivars of canola in which the plants endeavor to lessen the salinity effects by maintaining the PQ pool size. However, salinity induced reduction in I-P phase is due to the sudden decline in leaf water status that might reach to the threshold level of salinity acclimation.

Chlorophyll fluorescence at different steps of OJIP and ratios of chlorophyll fluorescence are potential indicator of PSII efficiency. Salt stress significantly decreased the minimum level of fluorescence (Fo) reveals increase excitation energy transfer from the antenna to the RC, leading to low Fo. In contrast, Kalaji et al. (2011) found that imposition of salt stress reduced Fo of two Syrian barley landraces after 7 days of salt stress. Exogenous foliar GB application also decreased the Fo under non saline condition also reveals increase transfer efficiency of energy absorbed from antenna chl.a to PSII reaction center. On the other hand, GB application increased the Fo under saline condition reveals reduced energy transfer efficiency from the antenna chl a to PSII reaction center. Salt stress also reduces the maximal fluorescence (F_M) reveals the salt stress reduces the transfer of electrons to the acceptor side of PS II. It induces changing in the rate of Q_A reduction. GB protects the PSI so the foliar application of GB protects the rate of oxidation of plastoquinone by electron transfer to PSI.

ABS/RC which indicates the antenna size of active RCs which is estimated as the ratio of total absorbance of photons by chlorophyl molecules to total active reaction centers that decreased in plants those are treated by foliar application of glycinebetaine due to increased in size of antenna of active reaction centers. Among the JIP parameters, PI is the most sensitive parameters. This parameter gives information about fluorescent changes that associated with antenna conformational changes and energy fluctuations. Therefore with the help of PI, the vitality of the plants is estimated with high resolution. The foliar application of glycinebetaine under saline condition increased the PI value and the relationship between ET_0/ABS and log PI_{ABS} which demonstrates that in natural environment plants have high efficiency to utilize the PAR which reduce CO_2 to carbohydrate. Similar results are reported by Shine and Guruprasad (2012) that shows that pre-sowing magnetic field exposure of seed increased the PI and the relationship between log PI_{ABS} (=DF).

The analysis of polypeptide banding obtained on SDS-PAGE have shown that a 14-day treatment of GB and NaCl resulted in the change of the leaf water soluble proteins in comparison with that of control leaves. Both substances have induced the accumulation and synthesis of many different proteins inside the leaf tissue. Out of the three observed protein bands, the 100 kDa protein was significantly present in treated plants as compare to control. Maslenkova et al. (1992) reported the higher concentrations of many polypeptides in Barley seedlings as a result of salinity and Jasmonic acid treatments. They have observed a substantial enrichment of one polypeptide with an apparent molecular mass of 55 to 57 kDa.

The exact function of these induced proteins is still unclear for us and it is very difficult at this stage with this minimal data to say clearly about the identification and characterization of these water soluble proteins. However, it can be assumed that the same proteins present in control sample, have shown higher expression in leaf tissues of the treated plants. It is therefore of immense interest to go for further characterization of these important proteins either through Mass Spectrometry or molecular structure determination through NMR/X-ray crystallography.

