Physiological, Biochemical, Molecular and Hormonal Studies to Confirm Growth and Development Regulating Actions of Brassinosteroids in `*Phaseolus vulgaris* L.*cv*. Bronco Seedlings

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Abstract: In response to the addition of epibrassinosteroids as 24- epibrassinolide (EpiBL) at different concentrations (0.1,0.5 and 1.0 µM) there was appreciable increase in germination percentage. Addition of EpiBL to MS-growth medium of *Phaseolus vulgaris* L.*cv*. Bronco lead to significant increase in major growth attributes (percent germination, length of shoots and roots, and their fresh and dry weights). Changes at the metabolic level included significant increase in the content of chlorophyll *a*, chlorophyll *b*, carotenoids, soluble proteins, nitrate reductase (NR) activity, free proline, DNA and RNA content referred to control. The oxidative stress which is usually associated with active primary metabolism prerequisite for active growth was ameliorated by EpiBL via increasing activity level of superoxide dismutase (SOD), catalase (CAT), ascorbic acid peroxidase(APX)glutathione reductase(GR), total phenol(TP),polyphenol oxidase(PPO) concomitant with decrease in activity of peroxidase(POD) and ascorbic acid oxidase(AAO) compared with non-treated control. Such powerful scavenging systems protect membrane lipids being attracted as malonedialdehyde and H_2O_2 levels were depressed. Plant response to exogenous EpiBL application at endogenous hormonal levels refer to increase in the content of indole-3-acetic acid (IAA),naphthalene acetic acid(NAA), gibberellins (GAs), cytokinins(CK) and jasmonic acid(JA) associated with appreciable decrease in abscisic acid (ABA) content in respect with control seedlings. At the molecular level, peroxidase isozymes indicated total eight isozymes in bean seedlings manifested low level in the banding patterns polymorphism (25%) recording disappearance of isozymes at R*f*: 0.6, 0.7, while PPO expressed total five bands exhibiting monomorphic style. Also, SDS-proteins revealed total number of 20 bands with molecular weights ranging from 11.7 KDa to 106.7 KDa. Sixteen bands were observed as monomorphic, while four bands were polymorphic, giving 20% polymorphism. Protein has 44.8 KDa molecular weight was EpiBL marker regardless of its concentration. The results obtained in the present study point to the possibility of using brassinosteroid to tackle and manipulate plant seedling stage especially during early growth period, which is very sensitive to environmental threatens() interfere with further developmental stages of growth. Moreover, the study reveals some mechanisms of brassinosteroids during vigorous metabolism, which make present, step forward to manipulate EpiBL genes that may control metabolism and increase yield.

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1. Introduction

Brassinosteroids (BRs) due to their antistress and immunomodulatory properties are becoming promise candidates for new generation of phytohormones. BRs have been reported to modulate almost every aspect of plant growth and development. There are two major contributors to cell growth: cells division (increases number of cells in an organ) and, expansion of those cells. The marvelous increment in volume in plant cells manifested as cells transit from meristems to the differentiated state of the mature organ which explain cells growth importance as an essential component of plant development (Sugimoto-Shirasu and Roberts, 2003).Reactive oxygen species (ROS) are emerging as important regulators of plant development (Cao et al. 2005; Gapper and Dalon 2006). There is now abundant evidence that ROS play roles in cell growth and that spatial regulation of ROS production is an important factor controlling plant form. To mitigate high ROS production, plants have developed antioxidant defense system operating at cellular level. The operating antioxidant defense strategy proceeded via non-enzymatic metabolites as ascorbic acid, glutathione, proline, phenolics, carotenoids and enzyme systems as superoxide dismutase (SOD), catalase (CAT), ascorbic acid peroxidase (APX), glutathione reductase (GR) ,Peroxidase(POD) ,polyphenol oxidase(PPO) ,ascorbic acid oxidase (AAO) (Asada and Takahashi 1987; Salin 1988). Brassinosteroids showed a positive impact in modulating such stresses by regulating the non-

enzymatic and the enzymatic system and by interplay with the plant endogenous hormones at the biochemical and molecular levels. These compounds, when applied to plants, improve their quality and yield and can protect them against various stresses (Sirhindi et al. 2009).

Legumes account for 27% of the world's major crop production, with grain legumes providing more than one-third of humankind nutritional nitrogen requirement (Arellano et al. 2008). However, its average yield per hectare in 2008 was lower in Africa (0.61 t/ha) than in North America and the European Union (2 t/ha and 1.7 t/ha, respectively) (FAO stat. 2005). Biotic and abiotic constraints limit yields (Hillocks et al.2006), particularly for small farmers in Africa and South America. So earlier application of BRs may present future opportunities for protective state against biotic and a biotic constrains and help humankind in food increments.

Studies have provided compelling evidence regarding the role of BRs at different stages of growth and development and its role in alleviating oxidative stress (Bajguz and Hayat, 2009, Bajuz 2012). Keeping this in mind, the present work was undertaken to study the effects of 24-EpiBL on certain seedling growth attributes and antioxidant enzyme activities, lipid peroxidation hydrogen peroxide levels, endogenous hormones (auxins, GA, abscisic acid,cytokinins , jasmonic acid content, the seedling SDS-proteins profile and PPO, POD isozymes patterns in *Phaseolus vulgaris* plants grown in MS medium under controlled growth condition.

2. Material and Methods

Germination experiment

A pure strain of *Phaseolus vulgaris* L.cv. Bronco seeds were obtained from Faculty of Agriculture, Cairo University, Egypt. A uniform lot of *Phaseolus vulgaris* seeds were selected and surface sterilized in 0.1 % Na hypochlorite, thoroughly washed with tap water and divided into four groups. First, 2nd and 3rd groups of seeds were set to germinate in Petri-dishes furnished with filter papers moistened with 0.1, 0.5 or 1.0 µM of epibrassinolide solutions respectively. The fourth group of seeds was set to germinate in Petri-dishes containing distilled water-moistened filter papers serving as control. The germination of the seed was monitored till eight days. The germination was measured according to routine germination rules (ISTA, 2004) expressed as germination percentage.
The germination percentage was calculated as follow:
Germination % =Number of seeds germinated / total number of seeds sown X100

Phaseolus vulgaris* L. *in vitro culture

Time course experiment

Basal medium contained Murashige and Skoog salts (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose was used for *in vitro* culture. pH of media either free or after supplemented with 24-EpiBL was adjusted to 5.8 with 1N KOH or 1 N HCl, then solidified with 7 g L^{-1} agar prior to autoclaving at 121ºC and 1.2 kg cm^{-2} for 20 minutes. Culture media were dispensed as 50 ml per jar. All cultures were incubated for three days in dark condition for clean media assurance. The seeds were washed with running tap water, taken to the laminar airflow and surface sterilized by dipping in 70% (v/v) ethanol for 3 min, washed in sterilized distilled water. Then seeds were disinfected with 10 % (v/v) commercial Clorox containing two drops of a wetting agent Tween 20 solution for 20 min, rinsed three times with sterilized distilled water according to Gatica et al.(2010**).** In complete aseptic conditions equal number from sterilized seeds were inoculated in control MS medium and in MS culture media supplemented with 0.1 ,0.5 or 1.0µM of 24-EpiBL.Cultures were incubated in a controlled growth chamber at 26 ±1°C for three weeks then harvested. Medium residues were removed, shoot and, root and leaves fresh samples were taken, subjected to certain parameters and the rest were frozen immediately and stored at -20 ^{o} C until need.

Measurement of growth attributes

Three weeks- old seedlings were harvested, carefully cleaned from all adhering foreign residues. The length of shoot and root of each plant was measured with the help of meter scale. Fresh weight of shoot and root per plant was accounted by using a scale with the precision of 0.001 thereafter dry weights was estimated by drying each plant parts at 70°C to a constant weight using a hot air oven. Each treatment was represented with five replicates. Water content was calculated according to:

$$\text{Water Content} = \frac{\text{Weight of fresh plant part- Weight of dry plant part}}{\text{Weight of fresh plant part}} \times 100$$

Physiological, biochemical and endogenous hormone analyses

Estimation of nucleic acids

The frozen sample (200 mg) was homogenized for DNA and RNA extraction follows the method of Ogur and Rosen (1950), depending on their insolubility properties in cold perchloric, ethanol and in ether to get ride from interfering compounds. DNA was estimated by employing the diphenylamine test, measured at 595 nm following the procedure of Burton (1968), and RNA was quantified by the

method of Schneider (1957) using Orcinol reagent, measured at 660 nm.

Estimation of chlorophyll and carotenoid

Fresh leaf segments (200 mg) taken from control and treated seedlings were macerated in 5 mL of 80% acetone under cool condition following Arnon (1949) method. Chlorophylls and carotenoids were evaluated in the extract from absorbance readings, using Lichtenthaler and Wellburn (1983) equation for calculating the pigments contents expressed as mg g^{-1} FW.

Enzymes extraction and assay

For estimating antioxidant activities, frozen tissues taken from each shoot and root (0.5 g) were ground in 5 mL of 100 mM cooled sodium phosphate buffer pH 7.0 containing 0.5 mM EDTA (Sairam et al.,1998) . The homogenate mixture was centrifuged at 15 000 xg for 20 min at 4 °C. The supernatant fraction was used to assay the activities of superoxide dismutase, catalase, peroxidase, polyphenol oxidase, ascorbate peroxidase, glutathione reductase and ascorbic acid oxidase.

Superoxide dismutase (SOD; EC 1.15.1.1) activity was assayed following a photochemical method published by Giannopolitis and Reis (1977). The reaction solution (3 mL) contained 50 µM p-nitro blue tetrazolium(NBT), 1.3 µM riboflavin, 13 mM methionine, 75 nm EDTA, 50 mM phosphate buffer (pH 7.8), and 500 µL of enzyme extract. The reaction was allowed to run under light for 15 min and stopped by switching the light off. The absorbance of the irradiated solution was read at 560 nm using a spectrophotometer. Blank and control were run in the same manner, but without irradiation and enzyme, respectively One unit of SOD activity was defined as the amount of enzyme that inhibited 50% NBT photo reduction. Values are given in unit g^{-1} FW.

Catalase (CAT; EC 1.11.1.6) activity was assayed following Aebi (1984) method. The reaction mixture consisted of, 1.5 mL sodium phosphate buffer 100 mM, pH 7, 0.5 mL of 75 mM H_2O_2, 50 µL enzyme extract, were increased to 3 mL using distilled water. The decrease in H_2O_2 absorbance was monitored at 240 nm and expressed as mmol min^{-1} g^{-1} FW.

Peroxidase (POD; EC 1.11.1.7) activity was measured in a reaction mixture consisting from 50 µl enzyme extract, 20 mM guaiacol and 100 mM sodium phosphate buffer pH 7 in total volume of 3 mL. Reaction was initiated by 20 µL H_2O_2, following Ranieri et al. (1995) method. The activity was determined by measuring the change in absorbance at 470 nm and expressed as Δ $min^{-1}g^{-1}$ FW.

Polyphenol oxidase (PPO; EC 1.14.18.1) was assayed according to Luh and Phithakpol (1972) method in which 1 mL of enzyme extract was mixed with 1 mL of 100 mM sodium phosphate buffer pH 7 and 1 mL of 50 mM catechol. Enzyme activity was estimated by measuring the change in absorbance at 410 nm and expressed as Δ $min^{-1}g^{-1}$ FW.

