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Ameliorative Effect of Chelating Agents on Photosynthetic Attributes of Cd stressed Sunflower

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Abstract: Excessive input of heavy metals in water reservoir and cultivated land primarily affect the growth and yield of crops. The aim of this work was to study the mechanism of Cd tocixity and damage to photosynthetic pigments and their efficiency and the potential of natural and synthetic chelators in assisting the phytoextractor sunflower plant. The pot experiment was laid out in a complete randomized way for Cd, chelators and hybrids at seedling, vegetative and reproductive stages with three replications. Cd affects the gas exchange parameters directly or indirectly by effecting the light and dark reactions, while indirect effect include inhibition of chlorophyll and carotenoids biosynthesis and degradation and alteration in Chl a/b ratio. Among two chelators, natural chelator OA found to be very supportive in ameliorating the Cd tocixity by phytoextractor in assistance to sunflower hybrid Hysun-33.

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Introduction

Environmental pollution has become a serious problem at global level due to human activities (Koptsik *et al*., 2003; Jarup, 2003). Urbanization and industrialization is proving hazardous to environment due to greater production of industrial wastes especially metallic elements (Wei and Yang, 2010; Yaylalı-Abanuz, 2011; Mireles *et al*., 2012).

Among metals, cadmium (Cd) is the most toxic pollutant of water, soil and atmosphere (Ranieri *et al*., 2005). Naturally it occurs in soil due to weathering of sedimentary parent rocks (Alloway, 1995). It exists in the form of sulphides, oxides, and carbonates in lead, zinc and copper ores (Anonymous, 2010). The industrial sources include phosphate fertilizers, nickel–cadmium batteries, pigments, plastics, ceramics and solar cells. The major culprits are phosphate fertilizers, Cd containing municipal waste and sewage sludge (ECB, 2007; Kabata-Pendias and Mukherjee, 2007; ATSDR, 2008). Other sources include burning of fossil fuels, mine tailings, waste and slag of smelter and urban refuse (Anonymous, 2010).

The irrigation of agricultural land with Cd containing waste water reduced the concentration of photosynthetic pigments such as chlorophyll a, chlorophyll b, total chlorophyll and carotenoids (Van Assche and Clijsters, 1990; Krupa *et al.*, 1996; Yang *et al*., 1996a; Macfarlane and Burchett, 2001; Tantrey and Agnihotri, 2010; Pooja *et al*., 2012). Exposure of plants to Cd causes leaf chlorosis which is most simple and visual indicator of toxicity of Cd (Hsu and Kao, 2003) as it disrupts the structure and function of chloroplast by inhibiting the functioning of chlorophyll biosynthesizing enzymes (Boddi *et al*., 1995; Krupa and Baszynski, 1995; Siedlecka *et al*., 1997; Pence *et al*., 2000).

The higher Cd concentrations decreased the photosynthetic activity (Kovacs *et al.,* 2005) by reducing chlorophyll synthesis, by reacting with porphobilinogen deaminase and d-ALA dehydratase –SH group, resulting in prophyrins and ALA (the intermediates of cholorophyll biosynthesis) (Padmaja *et al*., 1990; Shakya *et al*., 2008) and by producing chlorophyllide through protochlorophillide photoreduction (Stobart *et al*., 1985).

Chelating agents either synthetic (EDTA, DTPA, HEDTA) or natural (oxalic acid, citric acid, acetic acid) are commonly used as amendments to induced phytoextraction (Nascimento *et al*., 2006). EDTA is the most extensively used synthetic chelator for induced phytoextraction which dissolves the bonds between the metal and soil particles and promotes metal solubility and bioavailability for easy uptake by plants (Jean *et al*., 2008; Saifullah *et al*., 2009). Although the practice of using synthetic chelators such as EDTA, DTPA is very effective for phytoextraction of metals but their persistence in soil and low rate of degradation increases the risk of metals leaching and contamination of ground water (Luo *et al*., 2005; Saifullah *et al*., 2009)

Oxalic acid is a cheap environment friendly and quickly biodegradable natural chelator that can be used in phytoextraction without having a danger of metal leaching and contamination of ground water (Wu *et al*., 2003a; Niu *et al*., 2011). Its efficient role in Cd mobilization, translocation and phytoextraction has been extensively observed (de Melo *et al*., 2008; Oustan *et al*., 2011).

