Effect of Redroot Pigweed Interference on Active Oxygen Species, Antioxidative Defence System and Oxidative Damage of Common bean (Phaseolus vulgaris L.)

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Abstract

Redroot Pigweed (*Amaranthus retroflexus* L.) is an important weed that is highly competitive with common bean. The aims of this study were to evaluate the relative expression of a number of antioxidant enzyme and light response genes and investigate the activity of antioxidant enzymes, anthocyanins and photosynthetic pigments of several red common bean cultivar/lines (Sayad, Derakhshan, and Line D81083) in third trifoliate leaf (V4) and pod formation (R7) stages under Redroot Pigweed free and infested conditions. At V4, enzymatic activity and gene expression decreased while with the increase of common bean competition with weeds, the expression of genes and enzymes activity increased. The activity of antioxidant enzymes increased in the R7 as compared to the V4, indicating the production of reactive oxygen species (ROS) upon competition with weed and increased enzymatic activity for the elimination of ROS. Sayad and D81083 exhibited the highest catalase contents under weed interference whereas demonstrated the lowest catalase enzyme amount under weed-free conditions. Thus, response of common bean to competition with weed depends on bean growth stage and genotype.

Introduction

Common bean (*Phaseolus vulgaris* L.) as an annual summer crop is one of the most important crops in the legume family. The common bean cultivation was estimated to be 33066183 hectares in 2019 (FAO, 2020). It is also an important source of protein (16 to 37%), carbohydrates and calories (Mola and Belachew, 2015; Kouam and Tsagua-Zanfack, 2020). Therefore, common beans play an important role in human nutrition (Kouam and Tsagua-Zanfack, 2020).

Weed interference is one of the main biological constraints in common bean production (de Aguiar et al., 2020; Etminani et al., 2020). Weed interference reduces the dry grain yield of common beans by up to 85%. The vegetative growth stage is a critical period in which common bean is sensitive to weed interference (El-Wakeel and El-Metwally, 2020). Weed allelopathic activities early in the season may limit the crop root system development and growth (Mola and Belachew, 2015). The critical period of weed control has been reported to be from 3 to 5 or 6 weeks after planting in common beans (Burnside et al., 1998) and from the second trifoliate stage to early flowering in white common bean (Woolley et al., 1993). There are several predominant weeds commonly found in the common bean fields. Redroot Pigweed (*Amaranthus retroflexus* L.) is an important weed with physiological and morphological characteristics such as high plant height, abundant seed production, high growth rate, C4 photosynthetic pathway, high light extinction coefficient and deep and transverse root expansion, which make the species highly competitive with the crops (Wilson et al., 1980).

Abiotic factors, such as light quality and quantity, are reflected in the photosynthetic capacity of plants. The content of photosynthetic pigments such as chlorophyll a and b and carotenoids each absorb different light wavelengths (de Aguiar et al., 2020). Leaf chlorophyll content also largely determines the photosynthesis rate and biomass production in plants (dos Santos et al., 2013). In the study of Mckenzie-Gopsill et al. (2019) showed that weeds interference reduces the carotenoids and chlorophyll pigments in soybean leaf (*Glycine max* (L.) Merr.).

The study of changes in the production of reactive oxygen species (ROS) in crops in response to weed interference, has demonstrated changes in enzymatic activity and gene expression of antioxidant compounds in relation to oxidative stress. Stress signaling due to weed interference in crops (Gal et al., 2015) might be related to light conditions (Piasecki et al., 2018) and allelopathic compounds released from weeds. Far red light reflected from adjacent weeds induces changes in the expression profiles of scavenger genes of ROS (McKenzie-Gopsill et al., 2016). The high rate of far-red light results in the production of ROS (Afifi and Swanton, 2012) that affects both photosynthesis and carbon partitioning (McKenzie-Gopsill, 2016). Allelochemicals stimulation of ROS production and activation of antioxidant-mediated defense may lead to damage to DNA, proteins, and cell membranes (De Almeida et al., 2008; Caverzan et al., 2019). Competition of Italian ryegrass (*Lolium multiflorum*) has led to oxidative damage to and increased activity of SOD, CAT, and APX enzymes in soybean (Agostinetto et al., 2016). Therefore, it is important to quantify the dynamic expression of antioxidant genes, as well as changes in the activity of enzymes and compounds that serve as tools to combat ROS that finally affect the crop yield (Caverzan et al., 2019; Caverzan et al., 2016).

Genes in crop plants are irreversibly altered by the presence of weeds (Moriles et al., 2012; McKenzi-Gopsill et al., 2016). Weeds also cause crop developmental changes by reducing the regulation of genes necessary for nutrient uptake by roots (Hansen et al., 2013). Up and down and down-regulation of defense-related genes in plants has been reported (Masclaux et al., 2012; Biedrzycki et al., 2011; Geisler et al., 2012; Horvath et al., 2015; Bowsher et al., 2017). Down expression of photosynthetic genes due to interspecific competition has been reported in various studies (Horvath et al., 2006; Schmidt and Baldwin, 2006; Moriles et al., 2012). *Phytochrome B* (*phyB*) gene was upregulated in both barley and maize at high plant density (St. Pierre et al., 2011). PIFs (phytochrome interacting factor) inhibits carotenoid accumulation by down-regulating the expression of the Phytoene Synthase (*PSY*) encoding gene, which is the major enzyme that determines velocity in the carotenoid pathway (Ortiz et al., 2014). A study indicated roles for increased oxidative stress and jasmonic acid signaling responses during weed stress (Horvath et al., 2015). According to transcriptome studies shade avoidance was found to be necessary as part of soybean response to weeds during the critical weed control period (CWCP) (Horvath et al., 2015), while shade avoidance responses in similar transcriptome studies has not been shown (Horvath et al., 2006).

There is no information on changes in gene expression that occurred in common bean under weeds interference during the CWCP period. Therefore, the aims of this study were to evaluate the expression of a number of antioxidant enzyme and light response genes and investigate the activity of antioxidant enzymes, anthocyanins and photosynthetic pigments of several red common bean cultivars in third trifoliate leaf (V4) and pod formation (R7) growth stages under weed free and weedy conditions during the CWCP period.

Results

Gene expression of antioxidant enzymes

The results showed an increase in *APX*, *CAT1*, and *SOD* expression in common bean cultivars/lines except for the *SOD* gene in the Derakhshan cultivar (Fig. 1A, 1B, and 1C) in stage R7 compared to stage V4 upon competing with weeds. Upon weed interference and in the R7 stage, the level of *CAT1* gene transcript was increased 7.64 and 3.35 folds in lines D81083 and Sayad cultivar, respectively (Fig. 1B).

