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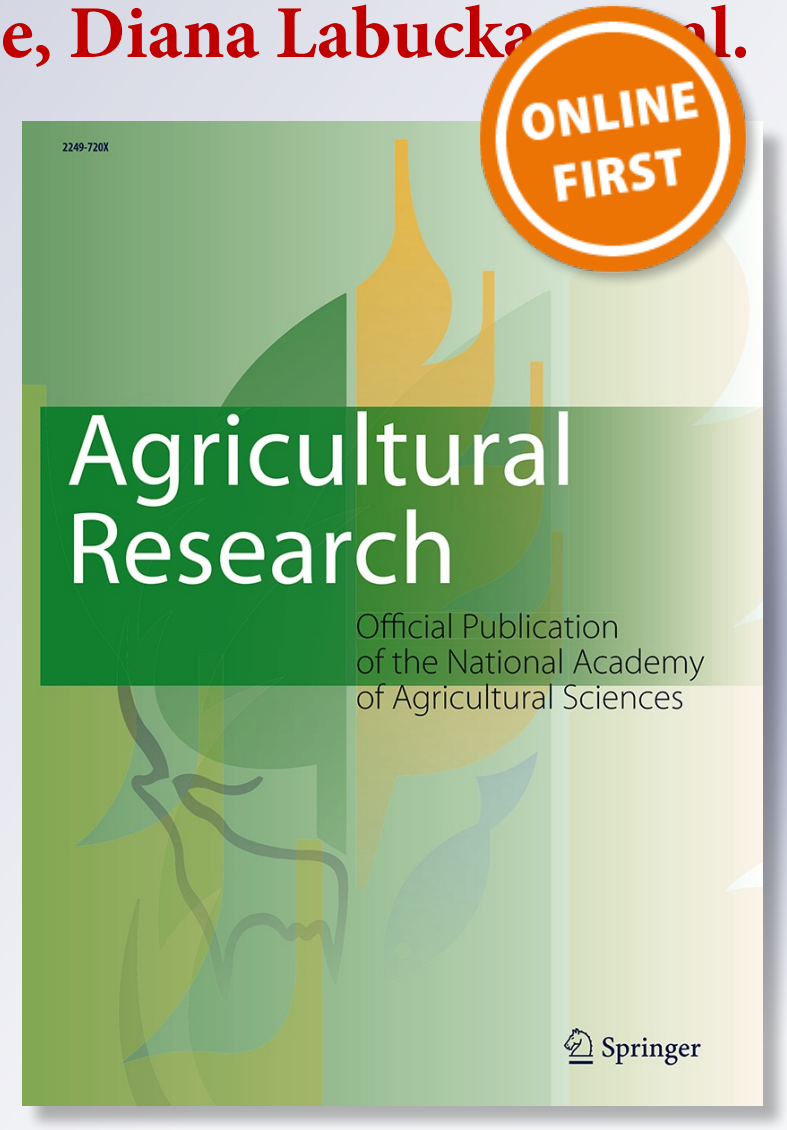
Agricultural Research

ISSN 2249-720X

Agric Res
DOI 10.1007/s40003-015-0154-x



Springer

Agric Res
DOI 10.1007/s40003-015-0154-x

FULL-LENGTH RESEARCH ARTICLE

Physiological Response at Different Plant Development Stages in *Glycine max* Exposed to Elevated CO_2 Concentrations and Fly Ash-Amended Soils

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Received: 13 August 2014 / Accepted: 22 January 2015

Abstract Increasing concentrations of carbon dioxide and heavy metals in soils through pollution are serious problems worldwide. In the present study, we investigated the impacts of elevated atmospheric CO_2 and fly ash (FA)-amended soil on the physiological response (chlorophyll content, non-structural carbohydrates, oil and total proteins) of soybean [*Glycine max* (L.) Merrill] at three growth stages (vegetative, reproductive and maturity). An increase in plant growth and biomass was observed at elevated CO_2 and for moderate concentrations of FA in amended soils in all development plant stages. In contrast to these results, a different response pattern was found for the chlorophyll content and non-structural carbohydrates in relation to the developmental stage, showing that even though in the vegetative growth stage the highest concentration of chlorophylls corresponded to elevated CO_2 conditions. An opposite result was observed during the grain filling stage (reduction of chlorophylls of 15 % at ambient CO_2 conditions for the treatments 10, 15, and 25 % of FA), which probably is related with the distribution of nutrients at this stage. Regarding to oil and total protein content an increase was observed at elevated CO_2 and high concentrations of FA in amended soils. Our findings demonstrate that elevated CO_2 and FA-amended soils alter the physiological response of soybean affecting the crop quality.

Keywords Plant physiology · CO_2 · Fly ash-amended soils · *Glycine max* · Plant development stages

Introduction

Global atmospheric carbon dioxide (CO_2) concentrations have risen from a pre-industrial value of about 280–379 ppmv in 2005, and it is expected to continue increasing in the future due to fossil fuel use and land-use change [39]. In addition, industrial, mining, and agricultural activities have polluted air, water, and soil with heavy metals, being a serious environmental problem [5, 32, 43]. It is important to note that heavy metals cannot be reduced via biochemical processes and they can be accumulated in plants as well as crops, which may be toxic to crop growth and then result in crop reduction [4]. Considering that in the future further increases in global CO_2 levels and contamination with heavy metals are likely, it is important to do more studies on the physiological response of crops. Although recently a few studies have assessed this problem, they were based on the accumulation of metals in

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Published online: 27 February 2015

plants without consideration of the physiological response in plants [9, 16, 17, 23, 26, 34, 41, 42, 45].