Ascorbate peroxidase (APX; EC 1.11.1.11) activity was assayed by the method of Nakano and Asada (1987).The reaction mixture contains 1.5 mL of 50 mM sodium phosphate buffer pH 7, 0.2 mL 5 mM ascorbic acid, 0.2 mL of 1 mM H_2O_2 and 200 µL from the enzyme extract was added to initiate reaction .The reaction was left for 10 min, after stopping, activity was measured as decrease in absorbance at 290 nm. Enzyme activity was quantified using the molar extinction coefficient for ascorbate (2.8 mM^{-1} cm^{-1}) and expressed as mmol oxidized ascorbate min^{-1} g^{-1} FW.

Glutathione reductase (GR; EC 1.6.4.2) activity was measured according to Foyer and Halliwell (1976), in which activity was measured by monitoring the decline in absorbance at 340 nm due to oxidation of $NADPHNa_4$. The reaction mixture contained 25 mM phosphate buffer (pH 7.8), 5 mM GSSG, and 1.2 mM $NADPHNa_4$. The reaction was carried out for 10 min and activity of GR was calculated by using the molar extinction coefficient of $NADPHNa_4$ (6.2 mM^{-1} cm^{-1}) and expressed as mmol min^{-1} g^{-1} FW.

Ascorbic acid oxidase (AAO; EC 1.10.3.3) activity was measured according to Vines and Oberbacher (1962) by spectrophotometric measurement depending on the ultraviolet properties of ascorbic acid absorption at 265 nm as ascorbic acid oxidase reduce the absorption peak as ascorbic acid was enzymatically oxidized. The assay mixture contained 0.5 µmol of ascorbic acid freshly prepared, 33 mmol of potassium phosphate at a pH 5.6 and 0.5 mL of enzyme extract in a total volume of 3 mL .Reaction was kept aside for 10 min at 25 ^{o} C with occasional shaking; 1.0 mL of 10% (v/v) trichloroacetic acid was added to stop the enzyme reaction .Enzyme activity was expressed as mmol g^{-1} FW.

Nitrate reductase (NR; EC. 1.6.6.1) activity in the seedling fresh material was determined by the intact tissue assay method of Jaworski (1971). Chopped leaves and root pieces (200 mg) were incubated for 2 h at 30ºC in a 5.5 mL reaction mixture, which contained 2.5 mL of 0.1 M phosphate buffer, 0.5 mL of 0.2 M potassium nitrate, and 2.5 mL of 5% isopropanol. The nitrite formed subsequently was calorimetrically determined at 540 nm after azo coupling with sulphanilamide and naphthylethylenediamine dihydrochloride .The NR activity was expressed as µg NO_2 g^{-1} FW.

Lipid peroxidation levels

The level of lipid peroxidation in seedling tissues is measured by estimating malondialdehyde (MDA) (Heath and Packer 1969) and other aldehydes (Meirs et al., 1992) breakdown products of lipid peroxidation. MDA content was determined with a thiobarbituric acid (TBA) reaction. Briefly, a 0.2 g frozen tissue sample was homogenized in 4 mL of 0.1% TCA. The homogenate was centrifuged at 4,000 ×g for 10 min. To a 1 mL aliquot of the supernatant was added 4 mL of 20% TCA containing 0.5% TBA. The mixture was heated at 95 ^{o} C for 15 min and cooled immediately. The absorbance was measured at 532 nm. The value for the non-specific absorption at 600 nm was subtracted. The level of lipid peroxidation was expressed as mmol g^{-1} FW.

H_2O_2 content

The content of H_2O_2 was determined according to Alexieva et al., (2001). A frozen sample (500 mg) was macerated with a mortar and pestle dipped in an ice bath with 5 mL of cold 0.1% trichloroacetic acid. The macerate was centrifuged at 12,000 ×g for 15 min under cool condition. From the supernatant 0.5 mL was added to 0.5 ml of 100 mM potassium phosphate buffer pH 7.0 and 1 mL of 1 M KI. The absorbance was read at 390 nm. H_2O_2 was expressed as mmol g^{-1} FW.

Proline content

The proline content was estimated according to Bates et al., (1973).The frozen plant material (100 mg) was homogenized in 5 mL of 30% aqueous sulfosalicylic acid and the homogenate was centrifuged at 4000 rpm. Supernatant was used for the estimation of free proline content. The reaction mixture which consisted of 1 mL supernatant, 1.5 mL from acidic ninhydrin, was heated at 90 ^{o} C for 1 h. After terminating of the reaction in ice bath, the reaction mixture was transferred to separating funnel and 5 mL toluene was added, vortexes several times and the upper phase was used to estimate proline concentration (520 nm) according to the following equation:

μmol proline g^{-1} FW = (μg proline mL^{-1} * mL of toluene / 115.5) / (g of sample)

Total phenolic compounds assay (TPC)

Frozen samples from each treatment (0.5 g) were extracted twice in 10 mL of 70% ethanol for 2 h at room temperature according to Yao et al. (2012) method. After filtration, the supernatants were combined and concentrated under reduced pressure in a rotary evaporator at 50 °C.TPC was measured using the Folin-Ciocalteu method as previously described by Zhou et al., (2009) and modified by Yao and Ren (2011). Briefly, 50 μL of the extract was mixed in 5 mL of distilled water followed by the addition of 500 μL of 1 N Folin-Ciocalteu reagent and 500 μL of a 20% (w/v) Na_2CO_3 solution. The reaction mixture was thoroughly mixed and incubated for 1h at room temperature before reading the developed blue absorbance at 765 nm. Quantification was performed with respect to a calibrated sample using gallic acid. The results were expressed as μmol of tannic acid equivalent g^{-1} FW.

Soluble proteins were determined as described by Lowry et al., (1951) using BSA (Merck) as standard.

Endogenous hormonal analysis

Extraction, fractionation, identification and determination of auxins, gibberellins, cytokinins and abscisic acid as described by Wasfy and Orrin, (1975), were practiced with some modifications.

Frozen leaves (5 g) per sample were macerated in cold 80% (v/v) aqueous methanol and equal (20 mL /g) volumes were added to the macerate before allowing tissue extraction overnight at 4 ˚C. The extract was vacuum filtered through a Whatman filter paper. The filter paper and the residue were returned to the container with fresh methanol, shaken for 1 h and filtered again. The previous step was repeated once more and the combined extracts were concentrated under reduced pressure in a rotary evaporator. The aqueous phase was adjusted to 2.5 with 1 N HCl and then partitioned with equal volumes of ethyl acetate 3 times, and then passed through anhydrous sodium sulfate. After that the combined acidic ethyl acetate phase was evaporated under vacuum and the dry residue containing hormones (fraction I) was dissolved in 2.0 mL of methanol and stored in vials at -20°C. This fraction was used for auxins, gibberellins, and abscisic acid determination. On the other hand, the aqueous phase fraction was adjusted to pH 8.0 with some drops of potassium hydroxide (1 M) and partitioned four times with N-butanol (1/4 of its volume each time). The n-butanolic phase, thereafter, was concentrated to 5 mL (fraction II) and stored at -20 ˚C for cytokinins determination. Kelen et al., (2004) detected auxins, gibberellins, and abscisic acid by HPLC isocratic UV analyzer, reverse phase C18 column (RP-C18 μ Bondapak, Waters). The column used included octadecylsilane (ODS) ultrasphere particle (5-μm), the mobile phases used were acetonitrile-water (26:74 v/v), pH 4.00; Flow rate: 0.8 mL/min, detection: UV 208 nm, the standard solution of the individual acid was prepared in the mobile phase and chromatographed. Cytokinins fraction were detected by HPLC isocratic UV analyzer ODS reverse phase C18 column, 20 min gradient from 0.1N acetic acid. pH 2.8 to 0.1 N acetic acid in 95% aqueous ethanol, pH 4. The flow rate: 1 mL / min, detection: UV 254 nm, standards of zeatin and zeatin riboside were used (Muller and Hilgenberg 1986).

Estimation and determination of jasmonic acid

Jasmonic acid (JA) was extracted using methanol 100% (Hong et al. 2011) following Dionex instructions for Accelerated Solvent Extraction (ASE) method.

A known weight of fresh leaves (500 mg) were frozen in liquid nitrogen and ground to a fine powder .Samples were placed in a Dionex ASE 150 and extracted using 100% methanol at 100 ºC and 1500 psi with 5 min static cycle, flush volume was 60% of the cell volume. Finally, the system was purged with nitrogen for 60 s. Detection and quantitation of JA extract was carried out using a GC (Agilent Technologies 7890A) interfaced with a mass-selective detector (MSD, Agilent 7000) equipped with a polar Agilent HP-5ms (5%-phenyl methyl poly siloxane) capillary column (30 m × 0.25 mm i. d. and 0.25µm film thickness). The carrier gas was helium with the linear velocity of 1 mL / min. The oven temperature was set at 85 ºC for 2 min. and then programmed until 250 ºC at a rate of 15ºC / min. The injector and detector temperatures were 150 ºC and 250 ºC respectively. Injection mode, split, split ratio 1:20, volume injected 1µL of the jasmonic acid extract. The MS operating parameters were as follows: Ionization potential 70 eV, interface temperature 250 ºC, and acquisition mass range 40-600. The identification of jasmonic acid in its extract was based on a comparison of its mass spectra and retention time with its authentic compound and by computer matching with NIST and WILEY library as well as by comparison of the fragmentation pattern of the mass spectral data with those reported in the literature.

SDS-protein electrophoresis

Sodium dodocylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to study the protein profiles of *Phaseolus vulgaris* L. Bronco under control and the hormone investments. Protein fractionation was performed following Laemmli (1970) as modified by Studier (1973) protocol. Fresh leaf sample 500 mg was macerated in 200 µL of protein extraction buffer shaken well and kept in the fridge overnight .The extracts were again vortexes and centrifuged at 14 000 xg under cooling condition for 15 min. Supernatant was decanted to new eppendorf and kept in -20 ^{o} C till used for SDS-PAGE analysis.

Protein extract (90 µL) from different tubes was taken and mixed with 30µL of denaturing buffer boiled for 3-4 minutes and incubated at 4°C for 30 minutes. The samples containing equal amount of protein were loaded into wells of 12% polyacrylamide gel. Electrophoresis was carried out at constant voltage of 80 volts until the bromophenol blue dye reached the resolving gel and then continued at 150 V till the running end and the apparatus was switched off. The gels were dipped in staining solution for one hour at room temperature under gentle shaking condition. Then the staining solution was discarded and the gel was washed once with distilled water and distained for 2 h under shaking condition, replaced every 30 min until bands appeared. Gel was photographed using a 35 mm colour film (100 ASA) and scanned with Bio-Rad Video densitometer Model 620. Software data analysis for Bio-Rad Model 620 densitometer and IBM compatible personal computer 165-2072, were used.

Native potein electrophoresis:

Extraction preparation and detection

Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted following Stegemann et al., (1985) method to identify isozyme variations under the study condition using peroxidase and polyphenol oxidase isozymes systems as molecular markers. For isozymes extraction 500 mg fresh leaves were homogenized in 1 mL extraction buffer(10% glycerol) using a mortar and pestle under 4°C. The extract was then transferred into clean Eppendorf tubes, centrifuged at 12 000 x g for 10 min. The supernatant was transferred to new clean Eppendorf tubes and stored at –20 ^{o} C until isozymes analysis.

