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Effects of cadmium and glufosinate ammonium contaminated water on wild strawberry plants

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Abstract

Fragaria vesca L. (cv. Annabelle) plants were cultivated in hydroponic system and treated for 28 days with control (Hoagland solution), 1 mg L^{-1} of Cd, 10 µg L^{-1} of glufosinate ammonium and the mix of glufosinate ammonium and Cd. Cd and glufosinate influenced the photosynthetic parameters starting from day 18. Cd influenced the fruit fresh weight after 28 days of exposure. The accumulation of Cd in roots was highest among all plant organs and was estimated being 200 µg g^{-1}$_{DW}$ followed by leaves at < 15 µg g^{-1}$_{DW}$ and reaching the lowest concentrations in fruits at < 3 µg g^{-1}$_{DW}$. Only the metabolite 3-(hydroxymethylphosphinyl) propionic acid (MPP) of glufosinate was detected over the detection limit in each organ analysed. Glufosinate exposure reduced Ca uptake (-35% compared to control) in fruits while Cd reduced the uptake of Fe and Ca in leaves and Zn, Mn and Ca in fruits. Thus, residual Cd and glufosinate ammonium, that could be present in wastewater used for irrigation, may affect wild strawberry physiology. The data indicates that at the concentrations mentioned above, the consumers have a low risk of Cd exposure but can be exposed to glufosinate metabolite MPP through consumption of fruits that are grown in contaminated wastewater.

Keywords Fruit quality · Chlorophyll · Health risk · Heavy metal · Pesticides

Introduction

The changes in precipitation have made the climate of many regions arid. Thus rain-fed crop production has become unsustainable and agriculture has become increasingly dependent on irrigation practices (Poustie et al. 2020). Since freshwater resources used in agriculture are becoming scarce and the freshwater is used for several other purposes besides agriculture, wastewater is a potential source for irrigation of agricultural fields (Rizzo et al. 2020). In several countries that are exposed to water scarcity, the use of treated wastewater is encouraged (Chang et al., 2012; Aziz et al., 2014). Despite its agronomic values, wastewater for crop irrigation may contain undesirable level of inorganic and organic xenobiotic contaminants that are derived from incorrect disposal and treatment systems. Heavy metals and pesticides/herbicides residues are classified as hazardous xenobiotic and for the safety and final quality of the produced food their presence must be avoided (Malakar et al. 2019). For the above-mentioned reasons and to avoid negative effects on the human health, the impact of wastewater on crop physiology and its edible products must be evaluated before confirming its safe and successful use (Salgot and Folch 2018). The capacity of crop plants to uptake pesticides/herbicides residues and heavy metals varies greatly between species (Handford et al. 2015; Romeh 2014) and significant interactions can occur between different xenobiotics. Macronutrients can be compartmentalized in specific roots tissues; when pesticides/herbicides and heavy metals are sequestered by plant roots, they can be stored or, in case of organic compounds, degraded in situ or translocated to the shoots where storage could occur (Eevers et al. 2017; Francini et al. 2018; Vannucchi et al. 2020, 2021; Acharya and Pesacreta 2022).

Among heavy metals, Cd has been classified as one of the ten most dangerous elements (Romè et al. 2016). This classification by the Agency for Toxic Substances and Disease Registry (ATSDR), consider its frequency, toxicity and

potential for human exposure. Under Cd stress, plants show symptoms like chlorosis, desiccation and necrosis. Chlorosis and chlorophyll breakdown are related to the alteration of the photosynthetic activities caused by Cd (Muradoğlu et al. 2015; Parmar et al. 2013). Cd has inhibitory effects on photosynthesis, and changes the activity of various enzymes involved in this process (Ying et al. 2010), altering the chloroplast ultrastructure and reducing stomatal conductance and leaf transpiration rates (Goussi et al. 2018; Souza et al. 2011). These toxic symptoms appear when the Cd concentration in plant organs reaches 3–30 mg kg^{-1} dry weight (Solís-Domínguez et al. 2007). Moreover, Cd could affect the uptake of other mineral nutrients such as Fe, Ca, Zn and Mn (Shiyu et al. 2020) inducing nutrition unbalance.

