IVAN LUIS ZENZEN

PHYSIOLOGICAL RESPONSES TO MILD CADMIUM STRESS OF DIFFERENT TOMATO GENOTYPES WITH CONTRASTING ABSCISIC ACID LEVELS

Dissertation presented to Universidade Federal de Viçosa as part of the requirement of the Post-Graduate Program in Plant Physiology for obtention of the degree of *Magister Scientiae.*

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Dissertation presented to Universidade Federal de Viçosa as part of the requirement of the Post-Graduate Program in Plant Physiology for obtention of the degree of *Magister Scientiae*.

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RESUMO

ZENZEN, Ivan Luis, M.Sc., Universidade Federal de Viçosa, novembro de 2010. **Respostas fisiológicas ao estresse moderado por cádmio em diferentes genótipos de tomateiro com níveis contrastantes de ácido abscísico.** Orientador: Marcelo Ehlers Loureiro. Co-orientadores: Fábio Murilo DaMatta e Francis Júlio Fagundes Lopes

Os metais pesados, especialmente o cádmio, têm se tornado um dos principais agentes de estresse abiótico em plantas superiores em função de sua alta toxicidade e aumento dos níveis de liberação no meio ambiente. Apesar toxicidade destes elementos, as plantas desenvolveram mecanismos que lhes conferem aumento na tolerância a esta condição de estresse através de adaptações físicas e ativação de uma rede integrada de respostas celulares e moleculares que começam a atuar após o início do estresse. Relatos de alterações fitohormonais durante o processo de aclimatação ao Cd envolvendo o ácido abscísico (ABA) são escassos na literatura, e os poucos existentes são relativos a toxicidade aguda, uma situação distinta daquela que normalmente ocorre no meio ambiente. Em vista disso, esta pesquisa propôs-se a elucidar um potencial papel do ABA sobre os mecanismos bioquímicos e fisiológicos de aclimatação e tolerância ao estresse crônico por Cd, utilizando para tanto o tomateiro mutante *notabilis* deficient em ABA, uma linhagem transgênica complementada *notabilis* $_{complemented\ 13}$, e seu tipo selvagem. Um padrão de resposta distinta das plantas notabilis pode ser apontado pelo aumento da absorção de Cd, uma elevada taxa de transpiração e redução do potencial hídrico foliar, combinado a inalterações da taxa de concentração de CO_2 entre a câmara sub-estomática e a ambiente (C_i/C_a), e da composição isotópica de carbono ($\delta^{13}C$), além de redução da condutância estomática (g_s) e da eficiência do uso da água (WUE), sob tratamento com este metal pesado. Apesar da maior atividade das enzimas antioxidantes superóxido dismutase (SOD) e catalase (CAT) na ausência de Cd, *notabilis* teve maior peroxidação lipídica em suas raízes. Limitações da g_s causadas pelo Cd aparentam ser o principal motivo da redução da taxa líquida de assimilação de carbono (A) em plantas do tipo selvagem e notabilis $_{complemented\ 13}$, ao passo que *notabilis* apresenta várias alterações negativas nos parâmetros fotoquímicos da fotossíntese, implicando em uma redução transitória no potencial de absorção da luz, reduzida conversão de energia para fotoquímica, e maior perda regulada de energia no fotossistema II, que podem explicar, pelo menos em parte, a redução da A. A complementação do mutante demonstrou recuperação do fenótipo para vários parâmetros para um patamar semelhante ao das plantas do

tipo selvagem, reforçando a hipótese que a síntese de ABA desempenha um papel chave na aclimatação das plantas ao metal.

ABSTRACT

ZENZEN, Ivan Luis, M.Sc., Universidade Federal de Viçosa, November 2010. **Physiological responses to mild cadmium stress of different tomato genotypes with contrasting abscisic acid levels.** Adviser: Marcelo Ehlers Loureiro. Co-advisers: Fábio Murilo DaMatta and Francis Júlio Fagundes Lopes

Heavy metals, especially cadmium have become one of the main abiotic stress agents for higher plants because of their high toxicity and increasing levels released in the environment. Despite the poisonous of these elements, plants have evolved mechanisms by which they increase their tolerance to this stress condition through both physical adaptations and activation of an interactive network of cellular and molecular responses that begin after the onset of stress. Information about phytohormonal changes during the Cd acclimation process involving abscisic acid (ABA) are scarce in literature, and the few existent depict the acute toxicity, a distinct situation from that which normally occurs in the environment. In view of that, this research purposed to find out a potential role of ABA on physiological and biochemical acclimation mechanisms and tolerance to chronic Cd stress, using the tomato plants ABA-deficient mutant *notabilis*, a transgenic complemented line *notabilis* $_{complemented\ 13}$, and their *wild type*. A different response pattern of *notabilis* plants could be pointed due increased Cd uptake, an elevated transpiration rate and reduced leaf water potential, combined with unaltered sub-stomatal-to-ambient CO_2 concentration ratio (C_i/C_a) and carbon isotopic composition ($\delta^{13}C$), a reduced effect on stomatal conductance (g_s), and on water use efficiency (WUE) under treatment with this heavy metal. Despite the higher activity of the antioxidative enzymes superoxide dismutase (SOD) and catalase (CAT) in absence of Cd, *notabilis* have higher lipid peroxidation in their roots. Limitations in g_s caused by Cd appear to be the main reason of reduction in net carbon assimilation rate (A) of *wild type* and *notabilis* $_{complemented\ 13}$ plants, whereas *notabilis* has several negative changes in photosynthesis photochemistry parameters that implicate in transient reduction in light absorption potential, lower photochemical energy conversion, and increased energy loss in photosystem II through a regulated non-photochemical mechanism that may explain, at least in part, the reduction in A. The complementation of the mutant showed to recovery several phenotype parameters close to *wild type* plants, strengthening the hypothesis that ABA synthesis has a key function in plant acclimation to Cd.

INTRODUCTION

Pollution of terrestrial and aquatic ecosystems by heavy metals has become a critical environmental concern due their potential hazards upon living organisms. Cadmium (Cd) is a non-essential and highly toxic metal pollutant that occurs naturally in soil at low concentrations, although global release by anthropogenic sources and its consequent widespread occurrence since the Industrial Revolution, increased drastically the level of this element in Earth crust, conferring a prominent position as soil contaminant (Sanita di Toppi and Gabrielli, 1999).

Bioconcentration in aquatic and terrestrial organisms, and a powerful multiplier effect of biomagnification through different trophic levels in the food chain, mean that even low levels of Cd deposition in environment may result in significant heavy-metal concentration, which can seriously threat human health (McConnel and Edwards, 2008). The main entry of this element in food chain is through plant crop systems, and it is often associated with the ability of the species to uptake and transport this element to the edible plant tissues (McLaughlin *et al.*, 2006). Cd acquiring process and the resulting content depends basically on its concentration in the soil and bioavailability, which are modulated by several intrinsic parameters, such as the presence of organic matter, pH, redox potential, temperature and concentrations of other elements. Uptake of Cd ions is known to be due by competition for the same binding site in transmembrane carrier of some nutrients, among which potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), manganese (Mn), copper (Cu), zinc (Zn), nickel (Ni) and other specific mineral transporters (Clarkson and Luttge, 1989; Clemens *et al.*, 1998; Williams and Salt, 2009).

Cytotoxic and genotoxic effects of Cd on plant biological systems are associated with a dose-dependent manner. At cellular level, toxicity may result basically from the binding of Cd^{+2} ions to sulphydryl groups in proteins, leading to an inhibition of activity or disruption of structure; through mimic other divalent nutrient ions as Ca^{2+}, and therefore interfering in several physiological processes mediated by this element, e.g. elicitation of signaling cascades; and finally through generation and accumulation of free radicals and reactive oxygen species (ROS) within the tissues (Benavides *et al.*, 2005). In addition, toxic effects of this pollutant include growth inhibition, losses in production yield, imbalance in nutrient uptake and homeostasis, leaf chlorosis caused by pigment degradation, inhibition of enzyme activities, alterations in stomatal behavior, transpiration, photosynthesis, and plasma membrane permeability, followed by reduction in water content, besides being they mutagenic potential, that causes an inactivation of

DNA mismatch repair system in yeast, human and plants cells (Barcelo and Poschenrieder, 1989; Stobart *et al.*, 1995; Hernandez *et al.*, 1996; Sandalio *et al.*, 2001; Jin *et al.*, 2003; Liu *et al.*, 2008; Solti *et al.*, 2008).

Overall, the susceptibility or tolerance to Cd in plants reflects the accomplishment of an interrelated network of physiological and molecular mechanisms that includes uptake, accumulation and detoxification of Cd ions in a coordinated action of multiple stress responsive genes, which also cross-talk with other components of stress signal transduction pathways (Benavides *et al.*, 2005; Tuteja, 2007). As a whole, plants need to make use of several mechanisms together, in order to establishing a homeostatic metabolic pattern to be able to cope with stress caused by heavy metals, implying in resort different strategies including epigenetics changes, induction of signaling events responsible for recruit a complex and robust cellular antioxidative system, adjustment of morpho-anatomical traits, and even activate or modify plant metabolism to allow adequate functioning of metabolic pathways and rapid repair of damaged structures (Prasad, 1999; Sanita di Toppi and Gabrielli, 1999; Cho *et al.*, 2003; Blanvillain *et al.*, 2008).

Usually, sensing and responses of plants to nonessential metal and/or metaloids are thought to be triggered by the damage occurring as a consequence of excessive exposure to these substances (Verbruggen *et al.*, 2009). Many studies on plant tolerance to heavy metals point distinct pathways of sensing and signaling, involving mainly ROS, Ca^{2+}, protein kinases, and phytohormones, that act alone or in an integrated agreement, resulting in biological changes at cell and organism levels responsible for an acclimation to the stress and consequent increased ability to survive in presence of these elements (Jonak *et al.*, 2004; Mithofer *et al.*, 2004; Maksymieck, 2007; Maksymieck and Kupra, 2007). In fact, phytohormones are important elicitors of signal transduction cascades. These molecules are associated with several developmental processes during plant life cycle, including responses to biotic and abiotic stress (McCourt, 2001; Wittenmayer and Merbach, 2005; Soldatova and Khryanin, 2010). The advent of molecular biology and genetics in recent decades, associated with several mutagenesis and mutant screening programs, provided a wide range of hormone-related phenotypes, a useful resource in studies of functional genomics to elucidate hormonal action mechanisms in plant biological systems. Study of mutants that have acquired changes or deletions in their nucleotide sequences is a advantageous practice in biology; since mutations can interrupt cellular processes, these organisms often hold the key to understanding gene function (McCourt

2001; Gazzarrini and McCourt, 2003). Experiments conducted with exposure of plants to the heavy metal Cd demonstrated alterations in metabolism of several phytohormones, such as jasmonate, abscisic acid, ethylene, and salicylic acid, however the mechanisms by which these substances act in response to heavy metals is rather controversial (Zhu, 2002; DalCorso *et al.*, 2008; Tamaoki, 2008).

Broadly, in a first step, the extracellular stress signal is perceived at membrane level by specific membrane receptors, ion channel, receptor-like kinase, histidine kinase among others, which activate intracellular complex signaling cascades including the generation and elicitation of secondary signal molecules such as Ca^{2+}, inositol phosphates, ROS and ABA that transduces inside the nucleus to induce multiple stress responsive genes, the products of which ultimately lead to plant adaptation and stress tolerance, directly or indirectly (Mahajan and Tuteja, 2005; Tuteja, 2007). A variety of stresses induce abscisic acid (ABA) synthesis, reason why it is considered the plant stress hormone. The ABA-mediated perceive and adaptive response to abiotic stress imposed by salt, cold, drought and wounding, is precisely due to the action of this phytohormone in a coordinated induction of many genes, which may cross-talk each others, whose resulting product reflects in the physiological or biochemical response to plant metabolism adjustment (Davies and Zhang, 1991; Shinozaki and Yamaguchi-Shinozaki, 1997; Swamy and Smith, 1999). Several transcription factors are known to regulate the ABA-responsive gene expression, since ABA-induced gene expression often relies on the presence of cis-acting elements called ABRE elements, demonstrating the importance of this phytohormone in the systemic process of plant acclimation to a stress circumstance (Yamaguchi-Shinozaki and Shinozaki, 1994; Uno *et al.*, 2000).

Currently, the pathway for ABA biosynthesis in higher plants is known in great detail, with all the major genes for the enzymes in the biosynthesis pathway identified, a credit of genetic and biochemical studies employing ABA-deficient mutants with impairment at some point of the ABA-biosynthesis pathway (Xiong and Zhu, 2003; Schwartz *et al.*, 2003). In the same way, functional analysis of ABA to determine physiological and metabolic responses to environmental factors implies in the use hormonal mutants and transgenic plants with altered expression patterns for specific pathway-step genes. Of special concern in these studies is a tomato mutant called *notabilis*, which has a genetic lesion affecting the oxidative cleavage step in ABA biosynthesis that generates the first C_{15}-specific intermediate of ABA. This mutation in nine-*cis*-epoxycarotenoid dioxygenase (*NCED*) enzyme, responsible for the oxidative cleavage

of the C_{40} carotenoid precursor of ABA, results in a deficiency of the plant hormone, with a consequently wilty phenotype caused by their susceptibility to water stress (Burbidge *et al.*, 1999). Complemented transgenic lines of this mutant with *LeNCED1* provide different degrees of phenotype recovery and corroborate with the relevance of ABA under some stress conditions (Thompson *et al.*, 2004).

Although well known benefits of ABA in plants under drought and salt conditions, very scarcely and superficial studies assessed the significance of this phytohormone in heavy metal stress tolerance, especially by Cd. In addition, studies of this nature often were done with high doses of this metal through short-term exposure, characterizing chronic toxicity and tolerance responses in plants. In this research, we aim to explore the role of ABA in Cd responses using ABA-deficient, normal and complemented plants, as well to detail plant response in long-term Cd exposure (28 days) under a very low toxicity level (12.5 µM), to uncover possible physiological and biochemical mechanisms of Cd tolerance and acclimation.

