Physiol Mol Biol Plants (April–June 2013) 19(2):189–198
DOI 10.1007/s12298-013-0165-7

RESEARCH ARTICLE

The role of calcium in improving photosynthesis and related physiological and biochemical attributes of spring wheat subjected to simulated acid rain

Aria Dolatabadian · Seyed Ali Mohammad Modarres Sanavy · Majid Gholamhoseini · Aydin Khodaei Joghan · Mohammad Majdi · Arman Beyraghdar Kashkooli

Published online: 22 February 2013

Abstract The response of photosynthesis parameters, catalase, superoxide dismutase and peroxidase activity, malondialdehyde, proline, chlorophyll, yield and yield components to foliar application of calcium and simulated acid rain in wheat were investigated. Foliar treatment of calcium led to significant increases in the photosynthesis rate, transpiration rate, stomatal conductance, proline, chlorophyll, yield and yield components in plants subjected to acid rain. Antioxidant enzyme activity and lipid peroxidation in the wheat leaves decreased because of calcium foliar application. Calcium hindered degradation of the rubisco subunits under acid rain treatment compared with water-treated plants. Results suggest that acid rain induces the production of free radicals resulting in lipid peroxidation of the cell membrane so that significant increase in antioxidant enzyme activity was observed. In addition, photosynthetic parameters i.e. photosynthesis rate, transpiration rate and stomatal conductance were drastically suppressed by acid rain. The cellular damage caused by free radicals might be reduced or prevented by a protective metabolism including antioxidative enzymes and calcium. We report that foliar application of calcium before acid rain may ameliorate the adverse effects of acid rain in wheat plants.

A. Dolatabadian · S. A. M. M. Sanavy (✉) · M. Gholamhoseini · A. K. Joghan
Agronomy Department, Faculty of Agriculture, Tarbiat Modares University, Jallal-Al-Ahmad Highway, Nasr Bridge, Tehran, Iran Zip code: 1411713116, P.O. Box: 14115-336
e-mail: modaresa@modares.ac.ir

M. Majdi
Agronomy and Plant Breeding Department, Faculty of Agriculture, University of Kurdistan, Sanandaj, Iran

A. B. Kashkooli
Horticulture Department, Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran

Keywords Acid rain · Antioxidant enzymes · Calcium · Protein pattern · Wheat

Introduction

Acid rain is an important abiotic stress factor that can limit the growth and yield of the crops. Acid rain has come in light after industrial revolutions as one of the main types of pollution causing a major problem for the countries in Europe, Asia and North America (Bouwman et al. 2002). Although, it was known for long time probably from 1730's, but it was discovered in the 1950's and started being noticed in the 1960's. Generally, sulfur dioxide and nitrogen oxides are gases released when fossil fuels are burned. When constituents of these gases react with water in the atmosphere, sulfuric acid and nitric acids can form and fall as rain, commonly referred to as acid rain. There are many problems and effects caused by acid rain in nature. Acid rain harms lakes and rivers and disrupts ecosystems and kills wildlife in affected ecosystems. Acid rain also damages plants directly and indirectly. Plant roots are damaged under low pH soils and acidity makes plants unable to draw in enough nutrients to support the plant. Acid rain turn leaves to brownish-yellow in color and interrupts photosynthesis. Previous researches showed that acid rain can also reduce crop yields both by direct injuring and by minerals leaching from the soil (Environmental Protection Agency 1980). As a first effect of exposure to acid rain, immediate cuticle destroying and impairment in stomata functions has been reported (Barnes and Brown 1990; Hogan 1992). In addition, increase in proton level in the stroma due to acid rain (acids act as proton donor), suppresses the enzymic reactions in Calvin's cycle (Woodrow et al. 1984). It has been reported that increased acidity in chloroplasts leads to the denaturing of proteins and enzymes

involved in photosystem II (Siefermann-Harms 1992). Moreover, Munzuroglu and co-researchers (2003) have shown that acid rain has serious effects on vegetative and reproductive parts of plants. Also, direct contact of acid rain with leaves causes changes in plant anatomy (Stoyanova and Velikova 1997/1998), structure, physiology and biochemistry (Gabara et al. 2003). In general, the biological effects of acidification in plants are divided to two visible (chlorosis and/or necrosis) and invisible symptoms (reduced photosynthesis, nutrient loss, altered water balance, and variation of several enzyme activities) by Evans (1982). It has been reported that transpiration and photosynthesis were inhibited by acid fog (pH 2.5 or 3.2) (Temple et al. 1987), but there is scarce data available on alterations of antioxidant enzyme when plants are subjected to acid rain. Therefore, we have investigated the effects of acid rain on antioxidant enzymes. Since calcium ion chemically reacts with acids so we hypothesized that if calcium concentration in apoplastic and symplastic spaces would increase, it may increase plant resistance to acidity due to acid rain. Moreover, there are many reports stating that calcium play many different roles in plants, for example stromal calcium may regulate several enzyme activities, while intra thylakoid calcium may constitutively promote photosystem II (Brand and Becker 1984). In addition, calcium is an important element in plants as a second messenger and it can both delay and promote leaf senescence (Bowler and Chua 1994). For instance, calcium has been shown to be involved in a light-mediated delay of senescence in detached corn leaves (Huang and Kao 1992). Calcium has a well-established role in strengthening the cell wall (Poovaiah et al. 1988). It is well known that insufficient calcium levels, however, lead to deterioration of the cell membrane; the cells become leaky, resulting in the loss of cell compounds and eventually death of the cell and plant tissue (Marschner 1995). Calcium is also believed to have an influence on the development of heat shock proteins that help the plant tolerate the stress of prolonged heat (Chang et al. 2006).

