Acta Physiol Plant (2011) 33:593–600
DOI 10.1007/s11738-010-0585-8

ORIGINAL PAPER

Molecular aspects in elevation of sunflower tolerance to drought by boron and calcium foliar sprays

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Received: 27 August 2009 / Revised: 19 July 2010 / Accepted: 18 August 2010 / Published online: 10 September 2010

Abstract Sunflower (*Helianthus annuus* L.) hybrid Hysun 333 (black seeded) was cultivated in a greenhouse and subjected during flowering stage to drought with or without the application of boron (B) and calcium (Ca) foliar sprays alone or in combination. The results revealed that drought induced a sharp decrease in seed fresh weight, seed protein and lipid contents. Application of B and Ca combined sprays overcame the drought effects on seed weight and seed lipid contents. Catalase expression was detected as a single band for all treatments where peroxidase isozymes were increased to seven, six of them were expressed when B sprays were applied with drought indicating that B has a major role in peroxidase up-regulation under drought conditions. SDS-PAGE analysis showed differential changes in protein profile with an appearance and/or disappearance of polypeptide protein bands, some of which were concluded to be drought-related proteins. The combined sprays of B and Ca seemed to overcome the effects of drought through minimizing band alterations (disappearance and/or appearance). The DD-RT PCR showed a variation in gene expression between the control and the other treatments. Sprays of B and Ca in combination seemed to be the most effective in band up-regulation and/or down-regulation that might play a possible role in improving tolerance of sunflower to overcome the drought deleterious effects.

Communicated by J.-H. Liu.

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Keywords Boron · Calcium · DD-RT PCR · Drought · *Helianthus annuus* · SDS-PAGE electrophoresis

Abbreviations

ANOVA	Analysis of variance
AOCS	American Oil Chemists Society
bp	Base pair
DAA	Days after anthesis
DD-RT PCR	Differential display reverse transcriptase polymerase chain reaction
kDa	Kilodalton
LSD	Least significant differences
RAPD	Random amplified polymorphic DNA
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

Introduction

Soil moisture is a main factor for sunflower infection by many pathogens as *Sclerotinia sclerotium* (Purdy 1979). So, it is recommended to withhold watering shortly after flowering until harvesting. On the other hand, water stress was more harmful when it was subjected during pre-anthesis and anthesis stages (Yegappan et al. 1982). Drought stress induces a range of physiological and biochemical responses in plants at both cellular and molecular levels. (Shinozaki et al. 2003; Bartels and Sunkar 2005). Nutrient foliar sprays specially B and Ca are most commonly used to correct these problems (Christensen 2005). Many roles for B in plants have been proposed (Blevins and Lukaszewski 1994). Ca functions as a regulator of plant cell metabolism and could also

participate in the regulating mechanism in plants adjusting to adverse conditions such as drought stress (Pietrobon et al. 1990; Bowler and Fluhr 2000). Moreover, Ca^{2+} induces increases in antioxidant enzyme activities in response to water stress (Shu and Fan 2000). The link between tolerance to oxidative stress and rise in antioxidants in plants has been established (Prince and Hendry 1991; Nemat Alla and Hassan 2006; Nemat Alla et al. 2008). Plants are well endowed with antioxidants and scavenging systems by catalase and peroxidase (Pyon et al. 2004; Nemat Alla et al. 2008). The numbers of available molecular markers includes isozymes, total protein, seed protein and RAPDs. They are used to define stress tolerance genes or their products which contribute positively to stress tolerance (Bayoumi et al. 2008). Changes in the protein profile are due to changes such as transcription rate, RNA stability, posttranscriptional control, and protein turnover (Smirhoff and Colombe 1989). This study was aimed at evaluating the molecular responses in sunflower seeds to the application of boron and/or calcium foliar sprays to ameliorate the adverse effects of drought.