MATERIAL AND METHODS

Plant material and growth conditions

Seeds of tomato (*Solanum lycopersicon* Mill.) cv. Ailsa Craig (*wild type*), the mutant *Notabilis*, and transgenic line Notabilis complemented 13 ($Not_{.comp.13}$), were kindly provided by Prof. Andrew Thompson (Warwick University, England). *Notabilis* is a null mutant induced by a programme of X-ray mutagenesis (Stubbe, 1957), characterized by a frameshift mutation, consequence of a specific A/T base pair deletion of *LeNCED1* gene coding sequence (Burbidge *et al.*, 1999), whereas $Not_{.comp.13}$ is a *Notabilis* construction from a tomato genomic cosmid library, with a vector suitable for *Agrobacterium*-mediated plant transformation, and a insert size of 19 kb containing the *LeNCED1* full-length cDNA that aligns with region 5404-7570 bp; the putative transcription start site for *LeNCED1* in $Not_{.comp.13}$ is therefore at bp 5404, without introns within the region of cDNA (Thompson *et al.*, 2004).

The seeds were sown in styrofoam multi-cell trays (26 cm^3) with one seed per cell. The trays were filled with commercial substrate PlantMax® and placed in controlled environment chambers with a day length of 14 h, day/night temperatures of 25/20°C, and a photosynthetic photon flux density of 350 µmol photons m^{-2} s^{-1} (supplied by cool-white fluorescent lamps), at 18 cm above the base of the trays. At 6 days after emergence (DAE), seedlings were transplanted into 5000 cm^3 styrofoam boxes irrigated with one quarter strength Hoagland solution with 45 µM FeIII-EDTA (Hoagland and Arnon, 1950) for acclimation in greenhouse. The nutrient solution was aerated continuously, the pH was maintained around 6.0 by daily adjustment, the volume replaced every day with deionized water, and the complete solution renewed once a week. After one week, the plants were transferred into half-strength Hoagland nutrient solution with 45 µM FeIII-EDTA. Cadmium treatment were done stepwise: firstly at 6.25 µM Cd ($CdCl_2.4H_2O$) for 24 hours, and then at 12.5 µM Cd, being cultivated for 26 days under heavy-metal treatment, and the complete solution renewed once a week. Upon harvest, each plant was divided into root, stem, leaf and fruits. Root and leaf samples were immediately frozen in liquid nitrogen and stored at -80°C for biochemical analyses, and the remaining was dried at 65 °C during 72 hours for growth and elemental analysis. Each treatment was conducted in 6 biological samples, everyone represented by one single plant.

Growth parameters

Fruit production was determined by direct counting at harvest, taking to account only fruits with more than 20 mm of diameter. Dry weight (DW) of each plant fraction, root, stem, leaf and fruits were also determined.

Gas exchange and chlorophyll fluorescence measurements

Leaf gas-exchange parameters were determined using an infrared gas analyzer – IRGA (Li-6400XT, LI-COR Inc., Lincoln, NE, USA). The readings were taken once a week between 8:00 and 10:30 a.m., which was presumed to be the diurnal period when photosynthetic rates would be maximal, in a total of five points over 26 days. The IRGA chamber was attached to the weekly youngest fully expanded leaf, careful as possible to not disturb the leaf position. The process was conducted under ambient CO_2 (370 ± 15 μmol mol^{-1}), natural temperature and photosynthetic active radiation (PAR) conditions, providing data used for calculation of the transpiration rate (*E*), net carbon assimilation rate (*A*), stomatal conductance (g_s), leaf temperature (T_{leaf}) and sub-stomatal-to-ambient CO_2 concentration ratio (C_i/C_a).

Chlorophyll *a* fluorescence light-adapted parameters were reached simultaneously to gas exchange, over the same leaf area, using a LI-Cor 6400-40 leaf chamber fluorometer integrated to IRGA system, providing the minimal fluorescence (F_0'), maximal fluorescence (F_m') and electron transport rate through photosystem II (ETR). The dark-adapted fluorescence parameters were done at predawn of the respective day, on the same previously labeled leaves posterior used in gas exchange analysis as described. Dark-adapted leaves were first irradiated with a weak modulated measuring beam to obtain minimal fluorescence (F_0), while the maximal fluorescence (F_m) was determined following a light saturating pulse. The yield of variable fluorescence (F_v) was calculated as F_m-F_0. Maximum quantum yield of PSII (F_v/F_m) was also obtained from the previous chlorophyll fluorescence parameters mentioned above. In addition, quantum yield of photosystem II photochemistry (Φ_{PSII}), quantum yield of ΔpH and xanthophyll-regulated thermal energy dissipation (Φ_{NPQ}) and quantum yield of constitutive thermal dissipation (Φ_{NO}) were also calculated (Genty *et al.*, 1989; Hendrickson *et al.*, 2004). All fluorescence parameters were determined when the rate of change in the fluorescence signal (dF/dt) approached zero and CO_2 concentrations in the leaf chamber attain a steady state (60-120 s).

Water relations

Leaf water potential (Ψ) was obtained 26 days after the beginning of Cd treatments on young fully expanded leaves (at the 6^{th}-8^{th} nodes from the base). Measurements were taken at predawn (Ψ_{pd} = 04:30-06:30 h) and midday (Ψ_{md} = 12-14:00 h) on the 6 plant replicate for each treatment with a pressure chamber (Scholander *et al.*, 1965). Photosynthetic water-use efficiency (WUE) was calculated as the relative rates of exchange of CO_2 and water vapor between photosynthesizing leaves and the surrounding atmosphere (µmol CO_2 mol^{-1} H_2O). The instantaneous WUE can be expressed as the quotient of the diffusive fluxes of CO_2 into the leaf and water vapor out of the leaf during photosynthesis (Farquhar & Richards, 1984).

Analysis of Cd and mineral micronutrient concentrations

Plants were separated into root, stem, leaf, and fruit fractions. The roots were washed with distilled water immediately after shoot harvesting in order to eliminate external soluble metal residues present in tissue surface. For Cd and the micronutrients Fe, Mn, Zn, and Cu analysis, samples were oven dried at 65 °C for 72 h, subsequently weighed and ground with glass beads in a bead mill homogenizer (Mini-Beadbeater-96, BioSpec Products, Bartlesville, OK, USA). The digestion was accomplished in a microtube dry block at 100°C with nitric:perchloric acid (4:1) until sample colorless, and the residues resulting from this process were diluted to 5 ml in water filtered through a MilliQ system to at least 18 MΩ resistivity. Mineral elemental analysis was carried out by optical inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 3300 DV, PerkinElmer, Waltham, MA, USA). The experiment was run with 6 biological samples per treatment and 3 replicates for each sample. The root-to-shoot translocation was estimated as the percentage of Cd present in the shoot compared with the whole plant.

Lipid peroxidation

Lipid peroxidation was determined by estimating the content of thiobarbituric acid reactive substances (TBARS), following the method proposed by Heath and Packer (1968), and adapted by Cakmak and Horst (1991). Approximately 0.05 g of frozen root and leaf tissue samples were ground in liquid nitrogen with a bead mill homogenizer, homogenized with 0.5 ml

of 1% trichloroacetic acid, and centrifuged at 15.000 g for 10 min at 4 °C. Aliquots of 0.25 ml from each supernatant were added to 0.75 ml of 0.5% thiobarbituric acid in 20% trichloroacetic acid in a new tube, and the mixture was heated at 90 °C for 25 min in a shaking water bath. After this incubation period, tubes were transferred into an ice bath and then centrifuged at 10.000 g for 5 min at 4 °C. The absorbance from the resulting supernatant was read at 532 nm, and corrected for non-specific absorption and turbidity by subtracting the absorbance at 600 nm. The concentration of malondialdehyde (MDA) equivalents were calculated using an extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as total thiobarbituric acid-reacting substances (TBARS=MDA) in terms of nmol mg^{-1} protein present in plant extract.

Carbon isotope composition ratio

Carbon isotope composition ratio ($\delta^{13}C$) was determined in leaf tissues from whole plants. For this purpose, samples were oven dried at 65 °C for 72 h, subsequently powdered in a bead mill homogenizer and weighed. Relative abundance of stable carbon isotopes was measured using isotope ratio mass spectrometer (IR-MS, SerCon ANCA-GSL, UK), and $\delta^{13}C$ was expressed as $^{13}C/^{12}C$ ratio ($\delta^{13}C$) relative to Pee Dee Belemnite standard (PDB), in parts per thousand (‰), according Farquhar *et al.* (1989).

Pigment analysis

Leaf samples of approximately 0.2 g from natural radiation illuminated leaves were frozen in liquid nitrogen and stored at -80°C until pigment analysis. Each frozen sample was homogenized in 2 ml of cold acetone (85%), the homogenate bubbled with N_2 gas for 1 min, and stored in darkness for 30 min at 0°C in a closed flask prior to centrifugation for 5 min at 3.000 *g*. The supernatants obtained were filtered through a 0.45 µm Millipore filter, and diluted to a known volume with cold acetone (85%). A reversed-phase HPLC (Hewlett Packard, 1050 Series HPLC System, CA, USA) analysis of the pigments was carried out on a C_{18}, 5 µm Spherisorb ODS-2 column (250 x 4.6 mm), according to method described by Young and Britton (1990) and Johnson *et al.* (1993). The elution of carotenoids and chlorophylls was performed at room temperature (23-24°C) over 24 min, with a 0.8 ml min^{-1} flow rate, using an optimized non-linear

gradient 25-100% ethylacetate in acetonitrile/water (9:1 [v/v], containing 0.1% triethylamine). The column was allowed to re-equilibrate for 5 min in 25% ethylacetate in acetonitrile/water (9:1 [v/v], containing 0.1% triethylamine), at a flow rate of 1 ml min^{-1}. This system resulted in a satisfactory separation of carotenoids, including neoxanthin, violaxanthin, antheraxanthin, zeaxanthin, lutein, α and β-carotene, chlorophyll *a* and *b*. Compounds were identified by their absorption spectra and retention times relative to respective standards (VKI, Horsholm, Denmark)

Antioxidative enzyme activities

The enzyme extraction steps were carried out at 4°C unless stated otherwise. Approximately 0.1g of frozen leaf and root plant tissues (-80°C) were weighed and homogenized in liquid nitrogen with glass beads in a bead mill homogenizer (Mini-Beadbeater-96, BioSpec Products, Bartlesville, OK, USA) with 0.1 M potassium phosphate buffer pH 6.8 (3:1 buffer volume:fresh weight) containing 0.1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulphonylfluoride (PMSF) and 1% (w/v) polyvinylpolypyrrolidone (PVPP), according to Peixoto *et al.* (1999). The homogenate was centrifuged at 12.000 *g* for 15 min, and the supernatant stored in separate aliquots at -80°C, prior to superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and total peroxidase (POX) analyses. Total protein was determined by the Bradford method (Bradford, 1976).

The assay system for SOD was adapted from the method of Giannopolitis and Ries (1977) for use with a spectrophotometric microplate reader. The assay mixture was composed by 0.05 M potassium phosphate buffer (pH 7.8), containing 13 mM methionine, 75 µM *p*-nitroblue tetrazolium chloride, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 2 µM riboflavin, that was added to 10 µl of crude enzymatic extract, reached a total volume of 250 µl per plate well. The reaction was started by exposing the microplate to light, provided by cool-white fluorescent lamps in a reaction chamber at 25°C during 5 min, afterward illumination was suspended. The color intensity of blue-formazan formed in the reaction mixture was measured at 560 nm in an ELISA microplate reader (VersaMax Tunable Microplate Reader, Molecular Devices Inc., CA, USA). In this assay, an additional non-illuminated reaction with the same mixture was taken as a blank for each sample, and a system devoid of enzymes served as negative chromogen formation

control. The activity was expressed as units SOD mg^{-1} protein (Beauchamp and Fridovich, 1971).

CAT activity in roots and leaves was determined according to the method of Havir and McHale (1987). The assay medium consisted of 0.05 M potassium phosphate buffer (pH 7.0) containing 12.5 mM H_2O_2, in a final volume of 750 µl. The reaction was initiated by the addition of 50 µL of crude enzymatic extract, and decrease in absorbance of H_2O_2 was recorded at 240 nm for 2 min at 25°C on a double-beam spectrophotometer (Shimadzu UV-2550, Shimadzu Corp., MD, USA) against 0.05 M potassium phosphate buffer (pH 7.0), used as the blank. The enzyme activity was calculated from the constant rate of the enzyme activity, using the extinction coefficient of H_2O_2 of 36 M^{-1} cm^{-1} at 240 nm, and expressed as µM H_2O_2 min^{-1} mg^{-1} protein.

APX activity was measured according to Nakano and Asada (1981), modified by Koshiba (1993). The assay reaction solution contained 0.05 M potassium phosphate buffer (pH 6.0), 0.8 mM ascorbic acid, and 1 mM H_2O_2, and reaction started with addition of 50 µL enzyme extract, in a final volume of 750 µl. The decrease in the concentration of ascorbic acid was recorded at 290 nm for 2 min at 25°C on a double-beam spectrophotometer (Shimadzu UV-2550, Shimadzu Corp., MD, USA) against 0.05 M potassium phosphate buffer (pH 6.0), used as the blank. The enzyme activity was calculated from the constant rate of activity using the extinction coefficient of ascorbic acid of 2.8 mM^{-1} cm^{-1} at 290 nm, and expressed as µM ascorbic acid min^{-1} mg^{-1} protein.

Activity of POX enzymes was measured in a reaction medium composed by 0.025 M potassium phosphate buffer (pH 6.8), 20 mM pyrogallol, and 20 mM H_2O_2 (Kar and Mishra, 1976). Reaction started with addition of 25 µL enzyme extract, in a final volume of 750 µl. Increase in purpurogallin concentration was recorded at 420 nm for 2 min at 25°C on a double-beam spectrophotometer (Shimadzu UV-2550, Shimadzu Corp., MD, USA) against 0.05 M potassium phosphate buffer (pH 6.8), used as the blank. The enzyme activity was calculated from the constant rate of activity using the extinction coefficient of purpurogallin of 2.47 mM^{-1} cm^{-1} at 420 nm, and expressed as µM purpurogallin min^{-1} mg^{-1} protein.

Data analysis

Statistical analysis was performed using a PC-based SAS program (SAS Institute, 1996) and Excell (Microsoft Office 2007). The t-test (Excell 2007 algorithm, Microsoft Office 2007) was employed to compare changes between Cd treatment and Tukey's test (SAS Institute, 1996) to compare different genotypes within the same heavy metal treatment. Statistical significant differences were considered in both analysis when the P value ≤ 0.05. All values reported in this work are means of six independent biological samples, except for pigments HPLC analysis were three independent biological samples represent mean values. All results are expressed as means followed by corresponding standard deviations and index letters indicating statistical differences between the means.