In light of these critical roles for calcium and because information regarding the effect of foliar application of calcium on increasing resistance to acid rain is not available, this experiment was conducted. In this study, we focused on the influence of foliar application of calcium and simulated acid rain on photosynthetic parameters, antioxidant enzyme activity and yield in spring wheat. The purpose of this research is to investigate if foliar application of calcium can reduce the adverse effects in plants induced due to acid rain.

Materials and methods

Site of study

The experiment was performed at the research farm of the Faculty of Agriculture, Tarbiat Modares University, Tehran, Iran (35°41′ N latitude, 51°19′ E longitude and an altitude of 1,215 m) during the 2009 growing seasons. The yearly average precipitation (30-year long-term period) was estimated 298 mm. The mean annual temperature was 18.8 °C. The meteorological data recorded during the trial period are provided in Fig. 1.

Soil sampling and analysis

Before planting, a composite soil sample was collected at a depth of 0–30 cm to determine its physical and chemical properties. The sample was dried at room temperature and then crushed, and analyzed for pH, electrical conductivity, saturation percentage, organic carbon through sulfuric acid using the Walkley and Black method (Walkley and Black 1934), total N by the Kjeldahl method (Houba et al. 1989), available P by the Olsen procedure (Olsen et al. 1954) and available K after extraction with ammonium acetate was determined according to Schollemberger and Simon (1954). The research field had a soil with a texture of sandy loam. Details of the soil properties are shown in Table 1.

Field and plot preparation

After plowing and disking in the early spring, the plots were prepared. The experimental design was a completely randomized block arrangement in factorial with ten treatments (water, H_2SO_4 pH 3, H_2SO_4 pH 2, HNO_3 pH 3, HNO_3 pH 2, water+1 % Ca^{+2}, H_2SO_4 pH 3+1 % Ca^{+2}, H_2SO_4 pH 2+1 % Ca^{+2}, HNO_3 pH 3+1 % Ca^{+2} and HNO_3 pH 2+1 % Ca^{+2}) in three replications. The experimental plots were 5 m×3 m, with fifteen sowing rows. Between all plots, a 2-m alley was kept to prevent the acid or calcium from dropping on different treatments. According to the results of the soil analysis, no P and K fertilizer was needed, but prior to seed sowing; 50 kg ha^{-1} N from urea was spread and incorporated into the soil. No organic manure was applied.

Seed sowing and agronomic management

Seeds of spring wheat (*Triticum aestivum* L. cv. Verinak) were obtained from the Seed and Plant Improvement

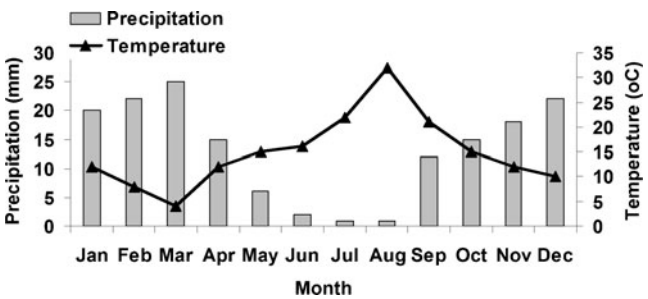

Fig. 1 Monthly precipitation and air temperature during the period January–December for 2009

Table 1 Physical and chemical soil properties

Physical	Depth	Sand	Silt	Clay	Texture	F.C	C.E.W	A.W	B_d	
	0–30 cm	69 %	20 %	11 %	Sandy loam	21 %	9 %	12 %	1.4 g cm^{-2}	
Chemical	Depth	CEC	pH	O.M	N	P	K	Fe	Cu	Zn
	0–30 cm	4.6 meq/100g	7.7	1.06 %	0.07 %	>12 ppm	>350 ppm	7.6 mgkg^{-1}	0.7 mgkg^{-1}	1 mgkg^{-1}

F.C Field Capacity; *C.E.W* Crop Extractable Water; *A.W* Available Water; B_d Bulk Density; *CEC* Cation Exchange Capacity; *O.M* Organic Matter; *N* Total Nitrogen; *P* Absorbable Phosphorous; *K* Absorbable Potassium

Institute (SPII), Karaj, Iran. The seeds were disinfected by fungicide (Vitavax) prior to sowing. The wheat seeds were hand-planted during the last 10 days of May at a rate of 120 kgha^{-1}, and then, irrigation was performed immediately. Weeds were effectively controlled during the growing season. The plots were top dressed by 25 kgha^{-1} rest nitrogen fertilizer from urea at the jointing stage.

Calcium and acid rain treatments

Treatments were induced when the plants reached the booting stage. At first, Ca^{2+} was applied in the form of $Ca(NO_3)_2 : 4H_2O$ containing 18.3 % Ca^{2+}. $Ca(NO_3)_2 : 4H_2O$ was selected as the calcium source because it is the most widely used form for foliar applications. In the calcium treatment plots, the entire shoot of the plants were foliar fertilized with 1 % $Ca(NO_3)_2 : 4H_2O$ solution using a calibrated pressurized backpack sprayer (20-l capacity).

