ANNEXURE-III

"EFFECT OF 28-HOMOBRASSINOLIDE ON OXIDATIVE AND SUGAR METABOLISM AND REPRODUCTIVE POTENTIAL OF *BRASSICA JUNCEA* L. UNDER THERMAL STRESS"

(1 April, 2013 to 31st March, 2017)

Final
PROGRESS REPORT
of Major research project
F. No. 42-920-2013(SR)

Submitted to

University Grant Commission
New Delhi-110002

Principal Investigator:

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PROGRESS REPORT

From 1 April, 2013 to 31st March, 2017

1. Title of Project: Effect of 28-homobrassinolide on oxidative and sugar metabolism and reproductive potential of *Brassica juncea* L. under thermal stress.

2. Date of Sanction: 1st April, 2013

3. Name of Principle Investigator:

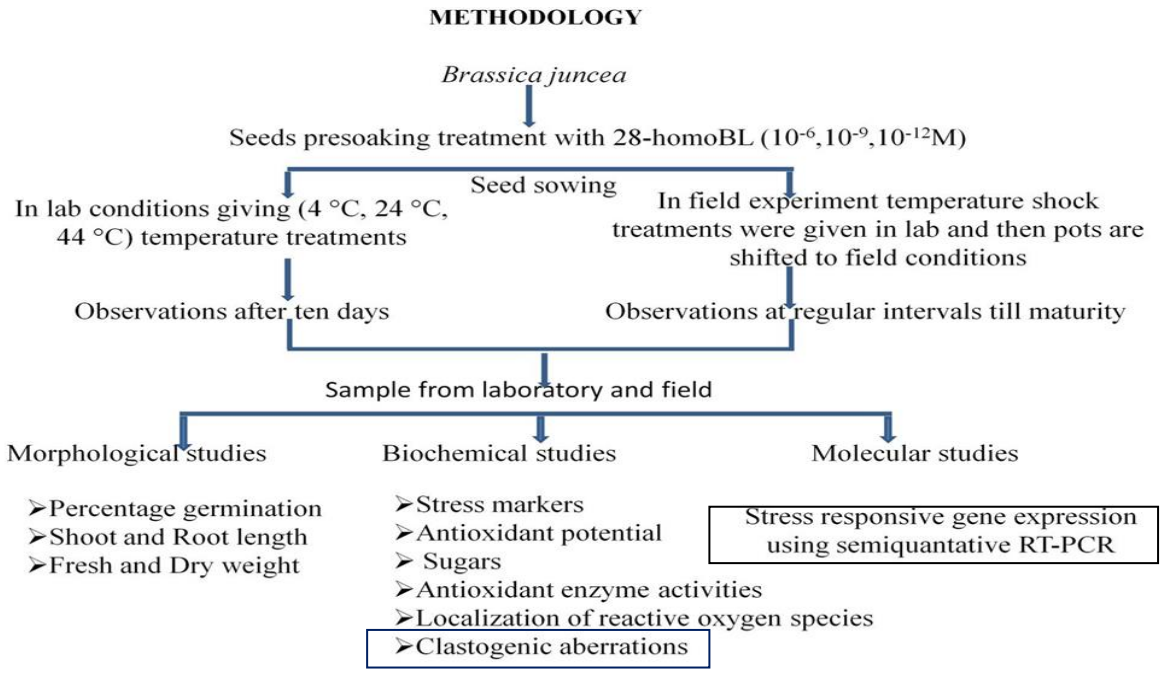
Dr. Geetika Sirhindi
Assistant Professor in Plant Physiology
Department of Botany,
Punjabi University,
Patiala-147002

4. Significant Achievements:

- Plants exposed to extreme temperature (4 ºC and 44 ºC) treatments alone showed stress symptoms in terms of decreased rate of shoot length, root length and fresh as well as dry weights as compared to control (DW) seedlings raised under normal conditions (25 ºC).
- Plants raised under controlled laboratory conditions after giving different temperatures showed increased level of proline, glycine betaine, H_2O_2 and MDA content. Which was further hiked when supplemented with different concentrations of 28-homoBL as compared to control (distilled water) seedlings.
- Antioxidant Defense System of *Brassica juncea* L. seedlings was enhanced under temperature treatments. This enhancement was further hiked when seedlings were supplemented with different concentrations of 28-homobrassinolide.
- Plants exposed to extreme temperatures (4 ºC and 44 ºC) and (10^{-9}M) treatment of 28-homobrassinolide and their combination showed up regulation of gene expression of antioxidants such as SOD, CAT, APOX, GR, DHAR and MDHAR in *Brassica juncea* L.
- Plants exposed to extreme temperature (4 ºC and 44 ºC) treatments alone showed stress symptoms in terms of increased oxidative damage and dead cells assay as compared to control (DW) seedlings raised under normal conditions (25 ºC).

- ❖ Plants raised under controlled laboratory conditions after giving different temperatures showed increased level of sugars. Which was further enhanced when supplemented with different concentrations of 28-homoBL as compared to control (distilled water) seedlings.
- ❖ Plants raised under controlled laboratory conditions after giving different temperatures showed increased level mitotic index. Which was further regulated when supplemented with different concentrations of 28-homoBL as compared to control (distilled water) seedlings.
- ❖ Clastogenic aberrations was increased under temperature treatments (4 ºC and 44 ºC) and decreased under 28-homoBL treatment alone but when supplementation of both is given then less number of meiotic aberrations was observed.

5. Flow Chart of Work Plan and Methodology:



6. Work done (1 April 2013 to 31st March 2017)

Seed germination (%)

Seed germination under laboratory conditions was measured on 3rd day after sowing while Percentage of germination was calculated using the formula:

$$\text{Percentage germination} = \frac{\text{Germinated Seeds}}{\text{Total Seeds}} \times 100$$

Shoot length (cm)

In laboratory shoot length (cm) was measured using scale centimeter on 10^{th} DAS in all treatments.

Root length (cm)

Root length (cm) was measured using standard centimeter scale on 10^{th} DAS.

Fresh weight and Dry weight (mg)

In laboratory experiments, 25 seedlings were taken for the measurement of fresh weight on 10^{th} DAS and then samples were dried in oven at 60±2 ºC for overnight for dry weight (mg) measurement. For weighing digital balance of Adventurer ^{TM}Ohaushingreadiability 0.001 g was used.

O^2- Radical Detection

Superoxide oxide anion (O^2-**)** radical was detected by Doke (1983) using nitro blue tetrazolium (NBT). Leaves of *B. juncea* was taken and vacuum-infiltrated in 0.05M sodium phosphate buffer (pH 7.5) containing 0.05% NBT (Nakarai) and then were incubated with NBT solution for 1 h. The incubated specimens were fixed in ethanol to stop the NBT reaction. The specimens were maintained in ethanol at room temperature overnight to remove chlorophyll components.

H_2O_2 Radical Detection

H_2O_2 Radical was detected following Thordal-Christensen *et al*. (1997). The leaves of *B. juncea* were vacuum-infiltrated in DAB (Nakarai) solution (1 mg/ml) and were incubated with DAB solution for 8h at room temperature in the dark and then fixed in a mixture of ethanol and acetic acid (96: 4, v/v) overnight.

Studies on cell viability (Yang, 1986)

For this, root tips of fresh seedlings were taken and then dipped in 10 µM solution of FDA for 20 min. These root tips were placed on glass slide covered with cover slip and fluorescence of fluorescein diacetate was measured with the help of Nikon Laser Scanning Confocal microscopy using emission/excitation maximum 488-494 nm.

Studies on cell non viability (Truernit and Haseloff, 2008)

For this, root tips of fresh seedlings were taken and then stained with 25 µM (PI) propidium iodide solution that can penetrate cell membranes of dead or dying cells. The coverslips were placed on root tips. Then, the fluorescence was measured with the help of Nikon Laser Scanning Confocal Microscopy using emission/excitation maximum is 535–617 nm.

MDA content

MDA Content was estimated according to Heath and Packer (1968) involving the principle that MDA is formed through auto-oxidation and enzymatic degradation of polyunsaturated fatty acids. This secondary end product of the oxidation of polyunsaturated fatty acids reacts with two molecules of thiobarbituric acid (TBA) via an acid-catalyzed nucleophilic-addition reaction yielding a pinkish-red chromagen with an absorbance at 532 and 600 nm.

H_2O_2 content

H_2O_2 content was estimated according to Velikova *et al.* (2000). Fresh plant tissue was crushed and centrifuged in 0.1 % trichloroacetic acid for 15 minutes. Then in the supernatant 10 mM Potassium phosphate buffer was added followed by 1 ml addition of potassium iodide and the absorbance was taken at 390 nm.

Proline Content

Proline content was estimated by the method of Bates *et al.* (1973) involving the principal that proline is made to react with ninhydrin in acidic condition to form the chromophore and the absorbance was taken at 520 nm.

Glycine betaine estimation

Glycine betaine estimation will be done according to the method of Greive and Grattan (1995). Plant tissue was ground and mechanically shaken with 20 ml deionized water for 24 hours at 25 ºC. Samples were filtered and centrifuged and the filtrates were diluted 1: 1 with 2N H_2SO_4 and aliquots were cooled in ice water for 1 hr. then cold KI-I_2 reagent was added and the reactants were gently stirred with a vortex mixture. Then tubes were stored at 4 ºC for 16 hours after this centrifuged at 0 ºC and into the pellet 9ml 1, 2-Dichloroethane was added and O.D was noted at 365nm.

Total sugars content

Total sugars content was estimated by following Loewus. (1952). Known weight of dried plant material was homogenised in 80% of ethanol then centrifuged at 3000x g for 15 minutes

and the extract was collected for sugars estimation. For total sugars 0.05ml of extract was diluted to 2ml by distilled water and adds 3ml cold anthrone reagent was added into it and mixed thoroughly. Then mixture was heated for 10 min in boiling water bath and cooled rapidly at room temperature. O.D. was recorded at 630 nm.

Analysis of sugars in Brassica juncea* L. *through HPLC

Sample preparation

The seedlings were collected at tenth days after sowing for sugar profiling analysis. The samples were extracted by crushing 1 gm plant material in 3 ml double distilled water. Then homogenate was centrifuged for 30 minutes. The extracts were filtered through 0.45 µm filter and injected (20µl) into HPLC for analysis.

Chemicals and reagents

Ultra pure water was purchased from Merck and the sugar reference compounds were purchased from himedia. All the references were 98 % pure for HPLC analysis.

Standard preparation

For the quantitative analysis a mixed standard solution containing raffinose, sucrose, maltose, lactose, glucose, dextrose, xylose, rhamnose, galactose, fructose, inositol, mannitol, sorbitol and ribose (500 ppm) was prepared. The solutions were stored in dark glass bottles at 4°C. The standard solutions were filtered through 0.45 µm filter and injected (20µl) into HPLC for analysis.

Analytical determination

Samples were analyzed by HPLC on a waters system, comprising of HPLC pump, autosampler, photodiode array detector and Nucleosil 100-5, and sugar pack-1 column 5.0µm (250× 4.6mm). Single mobile phase containing water HPLC fitted with RI detector was used. The flow rate was 1ml/min and the injection volume was 20 µl. Water gave a complete separation of raffinose, sucrose, maltose, lactose, glucose, dextrose, xylose, rhamnose, galactose, fructose, inositol, mannitol, sorbitol in *Brassica juncea* L. extracts.

Total protein content

Total protein content was determined by following the method of Lowery *et al.* (1951)

Superoxide Dismutase (SOD) (EC 1.15.1.1)

Superoxide Dismutase (SOD) activity was estimated according to the methodology of Kono *et al*. (1978). The activity of SOD was determined by monitoring its ability to inhibit

photochemical reduction of nitro blue tetrazolium (NBT) dye by superoxide radicals, which was generated by the auto oxidation of hydroxylamine hydrochloride. The reduction of NBT was followed by an absorbance increase at 540 nm. One unit of the enzyme activity is the enzyme concentration inhibiting reduction of NBT by 50 %.

Guaiacol Peroxidase (POD) (EC 1.11.1.7)

Guaiacol Peroxidase (POD) was estimated according to the method given by putter (1974). POD catalyzes the reduction of H_2O_2 to water and oxygen, using guaiacol as a substrate. The rate of formation of oxidized guaiacol was followed spectrophotometerically at 436 nm. Enzyme activity was calculated using the extinction coefficient of 25 nM^{-1} cm^{-1}.

Catalase (CAT) (EC 1.11.1.6)

Catalase (CAT) activity was determined as per the method of Aebi (1983). Catalase catalyzes the decomposition of H_2O_2 to water and oxygen. The rate of decomposition of H_2O_2 was followed by decreased in absorbance at 240nm. Enzyme activity was determined using the extinction coefficient of 6.93×10^{-3} $Mm^{-1}Cm^{-1}$.

Ascorbate Peroxidase (APOX) (EC 1.11.1.11)

Ascorbate peroxidase (APOX) activity was estimated according to the method of Nakano and Asada (1981). Ascorbate peroxidase (APOX) catalyzes the reduction of H_2O_2 involving the oxidation of ascorbate. APOX activity was assayed by following the decrease in absorbance at 290 nm. Enzyme activity will be determined using the extinction coefficient of 2.8 mM^{-1} cm^{-1}.