Materials and methods

Plant material and growth conditions

Sunflower (*Helianthus annuus* L.) seeds (hybrid Hysun 333, black seeded) were soaked for 4 h and then germinated in a greenhouse in clay soil texture with pH of 7.3 under field conditions. The plots were furrow-irrigated for uniform emergence as necessary during the growing season. Plants were 30 cm apart in rows with 70 cm between rows. Nitrogen was applied once as urea, at 50 kg ha^{-1}, 20 days after emergence. Plots were weeded manually until the canopy closed. All plants were irrigated weekly from beginning of emergence until flowering stage. After all flowers had opened, plots were divided into five groups: one group was left as control and irrigated with water once every week until the end of the experiment (30 days after anthesis, 30 DAA). The other four groups were subjected to drought treatment (irrigated every 2 weeks until the end of the experiment). One of the drought groups was sprayed with B foliar spray (borax 1 g L^{-1}), the second was sprayed with Ca ($CaCl_2$ 1.2 g L^{-1}), the third was sprayed with B and Ca in combination and the fourth was left without any additive sprays. Foliar sprays were carried out on the 60th day after emergence. Each plant received an adequate amount of the spray so as to cover the whole plant once weekly for the following 4 weeks. At harvest (30 DAA), seeds were collected and the number per gram was recorded as well as the weight per seed.

Determination of lipid content

The seeds were oven-dried at 40°C for 4 h, using a ventilated oven, up to a moisture content of about 5%, then ground with a Warring blender. Four grams of the powder were extracted with petroleum ether for 16 h in a Soxhlet system according to AOCS (1993). The oil extract was evaporated in a rotary evaporator at 40°C and the weight of total lipids was recorded.

SDS and native-PAGE analysis of proteins, catalase and peroxidase

Naked seeds (about 0.5 g fresh weight) were homogenized in 2 ml of potassium phosphate buffer (0.1 M, pH 7.8) containing 1 mM PMSF, 2 mM dithiothreitol, 0.1 mM EDTA, 1.25 mM polyethylene glycol 4000 and 20% polyvinyl polypyrrolidone using a homogenizer for 1 min and centrifuged at 5,000*g*. The supernatant was transferred to a new Eppendorf cup and stored at −20°C for separation on denaturing and nondenaturating polyacrylamide gels (Laemmli 1970) using the BioRad Mini Protean 3 equipment. For the denaturating gel, the resolving gel contained 12% acrylamide and the stacking gel contained 5% acrylamide. Ten micrograms of protein were applied to each lane. After electrophoresis, gels were stained with Coomassie Brilliant Blue R-250 and destained with 20% methanol. For the native gel, the resolving gel contained 10% acrylamide and the stacking gel contained 4% acrylamide. Electrophoretic separation was performed at 4°C. Twenty micrograms of protein samples were loaded onto the gel.

To visualize the catalase profile, gels were stained according to the procedures of Anderson et al. (1995). Staining for peroxidase was achieved according to the method of Larsen and Benson (1970). Protein content was determined in the extract according to the method of Bradford (1976).

DD-RT PCR for seed RNA

Total RNA was isolated from the mature seeds as described by Jianwel et al. (2001). The total RNA (DNase treated) was reverse transcribed to cDNA. The first strand cDNA was used as a template for second strand synthesis. Reverse transcriptase polymerase chain reaction was performed in a total volume of 50 µl containing 2.5 µl RT Enhancer, 25 µl 1-Step PCR Reddy Mix (2X), 7 µl 10 pmol T_s primer, 3 µl RNA and 1 µl Verso Enzyme Mix. The volume was completed using water PCR grade. PCR amplification programmed for one cycle at 50°C for 15 min (for cDNA Synthesis), then 95°C for 2 min (for Verso inactivation). Then 40 cycles were performed as follows: 20 s at 95°C for

denaturation, 1 min at 30°C for annealing and 1 min at 72°C for elongation. The reaction mixture was then incubated at 72°C for 5 min for final extension. Three different random primers provided from (MuCSAT) were used in this investigation singly A1A13 (5′CAGGCCCTTCCAGC ACCCAC3′), EZ351 (5′AACTGGAGGAAGGTGGGG3′) and NS1 (5′GTAGTCATATGCTTGTCTC 3′). The PCR products were separated by electrophoresis on a 1.5% agarose gel and stained by ethidium bromide. Finally the gel was visualized using a UV transilluminator and photographed.