Artificial acid rain was made by mixing sulfuric acid or nitric acid into double-distilled water for a range of solutions with pH values of 3 and 2. Three days after foliar application of the calcium, acid rain was simulated using the backpack sprayer. The droplet size of the artificial rain averaged 1.5 mm, and the intensity of the rain was approximately 10 mmh^{-1}. The vegetative and generative parts of the plants were subjected to different treatments of acid rain. One liter of acid rain was sprayed onto the plants two times successively at 20 min intervals using 0.5 l each time. For all control treatments water, instead of the Ca solution or pH solutions, was sprayed onto the control plants.

Collection of data and leaf sampling

After 24 h, when the acid rain symptoms appeared, the net photosynthesis rate [Pn, μmol$m^{-2}s^{-1}$], transpiration rate [E, mmol$m^{-2}s^{-1}$] and stomatal conductance [mol$m^{-2}s^{-1}$] were measured on fully expanded leaves. Pn, E and gs were measured using a portable system (Ciras-1, PP system, Hitchin, Herts SG5 1 RT, United Kingdom). Leaves were sampled from each plot, frozen in liquid nitrogen and stored at −80 °C until biochemical analysis.

Harvesting

At the end of the growing season on the last 5 days of September, 1 m^2 of plants were harvested and the seed number per ear, 1,000-seed weight and final yield were estimated.

Extract preparation

Frozen leaves (0.2 g) were grounded in a ceramic mortar and pestle with 3 ml ice-cold sodium phosphate buffer (25 mM, pH 7.8). The extraction was centrifuged at 18,000 g for 30 min at 4 °C, and then the supernatant was passed through filter paper. The supernatant was collected in micro tubes and stored in −24 °C freezer to future analysis. All the procedures were performed at 4 °C.

Determination of the activities of antioxidant enzymes in crude extracts

The catalase activity was estimated using the method of Cakmak and Horst (1991). 100 μl crude extract, 500 μl 10 mM H_2O_2 and 1,400 μl 25 mM sodium phosphate buffer were mixed as reaction mixture in a 3 ml quartz cell. The decrease in the absorbance was monitored at 240 nm for 1 min using a spectrophotometer. The catalase activity was calculated as ΔAbs mg protein min^{-1}.

According to the method of Giannopolitis and Ries (1977), superoxide dismutase activity was determined by measuring the ability of the enzyme extract to inhibit the photochemical reduction of nitroblue tetrazolium (NBT). The reaction mixture contained 100 μl 1 μM riboflavin, 100 μl 12 mML-methionine, 100 μl 0.1 mM EDTA (pH 7.8), 100 μl 50 mM Na_2CO_3 (pH 10.2), 100 μl 75 μM NBT in 2,300 μl 25 mM sodium phosphate buffer (pH 6.8), and 200 μl crude enzyme extract, in a final volume of 3 ml. Reaction mixtures were illuminated with a fluorescent lamp (180 W). Simultaneously, one tube free of enzyme was considered as blank. After illumination for 15 min, the absorbance was measured at 560 nm in all samples. One unit of superoxide dismutase activity was defined as the amount of enzyme that caused 50 % inhibition of the photochemical reduction of NBT. The superoxide dismutase activity of the extract was expressed as ΔAbs mg protein min^{-1}.

The peroxidase activity was estimated according to the method of Ghanati et al. (2002). The peroxidase activity was

determined by measuring of guaiacol oxidation in the presence of H_2O_2. The increase in absorbance due to oxidation was recorded at 470 nm for 1 min. The reaction mixture for this enzyme consists 100 µl crude extract, 500 µl 5 mM H_2O_2, 500 µl 28 mM guaiacol and 1,900 µl 60 mM potassium phosphate buffer (pH 6.1). The peroxidase activity of the extract was expressed as ΔAbs mg protein min^{-1}.

Protein extraction

The total soluble proteins were extracted from the wheat leaves using the method described by Guy et al. (1992). The leaves were fine powdered using liquid nitrogen, and homogenization was done with a chilled mortar and pestle using a buffer containing ice-cold 50 mM Tris-HCl, pH 7.5, 2 mM EDTA and 0.04 % (v/v) 2-mercaptoethanol. The homogenate was centrifuged at 12,000 g for 20 min at 4 °C, and the supernatant was re-centrifuged for 20 min at 4 °C and stored at −20 °C for electrophoresis analysis (Guy et al. 1992).

Quantification of protein by Bradford assay method

The protein concentration was determined based on the method described by Bradford (Bradford 1976), using Bio-Rad protein assay dye reagent. Test tubes containing 100 µl aliquots of bovine serum albumin (BSA; 0.156 $mg l^{-1}$ to 10 mg l^{-1} in 0.15 M NaCl) were prepared. Blank tubes containing 100 µl of 0.15 M NaCl were also prepared. Then, 1 ml of Coomassie brilliant blue solution was added to each tube. The reactions were mixed and left at room temperature for 2 min. The absorbance at a wavelength of 595 nm was determined against the blank, and the standard curve of absorbance versus protein concentration was plotted. Reactions containing dilutions of the soluble protein extracts (unknown concentrations) were set up using the same methods as above, and the absorbance at 595 nm was determined. The protein concentration of the extracts was determined from the standard curve, using a Unicam 8620 UV/VIS (USA) Spectrophotometer. According to the Bradford assay, an equal amount of protein (10 µg) was used for electrophoresis analysis.