Dehydro Ascorbate Reductase (DHAR; EC 1.8.5.1)

Dehydro ascorbate reductase was estimated by using the method of Dalton *et al*., 1986. 3 ml reaction mixture was prepared by mixing 50mM potassium phosphate buffer (pH 7.0), 0.2 m Mdehydroascorbate, 0.1 mM EDTA, 2.5 mM reduced gluthathione (GSH) in 100 µl enzyme extract. DHAR activity was measured by following the increase in absorbance at 265 nm.

Monodehydro Ascorbate Reductase (MDHAR; EC 1.6.5.4)

Monodehydro ascorbate reductase was estimated by using the method of Hossain *et al.*, 1984. The reaction mixture in the test tube contained 1.5 ml of potassium phosphate buffer (50 mM), 300 µl EDTA (3 mM),300 µl of NADPH (0.1 mM), 300 µl GSSG (1 mM) and600 µl of enzyme extract instead of which phosphate buffer (50 mM) was added. The activity of GR was calculated using extinction coefficients of 6.22 $mM^{-1}cm^{-1}$for NADPH at 340 nm.

Glutathione Reductase Activity (EC 1.8.1.7)

Glutathione Reductase Activity was determined by Carlberg and Mannervik, 1975. The reaction mixture in the test tube contained 1.5 ml of potassium phosphate buffer (50 mM), 300 µl EDTA (3 mM),300 µl of NADPH (0.1 mM), 300 µl GSSG (1 mM) and 600 µl of enzyme extract instead of which phosphate buffer (50 mM) was added. The activity of GR was calculated using extinction coefficients of 6.22 $mM^{-1}cm^{-1}$for NADPH at 340 nm.

Antioxidant potential

Antioxidant potential was determined by using DPPH according to the Miliauskas *et al*. (2004). Fresh leaves were taken homogenized and centrifuged at 4 ºC for 10 minutes. Then 400 µl aqueous extract was taken and 2.8 DPPH (80 µM in ethanol) was added into it and the absorbance was noted at 515nm.

Molecular analysis

RNA Isolation and quantitative real-time PCR (qRTPCR)

Total RNA was isolated from leaves of stressed and control samples with RaFlex™ solution as per the instructions (GeNei, India) and quantified spectrophotometrically. For real-time PCR, 250 µg total RNA was used for subsequent isolation of poly$(A)^+$-RNA using biotin labeled oligo-dT primer, which was used for making cDNA using RevertAid H minus first strand cDNA synthesis kit (Fermentas, Life Sciences). Primers for the real-time PCR were designed using MacVector 8.0 software (Table 1). The real-time PCR reaction was performed in 20 µl reaction mixture containing diluted cDNA sample as template and Power SYBR® Green PCR master mix, and 200 nM each of forward and reverse gene specific primers (Sigma-Aldrich St. Louis, MO). The reaction was performed using StepOne™ real-time PCR System (Applied Biosystems) with the following program: 95 °C (90 s) [94 °C (30 s), 55 °C (30 s), 72 °C (30 s)] × 40 cycles. To normalize the variance in the RNA quality and cDNA input, β-actin gene was used as the internal control in each case (Jain et al., 2006). The C_t values of samples in different RNA samples were normalized with Ct values of β-actin. The relative expression ratio under stress condition was calculated with respect to unstressed sample using REST 2005 version 1.9.12 software (Pfaffl et al., 2002).

Mitotic index

Roots were fixed and analyzed for Chromosomal Behavior in *Brassica juncea* L. Mitotic study revealed that 28-homoBL regulates the cell cycle without causing any irregularities and increases the mitotic activity. 28-homoBL regulates mitotic index to highest level in temperature (4 °C and 44 °C) treated seedlings as compared to alone temperature and controlled untreated seedlings. 28-homoBL increase the Mitotic index significantly in *B. juncea* L. as shown in (Table. 2).28-homoBL at nanomolar concentration increases the mitotic activity up to many fold as compared with controlled ones.

Meiotic aberrations

For meiotic chromosome counts, unopened floral buds of suitable sizes were fixed in a freshly prepared Carnoy's fixative (6:3:1 of alcohol: chloroform: glacial acetic acid) for 24 h at room temperature. The material was subsequently transferred to 70% alcohol and stored in a refrigerator until analyzed. Meiocytes were prepared by squashing the developing anthers, and staining them with acetocarmine (1%). Chromosome number was determined at diakinesis, metaphase and anaphases stage from the freshly prepared slides with a light microscope Olympus. 200-300 pollen mother cells (PMCs) were analyzed for meiotic behaviour at different stages, early prophase-I, metaphase-I/II, anaphase-I/II, telophase-I/II. To observe the effect of meiotic irregularities on microspore formation, sporad analysis was also carried out. Meiotic study revealed that 28-homoBL regulates the cell cycle without causing any irregularities but temperature (4 °C and 44 °C) treated seedlings as compared to alone 28 homoL causes meiotic aberrations such as laggard formation, disordered anaphase, cytomictic channel, monad, diad, triad and tetrad formation (Figure 4) and controlled untreated seedlings. 28-homoBL decrease the meiotic irregularities significantly in *B. juncea* L. as shown in (Table 3-5). 28-homoBL at nanomolar concentration showed significant reduction in meiotic aberrations as compared with controlled ones.

Statistical Analysis

The statistical analysis was executed by one way analysis of variance (ANOVA). The values represent the mean ± SE (n=5). $P \leq 0.05$ differ significantly.

7. Achievements of Defined Objectives:

OBJECTIVE DEFINED	OBJECTIVE MET
❖ Effects on changes in the level of stress markers: proline, glycinebetaine, H_2O_2 and MDA	

content in 10 day old seedlings (before, during and after giving temperature shock treatments (4, 24, 44 °C) to 7 day old seedlings upto 3 consecutive days for 5 hours daily and 24 hour recovery period) giving pre-sowing soaking treatments of brassinosteroids (28-homobrassinolide (10^{-6}, 10^{-9} and 10^{-12} M) to seeds of *B. juncea*.	Done
❖ To study the localization of reactive oxygen species (ROS) in cell organelles and antioxidant potential in 10 day old seedlings (before, during and after giving temperature shock treatments (4, 24, 44 °C) to 7 day old seedlings upto 3 consecutive days for 5 hours daily and 24 hour recovery period) giving pre-sowing soakingtreatments of brassinosteroids 28-homobrassinolide (10^{-6}, 10^{-9} and 10^{-12} M) to seeds of *B. juncea*.	Done
❖ Estimation and characterization of sugars in 10 day old seedlings (before, during and after giving temperature shock treatments (4, 24, 44 °C) to 7 day old seedlings upto 3 consecutive days for 5 hours daily and 24 hour recovery period) giving pre-sowing soaking treatments of brassinosteroids (28-homobrassinolide (10^{-6}, 10^{-9} and 10^{-12} M) to seeds of *B. juncea*.	Done
❖ Gene expression of antioxidant enzymes and total proteins using semi-quantitative RT-PCR in 10 day old seedlings (before, during and after giving temperature shock treatments (4, 24, 44 °C) to 7 day old seedlings upto 3 consecutive days for 5 hours daily and 24 hour recovery period) giving	

pre-sowing soaking treatments of brassinosteroids (28-homobrassinolide (10^{-6}, 10^{-9} and 10^{-12} M) to seeds of *B. juncea* using β-actin as marker.	Done
❖ Study the clastogenic aberrations at mitotic and meiotic stage upto F_1 generation and reproductive potential in plants raised under field conditions after giving pre-sowing soaking temperature (4, 24, 44 °C) and brassinosteroids (28-homobrassinolide (10^{-6}, 10^{-9} and 10^{-12} M) treatments to seeds of *B. juncea*.	Done

8. Summary of Work done so far:

1. Literature consulted: Literature relevant to the problem has consulted and collected during first year of project, in form of soft copies and print outs of research papers relevant to the work, from library of Botany Department, Punjabi University, Patiala and Internet.

2. Biochemical activities were done under laboratory conditions and localization of reactive oxygen species from field grown treated and untreated seeds and raised seedlings using standardized protocols and techniques.

3. Effect of temperature alone and supplemented with different concentrations of 28-homoBL on lipid per oxidation, proline, glycine betaine and H_2O_2 content were done from laboratory using standardized protocols and techniques.

4. Antioxidant gene expression was done under laboratory conditions using real time PCR technique.

5. Effect of temperature alone and supplemented with different concentrations of 28-homoBL on mitotic index were done from laboratory using microscopic techniques.

6. Effect of temperature alone and supplemented with different concentrations of 28-homoBL on meiotic irregularities were done from laboratory using microscopic techniques.

9. Results:

a) Laboratory experiments

Two sets of experiments were conducted under controlled lab conditions in Plant growth chamber and following results were found:

- ✓ Extreme temperature treatments retarded growth to significant levels including plant height, fresh as well as dry weights.

- ✓ All 28-homoBL treatments help in mitigating negative effect of extreme temperature (4 ºC and 44 ºC) stress and help in improving plant height and fresh as well as dry weights when compared with only temperature treated seedlings and 10^{-9}M 28-homoBL was found to be the best.
- ✓ Oxidative damage was more pronounced under stress conditions compared to that of distilled water and 28-homoBL alone.
- ✓ Proline, Glycinebetaine, H_2O_2 and MDA contents were decreased with 28-homoBL treatment which otherwise hiked under extreme temperatures (4 ºC and 44 ºC).
- ✓ Sugar content and profiling was increased under extreme temperature treatments which was further increased when supplemented with different concentrations of 28-homoBL.
- ✓ Degradation in total protein quantity, under high temperature treatment has been observed.
- ✓ Activities of antioxidant enzymes (SOD, CAT, APOX, GR, DHAR and MDHAR) increased with temperature treatment.
- ✓ Gene expression antioxidant enzymes (SOD, CAT, APOX, GR, DHAR and MDHAR) was increased under extreme temperature treatments which was further increased when supplemented with different concentrations of 28-homoBL.

b) Field experiments

Field experiments were done in first year by sowing the seeds of *Brassica juncea* L. after treated them with said treatments of temperature and 28-homoBL, in month of September and observations were taken as per plan of work. The results are summarized as:

- ✓ Formation of reactive oxygen species such as superoxide radical ($O.^2$) and hydrogen peroxide (H_2O_2) were more pronounced under temperature treatments.
- ✓ 28-homoBL treatments helped to reducing the negative effect of extreme temperature (4 ºC and 44 ºC) stress and hence lesser formation of reactive oxygen species in seedlings treated with different concentrations of 28-homobrassinolide.
- ✓ Mitotic index was enhanced under temperature treatment and reduces under 28-homoBL alone and their supplementation with temperature treatment.
- ✓ Meiotic irregularities were more pronounced under stress conditions compared to that of distilled water and 28-homoBL alone.

10. Conclusion

The present study reveals that temperature is one of the important environmental factors which is responsible for the normal functioning of plant growth and development and regulating the metabolism of plant in very efficient manner. Extreme temperature stress both low and high has substantial influence on metabolism and thus alter gene expression of antioxidant genes and ultimately lead to creation of imbalance between synthesis of free radicals and their scavenging. 28-Homobrassinolide, an isoform of brassinosteroides, a plant growth regulator of new generation, help in regulating the plant growth and development through various processes related to normal growth of plant. Latest report explored stress-ameliorative properties of 28-homoBL under various biotic and abiotic stress conditions in large number of crops. In present investigations, it has been explored that these 28-homoBL also work efficiently in protecting the plants from extreme temperatures in *B. juncea* L. by modulating Asada-Halliwell pathway and also by controlling mitotic and meiotic abnormalities under stress conditions.