Materials and methods

A pot experiment was performed in Old Botanical Garden, University of Agriculture, Faisalabad, Pakistan. The prevailing climatic conditions at the time of experiment were 34 °C with 66% relative humidity in month of July 2015. Pots were filled with 10 kg soil and properly irrigated with water for maintaining suitable moisture content. Ten surface sterilized achenes (with 0.1% mercuric chloride) of two sunflower hybrids (Hysun-33 and FH-533) were sown at 1 inch depth in plastic pots. Treatments i.e. 0, 250 and 450 mg Cd/kg soil along with and without EDTA and OA @ 1g/kg soil each were applied in the rooting medium. Experiment was completely randomized with three factors factorial and three replications. After complete germination, four plants were kept for determination of chlorophyll and carotenoids pigments and gas exchange attributes. All the physiological and photosynthetic attributes were determined at seedling, vegetative and reproductive stages of plants

Gas exchange parameters includes net assimilation rate (*A*), transpiration rate (*E*), sub-stomatal CO_2 concentration (*Ci*), stomatal conductance (*gs*) and water use efficiency (*A/E*) were measured from a fully expanded youngest leaf by using an open system LCA-4 ADC portable infrared gas analyzer (Analytical Development Company, Hoddeson, England). The specifications /adjustments of IRGA were as follows: leaf surface area 11.35 cm^3, ambient CO_2 concentration (Cref) 342.12 µmol/mol, temperature of leaf chamber (Tch) varied from 39.2 to 43.9°C, leaf chamber volume gas flow rate (v) 396 ml/min, leaf chamber molar gas flow rate (U) 251 µmol/sec, ambient pressure (P) 99.95 kPa, molar flow of air per unit leaf area (Us) 221.06 mol/m^2/sec, PAR (Q leaf) at leaf surface was maximum up to 918 µmol/m^2.

The concentration of chlorophyll (a, b and total) was calculated following the method of Arnon (1949) and whereas carotenoids were calculated following the method of Davis (1976). Fresh leaves (0.2 g) were grind well and extracted in 80% acetone at - 4°C. The extract was centrifuged at 10000 rpm for five minutes at 4 °C. The optical density of the supernatant was measured at 663, 645 and 480 nm on spectrophotometer (Hitachi-220 Japan).

Results

a. Photosynthetic Pigments

The chlorophylls concentration (chl-a, chl-b, Total) significantly reduced in the presence of Cd in growth medium in both hybrids. Hybrids varied significantly at all three growth stages. FH-533 showed maximum reduction of 91.89% in chlorophyll-a contents (chl-a) by application of 450 mg Cd/kg at seedling stage while in Hysun-33 this reduction was 66.66% respectively as compared to control. EDTA alone significantly affected chl-a at reproductive stage and caused 12.12% and 13.63% reduction in chl-a content of Hysun-33 and FH-533 respectively (Fig. 1). The OA alone significantly reduced 335.55% chl-a in Hysun-33 and 16.21% in FH-533 only at seedling stage (Fig. 1). Cadmium addition @ 250 and 450 mg/kg in growth medium in the presence of 1g EDTA/kg imposed more severe effects on photosynthetic pigments of FH-533 than Hysun-33 as compared to control treated plants at vegetative and reproductive stages (Fig. 1). Cd x OA interaction was significant for seedlings of two hybrids. In the presence of 1 g OA/kg soil maximum reduction was observed in combination with 450 mg Cd/kg soil which caused reduction in chlorophylls and carotenoids contents of Hysun-33 and FH-533 respectively (Fig. 1).

