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Effect of Abscisic acid on Legume Growth Rate, Seed Development and Protein Profile in *Pisum sativum* L.

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IN THIS work, cultivated pea (*Pisum sativum* L. cv. Master B) is used as a legume model crop plant to investigate the effect of abscisic acid (ABA) treatment on seed filling during embryogenesis. This was evaluated by using two exogenously applied concentrations (10& 20 µM) during three stages of seed developments (early, intermediate and late). Phenotypic characterization of the treated plants included measurement of seed / legume length and diameter at full maturation stages after treatment. The expression of major proteins of the treated plants was detected using SDS-PAGE electrophoresis technique. Our data showed a positive effect of ABA when applied at the late stage of seed development. ABA significantly increased legume size and elevated the expression of some major proteins such as convicilin (70-75kDa), vicilin (50, 34kDa) and legumin (40kDa). The impact of these results is further discussed.

Keywords: ABA, Embryogenesis, Pisum sativum, SDS- PAGE, Seed filling.

Introduction

Pisum sativum L., commonly known as pea, is one of the most important food crops for humans and animals. Pea is a source of protein, starch, complex carbohydrates, fibers, vitamins, minerals (Dahl et al., 2012), valuable phytochemicals like phenolics, terpenoids, and nitrogenous compounds (Fahim et al., 2019). It exhibits a great source of nutrients with high digestibility and less phytic acid (Amarakoon et al., 2012). It is a good candidate to decrease global warming impact due to its lower carbon footprint, as compared with other crops (Poore & Nemecek, 2018; Widi et al., 2021). In addition, its harvest ease and ability to increase soil fertility by N_2 fixation make it one of the hot subjects of research and development worldwide (Karkanis et al., 2016). Providing food with high protein content is a top priority in current research in order to feed the increasing population around the world (Small & Degenhardt, 2018). A plethora of recent research is working hard in this respect (Liu et al., 2015, Kozaki & Aoyanagi, 2022).

Seed filling is a critical stage in determining the quality and nutritional value of the crop yield. During this stage, all protein, lipid, oil and sugars or so called reserve products will accumulate in the developing seed to prepare seed for full maturation and prevent early germination (Borisjuk et al., 2004; Slater et al., 2013; Locascio et al., 2014). Seed filling and the nutrient content are directly proportional. Despite of unclear regulatory mechanism of seed filling at the molecular level, it is known that plant hormones are potential elicitors (Kamran et al., 2018). One of the major plant hormone that plays a role in seed development is abscisic acid (ABA). ABA is commonly known as a growth inhibitor and is currently recognized as a maestro in multiple plant growth, development, biotic/abiotic stresses and seed filling (Moustafa, et al., 2020; Younis, 2021). It exhibits a dual function on plant at different developmental stages and its effect is different from one trait to another. Recent research showed its impact on seed fillings in many plant species. However, the exact role of ABA in this respect at the molecular level is still ambiguous and elusive. Recent studies explored aspects and mechanisms

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of ABA in regulating seed morphogenesis and development during embryogenesis. Cross talk between ABA and other signaling molecules, regulators, cofactors and phytohormones can outline future perspectives to control seed development in plants. Yet, the complete network of ABA in many seed traits is still unclear (Faiza et al., 2021; Ali et al., 2022).

It is noteworthy to mention that ABA responses are different among plants and are mediated by unique molecular receptors that are distinguished according to the cell type and the stage of development (Fujii et al., 2007; Verslues & Zhu, 2007; Spartz & Gray, 2009). In most plants, ABA level is low during early embryogenesis but is elevated at later stages of seed development (Delahaie et al., 2013) indicating its crucial role in this respect (Radchuk et al., 2010).

To study the effect of ABA on seed fillings of peas during embryogenesis, we divided this work into three developmental stages: early, intermediate, and late seed embryogenesis. During early embryogenesis, cell division dominates to form the embryo (cellular proliferation). Seed filling takes place until the intermediate stage which is characterized by the accumulation of reserve materials and embryo expansion. At the late stage, embryo growth takes place until full maturation, then, the fully developed seed starts to lose water and desiccates (Bewley et al., 2012; Locascio et al., 2014). To the best of our knowledge, this is the first time to evaluate the effect of exogenously applied ABA treatment during seed embryogenesis in the cultivated pea (Master B). Master B trait is widely cultivated in Egypt at large scale and is certified by the Agriculture Research Centre, Ministry of Agriculture, as a true breeding line.

Materials and Methods

Plant material

Pea (*Pisum sativum* L.) seeds.cv. Master B as a true breeding certified line were provided by the Agricultural Research Center, Horticultural Research Institute, Giza, Egypt. Seed viability was tested to confirm vitality and assure homogenous germination. Seeds showed 100% germination percentage. Dry pea seeds were firstly soaked in water for imbibition overnight, then swollen green seeds were selected for planting out in pots. Selected seed were sterilized by soaking in 15% chlorex (15mL chlorex + 85mL H_2O) for 10min then rinsing with distilled water 4 times to remove any microorganisms that might be attached to their surface. Seeds were cultivated in green house during October in 5 inches pots containing peat moss: sand (2:1 ratio) + 2% NPK fertilizer. Plants were irrigated with equal amounts of water as needed until flowering. All analysis were carried out in cell and molecular genetics lab (Genetic unit, Faculty of Science, ASU, Cairo, Egypt). The field work was conducted in the green house of the Botany Department, Faculty of Science, Ain Shams University, Cairo, Egypt.

