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Biochemical and Physiological Mechanisms Related to Vernalization, Atonik and Benzyl Adenine in *Pisum sativum*

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ABSTRACT

Vernalization at 5°C for 5 days, either singly or in combination with a foliar application of atonik at 250, 500 and 1000 mg/l or 6-benzyl adenine (BA) at 25, 50 and 100 mg/l was studied on the growth parameters and flowering response, photosynthetic pigments, different carbohydrate and nitrogen fractions, ion contents and endogenous level of different phytohormones of *Pisum sativum* (cv. 'Master Bean'). All determined growth parameters (root and shoot length, fresh weight and dry weight; number of nodes/plant; number of leaves/plant; total leaf area/plant; relative water content; number of flowers/plant) decreased in response to vernalization treatment. In contrast, vernalization in combination with 1000 mg/l atonik or 50 mg/l BA led to a significant increase in these parameters. Vernalization, alone or in combination with atonik or BA, significantly increased all photosynthetic pigments and generally led to a significant increase in different carbohydrate and nitrogen fractions and ion content. On the other hand, vernalization led to a significant decrease in total auxins, gibberellic acid (GA_3) and different CK fractions (zeatin, kinetin and BA) in pea plant shoots; ABA increased significantly. In contrast, vernalization combined with atonik or BA at any concentration led to a progressive increase in total auxins, GA_3 and different CK fractions while ABA decreased significantly compared with control values.

Keywords: cold treatment, pea, plant growth regulators

INTRODUCTION

The aim of the present study was to investigate the effect of vernalization, either alone or in combination with atomic (250, 500 and 1000 mg/l) or 6-benzyl adenine (BA; 25, 50 and 100 mg/l) on different growth criteria and metabolic activities in pea plants (Pisum sativum cv. 'Master Bean'). Pea plants are widely grown as a cool season vegetable crop. The seeds may be planted as soon as the soil temperature reaches 10°C. The pea leaf weevil (Latin: Sitona lineatus) is an insect that damages peas and other legumes. The weevil larvae feed on the root nodules of pea plants, which are essential to the plant's supply of nitrogen, and thus diminish leaf and stem growth. Adult weevils feed on the leaves and create a notched "c-shaped" appearance on the outside of the leaves (Fisher and O'Keeffe 1976).

Vernalization

The transition in plants to flowering is a major developmental switch in their life cycle. In many plant species, this transition is strongly influenced by temperature and light as well as the long period of cold temperature known as vernalization (Macknight *et al.* 1997; Taiz and Zeiger 1998).

Vernalization reflects the ability of cold treatments to make a winter cereal behave like a spring cereal with respect to flowering behavior. Vernalization requirement is critical to winter habit plants as it prevents the transition to the reproductive phase in regions with cold winters (Loukoinov *et al*. 2005; Sung and Amasino 2005; Dennis and Peacock 2007; Hemming *et al*. 2008).

Atonik and benzyl adenine

A great deal of attention has focused on the application of several plant growth regulators (PGRs) in order to qualitatively and/or quantitatively improve the yield of many crop plants (Adam and Padmaja 2003). Atonik and BA are among the growth substances, which in minute quantities induce changes in the morphology, physiology and yield of plants. Atonik is a relatively new growth substance composed of 3 aromatic nitro compounds (sodium 5-nitroguaiacolate, sodium 1-nitrophenolate and sodium 4-nitrophenolate) that enhances growth and some essential metabolic processes of cotton plants (Guo and Oosterhuis 1995). BA is one of the synthetic cytokinins (CKs) which plays a permissive role in the regulation of various growth processes in the plant and mainly affects cell division through its effect on protein synthesis (Skoog *et al*. 1967).

Vernalization (at 5°C for 4 days) reduced the number of days to flowering, and increased nitrogen (N) and phosphorus (P) content *in Lupinus albus* cultivars compared to the control while dry matter increase was the same for both non-vernalized and vernalized treatments (Keeve *et al.* 2001). Galiba *et al.* (1997) found that the major changes in osmotic potential during cold treatment are due to changes in sugar concentration; they concluded that there is a correlation between sugar content and frost tolerance. The sugar identified in the apex of cauliflower plants increased in plants grown under 10°C (Fernández *et al.* 1997; Less *et al*. 2006).

Tsybul and Karpukhina (1991) observed that the activity of auxins and CKs in spring and winter wheat was higher in vernalized winter and spring plants than in slowly developing unvernalized winter plants. It was suggested that vernalization influences gibberellin (GA) content and GA metabolism in canola, with GAs serving as the probable regulatory intermediates between vernalization and subsequent stem growth (Taiz and Zeiger 1998). Also, II-Yashuk and Likholat (1989) found that exposing winter wheat plants, at the 3–leaf stage, to 3°C for 2 hrs increased abscisic acid (ABA) content in the shoots; Longer exposures (24

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hrs) significantly decreased the content of ABA.

Guo and Oosterhuis (1995) described atonik (at 0.1, 0.25 and 0.50%) as a yield and growth enhancer which increased cotton yield through enhanced assimilation (nutrient uptake, nitrate reduction and photosynthesis), improved the flow of assimilates (translocation and cytoplasmic streaming) and increased cell integrity (membrane leakage). Pulkrabek *et al.* (1999) concluded that atonik (0.6 l/ha) and CK R[3.10E(-6) M, NE(6)-(meta-hydroxybenzyl)adenosine] (in special literature denoted as mOH.BAR) at 3.10 E^{-6} M increased root and sugar yields of sugar beet. Atonik + urea gave the highest values for wheat grain yield, grain N content, total N and N use efficiency (Farahat 2002).

BA stimulated growth of *Kniphofia* (Nayak and Sen 1990) or *Lotus* spp. (Martinez *et al.* 2000). Flower initiation and differentiation were enhanced by 4.44 µM BA; however, many flower buds of *Boronia megastigma* reverted when the medium contained high levels of BA (10 mg/l) (Roberts *et al.* 1993). Also, Sivakumar *et al.* (2001) observed that BA increased the grain weight, grain number and partitioning of dry matter between ear and seed in both wheat and triticale. Zhang *et al.* (1997) found that 50 µmol/l BA increased the dry weight of plants and chlorophyll concentration in wheat leaves. When *Agrostemma githago* L. seeds were treated with BA (0.44 mM) the synthesis of 5 specific soluble proteins in the embryo axes and cotyledons increased (Bernhardt and Gerth 1994). Ni *et al.* (2000) found that BA increased the contents of TSS (total soluble solids), total sugars, reducing sugars and vitamin C in ponkan (*Citrus reticulata*) cv. 'Blanco' trees sprayed with 100-200 mg/l BA.

Vernalization and different PGRs like atonik and BA greatly affect the growth and metabolism of treated plants at different growth stages. Thus, we thought that it would be interesting to investigate further the effect of vernalization, either alone or in combination with atonik or BA, on pea plants and their growth parameters.

MATERIALS AND METHODS

The effect of atonik (250, 500 and 1000 mg/l) or BA (5, 50 and 100 mg/l) on different growth criteria and metabolic activities, as well as content of photosynthetic pigments, contents of carbohydrate, N fractions, total N and protein, was determined. In addition, ion content (K^+, Na^+ and Ca^{2+}), as well as PGRs (auxin, GAs, ABA and CKs) were determined in pea plants (*Pisum sativum* cv. 'Master Bean').

Atonik and BA were purchased from Sigma-Aldrich, St. Louis, MI, USA.