Native-PAGE with a 12% separating and 4.5% stacking gel at 4°C was carried out at 80 and 150 v in stacking and running gel, respectively. The crude extract from above mentioned treatments was used. From each extract 40 µL was taken mixed with 20 µL of sucrose and 10 µL of bromophenol blue, and then a volume of 50 µL from this mixture was added to each gel lane. After the dye front reached the end of the running gel, the gel was stained according to its enzyme system with its appropriate substrate and chemical solutions and was then incubated at room temperature in dark for complete staining. For peroxidase, benzidine-dihydrochloride HCl 0.125 g and 2 mL glacial acetic acid and was completed with distilled water up to 50 mL. Gel was placed into this solution and 5 drops of hydrogen peroxide was added then incubated at room temperature until bands representing POD activity appeared (Brown 1978). For polyphenol oxidase, the gel was soaked in 0.1 M sodium phosphate buffer pH 6.8, 100 mg of sulfanilic acid then mixed with 30 mg of cathecol dissolved in 1 mL of 99% acetone. The Gel was placed into this solution and incubated at 37°C until bands appeared (Flurkey and Jen 1978). Relative band mobility was measured in relation to the dye front and indicated by *Rf* values.

All gels resulted from isozyme electrophoresis were photographed, scanned using Gel Doc Vilber Lourmat system.

Statistical analysis

Study parameters were treated statistically using the one way analysis of variance as described by Snedecor and Cockran (1969). Means were compared by LSD using SPSS program version 16.

3. Results and Discussion

This study focused in particular on the impact of exogenous EpiBL application on certain growth attributes, nucleic acids content, photosynthetic pigments, lipid peroxidation levels, hydrogen peroxide content, and certain antioxidative enzymes namely SOD, CAT, APX, GR, POD, and PPO. Also it included assaying the activity level of nitrate reductase , protein, proline, total phenol content, SDS- protein and, Native-PAGE, endogenous hormones in *Phaseolus vulgaris* L. cv. Bronco seedling cultured on MS medium in controlled growth chamber.

The results obtained here refer to that 24-EpiBL particularly at the relatively highest applied concentration (1 µ M) significantly increased the seed germination percentage and generally all the studied growth traits of *Phaseolus vulgaris* (*Ph. vulgaris*) measured in terms of fresh, dry weight mass, and length of both shoots and roots of the developed seedlings over the control non treated (scored data tabulated in Table 1.

In accordance with the present results, Mahesh et al., (2013) found that brassinosteroids (EpiBL and 28-homobrassinolide) application caused marked increase in radish seed germination over untreated control. Belmonte et al. (2010) stated that beneficial effects recorded after BL application on embryo strength of *Brassica napus* might be due its ability to switch the glutathione and ascorbate pool towards an oxidized state which is required for germination. Also Leubner-Metzger (2001) found that tobacco seed germination was promoted by brassinosteroids application via enhancing the growth potential of the embryos of tobacco. Previously, Pugachev et al., (2000) explained the recorded significant increments in germination percentage of cherry plum and sloe embryos after 24-epibrassinolide addition as due to shortening the dormant period of embryos. In the current study increments in seed germination due to EpiBL inclusion in the culture medium increased the production of dehydroascorbic, thereby switching the redox state towards an oxidized environment which is needed for germination.

Brassinosteroids growth promoting effect might be attributed to their involvement in cell elongation and cell cycle progression (Gonzalez-Garcia et al.2011).The strategies employed by BRs, in general, in enhancing growth criteria like elongation of plant organs are very divers. In this connection, BR-induced elongation was envisaged by others in terms of the re-configuration of microtubules to the transverse orientation which permits and enhances longitudinal growth (Clouse and Sasse 1998, Metwally 2008).BRs –induced elongation may also be due to altering the mechanical properties of the cell wall which led to their weakening (Wang et al. 1993). BRs were also recorded to up regulate genes which encodes proteins like xyloglucan - endotransglucosylase/hydrolases, expansions, glucanases, sucrose synthase and cellulose synthase(Ashraf et al. 2010) which are implicated in cell wall loosening required for cell elongation (clous 1996). Also, elongation of plant organs in response to BR was conceived in terms of increasing the activity of vacuolar ATP ase which is implicated in regulating the transport of water via aquaporin (Morillon et al.2001; Ashraf et al. 2010). BRs may also influence length of plant organs through promotion of cell division (Sakurai et al. 1999; Hu et al. 2000).

The significant increase obtained in fresh weight of shoots and roots of *Ph. vulgaris* in response to the different applied concentrations of EpiBL could partially be due to the accelerated commencement of the plant tissues response. Increase obtained in water content particularly on applying a relatively high concentration of EpiBL, suggested facilitating via hydrolases enzymes, the mobilization of reserves to their translocatable form participate in different metabolic activities enhancement (Table 1). In this regard brassinolide has been shown to be involved in the modification of the water transport properties of cell membranes (Morillon et al. 2001).Also, previously Takeuchi et al., (1995) reported that BRs increase water inflow into tissues.

The significant increase in dry weight of both shoots(1.0 µM EpiBL) and roots of *Ph. vulgaris* (Table 1) encountered in the current study in response to EpiBL was associated with a concomitant significant increase in chlorophyll *a*, chlorophyll *b* and carotenoids content of leaves (Table 2). Brassinosteroids were found to induce an improvement in photosynthesis efficiency by stomatal or/and non-stomatal factors. Non-stomatal factors which lead to limitations of photosynthesis implicate photosynthetic pigments concentration, and activity of enzyme Ribulose-1, 5-bisphosphate carboxylase oxygenase (RuBisCo) and the use of its assimilative products. Thus, the significant increase of chlorophyll and carotenoids content induced by EpiBL is expected to enhance photosynthetic

efficiency of *Phaseolus vulgaris* and improve rate of carbon dioxide assimilation leading to motivated Calvin cycle. The increase in photosynthetic pigment content in response to EpiBL treatments was reported to be the result of accelerating its rate of synthesis and / or delaying its rate of degradation (Hou and Li 2001; Liu et al. 2008; Yuan et al. 2010). Also, Maity and Bera (2009) found that spraying *Vigna radiata* with brassinolide significantly increased chlorophyll *a, b*, and total chlorophyll and Hill activity in their leaves. In addition, efficiency of EpiBL in protecting pigment – protein complex which led to decrease in chlorophyll degradation was recorded by Farduddin et al. (2009). A role for BR in the regulation of photosynthesis in *Cucumis sativus* was detected where it significantly increased the light saturated net CO_2 assimilation rate accompanied by an increase in the maximum carboxylation rate of Rubisco and in the maximum rate of ribulose biphosphate regeneration (Baker et al. 1997, Hayat et al. 2007; Yuan et al. 2010). Prakash, et al.,(2008) reported that BR increase the activities of meristematic tissues of sesame, via increasing number and size of cell, which increased photosynthetic surface area, and leaf area index and ultimately increased photosynthetic efficiency of EpiBL-treated sesame plants . Also, Bajguz and Hayat (2009) found that treating *Chlorella vulgaris* with brassinolide increases carotenoids content.

Nitrate reductase(NR) activity is considered as a measure of the habitat dependent nitrate utilization of a plant (Larcher 1995).Also, nitrate reductase plays a pivotal role in the plant nitrogen supplement for growth, development and productivity, in the initiation of nitrogen metabolism and the level of protein synthesis. Data collected from the treated seedling *Ph*. *vulgaris* in the present study (Table 1) compared to the control, in both shoot and root, refer to significant increase in NR activity in response to the three applied concentrations of EpiBL. Similarly, Anuradha and Rao (2003) reported increase in nitrate reductase activity of rice plants by application of brassinosteroids. Mai et al. (1989) suggested that increment in NR induced by EpiBL treatment could be due to nitrate mobilization increments.

DNA and RNA of *Ph. vulgaris* shoot showed significant increase in the present study (Table 3) and effectively played a key role in enhancement of their growth via activation of cell division and their synthetic capacity for proteins and enzymes. In this regard, the growth promotion in radish seedlings by BRs in unstressed and water stressed conditions was associated with enhanced levels of DNA and RNA (Mahesh et al. 2013). Activation of synthesis of DNA, RNA and proteins was reported among the wide range of physiological responses elicited by BRs (Khripach et al. 2003, Hayat and Ahmad 2003, Sasse, 2003, Yu et al. 2004, Vardhini et al. 2012).Previously, Sairam (1993) recorded an increase in RNA and protein synthesis in wheat treated with BRs.

Changes in content of soluble proteins of *Ph. vulgaris* in the present study and after exogenous EpiBL application clearly exhibit elevated levels referred to their control (Table 3) which may be due to BR function in accelerating cell division and cell elongation commitments to their specific enzymes synthesis. In accordance with the study results in *Pisum sativum* and radish they exhibit improvement in soluble protein content after BRs supplementation (Shahid et al. 2011; Mahesh et al. 2013) respectively. Moreover several studies have shown that BRs stimulate protein synthesis in the leaves of plants growing under both control conditions and those treated with stressors (Anuradha and Rao, 2001; Sirhindi et al., 2009).Investment of *Chinese cabbage* protoplasts culture media with 24-epibrassinolide increased cell division rate and soluble protein (Nakajima et al., 1996). In this regard Sasse (1990) suggested that BRs growth enhancement can proceeded through the synthesis of particular proteins associated with growth. Kalinich et al., (1986) suggested that increase in protein content under brassinosteroid influence is a result of the enhanced activity of RNA and DNA polymerases that are engaged in a physiological response to the BR application.

Some of the activated oxygen species are highly reactive and, in the absence of protective mechanisms, induce damage to cell structure and function (Halliwell and Gutteridge, 1989). Under non stressful conditions the antioxidative defense system provides adequate protection against activated oxygen species (Foyer and Halliwell 1976), prevailing with primary metabolism.

Recent discoveries suggest that ROS may control growth and development through their role in regulating cell growth rate and regulate certain phenotypes(apical dominance, leaf shape) other than cell expansion (Gapper and Dolan 2006). The mechanism by which ROS act on these different facets of development remains mysterious. The discrete increments in major growth attributes of *Ph. vulgaris* in response to EpiBL, are hypothesized to be associated with and / or the result of highly oxidizing metabolic activities, that cope with the in actively growing plants induced by EpiBL . Cell elongation and expansion concomitant with active metabolism of carbon and nitrogen, prevailed during vigorous growth period is usually associated with the release of active oxygen species and free radicals like superoxide radical, hydroxyl and H_2O_2.

Reactive oxygen species can attack the hydrogen moiety in the unsaturated carbon to form water. This leaves an unpaired electron on the fatty acid that is then capable of capturing oxygen, forming a peroxy radical. Lipid peroxides are unstable and decompose to form a complex series of compounds, which include reactive carbonyl compounds, such as malondialdehyde .Thus, MDA increment reflect lipid peroxidation and loss of membrane integrity. The data obtained in the present study, indicated a reduction in MDA content of both shoot and root of *Ph. vulgaris* in response to EpiBL (Table 4).Hydrogen peroxide levels and ascorbic acid oxidase (AAO) activity were subjected to significant reduction in both shoots and roots of *Ph. vulgaris* (Table 4). It is likely that BRs play crucial role in catching ROS by implicating more than one mechanism, still among them, MDA and H_2O_2 represents the best explored markers of oxidative burst (Behnamina et al. 2009, Heidari 2010, Filova, et al. 2013). Reduction in AAO activity under brassinosteroids supplementation to bean plants (Table 4) was suggested as adaptive strategy to modulate ascorbic acid content as protective compound. Vardhini and Rao (2003) found that BRs in sorghum reduce ascorbic acid oxidase activity. Also, Sarkar and Das (2000) observed in stressed rice plants that brassinosteroids application lowered AAO activity suggesting as adaptive feature.