The experimental design was a randomized experiment or true experiment based on the random assignment. It was a simple factorial combination involving five treatments. All values were means of at least six replications (±SE) from two independent experiments. The full data were first subjected to analysis of variance (ANOVA) followed thereafter by least significant differences (LSD) test at 5% level (Snedecor and Cochran 1980).

Results and discussion

Drought significantly decreased seed fresh weight of sunflower even with the application of either B or Ca sprays as compared to control values (Fig. 1a). The magnitude of decrease was most pronounced with drought alone. Nevertheless, this decrease was overcome by the application of B and Ca combined sprays. The yield loss caused by drought stress could be attributed to an increased rate of flower and pod abortion. Also, disturbed nutrient uptake efficiency and photosynthate translocation within the plant could lead to yield reduction (Riaz and Chowdhry 2003). The application of B or Ca sprays to plants grown under drought conditions seemed to have slight effects. The combined sprays of B and Ca increased the seed fresh weight to a level nearly similar to that of control. These increases might be attributable to the necessity of these nutrients to plants. Hernandez-Munoz et al. (2006) concluded that these elements take part in several vital processes and affect many physiological disorders. Rajbir et al. (2007) reported that the concentration of Ca and B both in strawberry leaves and fruit increased with the application of Ca and B, attributing that these nutrients when applied through foliar means are readily available to plants and then translocated to different parts.

The contents of lipids in sunflower seeds were significantly reduced due to drought treatment as compared to control values (Fig. 1b). On the other hand, applications of B and Ca either separately or in combination overcame the drought-decrease lipids content. Similar results were obtained by Zubillaga et al. (2002) in sunflower. Oil yield of sunflower was increased under irrigation (Flagella et al. 2002). The highly positive effect of irrigation on seed yield confirms the key role of supplementary irrigation at critical growth stages, particularly those sensitive to water stress (Quaglietta and d'Andria 1994). In addition, mineral sprays have a remarkable effect on lipid content in many plants. Soybean yield benefits from B applied during reproductive stages (Freeborn et al. 2001). Other reports showed that crops grown in nutrient poor media or in conditions that limit Ca uptake produce lower yields than crops grown with continuous and adequate Ca nutrition (Smiciklas et al. 1989).

Drought resulted in a significant decrease in protein content as compared to control values (Fig. 2a). However, a recovery from the effects of drought was clearly pronounced after application of both B and Ca either alone or in combination. Moreover, B alone seemed to significantly increase protein content in seeds of drought-stressed plants

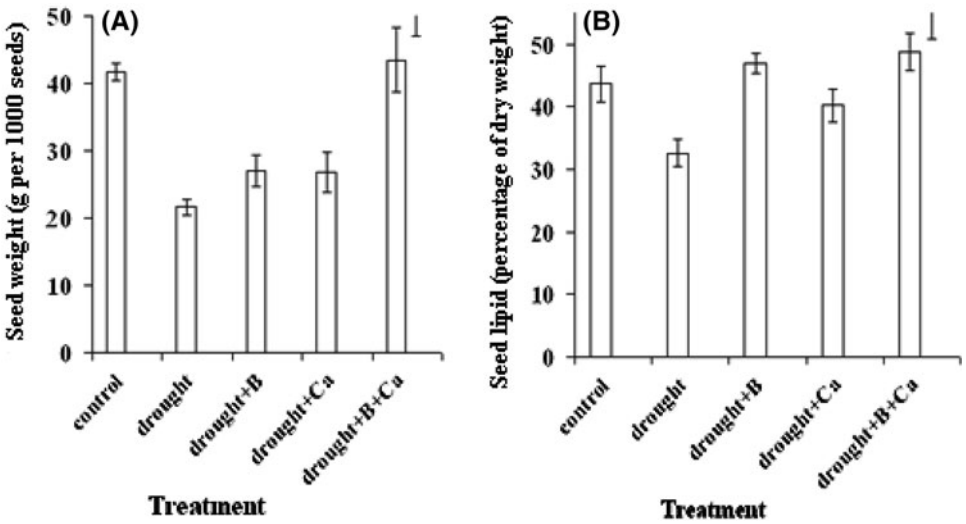

Fig. 1 Effect of drought either alone or in presence of B or Ca sprays separately or in combination on seed fresh weight (**a**) and seed lipid content (**b**) at harvest (30 days after anthesis). Data are means (±SE) of at least six replications from two independent experiments. *Vertical bar* represents LSD value at 5% level