Expression of genes related to light and photosynthesis

Decreased expression of the *CAB* gene (chlorophyll a/b-binding protein) was observed in both V4 and R7 stages. The highest expression of this gene was observed in the V4 stage in line D81083 (0.72 times) while its lowest expression was detected in the Derakhshan cultivar (0.024 times). The highest and lowest *CAB* gene expression was reported in the R7 stage in Sayad and Derakhshan cultivars (showing an increase by 0.90 and 0.19) respectively (Fig. 2).

Evaluation of *PHYB* (*Phytochrome B*) gene expression in weedy and weed-free conditions in the V4 stage (Fig. 3) showed that the expression of this gene was higher under complete weed interference in Sayad cultivar (2.38 times) as compared with the weed-controlled state. It was, however, lower in Derakhshan cultivar and line D81083. The highest and lowest expression levels of the *PHYB* gene at the R7 stage were 4.25 and 1.11 times, respectively, in D81083 and Sayad lines.

Under complete weed interference, decreased expression of the *IAA8* (auxin-responsive protein) gene was observed in the V4 stage of common bean growth in the three cultivars/lines studied (Fig. 4). The highest and lowest expression of this gene at the V4 stage were 0.40 and 0.24 times in Derakhshan and Sayad cultivars, respectively. The highest and lowest expression of the *IAA8* gene at the R7 stage were observed in line D81083 (3.98 times) and Sayad (0.65 times), respectively.

Decreased expression of the *PIF3* gene (phytochrome 3 interacting factor) was observed at the V4 stage under complete weed interference in the three studied cultivars/lines (Fig. 5). The highest and lowest expression of the *PIF3* gene at the V4 stage were observed in Sayad cultivar (0.51 times) and Derakhshan cultivar (0.019 times), respectively. At the R7 stage, an increase was detected in the expression of this gene in Sayad and D81083 cultivars under complete weed interference, while showing a slight decrease in Derakhshan cultivar. The highest and lowest expression of the *PIF3* gene at the R7 stage were observed in line D81083 (4.072 times) and Derakhshan cultivar (0.92 times), respectively.

Results of *HFR* gene expression (Long Hypocotyl in Far-Red light) under complete weed interference at the V4 stage (Fig. 6) showed that the expression of this gene decreased in Sayad (0.23 times) and Derakhshan (0.003 times) cultivars while exhibiting a slight increase in D81083 line (1.016 times). At the R7 stage, expression of this gene increased with incrementing weed interference pressure on the common bean plant. Increased *HFR* gene expression was also observed in Sayad cultivar and line D81083 under complete weed interference; whereas a decrease was observed in its expression in the Derakhshan cultivar. The highest and lowest expression of the *HFR* gene at the R7 stage were reported in the D81083 line (3.99 times) and Derakhshan cultivar (0.146 times), respectively.

Decreased expression of the *HAT4* gene (Homeobox-leucine zipper protein HAT4) was detected under complete interference with weeds (weedy) at the V4 stage of common bean growth in all three studied cultivars/lines (Fig. 7). The highest *HAT4* expression at the V4 stage was observed in the Derakhshan cultivar (0.509 times) while its lowest expression level was recorded in the Sayad cultivar (0.018 times). At the R7 stage, the expression of this gene increased with prolonging the weed interference duration. A significant rise was detected in the expression of this gene in Sayad cultivar and line D81083 exposed to complete weed interference increased but decreased while the Derakhshan cultivar showed a decline. The highest and lowest expression of the *HAT4* gene at the R7 stage were 5.167 and 0.653, respectively, observed in line D81083 and Derakhshan cultivar.

Investigation of the expression of the *PAR1* gene (rapid regulation of phytochrome) under complete weed interference at the V4 stage indicated decreased expression in the three studied cultivars/lines (Fig. 8). The highest and lowest expression of the *PAR1* gene at the V4 stage was observed in the Sayad cultivar (0.75 times) and the D81083 line (0.12 times), respectively. The expression level of this gene increased at the R7 stage by prolonging the weed interference period in the Derakhshan cultivar and D81083 line; while showing a decline in the Sayad cultivar. Decreased *PAR1* gene expression was observed under complete weed interference in Sayad cultivar (0.23 times) and D81083 line (0.25 times) whereas Derakhshan cultivar exhibited an increase (3.44 times).

Physiological traits

Antioxidant enzymes

Weed interference treatment with 0.0127 enzyme units per mg of protein led to the highest level of catalase in the V4 stage (Fig. 9A). The catalase and ascorbate peroxidase contents of the R7 stage were not affected by any of the treatments. At the V4 stage and under weed interference, the ascorbate peroxidase content (0.0599 units of enzyme per mg of protein) decreased compared to weed control conditions (0.03047 units of enzyme per mg of protein) (Fig. 9B).

At full interference with weeds and the V4 stage, the Derakhshan cultivar showed the highest superoxide dismutase content (12.525 enzyme units per gram of plant material) while the Sayad cultivar demonstrated the lowest amount of this enzyme (7.5608 enzymatic units per gram of plant material) (Fig. 9D). At the R7 stage, based on Fig. 9C, the content of superoxide dismutase enzyme was higher under the complete weed interference (14.41 units of enzyme per gram of plant material) as compared to the weed-free state (13,025 units of enzyme per gram of plant material).

Chlorophyll

At the V4 stage, Derakhshan cultivar and line D81083 had the highest (9.94 µg / ml) and the lowest (7.33 µg / ml) chlorophyll a (Fig. 10A), respectively. As shown in Fig. 10B, at the R7 stage, the chlorophyll-a content was lower under complete interference with weeds as compared to the weed-controlled conditions. At stage R7, the amount of chlorophyll-a respectively increased by 16.36% and 24.22% in the plants exposed to complete weed interference and weed-free control conditions as compared to the V4 stage. At V4 stage, the Sayad and Derakhshan cultivars had the highest (3.17 µg/ml) and lowest (2.47 µg/ml) chlorophyll-b contents, respectively (Fig. 10C). As shown in Fig. 10D, at the R7 stage, the chlorophyll-b content was lower under complete weed interference (3.13 µg/ml) compared to the weed-free state (3.94 µg/ml). Liters). In stage V4, Derakhshan cultivar and line D81083 had the highest (12.42 µg/ml) and lowest (9.84 µg/ml) total chlorophyll content (Fig. 10E), respectively. In both V4 and R7 stages, the total chlorophyll content was higher under completely controlled weed conditions as compared with the weed interference state. The total chlorophyll content respectively increased by 15.80 and 24.81% at the R7 stage under complete weed interference and the weed-free state as compared to the V4 stage (Fig. 10F). The highest and lowest relative chlorophyll levels at the R7 stage were observed in Derakhshan and Sayad cultivars, respectively. Relative chlorophyll content increased at the R7 stage compared to the V4 stage. Compared to the V4 stage, the relative chlorophyll content respectively increased by 14.30 and 7.66% in the R7 stage for samples exposed to complete weed interference and complete weed control (Fig. 11).