Glufosinate ammonium is a key herbicide that targets key enzymes in plants such as glutamine synthetase. The glufosinate induced foliar injury, is likely due to the accumulation of toxic levels of ammonia in leaves (Takano and Dayan 2020). However, there are also evidences that reactive oxygen species (ROS) could be the main drivers for the rapid phytotoxicity in glufosinate treated plants (Takano et al. 2019). Glufosinate uptake and translocation throughout the plant depend on transpiration rates and as a consequence glufosinate molecules tend to accumulate in older leaves with high transpiration rates (Singh et al. 2020) in which symptoms develop rapidly. Plants cells can convert glufosinate into five main metabolites: 4-methylphosphinico-2-oxo-butanoic acid (PPOB), 3-(hydroxymethylphosphinyl) propionic acid (MPP), 2-methylphosphinicoacetic acid (MPA), 4-methylphosphinico-2-hydroxybutanoic acid (MHB), and 4-methylphosphinicobutanoic acid (MPB) (Skora et al., 2000; Takano and Dayan 2020). Different rates of glufosinate metabolism in plants are reported in relation to sensitivity or resistance to this compound (Meyer et al. 2020; Brunharo et al. 2019). Glufosinate and its metabolites are highly soluble in water, they seem to not accumulate in the edible portions (US Environmental Protection Agency 2017; EFSA, 2017) and there are no indications for groundwater pollution risks (Laitinen et al. 2006). Although, MPP effect on fruit quality or edible plant tissues has not been documented in previous literature, yet its negative impacts on growth and physiological attributes have been studied (Zhang et al. 2019; Zhao et al. 2020). Moreover, glufosinate does not remain in the soil because it is rapidly degraded by bacteria, resulting in no residual activity (Boutin et al. 2014).

Numerous studies of the co-occurrence of heavy metals and herbicides demonstrated the importance to understand if there is a significant interaction between them that could modify the plants growth and productivity (Alengebawy et al. 2021).

Strawberries (Fragaria spp.) are highly profitable crops fruits of which are utilized as fresh or processed food (jam, juice, dry, and yogurt). In Mediterranean countries, strawberry production is around 1.6 million tons annually, comprising almost 18% of the world production (Chandler et al. 2012; FAOSTAT 2020). Strawberries cultivation is facing many challenges and climate changes induced drought is now limiting the profitability. Mediterranean area suffer for water scarcity and climatic changes will exacerbate this situation (IPCC, 2021). Since water demand in strawberry is very high, alternative source of water like wastewater will be precious (Pedrero et al. 2018; Renai et al. 2021). *Fragaria vesca* (L.) wild strawberry is a perennial herbaceous plant that grows naturally, produces edible fruits and can be easily cultivated. *Fragaria vesca* fruits are soft and strongly flavoured, they are an important source of bioactive phenolics, sugar and citric acid (Del Bubba et al. 2016; Doumett et al. 2011). The production of good quality fruits requires abundant freshwater (Hess and Chloe, 2018) and farmers are more frequently exploring the use of alternative water sources for strawberries irrigations.

Considering the importance of a high consumption of fruits, and in particular strawberries for their antioxidant and nutraceutical properties (Arias et al. 2022; Mezzetti et al. 2016), the evaluation of the consumers exposure level to heavy metal and pesticide, due to strawberry consumption is important.

In order to verify the potential impact of wastewater contaminated with Cd and glufosinate ammonium—individually or in combinations on *Fragaria vesca* (L.) *cv.* Annabelle growth and fruits quality, we investigated the following three questions: *i)* the uptake, translocation and storage of Cd and glufosinate ammonium in plant organs (roots, leaves, and fruits); *ii)* the impact on fruit growth and quality; *iii)* the consumers exposure level to Cd, glufosinate, and MPP metabolite.

Materials and methods

Plant material, Cd and glufosinate ammonium treatments

Plants of *Fragaria vesca* L. cv. Annabelle were bought at Falorni plant nursery situated at San Giuliano Terme (PI) and were cultivated in a single pot/plant hydroponic system under controlled environmental conditions (photoperiod of 16/8 h - day/night, temperature 23/18 °C - day/night and relative humidity of 55/75% —day/night). The composition of the nutrient solution was: Ca(NO$_3$)$_2$·4H$_2$O, 1062 mg L^{-1}; KNO$_3$, 28.8 mg L^{-1}; MgSO$_4$·7H$_2$O, 369 mg L^{-1}; NH$_4$NO$_3$, 80 mg L^{-1}; KH$_2$PO$_4$, 252 mg L^{-1}; FeEDTA, 9.2 mg L^{-1}; H$_3$BO$_3$, 2.86 mg L^{-1}; MnSO$_4$·H$_2$O, 4.2 mg L^{-1}; ZnSO$_4$·5H$_2$O, 1.14 mg L^{-1}; NaMoO$_4$, 0.12 mg L^{-1} and CuSO$_4$·5H$_2$O, 0.81 mg L^{-1}. Root aeration system (250 L