In the present study, a significant increase in SOD, CAT, APX, GR activity illustrated in (Fig.1 a-d) was observed in *Ph. vulgaris* seedlings reflecting enhanced O^-_2 scavenging mechanism. The activity of SOD which acts as first line of defense against ROS, dissimulating O^-_2 to H_2O_2 is further enhanced by EpiBL application compared with the control. Produced H_2O_2 as mobile component, can diffuses across the plasma membrane exerts toxic effect or acts as antioxidant as well as reductant (Foyer et al. 1997). Catalase (CAT) further breaks down the H_2O_2 to water and oxygen (Foyer et al. 1994). Enhanced CAT activity in bean seedlings treated with EpiBL might have resulted in the oxidation of such harmful substance and efficiently scavenge ROS which lead to protection and restoration of high growth rates. Prakash, et al., (2008) recorded significant increase in activity level of catalase in sesame as response to spraying with homobrassinolide. Current results agree with Behnamnia et al., (2009) who reported that 24-epibrassinolide increased the CAT activity in *Lycopersicon esculentum*. Ascorbate peroxidase mechanism in which reduced ascorbate as the first step of ascorbate-glutahione cycle is recognized as the most important peroxidase in H_2O_2 detoxification giving up dehydroascorbate and water (Michalak 2006).Elevated levels of H_2O_2 decomposition may be linked with acceleration of ascorpate peroxidase activity(APX), and efficient scavenging of H_2O_2 preventing the H_2O_2 - mediated cell damage. Hassan et al. (2008) reported that stress amelioration by BRs in *Cicer arietinum* operated via increasing the activity of CAT and APX. Pre-treatment with BL resulted in enhancing GR activity and preserve the active form of glutathione and ascorbate. In this connection, Ling-Yun et al. (2013) found that 24-epibrassinolide protected *Cucumis sativus* exposed to $Ca(NO_3)_2$ stress, by increasing ascorbic and GSH levels through enhancing activities of APX, glutathione reductase (GR), monodehydroascorbate reductase (MDAR) and dihydroascorbate reductase(DHAR) enzymes. Filova, et al., (2013) reported that BRs play crucial role in catching of ROS via increasing activity of the antioxidative enzyme systems.

The strategies employed by EpiBL in alleviating oxidative stress are really diverse. The data obtained in the present study refer to that one of the mechanisms of EpiBL in this connection relies on increasing the phenol content of both shoots and roots as both are faced with high growth rates which in need to vigorous metabolism which reflect the free- radical scavenging capability of EpiBL-treated plants (Fig.2, a) .In this regards, BRs was reported to enhance various secondary metabolites like phenolic compounds (Khripach et al., 2000; Ahammed et al., 2013).

Peroxidase (POD) protects cells against harmful concentrations of hydroperoxides and contributes effectively in a variety of cellular functions (Beauchamp and Fridovich 1971).In plants subjected to oxidative stress regardless of the initiators POD and PPO activity enhancement is a good weapon. However a decrease in POD activity due to EpiBL was obtained in the present study (Fig. 2, b), which was in accordance with the disappearance of certain POD isozymes (Fig 3a and Table 5). Vardhini and Rao(2003) and Anuradha and Rao (2007) recorded a similar decrease in POD activity in sorghum and radish respectively as result of BRs application .

PPO activity in the current study under EpiBL application was significantly increased, as compared to the control. In accordance with the present result Sirhindi et al., (2009) recorded significant increment in PPO after EBL addition into *Brassica juncea* seedlings. More recently also Verma et al., (2012) stated that in vitro effects of brassinosteroids (BRs) on polyphenol oxidase in *Arachis hypogaea* was found to increase in plantlets 4-5 weeks-old grown in controlled growth condition. Numerous studies indicate that BRs - as plant steroid hormones with anti-stress properties - stimulate the activity of antioxidant enzymes (Hasan et al., 2008). Less is

known about the impact of BRs on non-enzymatic antioxidants such as ascorbic acid and carotenoids; however the role of proline in this respect was partially evaluated.

The appreciable increase encountered in proline content in shoots and roots of *Ph. vulgaris* in response to EB (Fig.2, d) could participate in preventing oxidative burst initiation and so protect the cells proteins and membranes. Proline, beside other roles, has an action on lowering the generation of free radicals (Matysik et al.2002; Cao et al. 2005). This effect is consistent with a protective membrane against the attack of free radicals, which is in agreement with the results of Alia et al. (1997). In addition, the increased content of chlorophylls could result from protection by proline of thylakoids membranes against the attack of ROS as reported by Kavi Kishor et al. (2005). Farooq et al. (2009) also observed that application of BRs increased the free proline levels in rice under drought stress. Alleviation of Zn toxicity by 24-epibrassinolide was also found to be correlated with accumulation of proline in radish seedlings (Ramakrishna and Ra 2013). The findings of present investigation suggest that BRs play a protective role for maintaining cellular redox status balance.

Changes in isoenzyme profiles have come to be of fundamental significance in the investigation of molecular basis of growth and morphogenesis. Electrophoretic study of peroxidase and polyphenol oxidase isozymes of *Phaseolus vulgaris* L. cv. Bronco indicated either minor or no differences in isozyme patterns and intensity respectively. Data shown in Table 5 and Fig.3a, b illustrate that eight and five different POD and PPO isozymes were expressed respectively. Analyzing POD patterns, indicate the presence of there was 6 monomorphic bands in control and treated plants leaves three isozymes were expressed having R*f* : 0.3, 0.8 and 0.9 and other three bands R*f* : 0.1, 0.4 and 0.5 were absent, and two unique bands represents 25% polymorphism (Table5). EpiBL treatments resulted in the disappearance of peroxidase isozymes at R*f*: 0.6, 0.7 compared to the control which may be a reflection to the previously recorded retard activity in enzyme activity (Table 4). Al-Fiky et al., (2002) recorded disappearance of POD isozyme band R*f*: 0.7 in Bronco in alliance with data obtained in the present study. On the other hand PPO isozymes pattern exhibited monomorphic bands. Low levels of polymorphism in peroxidase were supported by Brown *et al.* (1982) and Bassiri and Adams (1978), who indicated that low levels of isozyme variation, were observed among *Phaseolus vulgaris* cultivars due to the recovery of low band numbers.

The protein banding profiles of the *Phaseolus vulgaris* L. cv. Bronco has been revealed by electrophoretic separation SDS-PAGE and illustrated in Table 6 and Figure 4. The total number of bands was 20 with molecular weights ranging from 11.7 KDa to 106.7 KDa. Control (lane 1) harvested the lowest number of bands 15 out of 20, while the highest number of bands was 18, identified in MS supplemented with 0.5 mg L^{-1} 24-EpiBL (lane 3). Demonstrative analysis for the presence and absence of bands were assessed with (1) and (0), respectively, as illustrated in Table 6.Generally sixteen bands were observed as monomorphic (106.7; 96.0; 87.5; 81.9; 79.6; 73.8; 68.4; 66.5; 60.3; 54.1; 48.7; 41.1; 38.9; 28.5; 24.0 and 11.7 KDa) while four bands were polymorphic, giving 20% polymorphism . Moreover, two bands (44.8 and 32.9KDa) were unique , protein have 44.8 KDa was EpiBL marker regardless of its concentration ,while the other protein 32.9KDa disappeared in the highest applied dose only referred with control. On the other hand 34.4 and 20.9 KDa proteins were expressed with the moderate and the highest applied dose only of EpiBL.

Growth-promoting effects of BRs in earlier analyses revealed that BRs interact with other plant hormones (Khripach et al. 1999). Moreover, molecular studies strongly support the nation that there is a crosstalk between BRs and auxins, gibberellins (GA), abscisic acid (ABA) and jasmonic acid (JA), includes alternation in the expression of hormone biosynthetic genes and / or signaling intermediates (Bouquin et al. 2001; Friedrichsen et al. 2000; Lin et al. 2003).

The results of HPLC and GC/MS analysis ,obtained in the present study, indicated appreciable increase in *Ph. vulgaris* content of auxins, gibberellins, cytokinins, jasmonic acid associated with marked decrease in ABA content(Table 7) in response to the applied EpiBL with a magnitude of response being obtained with the highest dose.

The marked increase obtained in auxins content of *Ph. vulgaris* plants in response to EpiBL is in alliance with those obtained by others where an appreciable increase in IAA content of squash seedlings was obtained upon application of BR (Eun et al. 1989). Moreover, Goda et al., (2002) revealed that most of the auxin-induced genes are induced by brassinolide application suggesting that a marked overlap exists between the BRs and auxin signaling pathways. Also, Nemhauser et al., (2004) provided evidence that BRs and auxins signaling pathways converge at the level of the transcriptional regulation of common target genes. In this connection also, previously Yang et al., (1999) reported that BRs increased auxin content via increasing rate of synthesis, de conjugation of auxins, or via increasing

the activity of auxin receptors, thus increasing sensitivity of tissues to auxins.

The appreciable increase encountered in gibbcrellins content of *Ph. vulgaris* in response to EpiBL treatment was associated with marked decrease in its content of ABA. Brassinosteroids were reported to probably act downstream of GAs because BRs are able to rescue germination of GA-deficient (Steber and Mc Court, 2001) and GAs response of mutant seeds (Steber et al. 1998).So, it was suggested that EpiBL interferes with branch points of metabolic control of gibberellins and ABA biosynthetic pathway. In this connection, BRs were previously reported to modulate the balance of inhibitors and promoters of the tissues by themselves or in association with other hormones (Takeuchi et al. 1995). Also, Krizek and Mandava (1983) recorded change induced by BRs in the balance of endogenous hormones as IAA, GA, ABA and ethylene.

The marked decrease obtained in ABA content of *Ph. vulgaris* in response to EpiBL treatment was in accordance with those obtained by Fujii et al., (1991) in rice and by Metwally (2008). Also, Steber and Mc Court (2001) showed that BR rescues the germination of GA-insensitive mutant by overcoming ABA-induced inhibition of growth. Some of the BR-related mutants display altered sensitivity to ABA, suggesting that BRs normally counteract the effect of ABA on root growth, seed germination and possibly stomatal movement (Haubrick et al. 2006).Molecular studies strongly support the notion that there is cross-talk between BRs and ABA includes alteration in the expression of hormone biosynthetic genes and / or signaling intermediates (Bouquin et al. 2001, Friedrichsen et al. 2000, Lin et al. 2003).

In response to EB treatment obvious increase in cytokinin (CK) content of *Ph. vulgaris* was obtained (Table 7). In alliance with this result, Kudryakova et al.,(2013) ,working on transgenic *Arabidopsis thaliana* plants provide the evidence for the involvement of BRs in the regulation of the genes of the CK signaling pathway through an increase in the CK levels. In this regard also, Hu et al. (2000) showed that EB can substitute for the effects of cytokinin in Arabidopsis cultured cells where it promoted their division. The obvious increase encountered in cytokinins content of *Ph. vulgaris* plants in response to EB (Table 7) could contributed in increased content of DNA, recorded in the present work, as cytokinins are known to induce replication of nuclear DNA via enhancement of DNA polyemerase (Reyes et al. 1991; Werner, et al. 2001).

In response to EB treatment appreciable increase in jasmonic acid content of the treated *Ph. vulgaris* was recorded. JA plays a crucial role in different aspects of plant growth and disease resistance. In this connection, Mei et al. (2006) stated that increased endogenous JA levels, up-regulated PR gene expression and enhanced disease resistance against rice blast. Also, in this regard, it was reported that jasmonate biosynthesis is up regulated by BR, so it was suggesting a relationship between BR and jasmonate signaling pathway (Song et al. 1995, Li and Chory, 1997).