Time course of experiment

Homogenously-sized seeds of 'Master Bean' were supplied by the Agricultural Research Center, Ministry of Agriculture, Giza, Egypt. These were selected and surface sterilized with 0.01% $HgCl_2$ solution then washed thoroughly with distilled water. The seeds were then divided into equal groups, each one containing 30 seeds. Each group was placed in a plastic box (21 × 14 cm) containing filter paper saturated with water. Boxes were incubated at 5°C for 5 days (after carrying out preliminary experiments at different temperatures in which 5°C was shown to be the lowest temperature at which germination occurred). This constituted the vernalization treatment. After vernalization, boxes were incubated at room temperature for 12 hrs before sowing. Other separate sterilized groups were placed in a plastic box contained filter paper saturated with water and were incubated at room temperature for 12 hrs before sowing. These served as the control.

All seeds were cultivated on November 3rd in pots (30 cm in diameter) containing equal amounts of homogenously mixed soil (sand: clay, 1: 2, v/v). Ten seeds were sown in each pot and irrigated, when required, by adding equal amounts of water to each pot. All plants were exposed to normal daylength and natural illumination. Super phosphate and urea fertilizers were added to the soil during the first week of cultivation. Sampling from control and vernalized plants took place at 20 days after sowing (DAS). After initial sampling, and on the same day, the pots containing vernalized plants were divided into 7 treatments, including the control. The first treatment was sprayed with water and considered as the vernalized treatment. The remaining treatments were sprayed separately with BA at 25, 50, 100 mg/l, and with atonik at 250, 500 and 1000 mg/l. At 7 DAS plants were sampled; this was referred to as stage I of growth and represented the vegetative stage. Sampling of stages II and III, which corresponded to the flowering and fruiting stages, took place on the 14th of December (21 DAS) and 7th of January (42 DAS), respectively. The samples collected at different stages were used to assess growth parameters as well as different metabolic activities (pigments, carbohydrate fractions and N fractions). In addition, the content of ions and PGRs were also determined.

All chemicals used in this study were purchased from Sigma-Aldrich, St. Louis, MI, USA.

Analytical methods

1. Estimation of the relative water contents

Based on the method described by (Pardossi *et al.* 1992), leaf samples from the third node were immediately sliced into 2-cm sections, weighed to obtain their fresh weight (FW), then floated on distilled water at 20°C in dim light for 4 hrs. After the 4-hr hydration period, turgid leaf slices were rapidly and gently blotted and weighed to obtain the turgid weight (TW). Leaf slices were then dried at 70°C until the constant dry weight (DW) was obtained. Leaf relative water content was calculated by the following formula:

$$\text{Relative water content \%} = (FW - DW)/(TW - DW) \times 100$$

2. Estimation of photosynthetic pigments

The plant photosynthetic pigments (chlorophyll (Chl) *a*, Chl *b* and carotenoids) were determined at different stages of plant growth using a spectrophotometric method as recommended by Arnon (1949) for Chl and (Horvath *et al*. 1972) for carotenoids as adopted by Kissimon (1999).

A known FW of plant leaves from the third node was cut in an ice-cold porcelain mortar, some quartz sand was added and about 10 mg of Na_2CO_3 were also used for reducing acidification. The leaves were ground with 1 ml of 80% acetone for 5 min.

After grinding, 3 ml of 80% acetone was added and the extract was stirred in the mortar; the pigment solution was then poured into a 15 mL Corning tube. The remaining pigments from the mortar were washed several times with ice-cold 80% acetone and the volume completed to 8 ml and mixed well. The tube was covered with parafilm to avoid the evaporation of acetone during centrifugation.

The extract was centrifuged for 3 min at 1000 × g. After centrifugation the colour was measured immediately (at 25°C) against a blank of pure 80% aqueous acetone at 3 wavelengths (λ = 480, 644 and 663 nm) using a spectrophotometer (Gilford Model 240). Taking into consideration the dilutions made, the concentration of the pigment fractions were calculated in µg/ml using the following equations as adopted by Kissimon (1999):

$$\text{Chl } a = 10.3\ E_{663} - 0.918\ E_{644}$$
$$\text{Chl } b = 19.7\ E_{644} - 3.87\ E_{663}$$
$$\text{carotenoids} = 5.02\ E_{480}$$

The fractions were then converted to µg/gm DW of the differently treated plant leaves.

3. Estimation of carbohydrates

The methods adopted in this investigation for extraction of the different carbohydrate fractions tested were essentially those of Yemm and Willis (1954) and Handel (1968).

Dry tissue samples were submerged overnight in 10 ml 80% (v/v) ethanol at 25°C with periodic shaking. The ethanolic mixture was filtered and the ethanolic filtrate was made up to a certain volume and kept in the refrigerator for analysis of the different sugar fractions.

***Estimation of glucose*:** Glucose content was estimated using the

O-toluidine procedure of Fetris (1965) as modified by Riazi *et al*. (1985) and described next. 1-ml aliquots of the alcoholic extract were heated with 5 ml *O*-toluidine reagent (60 ml *O*-toluidine + 2 g thiourea made up to 1000 ml with glacial acetic acid) and incubated for 15 min at 97°C. Absorbance was measured at λ = 630 nm using a Gilford Model 240 spectrophotometer.

***Estimation of sucrose*:** Sucrose content was determined using a modification of the Handel (1968) procedures by first degrading reactive sugars present in 0.1 ml of extract with 0.1 ml 5.4 N KOH at 97°C for 10 min. 3 ml of freshly prepared anthrone reagent (150 mg anthrone + 100 ml of 72% H_2SO_4) were then added to the cold reaction product, and the mixture was heated at 97°C for 5 min, then cooled. The colour that developed then was measured at 620 nm using a Gilford Model 240 spectrophotometer. With pure sucrose, a linear relation between the concentrations of sucrose and the optical density readings was obtained.

***Estimation of polysaccharides*:** The method used for estimation of polysaccharides in the present study was that of Thyermanavan and Sadasivam (1984). The plant leaves were treated with 80% ethanol to remove sugars, then starch was extracted with perchloric acid. In the hot acidic reaction, starch is hydrolysed into glucose and dehydrated to hydroxy methyl furfural. This compound forms a green coloured product with anthrone reagent. Dry tissues (0.1-0.5 g) were homogenized in hot 80% ethanol to remove soluble sugars, then centrifuged; the residue was retained. The residue was washed repeatedly with hot 80% ethanol until the washing did not form colour with anthrone reagent. The residue was dried well over a water bath. To the dry residue, 5.0 ml of distilled water and 6.5 ml of 52% perchloric acid were added, then extracted at 0°C for 20 min, centrifuged at 2000 × *g* for 5 min and the supernatant was saved. The extraction was repeated using fresh perchloric acid, centrifuged at 2000 × *g* for 5 min and the supernatant was cooled and made up to 100 ml. In a clean test tube, 0.1 or 0.2 ml of the supernatant was pipetted and made up to 1 ml with distilled H_2O. 4 ml of anthrone reagent were added to each tube. The mixture was heated for 8 min in a boiling water bath, then cooled rapidly. The intensity of the green to dark green colour of glucose units was measured at 630 nm using a Gilford Model 240 spectrophotometer. Polysaccharide content (i.e. starch) was determined from the standard curve of glucose.