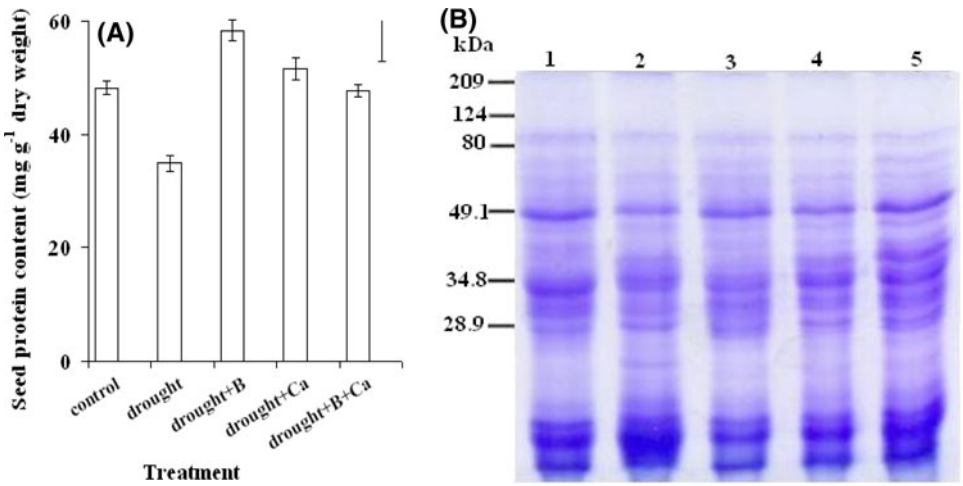

Fig. 2 Effect of drought either alone or in presence of B or Ca sprays separately or in combination on seed protein contents (**a**) and SDS-PAGE of seed proteins (**b**) at harvest (30 days after anthesis). Data are means (±SE) of at least six replications from two independent experiments. *Vertical bar* represents LSD value at 5% level. *1* control, *2* drought alone, *3* drought in presence of B sprays, *4* drought in presence of Ca sprays, *5* drought in presence of B and Ca combined sprays. Molecular masses of marker are shown to the *left*

relative to control level. Amutha et al. (2007) stated that proteins are altered in plants growing under water stress compared to plants growing under non-stress conditions. The increase of protein content due to mineral sprays appears to be due to increased synthesis of new proteins and/or inhibition of proteolytic activities. In accordance, Nayek et al. (1983) reported that Ca foliar sprays inhibited the decline of chlorophyll and protein contents in rice plants and inhibited the trend of rising protease and RNase activities that resulted in water stress.

Generally, protein content and profile analysis in sunflower seeds showed differential changes as a result of drought and minerals application treatments. The SDS-PAGE showed 22 bands of different molecular weights for proteins ranging from 9.1 to 95.4 kDa (Fig. 2b). The 76.5, 42.9 and 30.9 kDa bands disappeared from drought-treated plants whereas the 16.6 kDa protein band was expressed only under drought stress. The appearance and disappearance of these bands could suggest that they are drought-related proteins. Table 1 represents the detection of protein bands as a result of the different treatments. On the other hand, there was a disappearance of the three protein bands 59.9, 55.8 and 45.2 kDa in plants subjected to drought either alone or concomitantly sprayed with Ca alone or combined with B. These observations could suggest that drought could inhibit the expression of these protein bands. However, these bands were recovered with B sprays but not with Ca. On the contrary, drought either alone or in presence of Ca sprays with or without B showed expression of protein bands with molecular weights of 41.1 and 19.1 kDa. This induction might suggest that these proteins are drought-related proteins.