Table 1: Changes in germination percentage ,major growth attributes and nitrate reductase activity (NR) of shoot and root of *Phaseolus vulgaris* L seedlings (21- day- old) grown in MS culture medium as affected by exogenous application of 24-Epibrassinolide (EpiBL) in controlled growth chamber.

Parameters / Treatments	Germination %	Plant parts	Fresh weight (mg)	Dry weight (mg)	Water content %	Length (cm)	NR µmol g^{-1} FW
Control	27.5	Shoot	1.5c	0.46b	80.0	4.3c	203.75d
0.1 µ M Epi	40.0		1.62c	0.47b	80.9	4.5bc	272.32c
0.5 µ M Epi	50.0		1.64b	0.48b	80.5	5.6b	285.8b
1.0 µ M Epi	73.12		1.98a	0.59a	81.6	8.3a	300.73a
LSD at 5%			0.132	0.114	NS	1.25	12.26
Control		root	0.71c	0.07c	90.5	3.2b	66.61d
0.1 µM Epi			0.82bc	0.08bc	91.5	3.5b	82.28c
0.5 µ M Epi			0.84b	0.09b	91.4	3.6b	100.4b
1.0 µ M Epi			1.15a	0.11a	92.0	5.2a	171.42a
LSD at 5%			0.12	0.011	NS	1.33	10.48

Table 2 : Changes in chlorophyll *a*, *b* total chlorophyll and carotenoids contents leaves of *Phaseolus vulgaris* L. seedlings (21- day- old) grown in MS culture medium as affected by exogenous application of 24-Epibrassinolide (EpiBL) in controlled growth chamber.

Parameters / Treatments	Chl. *a* mg^{-1} g FW	Chl. *b* mg-^{1} g FW	Carotenoids mg-^{1} g FW
control	0.757d	0.559b	0.399c
0.1 µM Epi	0.956c	0.737b	0.511bc
0.5 µ M Epi	1.108b	0.99a	0.6184b
1.0 µ M Epi	1.321a	1.042a	3.286a
LSD at 5%	0.156	0.211	0.205

Table 3 : Changes in DNA ,RNA and total soluble protein contents of shoot of *Phaseolus vulgaris* L. seedlings (21- day- old) grown in MS culture medium as affected by exogenous application of 24-Epibrassinolide (EpiBL) in controlled growth chamber.

Parameters / Treatments	DNA mg^{-1} g FW	RNA mg^{-1} g FW	Total soluble protein mg^{-1} g FW
control	5.1d	5.95c	5.81d
0.1 µM Epi	7b	7.0b	9.34b
0.5 µ M Epi	7.8c	7.4b	7.5c
1.0 µ M Epi	8.17a	9.5a	11.05a
LSD at 5%	0.68	0.72	1.06

Table 4 : Changes in malondialdehyde (MDA) and hydrogen peroxide (H_2O_2) contents of shoot and root of *Phaseolus vulgaris* L. seedlings (21- day- old) grown in MS culture medium as affected by exogenous application of 24-Epibrassinolide (EpiBL) in controlled growth chamber.

Parameters / Treatments	Plant part	MDA mmol min^{-1} g^{-1} FW	H_2O_2 mmol g^{-1} FW	AAO mmol g^{-1} FW
control	shoot	0.30a	58.0a	0.34b
0.1 µM Epi		0.30a	50.4b	0.45a
0.5 µ M Epi		0.18b	41.0c	0.30b
1.0 µ M Epi		0.14b	30.5d	0.21c
LSD at 5%		0.084	6.45	0.098
control	root	0.20ab	56.0a	0.30a
0.1 µM Epi		0.23a	48.9b	0.27a
0.5 µ M Epi		0.16b	35.0c	0.26a
1.0 µ M Epi		0.10c	30.0d	0.14b
LSD at 5%		0.057	4.58	0.115

Table 5: The presence (+) and absence (-) of bands and relative mobility (R*f*) in two isozymes, peroxidase (POD) and Polyphenol oxidase (PPO) of leaves of *Phaseolus vulgaris* L. seedlings (21- day- old) grown in MS culture medium as affected by exogenous application of 24-Epibrassinolide (EpiBL) in controlled growth chamber.

Peroxidase(POD) Group	Relative Mobility	Treatments			
		Lane1	**Lane2**	**Lane3**	**Lane4**
POD 1	0.1	-	-	-	-
POD 2	0.3	+	+	+	+
POD 3	0.4	-	-	-	-
POD 4	0.5	-	-	-	-
POD 5	0.6	+	-	-	-
POD 6	0.7	+	-	-	-
POD 7	0.8	+	+	+	+
POD 8	0.9	+	+	+	+
Total bands		**5**	**3**	**3**	**3**
Polyphenol Oxidase(PPO) Group	Relative Mobility	1	2	3	4
PPO1	0.3	+	+	+	+
PPO2	0.5	+	+	+	+
PPO3	0.6	+	+	+	+
PPO4	0.7	+	+	+	+
PPO5	0.8	+	+	+	+
Total bands		**5**	**5**	**5**	**5**

Lane 1: from control; lane 2: seedlings treated with 0.1 mg L^{-1} EpiBL ; lane 3: 0. 5 mg L^{-1} EpiBL ; lane 4: 1.0 mg L^{-1} EpiBL.

Table 6: Data matrix illustrating the presence or absence of protein bands and their molecular weights (MW) of leaves of *Phaseolus vulgaris* L. seedlings (21- day- old) grown in MS culture medium as affected by exogenous application of 24-Epibrassinolide (EpiBL) in controlled growth chamber.

Band number	MW	Lane1	Lane2	Lane3	Lane4
1	106.7	1	1	1	1
2	96.0	1	1	1	1
3	87.5	0	0	0	0
4	81.9	1	1	1	1
5	79.6	1	1	1	1
6	73.8	1	1	1	1
7	68.4	1	1	1	1
8	66.5	0	0	0	0
9	60.3	1	1	1	1
10	54.1	1	1	1	1
11	48.7	1	1	1	1
12	44.8	0	1	1	1
13	41.1	1	1	1	1
14	38.9	1	1	1	1
15	34.4	0	0	1	1
16	32.9	1	1	1	0
17	28.5	1	1	1	1
18	24.0	1	1	1	1
19	20.9	0	0	1	1
20	11.7	1	1	1	1
Total No. of Bands		**15**	**16**	**18**	**17**

Lane 1: from control; lane 2: seedlings treated with 0.1 mg L^{-1} EpiBL ; lane 3: 0. 5 mg L^{-1} EpiBL ; lane 4: 1.0 mg L^{-1} EpiBL.

Table 7: Changes in endogenous hormones of leaves of *Phaseolus vulgaris* L. seedlings (21- day- old) grown in MS culture medium as affected by exogenous application of 24-Epibrassinolide (EpiBL) in controlled growth chamber.

	Concentrations (µg g^{-1} FW)					
Hormones / 24-Epi µM	GA_3	NAA	IAA	ABA	Cytokinins	JA
control	25.50	12.90	1.06	33.83	0.824	0.524
0.1 µM Epi	20.01	2.17	0.47	25.79	0.407	0.876
0.5 µ M Epi	41.88	64.18	1.44	45.70	1.010	0.894
1.0 µ M Epi	42.91	6.87	2.24	1.17	1.108	1.525