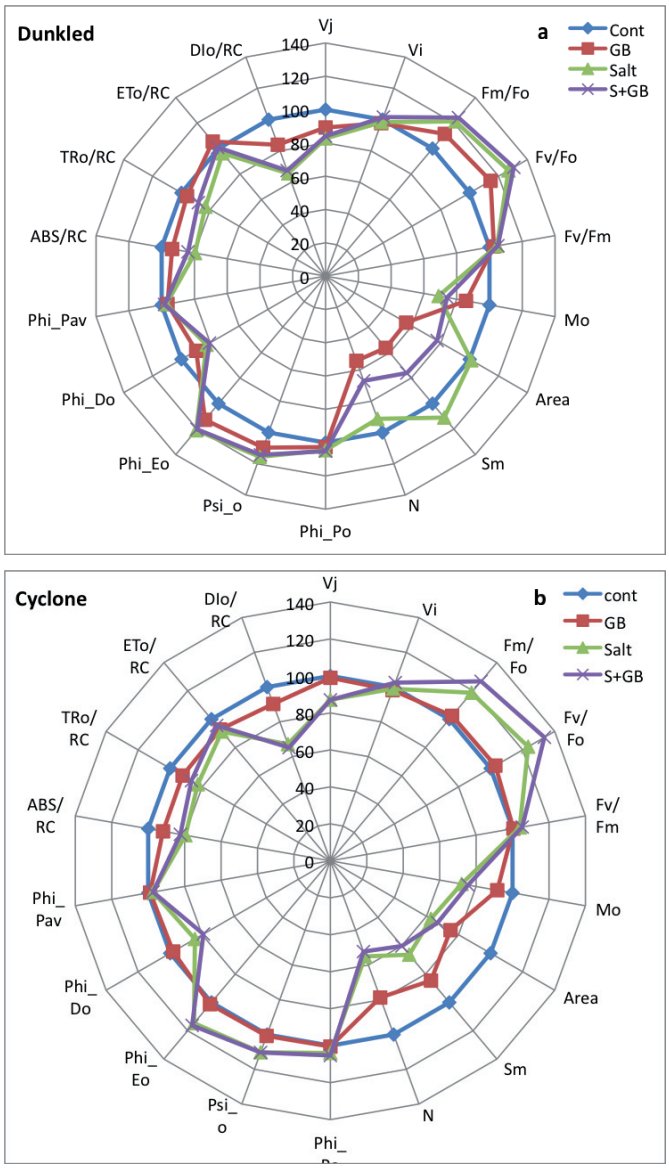

Fig. 6: Radar plot of JIP test parameters of two cultivars of canola (*Brassica napus* L.) when three week old plants were subjected to foliar application of glycinebetaine and salt stress for further two weeks.

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Tab. 1: Mean squares from analysis of variance of data for V_J, V_I, Fv/Fo, Fv/Fm and Fm/Fo in leaves of two cultivars of canola (*Brassica napus* L.) treated with or without foliar spray of GB to salt stressed and non stressed condition.

Source of variance	df	V_J	V_I	F_V/F_O	F_V/F_M	F_M/F_O
Salt	1	0.026***	6.125***	7.998***	0.0084***	7.998***
GB	1	4.205ns	3.001***	0.641**	7.801***	0.641**
Cultivar	1	2.101ns	1.125***	0.116ns	1.25ns	0.116ns
Salt* cultivar	1	2.88ns	1.8ns	0.111ns	1.361**	0.111ns
Salt*GB	1	3.781ns	0.0029***	0.0028ns	2.812ns	0.002ns
GB*Cultivar	1	0.0035***	1.512ns	0.3676*	1.8e-5ns	0.3676*
Salt*GB*Cultivar	1	0.0010*	1.012ns	0.185ns	3.125***	0.185ns
Error	24	0.0042	2.99	1.25	2.465	0.0524

ns = non-significant; *,**,*** significant at 0.05, 0.01 and 0.001 probability
GB = Glycinebetaine, Salt = Salt stress
$V_{J,}$ = relative variable fluorescence at phase J of fluorescence transient curve, V_I = relative variable fluorescence at phase I of fluorescence transient curve, F_V/F_O = maximum primary yield of photochemistry, F_V/F_M = maximum yield of photochemistry of PSII.

Tab. 2: Mean squares from analysis of variance of data for area, fix area, Sm, Ss and N in leaves of two cultivars of canola (*Brassica napus* L.) treated with or without foliar spray of GB to salt stressed and non stressed condition.

Source of variance	df	Area	Fix area	S_M	S_S	N
Salt	1	2.50*	4.069ns	107083.4***	0.054***	261404.0***
GB	1	4.98**	4.177ns	53670.7***	4.27ns	434800.2***
Cultivar	1	1.89ns	2.466ns	31302.4***	0.002*	2039.8ns
Salt* cultivar	1	1.26**	3.083ns	55587.0***	4.27ns	384013.7***
Salt*GB	1	5.89**	1.156***	60786.1***	0.007***	104372.5***
GB*Cultivar	1	1.82ns	6.131ns	4017.64*	2.82ns	95882.2***
Salt*GB*Cultivar	1	4.71ns	1.590**	40273.9***	1.015ns	923.9ns
Error	24	1.325	4.094	13294.1	0.010	32840.7

ns = non-significant; *,**,*** significant at 0.05, 0.01 and 0.001 probability
GB = Glycinebetaine, Salt = Salt stress
Area= size of reduced plastoquinone pool or the area above the chl. fluorescence between F_O and F_M, S_M=multiple turnover number, S_S=single turnover number, N= number of Q_A^- redox turnover until F_M.

Tab. 3: Mean squares from analysis of variance of data for ϕ_{Po}, ψ_o, ϕ_{Eo}, ϕ_{Do}, ϕP_{AV} in leaves of two cultivars of canola (*Brassica napus* L.) treated with or without foliar spray of GB to salt stressed and non stressed condition.

Source of variance	Df	ϕ_{Po}	ψ_o	ϕ_{Eo}	ϕ_{Do}	ϕP_{AV}
Salt	1	0.0091***	0.0269***	0.0358***	0.0091***	1073.8***
GB	1	7.1253*	4.205ns	0.001*	7.125**	501.882***
Cultivar	1	1.4878ns	2.101ns	3.445ns	1.487ns	225.234***
Salt* cultivar	1	1.4028ns	2.88ns	4.575ns	1.4028ns	493.506***
Salt*GB	1	2.5312ns	3.781ns	2.587ns	2.531ns	1452.05***
GB*Cultivar	1	5.3628**	0.003***	0.0036***	5.3628**	133.861**
Salt*GB*Cultivar	1	5.6640*	0.0010*	0.0013*	3.1878*	745.047***
Error	24	5.6281	1.754	1.926	0.0013	247.93

ns = non-significant; *,**,*** significant at 0.05, 0.01 and 0.001 probability
GB = Glycinebetaine, Salt = Salt stress
ϕ_{Po}= maximum quantum yield of primary photochemistry, ψ_o= capacity of PS II to transfer trapped excitation, ϕ_{Eo}= quantum yield of electron transport, ϕ_{Do}= quantum yield of dissipation energy, ϕP_{AV}= performance indux