Besides environmental consequences of global change, the increase of industrialization in countries with high population densities, such as India, can have severe impacts on the environment. Industrial growth is accompanied by an increase in industrial waste, such as fly ash (FA), the main waste generated by coal-fired power plants. Generally, FA components are plant macronutrients, micronutrients, and also toxic elements such as Hg, Pb, Cd, and Cr. The elemental composition of FA can differ according to the types and sources of coal used [6]. Numerous reports mention that the addition of FA to soil may improve the physical–chemical properties as well as nutritional quality of the soil, with the extent of the changes produced depending on the soil and FA properties [33]. However, other studies report about the contamination of soils with heavy metals through the dispersion of high amounts of FA disposal [10, 18, 22, 31].

Regarding to the importance of FA application to agricultural soils and their potential effect in combination with an increase of CO_2 concentrations, we conduct a previous study which analyzed this combined effect on the uptake and accumulation of trace elements in soybeans, finding a toxicological risk for human consumption [34]. In the present study, our research was focused on the evaluation of physiological processes that occur at different growth stages in soybean. Soybean (*Glycine max*) is one of the most widespread crops worldwide, and Argentina is one of the major producers [15]. Although no precedents exist about the use of FA-amended soils in Argentina, it is possible that this practice will be implemented as a result of increasing industrialization in the future. Moreover, pollutant emitting industries (mainly coal-fired power plants) and other emission sources contribute to the deposition of particulate contaminants to the agricultural soils. Regarding to this, we performed a recent study in agricultural sites cultivated with soybean and being affected by different human activities, in which a potential accumulation of heavy metals in the crop was found [36].

In relation to the physiological effects of global change on crops, numerous studies indicating that high levels of CO_2 are associated with increased plant productivity, especially in the case of the C3 crops, mainly based on the stimulation of photosynthesis [8, 24, 29], an increase in CO_2 concentrations may stimulate the carbon sequestration by promoting higher growth rates [19, 37]. However, currently little is known about the combined effect of increased greenhouse gases and pollutants such as heavy metals on crops physiology. Therefore, taking into account the facts mentioned above, the objective of this study was to assess the physiological response at different growth stages of soybean [*Glycine max* (L.) Merrill] growing at elevated CO_2 concentrations and FA-amended soils.

Materials and Methods

Plant Material, Environmental Conditions, Fly Ash Characteristics, and Elemental Content of Soybeans

Detailed information about plant material, environmental conditions, chemical characteristics of fly ash, and metal content in soybean has been previously described in Rodriguez et al. [34]. Briefly, soybean seeds [*Glycine max* (L.) Merrill, advanced line of the conventional J001730 INTA Marcos Juárez] were sowed in a soil:sand (3:1) substrate (macronutrients [mg L^{-1}]: 124–185 N, 120–179 P_2O_2, 190–284 K_2O; pH 5.5–6.1; salinity [g L^{-1}] 0.8–1.4) enriched in heavy metals through the incorporation of FA. Treatments were Control or 0 % FA; 1 % FA; 10 % FA; 15 % FA and 25 % FA. Plants with three replicates for each of the five soil treatments were exposed from germination up to seed maturation in environmentally controlled chambers (Vötsch - Bio Line, Type VB 151,415 with CO_2 and dosing adjustment device IR system 3600) at 400 (ambient) and 600 (elevated) ppm CO_2 whit the climatic conditions of Córdoba, Argentina (Fig. 1). Finally, three harvests were made at the vegetative (V5), reproductive/grain filling (R5, 5), and maturity (R8) stages as defined by Fehr and Caviness [12]. Subsequently, soil samples were analyzed for pH, concentrations of plant-available macronutrients and metals, while heavy metal concentrations were determinate in soybean seeds. Taking into account that only Pb showed concentrations above the maximum permitted levels in soybean, a summary of the values obtained for the main parameters measured in soils and soybean (pH, macronutrients and Pb content in soils and seeds) is shown in Table 1 corresponding to the study of Rodriguez et al. [34].

Morphological Parameters

With the purpose of analyzing differences in growth and development of soybeans, morphological parameters were determined in each of the above-mentioned growth stages (vegetative, reproductive/grain filling and maturity). The parameters were plant height, specific leaf area and biomass of leaves, stems, pods, seeds and roots. All determinations were expressed as dry weight (DW).

Table 1 Chemical characteristics of fly ash (FA)-amended soils (pH, P, K, Mg, N, Pb) and Pb concentrations in seeds of *G. max* exposed to ambient and elevated CO_2 concentrations

Treatment (CO_2 % FA)	Physical and chemical parameters					Soil	Seed
	pH	P (mg 100 g^{-1} DW)	K (mg 100 g^{-1} DW)	Mg (mg 100 g^{-1} DW)	N (mg 100 g^{-1} DW)	Pb (μg g^{-1} DW)	Pb (μg g^{-1} DW)
A 0	5.8	27	50	33	30	4.15	0.37
E 0	6.4	25	42	26	17	3.96	1.67
A 1 %	5.8	38	74	26	19	4.4	1.48
E 1 %	5.9	43	76	38	30	4.49	3.20
A 10 %	6.7	125	27	46	15	6.55	1.61
E 10 %	6.5	96	43	39	13	5.55	1.37
A 15 %	7.1	182	41	50	24	7.57	1.62
E 15 %	7.1	180	41	52	17	7.76	1.49
A 25 %	7.8	309	29	60	14	9.97	1.65
E 25 %	7.9	236	37	66	13	8.36	1.63

Original data reprinted from Rodriguez et al. [34]

Notes 0, Control (0 % FA/100 % S), 1 % (1 % FA/99 % S), 10 % (10 % FA/90 % S), 15 % (15 % FA/85 % S), 25 % (25 % FA/75 % S). *A* ambient CO_2 concentrations, 400 ppmv, *E* elevated CO_2 concentrations, 600 ppmv, *FA* fly ash, *S* standard substrate