ABA hormone preparation

An amount of 80µM ABA stock solution was prepared by dissolving 0.001g ABA in the least amount of ethanol then completed by 50mL sterile distilled water. 20 & 10µM concentrations were prepared by serial dilutions using sterile distilled water. The same amount of ethanol was diluted with distilled water without hormone and used as a control to determine if there is an effect of ethanol on plant response. Water control was used as well to determine the basal response in untreated plants.

Time course of ABA treatment

To determine the effect of ABA on embryogenesis, legumes were treated as early as embryo started to develop. Growing legumes were treated with 50µL of 10 (treatment A) and 20 (treatment B) µM ABA. The stages of the treated legumes are shown in Fig. 1. This Figure shows different developmental stages we focused on during this work. Three different developmental stages of the embryo were chosen: Early stage (6 days after blooming), intermediate stage (11 days after blooming) and late stage (14 days after blooming).

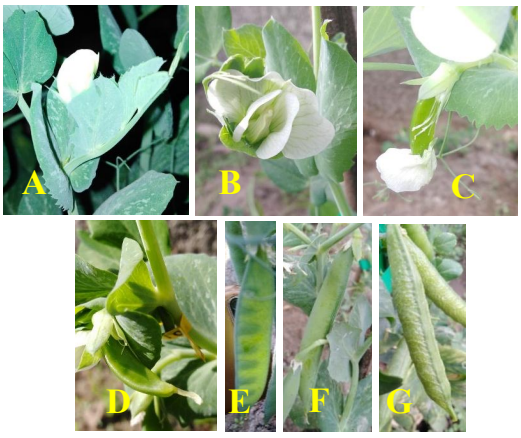


Fig. 1. Morphology of legume in pea plants during different embryogenesis stages in untreated plants (control). A: young flower, B: flower

during fertilization, C: Nascent legume, D: early stage, E: intermediate stage, F: late stage and G: fully mature legume with filled seeds

Application of ABA

Application of ABA with an accurate volume could not be achieved via spraying or using a cotton piece attached to the stigma of the growing legume. Therefore, Hamilton syringe, (a microliter syringe), was used to inject the hormone inside the petiole of the growing legume according to Eeuwens & Schwabe (1975) as shown in Fig. 2. ABA were applied in two concentrations of 10 and 20µM as recommended by Chandrasekaran et al. (2014), Mukherjee et al. (2015) and Zhang et al. (2017) but views on the role of ABA in kernel formation and abortion are not unified. The response of the developing maize kernel to exogenous ABA was investigated by excising kernels from cob sections at four days after pollination and culturing in vitro with different concentrations of ABA (0, 5, 10, 100µM). It is noteworthy to mention that we applied ABA at the concentrations of 2.5 & 5µM but the effect was nontangible. The legumes of the control plants were also injected with diluted ethanol that used in preparing hormone solution. Negative control was left untouched. We used 6 replicates for each treatment. The experiment was repeated 3 times to validate the data. The same ABA concentrations were used as well to spray the vegetative parts of 4 weeks old plants. This was done to determine the effect of ABA at the vegetative level.

Fig. 2. Injection of ABA hormone using Hamilton micro-syringe technique. Picture was taken in the green house of Botany Department, Faculty of Science, ASU, Egypt

Phenotypic characterization of the legume after ABA application

The three developmental stages used in this work to study the effect of ABA during seed embryogenesis are shown in Fig. 3. Legume length and diameter were measured every 3 days until the legume was fully mature (maximum seed filling). The legume length and diameter were measured each 3 days until the legume is fully mature.

Protein profile using SDS-PAGE gel electrophoresis

Total soluble proteins were extracted from the progeny of ABA treated plants by following the basic method developed by Laemmli (1970) and updated by Manns (2011). Protein for each treatment was pooled from 3 seeds. Testae were excluded from the analysis. The gels were scanned and analyzed with Gel analyzer software (Gel Analyzer 19.1, www.gelanalyzer.com) to quantify the relative intensity of protein bands.

Statistical analysis

The mean and standard deviation of replicates were calculated. The significance of results was determined using T-Test student. Calculations were executed using Microsoft Excel (Microsoft office professional plus 2019, version 2303).

Results

ABA sprayed on one month old plants during the vegetative stage resulted in slight increase in the stem length in treatment A (8.3 cm ± 1.16), B (9.2cm ± 1.16) compared with the control (8.5cm± 0.63). However, this increase in stem length was not statistically significant (Table 1). At early developmental stages of seed, A& B treatments did not exhibit a significant effect in treated plants as compared to the control . However, at later stages, both treatments resulted in an increase in the legume diameter as shown in Fig. 4. The highest concentration of ABA (treatment B) significantly increased the diameter of the treated legume at late stage (Fig. 5).