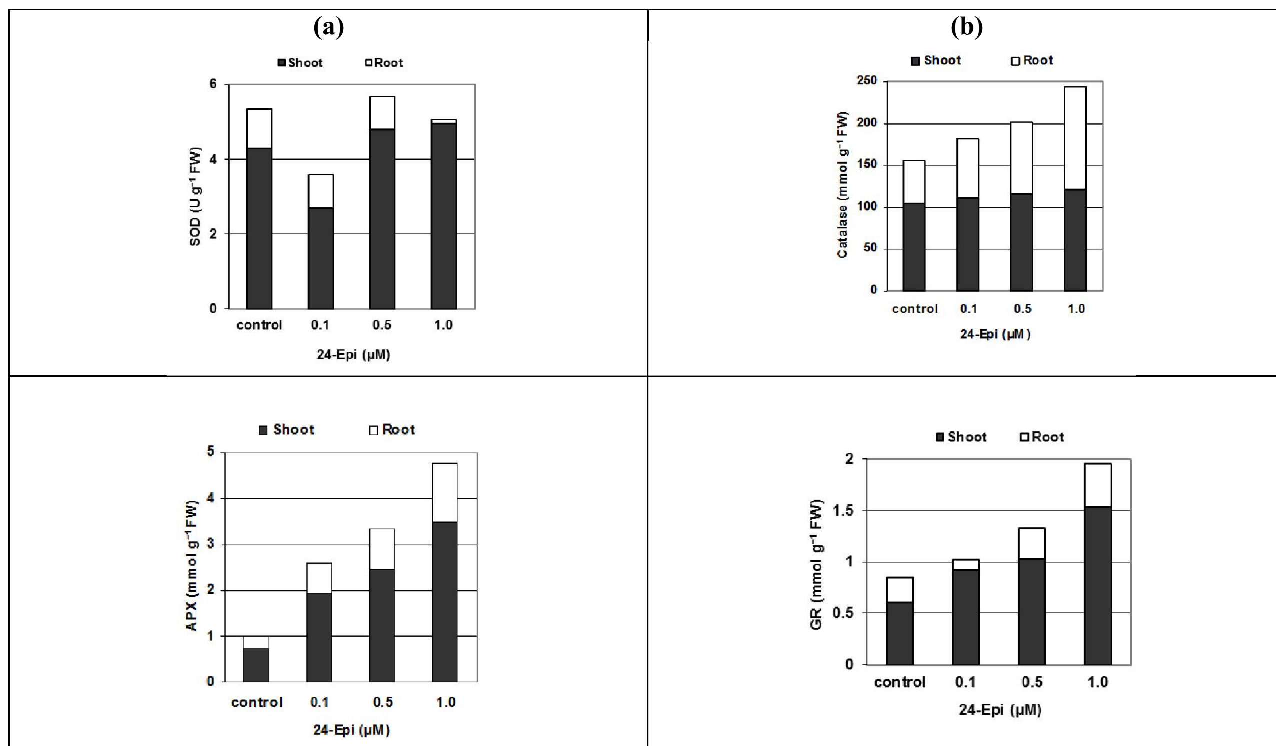

Fig. 1 : **Chang** **l) glutathione**
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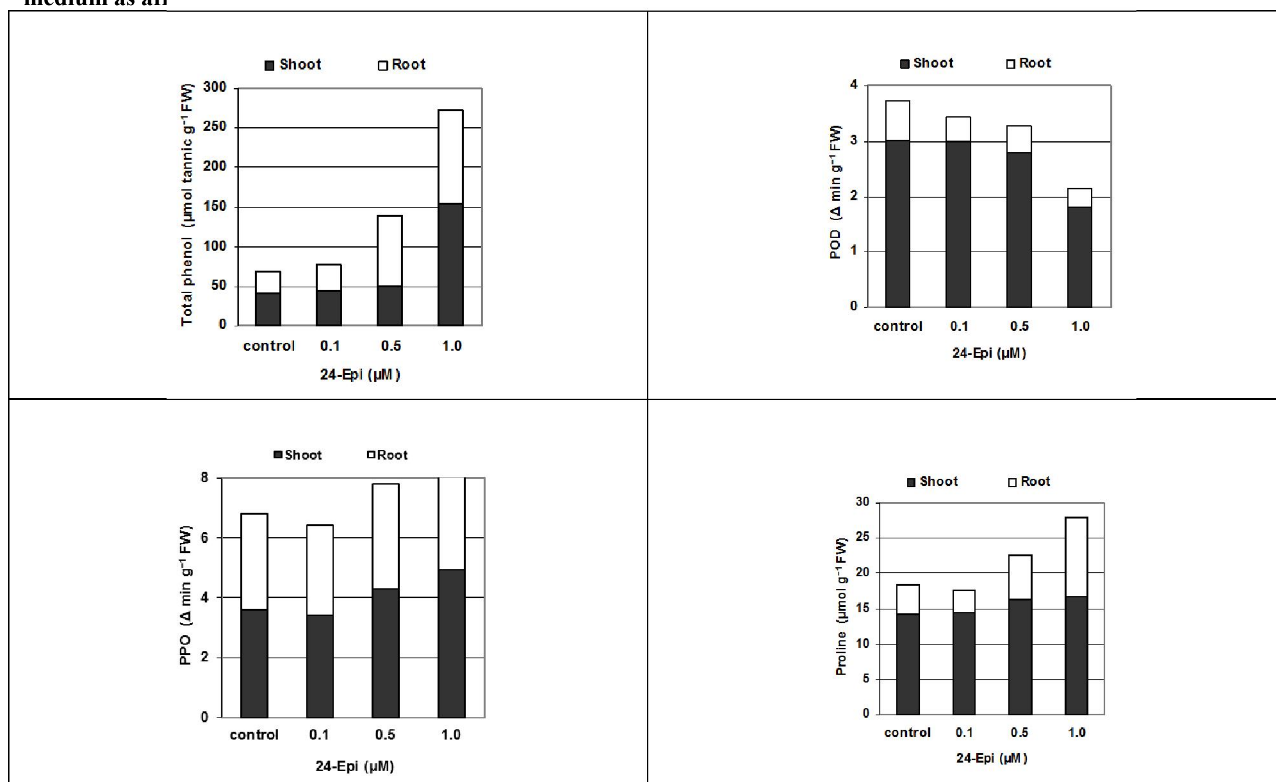

Fig. 2: Changes in(a) total phenol (TP) , (b) peroxidase (POD), (c) polyphenol oxidase (PPO) enzymes activity and (d) proline content of shoot and root of *Phaseolus vulgaris* L. seedlings (21- day- old) grown in MS culture medium as affected by exogenous application of 24-Epibrassinolide (EpiBL) in controlled growth chamber.

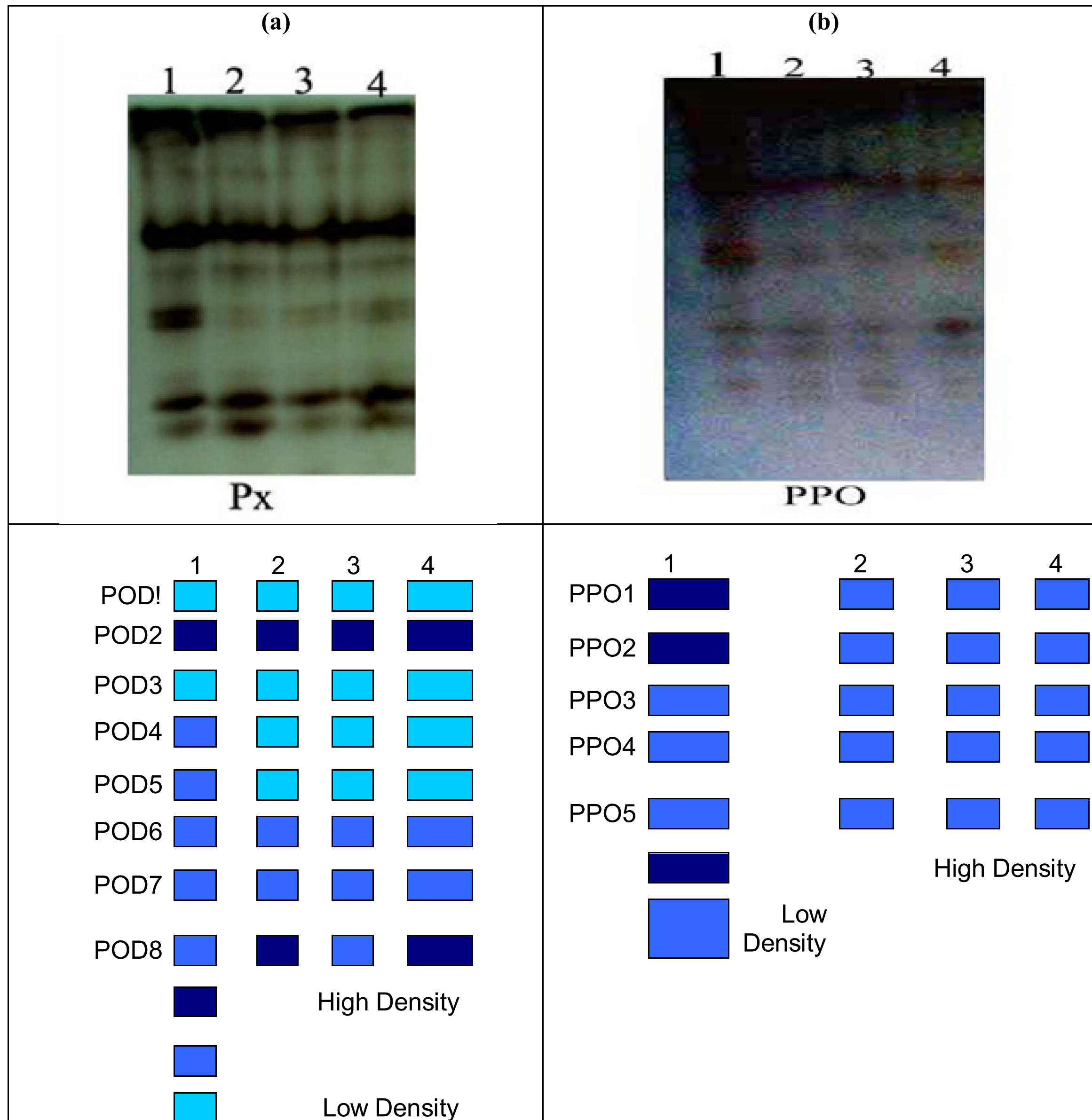

Fig. 3: Electrophoretic banding patterns and their density of (a) peroxidase (POD) and (b) polyphenol oxidase (PPO) isozymes of leaves of *Phaseolus vulgaris* L. seedlings (21- day- old) grown in MS culture medium as affected by exogenous application of 24-Epibrassinolide (EpiBL) in controlled growth chamber.

Lane 1: from control; lane 2: seedlings treated with 0.1 mg L^{-1} EpiBL ; lane 3: 0. 5 mg L^{-1} EpiBL ; lane 4: 1.0 mg L^{-1} EpiBL.

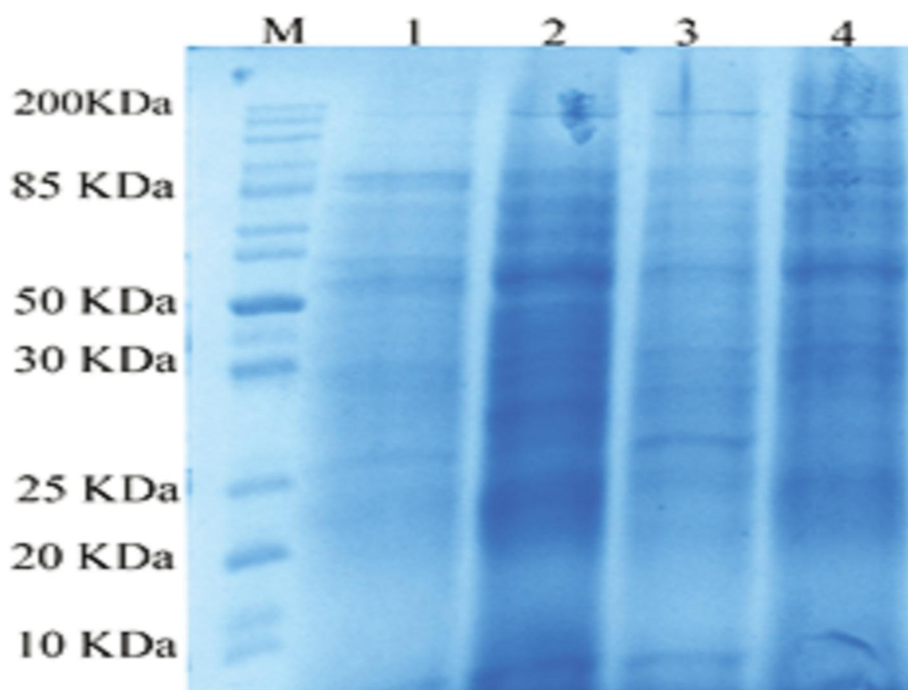

Fig. 4: Electrophoretic banding patterns, SDS-protein of total soluble protein of seedlings leaves (21- day- old) of *Phaseolus vulgaris* L. as affected by exogenous application of 24-Epibrassinolide (EpiBL) Into MS culture medium and germinated in controlled growth chamber.

M = Marker protein, lane 1: control; lane 2: seedling treated with 0.1 mg L^{-1} EpiBL; lane 3: 0.5 mg L^{-1} EpiBL; lane 4: 1.0 mg L^{-1} EpiBL.

References

1. Aebi H (1984) Catalase in vitro. Methods Enzymol 105:121–126.
2. Ahammed GJ, Zhou YH, Xia XJ, Mao WH, Shi K, Yu JQ (2013) Brassinosteroid regulates secondary metabolism in tomato towards enhanced tolerance to phenanthrene. - Biol. Plant 57: 154-158.
3. Alexieva V, Sergiev I, Mapelli S, Karanov E (2001) The effect of drought and ultraviolet radiation on growth and stress markers in pea and wheat. Plant Cell Environ 24: 1337-1344.
4. Alia B, Pardhasaradhi P, Mohanty P (1997) Involvement of proline in protecting thylakoid membranes against free radical-induced photodamage. J of Photochemistry and Photobiology 38: 253-257.
5. Anuradha S, Rao SSR (2001) Effect of brassinosteroids on salinity stress induced inhibition of seed germination and seedling growth of rice (*Oryza sativa* L.). Plant Growth Regul 33:151-153.
6. Anuradha S, Rao SSR (2003) Application of brassinosteroids to rice seeds (*Oryza sativa* L.) reduced the impact of salt stress on growth and improved photosynthetic pigment levels and nitrate reductase activity. Plant Growth Regul 40:29-32.
7. Anuradha S, Rao SSR (2007) The effect of brassinosteroids on radish (*Raphanus sativus* L.) seedlings growing under cadmium stress. Plant Soil Environ 53: 465 – 472.
8. Arellano J, Fuentes S, Castillo-Espan˜a P, erna´ndez G (2008) Regeneration of different cultivars of common bean (*Phaseolus vulgaris* L.) via indirect organogenesis. Plant Cell Tiss Organ Cult 96:11–18. DOI 10.1007/s11240-008-9454-1.
9. Arnon DI (1949) Copper enzyme in isolated chloroplasts: Polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24: 1-15.
10. Asada K, Takahashi M (1987) Production and scavenging of active oxygen in photosynthesis. In: Kyle DJ, Osmond CJ, Artnen CJ (eds) Photo inhibition: Topics in Photosynthesis Elsevier, Amsterdam, pp 227-287.
11. Ashraf M, Akram NA, Arteca RN, Foolad MR (2010) The Physiological, Biochemical and Molecular Roles of Brassinosteroids and Salicylic Acid in Plant Processes and Salt Tolerance. Critical Reviews in Plant Sciences (29)3:162-190.
12. Bajguz A (2012) Origin of Brassinosteroids and Their Role in Oxidative Stress in Plants. Phytohormones and Abiotic Stress Tolerance in Plants, 169-18
13. Bajguz A, Hayat S (2009) Effects of brassinosteroids on the plant responses to environmental stresses. Plant Physiol Biochem 47:1–8. doi: 10.1016/j.plaphy.2008.10.002.
14. Baker NR, Nogues S, Allen DJ (1997) Photosynthesis and photo inhibition In: Lumsden PJ (ed) Plants and UV- B: response to environmental change. Cambridge, UK: Cambridge University Press 95 – 111.
15. Bassiri A, Adams MW (1978) Evaluation of common bean cultivars relationships by means of isozyme electrophoretic patterns. Euphytica 27: 707-720.
16. Bates LS, Waldren RP, Teare ID (1973) Rapid determination of free proline for water stress studies. Plant and Soil 29: 205-207.
17. Beauchamp C, Fridovich I (1971) Superoxide dismutase: improved assay and an assay applicable to acrylamide gels. Anal Biochem 44: 276–287.
18. Behnamnia M, Kalantarl KM, Rezanejad F (2009) Exogenous application of brassinosteroid alleviates drought-induced oxidative stress in *Lycopersicon esculentum*. Generaland Applied Plant Physiology 35 (1–2): 22–34.
19. Belmonte M, Elhiti M, Waldner B, Stasolla C (2010) Depletion of cellular brassinolide decreases embryo production and disrupts the architecture of the apical meristems in *Brassica napus* microspore-derived embryos**.** J Exp Bot 61(10): 2779–2794.