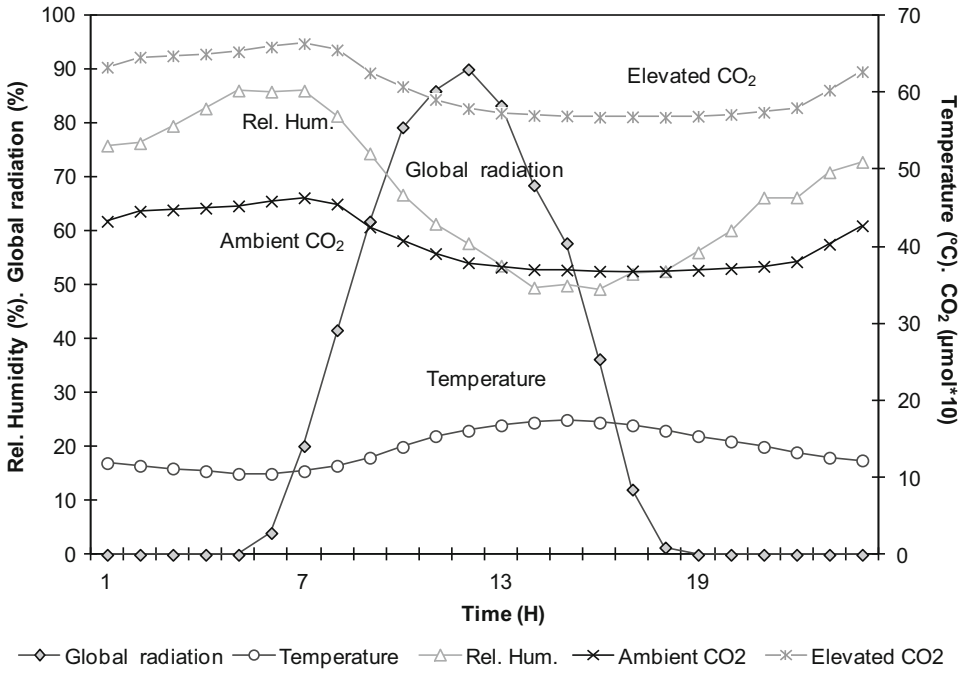

Fig. 1 Mean daily profile of climate and diurnal profiles of 'ambient' (×) and 'elevated' (*) CO_2 treatments simulated in the growth chambers. Data based on 15 min averages measured over 12 weeks

Biochemical Parameters in Leaves and Seeds

Chlorophyll Determinations

Sub-samples were separated to determine the chlorophyll in fresh samples of soybean leaves while another fraction was lyophilized for other analyses. Three sub-samples per treatment were taken.

Quantification of chlorophyll a (Chl *a*) and chlorophyll b (Chl *b*) concentrations in soybean leaves was performed with 100 mg of material, which was homogenized in 10 ml of ETOH at 96 % v/v with an Ultra Turrax homogenizer, T18. 1KA Works, Inc. USA. Subsequently, the supernatant was separated. Absorption of chlorophylls was measured with a spectrophotometer Beckman DU 7000, USA. Concentrations of chlorophylls were calculated on a dry weight basis [44].

Carbohydrates and Protein Determinations in Leaves and Seeds

An enzymatic method for non-structural carbohydrates in leaves and seeds was performed according to the technique

described by Högy [21], in which reducing carbohydrates (glucose and fructose), sucrose, and starch were extracted using 70 % ethanol and results expressed in % DW.

On the other hand, the quantity of soluble protein was determined in leaves as described in Högy [21], and results expressed in mg g^{-1} DW.

The total protein concentration in seeds was determined by the Kjeldahl method as % N × 6.25 [3] and expressed in mg g^{-1} DW. Finally, with the aim of evaluating changes in protein fractions, a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Meriles et al. [30] and the protein bands were identified from the literature [11, 28]. Gels were scanned and analyzed with image processing based on the Java Image J.

Soybean Oil Quality

Oil content in seeds (%) was determined according to Maestri et al. [28]. The composition of fatty acids was analyzed by gas chromatography (GC) according to Maestri and Guzman [27] and the theoretical iodine index was calculated according to Carreras et al. [7].

Statistical Analysis

Growth and biochemical parameters were submitted to an analysis of variance (ANOVA) for the to one and two criteria of classification, the latter being conducted to check the possible effect of interaction between the main factors (soils and CO_2) on each of the parameters. Taking into account that the two-way ANOVA did not show interactive treatment effects, a one-way ANOVA for each of the variables was performed. Whenever the one-way ANOVA indicated significant effects ($p \leq 0.05$), a pairwise comparison of means was undertaken using the Tukey test. The ANOVA assumptions were previously verified graphically (residual versus fitted values, box plots and steam leaf plots).

Seed quality parameters such as oil yield, non-structural carbohydrates in seeds, total proteins, and lead concentration, this last parameter described in Rodriguez et al. [34], were submitted to Pearson's coefficient of correlation in order to study the relationship among the lead and seed quality measured in soybean.

Results and Discussion

Morphological and Biochemical Parameters

Vegetative Growth Stage

Table 2 shows the results of plant height, stem biomass, root biomass, specific leaf area, Chl $a + b$ (mg g^{-1} DW), reducing sugars in leaves (% DW), sucrose in leaves (% DW), starch in leaves (% DW), and soluble proteins in leaves (mg g^{-1} DW) for the different amended soil treatments and CO_2 levels in the vegetative growth stage.

Comparison among different amended soils and CO_2 treatments for the morphological parameters analyzed showed only significant differences for the plant height in plants exposed to elevated CO_2 and the higher proportion of FA in soils (15 and 25 %). In addition, the stem biomass and specific leaf area showed the highest values at 1 % FA in soils and elevated or ambient CO_2, respectively. Although it has been widely established that higher CO_2 concentrations would promote the growth of C_3 plants since under current CO_2 levels the photosynthetic rate is not saturated [24], the biomass parameters analyzed in this study indicate that FA has a significant negative effect on biomass at ambient CO_2 concentration.

Regarding the physiological parameters, concentration of photosynthetic pigments showed higher content for 1 and 15 % of FA-amended soils at elevated CO_2, but no difference was found among FA-amended soils. In agreement, Koti et al. [25] observed a positive relationship between photosynthetic pigments and elevated CO_2 (720 ppm) in soybean.