4. Estimation of nitrogenous constituents

The method used in this investigation was essentially that adopted by Yemm and Willis (1956). The dried tissue samples were ground to a fine powder and a known weight of the powder was extracted in distilled water by grinding the samples for 30 min at room temperature in a glass mortar. The mixture was then quantitatively transferred to a boiling tube, brought quickly to a water bath and maintained at 80°C for 5 min. The insoluble residue was removed by filtration through Whatman filter paper. After filtration, the filtrate was made up to volume and used for the estimation of ammonia-, amino-, amide- and total soluble N fractions. Total N was determined directly using the powdered tissue. Subtracting total soluble N from total N gave the value of protein N.

***Estimation of ammonia-N*:** Ammonia-N was estimated spectrophotometrically by the method adopted by Delory (1949) using Nessler's reagent and modified by Naguib (1964) as follows: An aliquot of the tissue extract was mixed with 1.0 ml 1 N NaOH and 0.5 ml of 0.5% $ZnSO_4$. The mixture was made up to 14 ml with distilled water before 1 ml of Nessler's reagent was added, shaken well and allowed to stand for 5 min. The optical density was then measured at 450 nm using a Gilford Model 240 spectrophotometer. To prepare Nessler's reagent, 35 g of KI was dissolved in 100 ml of distilled water and then 4% $HgCL_2$ solution was added with continuous stirring till a persistent slight red precipitate was obtained (~325 ml is required). Also, while stirring, a solution of 120 g NaOH in 250 ml of water was introduced and the mixture was made up to 1 l with distilled water. Slightly excess $HgCL_2$ solution was added until permanent turbidity. The mixture was allowed to stand for 1 day and then filtered through Whatman filter paper. It was always kept in a stoppered dark brown bottle in the refrigerator but not for more than 14 days.

***Estimation of amide-N*:** The method used was that of Naguib (1964). 10 ml of the tissue extract was mixed with 2.5 ml of 10 N H_2SO_4. The mixture was refluxed for 4 hrs at 100°C, neutralized with 5 N NaOH and made up to 50 ml by distilled water. The ammonia-N was estimated using Nessler's reagent. The difference between this value and that for free ammonia is considered as equivalent to the amide-N.

***Estimation of amino-N*:** The method used in the present study was that of Muting and Kaiser (1963). The extract was deprotenised with ethanol/acetone and the free amino acids were then determined spectrophotometrically with ninhydrin reagent.

Reagents required: 1) Ethanol/acetone mixture, 1:1 (v/v); 2) Phosphate buffer, pH 6.5, 0.5 M Na_2HPO_4 solution (18 ml) + 0.5 M NaH_2PO_4 solution (32 ml), complete with distilled water to 20 ml; 3) Ninhydrin solution (0.5% ninhydrin dissolved in *n*-butanol). This solution keeps well for about 14 days in a well stoppered dark bottle in the refrigerator.

Procedure: The following solutions were pipetted into quick fit tubes:

Sample	Blank	Solution
0.1 ml	0	Sample
1.5 ml	1.6 ml	Ethanol/acetone
0.1 ml	0.1 ml	Phosphate buffer
2.0 ml	2.0 ml	Ninhydrin reagent

The tubes were stoppered with glass stoppers and placed in a boiling water bath for 20 min. The tubes were immediately cooled in ice water and methanol was added to 10 ml. The contents were mixed by turning the tubes over 2 to 3 times and the optical density of the colour that developed was measured at 580 nm using a Gilford Model 240 spectrophotometer. A standard curve was made using standard glycine solution.

***Estimation of total soluble-N*:** Total soluble N was determined by the conventional semi micromodification of Kjeldahl method (Pirie 1955). An aliquot of the extract was taken into a digestion flask and heated for at least 8 hrs with 0.5 g catalyst (80 g K_2SO_4, + 20 g $CuSO_4 \cdot 5H_2O$ + 0.3 g SeO_2), 2 ml of ammonia-free concentrated H_2SO_4 and 1 ml of distilled water. The solution was treated with 15 ml of 40% NaOH and steam distilled in a conventional manner into 5 ml of 0.05 N H_2SO_4. The distillate was made up to volume and used for estimation of total soluble N by estimating ammonia, using Nessler's reagent, as described before.

***Estimation of total-N*:** Total N was determined by the conventional semi-micropropagation of Kjeldahl method of Rees and Williams (1943). Exactly 0.02-0.03 g of the dry powdered tissue was heated for at least 2 hrs with 0.5 g catalyst (as for total soluble N), 2 ml of ammonia-free concentrated H_2SO_4 and 1 ml of distilled water. The solution was treated with 15 ml of 40% NaOH and steam distilled in a conventional manner into 5 ml of 0.05 N H_2SO_4. The distillate was made up to volume and used for estimation of total N by estimating ammonia as described before.

5. Determination of K^+, Na^+ & Ca^{++}

At the time of sampling, the plant roots were rinsed in distilled water for 30 sec to remove soil remains from the root surface. Thereafter, plants were separated into shoots and roots. Samples were dried in an oven at 80°C until constant weight and DWs of samples were recorded. The dried matter was digested in concentrated HNO_3 and made up to volume with deionized distilled water. Potassium, sodium and calcium were measured by atomic absorption spectrophotometry (ICP-AES-varian-Liberty series II) according to the method described by Chapman and Pratt (1978).

6. Estimation of PGRs

***Extraction and separation*:** Plant extract was obtained by grinding fresh growing shoot tips of plants in cold 80% methanol and kept

in brown glass-stoppered jars which were kept in a deep freezer for extraction. After the ground material was left in 80% methanol for 48 hrs, the methanolic extract was filtered, evaporated to an aqueous residue, acidified by 1 N H_2SO_4 to pH 2 and extracted 3 times with ethyl acetate. The ethyl acetate fractions contained gibberellins, auxins as IAA and abscisic acid while the aqueous fraction contained mainly CK. The acidic ethyl acetate extracts were combined and extracted 3 times by soaking with 5% (w/v) sodium bicarbonate in a separating funnel. The pH of the combined bicarbonate extract was adjusted to pH 2 and it was re-extracted 3 times with ethyl acetate. The ethyl acetate was dried to yield the acidic fractions of PGRs (Davis *et al.* 1968; Shindy and Smith 1975).

Methylation of plant hormones: Diazomethane was obtained from methylamine hydrochloride as reported by Vogel (1975) as follows: Methylamine solution (100 g) was placed in a 500-ml flask and concentrated hydrochloric acid (78 ml) was added. Water was added to bring the total weight to 250 g, then urea (150 g) was introduced and the mixture was boiled gently under reflux for 2 min and then vigorously for 15 min. The solution was cooled to room temperature, then sodium nitrate (55 g) was dissolved in it and a mixture of 300 g of crushed ice and 50 g concentrated sulfuric acid was prepared in a 1500-ml beaker surrounded by an ice bath and salt. Cold methyl urea-nitrate solution was added slowly with mechanical stirring at such a rate that the temperature did not rise above 0°C.

The crystalline nitrosomethyl urea was filtered at once then drained well and dried in a vacuum dessicator. An aqueous potassium hydroxide solution (50 ml, 50%) and ether (200 ml) were placed in a 500-ml round-bottomed flask. The mixture was cooled to 5°C. Nitrosomethyl urea (20.56 g) and ether (80 ml) were then added. The ethereal layer was separated using a separating funnel and dried over pellets of potassium hydroxide for 2-3 hrs.

***Methylation with diazomethane*:** PGR fractions and standard were dissolved in a little anhydrous ether and the ethereal solution of diazomethane was added in small portions until gas evolution ceased. The mixture acquired a pale yellow colour indicated the addition of excess diomethane. The reaction mixture was left for 10 min and ether was distilled off using a hot water bath. The residue was dissolved in a minimal amount of acetone and used for gas-liquid-chromatography (GLC) analysis using a Hewellet-Packard (HP-GC-MS model).