Table 1 Detection of seed protein polypeptide bands of sunflower at harvest (30 days after anthesis) due to drought treatments

kDa	1	2	3	4	5
76.5	+	−	+	+	+
59.9	+	−	+	−	−
55.8	+	−	+	−	−
45.2	+	−	+	−	−
42.9	+	−	+	+	+
41.1	−	+	−	+	+
37.3	−	+	+	+	+
30.9	+	−	+	+	+
28.9	+	+	−	+	+
24.5	+	−	+	+	+
19.1	−	+	−	+	+
16.6	−	+	−	−	−

1 control, *2* drought alone, *3* drought in presence of B sprays, *4* drought in presence of Ca sprays, *5* drought in presence of B and Ca combined sprays, + detected, − not detected

In addition, all treatments used in this study expressed the 37.3 kDa polypeptide band whilst drought either alone or combined with B sprays, respectively, led to loss of the 24.5 or 28.9 kDa bands. Generally, drought-induced metabolic changes related to protein turnover (alterations in protein synthesis, maintaining the level of some proteins or protein degradation) (Bray 1997). Han et al. (1997) detected a polypeptide of about 41 kDa in mature dry seeds of castor bean. Moreover, Riccardi et al. (2004) have demonstrated that plant response to water deficit shows some genetic variations. The efficacy of B and/or

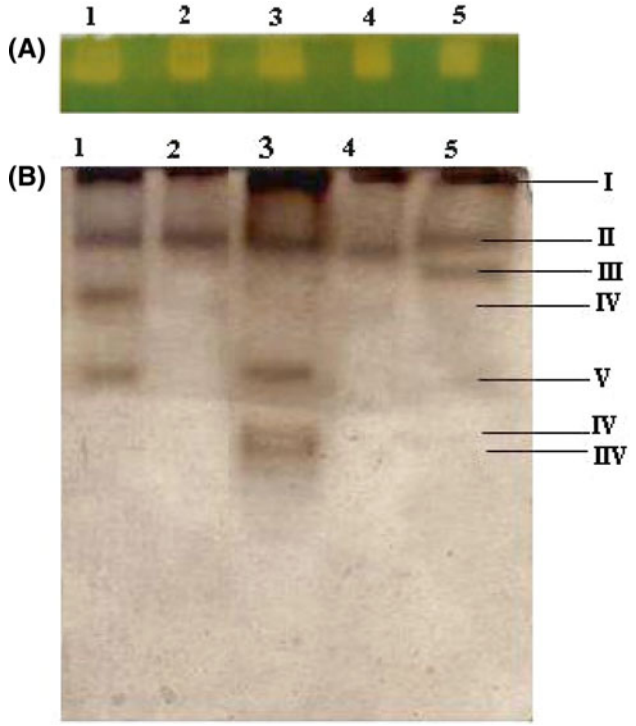

Fig. 3 Effect of drought either alone or in presence of B or Ca sprays separately or in combination on native-PAGE of seed catalase (**a**) and native-PAGE of seed peroxidase (**b**) at harvest (30 days after anthesis). *1* control, *2* drought alone, *3* drought in presence of B sprays, *4* drought in presence of Ca sprays, *5* drought in presence of B and Ca combined sprays

Ca in alleviation of the drought stress effects could be related to antioxidants such as catalase and peroxidase.

The catalase band was clearly recognized in all treated samples as well as in the control (Fig. 3a). It was reported that the catalase/peroxidase system might act cooperatively to remove H_2O_2 at a minimal expense of reducing power and at a maximal rate (Hilde et al. 1997). Catalase plays a key role in the mechanism of H_2O_2 scavenging in plant cells (Li et al. 2003). The effect of foliar sprays with B and/or Ca seemed to have no effect on catalase polypeptides. In accordance, Li et al. (2003) reported that catalase activity in liquorice cells was similar in both stress media containing and not containing Ca during the water stress acclimation period. In contrast, the induction of catalase under water stress is well documented and a positive relationship had been found for its up-regulation and stress tolerance (Ushimaru et al. 2001). Moussa and Abdel-Aziz (2008) reported that catalase activity increased under water stress conditions in both tolerant and susceptible genotypes of maize.