RESULTS

Cadmium accumulation and allocation

Cd was not detected in control plants from any genotype by the method employed, whereas all plants showed Cd accumulation when they were grown in presence of this metal. The ABA-deficient mutant *Notabilis* absorbed approximately 30% more Cd, compared to *wild type* and the transgenic line *Not.*$_{compl.13}$, which did not differ in accumulation (Fig. 1A). The highest amounts were found in roots, accounting for more than 95% of total Cd absorbed in each genotype, of which *Notabilis* showed the highest (Fig. 1B). The smallest amounts were detected in stems (Fig. 1D), and no differences between genotypes could be verified, as was also observed in leaves (Fig. 1C). In fruits, no Cd was detected under the experimental conditions adopted.

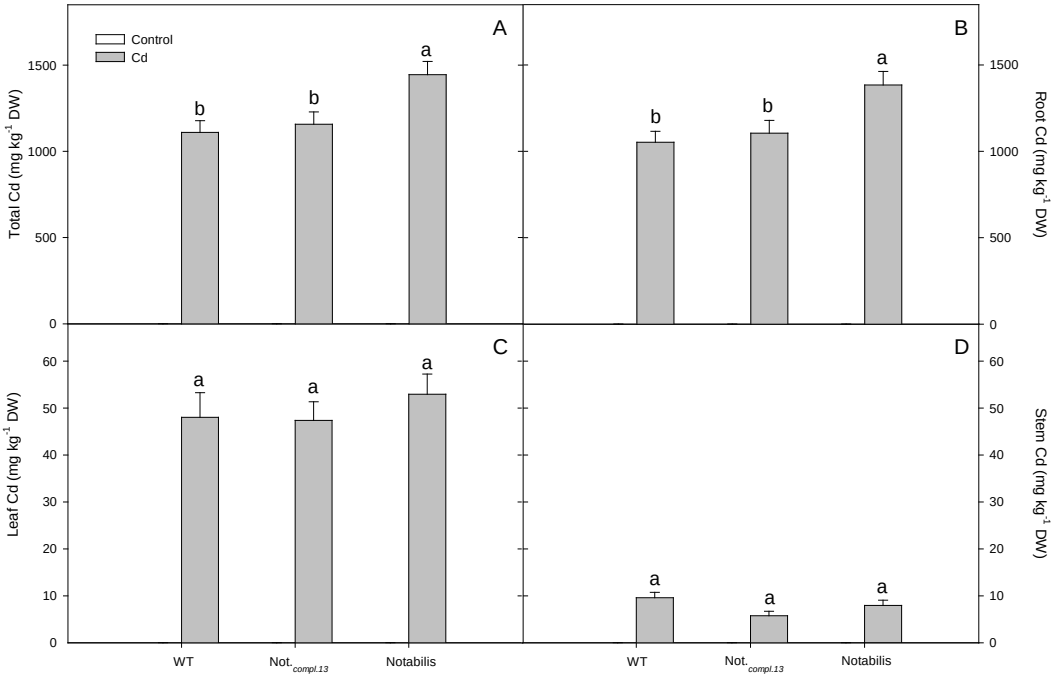

Fig. 1. Cd accumulation (mg kg^{-1} dry weight) in whole plant (A), roots (B), leaves (C) and stems (D) of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.$_{compl.13}$) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter are not significantly different at $P < 0.05$ (Tukey's test).

Lipid peroxidation

Comparing Cd-treated plants with their respective genotype controls, the metal concentration supplied did not lead to lipid peroxidation, neither in root (Fig. 2A) nor in leaf (Fig. 2B) tissues. However, the ABA-deficient mutant has higher oxidative injury in roots (Fig. 2A).

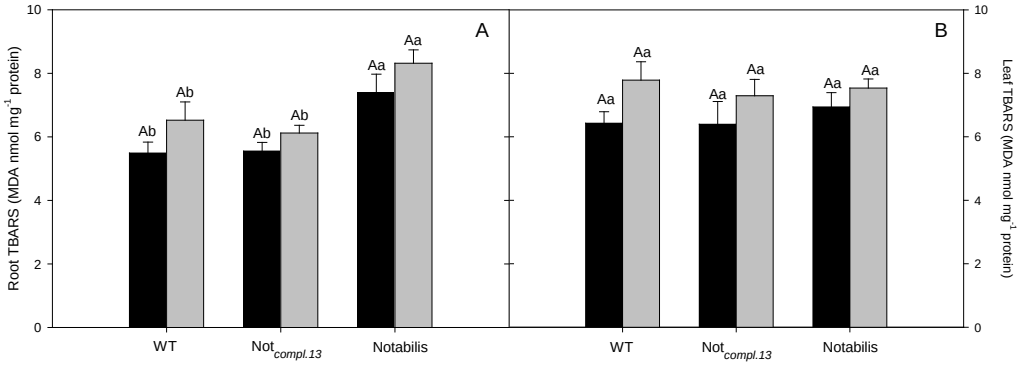

Fig. 2. Effect of Cd stress on lipid peroxidation (MDA nmol mg^{-1} protein) in roots (A) and leaves (B) of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.$_{compl.13}$) and *Notabilis* after 26 days of growth in hydroponics in absence (control – black) or in presence of Cd (12.5 µM Cd - grey). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test). Abbreviations: TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde.

Changes some micronutrients

In absence of Cd, the mutant *Notabilis* and its complemented genotype *Not.*$_{compl.13}$ had higher total iron and manganese (Fig. 3A and D), mainly due the higher levels of both metals in roots (Fig. 3B and E), since no difference was observed in their shoots (Fig. 3C and F). *Notabilis* did not differ from *wild type* plants for total, root and shoot Zn (Fig. 3G, H and I), as well as for total Cu (Fig. 3J), whereas it had a slight higher amount of Cu in root and a lower one in shoot (Fig. 3K and L). For both, iron and copper, Cd caused an increase in total and root concentrations in *wild type* and in the mutant *Notabilis* (Fig. 3A, B, J and K), while for *Not.*$_{compl.13}$ Cd increased only the total, root and shoot copper (Fig. 3J, K and L). Total and root concentration of zinc decreased by Cd treatment only for *Not.*$_{compl.13}$ (Fig. 3G and H), whereas for shoot this exposure is associated with reduction of Zn in all genotypes (Fig. 3I). The presence of Cd decreased total and root manganese in *Notabilis* and *Not.*$_{compl.13}$ (Fig. 3D and E), reaching levels similar to those observed in *wild type* plants, whereas Mn increased in shoots in both cited genotypes (Fig. 3F). Exposure of plants to Cd reduced iron and manganese content in young fruits of all plants analyzed, while zinc decreased only in *Not.*$_{compl.13}$ and *Notabilis*. Additionally,

copper concentration increased in *wild type*, but decreased in fruits of the other genotypes (Supplementary. Fig. 1E, 2E, 3E and 4E).

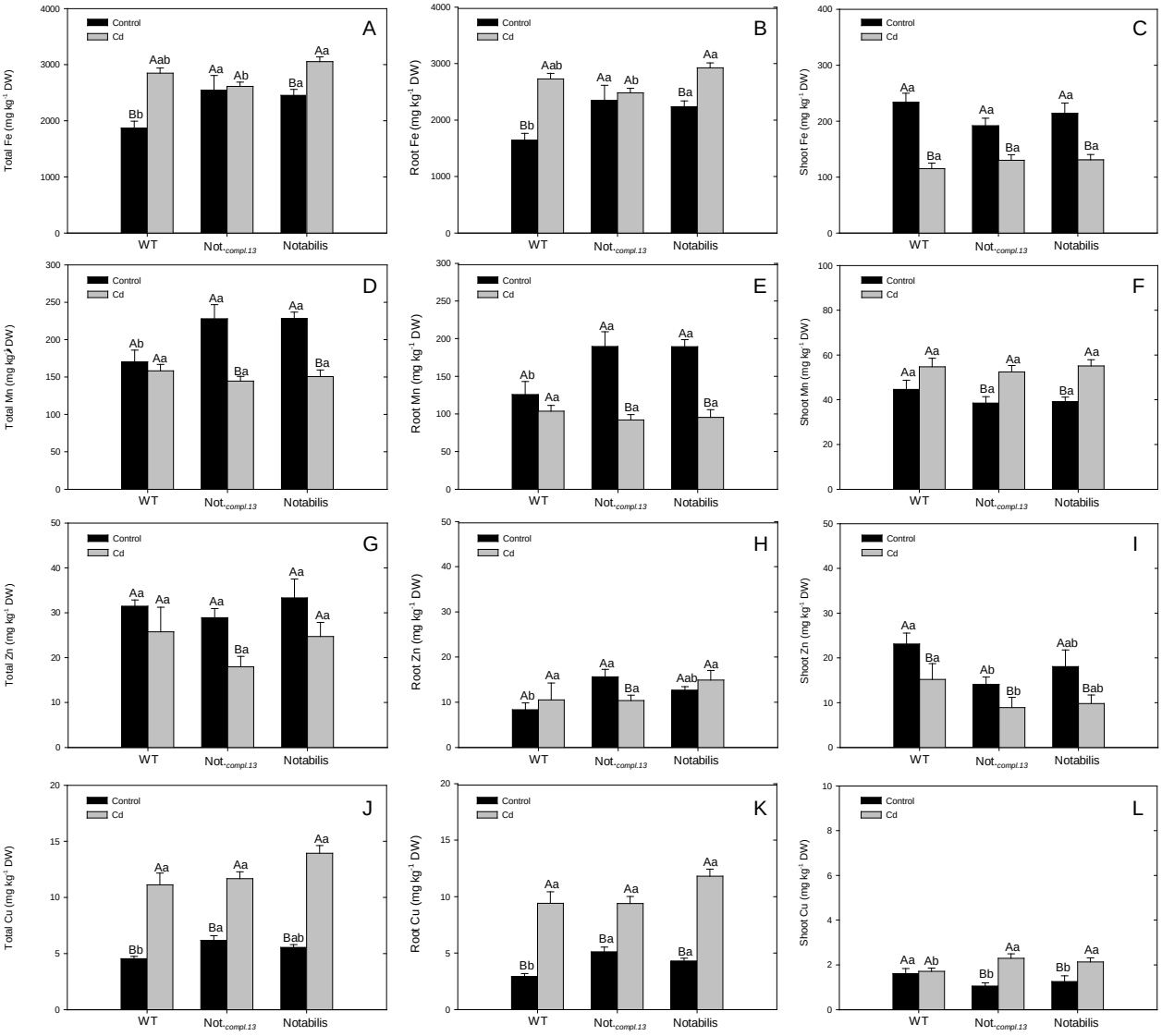

Fig. 3. Micronutrient concentration (mg kg^{-1} dry weight) in whole plant (A, D, G, J), roots (B, E, H, K) and shoots (C, F, I, L)) of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.*compl.13*) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

Gas exchange and leaf temperature

Cd treatment reduced stomatal conductance (g_s) in all genotypes in distinct patterns, and in a transient way for *Notabilis*. The maximal reductions reached 65 and 58% in *wild type* and *Not.compl.13*, respectively, and occurred at the 5th day after heavy metal exposure, even though a partial recovery occured, g_s of Cd-treated plants from these genotypes remained below their

controls (Fig. 4A and B). In *Notabilis*, decrease in g_s accomplished just 27%, and occurred only on the 12th day of Cd treatment, with a subsequent recovery to values similar to the control plants (Fig. 4C). In absence of Cd, *wild type* had lower g_s compared to *Notabilis*, whereas *Not.*$_{compl.13}$ showed intermediate values. Under Cd treatment, *wild type* and *Not.*$_{compl.13}$ did not show difference in g_s between them, being approximately 42% lower than *Notabilis* (Fig. 4A, B and C).

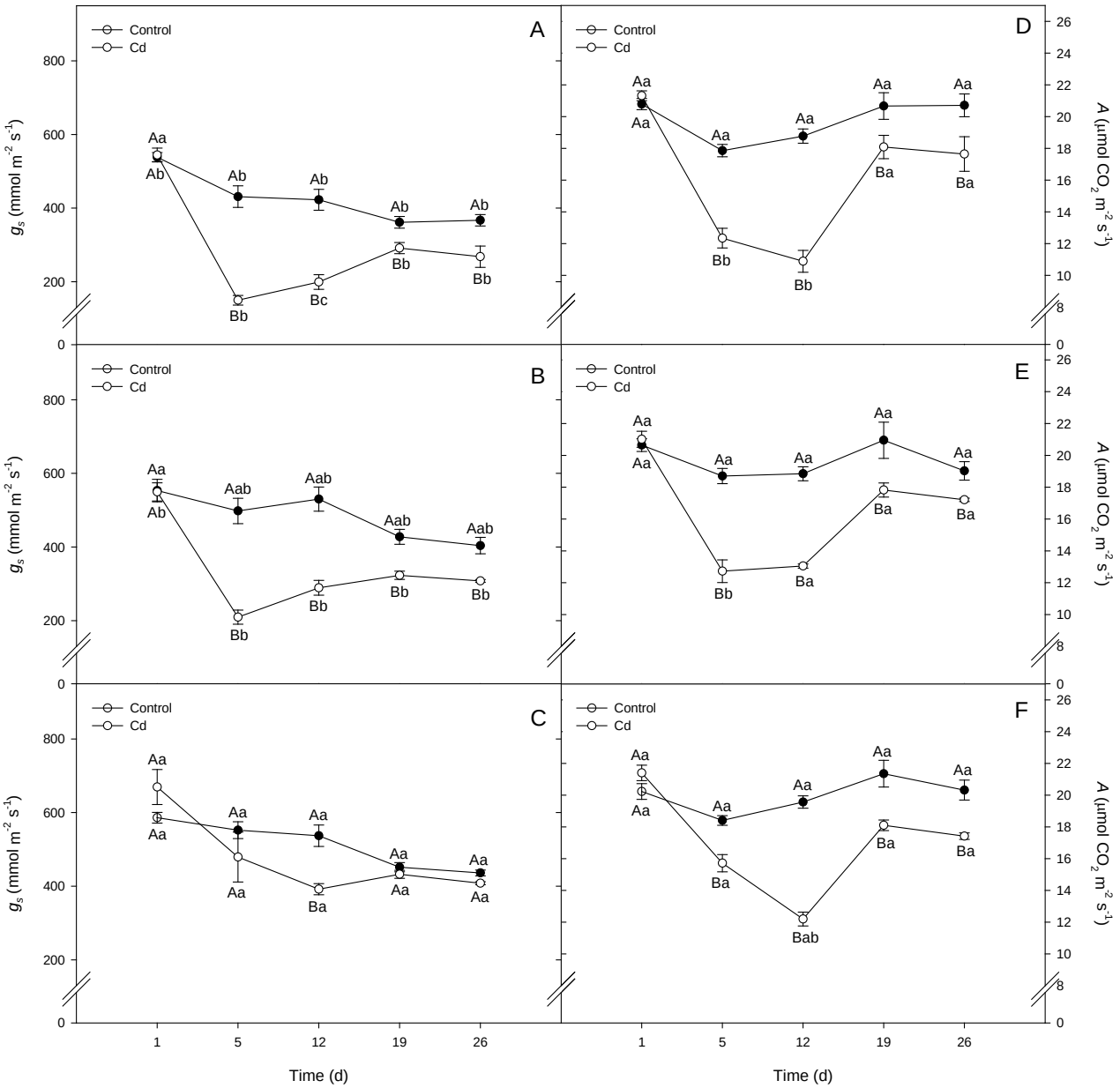

Fig. 4. Gas exchange parameters, stomatal conductance (g_s, in mmol m^{-2}s^{-1}), and net carbon assimilation rate (A, in µmol CO$_2$ m^{-2} s^{-1}) in leaves of *L. esculentum* plants *wild type* (A and D), *Notabilis complemented line 13* (B and E), and *Notabilis* (C and F) through 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at P < 0.05 (Tukey's test).