***Separation of methyl esters of plant hormones by gas liquid chromatography*:** Analysis of methyl esters of organic acids was performed on a Pye Unican GCV gas chromatograph equipped with a dual flame ionization detector. The gas liquid chromatographic conditions for isothermal work were as follows: 2.8 m × 4 mm glass column packed with acid, alkali and silanized diatomite C (100-120 mesh) and coated with 1% OV-17, temperature; injector 250°C, column 230°C, and detector 300°C flow rates; nitrogen 300 ml/min; hydrogen 33 ml/min, and air 330 ml/min range of 32 × 10^2 and chart speed of 3 cm/min.

***Identification and determination of auxins, GAs and ABA*:** Peak identification was performed by comparison of the relative retention time for each with those of standard PGRs (IAA, GA and ABA) from Sigma-Aldrich (Shindy and Smith 975).

***Identification and determination of CKs*:** Aqueous fractions were combined and then adjusted to pH 5.5 by 1% NaOH and partitioned 3 times with 50-100 ml of water-saturated *n*-butanol. Then *n*-butanol fractions were combined and reduced in volume to 5 ml, and stored at -20°C until GLC analysis for CKs (Horgan *et al.* 1973).

Statistical analyses

The results were first subjected to analysis of variance (ANOVA). If ANOVA showed a significant ($P < 0.05$) effect, the least significant difference (LSD) was used to compare treatments (Snecdor and Cochran 1980).

RESULTS AND DISCUSSION

With regard to floral transition, all higher plants share some common mechanisms that control this important switch from vegetative to reproductive growth (Baurle and Dean 2006; Imaizumi and Kay 2006; Turck *et al*. 2008). First, the shoot apical meristem (SAM), which gives rise to both vegetative and reproductive structures, is the part of the plant where the actual transition occurs. Second, the SAM must be competent to perceive inductive signals to make inflorescence and floral meristems. Third, although the SAM is the target of floral inductive signals, the signals themselves, in most cases, originate in vegetative tissues, usually the leaves (Colasanti and Coneva 2009).

The transition of plants to flowering involves major changes in the pattern of morphogenesis and cell differentiation at the SAM. Ultimately, this process leads to the production of the floral organs, sepals, petals, stamens and carpels (Taiz and Zeiger 1998). Thus, if seeds of winter wheat are germinated in an ice box and subjected to suitable conditions of light, moisture and air, they can be sown in the spring and will flower at the same time. The phenomenon is represented by the term vernalization (Phillips and Wareing 1977). Chouard (1960) defined vernalization as the acquisition or acceleration of the ability to flower by chilling treatment. Also, Dennis and Peacock (2009) stated that vernalization, a prolonged period of low temperature, is one environmental stimulus that ensures that flowering occurs in the appropriate season of the year – spring – and that many plant species, including both broadleaf plants (the dicots) and grass-like plants (the monocots), require vernalization to stimulate flowering

The aim of this study, as stated before, was to assess the effect of vernalization at 5°C either alone or in combination with a foliar application of atonik, at 250, 500 and 1000 mg/l) or BA (25, 50 and 100 mg/l) on growth parameters and the flowering response of pea as well as the endogenous level of different PGRs.

Changes in growth parameters

The estimated growth parameters (root length, root and shoot FW and DW, shoot length, number of nodes and leaves, total leaf area and relative water content) decreased significantly, in general, during the different growth stages of *P. sativum* when subjected to vernalization treatment, as compared to control values (**Table 1**).

Wang *et al.* (1995) applied vernalization treatments of 0 to 56 d to wheat (*Triticum aestivum* L.) plants at their first leaf stage. All plants headed irrespective of duration of vernalization treatment. Vernalization response was assessed through the change of final leaf number (FLN) on the main stem at heading. Many leaves can be reduced by one day of vernalization treatment. Sun *et al*. (1995) investigated the effect of vernalization time and gibberellin (GA_3) concentrations on flowering time, the number of leaves and plant height in early and late heading Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*). Their results indicate that the fewer the number of flowering days from sowing to flowering, fewer leaves and shorter plants were observed. GA_3 treatments gave the same effects as low temperature treatments.

China Vegetables stated that cold storage could lead to a reduction in stem length and FW of cut flowers and promote more uniform flowering. On the other hand, Mahfoozi *et al.* (2001) found that vernalization at 4°C led to a significant increase in leaf number of winter cereals. In the present study, it can be concluded that the reduction in growth in response to vernalization treatment may be related to the changes in the level of endogenous PGRss (**Table 7**). This is in accordance with the findings of Hare *et al*. (1997), who stated that CK levels tend to decrease under adverse environmental conditions. A general view has emerged that during stress, a reduction of CK supply from the root alters gene expression in the shoot and thereby elicits appropriate

Table 1 Effect of vernalization and different concentrations of Atonik or benzyladenine on growth parameters of *Pisum sativum* plant.

Stage	Treatment	Root length (cm)	Root fresh weight (g)	Root dry weight (g)	Shoot length (cm)	Number of nodes/ plant	Number of leaves/ plant	Leaf area /plant (cm^2)	Shoot fresh weight (g)	Shoot dry weight (g)	Relative water content
Initial	Control	6	0.231	0.022	5.254	3.853	3.203	16.763	0.525	0.084	91.1
	Vernalized	5.133*	0.204*	0.015*	4.627*	3.233*	2.746*	12.19*	0.473*	0.07*	88.433*
	LSD	0.489	0.016	0.004	0.403	0.492	0.338	0.956	0.02	0.004	1.802
Stage I	Control	7.243	0.373	0.037	7.3	5.06	3.738	22.5	0.666	0.126	82.833
	Vernalized	5.936*	0.302*	0.025*	6.09*	4.18*	3.41	20.493*	0.58*	0.117*	78.433*
	Vernalized+Atonik 250	8.136*	0.424*	0.042	7.8*	5.373	4.2*	23.88	0.716*	0.133*	85.433
	Vernalized+Atonik 500	8.82*	0.5*	0.05*	8.593*	5.913*	4.8*	26.046*	0.813*	0.151*	90.59*
	Vernalized+Atonik 1000	9.566*	0.587*	0.059*	9.403*	6.47*	5.07*	27.626*	1.128*	0.175*	94.786*
	Vernalized+BA 25	7.693*	0.413*	0.044*	7.66	5.143	3.95	23.376	0.701	0.131	87.333*
	Vernalized+BA 50	8.133*	0.46*	0.051*	8.496*	5.506*	4.8*	26.61*	0.874*	0.161*	90.55*
	Vernalized+BA 100	7.553	0.393	0.041	7.376	5.113	3.513	21.55	0.696	0.128	85.783
	LSD	0.37	0.032	0.004	0.388	0.332	0.323	1.205	0.04	0.005	3.32
Stage II	Control	11.086	0.78	0.041	9.5	6.54	5.84	30.133	1.113	0.322	78.7
	Vernalized	9.933*	0.68*	0.036*	8.013*	5.253*	4.733*	26.944*	0.993*	0.247*	72.1*
	Vernalized+Atonik 250	11.543	0.849	0.05*	10.633*	6.906	6.103	32.6*	1.33*	0.367*	84.333*
	Vernalized+Atonik 500	12.656*	1.006*	0.055*	11.426*	7.323*	6.79*	33.876*	1.682*	0.449*	86.836*
	Vernalized+Atonik 1000	13.75*	1.293*	0.064*	12.023*	7.766*	7.21*	36.7*	1.888*	0.634*	89.333*
	Vernalized+BA 25	11.73	0.903*	0.051*	10.54*	6.75	6.08	31.003	1.283*	0.389*	80.033
	Vernalized+BA 50	12.733*	1.181*	0.06*	11.25*	7.476*	6.9*	36.193*	1.583*	0.495*	86.4*
	Vernalized+BA 100	11.41	0.876	0.041	9.876	6.736	4.816*	28.166*	1.223*	0.293*	77.66
	LSD	0.594	0.084	0.002	0.382	0.332	0.445	1.493	0.086	0.004	3.174
Stage III	Control	13	1.21	0.053	12.88	8.253	7.683	39.116	1.856	0.521	77.623
	Vernalized	11.4*	0.89*	0.042*	10.103*	6.283*	5.643*	29.623*	1.613*	0.34*	74.2*
	Vernalized+Atonik 250	16.333*	1.573*	0.061*	14.11*	8.586	8.06*	41.183*	2.233*	0.633*	79.6
	Vernalized+Atonik 500	17.676*	1.714*	0.071*	15.143*	9.79*	8.75*	43.62*	2.562*	0.75*	81.843*
	Vernalized+Atonik 1000	19.226*	1.983*	0.079*	16.846*	10.366*	9.313*	45.753*	3.076*	0.921*	83.58*
	Vernalized+BA 25	13.833	1.296*	0.065*	14.633*	8.95*	8.143*	41.36*	2.12*	0.612*	80.4*
	Vernalized+BA 50	15.58*	1.49*	0.073*	15.91*	10.016*	8.686*	42.12*	2.33*	0.813*	82*
	Vernalized+BA 100	13.593	1.229	0.055	13.406	8.49	7.746	40.666*	2.106*	0.584*	76.426
	LSD	0.785	0.073	0.003	0.542	0.467	0.255	1.29	0.159	0.014	2.226