On the other hand, the peroxidase pattern showed up to a total of seven isozymal bands (Fig. 3b). Drought in the presence of B sprays exhibited the highest number of isozymes (6 bands). The number of these bands was only four both in the control and in response to drought either alone or in the presence of Ca sprays. The isozymal bands no. I and II showed peroxidase activity for all treatments including the control. Drought in presence of B and Ca combined sprays expressed band no. III but led to loss of expression of peroxidase band no. IV. The peroxidase isozymal band no. V disappeared upon drought treatment with or without the presence of Ca sprays. The bands no. VI and VII were only expressed by drought in the presence of B sprays. In this context, El-Sayed et al. (2002) reported that selected drought-tolerant tomato somaclones revealed one peroxidase band which was not present in their sensitive donor parents from a total of eight peroxidase bands. Peroxidase was reported to be enhanced under water stress; the activity was positively correlated with water stress tolerance (Hernandez et al. 2000; Ushimaru et al. 2001). Enhancement in peroxidase activity under various stress conditions has been linked with protection from oxidative damage, lignifications and cross-linking of the cell wall (Dalal and Khanna-Chopra 2001).

A total of 14 different sizes of PCR bands were recognized by using A1A13 primer ranging from 990 to 250 bp, three of which were detected and not varied among the different treatments and the control as well (Fig. 4a). Drought in the presence of B and Ca combined sprays down-regulated four bands (990, 850, 370 and 280 bp) but up-regulated two other bands (350 and 250 bp). Another two bands (690 and 600 bp) disappeared under drought in the presence of Ca sprays. A band of molecular weight of 730 bp was up-regulated by drought in presence of Ca sprays either alone or combined with B whilst the 650 and the 550 bp bands were up-regulated by drought in the presence of Ca sprays. Drought alone rather than the presence of the other additives had no effect on the down-regulation of the 480 bp band.

The differential display analysis using EZ351 primer reveals that the primer amplified 11 bands with size ranges from approximate 180 to 1,170 bp, three of which were detected and not varied among the different treatments and the control (Fig. 4b). The resulting data exhibited a down-regulation of four different bands with the molecular weights 1,170, 520, 450 and 320 bp by drought in presence of combined B and Ca sprays with a consequent up-regulation of the bands with the molecular weights 260 and 240 bp. On the other hand, drought in presence of B sprays up-regulated a band with approximate molecular weight 200 bp whereas drought in presence of Ca sprays resulted in down-regulation of the 350 bp band.

The NS1 primer showed a different pattern compared to A1A13 and EZ351 primers. It possessed 13 different sizes of regulated bands ranging from 160 to 1,190 bp, four of which were detected and not varied among the different treatments and the control (Fig. 4c). Table 2 represents the