20. Bouquin T, Meier C, Foster R, Nielson ME, Mundy J (2001) Control of specific gene expression by gibberellin and brassinosteroids. Plant physiol 127: 450 – 458.
21. Brown AHD (1978) Isozymes plant population genetic structure and genetic conservation. Theoretical and Applied Genetics 52(4): 145-157.
22. Brown JWS, McFerson JR, Bliss FA, Hall TC (1982) Genetic divergence among commercial classes of *Phaseolus vulgaris* in relation to phaseolin pattern. Hort Science 17:752-754.
23. Burton K (1968) Determination of DNA Concentration with Diphenylamine. In: Grossman L, Meidave M (eds) Methods in Enzymology, Academic Press, New York, p 163.
24. Cao SQ, Xu QT, Cao YJ, Qian K, Kun A, Zhu Y, Hu BZ, Zhao HF, Kuai B (2005) Loss of function mutations in DET2 gene lead to an enhanced resistance to oxidative stress in arabidopsis. Physiologia Plantarum 123 (1): 57-66.
25. Clouse SD (1996) Molecular genetic studies confirm the role of brassinosteroids in plant growth and development. Plant J 10: 1-8.
26. Clouse SD, Sasse JM (1998) Brassinosteroids: essential regulators of plant growth and development. Annu Rev Plant physiol Plant Mol Bio 49: 427-451.
27. Eun JS, Kuraishi S, Sakuai N (1989) Changes in levels of auxin and abscisic acid and the evolution of ethylene in squash hypocotyls after treatment with brassinolide. Plant cell physiol 30: 807 – 810.
28. FAO stat. (The Food and Agriculture Organization) [en ligne], (2005) Consulté 07/04/ 2010. Disponible sur World Wide Web: <http://faostat.fao.org/site/567/default.aspx#ancor, (07/04/10)>.
29. Fariduddin Q, Yusuf M, Hayat S, Ahmad A (2009) Effect of 28- homobrassinolide on antioxidant capacity and photosynthesis in *Brassica juncea* plants exposed to different levels of copper. Environ Exp Bot 66:418–424. doi: 10.1016/j.envexpbot.2009.05.001.
30. Farooq M, Wahid A, Basra SM, Din IU (2009) Improving the Water Relations and Gas Exchange with Brassinosteroids in Rice under Drought Stress. J of Agronomy and Crop Science 195 (4): 262-269.
31. Filova A, Sytar O, Krivosudska E (2013) Effects of brassinosteroid on the induction of physiological changes in *Helianthus annuus* L. under copper stress. Acta Universitatis Agriculturae Etsilviculturae Mendelianae Brunensis. LXI 68 (3).
32. Flurkey WH, Jen JJ (1978) Peroxidase and polyphenol oxidase activities in developing peaches. J Food Sci 43: 1826 – 1831.
33. Foyer CH, Descourvieres P, Kunert KJ (1994) Protection against oxygen radicals an important defense-mechanism studied in transgenic plants. Plant, Cell and Environment, 17(5):507-523.
34. Foyer CH, Halliwell B (1976) Presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133: 21-25.
35. Foyer CH, Looez-Delgado H, Dat JF, Scott IM (1997) Hydrogen peroxide and glutathione associated mechanisms of acclamatory stress tolerance and signing. *Physiologia Plantarum*, 100 (2): 241-254
36. Friedrichsen DM, Joazeiro CA, Li J, Hunter T, Chory J (2000) Brrassinosteroids – insensitive – 1 is a ubiquitously expressed leucine– rich repeat receptor serine / threonine Kinase. Plant physiol 123: 1247 – 1256.
37. Fujii S, Hirai K, Saka H (1991) Growth regulating action of brassinolide in rice plants. In: Culter HG, Yokota T, Adam G (eds) Brassinosteroids: Chemistry, bioactivity and applications. Washington, American Chemical Society, pp 306 – 311.
38. Gapper C,Dolan L (2006) Control of Plant Development by Reactive Oxygen Species. Plant Physiology (141): 341–345. American Society of Plant Biologists.
39. Gatica-Arias MA, Valverde JM, Fonseca PR, Melara MV (2010) In vitro plant regeneration system for common bean (*Phaseolus vulgaris*): effect of N6- benzyl amino purine and adenine sulphate. Electronic. J of Biotechnology .DOI:10.2225/vol13-issue1-full text-7.
40. Giannopolitis CN, Ries SK (1977) Superoxide dismutase I. Occurrence in higher plants. Plant Physiol 59:309–314.
41. Goda H, Shimada Y, Asami T, Fujioka S, Yoshda S (2002) Microaray analysis of brassinosteroids- regulated genes in Arabidopsis. Plant physiol 130 (3): 1319 – 1334.
42. Gonzalez-Garcia MP, Vilarrasa-Blasi J, Zhiponova M, Divol F, Mora-Garcia S, Russinova E, Cano-Delgado AI (2011) Brassinosteroids control meristem size by promoting cell cycle progression in *Arabidopsis* roots. Development 138: 849–859.
43. Halliwell B, Gutteridge JMC (1989) Free radicals in biology and medicine, Ed (2). Oxford University Press (Clarendon), Oxford, UK.
44. Hasan SA, Hayat S, Ali B, Ahmad A (2008) 28-Homobrassinolide protects chickpea (*Cicer arietinum*) from cadmium toxicity by stimulating antioxidant. Environ Pollut 151:60-66.
45. Haubrick LL, Torsethaugen G, Assmann SM (2006) Effect of brassinolide, alone and in concert with abscisic acid, on control of stomata aperture and potassium currents of *Vicia faba* guard cell protoplasts. Physiologia Plantrum 128: 134 – 143.
46. Hayat S, Ahmad A (2003) 28 – Homobrassinolide induced changes favored germ inability of wheat grains. Bulgarian J of Plant physiology 29 (1-2): 55 – 62.
47. Hayat S, Ali B, Hasan A, Ahmad A (2007) Brassinosteroid enhanced the level of antioxidants under cadmium stress. In: *Brassica juncea*. Environmental and Experimental Botany Department 60: 33 – 41.
48. Heath RL, Packer L (1969) Photo peroxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. Arch Biochem Biophys 125: 189-198.
49. Heidari M (2010) Nucleic acid metabolism, proline concentration and antioxidants enzyme activity in canola (*Brassica nupus* L.) under salinity stress. Agric Sci China 9: 504–511.
50. Hillocks RJ, Madata CS, Chirwa R, Minja EM, Msolla S (2006) Phaseolus bean improvement in Tanzania, 1959-2005. Euphytica, 150: (1-2) 215-231.
51. Hong FU Ji, Hong SX, De W Ji, Fang C Jin,Yu Y C (2011) Progress in quantitave analysis of plant hormones. Chinese Science Bulletin 56(4-5) 355-366.
52. Hou LP, Li ML (2001)Progress of studies on the plant growth promoting mechanism of brassinolide (BR). Chinese Bull Bot 5:560–566.
53. Hu YX, Bao F, Li JY (2000) Promotive effects of brassinosteroids on cell division involves a distinct Cyc D_3 induction pathway in Arabidopsis. The plant J 24: 693 – 701.
54. Jaworski EG (1971) Nitrate reductase assay in intact plant tissues. Biochem.Biophys. Res Commun 43: 1247-1279.
55. Kalinich JF, Mandava NB, Todhunter JA (1986) Relationship of nucleic acid metabolism to brassinolide-induced responses in beans. J Plant Physiol 125:345-353.
56. Kavi Kishor PB, Sangam S, Amruth RN, Sri Laxmi P, Naidu KR, Rao KR, Sreenath R, Reddy KJ, Theriappan P, Sreenivasulu N (2005) Regulation of proline biosynthesis, degradation, uptake and transport in higher plants: its implications in plant growth and abiotic stress tolerance. Current Science 88: 424-438.