Regarding carbohydrate content in leaves, reducing sugars (glucose + fructose) in leaves showed no significant differences between soil treatments, while the comparison between CO_2 conditions showed the higher sucrose values at elevated CO_2. As for starch content in leaves the higher values were found for the higher concentrations of FA (15 and 25 %) under elevated CO_2, and the comparison between CO_2 levels showed the higher values at elevated CO_2. According to that, numerous studies indicated an increase in foliar carbohydrate concentrations in soybean grown under elevated CO_2 (average 650 ppm) [1, 2, 35]. Furthermore, Mishra et al. [31] pointed out that the carbohydrate content increased in rice grown on FA-amended soils.

On the other hand, soluble proteins in leaves mostly presented the higher values for treatments with 0, 10, and 15 % FA-amended soils at both CO_2 levels, while the comparison of CO_2 conditions only showed differences in control samples with the highest values being found under elevated CO_2. These results indicated that the total soluble protein content was positively influenced by the intermediate concentrations of FA-amended soils, which are consistent with results reported by other authors using rice and chickpea, who indicate that low FA-amended soils may promote the synthesis of proteins [10, 18, 31].

Reproductive Stage/Grain Filling

Table 3 shows the results of plant height, stem biomass, root biomass, specific leaf area, Chl $a + b$ (mg g^{-1} DW),

Table 2 Mean values (±standard deviation, SD) and results of the analysis of variance (ANOVA) of the morpho-physiological parameters measured in *Glycine max* at different CO_2 concentrations and different proportions of fly ash (FA) in soils. Vegetative growth stage

Parameter	CO_2	Mean ± SD					ANOVA[a]
		Control	1 % FA	10 % FA	15 % FA	25 % FA	
Plant height (cm)	A	40.65 ± 7.28	36.25 ± 2.47	42.35 ± 4.74	44.65 ± 2.33	38.00 ± 2.83	ns
	E	45.85 ± 1.34 bc	41.40 ± 3.82 c	47.45 ± 4.45 abc	50.25 ± 0.35 ab	53.25 ± 1.06 a	*
	ANOVA[b]	ns	ns	ns	ns	*	
Stem biomass (g DW $plant^{-1}$)	A	0.67 ± 0.36	0.41 ± 0.06	1.16 ± 0.09	0.90 ± 0.21	0.37 ± 0.18	ns
	E	1.03 ± 0.28	0.74 ± 0.08	1.30 ± 0.28	1.19 ± 0.06	0.63 ± 0.04	ns
	ANOVA[a]	ns	*	ns	ns	ns	
Root biomass (g DW $plant^{-1}$)	A	0.53 ± 0.02	0.21 ± 0.07	1.22 ± 0.23	1.07 ± 0.53	0.49 ± 0.29	ns
	E	0.84 ± 0.64	0.46 ± 0.06	1.29 ± 0.06	1.33 ± 0.15	0.69 ± 0.01	ns
	ANOVA[a]	ns	ns	ns	ns	ns	
SLA (cm^2 g^{-1} DW)	A	202.79 ± 47.79	198.62 ± 0.63	165.61 ± 18.93	165.22 ± 13.48	156.20 ± 8.81	ns
	E	181.14 ± 77.94	165.78 ± 1.02	161.29 ± 17.45	144.96 ± 6.53	134.41 ± 2.43	ns
	ANOVA[a]	ns	***	ns	ns	ns	
Total chlorophylls (mg g^{-1} DW)	A	10.08 ± 0.63	7.73 ± 0.98	7.86 ± 1.04	9.97 ± 3.46	9.70 ± 2.05	ns
	E	10.74 ± 3.52	11.33 ± 1.72	12.97 ± 2.61	11.16 ± 2.58	11.04 ± 2.60	ns
	ANOVA[a]	ns	*	*	ns	ns	
Reducing sugars in leaves (% DW)	A	0.18 ± 0.01	0.21 ± 0.01	0.19 ± 0.003	0.19 ± 0.01	0.18 ± 0.003	ns
	E	0.20 ± 0.04	0.17 ± 0.01	0.18 ± 0.01	0.18 ± 0.003	0.19 ± 0.02	ns
	ANOVA[a]	ns	ns	ns	ns	ns	
Sucrose in leaves (% DW)	A	0.03 ± 0.001	0.04 ± 0.008	0.03 ± 0.002	0.03 ± 0.001	0.05 ± 0.01	ns
	E	0.09 ± 0.01	0.08 ± 0.01	0.09 ± 0.01	0.10 ± 0.002	0.09 ± 0.01	ns
	ANOVA[a]	**	*	**	***	ns	
Starch in leaves (% DW)	A	0.08 ± 0.004	0.12 ± 0.02	0.08 ± 0.005	0.12 ± 0.07	0.19 ± 0.08	ns
	E	0.14 ± 0.04 c	0.20 ± 0.003 bc	0.25 ± 0.004 b	0.48 ± 0.01 a	0.48 ± 0.06 a	***
	ANOVA[a]	ns	*	***	*	ns	
Protein in leaves (mg g^{-1} DW)	A	19.33 ± 1.05 c	21.87 ± 2.21 bc	26.24 ± 5.58 ab	29.58 ± 6.48 a	18.58 ± 1.40 c	**
	E	30.68 ± 7.83 a	27.44 ± 5.18 ab	32.90 ± 3.39 a	31.14 ± 9.10 a	18.67 ± 3.86 b	*
	ANOVA[a]	*	ns	ns	ns	ns	

Values in each row followed by the same letter do not differ significantly at $p < 0.05$. *ns* not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

SLA specific leaf area, *A* ambient, 400 ppmv CO_2, *E* elevated, 600 ppmv CO_2

[a] ANOVA between soil treatments

[b] ANOVA between CO_2 treatments

reducing sugars in leaves (% DW), sucrose in leaves (% DW), starch in leaves (% DW), and soluble proteins in leaves (mg g^{-1} DW) for the different amended soil treatments and CO_2 levels in the reproductive/grain filling growth stage of soybean.