11. Attach publications/patents/technology and product Annexure-VI

SIGNATURE OF THE PRINCIPLE INVESTIGATOR

ANNEXURE-V

EFFECT OF 28-HOMOBRASSINOLIDE ON OXIDATIVE AND SUGAR METABOLISM AND REPRODUCTIVE POTENTIAL OF *BRASSICA JUNCEA* L. UNDER THERMAL STRESS

Project: MRP F. NO. 42-920-2013 (SR) Dated: 14 March 2013

Objectives of the Project:

1. Effects on changes in the level of stress markers: proline, glycinebetaine, H_2O_2 and MDA content in 10 day old seedlings (before, during and after giving temperature shock treatments (4, 24, 44 °C) to 7 day old seedlings upto 3 consecutive days for 5 hours daily and 24 hour recovery period) giving pre-sowing soaking treatments of brassinosteroids (28-homobrassinolide (10^{-6}, 10^{-9} and 10^{-12} M) to seeds of *B. juncea*.
2. To study the localization of reactive oxygen species (ROS) in cell organelles and antioxidant potential in 10 day old seedlings (before, during and after giving temperature shock treatments (4, 24, 44 °C) to 7 day old seedlings upto 3 consecutive days for 5 hours daily and 24 hour recovery period) giving pre-sowing soaking treatments of brassinosteroids (28-homobrassinolide (10^{-6}, 10^{-9} and 10^{-12} M) to seeds of *B. juncea*.
3. Estimation and characterization of sugars in 10 day old seedlings (before, during and after giving temperature shock treatments (4, 24, 44 °C) to 7 day old seedlings upto 3 consecutive days for 5 hours daily and 24 hour recovery period) giving pre-sowing soaking treatments of brassinosteroids (28-homobrassinolide (10^{-6}, 10^{-9} and 10^{-12} M) to seeds of *B. juncea*.
4. Gene expression of antioxidant enzymes and total proteins using semi-quantitative RT-PCR in 10 day old seedlings (before, during and after giving temperature shock treatments (4, 24, 44 °C) to 7 day old seedlings upto 3 consecutive days for 5 hours daily and 24 hour recovery period) giving pre-sowing soaking treatments of brassinosteroids (28-homobrassinolide (10^{-6}, 10^{-9} and 10^{-12} M) to seeds of *B. juncea* using β-actin as marker.
5. Study the clastogenic aberrations at mitotic and meiotic stage upto F_1 generation and reproductive potential in plants raised under field conditions after giving pre-sowing

soaking temperature (4, 24, 44 °C) and brassinosteroids (28-homobrassinolide (10^{-6}, 10^{-9} and 10^{-12} M) treatments to seeds of *B. juncea*.

Year wise Plan of Work and Targets to be achieve:

1st Year:

It will include study of literature, standardization of the techniques involved for the qualitative and quantitative estimation of protein and sugars, ROS and antioxidant potential. The effects on stress markers, ROS localization in cell organelles and sugars (qualitative and quantitative) will be studied in seedlings of *B. juncea*, which will be exposed to different treatments of temperature after giving pre-sowing soaking treatments of different concentrations of brassinosteroids. The plants will be grown in Plant growth chamber for the study of above mentioned parameters.

2nd Year:

It will include the protein extraction and estimation of protein and antioxidant enzymes using SDS-PAGE. Protein profiling will be done along with gene expression analysis of proteins and antioxidant enzymes will be carried out using semiquantitaive RT-PCR. Pre treated seeds with temperature and BR will sown in plant growth chamber for mitotic studies and in field for meiotic and reproductive studies

3rd Year:

The remaining work of 2nd year will be continued. Further, the work done in 1st year will be reviewed. Comparative studies will be carried out to compare the affectivity of various brassinosteroids, their different concentrations and different methods. The statistical tools such as standard deviation, standard error, coefficient of variation, linear and non-linear regression and correlation analysis etc. will be applied. The significant work will be published in journals of National/International repute, presented in various conferences/symposia. Attempts will be made to patent the significant contributions.

ANNEXURE-VI

List of Paper Published:

1. Kaur, Harpreet, **Geetika Sirhindi**, Renu Bhardwaj, M. N. Alyemeni, Kadambot H. M Siddique and Parvaiz Ahmad (2018). 28-homobrassinolide regulates antioxidant enzyme activities and gene expression in response to salt- and temperature-induced oxidative stress in *Brassica juncea*. *Scientific Reports* 8:8735 | DOI:10.1038/s41598-018-27032-w.
2. **Sirhindi, G.**, Kaur, H., Bhardwaj, R., Sharma, P. and Mushtaq, R. (2017). 28-Homobrassinolide potential for oxidative interface in *Brassica juncea* under temperature stress. *Acta Physiol Plant*. 39:228. DOI 10.1007/s11738-017-2524-4.
3. Kaur, H., **Sirhindi, G.** and Bhardwaj, R. (2017). Influence of 28-homobrassinolide on photochemical efficiency in *Brassica juncea* under stress of extreme temperatures and salt. *Canadian Journal of Pure and Applied Sciences*. 11(2): 4205-4213.
4. Kaur H., **Sirhindi, G.**, Bhardwaj R. and Sharma, P. (2017). Interactive effect of 28-homobrassinolide and salinity on morpho-physiological attributes of 60 day old *Brassica juncea* plants. *International Journal of Advance Research in Science and Engineering*. 6(3): 315-324.
5. Kaur, H., **Sirhindi, G.**, Bhardwaj, R. and Sharma, P. (2015). 28-homobrassinolide modulation of osmolytes in *Brassica juncea* L. under salt stress. *International Journal of Scientific Research*. **4:** 27-29.
6. Harpreet, K., **Sirhindi, G.** and Bhardwaj, R. (2015). Alteration of antioxidant machinery by 28-homobrassinolidein *Brassica juncea* L. under salt stress. *Advances in Applied Science Research*. **6(4):** 166-172.
7. Kumar, S., **Sirhindi, G.** and Bhardwaj, R. (2014). 28-Homobrassinolide-Induced Exaggerated Growth, Biochemical Molecular Aspects of Brassica Juncea L. RLM-619 Seedlings under High Temperature Stress. *J Plant Biochem Physiol.* **2(2):** 127. doi:10.4172/2329-9029.1000127.
8. Kaur, H., **Sirhindi, G.**, Bhardwaj, R., Sharma, P. and Mudasir, M. (2014). 28-homobrassinolide modulate antenna complexes and carbon skeleton of *Brassica juncea* L. under temperature stress. *Journal of Stress Physiology and Biochemistry*. **10(3):** 186-196.

List of international/national conferences/workshops attended and paper presentation

1. 2 Days International Conference on Recent Innovations in Engineering, Science, Humanities and Management (ICRIESHM-17) held at Dev Samaj College for Women, Ferozepur, Panjab, 18-19 March 2017.

2. International symposium on plant signaling and behavior organized by Department of Botany Delhi University, 7-10 March 2014

3. National conferences on basic and applied researches in plants and microbes organized by Department of Botany, Punjabi university, Patiala. 3-5 November 2016.

4. 2nd national symposium on emerging trends in biological sciences organized by Multani Mal Modi College, Patiala, 12 November 2016.

5. 18th punjab science congress, Innovative trends of science and technology in current scenario organized by Punjab academy of sciences in Desh Bhagat University, Mandi Gobindgarh, February 7-9, 2015.

6. 7th national conference on recent adavances in chemical, biological and environmental sciences organized by Multani Mal Modi College, Patiala, 30-31 January 2015.

7. National conference on Perspective and trends in plant sciences and biotechnology organized by Department of botany, Punjab university, Chandigarh and society of plant research India, 21-23 February 2014.

8. National symposium on emerging trends in botanical sciences organized by Department of Botany, Punjabi university, Patiala. 17-18 February 2014.

9. National seminar on perspectives in plant and environmental sciences organized by Guru Nanak Dev University, Amritsar, 11-12 March, 2014.

SHORT TRAINING COURSS (WORKSHOPS)

Short training course on "Basic techniques in molecular biology" organized by Sher –e-Kashmir University of Agricultural sciences and technology of Jammu school of biotechnology, 6-12 November 2013.

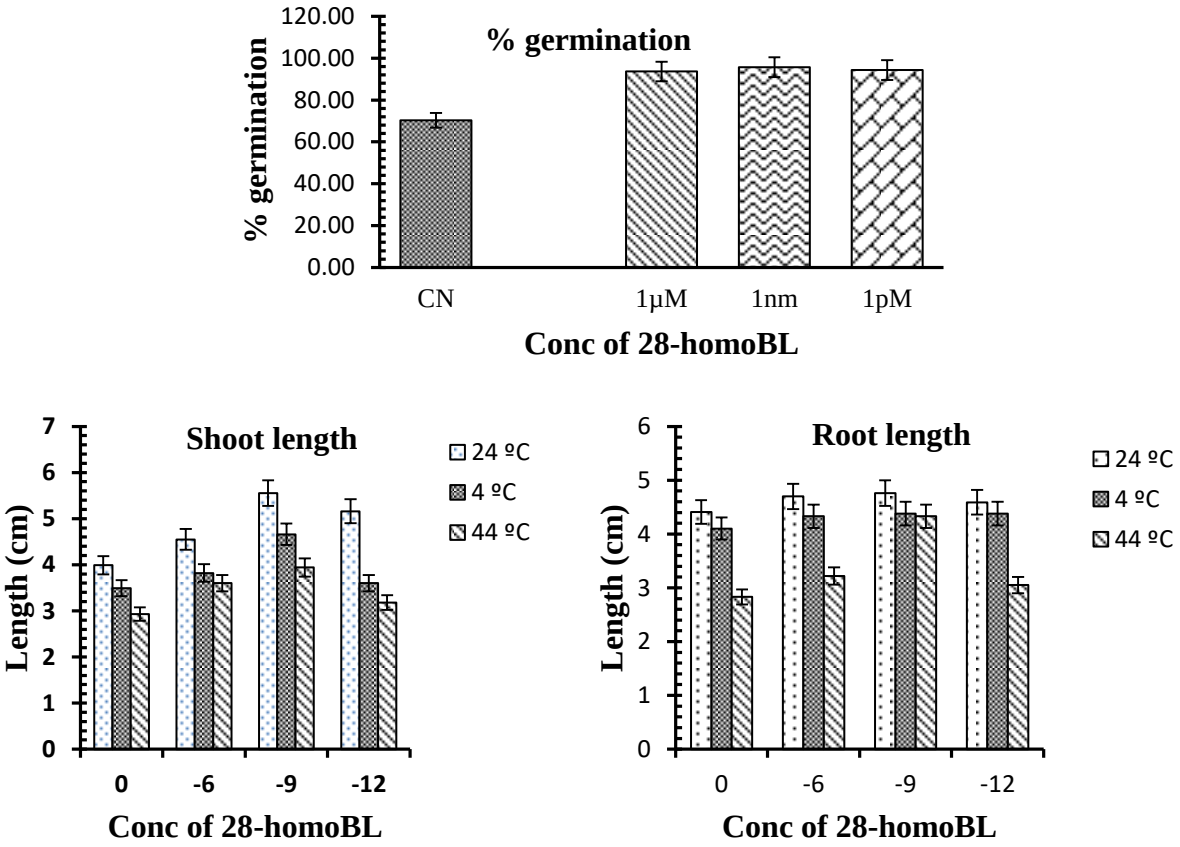


Figure: 1- Effect of 28-homoBL on % germination, shoot and root length of *Brassica juncea* L. under temperature stress

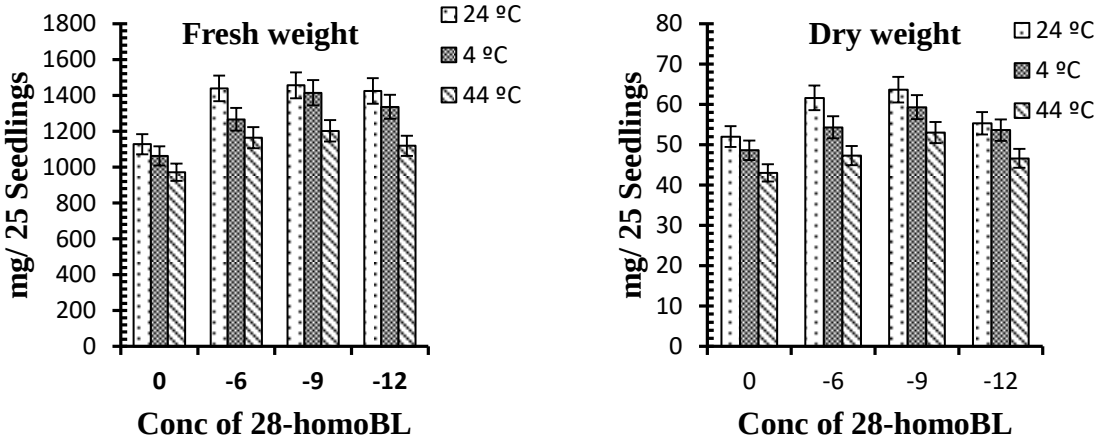


Figure: 2- Effect of 28-homoBL on fresh and dry weight of *Brassica juncea* L. under temperature stress