Anthocyanins

The highest and lowest anthocyanin levels at the V4 stage were observed in the Sayad cultivar (0.511 µmol / ml) and line D81083 (0.3758), respectively (Fig. 12A). Anthocyanin levels decreased at the R7 stage compared to the V4 stage (approximately 90%) (Fig. 12B).

Carotenoids

At the V4 stage, the Derakhshan cultivar had the highest carotenoids content (2.2350 µmol / ml) under complete weed control, while line D81083 exhibited the highest amount of carotenoids (2.091 µmol/mL) under weed interference (Fig. 13).

Discussion

In both stages, an increase was observed in *CAT1* gene expression of Sayad cultivar and line D81083 that was pronounced at R7 under weed conditions. However, no gene expression was observed in the V4 stage with slight expression in the R7 stage in the Derakhshan cultivar under weed interference (Fig. 1B). At the R7 stage, the content of Antioxidant enzymes under the complete weed interference was higher as compared to the weed-free state.Increased and decreased expression of ROS scavenging genes were observed in the hypocotyl stage and the single leaflet stage of soybean development, respectively (Mckenzie-Gopsill et al., 2016). The production of ROS under different types of stress is a common mechanism. Imbalance in the production and removal of ROS due to environmental stresses can lead to a rapid and unstable ROS rise and oxidative damage to the plants, resulting in the activation of defense mechanisms against oxidative stress and, hence, increased activity of antioxidant enzymes in plant cells (Agostinetto et al., 2016; Mullineaux and Baker, 2010; Piasecki et al., 2018). The presence of weeds at densities greater than or equal to crop can damage the cell membrane by lipid peroxidation and leakage of cell contents into the environment (Zhou et al., 2007; Agostinetto et al., 2017) suggesting weed control measures before the effects emerge. The R/FR ratio of light reflected from adjacent plants and transmitted to the crop can alter the amount of leaf antioxidants. Shade-compatible responses control the accumulation of antioxidants in leaves through the R/FR signal transmission system. The reflected R/FR ratio can act as a warning signal, informing the plants about the increased risk of photon inhibition and oxidative stress (Bartoli et al., 2009). Under FR-rich conditions, changes in the content of photosystem elements (PS) lead to the production of ROS as well as antioxidant responses. According to a study by Mckenzie-Gopsill et al. (2019), weed-filled treatment significantly increased H_2O_2 levels in monocotyledonous leaves (19%) by increasing the activity of waste-removing enzymes. ROS was associated with ascorbate peroxidase (30%) and glutathione peroxidase (14%) but catalase activity remained unchanged (Mckenzie-Gopsill et al., 2019).

The expression of *CAB* gene (chlorophyll a/b-binding protein) decreased at the R4 and R7 stage with enhancing the weed interference pressure on the common bean plant. A small number of selected genes were expressed in maize under weed stress at the V8 stage that included different members of the CAB family; a senescence-related protein, and the glycosyltransferase 8 family (Horvath et al., 2006). Decreased expression of genes involved in photosynthesis (such as ribulose bisphosphate carboxylase and chlorophyll a-b binding protein) was observed in corn under competition with velvetleaf (Horvath et al., 2007). Low expression of this gene confirms that weed stress and shade limit the photosynthetic abilities of the plant. In both V4 and R7 stages, the total chlorophyll content was higher under completely controlled weed conditions as compared with the weed interference state. FR-rich light generated by adjacent weeds affected the levels of chlorophyll precursors (such as Pchlide and Chlide a) (Sineshchekov et al., 2004; Bou-Torrent et al., 2015). However, the effect of increasing FR light on photosynthetic pigments varies between plant species and in-plant organs (Sineshchekov et al., 2004).

Under complete weed interference, prolonged weed competition enhanced the expression of *Phytochrome B* at the R7 stage in the Derakhshan cultivar (line D81083) while it showed a decline in the Sayad cultivar. R/FR light signals are received and transmitted by phytochrome light receivers. Arabidopsis contains several phytochrome receptors (*PHYA-E*; Franklin, 2008). These different receptors have somewhat common roles in shade avoidance syndrome (SAS), day and night regulation, seed germination, and seasonal growth changes such as bud dormancy and flowering (Horvath et al., 2015). Masclaux et al. (2012) identified the *PHYA* gene which is involved in the transmission of light signals. *PIF3* gene expression was constantly increased in response to the presence of weeds (Horvath et al., 2015).

Under complete weed interference, with increasing the duration of weed interference, the expression of the *IAA8* gene increased at the R7 stage compared to the V4 stage, however, the gene expression decreased compared to the complete weed-controlled Sayad and Derakhshan cultivars while showing a rise in

D81083 line. In the study of maize microarrays in competition with velvetleaf (Horvath et al., 2006), *Aux/IAA* gene expression decreased. Previous findings suggest that both the auxin and ethylene signal transduction responses are altered by shade avoidance responses (Devlin et al., 2003; Pierik et al., 2004).

The expression of *PIF3* gene increased at the R7 stage with enhancing the weed competition pressure on the common bean plant. PIF3 is a helix-loop-helix transcription factor (Ni et al., 1998) that is more regulated by post-transcriptional mechanisms (Soy et al., 2012). In a study on soybean RNAseq in competition with weeds, the weed-induced *PIF3* gene (*PIF3a*) was clearly increased at the presence of weeds, but if weeds were removed in the V3 stage, weed-induced *PIF3a* expression did not maintain its high level (Horvath et al., 2015).

HFR gene expression under complete weed interference at the V4 stage decreased while At the R7 stage, expression of this gene increased. *HFR1* is one of the well-known negative regulators induced by low R/FR treatment (Kim et al., 2016). This negative regulator prevents more elongation at a low R/FR ratio (de Wit et al., 2016). A comparison of Kim et al. (2016) study with previous reports showed no strong association with several marker genes for the shadow avoidance response such as *ATHB2*, *HFR1*, *FT*, and other auxin-related genes.

Expression of the *HAT4* gene under complete interference with weeds (weedy) at the V4 stage of common bean decreased but at the R7 stage increased. Masclaux et al. (2012) identified several specific genes which respond to low R/FR ratios, these genes are involved either in light signal transmission (*HFR1*, *PHYA*, *FHL*), or elongation processes regulated by auxin (*HAT2*, *XTR7*), or auxin transport (*ASA1*). This indicates that plants grown at high densities detect changes in light quality and initiate a growth response regulated by auxin.