Careful observation of the L.S sections of developing seed in the control and the treated plants did not exhibit a significant difference in the size of the embryo of the developed seed as shown in Fig. 6. The diameter of developing seeds at all stages was increasing with the applied concentrations of the ABA (Table 2). The peak

analysis using the gel analyzer program is shown in Fig. 7. SDS-PAGE protein profile indicates an increase of some major pea proteins in relation to treatments A and B (Figs. 7, 8 and Table 3). This effect was also the highest in applications at late stages. As shown in Fig. 8 and Table 3, the intensity of Convicilin (70-75kDa), Vicilin (50,34kDa) and legumin (40kDa) bands increased and were directly proportional with the concentration of ABA, as compared to corresponding control (Fig. 8). This increase was the highest when ABA was applied during the late stage (Fig. 8). On the other hand, other protein bands such as lipoxygenase (97kDa), Vicilin (30, 25kDa), Legumin β (20kDa) and Lectin β exhibited slight change in their expression, compared with the control (Fig. 8).

Discussion

This work is focused on the role of ABA in seed filling of pea plants (*Pisum sativum* var. Master B) applied at different stages of seed development during embryogenesis. The main focus is to connect the phenotypic changes related to seed filling (seed and legume size) to the expression level of major proteins (protein profile) in relation to ABA treatment. We treated the legumes with ABA at three developmental stages. In general, there are two major phases of seed embryogenesis that include pre-storage and maturation. The former is characterized by active cell division while the later is characterized by depositing protein reservoir before seed dehydration. The transition of these stages is partially controlled by ABA (Radchuk et al., 2010). The two stages are overlapping with no fine borderlines, thus, we decided to categorize our work into three different stages: early, intermediate and late as explained in the material and method section.

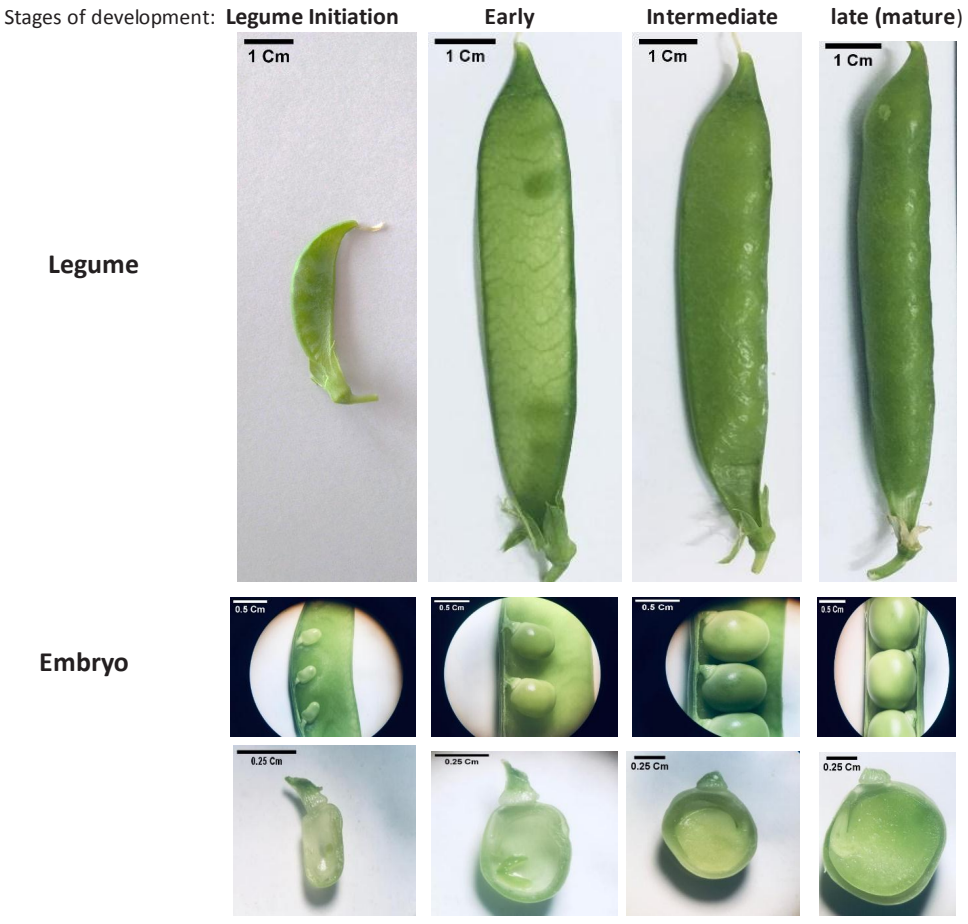
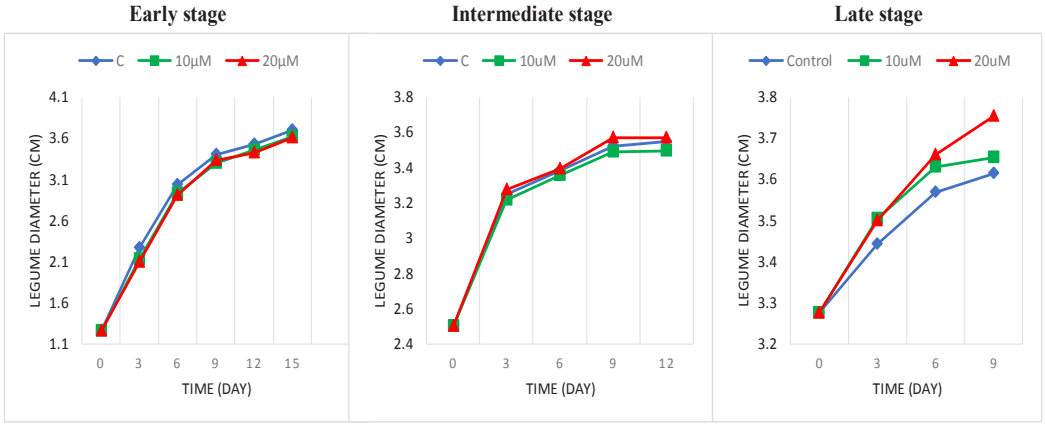