* = Significant increase or decrease at 0.05 LSD

responses to ameliorate the effects of stress. However, recent studies have indicated that transcription of many stress-inducible genes can also be caused by the application of CK. Menhenett and Wareing (2006) studied the effects of photoperiod (8 or 16 hr, SD or LD) and temperature (low or high, 8 or 20°C) on the growth and CK content of Norwegian and Portuguese populations of *Dactylis glomerata* (cocksfoot). In general, conditions which promoted active leaf growth (LD or high temperature) of plants of the Norwegian population tended to result in lower levels of CK bases and/or nucleosides and in higher levels of 'nucleotide' CKs than conditions which lead to reduced growth rate (SD or low temperature).

Hare *et al*. (1997) stated that CK levels tend to decrease under adverse environmental conditions. A general view has emerged that during stress: a reduction of CK supply from the root alters gene expression in the shoot and thereby elicits appropriate responses to ameliorate the effects of stress. However, recent studies have indicated that transcription of many stress-inducible genes can also be caused by CK application.

Compared to the control value, a general significant increase was detected during the stage of growth of pea plants in various growth parameters in response to treatment with either atonik or BA under vernalization condition (**Table 1**). The magnitude of increase seemed to be more pronounced when treated with 1000 mg/l atonik or 50 mg/l BA. Al-Badawy *et al.* (1984) found that atonik could increase *Chamomilla suaveolens* height, branch number, herb DW, flower number and weight and essential oil content. Also, Koupil (1996) observed that atonik increased the number of internodes of apple; hence shoot length was increased. On the other hand, Omon (1994) demonstrated that atonik treatment reduced rooting percentage, root number, shoot length and number of buds of *Pericopsis mooniana*. Concerning the effect of BA, numerous authors authenticated that BA, either alone or in combination with vernalization, stimulated the growth and metabolism of many plant species. Martínez *et al.* (2000) stated that BA treatment to *Lotus* spp. shortened internodes and promoted branching. In addition, Seyring and Vogt (2000) noticed the inhibition of shoot and root growth of *Argyranthemum frutescens* under the influence of 2.5 mg/L BA. In conclusion, the stimulation of pea growth as a consequence of atonik or BA treatments may be due to the excessive absorption of minerals and nutrients from soil (**Table 6**). Zraly *et al.* (1999) used atonik to induce an increase of mineral uptake (23.5, 22.2 and 27.8% for K, Ca and Mg, respectively) in cotton. Rawia and Bedour (2006) showed that BA at 75 mg/l had a slight effect on the K level of croton plants.

Changes in flowering response

With respect to the events of flowering (**Table 2**), flowering took place in the control plants after 41 days for *P. sativum*, after germination, yet the present results indicate that the treated plants, with vernalization alone or in combination with atonik or BA, appeared to interrupt juvenility and to cross the threshold to flowering. Thus, the start of flowering was earlier by one day in the vernalized pea plants, whereas vernalization in combination with either atonik or BA caused induction of flowering by 2, 2 and 3 days in response to treatment of pea plants with 250, 500 and 1000 mg/l atonik, respectively. Also, the start of flowering was earlier by 2, 3 and 1 days in response to pea treatment with BA at 25, 50 and 100 mg/l, respectively. Sheldon *et al.* (1999) and Kuittinen *et al.* (2008) also reported the induction of *Arabidopsis* flowering by vernalization treatment as did Hemming *et al.* (2008) and Karsai *et al.* (2008) in barley, in which the cold treatment induced gene activity, rather than repressing it as in *FLC* in *Arabidopsis*. *VRN1* is a promoter of the transition from the vegetative to reproductive

Table 2 Effect of vernalization and different concentrations of Atonik or benzyl adenine on date of flowering and number of flowers/plant of *Pisum sativum* plant during December.

Treatment	Date of flowering	No of flowers/plant
Control	14/12	1.053
Vernalized	13/12	1.273
Vernalized+Atonik 250	12/12	1.373
Vernalized+Atonik 500	12/12	1.473
Vernalized+ Atonik 1000	11/12	1.616
Vernalized+BA 25	12/12	1.413
Vernalized+BA 50	11/12	1.570
Vernalized+BA 100	13/12	1.313

state of the growing shoot apex. Induction of *VRN1* is accompanied by the repression of another genetically defined gene, *VERNALISATION 2* (*VRN2*), which, when active, prevents transcriptional activity of the *FT* gene and production of the mobile flowering signal (Trevaskis *et al*. 2006). Koupil (1996) found that atonik increased fruit size of apple. Rylott and Smith (1990) stated that the application of CKs to flowers caused active cell division of the embryo, and hence the attraction of assimilates to the new developing pods from other plant parts; Ni *et al*. (2000) stated that BA enhanced *Citrus reticulata* fruit enlargement and increased fruit FW. In addition, Guo and Oosterhuis (1995) stated that atonik increased the yield of cotton and improved quality through the stimulation of plant metabolism.

Changes in photosynthetic pigments

At all stages of *P. sativum* growth, the determined photosynthetic pigments (Chl *a*, Chl *b*, total Chl, carotenoids and total pigments) significantly increased under vernalization treatment, as shown in **Table 3**). Roberts *et al*. (1993) detected an increase in Chl synthesis in wheat under cold treatment. Throughout the experimental growth stages of *P. sativum* plants, vernalization in combination either with atonik or BA led to a significant increase in Chl *a*, Chl *b*, total Chl and carotenoids and consequently, in total pigments in relation to control value. On the other hand, the Chl *a/ b* ratio seemed to be unaffected in response to the above mentioned treatments. Guo and Oosterhuis (1995) fund a similar pattern in cotton plants.