h^{-1}) was set up in each pot using an air compressor system. After two weeks of acclimation in the climatic chamber, plants (with only flowers and no fruits) were randomly assigned to the following experimental treatments: (i) Control (0 mg L^{-1} Cd, 0 µg L^{-1} of glufosinate ammonium); (ii) 1 mg L^{-1} Cd; (iii) 10 µg L^{-1} of glufosinate ammonium and, (iv) a mix of glufosinate ammonium and Cd (1 mg L^{-1} Cd, 10 µg L^{-1} of glufosinate ammonium). Plants were treated for 28 days, and solutions were changed every three days adding 700 mL of Hoagland solution containing the four experimental treatments described above. The source of glufosinate ammonium was Pestanal® (Millipore, Sigma) and for Cd was Cadmium AAS Standard, Fluka™ Certified Reference Material, 1000 mg/L Cd in 2% HNO_3.

Gas exchange measurements and chlorophyll analyses

The leaf stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}) and the net CO_2 assimilation rate (Pn, µmol CO_2 m^{-2} s^{-1}) were measured following Sodini et al. (2022) at 5, 7, 15, 18, 22 and 27 days after the beginning of the treatments, with a portable photosynthesis system (Ciras-2, PP System International, MA, USA) operating at 100 ml min^{-1} flow rate, 420 ± 10 ppm ambient CO_2 and a photosynthetic flux density of 1000 µmol m^{-2} s^{-1}. SPAD data on leaves were recorded non-destructively with a SPAD-502 instrument at 1, 4, 6, 8, 11, 13, 18, 22 and 27 days after the beginning of the treatments in three mature leaves per plant. At the end of experiment fresh leaves samples were used for chlorophyll $a+b$ determination. Briefly, small discs (Ø=10 mm) were taken from leaves, weighted, and added to 1 mL of 100% methanol for overnight chlorophyll extraction. After extraction, the supernatant was analysed using a spectrophotometer (Tecan Infinite® 200 PRO) and the concentration of chlorophyll $a+b$ was calculated according to Lichtenthaler (1987) equation: chlorophyll $a+b$ (µg/mL) = $(1.44*A_{665.2})$ + $(24.93*A_{652.4})$.

Cadmium and mineral elements

At the end of treatments (28 days), roots, leaves, and fruits were sampled. Fresh weight (FW) was immediately recorded, and samples were lyophilised (freeze–dried for 72 h with LIO5P digital Bioclass, Italy). All lyophilised samples were stored at -80 °C until analyses. An aliquot of 300 mg (roots, leaves, or fruits) of each lyophilised samples was digested into 8.0 mL of 65% trace grade nitric acid using a 70 mL Teflon vessel and a microwave (COOLPEX Smart Microwave Reaction System—Yiyao Instrument Technology Development Co., Ltd., Shanghai, China) as in Francini et al. (2022). *Daucus carota* (L.) leaf tissue was used as analytical standard reference (WEPAL IPE, Wageningen University, Wageningen, Netherland). After the digestion, samples were diluted with milliQ water and analyzed for cadmium (Cd), iron (Fe), calcium (Ca), manganese (Mn) and zinc (Zn) concentrations in an atomic emission spectrometer (4210 MP-AES, Agilent). The value of calibration curve and the R squared value were $y = 3775.5x - 0.153$ ($R^2 = 0.99995$) for Cd, $y = 7952.54x - 6.099$ ($R^2 = 0.99988$) for Fe, $y = 63645.60x + 0.214$ ($R^2 = 0.99993$) for Mn, $y = 573104.32x + 7625.96$ ($R^2 = 0.99908$) for Ca, and $y = 21063.55x + 112.34$ ($R^2 = 0.99957$) for Zn.