57. Kelen M, Demiralay EC, Sen S, Ozkan G (2004) Separation of abscisic acid, Indole-3-acetic acid, Gibberellic acid in 99 R (*Vitis berlandieri* x *Vitis rupestris*) and Rose Oil (*Rosa damascena* Mill.) by Reversed Phase Liquid Chromatography. Turk J Chem 28: 603 -610.
58. Khripach V, Zhabinskii V, De Groot A (1999) Brassinosteroids. A new class of of plant hormones.Academic Press, San Diego.
59. Khripach V, Zhabinskii V, De Groot A (2000) Twenty years of brassinosteroids: Steroidal plant hormones warrant better crops for XXI century. Ann Bot (Lond.) 86:441–447.
60. Khripach V, Zhabinskii V, Khripach NB (2003) New practical aspects of brassinosteroids and results of their 10 – year agricultural use in Russia and Belarus. In: Hayat S, Ahmed A (eds) Brassinosteroids: Bioactivity and Crop Productivity. Kluwer Academic Publisher, Dordrecht, pp 189 – 230.
61. Krizek DT, Manava NB (1983) Influence of spectral quality on the growth response of intact bean plants to brassinolide, a growth – promoting steroidal lactone. 11. Chlorophyll content and partitioning of assimilate. Physide Plant 57: 324 – 329.
62. Kudryakova NV, Efimova MV, Danilova MN, Zubkova NK, Khripach VA, Kusnetsov VV, Kulaeva ON (2013) Exogenous brassinosteroids activate cytokinin signaling pathway gene expression in transgenic *Arabidopsis thaliana*. Plant Growth Regulation70: 61-69.
63. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4.Nature 227: 680-685.
64. Larcher W (1995) Physiological plant ecology. New York: Springer-Verlag Berlin Heidelberg.
65. Leubner-Metzger G (2001) Brassinosteroids and Gibberellins promote Tobacco seed germination by different pathways. Planta 213 (5):758-763.
66. Li J, Chory J (1997) A Putative lecture – rich repeat receptor Kinase involved in brassinosteroid signal transduction. Cell 90: 929 – 938.
67. Lichtenthaler H, Wellburn A (1983) Determinations of total carotenoids and chlorophylls a and b of leaf extracts in different solvents. Biochem. Soc. Trans 11: 591-592.
68. Lin J, Tarr PT, Yang R, Rhee J, Puigserver P, Newgrad CB, Spiegelman BM (2003) PGC – I beta in the regulation of hebatic glucose and energy metabolism. J Biol Chem 278: 30843 – 30848.
69. Ling-Yun Y, Du J, Yuan Ying-Hui, Shu S, Sun J, Guo Shi-Rong (2013) Effects of 24-epibrassinolide on ascorbate–glutathione cycle and polyamine levels in cucumber roots under $Ca(NO_3)_2$ stress. Acta Physiologiae Plantarum 35 (1): 253-262.
70. Liu DB, Wei JY, Li SP, Cui BM, Peng M (2008) Effects of Brassinolid on chilling resistance in banana seedlings. Bull Bot Res 2:195–221.
71. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chemistry 193: 265-275.
72. Luh BS, Phithakpol B (1972) Characteristics of polyphenol oxidase related to browning in cling peaches. J Food Sci 37: 264–268.
73. Mahesh K, Balaraju P, Ramakrishna B, Ram Rao S (2013) Effect of Brassinosteroids on germination and seedling growth of Radish (*Raphanus sativus* L.) under PEG-6000 induced water stress. American J of Plant Sciences 4: 2305-2313.
74. Mai Y, Lin S, Zeng S, Ran R (1989) Effect of brassinolide on nitrate reductase activity in rice seedlings. Plant Physiol Comm 2: 50–52.
75. Maity U, Bera AK (2009) Effect of exogenous application of brassinolide and salicylic acid on certain physiological and biochemical aspects of green gram (*Vigna radiata* L. Wilczek), Indian. J of Agricultural Research 43 (3): 194.
76. Matysik J, Alia B, Bhalu, Mohanty P (2002) Molecular mechanisms of quenching of reactive oxygen species by proline under stress in plants. Current Science 82: (5) 10.
77. Mei C, Qi M, Sheng G, Yang Y (2006) Inducible over expression of a rice allene oxide synthase gene increases the endogenous jasmonic acid level, PR gene expression, and host resistance to fungal infection. Mol Plant–Microbe Interact 19: 1127–1137.
78. Meirs S, Philosophhadas S, Aharoni N (1992) Ethylene-increased accumulation of fluorescent lipid–peroxidation products detected during senescence of parsley by a newly developed method. J of the American Society for Hort Science 117: 128-132.
79. Metwally MH (2008) Phsiological and molecular studies of brassinosteroids,24-Epibrassinolide on *Cucurbita popo* L.M.Sc. Botany Department ,Ain Shams University.
80. Michalak A (2006) Phenolic Compounds and their antioxidant activity in plants growing under heavy metal stress. Polish J of Environmental Studies 15 (4): 523-530.
81. Morillon R, Catterou M, Sangwan RS, Sangwan BS, Lassalles JP (2001) Brassinolide may control aquaporin activities in *Arabidopsis thaliana*. Planta 212: 199 – 204.
82. Muller P Hilgenberg W (1986) Isomers of zeatin and zeatinriboside in club root tissue: evidence for trans–zeatin biosynthesis by *Plasmodiophora brassicae*. Physiol Plant 66:245–250.
83. Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Phys Plant 15:473–479.
84. Nakajima N, Shida A, Toyama S (1996) Effect of Brassinosteroids on Cell Division and Colony Formation of Chinese Cabbage Mesophyll Protoplasts. Japanese Crop Science 65 (1):114-118.
85. Nakano Y, Asada K (1987) Purification of peroxidase in spinach chloroplasts: its inactivation in ascorbate-depleted medium and re-activation by mono dehydro ascorbate radical. Plant Cell Physiol 28: 131-140.
86. Nemhauser JL, Mockler TC, Chory J (2004) Interdependency of brassinosteroids and auxin signating in *Arabidopsis*. Plos Biol 2: 258 – 260.
87. Ogur M, Rosen G (1950) The Extraction and estimation of deoxypentose nucleic acid and pentose nucleic acids. Archive Biochemistry Biophysics 24: 262- 276.
88. Prakashy M, Suganthi S, Gokulakrishnan J, Sabesan T (2008) Effect of Homobrassinolide on growth, physiology and biochemical aspects of sesame. Karnataka J Agric Sci 20 (1): 110-112.
89. Pugachev RM, Matveev VA, Skorina VV (2000) Influence of mineral and hormonal composition of nutrient medium on pune, cherry plum and sloe embryos germination and growing in Vitro. Sodininkysteir Darzininkyste 19 (3): 454-463.
90. Ramakrishna B, Rao SS (2013) 24-Epibrassinolide maintains elevated redox state of AsA and GSH in Radish (*Raphanus sativus*) seedlings by under Zinc stress. Acta Physiologia Plantarum 35(4): 1291-1302.
91. Ranieri A, Nali G, D'Urso G (1995) Peroxidase activity in *Cucurbita pepo* L. leaves exposed to ozone. Agricoltura Mediterranea. (Special volume) 47-54.
92. Reyes J, Jimenez-Garcia LF, Gonzalez MA, Vazquez-Ramos JM (1991) Benzyl adenine stimulation of nuclear DNA synthesis and cell division in germinating maize. Seed Science Research 1: 113-117.
93. Sairam RK (1993) Effect of homobrassinolide on plant metabolism under moisture – stress in two varieties of wheat *Triticum aestivum* L. Indian. J of Experimental Biology.
94. Sairam RK, Deshmukh PS, Saxena DC (1998) Biologia Plantarum 41 (3): 387-394.

95. Sakurai A, Yokota T, Clouse SD (1999) Brassinosteroids. Springer Verlag, Tokyo, pp 253.
96. Salin ML (1988) Toxic oxygen species and protective systems of chloroplast. Physiol Plant 72: 681-728.
97. Sarkar RK, Das A (2000) Changes in anti-oxidative enzymes and antioxidants in relation to flooding tolerance in rice. J Plant Biol 27: 307–311.
98. Sasse JM (1990) Brassinolide-induced elongation and auxin. *Physiologia Plantarum* 80 (3): 401- 408.
99. Sasse JM (2003) Physiological actions of brassinosteroids: an update. J Plant growth Regul 22: 276 – 288.
100. Schneider WC (1957) Determination of nucleic acids in tissues by pentose analysis. In: Colowick SP, Kaplan WO (eds) Methods in Enzymology, Academic Press, New York, p 680.
101. Shahid MA, Pervez MA, Balal RM, Mattson NS, Rashid A, Ahmad R, Ayyub CM, Abbas T (2011) Brassinosteroid (24-epibrassinolide) enhances growth and alleviates the deleterious effects induced by salt stress in pea (*Pisum sativum* L.). Aust J Crop Sci 5: 500-510.
102. Sirhindi G, Kumar S, Bharadwaj R, Kumar R (2009) Effects of 24-epibrassinolide and 28-homobrassinolide on the growth and antioxidant enzyme activities in the seedlings of *Brassica junceae* L. Physiol Mol Biol Plants 15(4):335-341.
103. Snedecor GM, Cochran WC (1969) Statistical methods. 6th Ed. Iowa Univ. press. Ames. Iowa U.S.A.
104. Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wangs B, Zhao WX, Zhu LH, Fauquet C, Ronald P (1995) A receptors Kinase – like protein encoded by the disease resistance gene, Xa21.Science 270: 1894 – 1806.
105. Steber CM, Cooney SE, Mc Court P (1998) Isolation of the GA – response mutant Stylas a suppressor of AB11 1 in *Arabidopsis thaliana*. Genetics 149: 509 – 521.
106. Steber CM, Mccourt P (2001) Arole for brassinosteroids in germiniation in *Arabidopsis*. Plant physiol 125: 763 – 669.
107. Stegemann H, Afify AMR, Hussein KRF (1985) Cultivar identification of dates (*Phoenix dactylifera*) by protein patterns. Second International Symposium of Biochemical Approaches to Identification of Cultivars .Braunschweing ,West Germany pp: 44.
108. Studier FW (1973) Analysis of bacteriophage T7 early RNAs and proteins on slab gels. Journal of Molecular Biology 79(2): 237-242.
109. Sugimoto-Shirasu K, Roberts K (2003) Big it up end reduplication and cell-size control in plants. Curr Opin Plant Biol 6: 544–553.
110. Takeuchi Y, Omigawa Y, Ogasawara, M, Yoneyama, K, Konnai M, Worsham AD (1995) Effects of brassinosteroids on conditioning and germination of clover broomrape (*Orabanche minor*) seeds. Plant Growth Regulation 16: 153 – 160.
111. Vardhini BV, Rao SSR (2003) Amelioration of water stress by brassinosteroids on seed germination and seedling growth of three varieties of sorghum. Plant Growth Regul 41: 25–31.
112. Vardhini BV, Sujatha E, Rao SSR (2011) Effect of brassinolide on biochemical composition of radish (*Raphanus sativus*). Bioinfolet 8: 404-406.
113. Vardhini BV, Sujatha E, Rao SSR (2012) Influence of brassinosteroids on metabolites of *Raphanus Sativus* L. J of Phytology 4 (2): 45-47.
114. Verma A, Malik CP, Gupta VK (2012) In Vitro Effects of Brassinosteroids on the growth and antioxidant enzyme activities in groundnut. ISRN Agronomy, Volume 2012 , Article ID 356485.
115. Vines HM, Oberbacher F (1962) Citrus fruit enzymes. I. Ascorbic Acid Oxidase in Oranges. Published by American Society of Plant Biologists.
116. Wang TW, Cosgrove DJ, Arteca RN (1993) Brassinosteroid stimulation of hypocotyls elongation and wall relaxation in Pakchoi (*Brassica chinensis cv* lcichoi). Plant physiol 101:965-968.
117. Wasfy WS, Orrin ES (1975) Identification of plant hormones from cotton ovules. Plant Physiol 55: 550 – 554.
118. Werner T, Motyka V, Strnad M, Schmülling T (2001) Regulation of plant growth by cytokinin. Proc Natl Acad Sci USA 98: 10487–10492.
119. Yang YH, Zhang H, Cao RQ (1999) Effect of brassinotide on growth and shikonin formation in cultured on *Osoma paniculatum* cell. Plant growth regul 18: 89 – 92.
120. Yao Y , Cheng Xu-Zhen, Wang Li-Xia, Wang Su-Hua , Ren G (2012) Major phenolic compounds, antioxidant capacity and ant diabetic potential of Rice Bean (*Vigna umbellata* L.) in China. Int J Mol Sci 13(3): 2707–2716.
121. Yao Y, Ren GX (2011) Effect of thermal treatment on phenolic composition and antioxidant activities of two celery cultivars. LWT-Food Sci Techno 44:181–185.
122. Yu JQ, Huang LF, Hu WH, Zhou YH, Mao WH, Ye SF, Nogues S (2004) A role for brassinosteroids in the regulation of photosynthesis in *Cucumis sativas*. J Exp. Bot 55 (399): 1135 – 1143.
123. Yuan GF, Jia CHJ, Zhen L, Sun B, Zhang LP, Liu N, Wang QM (2010) Effect of brassinosteroids on drought resistance and abscisic acid concentration in tomato under water stress. Sci Hortic 126: 103–108.
124. Zhou S, Fang Z, Lü Y, Chen J, Liu D, Ye X (2009) Phenolics and antioxidant properties of bayberry (*Myrica rubra sieb*. et Zucc.) pomace. Food Chem 112:394–399.

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