The observed increase in the photosynthetic pigments in response to different concentrations of BA is of the same as that observed by Yokoyama *et al.* (1980) on young bean and Rani*et al.* (1988) on mung bean plants. Wittenbach (1977) found that kinetin retards Chl breakdown via the inhibition of chlorophyllase which leads to the retarded senescence of leaves. In addition, He and Pan (1994) stated that BA increases the rate of photosynthesis in rice. Moreover, Rulcová and Pospíšilová (2001) found that Chl content and net photosynthetic rate increased in *Phaseolus vulgaris* leaves after BA application. In conclusion, the enhancement of the production of Chl *a*, Chl *b* and carotenoids and total pigments during the growth and development of pea plants subjected to vernalization and various concentrations of atonik or BA may be due to an increment in endogenous kinetin level (**Table 7**) which delays the senescence of plants. Chernyad-ev (2000) stated that BA enhanced Chls, carotenoids, proteins and Rubisco in sugar beet by retarding senescence.

Changes in carbohydrates

In this investigation, glucose, sucrose and polysaccharide concentrations, in general, significantly increased at all the different growth stages of pea grown under the influence of vernalization with various concentrations of atonik or BA (**Table 4**) in relation to control values. Chel (1990) observed an increase in sucrose content of wheat during vernalization. Soluble sugars accumulated in *Arabidopsis* and tobacco (*Nicotiana tabacum*) after 3 days of cold acclimation at 4°C (Rajashekar *et al*. 2006; Zhao *et al*. 2009; Du *et al*. 2010).

Pulkrabek (1996) found that atonik treatment increase sugar yield of sugar beet. Erwin *et al*. (2002) suggested that

Table 3 Effect of vernalization and different concentrations of Atonik or benzyl adenine on photosynthetic pigments (chlorophyll (Chl) and carotenoids) content, in mg/g dry weight of *Pisum sativum* plant.

Stage	Treatment	Chl *a*	Chl *b*	Chl *a* + *b*	Chl *a*/*b*	Carotenoids	Total pigment
Initial	Control	0.1067	0.0553	0.1621	1.9338	0.0216	0.1837
	Vernalized	0.1224*	0.0629*	0.1853*	1.9458	0.0288*	0.2142*
	LSD	0.0075	0.0058	0.0117	0	0.0022	0.0112
Stage I	Control	0.1299	0.0757	0.2056	1.7157	0.0266	0.2322
	Vernalized	0.1531*	0.0848*	0.2379*	1.805	0.0361*	0.274*
	Vernalized+Atonik 250	0.1656*	0.0914*	0.257*	1.8127	0.0421*	0.2992*
	Vernalized+Atonik 500	0.176*	0.0992*	0.2752*	1.7742	0.0488*	0.3241*
	Vernalized+Atonik 1000	0.1933*	0.1082*	0.3015*	1.7887	0.0544*	0.3559*
	Vernalized+BA 25	0.1616*	0.0904*	0.2521*	1.7879	0.0419*	0.294*
	Vernalized+BA 50	0.188*	0.1121*	0.3001*	1.6775	0.0464*	0.3465*
	Vernalized+BA 100	0.1683*	0.0932*	0.2616*	1.8059	0.0378*	0.2994*
	LSD	0.0058	0.0037	0.0066	0.142	0.0022	0.0066
Stage II	Control	0.102	0.0577	0.1597	1.7687	0.0163	0.176
	Vernalized	0.1107*	0.0647*	0.1754*	1.7108	0.0203*	0.1957*
	Vernalized+Atonik 250	0.1172*	0.0739*	0.1911*	1.5879	0.0232*	0.2144*
	Vernalized+Atonik 500	0.1262*	0.0823*	0.2086*	1.5357	0.0287*	0.2373*
	Vernalized+Atonik 1000	0.1368*	0.093*	0.2298*	1.4732	0.0355*	0.2654*
	Vernalized+BA 25	0.1159*	0.078*	0.1939*	1.486	0.0262*	0.2201*
	Vernalized+BA 50	0.123*	0.0889*	0.2119*	1.3835	0.0322*	0.2441*
	Vernalized+BA 100	0.1146*	0.0666*	0.1813*	1.7195	0.0227*	0.204*
	LSD	0.0049	0.0037	0.007	0.08	0.0027	0.0076
Stage III	Control	0.0308	0.0129	0.0438	2.3786	0.0067	0.0505
	Vernalized	0.0421*	0.0185*	0.0607*	2.2736	0.0092*	0.0699*
	Vernalized+Atonik 250	0.0512*	0.0254*	0.0766*	2.017	0.0115*	0.0881*
	Vernalized+Atonik 500	0.0573*	0.033*	0.0903*	1.7383	0.0132*	0.1035*
	Vernalized+Atonik 1000	0.071*	0.0401*	0.1112*	1.7695	0.0167*	0.1279*
	Vernalized+BA 25	0.0559*	0.0243*	0.0802*	2.303	0.0118*	0.0921*
	Vernalized+BA 50	0.065*	0.0377*	0.1028*	1.7234	0.0135*	0.1164*
	Vernalized+BA 100	0.0487*	0.0236*	0.0723*	2.0807	0.0104*	0.0827*
	LSD	0.0032	0.0019	0.004	0.21	0.001	0.0041

* = significant increase or decrease at 0.05 LSD.

Table 4 Effect of vernalization and different concentrations of Atonik or benzyladenine on carbohydrates content, in mg/g dry weight of *Pisum sativum* plant.

Stage	Treatment	Glucose	Sucrose	Polysaccharides
Initial	Control	0.602	48.666	160.966
	Vernalized	0.831*	57.666*	185.333*
	LSD	0.079	3.803	19.35
Stage I	Control	1.8	80.533	323.978
	Vernalized	1.815	82.833	319.056
	Vernalized+Atonik 250	2.183*	90.25*	353.168*
	Vernalized+Atonik 500	2.6*	98*	411.557*
	Vernalized+Atonik 1000	3.28*	115.666*	462.416*
	Vernalized+BA 25	2.09*	83.546	344.133
	Vernalized+BA 50	2.476*	91*	396.533*
	Vernalized+BA 100	1.873	79.663	324.693
	LSD	0.179	6.701	21.2
Stage II	Control	2.625	107.035	365.333
	Vernalized	2.632	108.8	374.166
	Vernalized+Atonik 250	2.812*	122.766*	398.333*
	Vernalized+Atonik 500	3.483*	145.586*	436.866*
	Vernalized+Atonik 1000	4.436*	162.854*	506.133*
	Vernalized+BA 25	2.895*	120.666*	392
	Vernalized+BA 50	3.806*	140.666*	428.866*
	Vernalized+BA 100	2.836*	115.333	380
	LSD	0.145	8.866	26.17
Stage III	Control	3.485	80.883	272
	Vernalized	3.297	81.905	262.666
	Vernalized+Atonik 250	4.233*	82.98	326.333*
	Vernalized+Atonik 500	5.662*	88.959*	384.666*
	Vernalized+Atonik 1000	6.38*	105.717*	437.866*
	Vernalized+BA 25	4.194*	93.3*	329*
	Vernalized+BA 50	5.471*	102.949*	393.233*
	Vernalized+BA 100	3.583	85.95	292.866*
	LSD	0.307	4.93	15.207

* = Significant increase or decrease at 0.05 LSD.

the gene-regulated sucrose accumulation in Japanese radish (*Raphanus sativus*) was closely associated with the effect of vernalization. The stimulation of different carbohydrates in pea plants treated with atonik or BA under the effect of vernalization appears to coincide with the present stimulated changes in leaf area, DW and photosynthetic pigments as well as the content of PGRs. Chernyad-ev (2000) found that the rate of photosynthetic CO_2 assimilation on a leaf area basis increased in vernalized sugar beet plants.