b. Gas Exchange Attributes

Statistical analysis revealed that cadmium significantly affected the photosynthetic rate (A), transpiration rate (E), stomatal conductance (gs) and sub-stomatal CO_2 concentration (Ci) of treated plants at all three stages i.e. seedling, vegetative and reproductive stages. EDTA and OA alone had significant effects on gas exchange attributes only at seedling stage. Hybrids differed significantly throughout the studied period; Hysun-33 had better A, E, gs and Ci as compared to FH-533. Cadmium application @ 250 and 450 mg Cd/kg resulted in decrease of gas exchange attributes at seedling stage and this reduction was greater at reproductive stage of both the hybrids. EDTA significantly reduced the physiological attributes at seedling stage in both the selected hybrids i.e., Hysun-33 and FH-533 but found significant at reproductive stage for Ci. Cd x EDTA interaction was also significant for these parameters at seedling stage, so the addition of EDTA @ 1 g/kg in growth medium containing 250 and 450 mg Cd/kg slightly affected them as compared to Cd alone (Fig. 2). OA impact alone was statistically significant at vegetative and reproductive stage of two hybrids for Ci and OA plus Cd interactive effect was proved significant for A, E and gs only at seedling stage. The impact of OA alone was mild as compared to combined application with Cd and 1 g OA/kg + 450 mg Cd/kg affected the gas exchange attributes of two hybrids greater than 1 g OA/kg + 250 mg Cd/kg respectively (Fig 2-3).

From analysis of variance it is clear that all the main factors i.e., Cd, OA and EDTA proved non-significant alone and in combination for water use efficiency of sunflower hybrids throughout the studied. Only significant factor was hybrid at seedling stage. Minimum water use efficiency (1.11) µmol CO_2/mmol H_2O was measured in FH-533 under 250 mg Cd/kg application at vegetative stage and maximum (3.19) µmol CO_2/mmol H_2O was maintained by Hysun-33 under 250 mg Cd/kg application at seedling stage.

All the interactions for hybrids, Cd and chelating agents remained statistically non-significant for pigments and gas exchange attributes at seedling, vegetative and reproductive stages.

Discussion

a. Pigments

Reduction in the concentration of photosynthetic pigments such as chl-a, chl-b, chl-T and accomplice pigments like carotenoid has been found a common symptom of metal toxicity in a number of species (Van Assche and Clijsters, 1990; Krupa *et al.*, 1996; Macfarlane and Burchett, 2001; Tantrey and Agnihotri, 2010; Pooja *et al*., 2012). Decline in photosynthetic pigments (Chlorophyll a, b and carotenoids) was noticed in sunflower under stress condition (Haseeb *et al*., 2015). Exposure of plants to Cd causes leaf chlorosis which is most simple and visual indicator of Cd toxicity (Hsu and Kao, 2003).

During present study Cd caused significant reduction in chlorophyll a, chlorophyll b, chl-T and accomplice pigment carotenoids especially in first harvest of study. Both chealtors i.e., EDTA and OA helped in reducing the damaging effects of Cd on pigments; however OA had given more assistance in ameliorating the damaging effects of Cd than EDTA. The sensitive hybrid FH-533 experienced more reduction in pigment contents than Hysun-33 (Fig. 4.50 to 4.57). The present results are in accordance with previous findings of Miao *et al*. (2012) who documented better pigment status of *Arundo donax* L. in the presence of chelating agent i.e., EDTA growing in Cd, Pb and arsenic contaminated soil. Markovska *et al*. (2013) also reported the improving effects of EDTA on photosynthetic pigments of *Tribulus terrestris.* Chen *et al.* (2011b) reported that increasing Cd levels in soil decrease the total chlorophyll of plants. Similarly in pea plants the negative relation observed between chlorophyll a, b and carotenoid level and Cd concentration (Al-Hakimi, 2007).

The results can be defended by the argument that the better pigment status in chelators treated plants might be the result of increased concentration of Fe in the shoots which have influence on chlorophyll b structure furthermore formation of metal-chelator complex which is incapable to penetrate the membranes of plant hence chelators decrease the metal mobility and then its toxicity (Ruley *et al*., 2006).