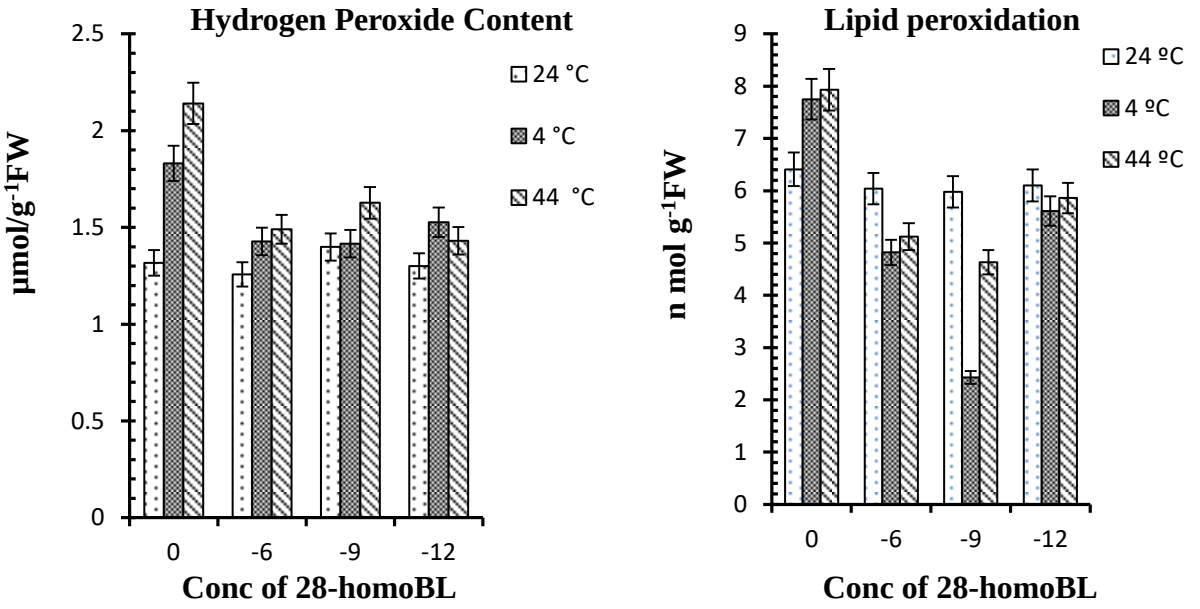


Figure: 3- Effect of 28-homoBL on hydrogen peroxide and MDA content of *Brassica juncea* L. under temperature stress

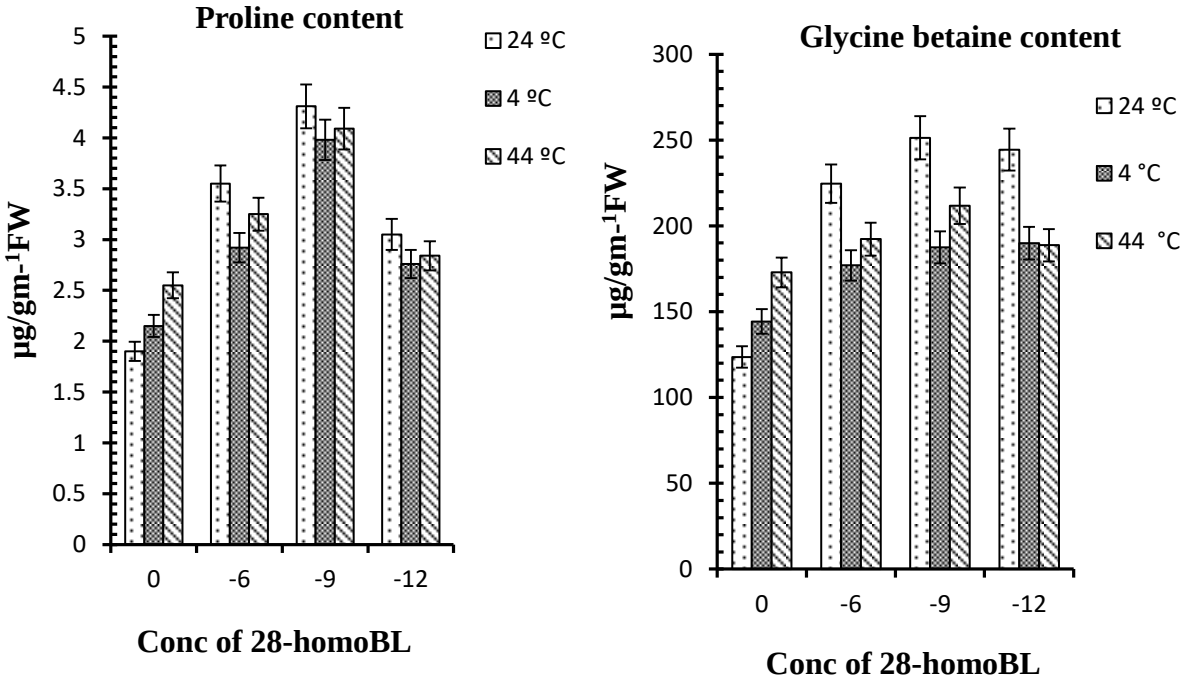


Figure: 4- Effect of 28-homoBL on proline and glycine betaine content of *Brassica juncea* L. under temperature stress

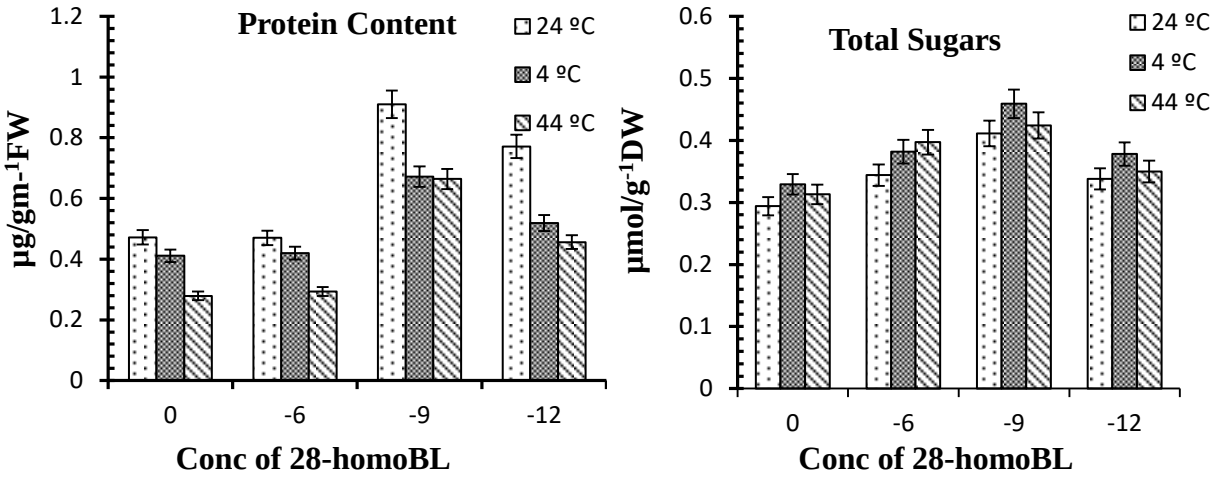


Figure: 5- Effect of 28-homoBL on protein and total sugars content of *Brassica juncea* L. under temperature stress

Treatments	**24 °C Temp**	**4 °C Temp**	**44 °C Temp**	**10^{-9}M HBL**	**10^{-9}+4 °C**	**10^{-9}+44 °C**
Raffinose (mg g^{-1} FW)	425.4	161.8	4.96	64.3	0	635.05
Sucrose (mg g^{-1} FW)	358.3	294.9	382.5	599.4	2500.1	0
Maltose (mg g^{-1} FW)	358.3	294.9	382.5	599.4	2500.1	0
Lactose (mg g^{-1} FW)	358.3	294.9	382.5	599.4	2500.1	0
Glucose (mg g^{-1} FW)	0	21.4	48.8	48.4	79.1	5.6
Dextrose (mg g^{-1} FW)	0	21.4	48.8	48.4	79.1	5.6
Xylose (mg g^{-1} FW)	0	139	339.6	7.4	58.9	134.9
Rhamnose (mg g^{-1} FW)	0	139	339.6	7.4	58.9	134.9
Galactose (mg g^{-1} FW)	0	139	339.6	7.4	58.9	134.9
Fructose (mg g^{-1} FW)	1067.7	495.7	2020.7	0	1302.3	492.4
Inositol (mg g^{-1} FW)	1067.7	495.7	2020.7	0	1302.3	492.4
Mannitol (mg g^{-1} FW)	0	0	227.7	327.4	72.08	50.2
Sorbitol (mg g^{-1} FW)	174.7	154.4	127.5	176.3	0	22.2
Ribose (mg g^{-1} FW)	0	0	0	0	0	0

Table: 1- Effect of different concentrations of 28-HBL (10^{-9}M) and temperature (4 °C and 44 °C and 24 °C as control) on the levels of different sugars in *Brassica juncea* L. seedlings and their combinations.

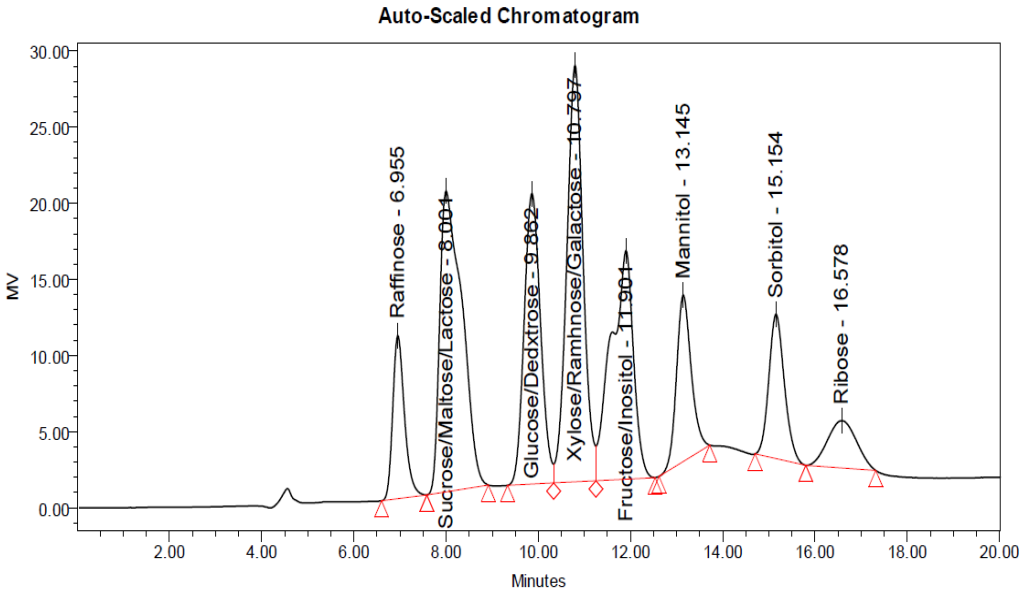


Figure 6- HPLC Chromatogram of standards viz. raffinose, sucrose, maltose, lactose, glucose, dextrose, xylose, rhamnose, galactose, fructose, inositol, mannitol, sorbitol and ribose using water as mobile phase.

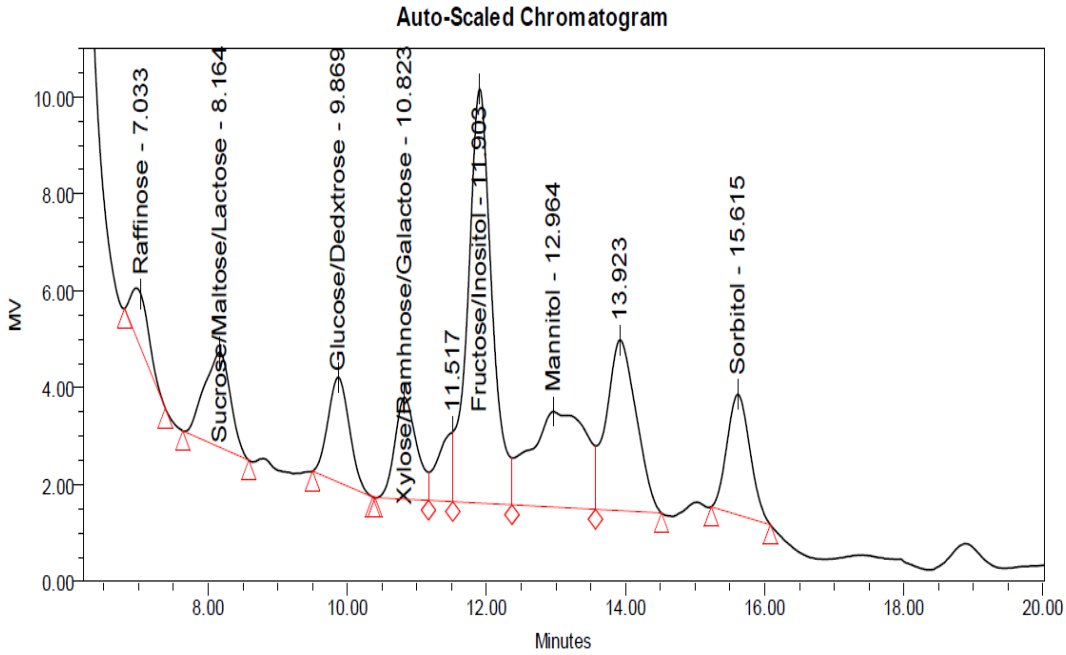


Figure 7-HPLC chromatogram of *Brassica juncea* L. seedlings treated with control distilled water (at 44 °C) showing the presence of raffinose, sucrose, maltose, lactose, glucose, dextrose, xylose, rhamnose, galactose, fructose, inositol and mannitol using water as mobile phase.