In addition, expression of the *PAR1* gene under complete weed interference at the V4 stage decreased and at the R7 stage increased. *PAR1* (*Phytochrome rapidly Regulated 1*) and *PAR2* are negative regulator genes that originated from avoiding low R/FR-induced shading in *Arabidopsis*, which may not be induced in response to weed competition (Horvath et al., 2015; de Wit et al., 2016).

Anthocyanin levels decreased at the R7 stage compared to the V4 stage. In weed-filled treatments, exposure to maize at low R/FR ratios decreased the stem anthocyanins while increasing lignin in stem tissues compared with weed-free treatments (Afifi and Swanton, 2012). Besseau et al. (2007) reported that the metabolic change in the phenylpropanoid pathway can occur through different pathways to produce anthocyanins or lignin. Anthocyanins are required to protect the plant against high light conditions and will be reduced under weed-induced shade conditions (Horvath et al., 2015).

Infrared-rich light (FR-E) was also increased under weed conditions. It has been shown to affect carotenoid levels (Sineshchekov et al., 2004; Bou-Torrent et al., 2015). However, the effect of FR-E light on photosynthetic pigments depends on the plant species and in-plant organs (Sineshchekov et al., 2004). In a study by Mckenzie-Gopsill et al. (2019), the levels of leaf carotenoids, as the strongest $^{1}O2$ waste remover, were significantly reduced under light conditions in weed-filled treatment (39%).

Conclusion

By enhancing the pressure of weed interference on the common bean plant, the expression of most studied genes increased at the R7 stage compared to the V4 stage. Sayad cultivar and line D81083 exhibited the highest catalase contents under complete weed interference whereas these varieties demonstrated the lowest amount of catalase enzyme under weed-free conditions and at the V4 stage. Despite no significant differences in ascorbate peroxidase of different treatments, the activity of this enzyme showed a 67% increase under competition with weeds during the R7 stage as compared to the V4 stage. In both stages, the total chlorophyll content was higher under weed-controlled conditions compared to the full interference with weeds. Under weed interference, line D81083 had the highest amount of carotenoids. It can be generally concluded that adjacent weeds alter the proportion of photosynthetic pigments, and induce specific hormonal and defense responses. Weeds negatively affect photosynthesis and growth. Changes in gene expression negatively influence the growth of the plant. Under competition with weeds, the common bean induces the expression of genes involved in light quality signal transmission processes (low R/FR). Moreover, weeds continuously alter the growth responses and gene expression in the common bean plant. The gene expression of the common bean plant alters with the developmental stage; the antioxidant system of the common bean plant behaves differently under weed stress. Adjacent weeds increase the level of leaf ROS and affect ROS scavenger network components. The highest increase in gene expression and enzymatic activity was observed at the R7 stage (pod formation). Thus, it can be concluded that the weed interaction with the common bean plant reached its highest level as the R7 stage. This study showed that crop-weed interactions are much more complex than previously thought. It can be concluded response of common bean to competition with weed depends on its growth stage and genotype. The study of transcriptomes and gene expression responses in common bean plants under competition with weeds along with physiological and morphological studies provides an opportunity to deeply explore key issues in weed science.

The relationship of studied genes to genes involved in shade avoidance responses in Arabidopsis and other listed plants, provide evidence that these genes may be important in the response of common been to weeds. These results suggest that the studied genes can will be a target for manipulating weed tolerance in common been. Various researchers have suggested manipulating the shade avoidance response as a means to improve weed tolerance in crops (Smith, 1992; Horvath et al., 2015). Such experiments could provide much information to improve the competitive ability of crop genotypes. In addition, this information is needed to develop of crop–weed interactions to better understand and predict the consequences of weed competition and interference. Future examination should provide us with a better model to gain new insights into the mechanism underlying the regulation of antioxidative defense system in common bean in the Redroot Pigweed Interference responses.

Methods

Plant materials and experimental setup

A factorial experiment was conducted based on completely randomized design with two factors and five replications at laboratory of Tarbiat Modares University in 2019 the red common bean cultivars / line were obtained from the Agricultural and Natural Resources Research Center of Markazi Province - Khomein National Common Bean Research Station, Iran (Table 1) were set as first factor and weedy and weed free conditions as second factor levels.

Table 1
Common bean cultivars used in the experiment

Cultivars	Origin	Average height	Growth period	Growing habit
Sayad	Colombia	60 – 55 cm	90 days	Unlimited and semi erect growth (Type 2)
Derakhshan	Colombia	40 – 35 cm	95–100 days	Limited and erect growth (Type 1)
D 81083	Colombia	35 cm	80 days	Limited and erect growth (Type 1)

The pots with diameter of 25 cm were filled with a mixture of soil, sand, gravel, peat moss and perlite in a ratio of 1: 1: 1: 1: 3, respectively. The soil mixture was autoclaved before filling the pots. Redroot pigweed seeds were stored 6 months at room temperature to reduce their dormancy (Khan et al., 2022). A naturally occurring weed population of redroot pigweed that was collected from a red common bean field in 2018 and used as the weed competition source. Redroot pigweed seed were sown in tray before common bean planting to provide seedlings and kept in the greenhouse until the four true leaves were emerged (Carvalho and Christoffoleti, 2008). One common bean seed was planted in the center of each pot at a depth of 4 to 5 cm from the soil surface and 4 redroot pigweed seedlings were transplanted around it (Horvath et al., 2015). The plants were grown in the growth chamber under photoperiod of 16 hours' light and 8 hours' darkness at a temperature of 25 ± 1 ° C, close to the range of optimum temperature for germination and growth of *A. retroflexus* (Guo and Al-Khatib, 2003). Irrigation was done once every four days by adding a constant amount of water to each pot.

Plant Sampling

Sampling of common bean plants were done at V4 (third trifoliate leaf stage) as beginning weed interference and at R7 stage (pod formation) in all treatments. Two leaf samples were selected from the top three leaves in each pot and sealed in sterile aluminum foil and immediately frozen in liquid nitrogen and then transferred to a -80°C freezer for RNA extraction and study the physiological traits.

Gene expression analysis by qRT-PCR

Extracting the total RNA was performed by powdering 0.1 g (100 mg) of frozen common bean leaf tissue in liquid nitrogen, using Qiagen kit (RNeasy Plant Mini Kit), according to manufacturer's instructions (Qiagen, www.qiagen.com). After RNA extraction, the quantity (concentration) of RNA was measured using Epoch microplate spectrophotometer (BioTek Company, USA). The quality of RNA samples was determined by electrophoresis of the samples on 1% agarose gel in TAE buffer. Synthesis of the first strand of cDNA from total RNA was performed using protocol of the company Fermentas (www.thermofisher.com).