Net carbon assimilation rate (*A*) was affected in all genotypes by Cd treatment, having a temporary effect similar to observed for changes in g_s for *wild type* and *Not.*$_{compl.13}$, but not at all for the mutant. On the 5th day of Cd exposure, *wild type*, *Not.*$_{compl.13}$ and *Notabilis* plants showed a decrease of 31, 32 and 15% in *A*, respectively (Fig. 4D, E and F). This reduction was intensified on the 12th day to 42 and 38% in *wild type* and *Notabilis* plants, although it remained unchanged in *Not.*$_{compl.13}$.

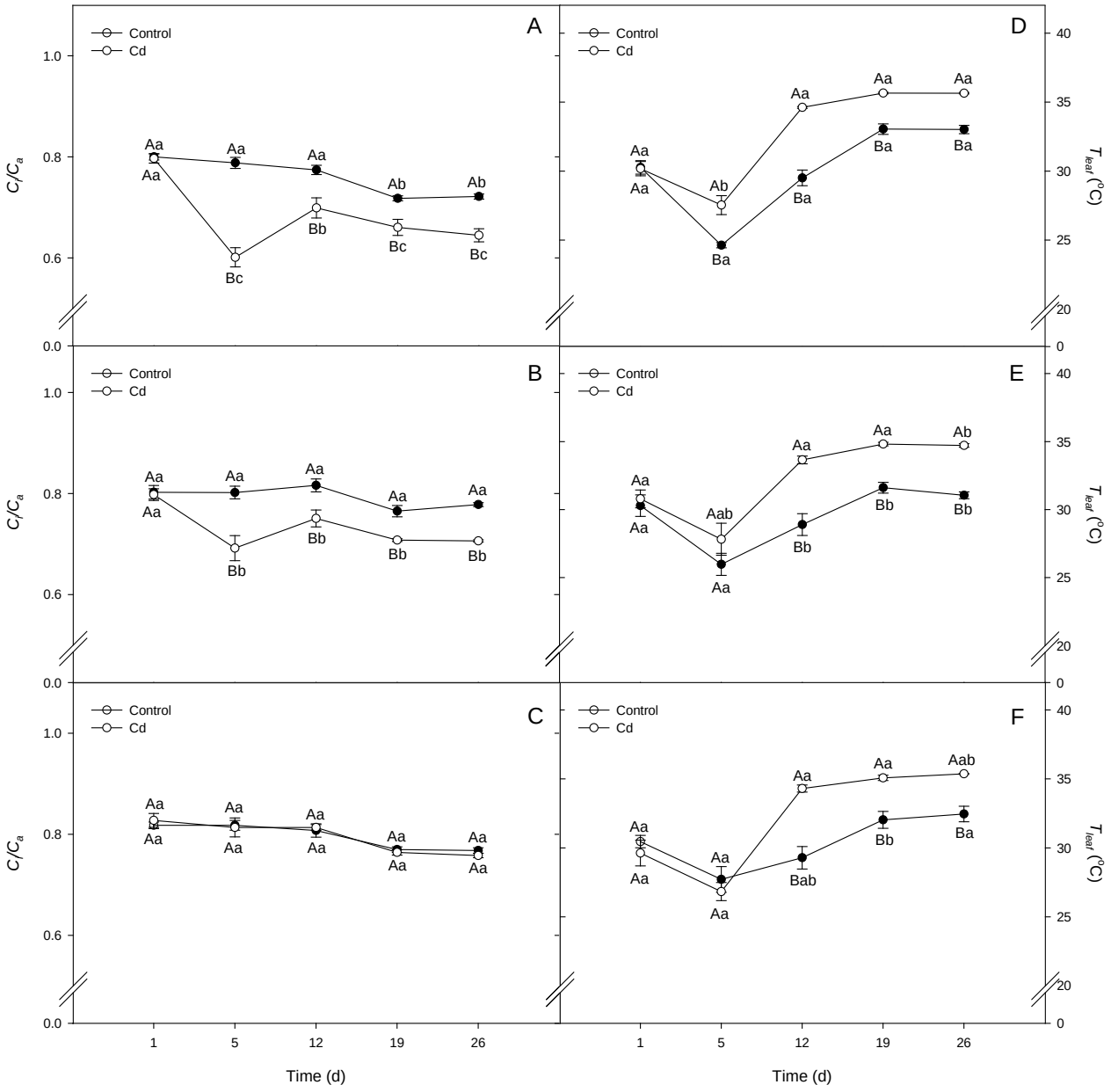

Fig. 5. Gas exchange sub-stomatal-to-ambient CO_2 concentration ratio (C_i/C_a ratio), and leaf temperature (T_{leaf}, in °C) in leaves of *L. esculentum* plants *wild type* (A and D), *Notabilis complemented line 13* (B and E), and *Notabilis* (C and F) after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

A partial recovery of *A* in Cd-treated plants was detected for all genotypes on the 19th day, when values reached approximately 15% below their respective control plants. *Notabilis* had a slower decrease in *A* compared to *wild type* and *Not.*$_{compl.13}$, and despite the fact that at the level used Cd produced no significant reduction in g_s at the 19th and 26th days, *A* on this period was significant lower (Fig. 4D, E and F). Cd caused a continuous decrease in C_i/C_a ratio in *wild type* and *Not.*$_{compl.13}$ plants after 5 days of treatment, a fact not observed for the mutant *Notabilis* (Fig. 5A, B and C). Increase in leaf temperature, especially in *wild type* and *Not.*$_{compl.13}$, was reflect of decrease in stomatal conductance. A significant increase of 3°C in *wild type* T_{leaf} was observed already on the 5th day of Cd exposure (Fig. 5D), although this change occurred only at the 12th day of Cd treatment in *Notabilis* and *Not.*$_{compl.13}$ plants (5E and F). This increase in T_{leaf} was maintained in all genotypes until the 26th day of heavy metal treatment.

Carbon isotopic composition

Figure 6 shows the effects of Cd treatment on $\delta^{13}C$ values in leaves of *wild type*, *Not.*$_{compl.13}$ and *Notabilis* plants. In absence of Cd, *Notabilis* showed higher $\delta^{13}C$, followed by the transgenic line *Not.*$_{compl.13}$, and then *wild type* plants. However, when *wild type* and *Not.*$_{compl.13}$ plants were grown in the presence of Cd, there was a substantial enrichment of ^{13}C in their leaf tissues, about 7 and 2‰ respectively, compared to their controls, as expected from decrease in stomatal conductance. Conversely, the ABA-mutant *Notabilis* did not show difference in ^{13}C discrimination between control and Cd-treated plants, and consequently $\delta^{13}C$ remain unchanged.

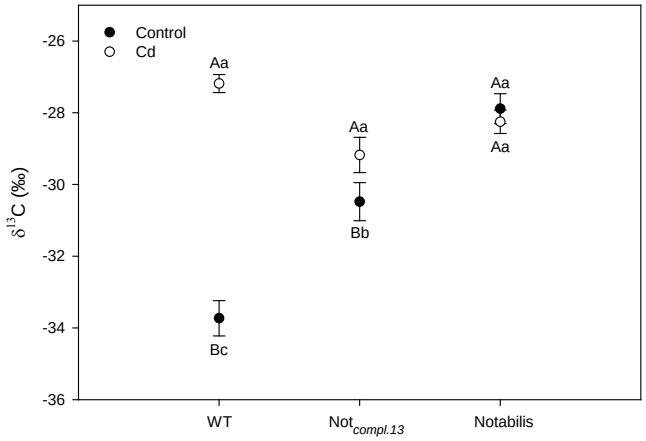

Fig. 6. Carbon isotopic composition ($\delta^{13}C$) in leaves of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.$_{compl.13}$) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

Water relations

Temporal changes in transpiration (*E*) differed between *Notabilis* and other genotypes. In presence of Cd, *wild type* and *Not.*$_{compl.13}$ plants had an initial decrease of approximately 40% in transpiration on the 5th day, with a subsequently recovery and maintenance of *E* levels similar that found in control plants until the 26th day (Fig. 7A and B). In contrast to other genotypes, *Notabilis* did not show this initial decrease in *E* caused by Cd, but an increase of 40% from the 12th day of treatment, sustaining these high rates until the end of the experiment (Fig. 7C).

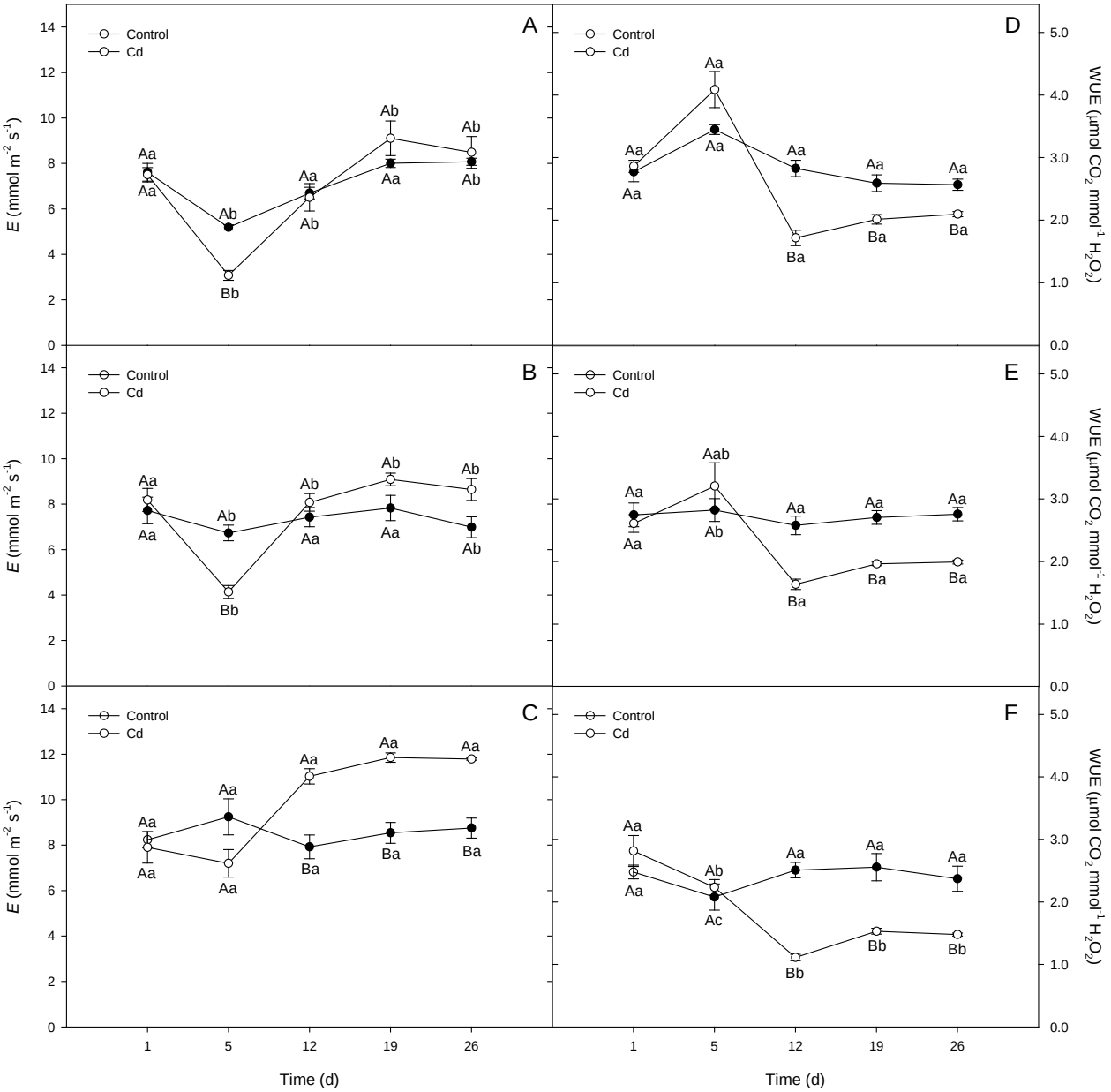

Fig. 7. Transpiration rate (*E*, in mmol m^{-2} s^{-1}), and water use efficiency (*WUE*, in µmol CO_2 $mmol^{-1}$ H_2O_2) of *L. esculentum* plants *wild type* (A and D), *Notabilis complemented line 13* (B and E), and *Notabilis* (C and F) after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

Plants grown without Cd did not display significant differences in *WUE* (Fig. 7D, E and F), although adverse effects of different intensity were observed among genotypes when plants were exposed to the heavy metal treatment. In *wild type*, Cd decreased *WUE* in 40% on 12th day, with a subsequent partial recovery to approximately 20% less than control plants on the 19th and 26th days (Fig. 7D). Similar to *wild type*, *Not.*$_{compl.13}$ had a decrease of 36% in *WUE* on the 12th day of Cd exposure, reaching to 27% less than their respective control plants on 19th and 26th days (Fig. 7E). *Notabilis* was more drastically affected by Cd, where *WUE* had a higher reduction, corresponding to 56% on 12th day, and recovery was less intense, to around 40% lower than control plants on the remaining period (Fig. 7F).