Protein electrophoresis

The homogenate was mixed with a buffer containing 62.5 mM Tris-HCl (pH 6.8), 2 % SDS, 5 % (v/v) 2-mercaptoethanol, 10 % glycerol and 0.01 % bromophenol blue and boiled at 100 °C for 5 min and centrifuged at 4,000 g for 30 min. The supernatant was used for electrophoresis analysis. The protein extracts were thawed and separated as polypeptides, using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10 % gels (Laemmli 1970); 10 µg of soluble total protein was loaded into each well. The gels were fixed in trichloroacetic acid and stained in 0.1 % (w/v) Coomassie brilliant blue G-250, 10 % (v/v) glacial acetic acid and 40 % (v/v) methanol. The gels were placed in freshly prepared destaining solution (40 % methanol, 10 % glacial acetic acid) with gentle shaking for 30 min. This process was repeated until the gels were sufficiently destained. The gels were then photographed. The molecular weights of the proteins were estimated using standard proteins from the Fermentas PageBlue™ Protein Staining Solution (#R0571).

Determination of malondialdehyde in crude extract

The level of membrane damage was determined by measuring the amount of malondialdehyde, which is the end product of lipid peroxidation, according to the method of De Vos et al. (1991). In brief, the samples were homogenized in 10 % trichloroacetic acid (w/v), and aliquots of the filtrates were heated (100 °C for 30 min) in 0.25 % thiobarbituric acid. The amount of malondialdehyde in the samples was determined from the absorbance at 532 nm, followed by correction for non-specific absorbance at 600 nm using spectrophotometry. The concentration of malondialdehyde was determined by extinction coefficient MDA [$\varepsilon = 155\ \mu M cm^{-1}$].

Proline content determination in crude extract

The proline content of the leaves was determined according to a modification of the method of Bates et al. (1973). Samples of the leaves (0.2 g) were homogenized in a mortar and pestle with 3 ml sulfosalicylic acid (3 %w/v) and then centrifuged at 18,000 g for 15 min. Then, 2 ml of the supernatant was then added to a test tube, to which 2 ml glacial acetic acid and 2 ml freshly prepared acid ninhydrin solution (1.25 g ninhydrin dissolved in 30 ml glacial acetic acid and 20 ml 6 M orthophosphoric acid) were added. The test tubes were incubated in a water bath for 1 h at 100 °C and then allowed to cool to room temperature, and 4 ml of toluene was then added to the tubes and mixed using a vortex mixer for 20 s. The test tubes were allowed to stand for at least 10 min to allow separation of the toluene and aqueous phases. The toluene phase was carefully separated by pipette into a glass test tube and its absorbance was measured at 520 nm in a spectrophotometer. Proline content was calculated from a standard curve and was expressed as $mmol g^{-1}$ fresh weight.

Determination of chlorophyll content in leaf extract

The chlorophyll from the leaf samples was extracted in 80 % acetone according to the method of Arnon (1949). The extracts were filtrated, and the content of total chlorophyll was determined by spectrophotometry at 645 and 663 nm. The content of the total chlorophyll was calculated by

Arnon equation (Eq. 1) and then corrected by Porra equation (Eq. 2) (Porra 2002).

$$\text{Total chlorophyll} = [20.2(D645) + 8.02(D663)] \times V/1000\,W \quad (1)$$

$$[\text{Chl a} + \text{b}]^{T} = 0.895[\text{Chl a} + \text{b}]^{A} \quad (2)$$

Statistical analysis

Each treatment was replicated three times in a completely randomized block design arrangement in factorial design. Data were analyzed with ANOVA following the GLM procedures in SAS. Differences among the means of treatments were compared by Duncan's Multiple Range Test at the 0.05 confidence level.

Results and discussion

The analysis of variance test showed that treatments of different dosage of acid rain and calcium foliar application were significantly affected in all the studied traits, while the calcium foliar application × acid rain interaction was noticeably significant only for the transpiration rate, photosynthetic rate, antioxidant enzyme activity, malondialdehyde, chlorophyll, 1,000-seed weight and number of seeds per ear (Table 2). Statistical comparison of the means showed that three critical photosynthetic parameters (transpiration rate, photosynthetic rate and stomatal conductance) were significantly affected by main effects of foliar application of calcium and simulated acid rain. The results are shown in Table 3. Figures 2 and 3 show the changes in the stomatal conductance after calcium foliar application and simulation of acid rain, respectively. In brief, the transpiration rate dramatically decreased in the plants that were treated with acid rain compared to the plants treated just with water. Interestingly, the transpiration rate improved in the plants pre-treated with calcium (Table 3). The highest stomatal conductance was observed in the treated plants by calcium (Fig. 2). Similar results were found for the photosynthetic rate (Table 3). We observed that acid rain had the negative effect on the photosynthetic rate, while calcium had an additive effect on the photosynthetic rate (Table 3). The ameliorative effects of calcium in the chlorophyll fluorescence parameters were reported by Misra et al. (2001) in mung beans, although they used calcium in a nutrient solution, not as a foliar application. Artificial acid rain caused a progressive and concomitant decrease in stomatal conductance in wheat leaves. In plants treated with artificial acid rain, HNO_3 at pH 2, H_2SO_4 at pH 3, H_2SO_4 at pH 2 and HNO_3 at pH 3, the stomatal conductance drastically decreased by 25 %, 30.5 %, 38.8 % and 50 %, respectively, compared with the control (Fig. 3). Foliar application of calcium appeared to have increased the stomatal conductance in plants by 42.8 % compared with the control (Fig. 2).