Multi-element standard control was prepared in 5% HNO_3 (v/v) medium. Concentration data were expressed on a dry mass basis (mg kg^{-1}_{DW}). The detection limit and the limit of quantification of the instrument for each element analysed are: $LOD_{Fe} = 0.041$, $LOQ_{Fe} = 0.135$; $LOD_{Ca} = 0.003$, $LOQ_{Ca} = 0.011$; $LOD_{Mn} = 0.0001$, $LOQ_{Mn} = 0.0005$; $LOD_{Zn} = 0.028$; $LOQ_{Zn} = 0.092$; $LOD_{Cd} = 0.0033$, $LOQ_{Cd} = 0.011$.

Glufosinate and MPP metabolite

Targeted quantitative analyses of glufosinate and MPP metabolite were performed in roots, leaves and fruits extracts. Lyophilized samples (0.5 g) were homogenized with Milli Q water containing 0.1% (v/v) formic acid following the procedure of Halim and Kuntom (2013) and centrifuged at $2500 \times g$ (Allegra 64R, Beckman) for 10 min at 10 °C, the supernatant was collected and filtered using a Whatman filters (0.22 µm). Filtered samples were diluted in water (1:5, v:v) and measurements performed on three technical replicates for each biological replication. Analysis were carried out by LC-MS/MS using a Sciex 5500 QTrap+ mass spectrometer (AB Sciex LLC, Framingham, MA, USA), equipped with a Turbo V ion-spray source and coupled to an ExionLC AC System custom made by Shimadzu (Shimadzu Corporation, Kyoto, Japan) which includes ExionLC Controller, ExionLC Degasser, 2 ExionLC AC Pumps, ExionLC AC Autosampler. The UHPLC chromatographic separation was performed on a Venusil Hilic HPLC Column 10 cm $\times$ 2.1 mm, 3 µm. The elution was carried out using acetonitrile/water 15/85 containing 0.1% (v/v) formic acid as a mobile phase at flow rate 400 µL min^{-1}. Injection volume was 5 µL and column oven temperature was setup at 40 °C. Analytical run was 3 min; from 0 to 0.8 min and from 2 to 3 min the flow was diverted to waste to minimize source contamination. MS/MS experiments were performed in Electrospray negative ion mode using nitrogen as collision gas, with the following operation source parameters: source type, Turbospray; nebulizer gas (GS1) 60 (arbitrary units); turbo gas (GS2) 60 (arbitrary units); curtain gas (CUR) 20 (arbitrary units); temperature (TEM) 500 °C; Ionspray Voltage (IS) -4500 V. Glufosinate was determined using Selected Reaction Monitoring (SRM) of the transition

m/z 180.4→62.9 using the following compound-related parameters, optimized for maximum signal: Declustering Potential (DP), − 91 V; Collision Energy (CE), 60 eV; Collision cell eXit Potential (CXP), − 6 V. Qualitative confirmation was achieved using Information Dependent Acquisition (IDA) criteria to switch from SRM to EPI (Enhanced Product Ions) obtaining the MS-MS spectrum using a CE of 35 eV with a CE spread of 15 eV. Data were normalized according to matrix effect and recovery percentage. Matrix effect was calculated as peak area of the sample spiked after extraction/peak area of the standard, while recovery was calculated as peak area of the sample spiked before extraction/peak area of the sample spiked after extraction. Calibration curve for quantitative analysis were done using glufosinate and MPP metabolite standard (Supelco®, Sigma) from 1 to 512 ppb.

Ethylene measurements

Fruit ethylene production was evaluated enclosing one ripe fruit in airtight glass bottles closed with plastic screw caps provided with rubber septa (Dias et al. 2022). Fruit was incubated for 1 h at 25 °C and the gas sample (2 mL) was taken from the top of the airtight glass bottles with a hypodermic syringe. The ethylene concentration in the sample was measured by gas chromatography (HP5890, Hewlett-Packard, Menlo Park, California), using a flame ionization detector (FID), a metal column (i.d. 150×0.4 cm) packed with Hysep T. Column and detector temperatures were set up at 70 and 350 °C, respectively. A nitrogen carrier gas at a flow rate of 30 mL min^{-1} was used.