The Cd concentration employed did not affect predawn leaf water potential within genotype, although significant differences were observed among them. In absence of Cd, in agreement with $\delta^{13}C$ measurements, both predawn water potential (Ψ_{wpd}) and midday water potential (Ψ_{wmd}), were approximately 170% lower in *Notabilis* than in *wild type* plants, while an intermediate behavior was observed for *Not.*$_{compl.13}$, with Ψ_{wpd} and Ψ_{wmd} of 46 and 26% lower than *wild type*, respectively (Fig. 8A and B). An effect of cadmium on leaf water potential was observed only at midday and for the mutant, which decreased 41% (Fig. 8B).

The hydration recovery at night, reflected by the Ψ_{wpd}, indicates a different potential among the genotypes, which was compromised in *Not.*$_{compl.13}$ and even more in *Notabilis*, both in presence or absence of Cd (Fig 8A and B).

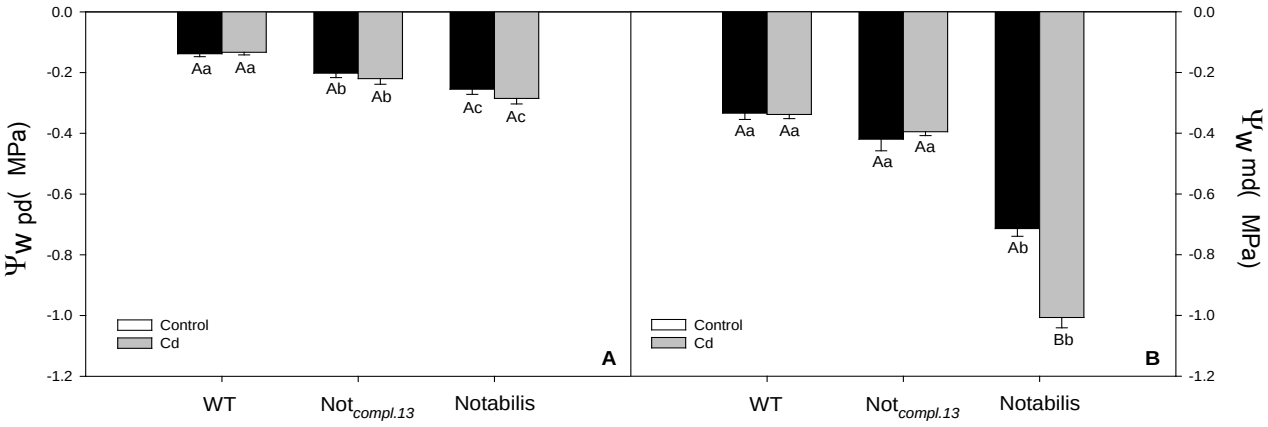

Fig. 8. Leaf water potential at predawn (A), and at midday (B) of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.$_{compl.13}$) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

Chlorophyll fluorescence

As shown in Fig. 9A, B and C, predawn potential quantum yield of photosystem II values (F_v/F_m) were always above 0.8 in all genotypes for both, control and Cd-treated plants. However a small but significant decrease in F_v/F_m was observed in *Notabilis* and *Not.*$_{compl.13}$ at the 19th and 26th day, a fact not observed in control plants.

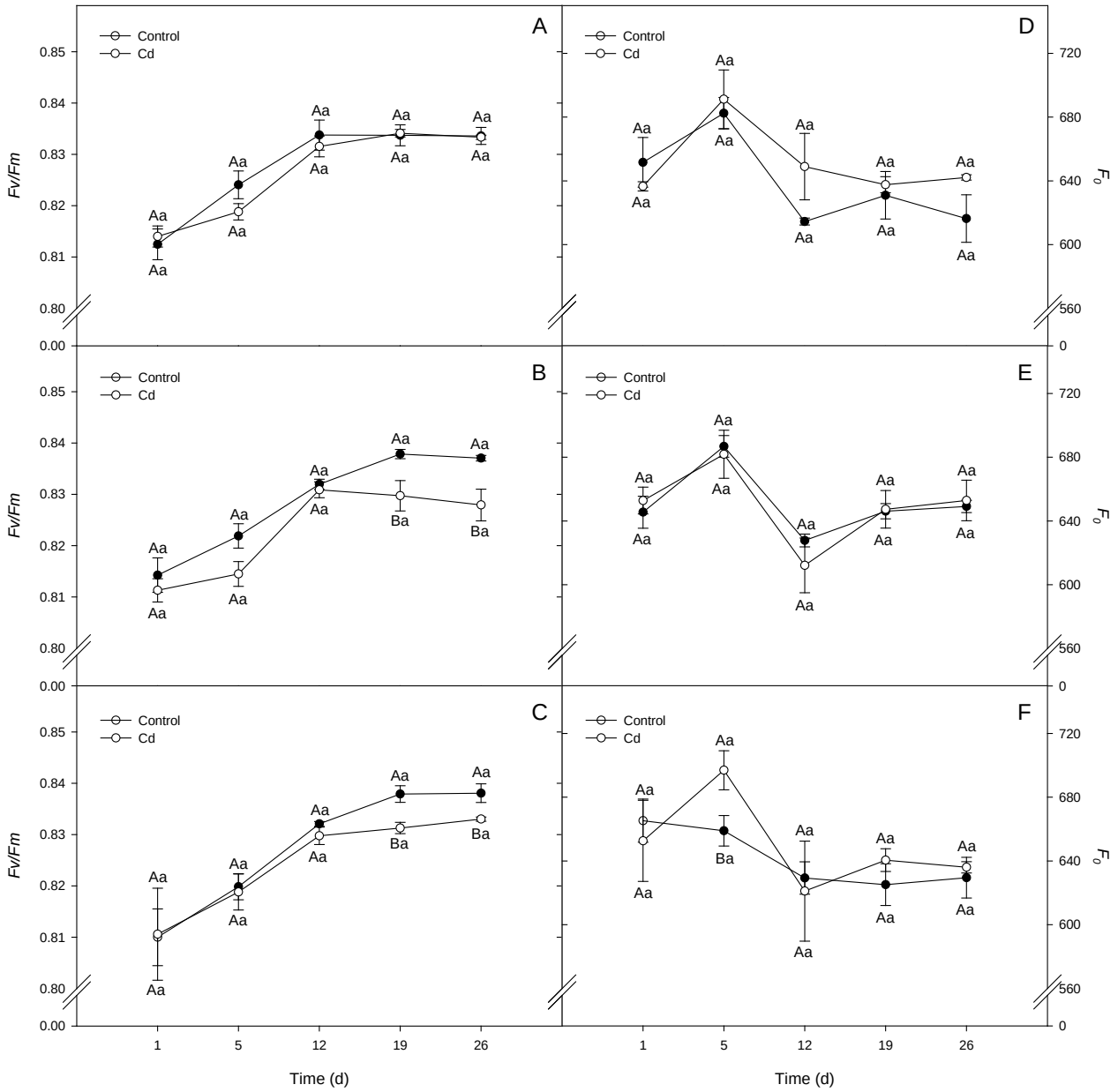

Fig. 9. Chlorophyll fluorescence parameters, potential quantum yield of photosystem II (F_v/F_m), and minimal fluorescence from dark adapted state (F_0) of *L. esculentum* plants *wild type* (A and D), *Notabilis complemented line 13* (B and E), and *Notabilis* (C and F) after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

Minimal fluorescence (F_0) from dark adapted leaves indicates a similar pattern for control and Cd-treated plants in *wild type* and *Not.*$_{compl.13}$ throughout the period evaluated (Fig. 9D and E), while in *Notabilis* Cd caused a transitory increase of 6% in F_0 on the 5th day, with a subsequent recovery to control plants levels (Fig. 9F).

Both, electron transport rate (ETR) and quantum yield efficiency of photosystem II (Φ_{PSII}) were affected transiently by Cd in a similar way, with differences between genotypes. Although no change occurred in these parameters for *wild type* plants (Fig. 10A and D), a decline of 34% in ETR and 14% in Φ_{PSII} was observed in *Not.*$_{compl.13}$ on the 5th day of Cd exposure (Fig. 10B and E).

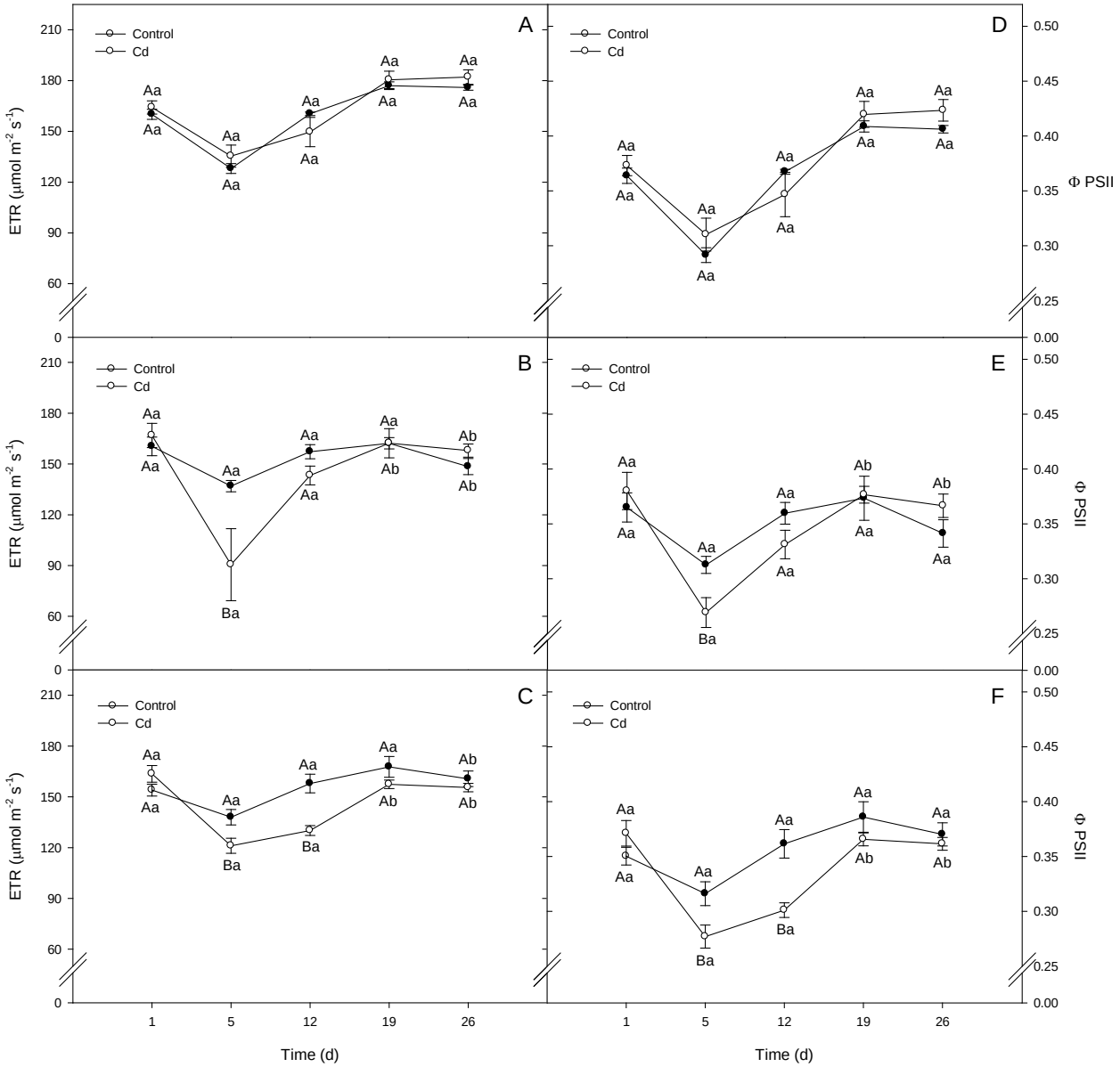

Fig. 10. Chlorophyll fluorescence parameters, electron transport rate (*ETR*, in µmol m^{-2} s^{-1}), and quantum yield efficiency of photosystem II photochemistry (Φ_{PSII}) of *L. esculentum* plants *wild type* (A and D), *Notabilis complemented line 13* (B and E), and *Notabilis* (C and F) after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

Despite Cd-induced changes in *Notabilis* were also observed on the 5th day for both parameters (reduction of 12 and 17% in ETR and Φ_{PSII}, respectively), an additional decrease of 13% in ETR and 26% in Φ_{PSII} occurred on the 12th day of treatment (Fig. 10C and F). Another difference between *Notabilis* and the transgenic line *Not.*$_{compl.13}$ was the recovery in ETR and Φ_{PSII} that occurred on the 12th day in transgenic plants, and only at the 19th day in *Notabilis* (Fig. 10B, C, E and F).