Acid rain can affect plants through contact with the cuticle; thus, the decrease in photosynthetic parameters in acid rain treated plants might be explained by damage to the guard cell membrane and the destruction of chloroplasts.

Direct contacts of acid rain with leaves are reported to injure assimilative organs (Hogan 1992) and to alter the cuticular surface (Barnes and Brown 1990) that may affect the function of the stomata. Consecutively, variations in cation leaching from foliage (Hogan 1992), photosynthesis, water relations (Barnes and Brown 1990), and carbon metabolism (Hampp 1992) can also be found. The results show that foliar applications of calcium overcome the inhibitory effect of acid rain stress on photosynthetic parameters. Calcium adjusts the activity of the phosphatase enzymes in the carbon reduction cycle and also regulates chloroplast NAD + kinase activity through a calmodulin-like protein (Brand and Becker 1984). Stromal calcium may regulate several enzyme activities, while

Table 2 Analysis of variance of the various measured parameters that were affected by simulated acid rain and foliar application of calcium in presence and in absence of acid rain

Sources of variation	d.f	TR	SC	PR	CAT	SOD	POX	MDA	PRO	CHL	SW	SN	Y
Replication	2	ns	ns	ns	ns	ns	ns	**	ns	ns	ns	ns	ns
Acid	4	**	**	**	**	**	**	**	**	**	**	**	**
Calcium	1	**	**	**	**	**	**	**	**	**	**	**	**
Acid × Calcium	4	**	ns	**	**	**	**	**	ns	**	**	**	ns
Error	18	0.03	0.00	0.29	12.06	50.98	23.50	0.00	0.00	0.00	2.34	2.77	2266.38
Coefficient variation (%)		10.58	18.44	4.65	1.94	0.70	1.83	0.40	11.40	3.57	5.44	7.22	1.83

d.f Degree of freedom; *TR* Transpiration rate; *SC* Stomatal conductance; *PR* Photosynthetic rate; *CAT* Catalase; *SOD* Superoxide dismutase; *POX* Peroxidase; *MDA* Malondialdehyde; *PRO* Proline; *CHL* Chlorophyll; *SW* 1,000 seed weight; *SN* Seed number (per ear); *Y* Yield; *ns* no significant

*significant at the 0.05 level of probability

**significant at the 0.01 level of probability

Table 3 Significant interaction of different acid rain and calcium on various parameters that were measured in this study

Treatments	TR	PR	CAT	SOD	POX	MDA	CHL	SW	SN
Water	3.39 b	20.51 b	103.43 f	722.20 h	162.33 d	1.13 h	2.55 b	35.23 b	33.00 b
HNO_3 pH 3	0.85 ef	4.46 e	162.14 d	1078.04 d	291.10 b	1.42 e	1.97 c	30.71 d	26.00 c
H_2SO_4 pH 3	0.56 fg	3.11 fg	245.03 a	1137.44 b	318.92 a	2.22 b	0.22 g	16.36 g	11.66 f
H_2SO_4 pH 2	0.73 fg	3.77 ef	174.70 c	1080.21 d	286.72 b	1.35 f	1.83 d	28.04 e	18.66 e
HNO_3 pH 2	0.46 g	2.32 g	250.73 a	1202.79 a	354.97 a	2.42 a	0.12 h	15.14 g	8.66 g
Water + Ca	4.51 a	26.39 a	87.14 g	705.33 i	127.57 e	1.13 h	2.96 a	38.86 a	45.33 a
HNO_3 pH 3+ Ca	2.36 c	15.56 c	146.99 e	986.03 g	268.11 c	1.35 f	1.87 d	34.02b c	31.66 b
H_2SO_4 pH 3+ Ca	1.28 d	13.34 d	232.38 b	1042.45 e	284.70 b	2.02 d	1.22 f	25.41 f	18.66 e
H_2SO_4 pH 2+ Ca	2.24 c	14.76 c	150.06 e	1011.02 f	261.49 c	1.23 g	1.69 e	32.38c d	23.00 d
HNO_3 pH 2+ Ca	1.15 de	12.44 d	235.94 b	1100.92 c	289.84 b	2.20 c	1.18 f	24.75 f	14.00 f

Within columns, means followed by a different letter are significantly different at $P \leq 0.05$

TR Transpiration rate [mmol m^{-2} s^{-1}]; *PR* Photosynthetic rate [μmol m^{-2} s^{-1}]; *CAT* Catalase [ΔAbs mg protein min^{-1}]; *SOD* Superoxide dismutase [ΔAbs mg protein min^{-1}]; *POX* Peroxidase [ΔAbs mg protein min^{-1}]; *MDA* Malondialdehyde [ε = 155 μM cm^{-1}]; *CHL* Chlorophyll [mg g^{-1} FW]; *SW* 1,000 seed weight [g]; *SN* Seed number (per ear)

intra thylakoid calcium may constitutively promote photosystem II function (Brand and Becker 1984). Moreover, calcium signals decoding elements are involved in ABA-induced stomatal closure and plant adaptation to drought, cold, salt and other abiotic stresses (Song et al. 2008). Calcium channel proteins such as AtTPC1 and TaTPC1 can regulate stomatal closure (Song et al. 2008). The results indicate that calcium ions are probably participating in the regulation of stomata behavior in wheat plants.