Cadmium reduced the chlorophyll concentration (Vijayaragavan *et al*., 2011; Touiserkani and Haddad, 2012) by removing the Mg ion from its binding position in chlorophyll (Kupper *et al*., 1995) resulting in degradation of chlorophyll molecule (Otero *et al*., 2006) or by inhibiting the activities of enzymes involved in cholorophyll biosynthesis like protochlorophyllide reductase (Mysliwa-Kurdziel *et al*., 2004) porphobilinogen deaminase (Walley, 2005) aminolevulinic dehydratase (Noriega *et al*., 2007) thus causing deficiency in Fe^{2+} and Mg^{2+} supply necessary for chlorophyll synthesis, and also causing inhibition of carbonic anhydrase activity due to Zn^{2+} scarcity (Van Assche and Clijsters, 1990).

Broadly, in interpretation of the high redox potential of Cd it is interpreted that during biosynthesis of photosynthetic pigments the reductive steps are inhibited due to Cd stress. In addition the activity of vital enzyme protochlorophyllide reductase, responsive for protochlorophyll reduction into chlorophyll known to be repressed (De Filippis and Pallaghy, 1994).

b. Gas exchange parameters

In the present investigated study photosynthetic rate (*Pn*), transpiration rates (*E*), stomatal conductance (*gs*) and substomatal CO_2 concentration (*Ci*) were decreased and water use efficiency (*WUE*) increased under the treatment of Cd, EDTA and OA in combination or by their separate application in all harvest but this increasing and decreasing pattern in gas exchange parameters were more dominating during first harvest time. These trends are more pronounced when Cd was separately added in growth medium. According to present findings EDTA and OA application along with Cd helped in improving the gas exchange parameters of sunflower hybrids. The comparison between chelating agents showed that OA proved more helpful in reducing the adverse effects of Cd on gas exchange parameters than EDTA (Fig. 2). The results of present research work are in line with the findings of Markovska *et al*. (2013) who reported improvement in gas exchange parameter i.e., *Pn, E, gs, Ci* and WUE under the influence of EDTA of *Tribulus terrestris* growing in metal contaminated soil. In present study the improved efficiency of gas exchange parameters by chelators (EDTA, OA) application might be due to formation of chelate with Cd which reduced the noxious effects of Cd as also reported by (Chen and Cutright, 2001). Markovska *et al*. (2013) also reported the beneficial effect of EDTA on gas exchange parameters including transpiration rate, stomatal conductance and net photosynthetic rate. Transpiration is a vital process for the enhancement of water soluble components or uptake of contaminants and flux to the upper plant parts. The greater WUE in (Cd+chelator) treated plants can be interpreted as plants effort to improve their water regime. Improved WUE is largely a meaning of reduced water use then overall/net improvement in production of plant or biochemistry of assimilation (Baszyfiski *et al*., 1980).

Some previous investigations showed the negative effects of Cd on *Pn*, *E* and *Ci* in *Brassica napus* (Wan *et al*., 2011), Pea and barley (Januskaitiene, 2010), Maize (Krantev *et al*., 2008). Reduction in photosynthetic rate due to Cd toxicity might be the result of reduction in chlorophyll contents by its reaction with porphobilinogen deaminase and d-ALA dehydratase –SH group, resulting in prophyrins and ALA the intermediates of cholorophyll biosynthsis (Padmaja *et al*., 1990; Shakya *et al*., 2008) by producing chlorophyllide through protochlorophillide photoreduction (Stobart *et al*., 1985). In mitochondria and chloroplast for heme and chlorophyll synthesis ALA formation is the rate controlling and regulating step during tetrapyrrole biosynthetic pathway (Porra and meisch, 1984). Cd restricts ALA synthesis (Parekh, 1990) reduce the chlorophyll synthesis and many other photosynthesis related reactions (Marschner, 1983; Siedlecka and Baszynski, 1993) by causing Fe insufficiency (Marschner, 1983). The heme and chlorophyll biosynthesis is interrupted by Cd, as it reacts with the –SH functional groups like ALA dehydratase, ALA synthase, protochlorophyllide reductase and PBG deaminase (Prasad and Prasad, 1987).