To analyze gene expression, ten genes of genes involved in crop and weed interference were selected (Table 2). Genes whose expression in common bean plants were examined in two V4 and R7 stages, were selected based on previous studies on the competition of different plant species with weeds (Horvath et al., 2015; Masclaux et al., 2012; Nohato et al., 2016; Geisler et al., 2012; McKenzie-Gopsill et al., 2016; de Wit et al., 2016; Kim et al., 2016).

Table 2
Primers characteristics used in the study

Full gene names	Primer name	Accession	Forward primer (F) (5'→3')	Reverse primer (F) (5'→3')	Product length (bp)	T_m (°C) Forward primer	T_m (°C) Reverse primer
Phytochrome B	*PHYB*	KF775132.1	CCTTTTCTGGTTCAGGTCGC	CCATTTCCGCATTCTCCCAT	167	56.3	55.5
chlorophyll a/b-binding protein	*CAB*	JX869947.1	ATGTTCGGGTTCTTCGTCCA	AACTCTCGAATCCACAAGTCATTC	162	56.4	55
opper/zinc superoxide dismutase	*SOD*	KF569535.1	GCTGTTGTTGTCCATGCTGA	CGCCCGTTTTCATGTGACTA	139	56	55.4
ascorbate peroxidase	*APX*	KF033563.1	CCTTCTTCGCTGATTACGCA	GGCAAACACACCCTCACATC	131	55.2	56.6
catalase1	*CAT1*	AF149283.1	GGCACATGGATGGTTTTGGT	GTGGCATGACTGTGGTTGAA	157	56.2	55.7
auxin-responsive protein IAA8	*IAA8*	KF033420.1	GGGATGTACCGTGGGAAATG	GATGAGACAAAAGGCAGTCCC	144	55.7	55.9
phytochrome interacting factor 3	*PIF3*	XM_007163190.1	GCAGAATCCAGTGTCCTTTCAT	ACCAAATTCTACAGTCGCCTCC	163	55.2	57
Long Hypocotyl in Far-Red Light	*HFR*	XM_007144565.1	CGAACGGGAAGAAGGTTAGC	ATACACATTAAGCCATTGTGATTGG	169	55.7	55.7
homeobox-leucine zipper protein HAT4	*HAT4*	KF569529.1	CCGCCACACGTCATCATC	TTCACACCCTTCCCTCCA	139	56.4	55.8
Phytochrome Rapidly Regulated	*PAR1*	-	CCTTGCAGTACCAGGTGAAAG	GCTGCTGCTTCCATAATCCATC	185	55.8	56.3
Actin11	*ACT11*	62703083	TGCATACGTTGGTGATGAGG	AGCCTTGGGGTTAAGAGGAG	190	55	56.4

In order to design the primers of the studied genes, their protected sequences (selection of the forward primer from the coding sequence (5'CDS) and the reverse primer from the 3'UTR (Untranslated Regions) of the gene from the NCBI site with the address http://www.ncbi.nlm.nih.gov was taken. Then, the primers related to these sequences were used using Primer3 software with the web address http://frodo.wi.mit.edu/primer3, Primer - Blast and Oligo 7 (Version: 7.54) were designed and the final evaluation and approval of the primers was performed using the online Oligoanalyzer software (https://www.idtdna.com/pages/tools/oligoanalyzer) and for primer synthesis was sent to the Metabion company in Germany. The *ACT11* reference gene was also used to correct and determine the relative expression of the target genes. This gene is usually involved in essential cell processes (Borges et al., 2011).

Quantitative analysis of gene expression was performed using a QIAGEN Rotor-Gene Q (5plex HRM Platform) real-time device. For quantitative evaluation of gene expression, CTs related to the main genes and internal control genes (five technical replicates and three biological replicates) obtained from real-time PCR, using Rest software and based on Levak equation (Eq. 1) (Livak and Schmittgen, 2001) were analyzed. GraphPad Prism software was also used to plot gene expression data.

$\Delta Ct = C_t^{Sample} - C_t^{control}$

Equation 1 $R = 2^{-[\Delta CtSample - \Delta CtControl]}$

Measurement of anthocyanin and photosynthetic pigments content

To measure the amount of chlorophylls a (Eq. 2) and b (Eq. 3), total chlorophyll (Eq. 4) (Porra, 2002), anthocyanin (Eq. 5)) Sims and Gamon, 2002) and carotenoid (Eq. 6) (Lichtenthaler and Wellburn, 1983), 200 mg of each selected green leaf were homogenized in 5 ml of 80% acetone. After centrifugation the samples at 3000 rpm and temperature of 4°C for 15 minutes, the supernatant was removed and the volume was increased to 10 ml with 80% acetone. Then, the absorption rate was measured at 663, 470, 646.6, 663, 647 and 537 wavelengths by UV-Visible spectrophotometer (Cary-50 model made by Varian company, Australia), and photosynthetic pigments were calculated using the following equations. A sample of 80% acetone was used as a control to adjust the device.

Chl.a ($\mu g.ml^{-1}$) = 12.25 (A 663.6) – 2.55 (A 646.6) Eq. 2

Chl.b ($\mu g.ml^{-1}$) = 20.31 (A 646.6) – 4.91 (A 663.6) Eq. 3

Chl.T (μg.ml^{-1}) = 17.76 (A 646.6) + 7.34 (A 663.6) = Chl.a + Chl.b Eq. 4 Anthocyanin (μmol ml^{-1}) = 0.08173 (A 537) – 0.00697 (A 647) – 0.002228 (A 663) Eq. 5 Carotenoid (μg.ml^{-1}) = [1000 (A 470) – 3.27 (Chl.a) – 104 (Chl.b)]/229 Eq. 6

Where, A is the rate of absorption of the extract solution at specified wavelengths. Chl.a, Chl.b and Chl.T are the concentrations of chlorophyll a, chlorophyll b and total chlorophyll, respectively. Carotenoids include carotene and xanthophyll.

Enzymatic activity analysis

For enzymatic extract, 200 mg of common bean leaf sample were prepared. The activity of catalase enzyme was measured at 25 ± 1°C using a spectrophotometer. Catalase activity was measured by calculating the reduction of H_2O_2 uptake at 240 nm. Enzyme activity was calculated in the form of the enzymatic unit according to the total protein (mg) obtained by Bradford (1976) method in 100 μl of enzyme extract per minute. A unit of enzymatic activity is considered as the amount of enzyme that breaks down 1 mmol of H_2O_2 in one minute (Sun et al., 2013).