After 24-hour applications of simulated acid rain on the wheat crops, the leaf samples were collected, and the changes in antioxidant enzyme activity were determined. The data indicates that the calcium treatment inhibited enzyme activity not only in the stressed plants but also in the control plants (Table 3). This implies that the calcium protected the plants against the oxidative stress induced by acid rain. We reported that calcium increased the photosynthesis rate in wheat leaves subjected to acid rain. Specifically, calcium is known to contribute to the maintenance of the structure and function of plant cell membranes under adverse conditions (Minorsky 1985), but there are few reports on calcium influencing a relationship between photosynthesis and the activity of antioxidant enzymes. Antioxidant enzymes are one of the important reactive oxygen species detoxifier systems in plant cells. Therefore, an induced increase in antioxidant enzyme activity can be considered an important mechanism in the cellular defense strategy against oxidative stress (Shi et al. 2006). The stress of acid rain can induce the production of free radicals, which can be removed by antioxidant enzymes (Wyrwicka and Sklodowska 2006). In this study, the catalase activity was increased considerably by the acid rain (Table 3). The highest was seen in plants treated with H_2SO_4 pH 3 or HNO_3 pH 2, and the lowest catalase activity was observed in plants treated with the calcium solution. The stress of acid rain can induce the production of free radicals, which can be removed by antioxidant enzymes (Wyrwicka and Sklodowska 2006). As the key enzyme in the removal of toxic H_2O_2, catalase is capable of scavenging large quantities of H_2O_2, thus protecting the photosynthetic apparatus against oxidative stress (Apel and Hirt 2004).

There was an increase in superoxide dismutase and peroxidase activity in all plants exposed to simulated acid rain compared to the control plants (Table 3). The activities of

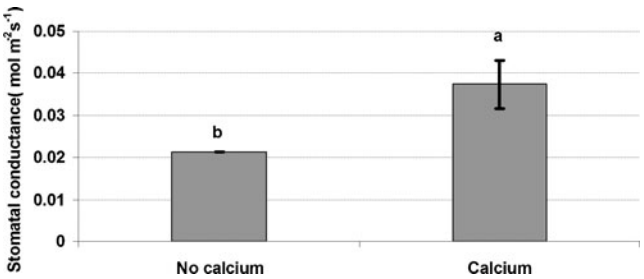

Fig. 2 Effect of calcium foliar application on stomatal conductance of wheat leaves. All the values followed by the same letter are not statistically different at the $p<0.05$ probability level

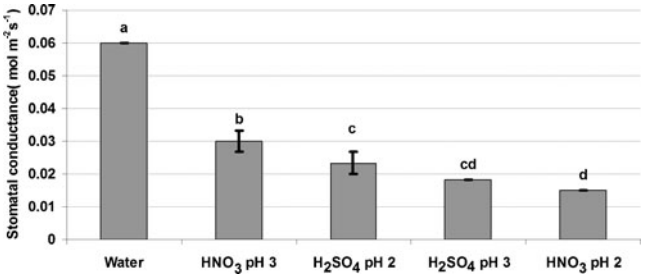

Fig. 3 Effect of different acid rains on stomatal conductance of wheat leaves. All the values followed by the same letter are not statistically different at the $p<0.05$ probability level

superoxide dismutase and peroxidase were inhibited by calcium treatment. Even during normal metabolism, reactive oxygen species are generated as a side product in electron transport processes such as photosynthesis and respiration. In this study, antioxidant enzyme activity in the control (water spraying) plots was much higher than in those plots which were treated with calcium solution. A decrease in antioxidant enzyme activity due to calcium solution application shows that foliar calcium spray can mitigate the reactive oxygen species. The most important increases in enzyme activities were established in plants sprayed with HNO_3 pH 2 and H_2SO_4 pH 3 (Table 3). We found considerable changes in chlorophyll content in the wheat plants treated with acid rain. Acid rain led to a decrease in chlorophyll content as compared to those treated with distilled water treatment. However, the calcium treatment prior to acid rain neutralized the adverse effects of the acid rain that resulted higher chlorophyll content as compared to those treated with acids alone. Therefore, we suggest that calcium does promote the photosynthesis rate through prevention of chlorophyll degradation by acid rain. Moreover, it has been reported that, among cations, K^+ and Ca^{2+} have noticeable effects on chlorophyll accumulation (Knypl and Rennert 1970). According to the results, higher level of chlorophyll in calcium treated plants indicates that calcium plays an important role in regulating leaf senescence. It has been reported that on one hand chloroplast is the location of more than half of the calcium content of the leaves (Stocking and Onget 1962), on the other hand, chlorophyll deterioration is one of the first symptoms of calcium deficit, therefore an increase in cellular calcium will lead to an increase in chlorophyll content of the plants.