Similar to the vegetative growth stage, the highest values for plant height were found in amended soils with higher concentrations of FA and elevated CO_2. The stem biomass values were lowest at 25 % FA-amended soils and higher values were found at elevated CO_2 concentrations. In addition, significant higher root biomass was found for 1 % FA-amended soil at elevated CO_2, while no significant differences were observed for specific leaf area. Consistently, Koti et al. [25] reported an increase in height and biomass in soybeans exposed to elevated CO_2 conditions.

With regard to Chl contents, the comparison among FA-amended soils showed the higher values in the treatments with 0, 1, and 10 % FA-amended soils, while in general the comparison between CO_2 conditions showed the higher values at ambient CO_2. In addition, reducing sugars content in leaves were higher for the 15 and 25 % FA-amended soil treatments at ambient CO_2, while the comparison of CO_2 conditions showed the highest values in control samples under elevated CO_2, and no differences were observed for sucrose. Regarding to starch content in leaves, the higher values were found for the higher FA-amended soil treatments.

Table 3 Mean values (±standard deviation, SD) and results of the analysis of variance (ANOVA) of the morpho-physiological parameters measured in *Glycine max* at different CO_2 concentrations and different proportions of fly ash (FA) in soils. Reproductive/grain filling stage

Parameter	CO_2	Mean ± SD					ANOVA[a]
		Control	1 % FA	10 % FA	15 % FA	25 % FA	
Plant height (cm)	A	56.00 ± 1.41 b	61.00 ± 5.66 b	61.50 ± 0.71 b	63.50 ± 2.12 b	71.50 ± 2.12 a	*
	E	67.00 ± 4.24 b	61.50 ± 3.54 b	67.00 ± 9.90 b	83.00 ± 4.24 a	89.50 ± 3.54 a	*
	ANOVA[b]	ns	ns	ns	*	*	
Stem biomass (g DW $plant^{-1}$)	A	2.54 ± 0.01	2.18 ± 0.35	2.10 ± 0.50	2.14 ± 0.22	1.46 ± 0.01	ns
	E	3.37 ± 0.05 a	2.96 ± 0.19 b	3.54 ± 0.06 a	3.63 ± 0.04 a	1.78 ± 0.08 c	***
	ANOVA[b]	**	ns	ns	*	*	
Root biomass (g DW $plant^{-1}$)	A	1.77 ± 0.13	1.52 ± 0.01	2.07 ± 0.29	2.16 ± 0.54	1.54 ± 0.07	ns
	E	2.77 ± 1.10	2.23 ± 0.06	2.48 ± 0.42	2.63 ± 0.26	1.61 ± 0.16	ns
	ANOVA[b]	ns	**	ns	ns	ns	
SLA (cm^2 g^{-1} DW)	A	275.41 ± 2.01	284.80 ± 9.93	275.81 ± 0.49	247.28 ± 14.19	252.37 ± 16.43	ns
	E	251.86 ± 32.60	272.36 ± 8.99	278.58 ± 25.45	231.82 ± 0.11	283.12 ± 78.78	ns
	ANOVA[b]	ns	ns	ns	ns	ns	
Total chlorophylls (mg g^{-1} DW)	A	11.37 ± 0.70 b	9.83 ± 0.31 c	13.19 ± 0.52 a	11.20 ± 0.98 bc	11.18 ± 0.55 bc	***
	E	11.50 ± 0.97 a	11.92 ± 0.30 a	11.14 ± 1.27 ab	9.49 ± 0.51 b	9.54 ± 0.38 b	***
	ANOVA[b]	ns	***	*	*	**	
Reducing sugars in leaves (% DW)	A	0.18 ± 0.002 c	0.20 ± 0.01 bc	0.18 ± 0.002 c	0.24 ± 0.02 a	0.22 ± 0.01 ab	*
	E	0.19 ± 0.01	0.20 ± 0.02	0.19 ± 0.002	0.21 ± 0.11	0.21 ± 0.008	ns
	ANOVA[b]	*	ns	ns	ns	ns	
Sucrose in leaves (% DW)	A	0.07 ± 0.03	0.08 ± 0.04	0.06 ± 0.01	0.09 ± 0.02	0.06 ± 0.03	ns
	E	0.06 ± 0.02	0.07 ± 0.03	0.05 ± 0.02	0.06 ± 0.03	0.05 ± 0.02	ns
	ANOVA[b]	ns	ns	ns	ns	ns	
Starch in leaves (% DW)	A	0.31 ± 0.15 b	0.32 ± 0.06 b	0.27 ± 0.08 b	0.52 ± 0.01 a	0.55 ± 0.02 a	**
	E	0.33 ± 0.09 b	0.21 ± 0.14 b	0.29 ± 0.09 b	0.51 ± 0.04 a	0.56 ± 0.02 a	***
	ANOVA [b]	ns	ns	ns	ns	ns	
Protein in leaves (mg g^{-1} DW)	A	18.15 ± 1.26 c	20.70 ± 2.15 b	17.01 ± 0.64 c	23.48 ± 1.15 a	21.39 ± 1.67 ab	***
	E	25.51 ± 0.70 a	18.95 ± 0.55 b	17.83 ± 0.92 b	14.17 ± 0.51 c	15.28 ± 2.15 c	***
	ANOVA[b]	***	ns	ns	***	**	

Values in each row followed by the same letter do not differ significantly at $p < 0.05$. *ns* not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