Fig. 3 . Different developmental stages of fruit development (legume, seed and embryo) in untreated plants (control) [Three different developmental stages of the embryo were chosen; early stage (6 days after blooming), intermediate stage (11 days after blooming) and late stage (14 days after blooming)]

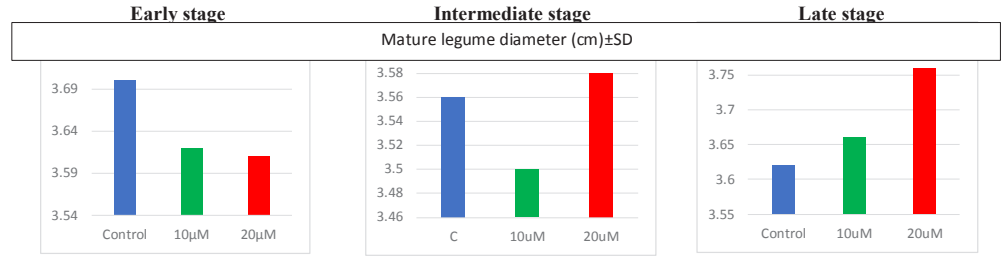
TABLE 1. Plant height after spraying one month old plants with 10µM and 20µM of ABA. Control plants were sprayed with same volume of water [Differences in height were non-significant among treated plants and control (n = 5)]

Treatments	Plant height (cm)
Control (0)	8.5 ± 0.63
10µM ABA	8.3 ± 1.16
20µM ABA	9.2 ± 1.16



Days	Control	10µM	20µM	Control	10µM	20µM	Control	10µM	20µM
0	1.26	1.26	1.26	2.5	2.5	2.5	3.28	3.28	3.28
3	2.26	2.14	2.1	3.26	3.22	3.28	3.44	3.5	3.5
6	3.04	2.94	2.92	3.4	3.36	3.4	3.56	3.64	3.66
9	3.4	3.3	3.34	3.52	3.5	3.58	3.62	3.66	3.76***
12	3.54	3.46	3.42	3.56	3.5	3.58			
15	3.7	3.62	3.6						

Fig. 4. Effect of ABA on legume diameter expansion rate: Measurements were taken every 3 days for plants treated with 10µM and 20µM ABA along with their controls at early, intermediate and late developmental stages. n=3. Note that measurements were adjusted according to the treatment stage (plants in early stage, intermediate stage and late stage were treated for 15 days, 12 days and 9 days respectively to attain full maturation



treatment	Early stage	Intermediate stage	Late stage
Control	3.7± 0.19	3.56± 0.07	3.62± 0.02
10µM ABA	3.62± 0.1 P=0.35	3.5± 0.1 P=0.38	3.66± 0.09 P=0.35
20µM ABA	3.6± 0.03 P=0.17	3.58± 0.05 P=0.67	3.76*** ± 0.08 P=0.006

Fig. 5. Overall effect of ABA on legume diameter after maturation: Measurements were taken for fully mature legume. n=3 [* Indicates values that are significantly different at level P = 0.1; ** Indicates values that are more significantly different at level 0.05 < P < 0.1; * Indicates values that are highly significantly different at level P ≤ 0.05]**