The plants subjected to acid rain stress showed significantly higher malondialdehyde content than the control plants (Table 3). This suggests that acid rain results in an increased rate of reactive oxygen species production and membrane lipid peroxidation in wheat leaves. In this research, when calcium was applied before the application of acid rain, there was a significant decrease in malondialdehyde content. Calcium, as an insoluble salt of pectic acid, acts as a binding agent in the middle lamella between the primary cell walls. More recently, it has been suggested that calcium may be required for the maintenance and formation of cellular membranes (Marinos 1962). Membrane permeability may be increased if there is an insufficient supply of calcium (Rehfeld and Jensen 1973). Our results agree with the findings of Wyrwicka and Sklodowska (2006) that treatment with acid rain induces the production of free radicals, which results in the lipid peroxidation of the cell membrane, and produces the toxoid malondialdehyde. Therefore, it could be deduced that treatment with calcium might change the intracellular pH, which made the plants resistant to the acidity of the acid rain.

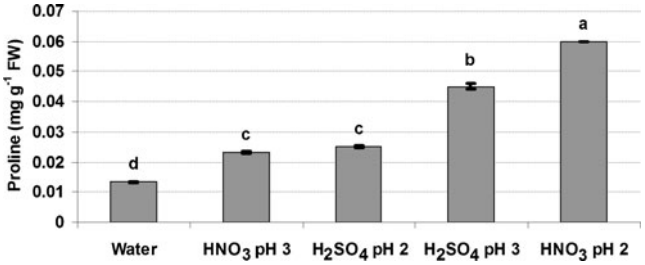

Fig. 5 Effect of different acid rains on proline accumulation of wheat leaves. All the values followed by the same letter are not statistically different at the $p<0.05$ probability level

Proline is a major osmoprotectant, and abiotic stress, such as drought and salt, often results in an increase in proline content in plants (Zhang et al. 2004). However, opinions vary on the possible relationship between calcium and proline. It has been shown that exogenous calcium correlated with an increase in proline content in plants under low temperature (De et al. 1996). Our results agree with the former reports. Calcium-treated plants showed a significantly higher proline content than water-treated plants (Fig. 4). Moreover, acid rain treatments produced higher proline content (Fig. 5). Our results are consistent with the notion that calcium is a messenger that participates in osmoregulation and that calcium plays a positive role in osmoregulation in wheat leaves in response to acid rain. The increase of proline accumulation in wheat leaves because of acid rain can be due to cell water status alteration.

The yield and its components were dramatically affected by foliar application of calcium and simulated acid rain (Figs. 6 and 7, Table 3). Interaction between the calcium treatment and

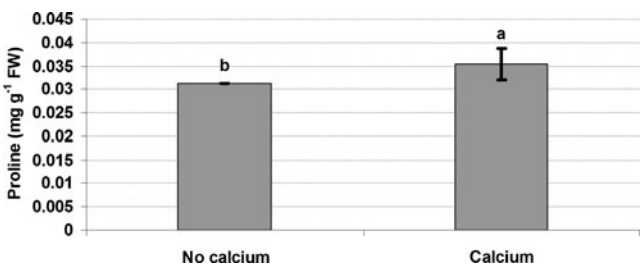

Fig. 4 Effect of calcium foliar application on proline accumulation of wheat leaves. All the values followed by the same letter are not statistically different at the $p<0.05$ probability level

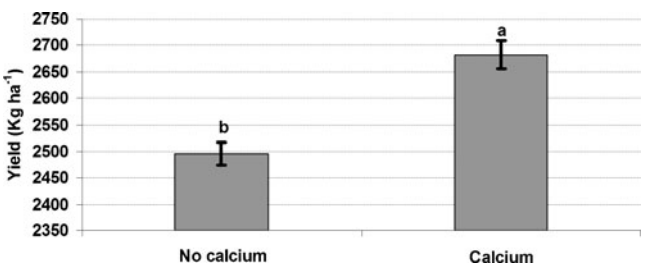

Fig. 6 Effect of calcium foliar application on final seed yield of spring wheat. All the values followed by the same letter are not statistically different at the $p<0.05$ probability level

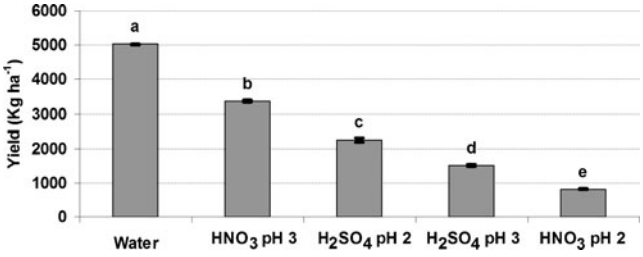

Fig. 7 Effect of different acid rains on final seed yield of spring wheat. All the values followed by the same letter are not statistically different at the $p<0.05$ probability level

acid rain was significant on the 1,000-seed weight and seed number per ear but not on the final seed yield. The highest and lowest 1,000-seed weight and seed number per ear were obtained from the calcium- applied plots and those plots that were treated by HNO_3 pH 2, respectively (Table 3). The final seed yield significantly decreased as a result of the different treatments of acid rain, especially due to HNO_3 pH 2 (Fig. 7). By contrast, we observed a significant increase in the final seed yield when calcium was applied on the wheat plants (Fig. 6). The reduction in seed yield correlates with the decrease in seed number per ear and seed weight as yield components. Simulated acid rain at the booting stage tears down reproductive organs and thus decreased pollination, zygosis and finally seed setting. We observed that acid rain caused enhanced death of the florets. In addition, the leaves, especially the flag leaf as most important leaf in the seed filling process, were injured because of the acid rain, therefore photosynthesis was impaired and eventually seed weight was decreased. However, foliar application of calcium improved the yield components and subsequently the final seed yield. It seems that foliar application of calcium before the simulation of acid rain made the plants resistant by neutralizing the adverse effects of acidification by HNO_3 and H_2SO_4. Calcium is known to act as a regulator of many physiological and biochemical processes in response to abiotic stresses in plants (Bowler and Fluhr 2000). Ai et al. (2006) showed that 10 mM $CaCl_2$ increased photosynthetic capacity in cucumbers. In our study, foliar application of calcium overcame acid rain's detrimental effects. Furthermore, we observed that transpiration rates, photosynthesis rate, stomatal conductance and chlorophyll content were increased in those plants that were pre-treated with calcium before treatment with acid rain. Additionally, the suppressed antioxidant enzyme activity proves the positive effect of calcium foliar application in the scavenging of reactive oxygen species. In sum, the increase of yield can be described by the beneficial effect of calcium on the physiological and metabolic processes and calcium's participation in removing free radicals.