SLA specific leaf area, *A* ambient, 400 ppmv CO_2, *E* elevated, 600 ppmv CO_2

[a] ANOVA between soil treatments

[b] ANOVA between CO_2 treatments

These results were opposite than those reported for the vegetative stage, which could be due to differences in accumulation of total non-structural carbohydrates, which indicate difference in the synthesis of photosynthetic pigments in relation with the growth stage, implying a higher accumulation of carbohydrates in the R2–R5 stages as observed by Allen et al. [2]. Moreover, it has been reported that in the second half of the grain filling stage (>R5, 5) the starch reserves are mobilized, resulting in a decrease in photosynthetic rate [13]. In our study, a decrease in photosynthetic pigment contents during the reproductive stage/grain filling for the plants grown at elevated CO_2 and 10, 15, and 25 % FA-amended soils may have occurred due to a significant mobilization of nutrients to the seed, which in turn would have been induced by greater sink demand due to the increased seed biomass in the high CO_2 treatment. Taking into account the differential behavior between vegetative and reproductive growth stages in relation to photosynthetic pigments, an ANOVA was performed for each soil treatment (Control, 1, 10, 15, and 25 % FA) and exposure level of CO_2 (ambient and elevated) between the values obtained for the vegetative and reproductive growth stages. Results showed significantly higher values in the reproductive stage (data not shown) for all cases, which would indicate that although the synthesis of chlorophylls during the reproductive stage was lower under elevated

CO_2 than for ambient concentrations, total chlorophyll content was increased significantly in the reproductive stage compared to the vegetative growth stage.

On the other hand, higher values of soluble leaf protein were noted for the treatments with the higher FA-content in soils at ambient CO_2, while in the control samples the highest values were found at elevated CO_2. In addition, the comparison between CO_2 conditions showed with the higher values at elevated CO_2 corresponding to the largest concentrations of FA-amended soils, while the control samples were higher at ambient CO_2.

Maturity Stage

Table 4 shows the results of plant height, stem biomass, pod biomass, seed biomass, root biomass, reducing sugars in seeds (% DW), sucrose in seeds (% DW), starch in seeds (% DW), total proteins in seeds (mg g^{-1} DW), and oil content in seeds (% DW) for the different amended soil treatments and CO_2 levels at maturity.

Regarding to morphological parameters in a similar way to the previous stages, the higher values of plant height, stem, pods, seeds, and roots biomass were found at

Table 4 Mean values (±standard deviation, SD) and results of the analysis of variance (ANOVA) of morpho-physiological parameters measured in *Glycine max* at different CO_2 concentrations and different proportions of fly ash (FA) in soils. Maturity stage

Parameter	CO_2	Mean ± SD					ANOVA[a]
		Control	1 % FA	10 % FA	15 % FA	25 % FA	
Plant height (cm)	A	56.50 ± 2.12	59.50 ± 6.36	72.50 ± 12.02	64.00 ± 5.66	68.50 ± 7.78	ns
	E	66.50 ± 2.12	69.50 ± 3.54	68.50 ± 2.12	89.00 ± 25.46	78.50 ± 2.12	ns
	ANOVA[b]	*	ns	ns	ns	ns	
Stem biomass (g DW $plant^{-1}$)	A	3.50 ± 0.22 a	2.83 ± 0.21 ab	3.48 ± 0.09 a	2.62 ± 0.02 b	2.48 ± 0.24 b	**
	E	4.82 ± 0.05	3.61 ± 0.50	5.06 ± 0.13	4.77 ± 0.94	3.58 ± 0.91	ns
	ANOVA[b]	*	ns	**	ns	ns	
Pods biomass (g DW $plant^{-1}$)	A	3.02 ± 0.02	2.83 ± 0.07	3.07 ± 0.78	2.25 ± 0.14	2.38 ± 0.61	ns
	E	3.91 ± 0.39 a	3.90 ± 0.21 a	3.53 ± 0.32 ab	2.91 ± 0.23 ab	2.45 ± 0.22 b	*
	ANOVA[b]	ns	*	ns	ns	ns	
Seeds biomass (g DW $plant^{-1}$)	A	12.34 ± 0.16 a	12.14 ± 0.30 a	10.51 ± 1.17 ab	9.00 ± 0.13 b	9.27 ± 1.65 b	*
	E	15.91 ± 0.40 a	15.43 ± 1.31 a	13.57 ± 1.32 ab	12.12 ± 0.02 bc	10.25 ± 1.33 c	*
	ANOVA[b]	**	ns	ns	***	ns	
Roots biomass (g DW $plant^{-1}$)	A	1.96 ± 0.94	0.80 ± 0.06	2.58 ± 0.19	2.13 ± 0.10	2.53 ± 0.45	ns
	E	1.48 ± 0.07	1.61 ± 0.19	1.53 ± 0.31	2.97 ± 1.77	3.83 ± 1.36	ns
	ANOVA[b]	ns	*	ns	ns	ns	
Reducing sugars in seeds (% DW)	A	0.17 ± 0.002	0.16 ± 0.005	0.16 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	ns
	E	0.15 ± 0.01	0.16 ± 0.004	0.17 ± 0.01	0.16 ± 0.005	0.16 ± 0.01	ns
	ANOVA[b]	ns	ns	ns	ns	ns	
Sucrose in seeds (% DW)	A	0.37 ± 0.05	0.37 ± 0.01	0.30 ± 0.14	0.35 ± 0.02	0.37 ± 0.03	ns
	E	0.37 ± 0.02	0.37 ± 0.03	0.39 ± 0.01	0.35 ± 0.03	0.40 ± 0.01	ns
	ANOVA[b]	ns	ns	ns	ns	ns	
Starch in seeds (% DW)	A	0.05 ± 0.001	0.05 ± 0.004	0.05 ± 0.004	0.05 ± 0.003	0.05 ± 0.002	ns
	E	0.04 ± 0.002	0.5 ± 0.003	0.05 ± 0.002	0.05 ± 0.001	0.05 ± 0.002	ns
	ANOVA[b]	ns	ns	ns	ns	ns	
Protein in seeds (mg.g^{-1} DW)	A	45.86 ± 0.26 bc	44.93 ± 0.77 c	46.64 ± 1.29 ab	48.10 ± 0.51 a	46.40 ± 1.07 bc	*
	E	45.30 ± 1.03 cd	46.45 ± 0.56 b	45.02 ± 0.42 d	46.29 ± 0.36 bc	48.38 ± 0.56 a	***
	ANOVA[b]	ns	ns	ns	**	*	
Oil in seeds	A	16.14 ± 0.91 b	16.63 ± 4.07 b	22.08 ± 1.33 ab	28.07 ± 0.61 a	25.75 ± 5.30 a	*
(% DW)	E	17.25 ± 2.47 b	21.13 ± 2.30 b	31.75 ± 1.06 a	19.75 ± 3.18 b	21.63 ± 3.71 b	*
	ANOVA [b]	ns	ns	**	ns	ns	