One enzymatic unit of ascorbate peroxidase is the amount of enzyme that oxidizes one millimole of ascorbate per minute. Using absorption changes at 290 nm, ascorbate extinction coefficient (2.8 mMol^{-1}cm^{-1}) and formula A = εbc, the amount of ascorbate remaining was calculated after 2 minutes of enzymatic reaction. Over time, the rate of absorption has a decreasing trend. Thus an enzyme unit of ascorbate peroxidase is the amount of enzyme that oxidizes one millimole of ascorbate in one minute. Enzyme activity according to enzyme units was reported in the amount of total protein (mg) obtained by Bradford method (1976) in 100 μl of enzyme extract (Nakano and Asado, 1981).

Superoxide dismutase (SOD) activity was determined by measuring the photochemical reduction inhibition of nitroblue tetrazolium (NBT) at 560 nm (Giannopolitis and Ries, 1977). Sample adsorption divided by blank absorption shows the percentage of inhibition as SOD activity. One unit of SOD activity is defined as the amount of enzyme required to inhibit the photochemical reduction of NBT by nearly 50%. The amount of SOD activity was obtained as the unit of enzyme per gram of plant material.

Statistical analysis

The normality and homogeneity of variances in data were assessed using SPSS 22 software. Analysis of variance for the physiological data was performed by a factorial design based on a completely randomized design using SAS 9.4 software. The mean comparison s of the studied traits was performed using the least significant difference (LSD) test in SAS 9.4 software at the significant level of 5%.

Declarations

Acknowledgments

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Conflict of interest

The authors declare that they have no conflict of interest.

Authors' Contributions

Seyede Zahra Tabatabaiepour: Collection of experimental data and writing of manuscript. Zahra Tahmasebi: supervision of the study and writing of manuscript. Alireza Taab: Advisors of the study and review of the manuscript. Sajad Rashidi Monfared: Advisors of the study.

Data availability

All data analyzed during this study are included in this article (and its Supplementary Information files). The genetic resources are available (in the Agricultural and Natural Resources Research Center of Markazi Province - Khomeini National Common Bean Research Station, Iran)

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Figures

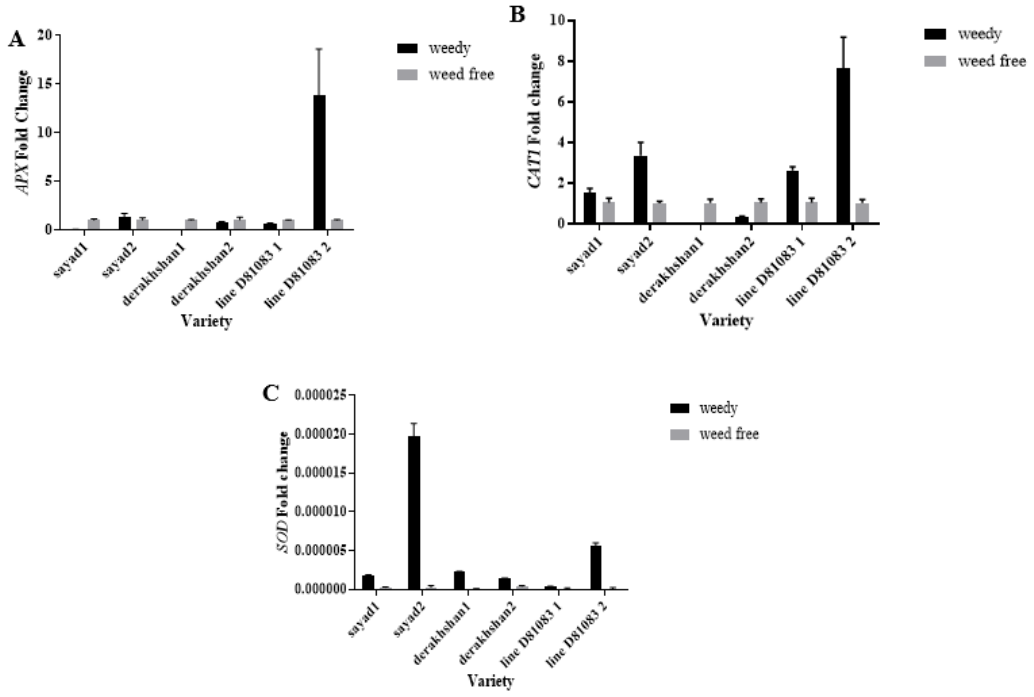

Figure 1

Real-time PCR (qRT-PCR) analysis of transcription levels of *APX* (A), *CAT1* (B) and *SOD* (C) genes in two treatments: weedy and weed free, in the three cultivars/lines studied in two stages of sampling V4 and R7 (data are the mean ± standard error in five biological replications and five technical replications, numbers 1 and 2 next to the name of the cultivar represent the V4 and R7 stages, respectively).

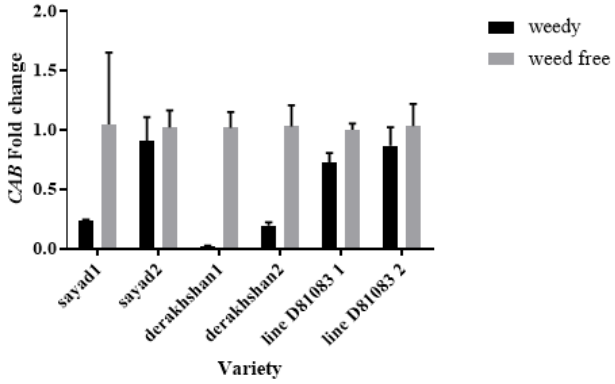

Figure 2

Real-time PCR (qRT-PCR) analysis of *CAB* gene transcript levels in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7 (data are the mean ± standard error in five biological replications and five technical replications, numbers 1 and 2 next to the name of the cultivar represent the V4 and R7 stages, respectively).

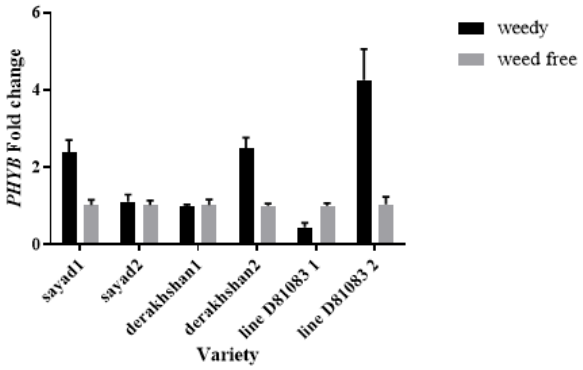

Figure 3

Real-time PCR (qRT-PCR) analysis of *PHYB* gene transcript levels in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7 (data are the mean ± standard error in five biological replications and five technical replications, numbers 1 and 2 next to the name of the cultivar represent the V4 and R7 stages, respectively).