SDS-PAGE analyses on the leaves were performed after the calcium and acid rain treatments and compared to the respective controls (Fig. 8). The protein pattern was conserved in the area of 20–50 kDa. The rubisco (1, 5-bisphosphate carboxylase/oxygenase) large subunit was indicated as a predominant band of 50–60 kDa. The rubisco large subunit showed a considerable decrease in band intensity caused by HNO_3 (pH 2) and H_2SO_4 (pH 3) compared to the control treatment, and those plants had been treated by calcium. In addition, other proteins were completely destroyed by HNO_3 (pH 2). An intact rubisco large subunit was recognized when calcium was applied before the HNO_3 (pH 2) treatment. In other words, the calcium foliar application prevented protein degradation caused by lowering the pH. Similar results were observed regarding the rubisco small subunit., There was a significant decrease in intensity for the rubisco small subunit band of 15 kDa as result of the HNO_3 (pH 2) and H_2SO_4 (pH 3) applications. Obviously the rubisco subunits could be directly degraded by active oxygen, probably via the hydroxyl radicals and superoxide radicals generated in the wheat chloroplasts. Acid rain is connected with oxidative stress; hence, it could be considered that some fragmentation of the rubisco large subunit and rubisco small subunit might occur. Degradation of the rubisco subunits may be accelerated under various environmental stresses, such as ultraviolet-B radiation (Hidema and Kumagai 2006), ozone treatment (Landry and Pell 1993), rain treatment under weak illumination (Hanba et al. 2004) and copper stress (Mehta et al. 1992), resulting in decreased photosynthetic capacity. Under stress conditions,

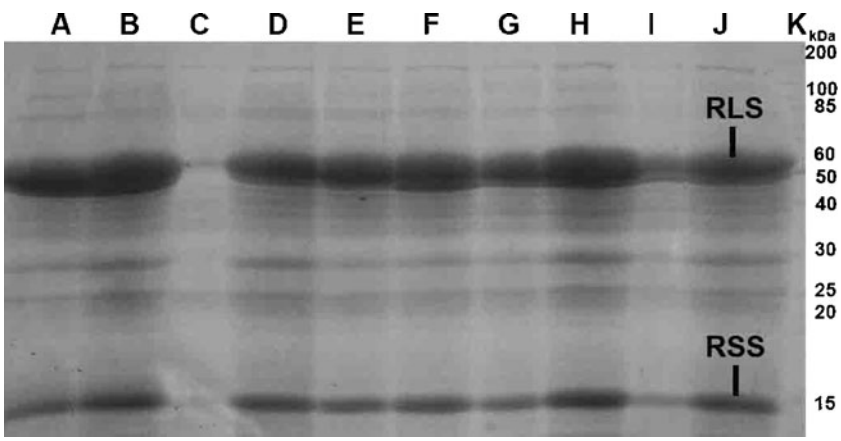

Fig. 8 Wheat leaf protein pattern (SDS-PAGE) in water (**a**), Water + Ca (**b**), HNO_3 pH 2 (**c**), HNO_3 pH 2 + Ca (**d**), HNO_3 pH 3 (**e**), HNO_3 pH 3 + Ca (**f**), H_2SO_4 pH 2 (**g**), H_2SO_4 pH 2 + Ca (**h**), H_2SO_4 pH 3 (**i**), H_2SO_4 pH 3 + Ca (**j**), Ladder kDa (**k**). Samples with protein quantity equivalent to 5 mg FW were loaded per lane. The position of rubisco subunits are indicated in the right—rubisco large subunit (RLS) and rubisco small subunit (RSS)

the production of reactive oxygen species is enhanced in chloroplasts, causing damage to various biomolecules, especially proteins and enzymes (Moller et al. 2007). Therefore, it has been postulated that reactive oxygen species generated by acid rain represents one trigger for rubisco degradation. However, calcium treatment apparently may reduce protein degradation or may increase protein synthesis in plants (Cheruth et al. 2008).

Conclusion

In summary, calcium foliar application enhanced the adaptation of wheat plants to acid rain, as shown by the increase in photosynthetic parameters, chlorophyll and proline content, reduction in antioxidant enzyme activity and malondialdehyde content and prevention of rubisco degradation as a key enzyme of photosynthesis. Therefore, our main objective to determine how to minimize the adverse effect of acid rain on wheat plants through foliar application of calcium was achieved, suggesting the mitigating effect of calcium foliar treatment applied to wheat plants before the possibility of acid rain.

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