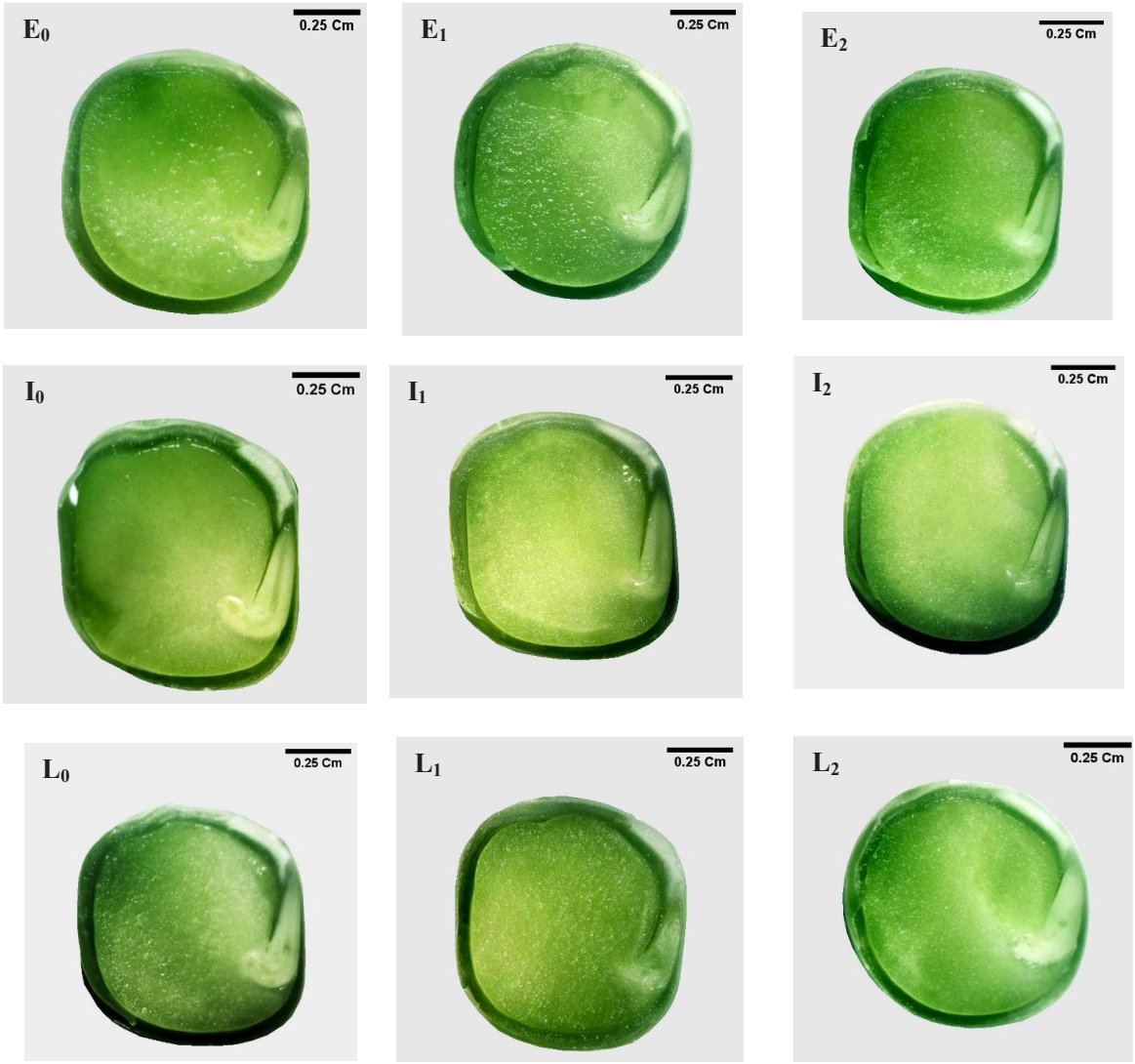


Fig. 6. L.S of pea seeds during different developmental embryogenesis stages in control and ABA treated plants [E0): early stage control, I0): Intermediate stage control, L0): late stage control, E1): 10µM ABA in early stage, E2): 20µM ABA at Early stage, I1): 10µM ABA in intermediate stage, I2): 20µM ABA at intermediate stage, L1): 10µM ABA at late stage and L2): 20µM ABA at late stage. No obvious difference was observed]

TABLE 2. Seed diameter (cm) of the control and ABA treated pea plants at the three developmental stages under study

Treatments	Early stage	Intermediate stage	Late stage
Control (0)	0.83	0.78	0.79
10µM ABA	0.81	0.76	0.80
20µM ABA	0.75	0.79	0.82