Previous reports confirmed the increase in transpiration rate of plants due to low Cd levels, which, however, decreased due to higher Cd concentrations. In *Phragmites australis* 50 µM Cd had no effect on this parameter, however it decreased under 100 µM Cd treatment (Pietrini *et al.*, 2003). Higher Cd concentrations caused structural abnormalities in stomata resulting in rudimentary flawed stomata with reduced size and number, leading to depreciate stomatal conductance and modified rate of transpiration (Perfus-Barbeoch *et al*., 2002; Greger and Johansson, 2006; Kaznina, *et al*., 2011).

Under high Cd stress plants close their stomata to decrease the transpiration rate (Greger and Johansson, 2006). Its physiological explanation is that plants store the water reserves when Cd limits the water uptake efficiency through roots (Veselov *et al*., 2003)

Cadmium (10 and 100 µM) affects the *gs* in tomato plants (Lopez-Millan *et al*., 2009). The defective stomata of Cd stressed plants do not function appropriately (Greger and Johansson, 2006) so inverse proportion found between increasing Cd concentration and decrease in *gs* (Dong *et al*., 2005).

Cadmium disturb the water relations of pea plants and reduce the WUE and consequently caused reduction in water uptake and its translocation to above ground parts (Singh *et al.*, 2008). Contradictory reports were documented by Januskaitiene (2010) that WUE increased as a result of more suppression in transpiration rate than *Pn*.

Present results advocate that Cd may have a straight inhibitory influence on stomatal closing and opening that may lead to reduced stomatal conductance thus disturbing gasses exchange and eventually cause reduction in photosynthetic rate (Vassilev *et al*., 2004). Higher Cd concentration has also been reported to result in photosynthetic inhibition nonspecifically due to smashed structure of chloroplast (Fediuc and Erdei, 2002), impaired chlorophyll synthesis or its elevated breakdown (Baryla *et al*., 2001), disturbed electron transport chain (Krupa *et al*., 1996), diminished activities of enzymes of Calvin cycle (Vangrosveld and Clijsters, 1994) and CO_2 deficiency in chloroplasts due to stomatal closure (Singh and Singh, 1987; Chen *et al*., 2011).This can eventually results in severe decrease in photosynthetic rate under Cd stress as observed in present study. Furthermore, the K ion concentration drops down by elevation in external Cd concentration. It is now well reported that K is a crucial nutrient required for stomatal regulation (Taiz and Zeiger, 2006). The decrease in K concentration, as experienced in present investigation could directly modify the K fluxes through membranes of guard cell thus imiting the stomatal conductance and finally photosynthesis (Sharma and Agrawal, 2005).

Conclusion

Cd affects the gas exchange parameters directly or indirectly by effecting the light and dark reactions. The direct effect of Cd on light reactions is on photosynthesis O_2 evolution, photophosphorylation and reduction of NADP, while indirect effect include inhibition of chlorophyll biosynthesis and degradation and alteration in Chl a/b ratio.