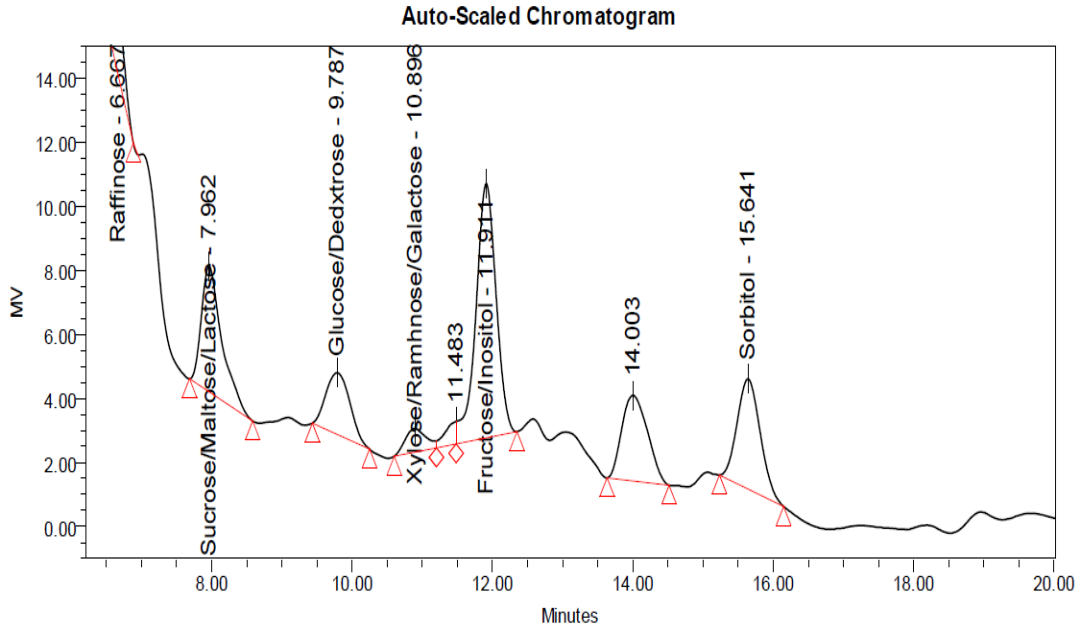


Figure 8- HPLC Chromatogram of *Brassica juncea* L. seedlings treated with (10^{-9}M HBL) showing the presence of raffinose, sucrose, maltose, lactose, glucose, dextrose, xylose, rhamnose, galactose, fructose, inositol and mannitol using water as mobile phase.

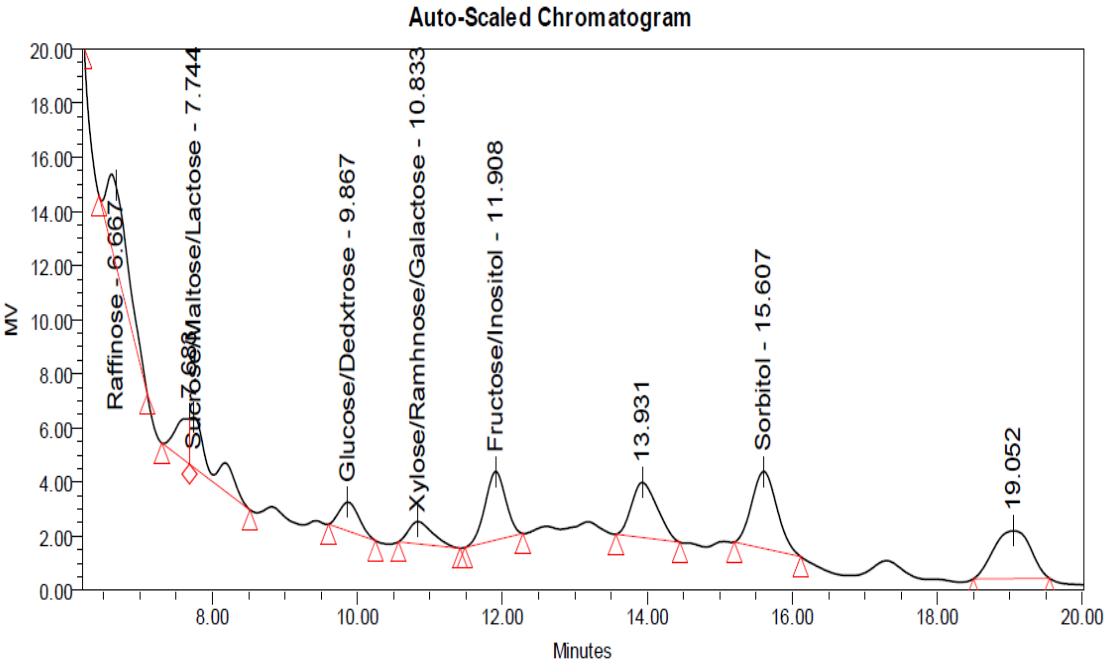


Figure 9- HPLC Chromatogram of *Brassica juncea* L. seedlings treated with (4 °C) temperature showing the presence of raffinose, sucrose, maltose, lactose, glucose, dextrose, xylose, rhamnose, galactose, fructose, inositol and mannitol using water as mobile phase.

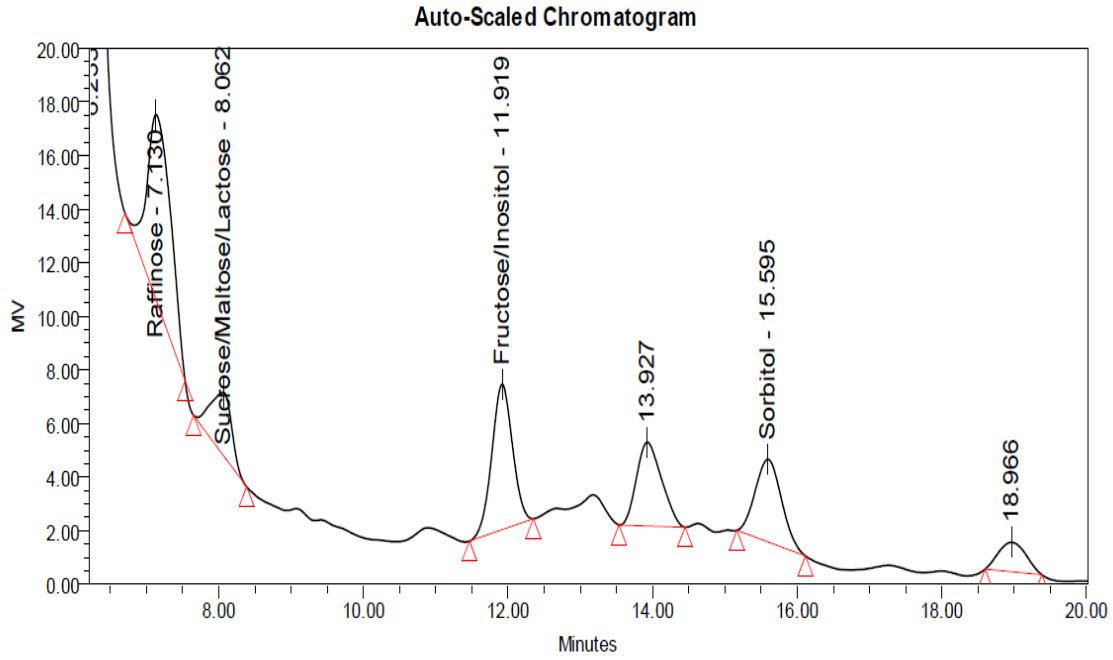


Figure 10- HPLC Chromatogram of *Brassica juncea* L. seedlings treated with (24 °C) temperature showing the presence of raffinose, sucrose, maltose, lactose, fructose, inositol and sorbitol using water as mobile phase.

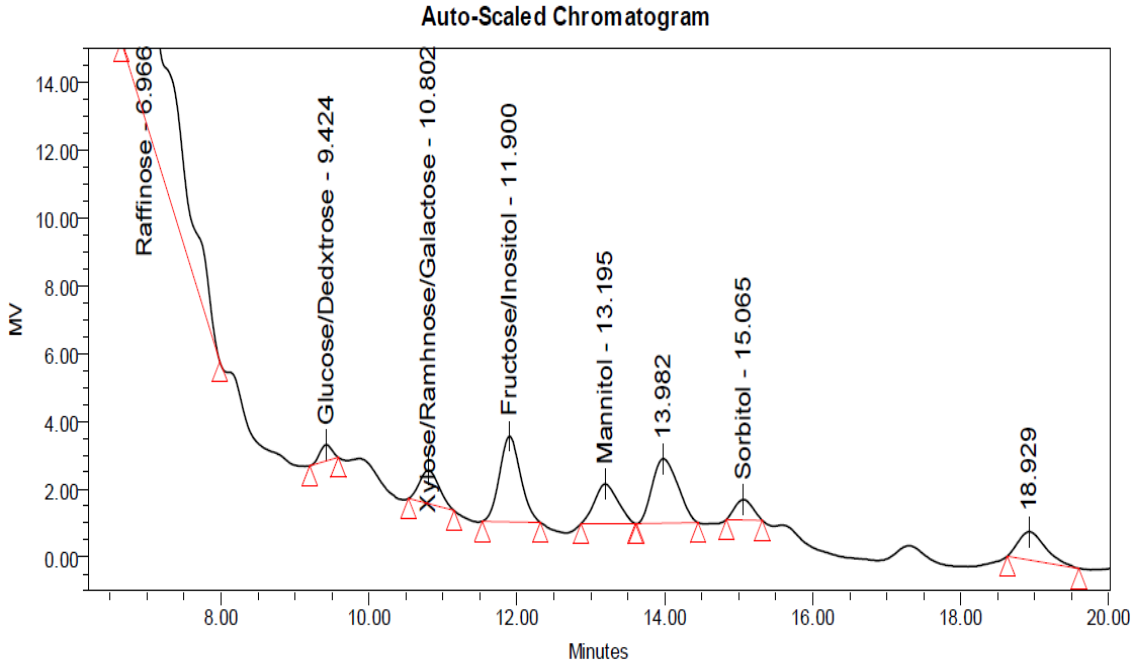


Figure 11- HPLC Chromatogram of *Brassica juncea* L. seedlings treated with (10^{-9}+44 °C) temperature showing the presence of raffinose, glucose,dextrose, xylose, rhamnose, galactose, fructose, inositol, mannitol and sorbitol using water as mobile phase.

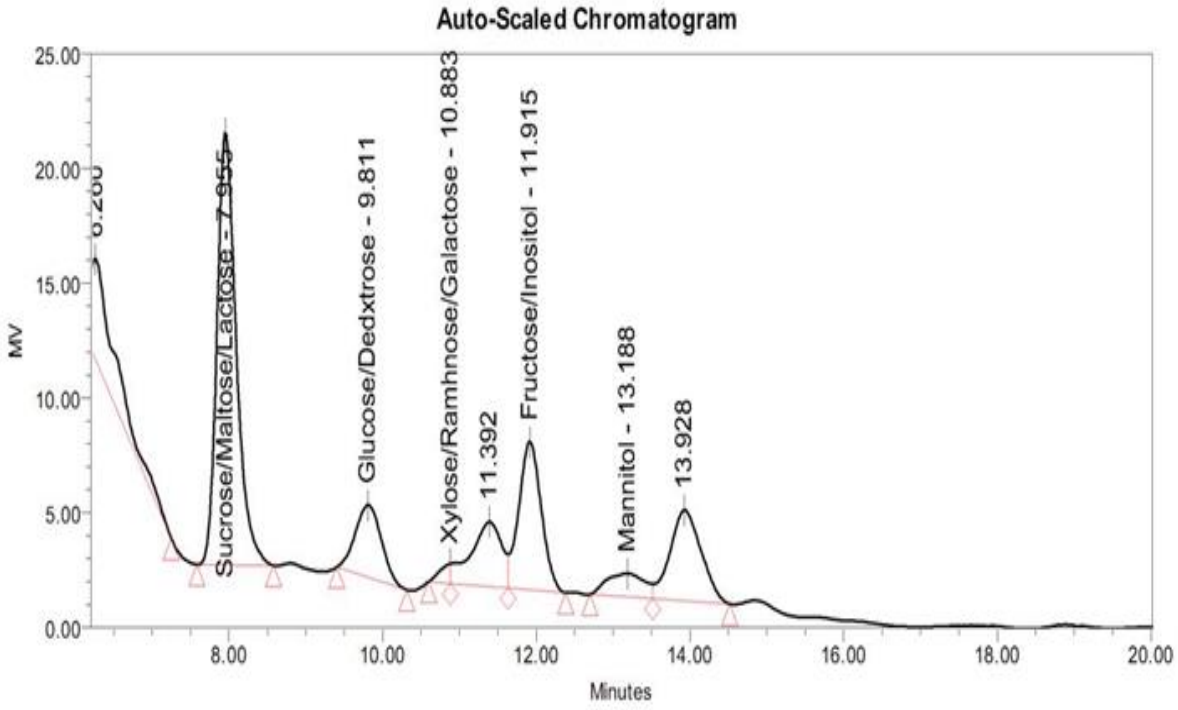


Figure 12- HPLC Chromatogram of *Brassica juncea* L. seedlings treated with (10^{-9}+4°C) temperature showing the presence of sucrose, maltose, lactose, glucose, dextrose, xylose, rhamnose, galactose, fructose, inositol and mannitol using water as mobile phase.