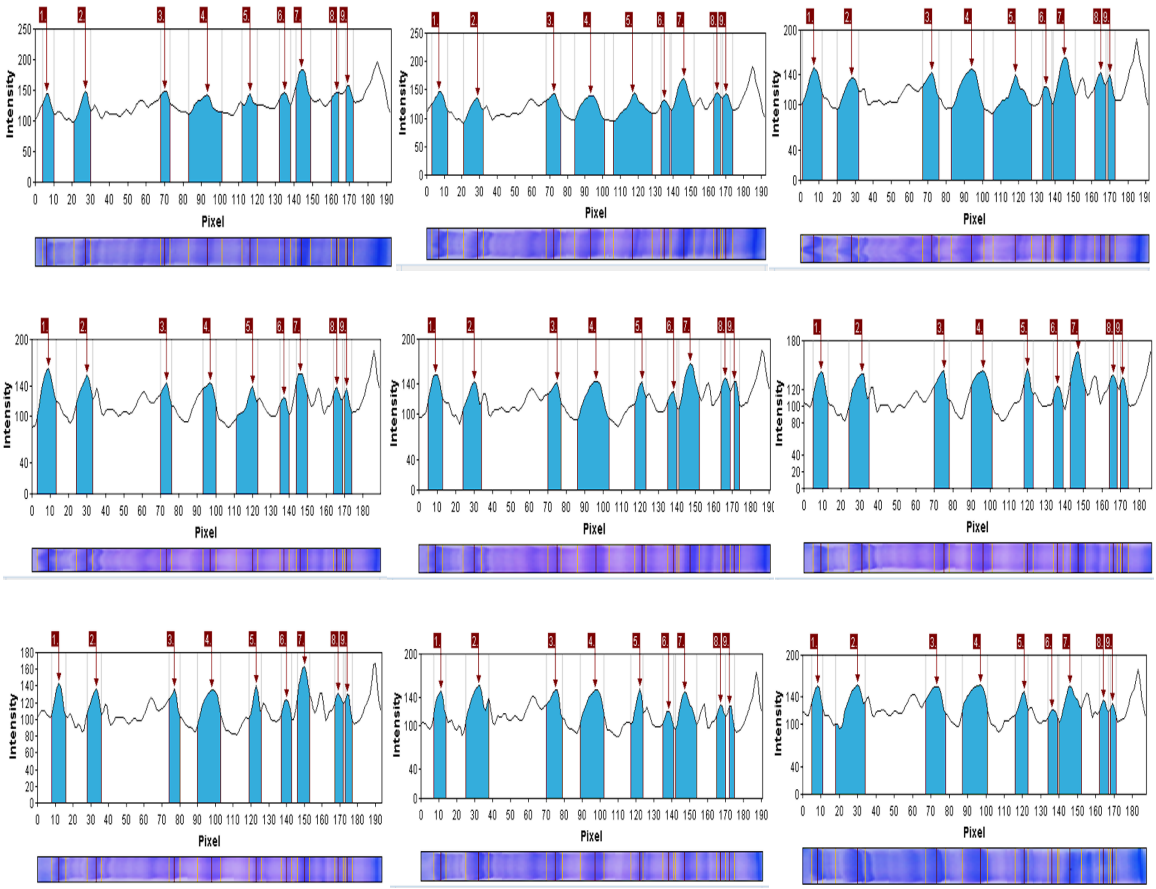


Fig. 7. The intensities of nine major protein bands as revealed using gel analyzer document system

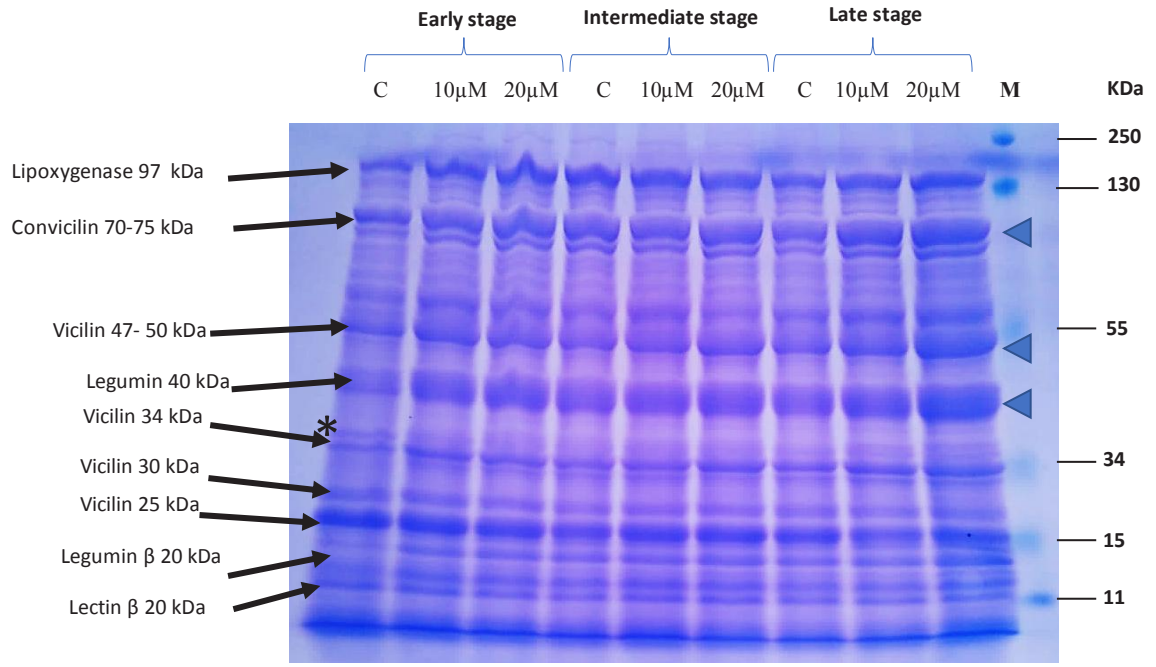


Fig. 8. SDS-PAGE of total proteins profile from pea seeds (master B cultivar) at the three designated stages of embryogenesis as indicated on the image [All samples were having the same concentrations of 20µg/lane as measured by Bradford protein assay]