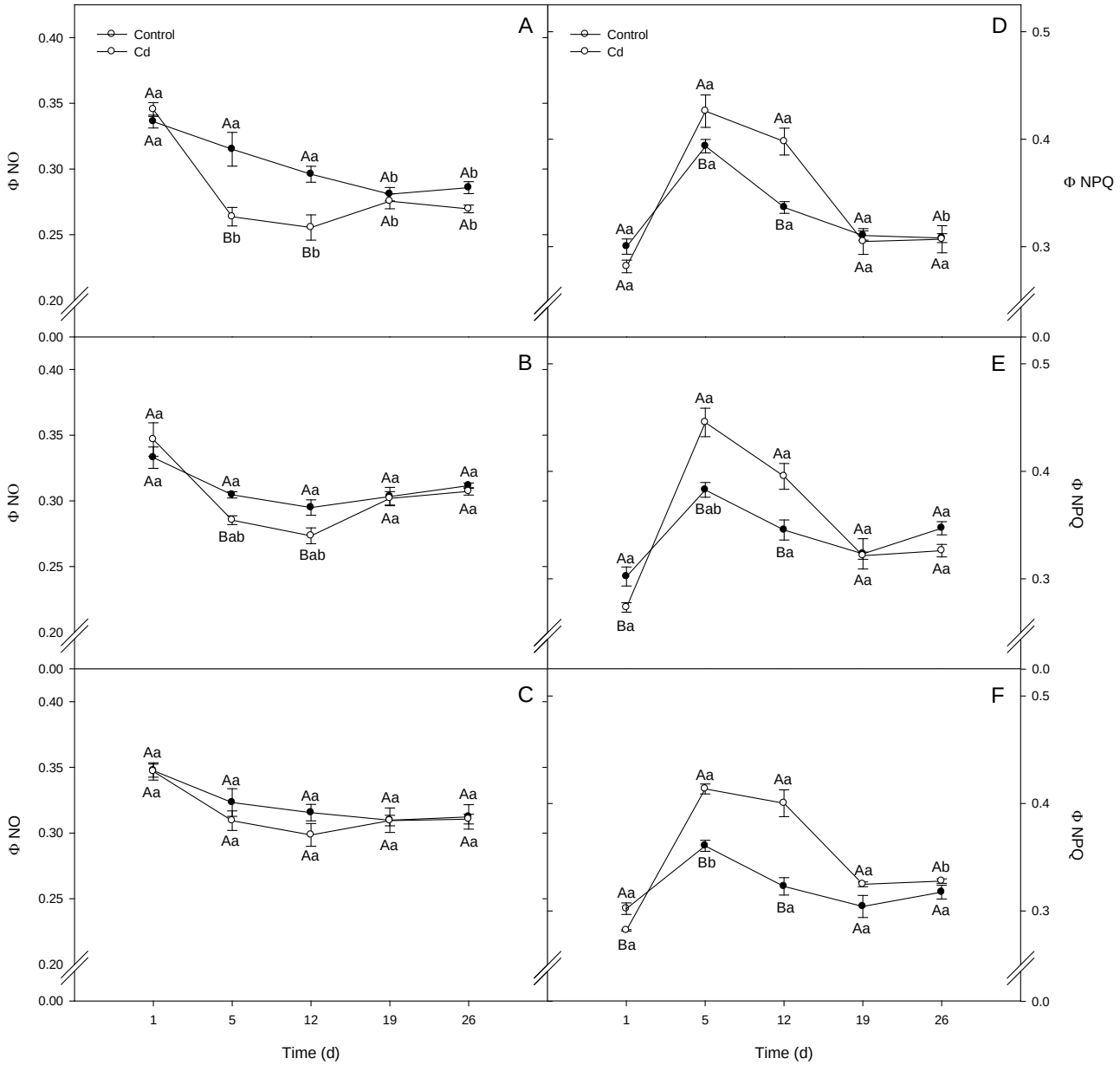

Fig. 11. Chlorophyll fluorescence parameters, quantum yield of constitutive thermal dissipation (Φ_{NO}), and quantum yield of ΔpH and xanthophyll-regulated thermal energy dissipation (Φ_{NPQ}) of *L. esculentum* plants *wild type* (A and D), *Notabilis complemented line 13* (B and E), and *Notabilis* (C and F) after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

Cadmium decreased the quantum yield of constitutive thermal dissipation (Φ_{NO}) both on day 5 and 12 in *wild type* and *Not.*$_{compl.13}$ (16 and 14%, and 6 and 7%, respectively), but this effect was transitory and not observed later (Fig. 11A and B). No changes were observed in Φ_{NO} for *Notabilis* in response to Cd (Fig. 11C). Among Cd-treated plants, *wild type* showed the lowest Φ_{NO} values from the 5th until the 26th day, although they did not differ from other genotypes when grown in absence of this heavy metal (Fig. 11A, B and C).

The exposure of plants to Cd also increased transiently the quantum yield of ΔpH and xanthophyll-regulated thermal energy dissipation (Φ_{NPQ}) in all genotypes under study. Fig. 11D, E and F shown the increases in Φ_{NPQ} on the 5th and 12th day occurred in *wild type* (8 and 18%), *Not.*$_{compl.13}$ (14 and 16%), and *Notabilis* (15 and 24%). *Notabilis* plants had a significant lower Φ_{NPQ} among control plants on the 5th day, while *Not.*$_{compl.13}$ showed the highest Φ_{NPQ} on 26th day (Fig. 11D, E and F).

Photosynthetic pigments content

In the absence of Cd, differences in pigments between *wild type* and *Notabilis* were not significant, except for zeaxanthin, which was higher in *wild type* plants (Fig. 12F). On the other hand, the complemented mutant *Not.*$_{compl.13}$ showed a higher content of chlorophyll *b*, neoxanthin and lutein (Fig. 12B, C and G), and a strongly lower concentration of β-carotene, when compared to *wild type* (Fig. 12H). In presence of Cd, compared to *wild type*, *Notabilis* showed higher levels of neoxanthin, violaxanthin and lutein (Fig. 12C, D and G), while *Not.*$_{compl.13}$ differed only on zeaxanthin content that was significant lower (Fig. 12F).

No changes in chlorophyll *a* and *b* content were observed in *wild type* and *Not.*$_{compl.13}$ when plants were grown in presence of Cd, although an increase only for chlorophyll *a* was observed for the ABA-deficient mutant *Notabilis* (Fig. 12A and B). A different pattern response to Cd was observed between *Notabilis* and *Not.*$_{compl.13}$, while *Not.*$_{compl.13}$ showed a dramatic decrease in zeaxanthin, moderate reductions in neoxanthin and lutein, and no changes for violaxanthin, the opposed was observed for *Notabilis* (Fig. 12C, D, F and G). Antheraxanthin content was decreased by Cd in all genotypes (Fig. 12E), conversely, β-carotene increased considerably in *wild type* and especially in *Not.*$_{compl.13}$ (Fig. 12H).

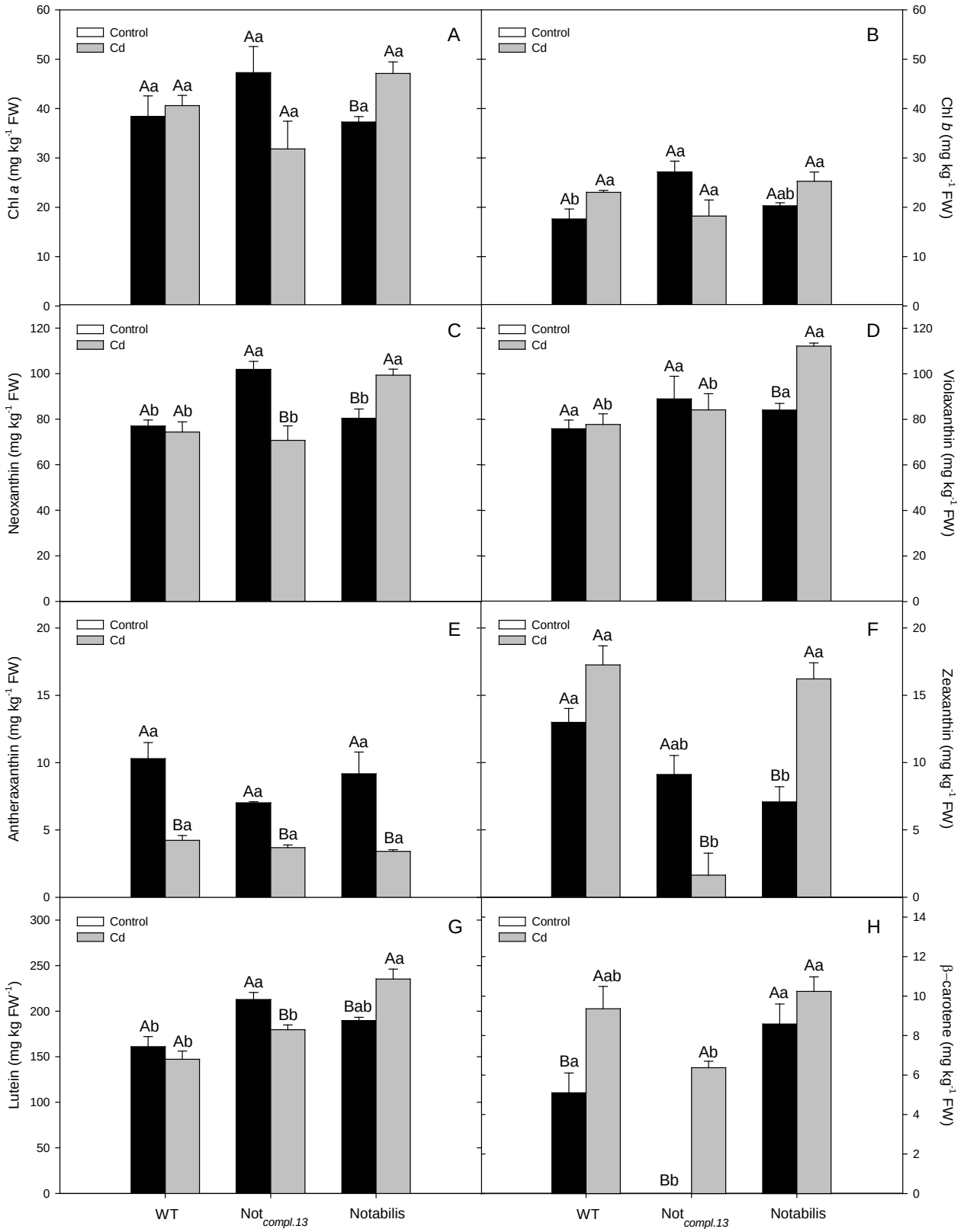

Fig. 12. Pigment content (mg kg^{-1} fresh weight), chlorophyll *a* (A), chlorophyll *b* (B), neoxanthin (C), violaxanthin (D), antheraxanthin (E), zeaxanthin (F), lutein (G) and β-carotene (H) of *L. esculentum* leaves from *wild type* (WT), *Notabilis complemented line 13* (Not.$_{compl.13}$) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

Antioxidant enzymatic activity

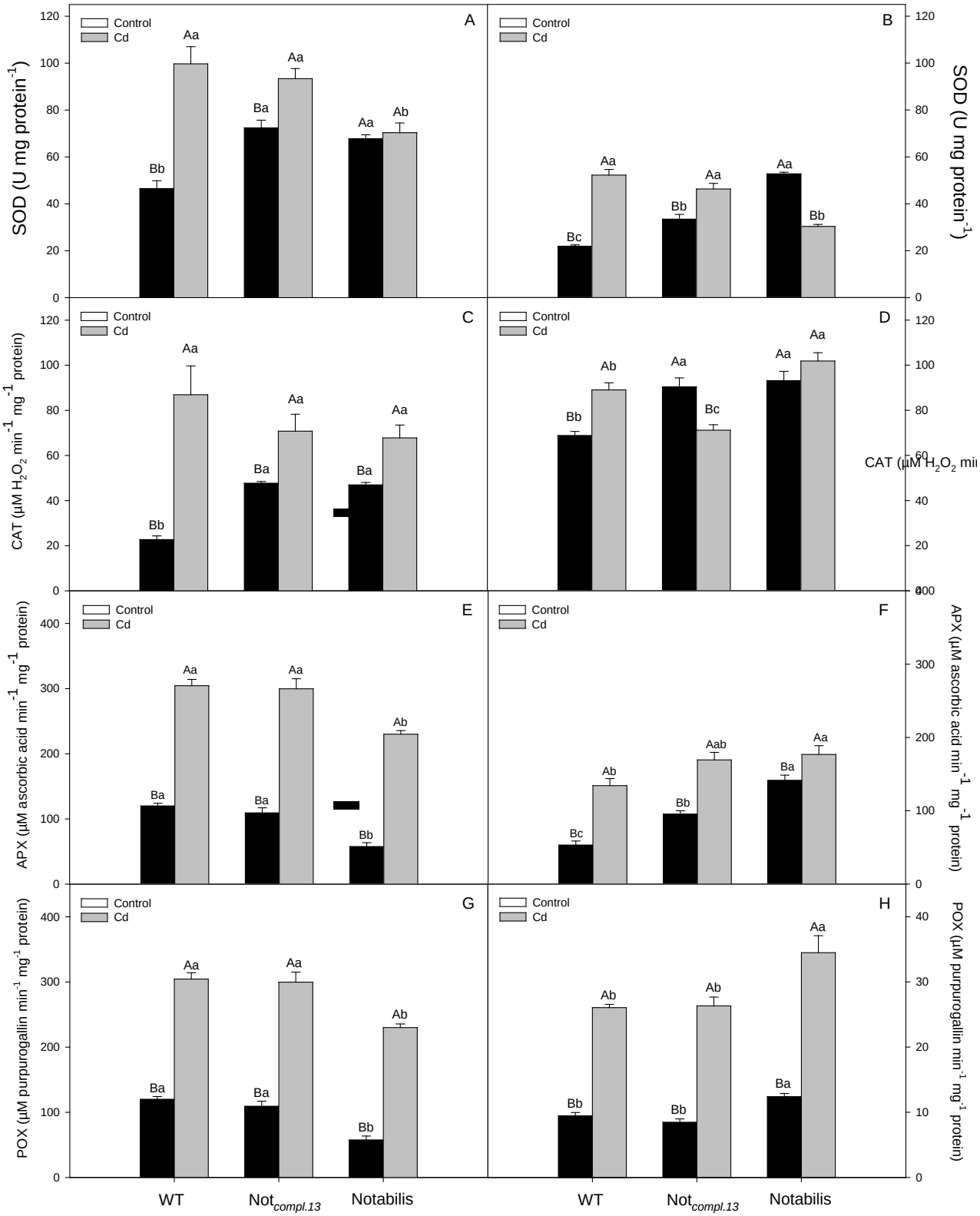

Fig. 13. Antioxidative enzyme activity superoxide dismutase (A, roots and B, leaves), catalase (C, roots and D, leaves), ascorbate peroxidase (E, roots and F, leaves) and total peroxidases (G, roots and H, leaves) of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.$_{compl.13}$) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

Superoxide dismutase (SOD) activity increased with Cd treatment in both, root and leaf tissues of *wild type* and *Not.*$_{compl.13}$ plants, whereas for *Notabilis* no changes and even a decrease was observed in root and leaf of plants (Fig. 13A and B). Regardless plant organ, among control plants, *Not.*$_{compl.13}$ and *Notabilis* had higher SOD activity, while in presence of Cd *Notabilis* showed the lowest level. Exposure of plants to Cd also interferes in catalase (CAT) activity, which increased in roots of all genotypes, especially from wild type plants (Fig. 13C). Distinct activity patterns were detected in leaf, with increase for *wild type* plants, decrease in *Not.*$_{compl.13}$ and no changes in *Notabilis* (Fig. 13D). In absence of Cd, wild type plants showed the lowest CAT activity in both, root and leaf. However, under Cd treatment, leaves from the mutant *Notabilis* had the highest activity of this enzyme, followed by *Not.*$_{compl.13}$ and then *wild type* plants, without differences among genotypes in roots. Ascorbate peroxidase (APX), another enzyme involved in detoxification of reactive oxygen species (ROS), also had an appreciable increase on activity in root and leaf of all genotypes when plants grown in presence of Cd (Fig. 13E and F). Despite the mutant *Notabilis* had the lowest APX activity in root, in leaf it was the highest irrespective of treatment with heavy metal. Total peroxidase (POX) followed a similar pattern of APX, with considerable increase in activity caused by Cd in root and leaf of all genotypes. Likewise APX, POX activity was lower in root tissues of *Notabilis* plants, although it was higher in leaf (Fig. 13G and H).