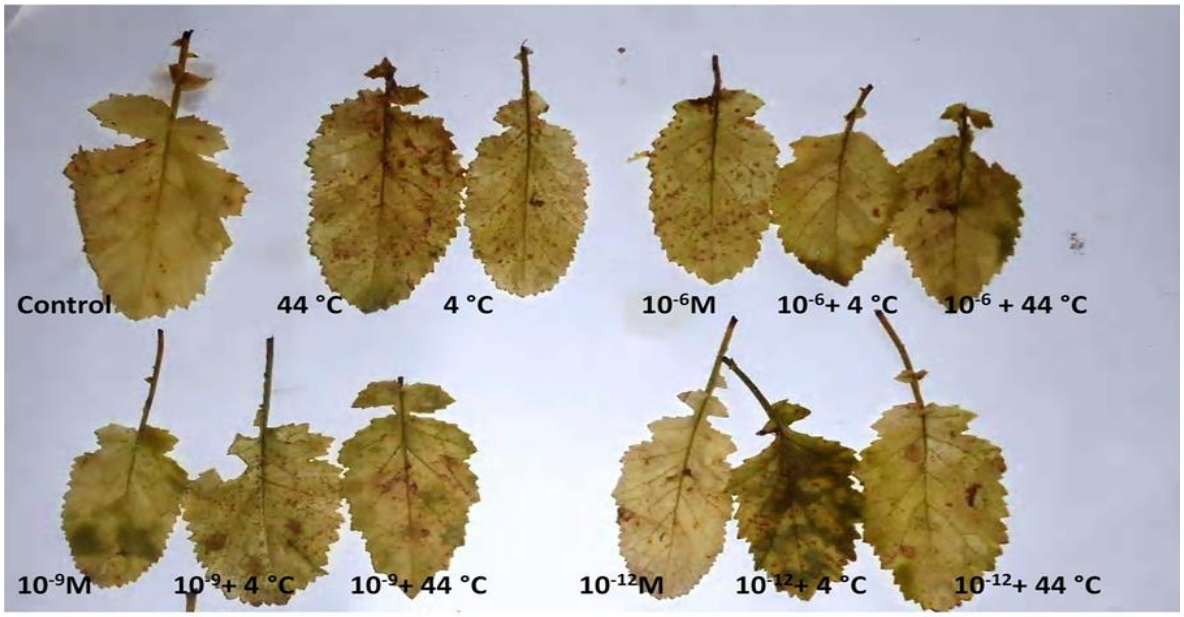


Figure 13- Effect of 28-homoBL on localization of hydrogen peroxide species in leaves of *Brassica juncea* L. under temperature stress

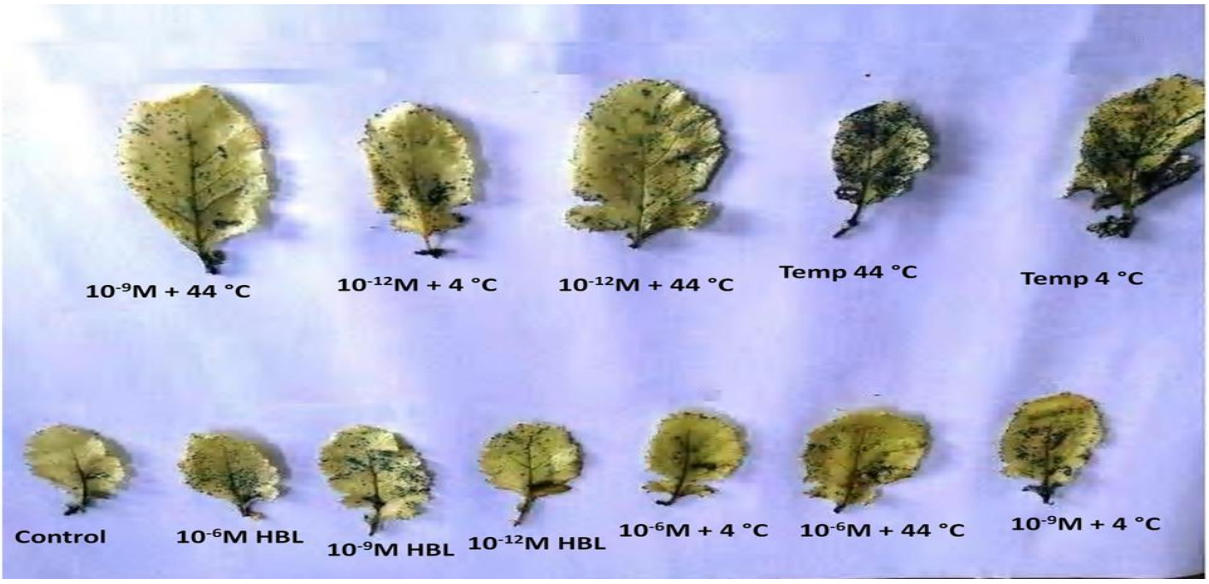


Figure 14- Effect of 28-homoBL on localization of superoxide species in leaves of *Brassica juncea* L. under temperature stress

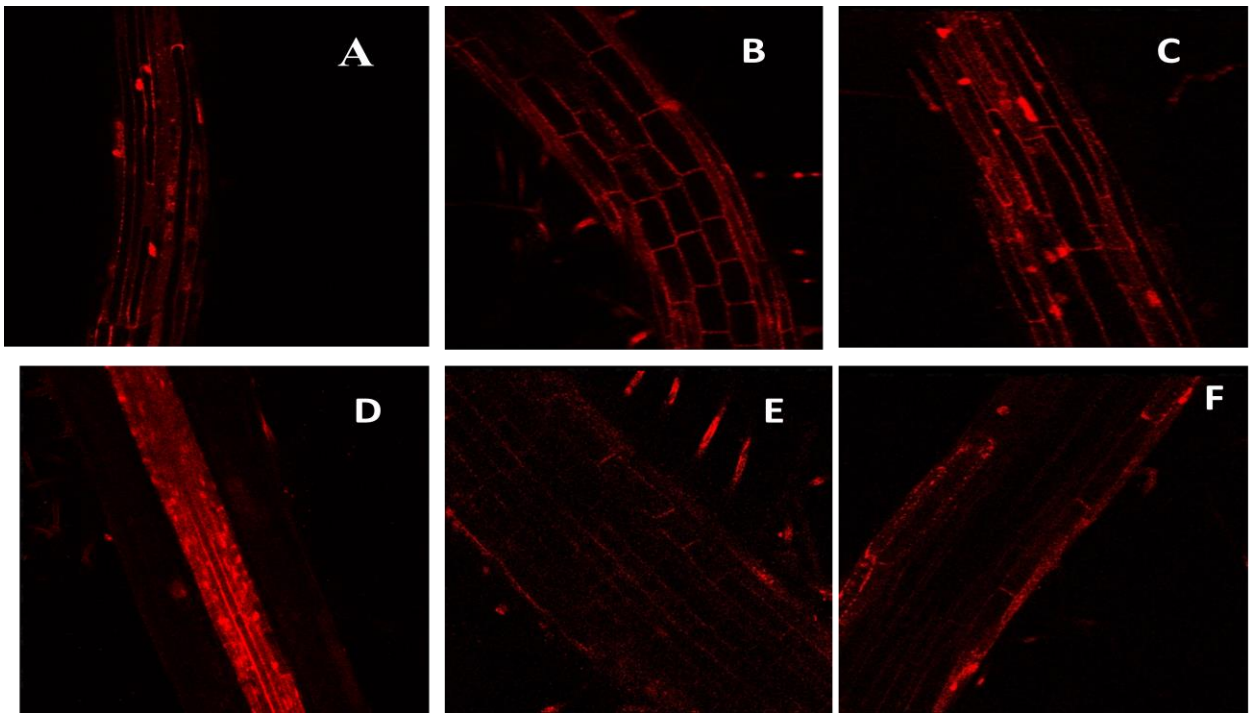


Figure 15- Cell non viability assay of *Brassica juncea* L. root tip after seed conditioning in water at 25 °C or solution of 10^{-9}M 28-homobrassinolide (28-homoBL) **A** control **B** 10^{-9}M (28-homoBL) **C** 4 °C temperature **D** 44 °C temperature **E** 10^{-9}M + 4°C temperature **F** 10^{-9}M + 44°C temperature The seedlings were grown at 25 °C in plant growth chamber for 7 days after 7 days seedlings were temperature shocked 4 °C, 44 °C for 5 hrs daily. Total oxidative damage were determined after seedlings were returned from temperature stress to 25 °C for 24 hrs

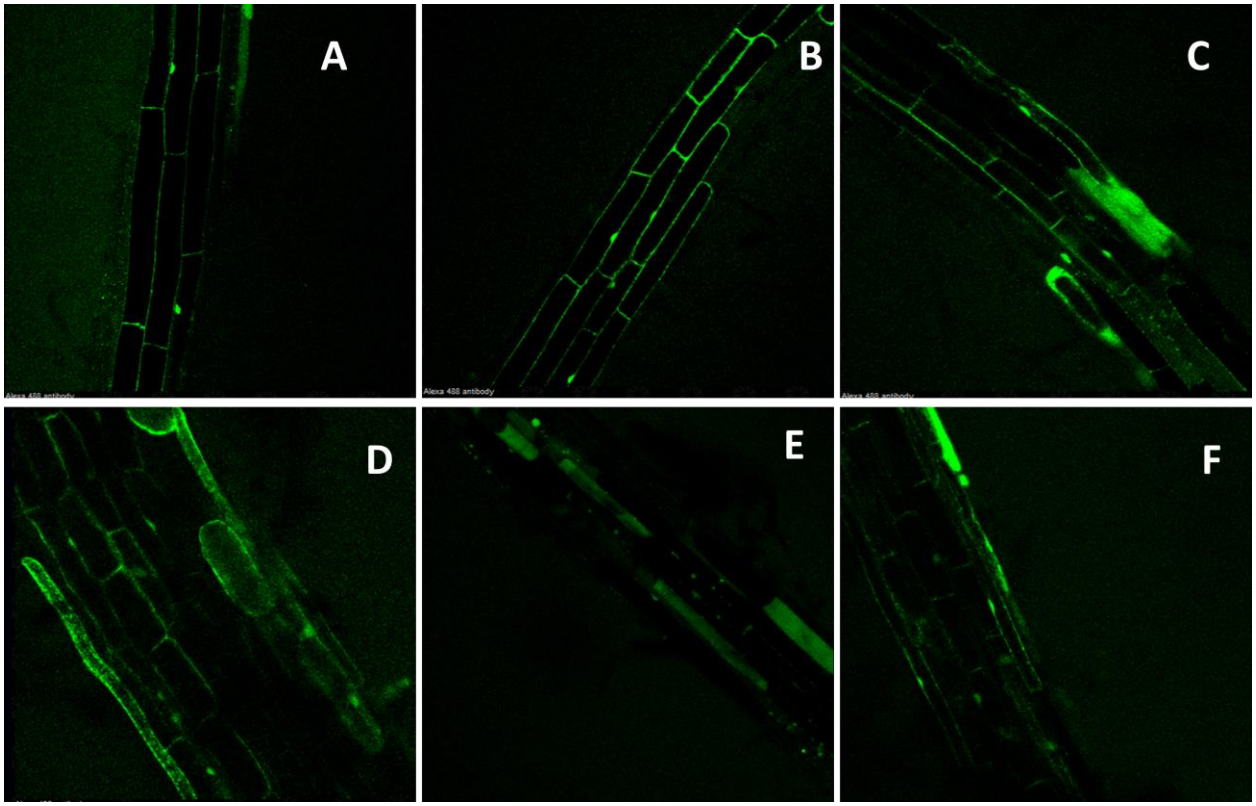


Figure 16- Cells viability assay of *Brassica juncea* L. root tip after seed conditioning in water at 25 °C or solution of 10^{-9}M 28-homobrassinolide (28-homoBL) **A** control **B** 10^{-9}M (28-homoBL) **C** 4 °C temperature **D** 44 °C temperature **E** 10^{-9}M + 4°C temperature **F** 10^{-9}M + 44°C temperature The seedlings were grown at 25 °C in plant growth chamber for 7 days after 7 days seedlings were temperature shocked 4 °C, 44 °C for 5 hrs daily. Total oxidative damage were determined after seedlings were returned from temperature stress to 25 °C for 24 hrs