TABLE 3. Band intensities of major nine proteins of pea seeds of the control and ABA treatments (10 & 20µM) at three different stages of legume development, resolved on SDS [PAGE gel electrophoresis as revealed by Gel analyzer software]

Protein band	Cont.	10µM	20µM	Cont.	10µM	20µM	Cont.	10µM	20µM
Lipoxygenase	919	1333	1552	1519	1249	1150	1112	1061	1000
Convicillin	1247	1304	1682	1128	1384	1486	1363	1794	1868
Vicilin (47-50kDa)	852	1203	1302	926	1053	1160	1333	1359	1710
Legumin (40kDa)	1496	1883	2071	1819	1715	1810	1707	1882	2265
Vicilin (34)	2585	2326	2066	2132	2348	2287	2101	2115	2493
Vicilin (30)	2397	2586	2834	2674	2663	2617	2404	2715	3047
Vicilin (25)	971	756	706	699	732	697	900	783	697
Legumin β (20kDa)	713	705	936	779	839	661	748	730	756
Lectin β	745	925	632	615	530	616	591	463	490

Legume diameter of plants treated during early to mid-stages exhibited non-significant change, as compared with their control (Fig. 5). We observed that during the early stage, treatments A and B resulted in a slight decrease in legume diameter, compared with that of the control. We assumed that ABA might affect the rate of cell division when applied during early to mid-stages. This assumption was in agreement with the conclusion of Raz et al. (2001). However, plants treated with the highest concentration of ABA during late stage showed a significant increase in legume diameter (Figs. 4, 5). Such increase in legume diameter was in agreement with previous work done with other plants such as wheat, maize (Liu et al., 2016), castor bean (Chandrasekaran & Liu, 2014) and rice (Ali et al., 2018). This result might be attributed to that applied treatments had elevated the concentration of ABA above its normal level and might have resulted in reduced cell division during early stages of seed development (Raz et al., 2001). On the other hand, the accumulation of storage protein at late stages following ABA treatment is concomitant with the increase in the size of seed and legume of treated plants (Sreenivasulu et al., 2006; Radchuk et al., 2010). Thus, it can be concluded that there are contrasting roles of ABA actions at early embryo differentiation and later maturation. This result is in agreement with the study of Sreenivasulu et al. (2006).

To test the effect of ABA on protein accumulation, storage protein profile was analyzed using SDS- PAGE. Major proteins resolved in this work are shown in Fig. 8. Our data detected an increase in the expression of some major proteins in the treated plants, as compared with their control (Fig. 8), while the expression of some other proteins remained elusive. In general, legumes are characterized by high content of proteins that do not possess any enzymatic or structural role during seed development (Duranti, 2006). Three types of proteins are usually present, namely, albumins, globulins and prolamins (Duranti, 2006). Pea seeds consists of 70–80% of globulin proteins that include legumin, vicilin and convicilin (González-Pérez & Arellano, 2009). It had been reported that, ABA can suppress the expression of some proteins while enhancing others (Weber et al., 2005). Thus, it was expected to see different pattern of protein expression from one stage to another without linear correlation with the applied ABA concentration. Therefore, for accurate analysis, we looked at resolved proteins as case-by-case study. We observed that the expression of Lipoxygenase protein (97kDa) was directly proportional with ABA concentration at early stages while the case was reversed in intermediate and late stages, as compared with the corresponding controls (Figs. 7, 8 and Table 3). It had been shown that the exogenous application of ABA resulted in no change to slight downregulation in lipoxygenase (LOX) in tea plants (Gai et al., 2020) as the case of our study. Thus, lipoxygenase accumulation is likely to occur

in seeds of the ABA treated plants at early stage of development and reduced during the intermediate or late stages. This might give an insight toward the possible use of ABA to reduce the expression of LOX proteins which are the main reason for the undesirable off-flavor/ odor release in cooked and stored peas.