Brix and total phenols concentration

The soluble sugar concentration in fruit was measured using a portable refractometer (Shodex, West Berlin, NJ, USA) and expressed in °Brix. Total polyphenols were extracted from 2 g fresh material using 7 mL of 80:20 (v/v, methanol/MilliQ). The samples were stirred in the dark for one hour, filtered through a 0.45 µm cellulose filter (Millipore, Milan, Italy) and stored at − 20 °C. Total phenols concentration was determined in technical triplicates from the reduction of Folin-Ciocalteau reagent by phenolic compounds following Singleton et al. (1999). Folin–Ciocalteu's phenol reagent (500 µL) was added to 100 µL of extract, or standard solution of gallic acid, and the mixture was shaken. After, 2 mL of 2% (w/v) Na_2CO_3 solution and 6.5 mL of Milli Q water were inserted in the solution and mixed thoroughly. The absorbance was measured at 750 nm versus water blank after 60 min of incubation at 27 °C. Total phenolic content was expressed as mg gallic acid equivalents (GAE) per 100 g of fresh material.

Leaves malondialdehyde assay

Lipid peroxidation was determined in leaves sampled at 28 days following the malondialdehyde (MDA) protocol (Heath and Packer, 1968). Briefly, leaf samples (0.3 g) were homogenized in 2.5 mL of trichloroacetic acid 0.1% and centrifuged at $10,000 \times g$ for 10 min. The supernatant was collected, and 1 ml was mixed with 4 ml of 20% trichloroacetic acid and 0.5% thiobarbituric acid. The mixture was heated at 95 °C (30 min), quickly cooled and centrifuged at $10,000 \times g$ for 10 min. The supernatant was used to determine MDA concentration at 532 nm using a UV–vis spectrophotometer (Tecan Infinite® 200 PRO).

Health risk assessment of Cd and glufosinate

A potential health risk to the consumers ingestion of Cd and glufosinate derived from consumption of wild strawberries was calculated following Melai et al. (2018) for the estimated daily intake (EDI): EDI (mg metal or pesticide/ kg per body weight per day) $= C_{metal\ or\ pesticide} \times Q/BW_{average}$.

Where $C_{metal\ or\ pesticide}$ is the measured concentration of metal or pesticide in fresh fruit (mg kg^{-1}), Q is the quantity of daily ingested fruit (kg) and $BW_{average}$ is the estimated average body weight (52.6 kg) considering the more exposed consumers (from 10 to 18 years).

Statistical analysis

The experiment was set up in a completely randomized design (n = 5). Data were subjected to 2-way ANOVA, followed by Bonferroni test, for statistical evaluation of the effects produced by Cd, glufosinate ammonium and the interaction of these two factors. Data represent mean ± SD. *t-test* was performed comparing control data and treatment in mineral element analyses. In each graph, different letters indicate significant difference among treatments. Statistical elaboration was performed using the NCSS 2000 Statistical Analysis System Software (NCSS, LLC., Kaysville, Utah, USA). Results and graphs were made using Prism (GraphPad Software, San Diego, USA).