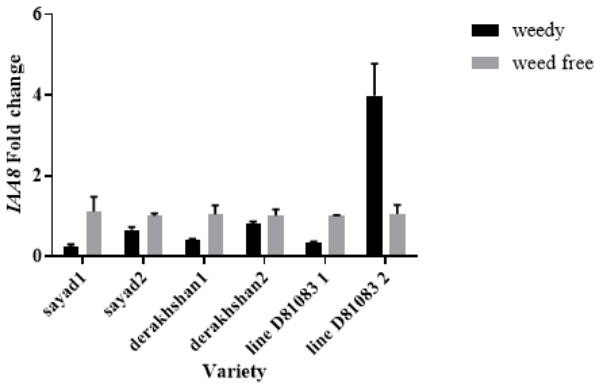

Figure 4

Real-time PCR (qRT-PCR) analysis of *IAA8* gene transcript levels in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7 (data are the mean ± standard error in five biological replications and five technical replications, numbers 1 and 2 next to the name of the cultivar represent the V4 and R7 stages, respectively).

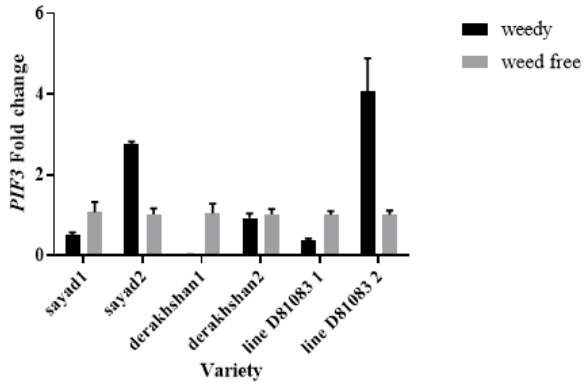

Figure 5

Real-time PCR (qRT-PCR) analysis of *PIF3* gene transcript levels in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7 (data are the mean ± standard error in five biological replications and five technical replications, numbers 1 and 2 next to the name of the cultivar represent the V4 and R7 stages, respectively).

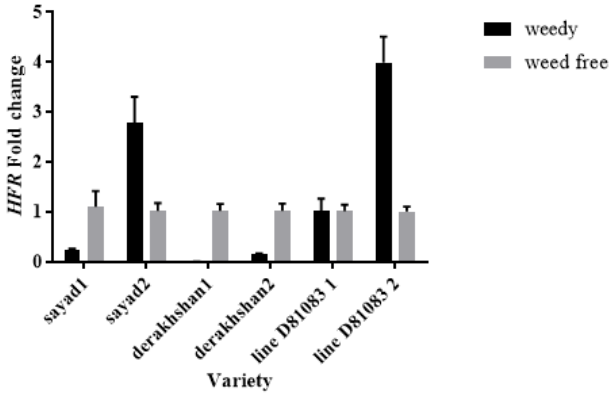

Figure 6

Real-time PCR (qRT-PCR) analysis of *HFR* gene transcript levels in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7 (data are the mean ± standard error in five biological replications and five technical replications, numbers 1 and 2 next to the name of the cultivar represent the V4 and R7 stages, respectively).

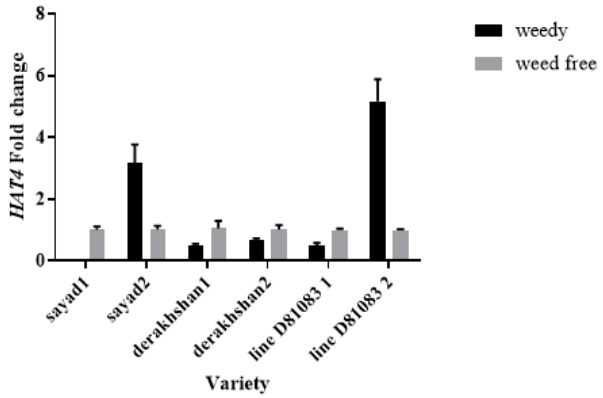

Figure 7

Real-time PCR (qRT-PCR) analysis of *HAT4* gene transcript levels in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7 (data are the mean ± standard error in five biological replications and five technical replications, numbers 1 and 2 next to the name of the cultivar represent the V4 and R7 stages, respectively).

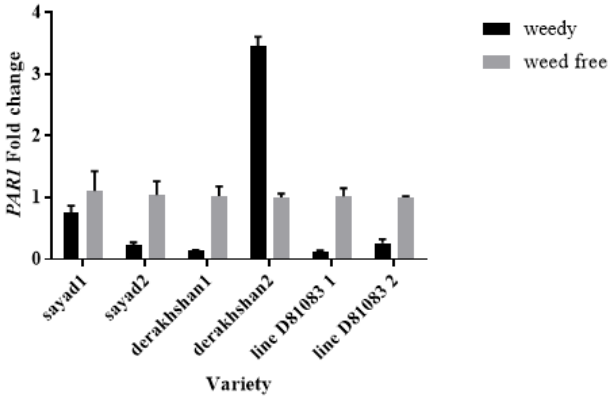

Figure 8

Real-time PCR (qRT-PCR) analysis of *PAR1* gene transcript levels in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7 (data are the mean ± standard error in five biological replications and five technical replications, numbers 1 and 2 next to the name of the cultivar represent the V4 and R7 stages, respectively).

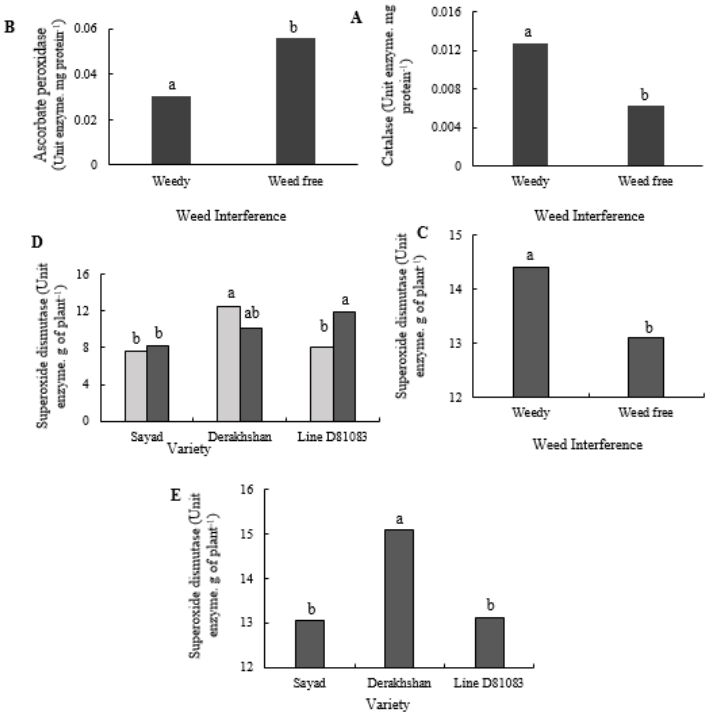

Figure 9

The activity of antioxidant enzymes in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7.