Band intensities of convicilin (70-75kDa), vicilin (50, 34kDa) and legumin (40kDa) were upregulated. This increase was directly proportional to the concentration of ABA, as compared with that of the control within the same stage (Fig. 8). Interestingly, this increase was highest when ABA was applied at late stage (Fig. 8). It is known that convicillin, vicillin and legumin are storage globulin proteins acting as nitrogen and carbon reserve compounds in seeds. These proteins can be used as markers for storage protein accumulation during seed development (Müntz, 1998). Thus, the upregulation in the expression level of these particular proteins can be used as direct measure /molecular marker to indicate the ability of exogenously applied ABA in enhancing the seed filling during late embryogenesis. Interestigly, the presence of a protein band with a size range of 35-40kDa above the 34kDa vicilin was observed in this work. This band is highlighted by * in Fig. 8. This protein band was expressed in the control at relatively low concentration, but yet detectable, and then was downregulted in plants treated with ABA. One explaination for this scenario could be related to the proposed model of vicilin postranlation modification (Le signor et al., 2017). Vicilin can undergo post-translational proteolysis, resulting in different fractions with variable mobilty rates (Casey & Domoney, 1999; Bourgeois et al., 2009). Vicilin processing starts at the ER and ends within storage vacuoles. In general, post-translational proteolysis of vicilin can generate different polypeptides with mobility rates of 50, 34, 30–33, 25–30, 18–19, 16, 14, 13–13.5, and 12–12.5kDa (Gatehouse et al., 1982). The high modification rate of vicilin is due to the lack of sulfur containing amino acids such as cysteine. The disulfide bonds generated by cysteine are crucial for protein stability (Bourgeois et al., 2009). Logically, this does not apply on the cysteine containing convicilins which are neither post-translationally processed nor glycosylated (Casey & Demoney, 1999). We assume according to our results that ABA treatment might have interfered with the postrranslational mechanism of vicilin. The effect of ABA on vicilin gene was documented before (Radchuk et al., 2006). Therefore, it is tempting to assume that vicilin gene expression is likely regulated via an ABA-dependent pathway (Radchuk et al., 2007). Whereas legumin gene expression, on the otherhand, is more under a nutritional control (Radchuk et al., 2007).

Other protein bands such as Legumin β (20kDa) and Lectin β did not change in their expression compared with the control (Fig. 8). In conclusion, this work can give new insights toward the use of ABA or one of its analogues to increase the protein content in pea crop plants.

Conclusion

Pisum sativum plants were treated with two concentrations of ABA (10μM as treatment A & 20μM as treatment B) in both vegetative and fruit stages. Vegetative stage was treated via spraying of a one-month-old plant with ABA twice through 10 days interval between both sprays. Fruit stage was treated via injection of the legume with ABA at three different developmental stages (early, intermediate, and late). Their corresponding controls were sprayed/injected with diluted ethanol. The main objective of this study is to decipher the role of ABA (i.e., seed formation in pea plants and correlate their impact on the physiological aspects and the expression of some selected genes responsible for the accumulation of seed storage proteins. When ABA was sprayed on one month old plants during vegetative stage, it resulted in slight increase in the stem length in treatment B and decrease in treatment A compared with the control. However, these changes in stem length in both treatments were not statistically significant. The highest concentration of ABA (treatment B) significantly increased the diameter of the treated legume at late stage. SDS-PAGE protein profile indicates an increase of some major pea proteins in relation to treatment A and B application. The effect was also the highest in applications of late stages. The intensity of CONVICILIN, VICILIN and LEGUMIN bands increased and were directly proportional with the concentration of ABA as compared to the control within the same stage. This increase was the highest when ABA was applied during the late stage. On the other hand, other protein bands such as LIPOXYGENASE, VICILIN, LEGUMIN B and LECTIN B exhibited slight change in their expression compared with the control.

Competing interests: The authors report no conflicts of interest regarding this work.

Authors' contributions: The main idea, the experimental design, the overall data analysis and the writing of the manuscript of this work was done by the third author. The third author also supervised, provided the facility, data collection for analysis & discussion, trained the first author to carry out all experiments related to all parts except the physiology part. The first author did all the practical work and collected data into tables and figures. The second author provided the facility to execute all the experiments related to the physiological part of this research with data analysis and discussion.

Ethics approval: Not applicable.

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تأثير حمض الأبسيسيك على معدل نمو القرون وتطور البذور وملف البروتين في نبات البسلة

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تم في هذه الدراسة استخدام نبات البسلة المنزرعة كنموذج لمحصول البقوليات لدراسة تأثير معاملة حمض الأبسيسيك على امتلاء البذور أثناء مرحلة تخليق الجنين في نبات البسلة. تم تقييم ذلك بتحضير تركيزين من حمض الأبسيسيك (10 و 20 ميكرومول)، ثم تطبيقيه أثناء ثلاثة مراحل من تطور البذرة (مرحلة مبكرة، ومرحلة متوسطة، ومرحلة متأخرة). تضمن التوصيف الظاهري للنباتات المعالجة قياس طول النبات، وقطر كل من البذور والقرون المعالجة بعد تمام نضجها. تم الكشف أيضا على التعبير الجيني لبعض البروتينات المختزنة بالبذور المعالجة باستخدام تقنية التفريد الكهربائي للبروتينات على هلام عديد الأكريلاميد. أظهرت النتائج ان هنالك تأثيرا إيجابيا لحمض الأبسيسيك عند تطبيقه أثناء المرحلة المتأخرة من تطور البذرة. حيث أدى تطبيق حمض الأبسيسيك الى زيادة قطر القرن وارتفاع التعبير الجيني لبعض البروتينات المختزنة كالفسيلين والليجيومين والكونفيسيلين. وتمت مناقشة هذه النتائج بمزيد من التفاصيل.