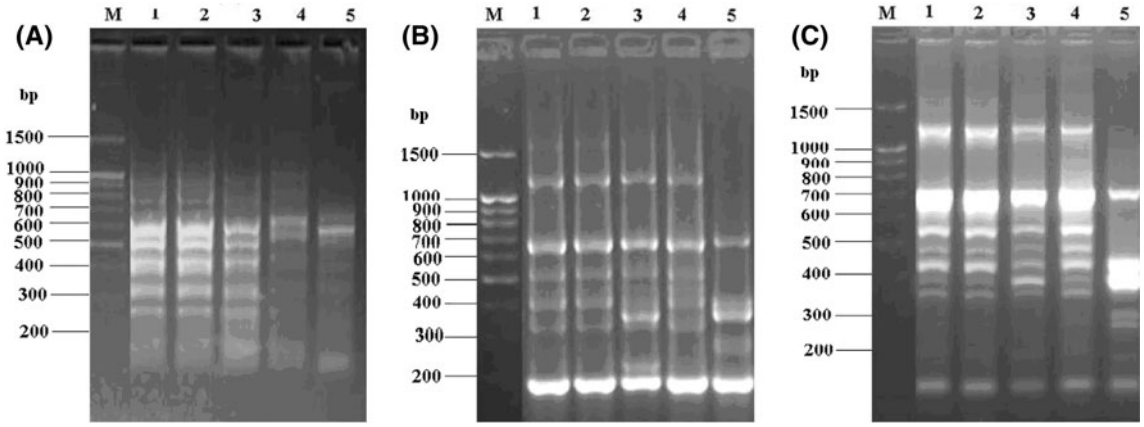

Fig. 4 Effect of drought either alone or in presence of B or Ca sprays separately or in combination on differential display of mature sunflower seeds using **a** A1A13 primer, **b** EZ351 primer and **c** NS1 primer. *1* control, *2* drought alone, *3* drought in presence of B sprays, *4* drought in presence of Ca sprays, *5* drought in presence of B and Ca combined sprays

Table 2 Up-regulation (+) and down-regulation (−) of seed protein PCR bands of sunflower at harvest (30 days after anthesis) due to drought treatments by using A1A13, EZ351 and NS1 primers

A1A13						EZ351						NS1					
bp	1	2	3	4	5	bp	1	2	3	4	5	bp	1	2	3	4	5
990	+	+	+	+	−	1,170	+	+	+	+	−	1,190	+	+	+	+	−
850	+	+	+	+	−	520	+	+	+	+	−	1,090	+	+	+	+	−
730	−	+	−	+	+	450	+	+	+	+	−	650	+	+	−	+	−
690	+	+	+	−	+	350	+	+	+	−	+	530	+	+	+	+	−
650	−	−	−	+	−	320	+	+	+	+	−	470	+	+	+	+	−
600	+	+	+	−	+	260	−	−	−	−	+	340	+	+	+	+	−
550	−	−	−	+	−	240	−	−	−	−	+	280	−	−	−	−	+
480	+	+	−	−	−	200	−	−	+	−	−	260	−	−	−	−	+
370	+	+	+	+	−							190	−	−	−	−	+
350	−	−	−	−	+												
250	−	−	−	−	+												

1 control, *2* drought alone, *3* drought in presence of B sprays, *4* drought in presence of Ca sprays, *5* drought in presence of B and Ca combined sprays

detection of up- and down-regulation of PCR bands recognized by using the different primers. Generally, the response of drought in presence of combined B and Ca sprays appeared to be completely different from control and other treatments. Five bands (1,190, 1,090, 530, 470 and 340 bp) were down-regulated, while other three bands (280, 260 and 190 bp) were up-regulated. The 650 bp band was down-regulated by drought in presence of B sprays as well as combined B and Ca sprays.

Jagadeesh et al. (2009) found that the most drought-tolerant line of *Gossypium hirsutum* L. cv. KC3 showed up-regulation of the 670 bp band which was not present in the less tolerant line MCU12 using a random primer. Moreover, when they extracted and sequenced this band, they concluded that there was a significant similarity with some drought-related genes like class III peroxidase. Generally, the mRNA differential analysis at seed maturity showed a variation in gene expression between the control and the other drought-treated samples especially in presence of combined B and Ca sprays. Also, drought-tolerant tomato somaclones revealed DNA fragments with different sizes which were not present in their sensitive donor parents (El-Sayed et al. 2002). Chen et al. (2002) suggested that transcription factors play an important role in signal transduction and gene expression under environmental stress.

Conclusion

Sunflower could be adapted to an array of soil types and climatic conditions. Overhead irrigation and lake of water are very harmful to sunflower plants especially during the

seed filling period as they cause infection by pathogens or severe loss in yield. So, withholding water during this period and supplying foliar spray mixtures of B and Ca to plants could overcome this problem through variations in gene expression. This would improve the performance of sunflower during this period and avoid pathogen infections. These variations in gene expression seemed to be stated from up-regulation and/or down-regulation of protein bands that probably related to antioxidant enzymes as well as polypeptides and base sequences that might serve in drought tolerance and resistance. The present results point to the conclusion that B and Ca combined sprays seemed to overcome the deleterious effects of drought by overcoming the drought-related molecular responses and consequently appeared to meliorate sunflower growth under insufficient water conditions.

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