Values in each row followed by the same letter do not differ significantly at $p < 0.05$. *ns* not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

A ambient, 400 ppmv CO_2, *E* elevated, 600 ppmv CO_2

[a] ANOVA between soil treatments

[b] ANOVA between CO_2 treatments

elevated CO_2 and low concentrations of FA in soils (Control, 1 and 10 % FA treatments). These results are consistent with Singh et al. [38], who reported that the application of low concentrations of FA to agricultural soils provide good conditions for plant growth. Such findings suggest that high proportions of FA in soils have a negative effect on the biomass production of soybean. In addition, Heinemann et al. [20] found a direct relationship between increased seed biomass and elevated CO_2.

In relation to seed carbohydrates (reducing sugars, sucrose, and starch), no significant differences were found between the two CO_2 conditions or between treatments with different proportions of FA-amended soils. This could be due to a decrease in the concentration of total non-structural carbohydrates in seeds, which presumably resulted from a higher oil and protein synthesis as indicated by Streeter and Jeffers [40], whereas the total protein concentration in seeds did not show a clear pattern.

On the other hand, the oil yield showed the highest values in the 15 and 25 % FA-amended soil treatments at ambient CO_2, while the comparison between CO_2 conditions showed the highest value at elevated CO_2 for the treatment with 10 % FA-amended soils. Consistently, Hao et al. [19] reported an increase in oil yield of soybean exposed to elevated CO_2 conditions. Thus, our results could imply a combined fertilization effect of CO_2 and FA since previous studies have indicated an improvement of the grain quality at higher concentrations of FA in amended soils [31]. However, there are no studies available regarding the interaction or additive effects of FA-amended soils and elevated CO_2. Therefore, in order to evaluate the relationship between seed quality parameter (oil yield, protein content and carbohydrates) and lead content in seeds, which showed food safety risk values described in Rodriguez et al. [34], we conducted a Pearson correlation analysis. Results showed no correlation between the content of lead and seed quality parameters. However, few correlations among the quality parameters were observed, which indicated a negative correlation between protein content and starch ($p < 0.06$, correlation coefficient

Table 5 Mean values (±standard deviation, SD) and results of the analysis of variance (ANOVA) of fatty acid composition (% of total fatty acids), oleic/linolenic (O/Ln) ratios, and iodine values (IVs) of *Glycine max* seed oils at different CO_2 concentrations and different proportions of fly ash (FA) in soils. Maturity stage

Fatty acids	CO_2	Mean ± SD										ANOVA[a]
		Control		1 % FA		10 % FA		15 % FA		25 % FA		
Palmitic (16:0)	A	1.450	0.003	1.453	0.008	1.451	0.005	1.452	0.010	1.446	0.008	ns
	E	1.451	0.003	1.447	0.002	1.454	0.002	1.456	0.000	1.456	0.008	ns
	ANOVA[b]	ns		ns		ns		ns		ns		
Stearic (18:0)	A	1.549	0.007	1.545	0.001	1.545	0.001	1.544	0.009	1.545	0.003	ns
	E	1.544	0.001	1.543	0.000	1.542	0.001	1.541	0.000	1.539	0.000	ns
	ANOVA[b]	ns		ns		ns		ns		ns		
Oleic (18:1)	A	1.355	0.019	1.306	0.038	1.306	0.027	1.340	0.016	1.330	0.009	ns
	E	1.354	0.022	1.317	0.031	1.341	0.005	1.315	0.039	1.310	0.006	ns
	ANOVA[b]	ns		ns		ns		ns		ns		
Linoleic (18:2)	A	0.979	0.015	1.039	0.001	1.040	0.029	1.025	0.046	1.035	0.031	ns
	E	0.993	0.011	1.003	0.034	1.010	0.002	1.048	0.044	0.987	0.060	ns
	ANOVA[b]	ns		ns		ns		ns		ns		
Linolenic (18:3)	A	1.484	0.009	1.483	0.046	1.484	0.004	1.464	0.025	1.472	0.020	ns
	E	1.479	0.009	1.510	0.004	1.476	0.006	1.467	0.000	1.490	0.014	ns
	ANOVA[b]	ns		ns		ns		ns		ns		
O/Ln	A	0.913	0.018	0.881	0.053	0.880	0.016	0.916	0.005	0.904	0.006	ns
	E	0.916	0.021	0.872	0.023	0.909	0.007	0.897	0.026	0.879	0.012	ns
	ANOVA[b]	ns		ns		ns		ns		ns		
IVs	A	144.084	3.160	138.934	0.940	141.127	1.400	136.646	3.543	141.557	3.425	ns
	E	139.602	9.239	144.019	1.019	143.511	2.239	142.883	0.746	145.103	5.886	ns
	ANOVA[b]	ns		ns		ns		ns		ns		

Values in each row followed by the same letter do not differ significantly at $p < 0.05$. *ns* not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