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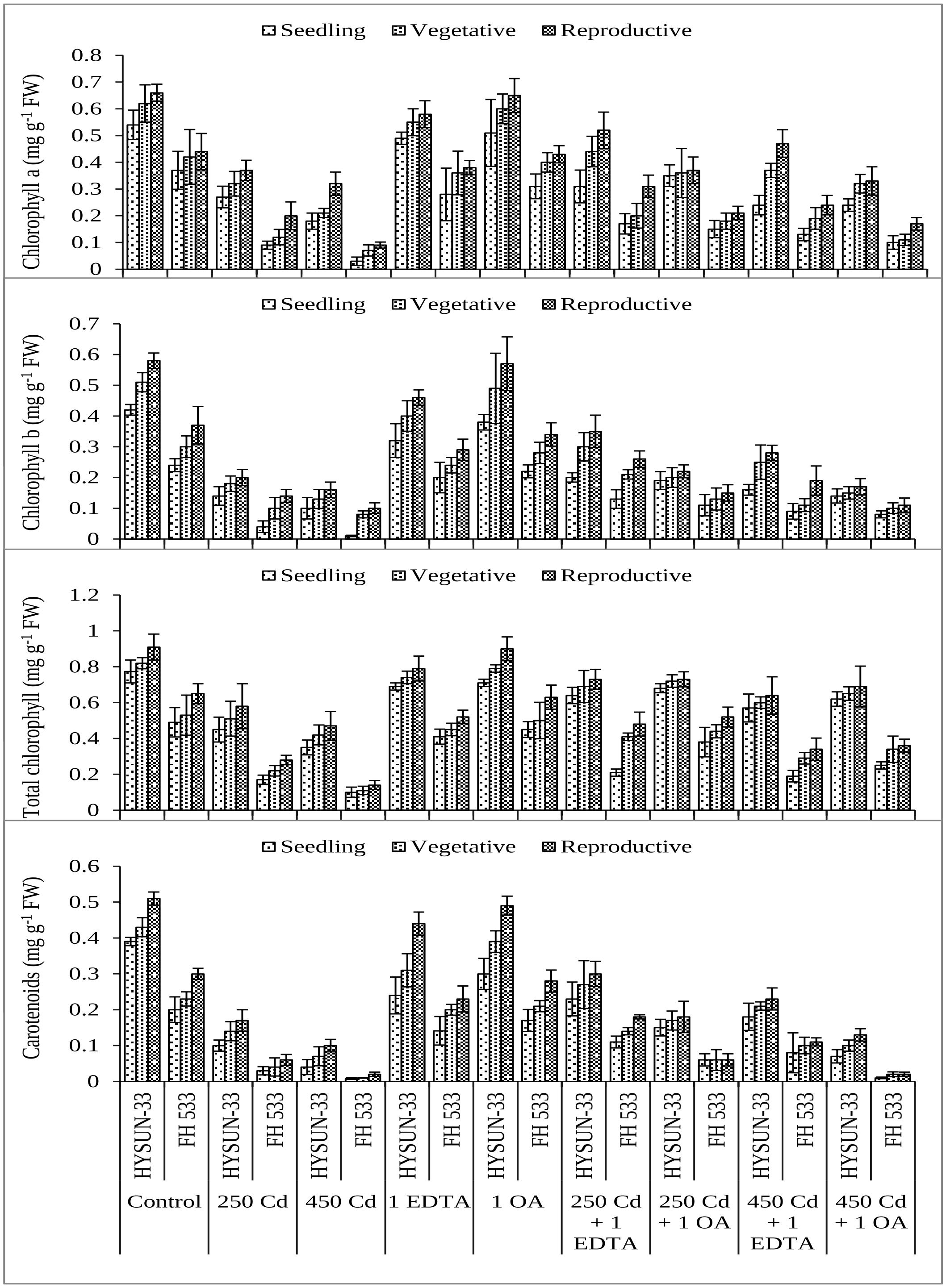

Fig 1. Effect of 250 and 450 mg/kg Cd along with EDTA (1 g/kg) and OA (1 g/kg) on pigments concentration of two sunflower hybrids (HYSUN-33 and FH-53) at seedling, vegetative and reproductive stages.