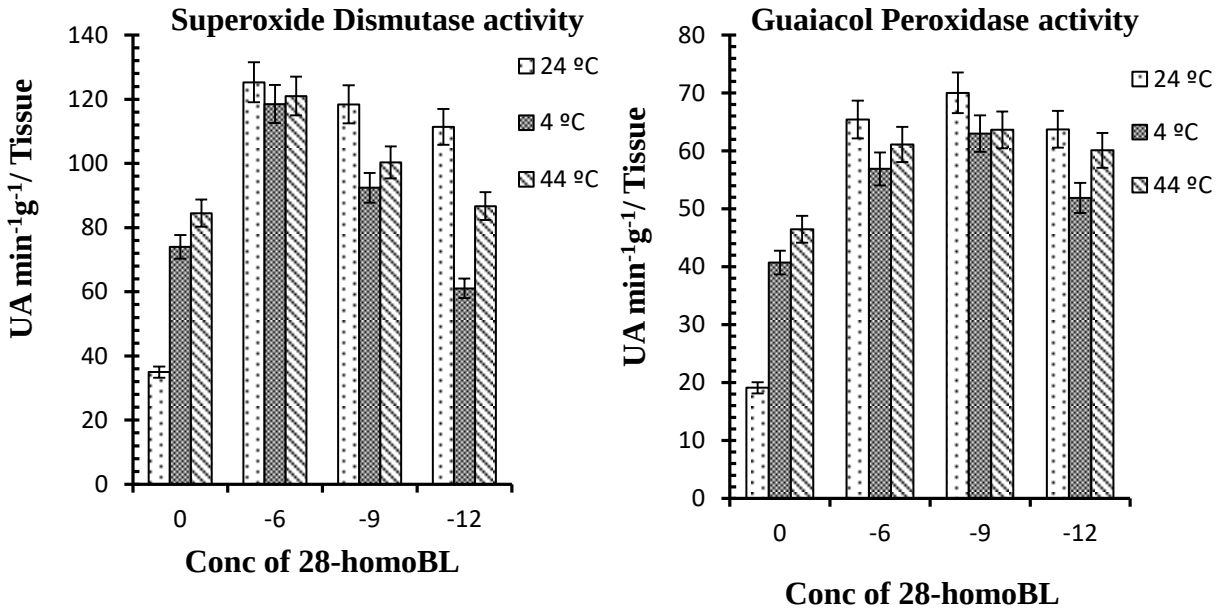


Figure 17- Effect of 28-homoBL on superoxide dismutase and guaiacol peroxide activities of *Brassica juncea* L. under temperature stress

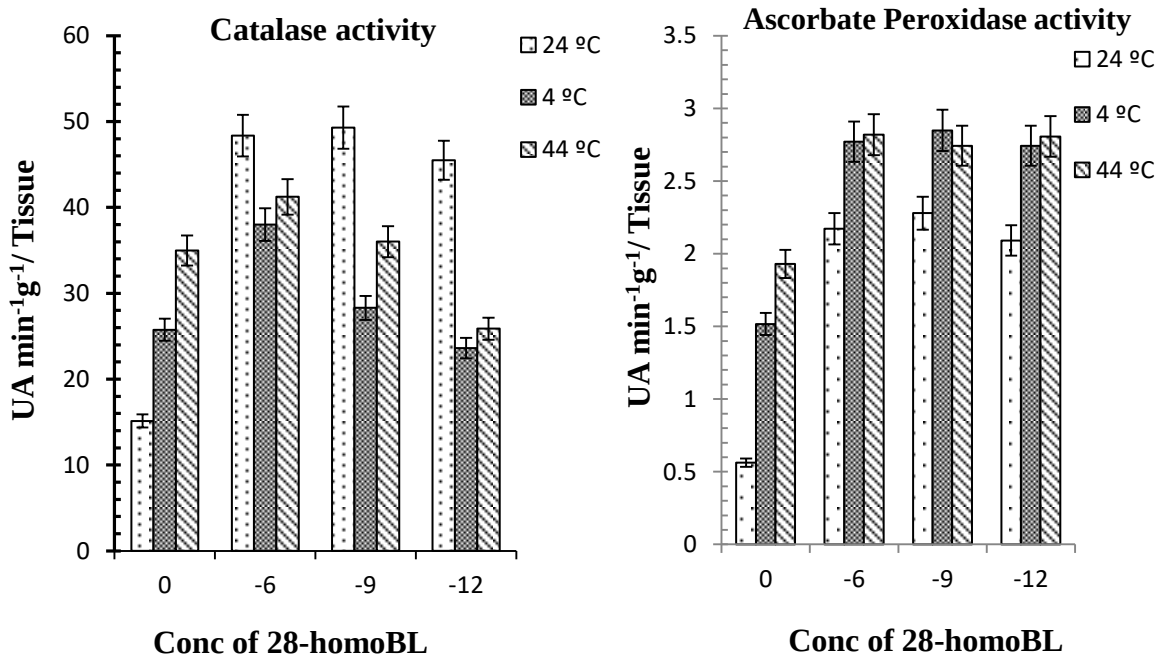


Figure 18- Effect of 28-homoBL on catalase activity and ascorbate peroxidase activity of *Brassica juncea* L. under temperature stress

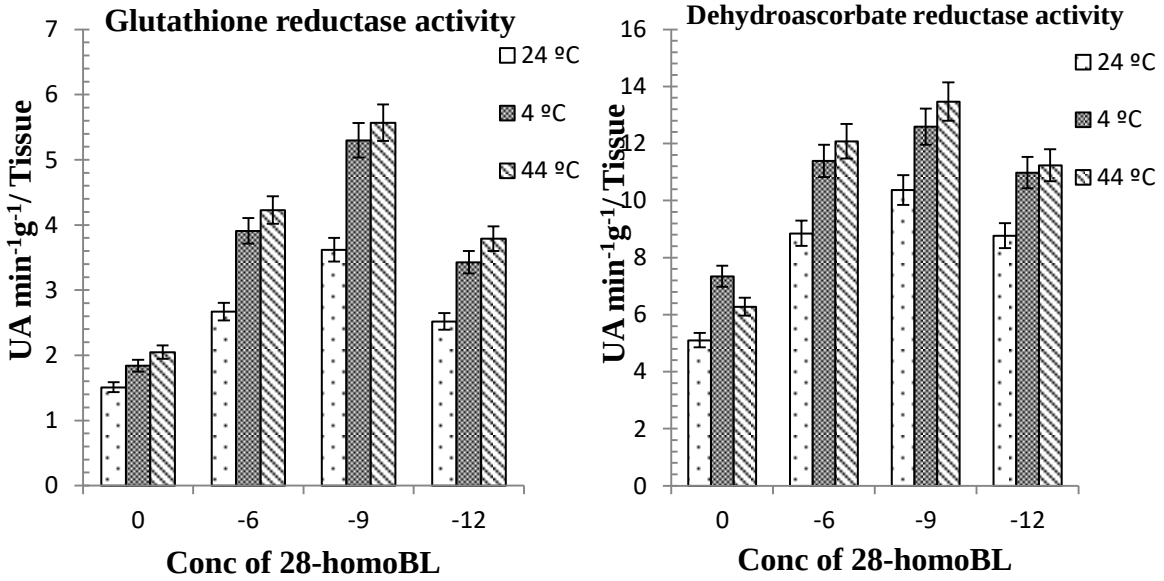


Figure 19- Effect of 28-homoBL on glutathione reductase activity and dehydroascorbate reductase activity of *Brassica juncea* L. under temperature stress

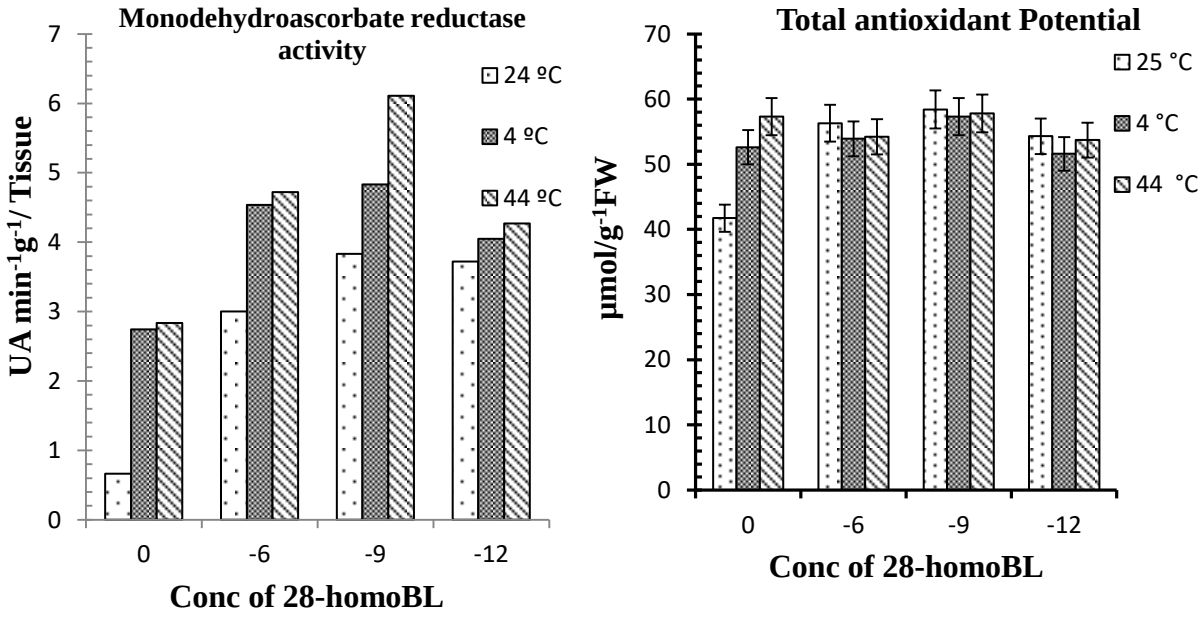
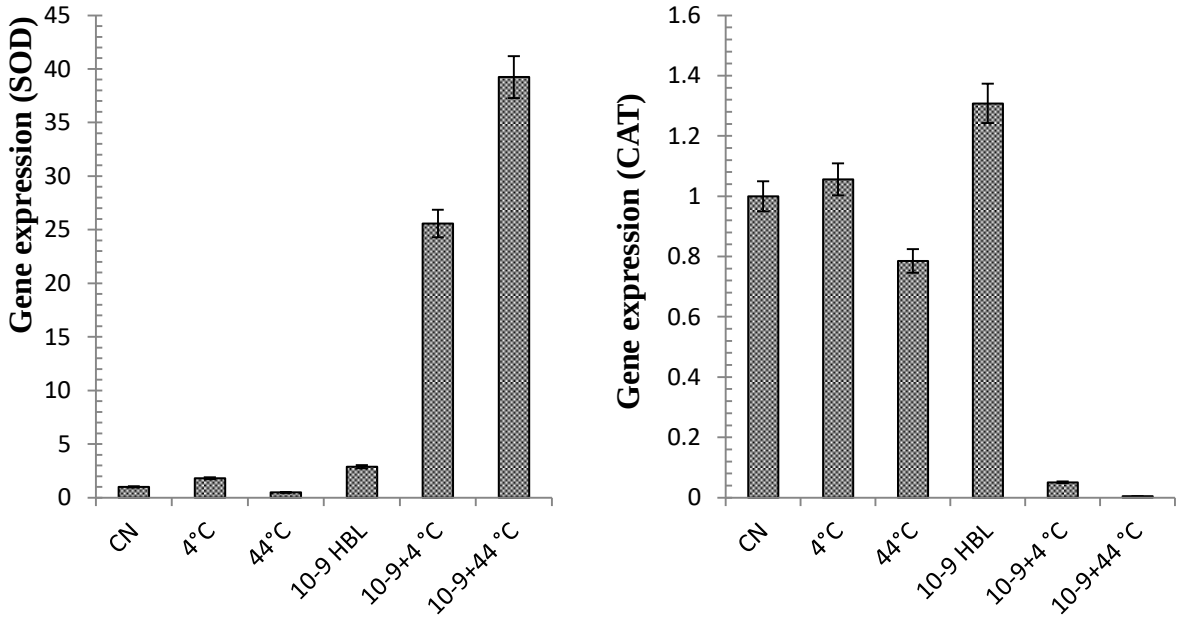


Figure 20- Effect of 28-homoBL on monodehydoreductase activity and total antioxidant potential of *Brassica juncea* L. under temperature stress

Table 2. The primer sequences for real time qPCR analysis of Cu/ZnSOD, CAT, APOX, GR, DHAR, MDHAR and β-actin genes.