Changes in nitrogen fractions

Except for amide-N in vernalized plants, which decreased significantly, a remarkable increase in ammonia, amino-N, total soluble N, protein and consequently total N were observed as a result of treatment of pea plants with vernalization or vernalization and different concentrations of atonik or BA during different stages of growth and development, as compared with control values (**Table 5**). Mey *et al.* (1992) also found that vernalization treatment increased the N content of *Lupinus albus* plants. Also, Nowak *et al.* (1997) observed that BA increased the N content in seeds, pods and stems of bean plants. Ammonia is the unit of N metabolism from which different amino acids are produced, these being further incorporated into protein synthesis (Sheldon *et al.* 1999). The progressive increase in ammonia content in pea plants treated with various applications (**Table 5**) may explain the massive increases in different nitrogenous fractions.

Downs *et al.* (1997) found that BA treatment delayed the decline in amino acids and soluble protein as well as the increase in ammonia and aspargine and glutamine concentrations in broccoli florets. They suggested that this effect of BA may be due to the effect of CK on the possible mechanisms regulating senescence. This conclusion is in a good agreement with our results (**Tables 5, 7**). The above mentioned progressive increments in protein of pea plant in response to treatment with atonik or BA under the influence of vernalization is supported by Sheldon *et al.* (1999) who found that cold treatment induced new protein synthesis in *Arabidopsis*. Also, Metho *et al.* (1999) detected that low temperature induced higher grain protein content in wheat (*Triticum aestivum* L.). Further, Shadi *et al.* (2001) stated that BA inhibited the biosynthesis of high molecular weight protein and stimulated biosynthesis of mid- and low-molecular weight protein in some maize inbred lines. The pattern of changes in different N and carbohydrate fractions in pea plants by various treatments (**Tables 4, 5**) may be explained on the basis of the role of these treatments in increasing the synthesis of the substances necessary for floral induction. To support this conclusion, Hume *et al.* (1995) studied the influence of temperature upon physiological processes of *Thlaspi arvense* and suggested that the net photosynthesis and respiration rate of the treated plants may influence the relative amounts of carbohydrates and N available to the shoot apices and therefore affect the time to floral transition.

Changes in ionic content

Treatment of pea plants with vernalization and different concentrations of atonik or BA led to a significant increase in K^+, Na^+ and Ca^{++} contents in both roots and shoots at different stages of growth (**Table 6**). The magnitude of increase in element contents was most pronounced as atonik concentration increased and with 50 mg/l BA, as compared to the control value. Taeb *et al.* (1992) found that dominant alleles of vernalization decrease Na content. In addition, Koupil (1996) studied the effect of a foliar spray of 0.05% atonik on the growth of apple and recorded general changes in N, P, K, Ca and Mg. The results also have a good link with final shoot length (**Table 1**). Nowak *et al.* (1997) studied the effect of BA application on *Vicia faba* and found that BA application increased the uptake of P, K, Na, Ca and Mg. The observed changes in the inorganic ions contents (K^+, Na^+ and Ca^{++}) in shoots and roots of pea plants in response to various treatments is expected to be the influ-

Table 5 Effect of vernalization and different concentrations of Atonik or benzyl adenine on different nitrogen fractions in, mg/g dry weight of *Pisum sativum* plant.

Stage	Treatment	Ammonia	Amide Nitrogen	Amino Nitrogen	Total soluble Nitrogen	Total Nitrogen	Protein
Initial	Control	0.776	0.73	0.503	3.654	25.833	22.179
	Vernalized	0.936*	0.43*	0.657*	4.212*	32.05*	27.838*
	LSD	0.1	0.041	0.028	0.071	2.068	2.018
Stage I	Control	1.261	1.058	0.6	3.821	39.36	35.538
	Vernalized	1.492*	0.753*	0.716*	4.462*	44.64*	40.177*
	Vernalized+Atonik 250	1.637*	1.456*	1.134*	4.771*	47.406*	42.635*
	Vernalized+Atonik 500	1.829*	1.224*	1.492*	4.97*	50.696*	45.726*
	Vernalized+Atonik 1000	1.997*	1.083	1.609*	5.366*	53.916*	48.55*
	Vernalized+BA 25	1.544*	1.216*	1.027*	4.607*	43.59*	38.983*
	Vernalized+BA 50	1.65*	1.162*	1.18*	5.021*	47.371*	42.35*
	Vernalized+BA 100	1.441*	1.046	1.386*	4.586*	43.615*	39.028*
	LSD	0.058	0.044	0.045	0.116	1.407	1.445
Stage II	Control	1.162	0.815	0.4	3.602	37.591	33.989
	Vernalized	1.295*	0.631*	0.606*	3.965*	40.848*	36.882*
	Vernalized+Atonik 250	1.574*	1.279*	0.991*	4.361*	44.766*	40.405*
	Vernalized+Atonik 500	1.808*	1.139*	1.127*	4.676*	47.775*	43.098*
	Vernalized+Atonik 1000	1.855*	0.912*	1.393*	4.874*	51.483*	46.609*
	Vernalized+BA 25	1.414*	1.024*	0.938*	4.081*	43.875*	39.794*
	Vernalized+BA 50	1.557*	0.986*	1.097*	4.386*	45.568*	41.181*
	Vernalized+BA 100	1.385*	0.833	0.853*	4.058*	42.083*	38.025*
	LSD	0.059	0.039	0.032	0.118	1.701	1.668
Stage III	Control	0.945	0.8	0.333	2.873	32.798	29.925
	Vernalized	1.135*	0.536*	0.518*	3.158*	36.626*	33.468*
	Vernalized+Atonik 250	1.238*	0.913*	0.8*	3.656*	41.275*	37.619*
	Vernalized+Atonik 500	1.479*	0.718*	0.914*	3.931*	43.191*	39.26*
	Vernalized+Atonik 1000	1.771*	0.662*	1.138*	4.179*	46.873*	42.694*
	Vernalized+BA 25	1.117*	0.941*	0.837*	3.926*	39.173*	35.246*
	Vernalized+BA 50	1.373*	0.848*	0.969*	4.155*	42.08*	37.925*
	Vernalized+BA 100	1.166*	0.63*	0.736*	3.529*	36.388*	32.858
	LSD	0.078	0.029	0.023	0.09	2.69	2.29

* = Significant increase or decrease at 0.05 LSD.

Table 6 Effect of vernalization and different concentrations of Atonik or benzyl adenine on potassium, sodium and calcium contents, in mg/g dry weight of *Pisum sativum* plant.