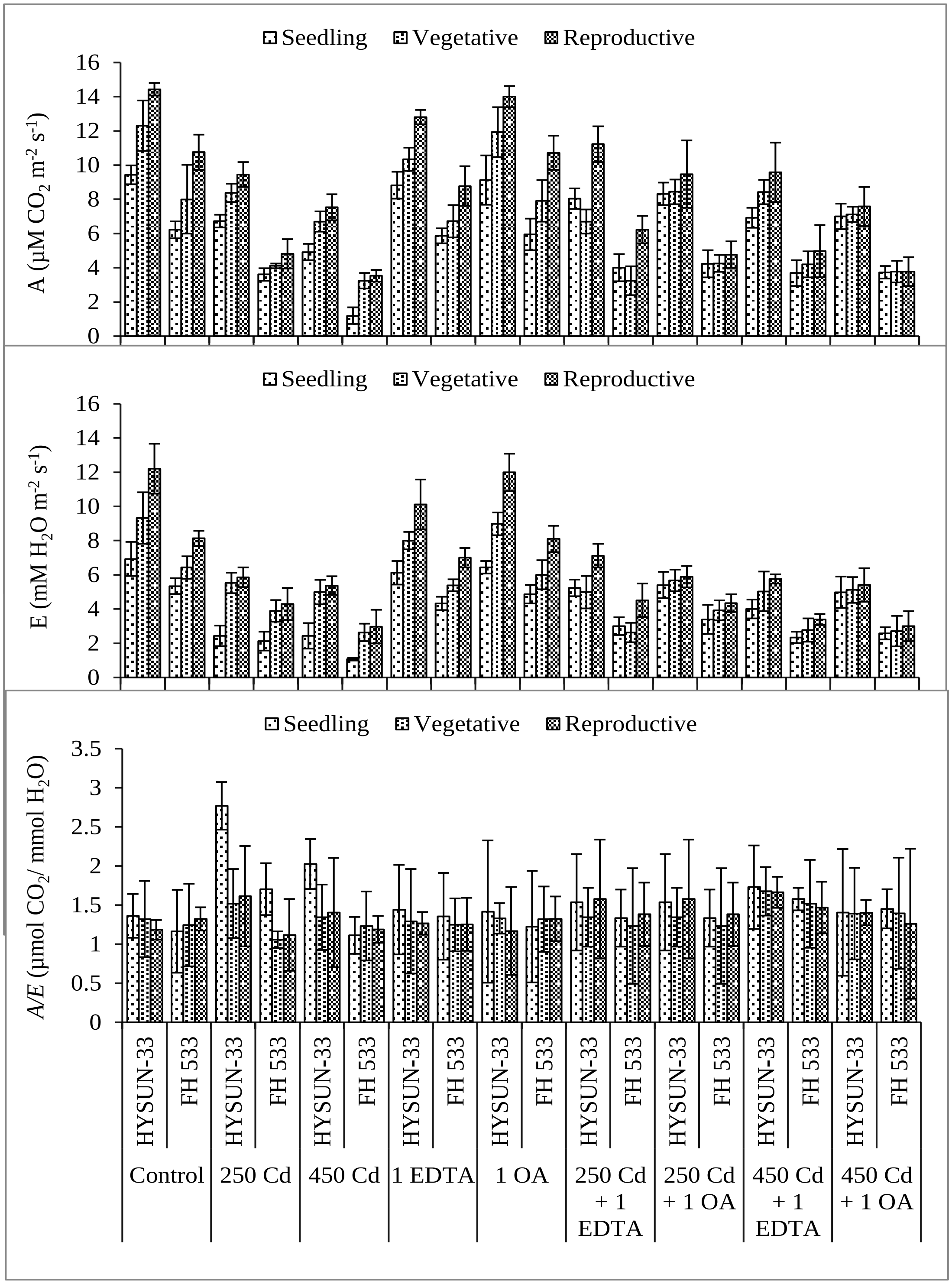

Fig 2. Effect of 250 and 450 mg/kg Cd along with EDTA (1 g/kg) and OA (1 g/kg) on net assimilation rate (*A*), transpiration rate (*E*) and water use efficiency (*A/E*) of two sunflower hybrids (HYSUN-33 and FH-53) at seedling, vegetative and reproductive stages.