Effects of Cd on growth

In the absence of Cd, *Notabilis* had lower total dry weight compared to *wild type* plants (Fig. 14A). This was due to lower root, stem and fruit dry weight (Fig. 14B, D and E), with no changes in leaf dry weigh (Fig. 14 C). In contrast, *Not.*$_{compl.13}$ did not differ from *wild type* for any of these growth parameters. In the presence of Cd, *Notabilis* and *wild type* plants showed a decrease in total and leaf dry weight (Fig. 14A and C), whereas no change was observed for *Not.*$_{compl.13}$. *S*pecific responses to Cd in *wild type* plants include a decrease in root, leaf, stem and fruit dry weight, which were not observed for the other two genotypes (Fig. 14 B, C, D and E). A third pattern in the genotypes responses to Cd was observed for stem dry weight, where a decrease was observed for *wild type* and *Not.*$_{compl.13}$, but not for *Notabilis* (Fig. 14D). Altogether, it could be assumed that Cd had a smaller effect in *Not.*$_{compl.13}$ than in other genotypes. Fruit

number was affected by Cd only in *wild type* plants, which consequently lead this genotype to the lowest fruit number among Cd-treated plants (Fig. 14F).

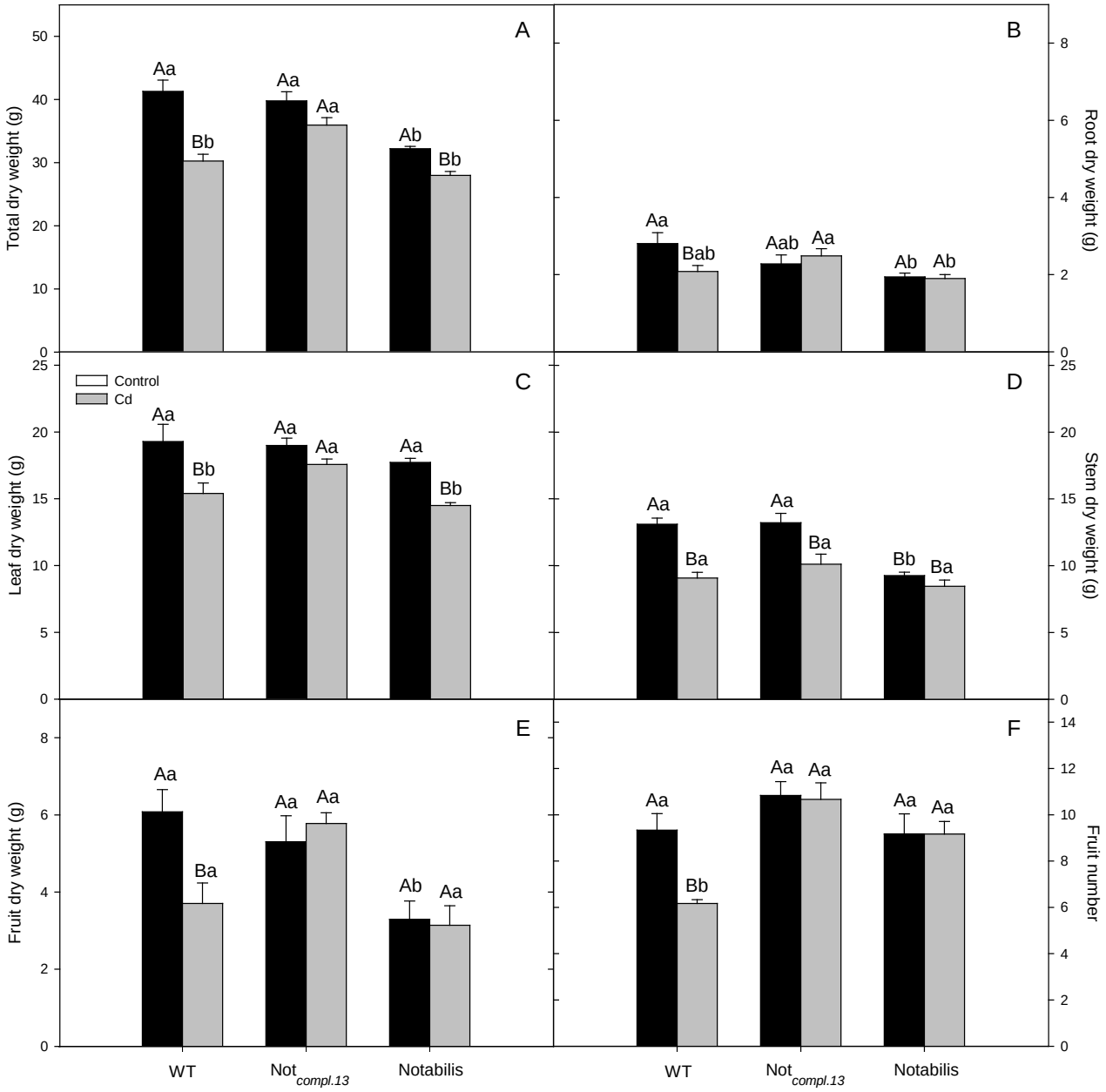

Fig. 14. Plant growth (g DW) whole plant (A), root (B), leaf (C), stem (D) and fruit (E) and fruit number (F) of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.$_{compl.13}$) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

DISCUSSION

Cadmium levels and toxicity

Cd accumulate in roots, leaves and stems of tomato plants, but the translocation of this heavy metal was sensible low, since the levels in roots were about 30-fold higher in this organs in comparison with shoots, and no changes in lipid peroxidation were observed in leaves of any genotype. Despite the low translocation to shoots, the majority of Cd mobilized was allocated in leaves, given that content in stem was about 80% lower, and it was undetectable in young fruits. The pattern of Cd accumulation observed in tomato plants demonstrate a typical mechanism used to cope with heavy metals that results in a relative tolerance, since many metal-tolerant species had a diminished accumulation of the respective metal in shoots, and accumulate higher amounts in roots as compared to non-tolerant ones, which is probably associated with compartmentalization of the metal as soon it enters into the cell, as a form to protect the shoots from excess of Cd (Baker, 1984; Hertstein and Jager, 1986; Tong *et al.*, 2004). This implies that possibly a higher toxicity of Cd occurs in roots. Comparing the genotypes, highest root Cd content was observed for the mutant *Notabilis*, which could be explained by the higher constitutive stomatal conductance (Hussain *et al.*, 2000) and carbon isotopic composition observed here, that probably imply a higher water consumption and consequently higher Cd uptake. This phenotype of *Notabilis* could explain why a higher lipid peroxidation occurs only in the roots of this mutant and not in the others.

Effect of cadmium on micronutrient content

Hormonal signals involving abscisic acid (ABA) are associated with mineral imbalances and perturbations of nutrient supply. Not surprisingly, in the absence of Cd, the ABA-deficient mutant *Notabilis* had higher iron, manganese and copper in their roots, which can also be an additive effect of the higher transpiration and water uptake. As before, the higher levels in roots were not translated to changes of concentration of these micronutrients in shoots. Alternatively, changes as occurred for some micronutrients in roots and shoots of *Notabilis* did not occur for zinc, may be due to a divergent effect caused by ABA deficiency in zinc homeostasis, as

predicted by this hormone to act at transcriptional regulation of ZIP1 genes, which encode a zinc transporter protein (Yazaki *et al.*, 2004).

Surprisingly, in the presence of Cd, the levels of Fe (*Notabilis* and *wild type*) and Cu (all genotypes) increase in roots, a behavior contrary to expected, since some mineral transporters are able to transport Fe/Cd, Cu/Cd, e.g. specific *P-type* heavy metal ATPases, IRT, ZIP and/or NRamp-proteins (Korshunova *et al.*, 1999; Thomine *et al.*, 2000), and that this could be a competitive process. However, some researchers state that a competition between Fe/Cu and Cd by the specific site in the transporter causes a deficiency, which in turn activates the respective uptake system and lead to an enhanced uptake of both, Cd and the micronutrient, as described by Fe (Nakanishi *et al.*, 2006). Since ZIP and ZnT mineral transporters have been also reported that could transport both Cd and Zn, a competition between both to be transported may occurs, and possible leading to a reduction in Zn concentration in leaves in the presence of Cd (Williams *et al.*, 2000; Hall and Williams, 2003).

Gas exchange and chlorophyll fluorescence

Inhibitory effects of heavy metals on ecophysiological parameters and biochemistry reactions associated to gas exchange and CO_2 fixation are widely reported in literature, although potential signaling mechanisms involving these processes have not yet been clearly determined. Despite a general negative effect of Cd on stomatal conductance in all genotypes, in *Notabilis*, contrary to other genotypes, the reduction in g_s was very low and transitory, without alteration in C_i/C_a ratio, and consequently $\delta^{13}C$ did not change. It might be a consequence of the negative effects exerted by Cd on stomatal regulation, where Cd^{2+} ions mimic Ca^{2+} and permeate through voltage-dependent Ca^{2+} channels in guard-cells, causing perturbations of intracellular Ca^{2+} signaling, and inducing stomatal closure (Perfus-Barbeoch *et al.*, 2002). Calcium signaling is a key element in guard cell osmoregulation (Allen *et al.*, 2000; Blatt, 2000), being an important signal linked to the role of ABA in the stomatal closure mechanism. The effect of Cd on stomatal behavior is a controversial issue. Whereas plant wilting has been described as a consequence of Cd toxicity in some species (Barcelo and Poschenrieder, 1990), in others a considerable increase in endogenous leaf ABA concentration was responsible for maintenance of the turgor pressure, avoiding plant wilting and conferring tolerance to the heavy metal stress (Poschenrieder *et al.*,

1989; Hsu and Kao, 2003). In agreement, our results show that higher $\delta^{13}C$ in plants with normal ABA synthesis could be evidence of an ABA-dependent response on long-term stomata conductance reduction.

The reduction in net carbon assimilation rate (*A*) in response to Cd seems to be basically due the decrease in g_s in *wild type* and in transgenic plants *Not.*$_{compl.13}$. However this does not seems to be the major factor leading to a decreased *A* in *Notabilis*, since no significant change in the *Ci/Ca* ratio was found in this genotype. Alterations in chlorophyll *a* fluorescence parameters, could explain the reduced *A* in the mutant *Notabilis*. Specific changes in this genotype, such as transitory decrease in minimal fluorescence on dark adapted state (F_0) and decrease in quantum yield of constitutive thermal dissipation (Φ_{NO}), together with sharper reductions in electron transport rate (ETR) and decreased potential quantum efficiency of photosystem II photochemistry (Fv/Fm), suggest that damages to antenna in PSII occurs in the mutant, and that could led to an increase in leaf temperature and a specific increase in transpiration in the absence of changes in g_s and C_i/C_a. Comparing genotypes a slight higher quantum yield of ΔpH and xanthophyll-regulated thermal dissipation (Φ_{NPQ}) was observed in *Notabilis*, which probably could also contribute the higher leaf temperature and transpiration. Altogether, decrease in light capture efficiency at PSII and lower ETR, explains the decrease in *A* in the absence of significant changes in stomatal behavior.

Water relations

A sharper reduction in WUE in the mutant *Notabilis* in response to Cd could be explained by the higher transpiration in this genotype, since differences in *A* among them are negligible. In the absence of Cd, the lower predawn water potential (Ψ_{wpd}) and midday water potential (Ψ_{wmd}) in this plant could be explained by the lower $\delta^{13}C$, which indicates a constitutively higher stomata aperture, and consequently, higher dehydration. In fact, *Notabilis* has lower ABA content, even in well hydrated leaves, which led to a deficiency in control of stomatal closure, and consequently causes higher water loses by the plant, decreasing leaf water potential (Thompson *et al.*, 2004). The specific decrease in Ψ_{wmd} in *Notabilis* could be explained by the increased transpiration in response to this heavy metal, while the recovery at night from dehydration seems to be deficient in this genotype, as well for the transgenic plants *Not.*$_{compl.13}$. Many disorders in plant water

relations are attributed to Cd and other heavy metals that compromise directly the water content of aerial tissues, including significant increases in transpiration rate and stomatal conductance, which can lead to a permanent dehydration state (Poschenrieder *et al.*, 1989; Zhang *et al.*, 2007; Vernay *et al.*, 2007).

Pigments analysis

A fine tuning balance among chlorophylls and carotenoids composition and content in higher plants is essential for an adequate operation of photosynthetic process, from the acquiring to the management of the light energy, and also to cope with distinct abiotic stresses through plant cycle (Wentworth *et al.*, 2006). Pigments serve also as intermediate metabolites for many different biosynthetic pathways as well for ABA synthesis. Therefore, alterations in any metabolic route might alter they respective precursor metabolite profile, notwithstanding it can change others too (Schmidt-Dannert *et al.*, 2000). Between the changes in pigments, the more interesting result is that in the presence of Cd an increase in zeaxanthin, neoxanthin and violaxanthin is observed only for the mutant *Notabilis*. This result, taken together with a significant higher ΦNPQ in this genotype, suggests that the xanthophyll cycle could contribute to dissipate excess of energy in PSII and prevent further damage on this photosystem. Since this difference is only seen in the presence of cadmium, the alternative explanation that reduction in ABA biosynthetic fluxes could lead to increase of its intermediate metabolites (violaxanthin and zeaxanthin) should be ruled out. That fact that also lutein is higher only in *Notabilis* in the presence of Cd could be interpreted also as evidence that an increase in xanthophylls cycle is induced in the mutant.