A ambient, 400 ppmv CO_2, *E* elevated, 600 ppmv CO_2

[a] ANOVA between soil treatments

[b] ANOVA between CO_2 treatments

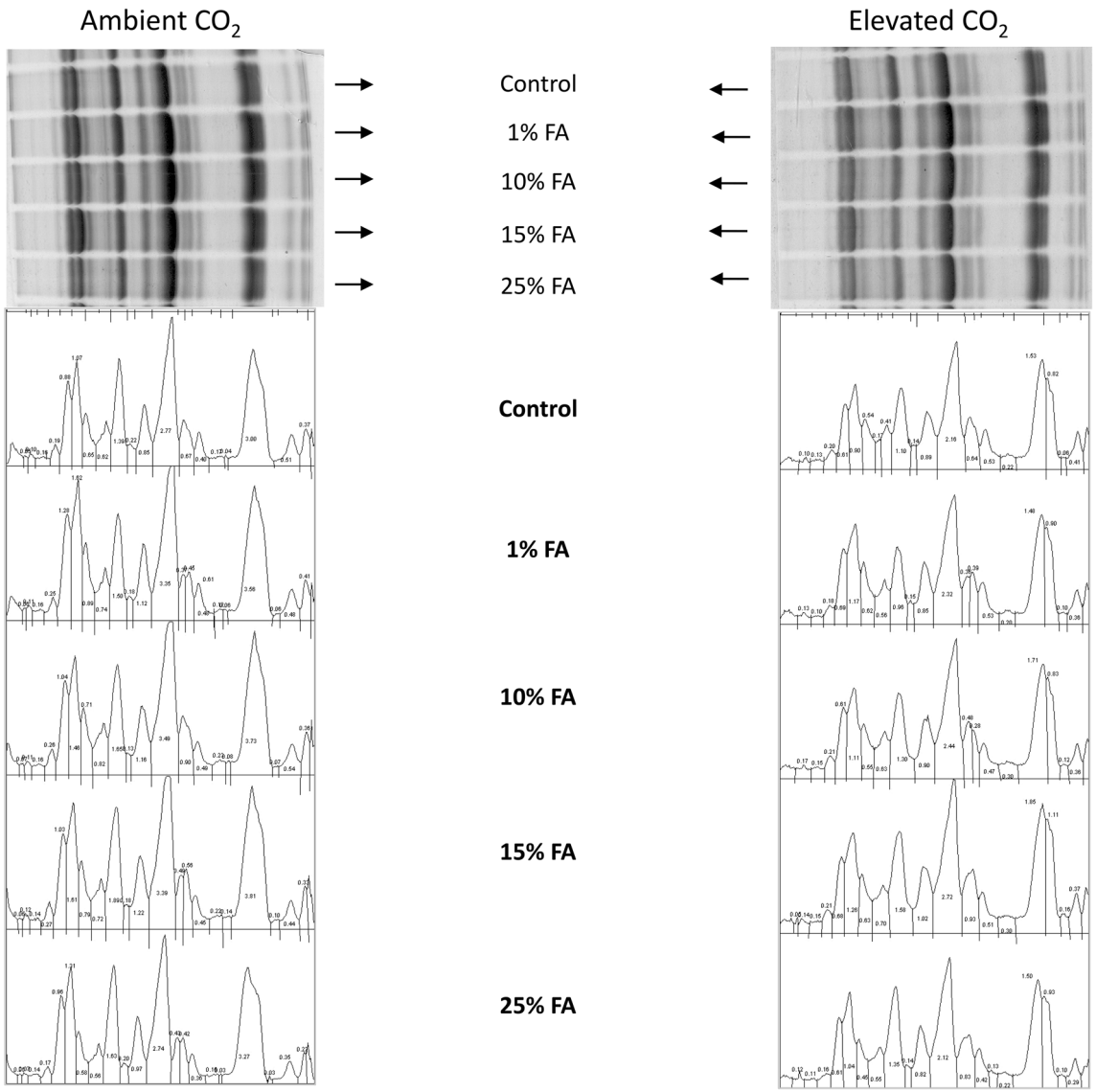

Fig. 2 Protein profile of soybean seeds obtained by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE) corresponding to different FA-amended soils and CO_2 levels

−0.62), and between oil yield and reducing sugars ($p < 0.03$, correlation coefficient −0.70), and a positive correlation between the content of starch and reducing sugars ($p < 0.03$, correlation coefficient 0.67).

It should be noted that no significant differences in the fatty acid composition (palmitic 16:0; stearic 18:0; oleic 18:1; linoleic 18:2; linoleic 18:3), oleic/linoleic ratios, or iodine values were observed in seeds (Table 5). Similarly, the protein profile of seeds obtained by SDS-PAGE showed no differences in the intensity or presence/absence of bands between both treatments (amended soils and CO_2 levels) (Fig. 2). Thus, bulk of proteins examined (storage proteins), which are the major constituents of seed proteins in soybean [14], showed not significant alterations under different CO_2 concentrations and FA-amended soils.

Conclusions

The growth parameters of soybean generally had direct positive relationships with elevated CO_2 and intermediate concentrations of FA in amended soils. On the other hand, pigment concentrations and carbohydrates showed different response patterns in relation to the growth stage and the association between amended soils and CO_2 condition. The quality parameters of soybeans (oil yield and total protein) were increased at high concentrations of FA-amended soils. Moreover, a synergistic effect between elevated CO_2 and FA-amended soils was observed for oil yield. However, as these results indicate that future CO_2 enrichment (600 ppm) and moderate proportions of FA-amended soils might improve some physiological parameters in soybean,

it would be necessary to evaluate the response of enzyme and compounds associated with the antioxidant defense system of soybean at different growth stages.

Acknowledgments This work was funded by the German Academic Exchange Service (DAAD) and Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) through scholarships to the author J. H. Rodriguez. Special thanks to the EnBW (Energie Baden-Württemberg company), Germany for providing the fly ash, to Agronomic Engineer L. Salines (INTA Marcos Juarez), for providing the soybean seeds and to Dr. J. Franzaring for setting the test program in the environmental chambers. The staff of the Institute of Landscape and Plant Ecology, Section Plant Ecology and Ecotoxicology, is thanked for the lab assistance and Mr. S. Weller for language revision.

Conflict of interest The authors declare that they have no conflict of interest.

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