Stage	Treatment	Root			Shoot		
		K^+	Na^+	Ca^{++}	K^+	Na^+	Ca^{++}
Initial	Control	2.166	11.716	1.393	6.873	0.646	2.000
	Vernalized	3.376*	13.763*	1.803*	8.143*	1.326*	2.423*
	LSD	0.219	0.662	0.051	0.664	0.076	0.157
Stage I	Control	4.753	14.480	2.073	9.150	1.960	5.300
	Vernalized	5.58*	16.643*	2.426*	10.103*	2.473*	6.613*
	Vernalized+Atonik 250	6.008*	19.97*	2.963*	11.586*	3.35*	7.326*
	Vernalized+Atonik 500	9.625*	21.853*	3.346*	17.179*	4.116*	8.586*
	Vernalized+Atonik 1000	11.25*	26.363*	4.69*	20.743*	4.89*	10.02*
	Vernalized+BA 25	7.466*	18.32*	3.586*	14.613*	3.29*	7.34*
	Vernalized+BA 50	9.732*	20.223*	4.276*	18.833*	4.296*	8.52*
	Vernalized+BA 100	4.508	16.093*	3.756*	10.093*	2.6*	6.45*
	LSD	0.435	0.871	0.173	0.722	0.152	0.368
Stage II	Control	6.720	16.400	2.466	9.467	2.778	9.056
	Vernalized	8.36*	20.066*	3.483*	10.4*	3.536*	10.27*
	Vernalized+Atonik 250	9.46*	22.228*	3.846*	13.236*	4.48*	11.746*
	Vernalized+Atonik 500	11.076*	25.566*	5.08*	18.2*	5.856*	12.893*
	Vernalized+Atonik 1000	15.93*	29.73*	8.903*	22.146*	7.25*	20.29*
	Vernalized+BA 25	8.213*	19.77*	3.926*	14.298*	4.236*	11.536*
	Vernalized+BA 50	10.613*	23*	5.123*	17.7*	6.72*	14.26*
	Vernalized+BA 100	6.250	19.58*	4.16*	11.07*	3.866*	9.646
	LSD	0.542	1.123	0.243	0.748	0.287	0.603
Stage III	Control	7.826	17.971	3.270	10.278	4.881	18.526
	Vernalized	9.816*	21.53*	4.073*	12.373*	5.733*	23.05*
	Vernalized+Atonik 250	12.373*	24.73*	4.533*	17.546*	7.8*	25.756*
	Vernalized+Atonik 500	13.44*	28.036*	5.736*	20.866*	10.106*	29.876*
	Vernalized+Atonik 1000	18.136*	32.603*	10.153*	24.381*	11.633*	34.88*
	Vernalized+BA 25	10.81*	23.103*	4.93*	19.74*	6.126*	24.383*
	Vernalized+BA 50	12.658*	27.59*	6.136*	21.755*	7.613*	27.383*
	Vernalized+BA 100	8.293	21.442*	4.503*	17.42*	4.990	18.906
	LSD	0.574	1.430	0.353	0.950	0.392	1.023

* = Significant increase or decrease at 0.05 LSD.

Table 7 Effect of vernalization and different concentrations of Atonik or benzyl adenine on plant growth regulators content, in µg/g fresh weight of *Pisum sativum* plant in Stage I.

Treatment	Total auxins	GA_3	ABA	Zeatin	Kinetin	Benzyladenine
Control	176.666	13.146	93	3.253	5.463	3.77
Vernalized	131.666*	9.23*	119.333*	1.72*	4*	2.453*
Vernalized+Atonik 250	201.333*	15.89*	65.666*	6.36*	6.756*	6.85*
Vernalized+Atonik 500	226.333*	19.53*	47.333*	10.336*	9.706*	10.736*
Vernalized+ Atonik 1000	271.666*	23.48*	28*	12.493*	14.26*	13.54*
Vernalized+BA 25	219.666*	17.39*	72*	3.986*	7.723*	16.4*
Vernalized+BA 50	238.666*	19.7*	56.666*	7.803*	10.52*	20.573*
Vernalized+BA 100	257.666*	11.676*	86.666	3.54	6.86*	25.933*
LSD	9.456	1.091	6.167	0.603	0.49	0.705

* = Significant increase or decrease at 0.05 LSD.

ence of these compounds on protein synthesis (**Table 5**). Schreoder *et al.* (1999) pointed out that proteins are required to transport protons, inorganic ions and organic solutes across the plasma membrane and tonoplast at rates sufficient to meet the needs of the cells.

Changes in PGRs

During vegetative growth, vernalization led to a significant decrease in total auxins, GA_3 and different CK fractions in the shoots of pea plants (**Table 7**). In contrast, ABA increased significantly by this treatment compared to the control level. Rietveld *et al.* (2000) suggested that the essential developmental step of vernalization is to increase the sensitivity to the auxin, IAA. Moreover, II-Yashuk and Likholat (1989) observed a high level of ABA at the end of vernalization of wheat plants. Also, the increase in ABA levels in pea plants caused by vernalization may probably be due to interference of amide with hormone metabolism by preventing ABA catabolism in *Phaseolus vulgaris* (Walton 1980).

In contrast, a reverse pattern was observed in response to vernalization with all atonik or BA concentrations; these treatments led to a general progressive increase in total auxins, GA_3 and different CK fractions (zeatin, kinetin and BA) in pea plants. On the other hand, [ABA] was significantly decreased by vernalization and the concentrations of atonik or BA used, compared with control values. Mohsen and Zaki (1998) found that low concentrations of BA increase the activity of auxin and GAs in wheat, while a reverse pattern was obtained for the activity of ABA.

In this study, the application of atonik or BA under the influence of vernalization, especially 1000 mg/l atonik and 50 mg/l BA, played an important role in flower induction of pea plants.

CONCLUSIONS

In conclusion, the determined metabolic responses (pigment content, carbohydrate content, nitrogenous fractions and ions content) and especially growth parameters (root length, root fresh weight, root dry weight (DW), shoot length, number of nodes, number of leaves, total leaf area, shoot fresh and dry weights and relative water content) as well as PGRs (auxins, gibberellins, ABA and CK) appeared to promote flowering in pea. This conclusion is supported by Sorlino (1997) who stated that vernalization affected total flower number of linseed, particularly when sown late through its effect on vegetative growth. Also, Pharis and King (1985) stated that the transition from vegetative to flowering state may actually be controlled by changes in the levels of endogenous PGRs such as auxins, gibberellins and CK or by the balance between these PGRs.

Common to both *Arabidopsis* and cereals, the vernalization response readies the *FT* gene to be induced into activity by longer daylengths (Dennis and Peacock 2009). If *FT* cannot be induced, because of a deletion or some other mutation, flowering might not occur. This is, however, not the case in either *Arabidopsis* or cereals. In the absence of *FT* activity, flowering is delayed in long-day conditions, but if short days are imposed experimentally, there is no effect on flowering time. In a recent analysis of genome-wide gene transcription during vernalization in wheat, Winfield *et al*. (2009) showed that the activity of key GA biosynthetic genes also increases in short-day vernalization in cereals. Consistent with GA activity, the cereal shoot apex lengthens during vernalization and subsequent growth in short days, so these results suggest that GA may be a back-up mechanism to the *FT* pathway in short days in cereals as well.

MacMillan *et al*. (2005) found specific effects of vernalization or LD on GA synthesis, content, and action when using four treatment pairs: nonvernalized or vernalized plants exposed to SDs or LDs. Irrespective of the vernalization status, exposure to two LDs increased expression of *L. perenne GA 20-oxidase-1* (*LpGA20ox1*), a critical GA biosynthetic gene, with endogenous GAs increasing by up to 5-fold in leaves and shoots.

The tested plant (*Pisum sativum* var. 'Master bean') in this study is a SD plant which is rarely affected by vernalization. Phillips and Wareing (1977) noted that flowering in the majority of SD plants and some LD plants cannot be induced by vernalization or any combination of the known naturally occurring hormones. Furthermore, there is evidence that the flowering stimulus (e.g. gibberellins) is identical in LD plants although gibberellins are quite ineffective in inducing flowering in most SD plants (Taiz and Zeiger 1998).

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