Tab. 4: Mean squares from analysis of variance of data for ABS/RC, TR_o/RC, ET_o/RC and DI_o/RC in leaves of two cultivars of canola (*Brassica napus* L.) treated with or without foliar spray of GB to salt stressed and non stressed condition.

Source of variance	df	ABS/RC	TR_o/RC	ET_o/RC	DI_o/RC
Salt	1	0.8613***	0.3224***	0.005ns	0.1297***
GB	1	0.006ns	7.8125e-5ns	0.0011ns	0.0048**
Cultivar	1	0.0411**	0.0192**	0.0032ns	0.0040**
Salt* cultivar	1	0.0136ns	0.0047ns	7.2e-5ns	0.0023*
Salt*GB	1	0.0716***	0.0438***	0.0080*	0.0033*
GB*Cultivar	1	5e-5ns	0.0017ns	0.0144**	0.0023*
Salt*GB*Cultivar	1	0.0037ns	1.5313e-4ns	0.0023ns	0.0023*
Error	24	0.0038	0.0023	0.0012	4.5569e-4

ns = non-significant; *,**,*** significant at 0.05, 0.01 and 0.001 probability
GB = Glycinebetaine, Salt = Salt stress
ABS/RC = absorbance flux per reaction center, TR_o/RC = trapped energy flux per reaction center, ET_o/RC = electron transport flux per reaction center, DI_o/RC = dissipation energy flux per reaction center.

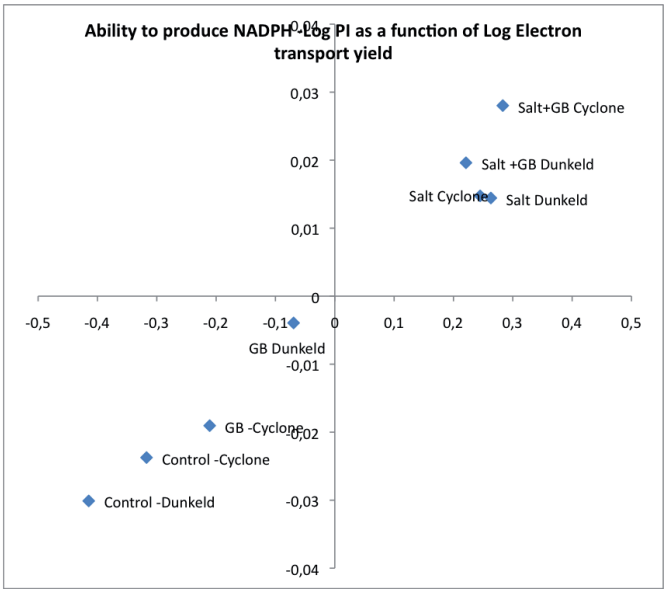

Fig. 7: Ability to produce NADPH of two cultivars of canola (*Brassica napus* L.) when three week old plants were subjected to foliar application of glycinebetaine and salt stress for further two weeks.

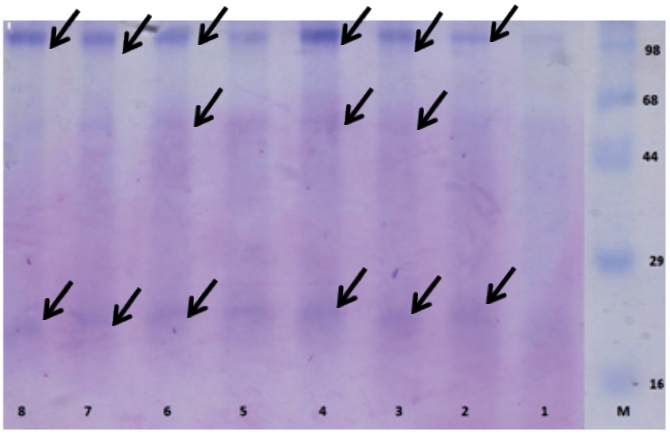

Fig. 8: Comparative analysis of protein banding pattern of the two canola cultivars. Lanes 1-4, Dunkled; Lanes 5-8, Cyclon. Lane 1: Control, Lane 2: GB (100 mM), Lane 3: Salt (150 mM), Lane 4: GB + Salt. Similar sample arrangement is for Cyclon cultivar in Lanes 5-8. Molecular weights of the marker proteins are given in kDa.

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