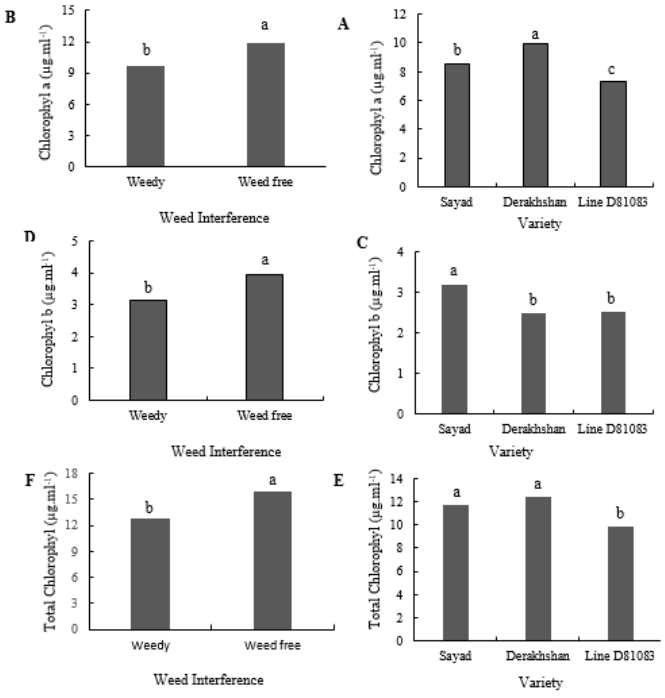

Figure 10

The chlorophyll-a, chlorophyll-b and total chlorophyll content in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7.

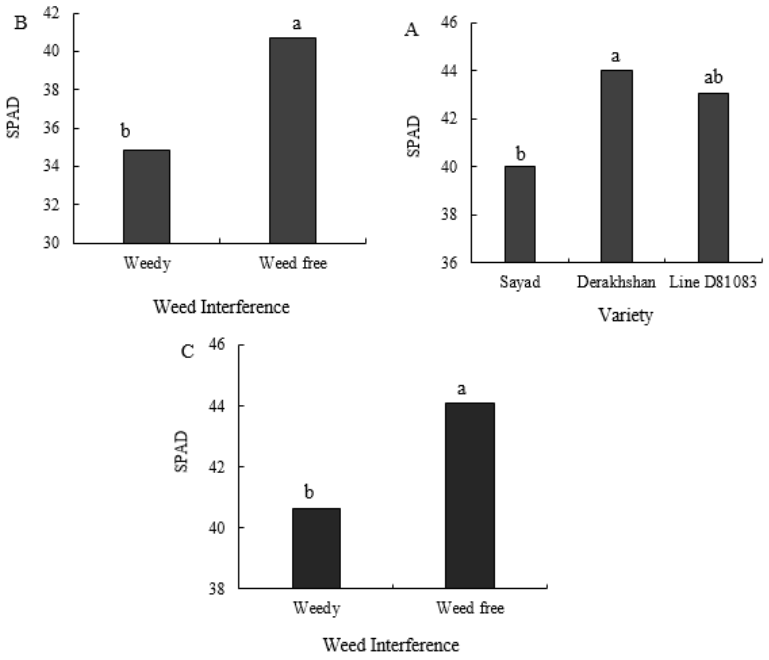

Figure 11

Relative chlorophyll content in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7.

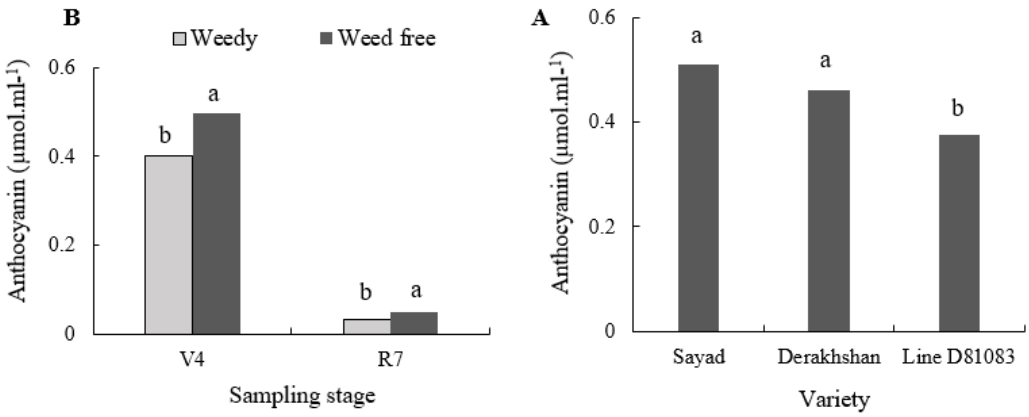

Figure 12

The anthocyanin content in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7.

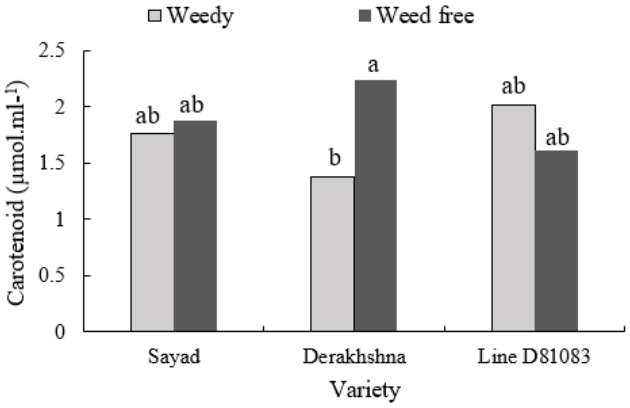

Figure 13

The carotenoid content in two treatments weedy and weed free, in the three cultivars/lines studied and in two stages of sampling V4 and R7.