In the absence of Cd, the surprising non-detectable foliar β-carotene levels in the transgenic *Not.*$_{compl.13}$ could be linked to a possible conversion of this pigment in another one, or even through a constitutive cycle of synthesis and degradation/conjugation of ABA, since this hormone level is similar to *wild type* plants (Thompson *et al.*, 2004). β-carotene, and zeaxanthin are intermediate metabolites of ABA synthesis (Botella-Pavia *et al.*, 2004), and the low levels could be interpreted as depletion due the high synthesis of this hormone. The small increase in Chl*b* and lutein observed could be speculated as a pleiotropic effect, since NCED had different

isoforms targeted to plastids, with different binding activity to thylacoid membrane, where also other enzymes of carotenoid biosynthesis are located (Tan *et al.*, 2003).

Antioxidative responses and oxidative damage

Cd is a well known oxidative stress elicitor within the cell, involved in reactive oxygen species (ROS) generation, and a potential protein structure disrupter. In order to minimize these effects, plants resort to distinct antioxidative mechanisms, especially an enzymatic system able to scavenging toxic substances from the cell (Dixit *et al.*, 2001). The higher Cd content in roots of *Notabilis* and the lowest level of SOD, APX and POX, could together explain why only in this genotype Cd triggers lipid peroxidation. The interesting fact that, in the absence of Cd *Notabilis*, in comparison to *wild type*, has a higher activity of SOD, CAT, APX (only in leaves), and POX (only in leaves), suggest that ABA deficiency could be associated to increases in ROS production (Sharp *et al.*, 2004). Additionally it could be seen, that despite these higher activities, higher malondialdehyde (MDA) content was still observed in this condition.

Although no increase in lipid peroxidation could be observed in response to Cd in *wild type* and in the transgenic *Not.*$_{compl.13}$, despite sharp increase of this metal in roots, a sharp increase in SOD, CAT, APX and POX in both plants was observed, indicating the level of Cd used in this experiment could be sufficient to increase the production of ROS. Antioxidant enzymes are also induced by production and/or accumulation of ROS, and they play important roles in adaptation and, ultimately, in the survival of plants under stress, which justify the enhanced activity of the performed enzymes in *wild type* and *Not*$_{.compl.13}$ (Chandru *et al.*, 2003). Alternatively, the increase in Fe, Mn and Cu in roots could contribute to higher total activity of SOD, given that these micronutrients are necessary for protein folding and consequently to establish a functional catalytic structure, besides the role of Cu e.g., which acts in transcriptional and translational expression regulation (Kim *et al.*, 1999; Cohu *et al.*, 2009; Beauchlair *et al.*, 2010). In agreement with this hypothesis, the decrease in Fe and Zn observed in the presence of Cd in leaves, occurred in parallel with a decrease in total SOD activity.

Growth parameters

The mutant, as previously reported, has impaired growth when compared to the transgenic *Not*$_{.compl.13}$ and *wild type* plants (Thompson *et al.*, 2004). Further evidence that the Cd dose used was sufficient to be toxic, could be inferred by the effects in the growth observed mainly in *wild type* and *Notabilis*. Curiously, the transgenic plants *Not*$_{.compl.13}$, which have a different pattern of *LeNCED1* expression than would be produced using the wild type *LeNCED1* natural promoter, do not have change in total, root, leaf, stem and fruit dry weight, in contrast to *wild type*, supporting the full complementation for these parameters, except for root dry weight, where a intermediate pattern between *wild type* and *Notabilis* was observed (Thompson *et al.*, 2004). These results suggest that the changes in NCED expression could help to decrease the toxicity of Cd by enhance development of root system, since the ability to renew periodically the most active parts of their below-ground biomass represents a characteristic mechanism of tolerance to heavy metals (Das *et al.*, 1997). Although no changes in ABA have been reported for this transgenic plant (Thompson *et al.*, 2004), samples from these plants need to be analyzed to verify a possible increase in ABA content, linked to the specificity of Cd responses and the possibility that more than one T-DNA insertion had occurred in these plants.

CONCLUSIONS

In summary, the accumulated evidence suggests that the mutation in *Notabilis* lead to an increase in Cd uptake without changes in the translocation pattern, given that the main content is retained in roots as is the case in *wild type* plants. Higher lipid peroxidation in root tissues occurred regardless of the presence of Cd, in addition to higher constitutive transpiration and lower leaf water potential. No alteration of C_i/C_a ratio and $\delta^{13}C$, and a subtle effect on g_s were detected for *Notabilis* under Cd treatment, in comparison with *wild type*. Although Cd reduces *A* in all genotypes, this cannot be explained by changes in g_s for the mutant *Notabilis* as it can be for the other genotypes. However, despite the minimal changes in stomatal behavior, *Notabilis* has a transitory change in F_0, sharp reductions in ETR and F_v/F_m, a decrease in Φ_{PSII}, and an increase in Φ_{NPQ}, besides higher leaf temperature and an increase in several xanthophylls. Another interesting phenotype of *Notabilis* is that in the absence of Cd, roots have higher SOD and CAT activities, and despite this, have the highest lipid peroxidation, indicating that there are increased levels of ROS production in roots of mutant plants. Taken together, these results put forward a reduction in photosynthetic photochemical reactions of *Notabilis* plants grown in the presence of low Cd concentrations which might play a protective role against photodamage, reducing the risk of overburdening the electron transport chain and probably dissipating excessive energy through xanthophyll cycle to avoid higher damage in these plants. The several intermediate phenotypes of *Not.*$_{compl.13}$ in presence of Cd, curiously ultimately do not lead to reductions in growth as might be expected.

Altogether, these results indicate that in the absence of normal levels of ABA there was a reduced acclimation response of the plants to Cd, and the appearance of other mechanisms linked to changes in photochemistry that appear less effective in cope with the stress caused by the metal. It also appears that probable changes in spatial and/or quantitative pattern of NCED gene expression contribute to alleviate the toxicity caused by Cd, as suggested by the complemented mutant *Not.*$_{compl.13}$. This could be interpreted as additional evidence to support an important role of ABA in Cd toxicity responses, not yet described in the literature.

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SUPPLEMENTARY MATERIAL

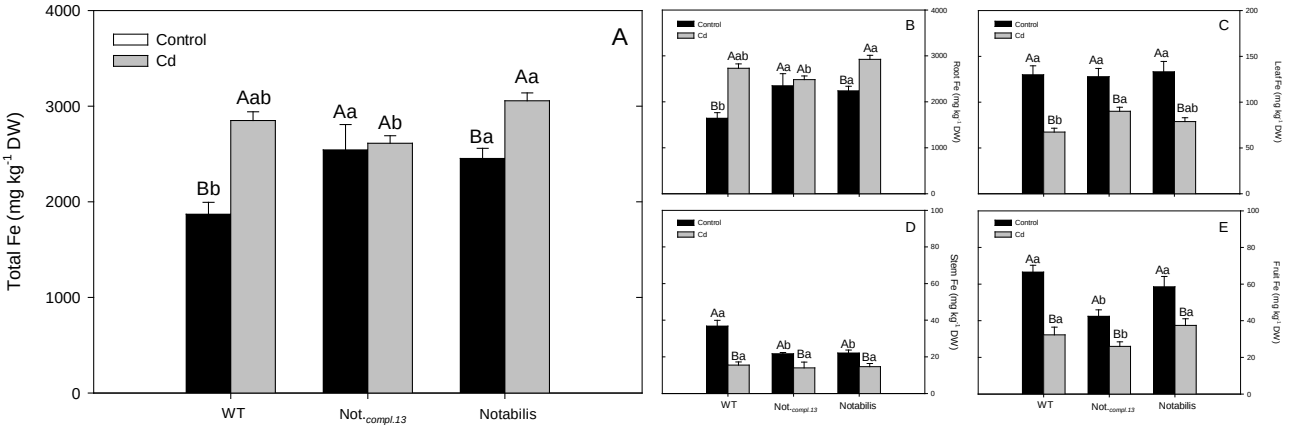

Fig. 1. Fe allocation in plant tissues. Concentration (mg kg^{-1} dry weight) in whole plant (A), roots (B), leaves (C), stems (D) and fruits (E) of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.*compl.13*) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

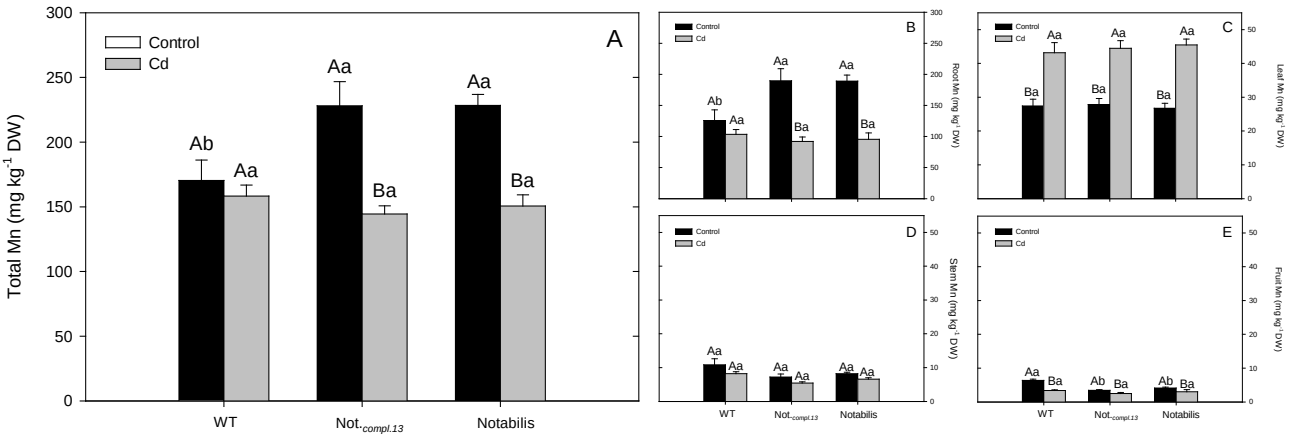

Fig. 2. Mn allocation in plant tissues. Concentration (mg kg^{-1} dry weight) in whole plant (A), roots (B), leaves (C), stems (D) and fruits (E) of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.*compl.13*) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

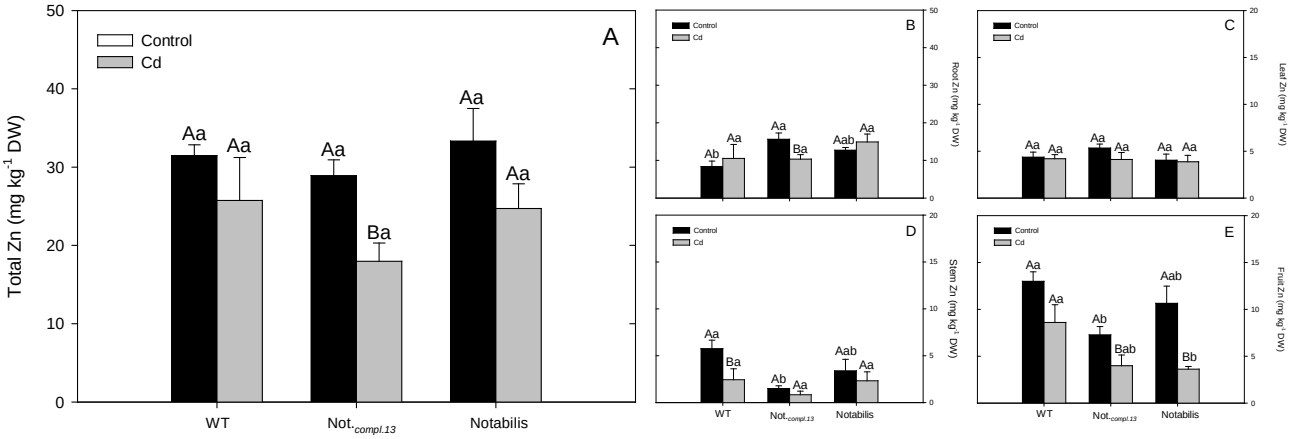

Fig. 3. Zn allocation in plant tissues. Concentration (mg kg^{-1} dry weight) in whole plant (A), roots (B), leaves (C), stems (D) and fruits (E) of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.*compl.13*) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).

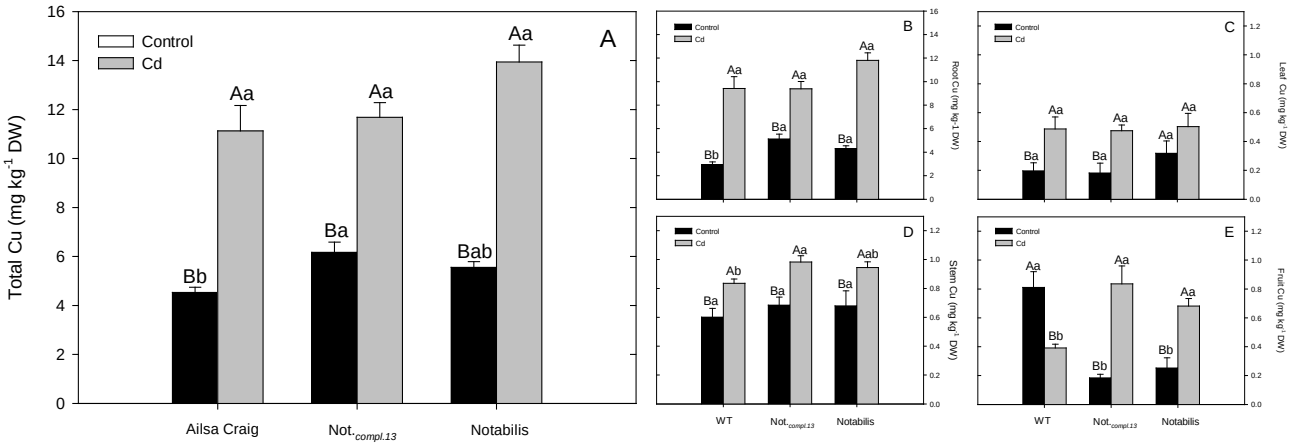

Fig. 4. Cu concentration (mg kg^{-1} dry weight) in whole plant (A), roots (B), leaves (C), stems (D) and fruits (E) of *L. esculentum* plants *wild type* (WT), *Notabilis complemented line 13* (Not.$_{compl.13}$) and *Notabilis* after 26 days of growth in hydroponics in absence (control) or in presence of Cd (12.5 µM Cd). Values (mean ± SEM) followed by the same letter, uppercase for the genotype, and lowercase for equal metal treatment, are not significantly different at $P < 0.05$ (Tukey's test).