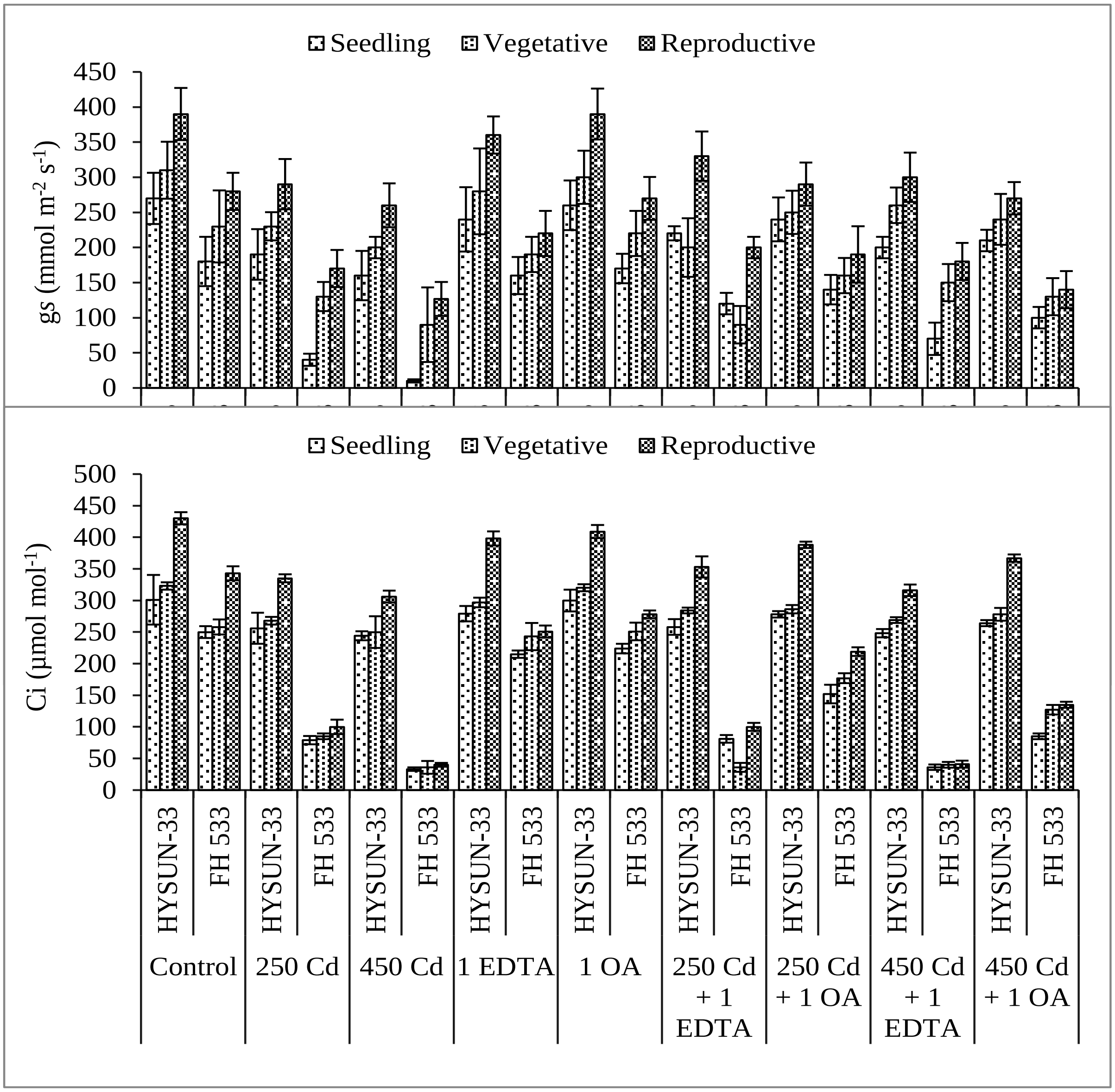

Fig 3. Effect of 250 and 450 mg/kg Cd along with EDTA (1 g/kg) and OA (1 g/kg) on sub-stomatal CO_2 concentration (*Ci*) and stomatal conductance (*gs*) of two sunflower hybrids (HYSUN-33 and FH-53) at seedling, vegetative and reproductive stages.

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