Gene	Species	Accession	Sequence of primers
Cu/ZnSOD	*Brassica juncea*	1204051	F: 5'- GTCCACGCAGACCCTGATGT-3' R: 3'-GAAGACCAATAATACCGCAAGCA-5'
CAT	*Brassica juncea*	4336751	F: 5'- CTGACCCCCGCATCACA-3' R: 3'-ACGTTCAGACGGCTTGCAA-5'
APOX	*Brassica juncea*	2746726	F: 5'-CCGGTGAGAAGGAAGGTCTTC-3' R:3'-CTTCCTCGTCAGCAGCGTATT-5'
GR	*Brassica juncea*	4704610	F:5'-CACAGCAGCTGAGGAGTTTGTC-3' R:3'-GACAGCTGTTTTAGCCTCAAGACTT-5'
DHAR	*Brassica juncea*	22653412	F: 5'-CCAAAGGTGATGGGCTAAAGAG-3' R:3'-ACATTGTACTAAAAGAAAGCAAGAGAAAAG-5'
MDHAR	*Brassica juncea*	gi|4704612	F:5'-CCCAAAGCTAGCAAGAAGTCAAC-3' R:3'- CTGTAGAGCGGCTTGAGCAA-5'
β-actin	*Brassica juncea*	gi|4139263	F:5'-GGATCTCGAAGGGAGAGTACGA-3' R:3'-TACCACACTCACCACCACGAA-5'



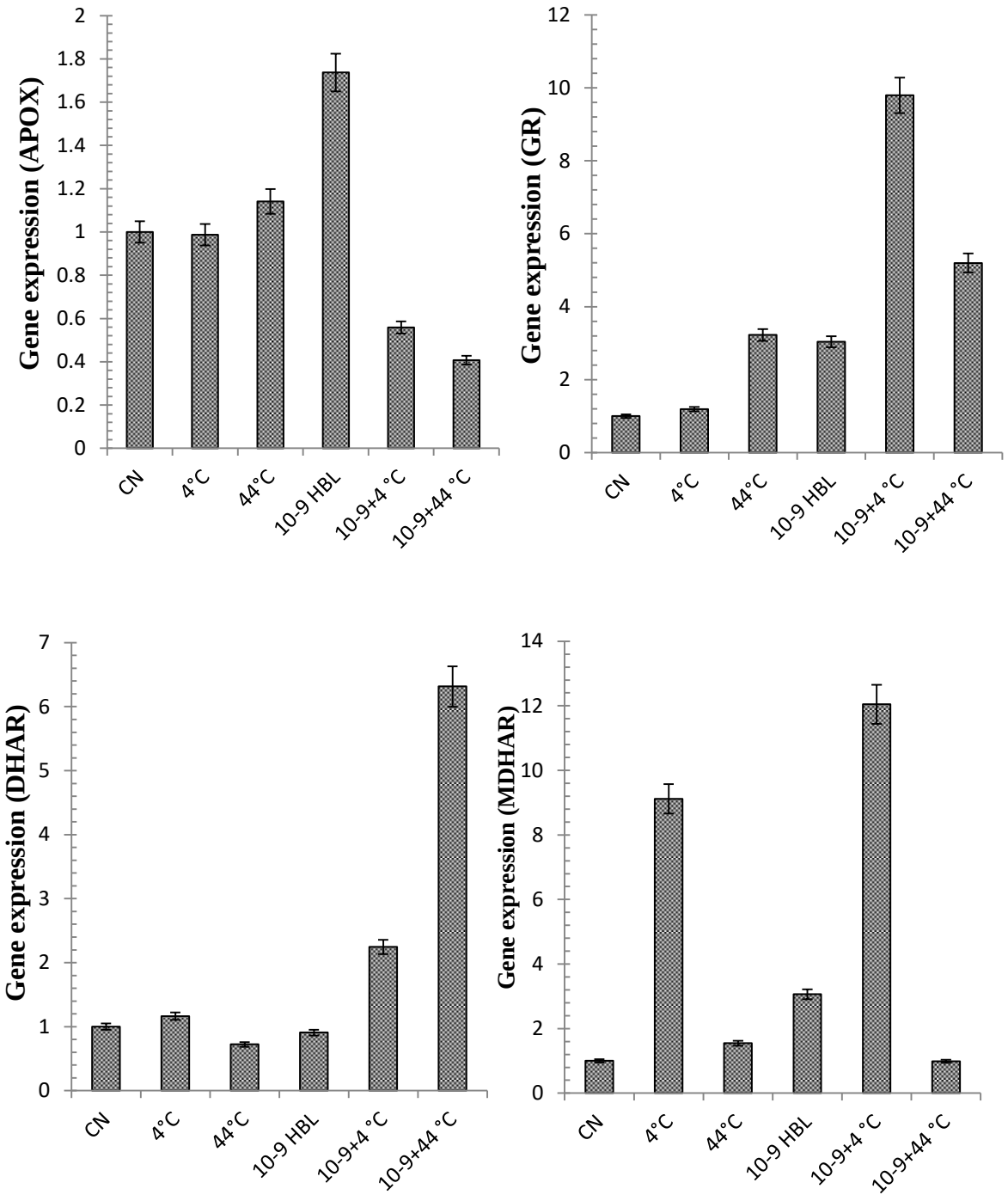


Figure 21- Effect of 28-homoBL on up regulation/down regulation of gene expression of antioxidants (SOD, CAT, APOX, GR, DHAR and MDHAR) of *Brassica juncea* L. under temperature stre

Table: 3. Effect of 28-homoBL (10^{-6}, 10^{-9}, 10^{-12}M) and temperature (4 °C and 44 °C) alone and in combinations on Mitotic index of *B. juncea* L. growing under controlled conditions

Treatments	Mitotic index (Mean± S.E)
Control	5.73 ± 0.13^{b}
10^{-6}M 28-homoBL	8.89 ± 0.66^{bc}
10^{-9}M 28-homoBL	9.31 ± 0.09^{bc}
10^{-12}M 28-homoBL	8.56 ± 0.60^{bc}
4 °C Temp	4.87 ± 0.05^{b}
44 °C Temp	3.21 ± 0.38^{a}
10^{-6} M+ 4 °C Temp	7.63 ± 0.12^{bc}
10^{-6} M+ 44 °C Temp	7.59 ± 0.09^{bc}
10^{-9} M+ 4 °C Temp	8.46 ± 0.06^{bc}
10^{-9} M+ 44 °C Temp	7.67 ± 0.11^{bc}
10^{-12} M+ 4 °C Temp	6.12 ± 0.06^{bc}
10^{-12} M+ 44 °C Temp	6.02 ± 0.10^{bc}

The data represented above are Mean S. E ± (n=3). Different superscripted alphabetical letters (a, b, c) within a column indicate significant difference from each other in all combinations (Tukey's test, $p \leq 0.05$).

Table: 4- Effect of 28-homoBL (10^{-6}, 10^{-9}, 10^{-12}M) and temperature (4 °C and 44 °C) alone and in combinations on clastogenic aberrations (Metaphase I/II) of *B. juncea* L. flower buds

	Metaphase I/II					
Treatments	**No. of PMC observed**	**Stickiness**	**Stray**	**Bivalents**	**Multivalents and Univalents**	**Abnormal PMC**
Control	200	-	-	-	-	0
10^{-6} M 28-homobL	200	1	2	-	3	06
10^{-9}9M 28-homobL	200	-	1	3	5	09
10^{-12}M 28-homobL	200	2	-	5	6	13
4 °C Temp	200	8	5	13	17	43
44 °C Temp	200	9	8	7	13	37
10^{-6}M + 4 °C	200	4	6	5	9	24
10^{-6}M + 44 °C	200	4	6	9	6	25
10^{-9}M + 4 °C	200	3	-	5	6	14
10^{-9}M + 44 °C	200	3	4	7	6	20
10^{-12}M + 4 °C	200	5	6	5	11	27
10^{-12}M + 44 °C	200	3	7	5	9	24

Table: 5- Effect of 28-homoBL (10^{-6}, 10^{-9}, 10^{-12}M) and temperature (4 °C and 44 °C) alone and in combinations on clastogenic aberrations (Anaphase I/II) of *B. juncea* L. flower buds

Anaphase I/II						
Treatments	**No. of PMC observed**	**Laggard**	**Bridges**	**Cytomixis**	**Disturbed anaphase**	**Abnormal PMC**
Control	200	-	-	-	-	0
10^{-6} M 28-homobL	200	4	3	-	1	8
10^{-9}M 28-homobL	200	1	2	1	-	4
10^{-12}M 28-homobL	200	3	3	2	-	8
4 °C Temp	200	6	6	7	3	22
44 °C Temp	200	5	9	7	3	24
10^{-6}M + 4 °C	200	5	7	8	5	25
10^{-6}M + 44 °C	200	6	7	9	4	26
10^{-9}M + 4 °C	200	4	8	5	-	17
10^{-9}M + 44 °C	200	3	2	4	3	12
10^{-12}M + 4 °C	200	2	3	3	4	12
10^{-12}M + 44 °C	200	6	6	5	6	23

Table: 6- Effect of 28-homoBL (10^{-6}, 10^{-9}, 10^{-12}M) and temperature (4 °C and 44 °C) alone and in combinations on clastogenic aberrations (Telophase I/II) of *B. juncea* L. flower buds

Telophase I/II					
Treatments	**No. of PMC observed**	**Cytomixis**	**Stickines**	**Micronuclei**	**Abnormal PMC**
Control	200	1	-	-	1
10^{-6} M 28-homobL	200	2	1	3	6
10^{-9}M 28-homobL	200	2	-	1	4
10^{-12}M 28-homobL	200	2	2	-	4
4 °C Temp	200	7	9	8	24
44 °C Temp	200	10	7	9	26
10^{-6}M + 4 °C	200	5	6	5	16
10^{-6}M + 44 °C	200	6	-	11	17
10^{-9}M + 4 °C	200	4	5	4	13
10^{-9}M + 44 °C	200	3	5	6	14
10^{-12}M + 4 °C	200	5	6	8	19
10^{-12}M + 44 °C	200	4	8	9	21

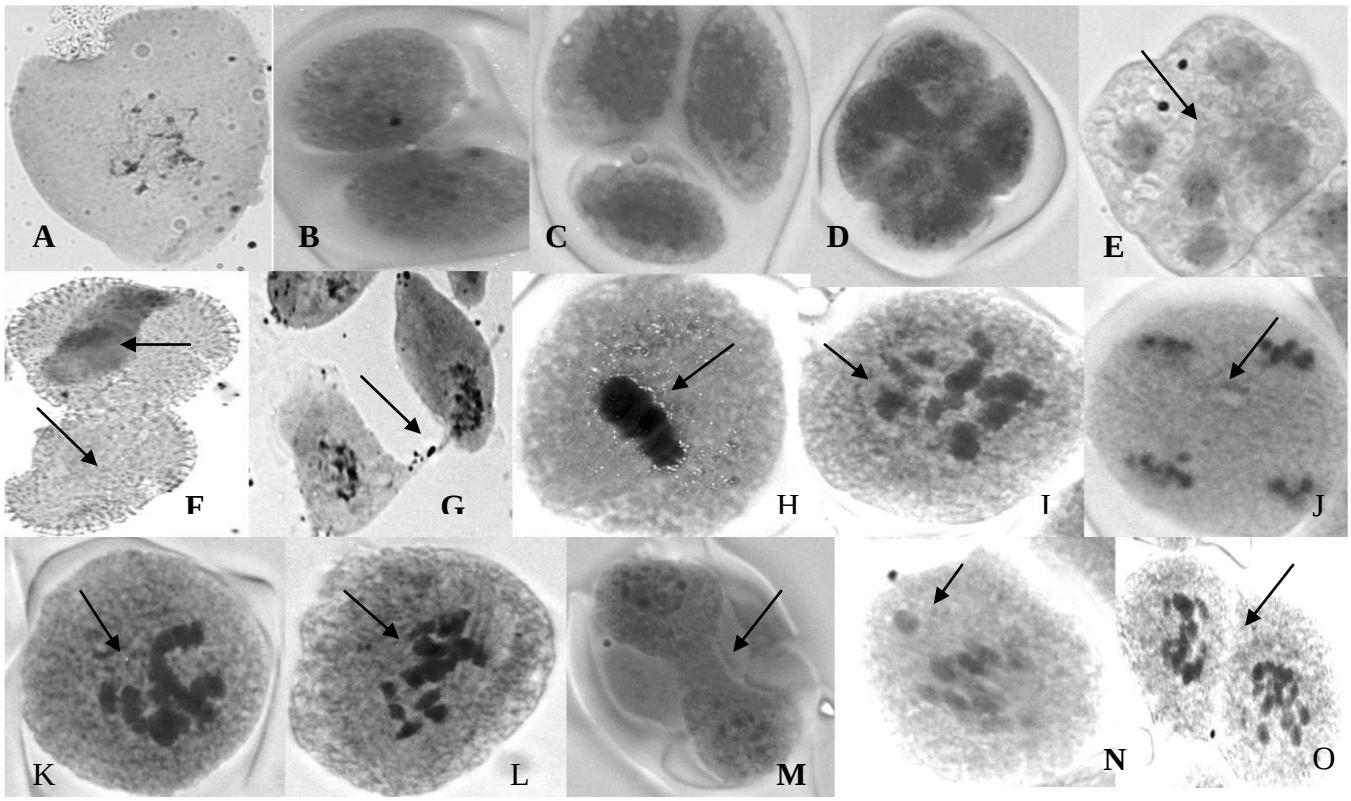


Figure: 21- Cytological features in *Brassica juncea* L. (A) PMC at prophase stage (B) PMC showing diad (c) PMC showing triad (D) PMC showing tetrad (E) PMC showing polyad (F) PMC showing sterile and fertile pollens (G) PMC showing cytomictic channels (H) PMC showing chromosomal stickiness at metaphase stage (I) PMC showing unoriented bivalents at Diakinesis stage (J) PMC at telophase stage (K) PMC showing multivalent (chain formation) (L) PMC at disordered telophase (M) PMC showing telophase stage (N) PMC showing laggard formation at metaphase stage (O) PMC showing 2n=18 chromosome number