Results

Gas exchange, chlorophyll measurements, growth parameters and MDA in leaves

Annabelle plants were investigated using non-destructive methods (Fig. 1). The hand-held meter for measuring the
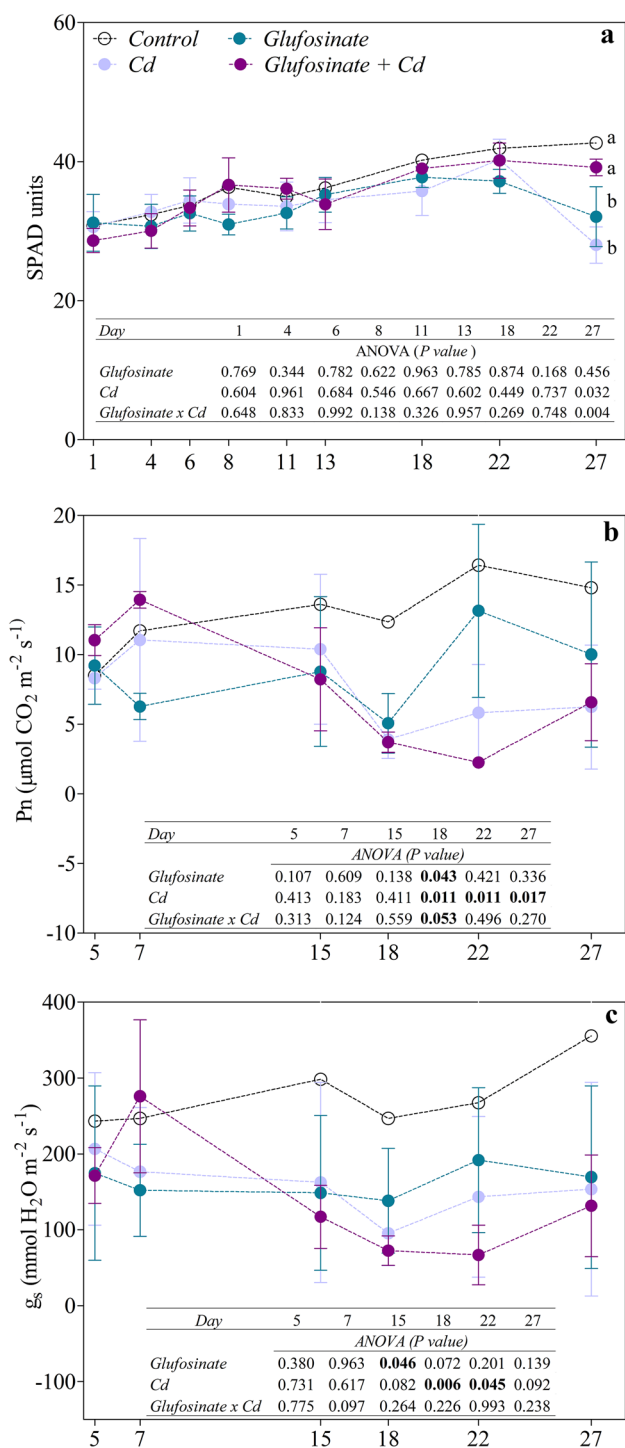
Fig. 1 SPAD units and gas exchange (Pn and gs) parameters in leaves of *Fragaria vesca* L. cv. Annabelle at different time. Control, 1 mg L^{-1} of Cd, 10 μg L^{-1} of glufosinate ammonium and mix of 1 mg L^{-1} of Cd and 10 μg L^{-1} of glufosinate ammonium. Statistical significance was determined with two-way ANOVA followed by Bonferroni test (n = 5) at each sampling time. Data represent mean ± SD

chlorophyll content of leaves (SPAD) showed that treatments did not affect significantly the chlorophyll $a + b$ content during the experiment until the 22 day then, a decrease of SPAD value has been observed under Cd treatments (Fig. 1a). There was a significant and stable effect of Cd treatment alone on photosynthetic parameters starting from day 18 (Fig. 1b). The glufosinate effects on photosynthetic parameters appeared at 18 (P$_n$) and 15 (g$_s$) day but then plant seem to recover. Finally, the interaction between Cd and glufosinate was never significant.

The fruit fresh weight per plant was negatively affect by Cd treatment after 28 days, while the number of fruits per plant was not significantly modified (Table 1). Similarly, leaves and roots FW did not change under treatments. The interaction of glufosinate ammonium and Cd was significant on chlorophyll $a + b$ data (Table 1) with the lowest chlorophyll $a + b$ concentrations in glufosinate (875 ± 284 μg g^{-1} FW) followed by Cd (1013 ± 336 μg g^{-1} FW), glufosinate + Cd (1172 ± 277 μg g^{-1} FW), and control (1340 ± 269 μg g^{-1} FW) treated plants. After 28 days of treatments the degree of plasma membrane damage (MDA) in leaves of treated plants remain in the range of control leaves (Table 1).

Cadmium and mineral elements in plants

Application of 1 mg L^{-1} of Cd without or with glufosinate induce a significant increase of this element in all organs (Fig. 2) and Cd concentrations decreased sharply from roots (> 200 μg g^{-1}$_{DW}$) to leaves (< 15 μg g^{-1}$_{DW}$) reaching the lowest concentrations in fruits (< 3 μg g^{-1}$_{DW}$). When glufosinate was analysed in each organ, only the metabolite MPP has been detected over the detection limit (Fig. 3). As for Cd, the MPP concentrations decreased from roots (> 80 ng g^{-1}$_{DW}$) to leaves and fruits (< 20 μg g^{-1}$_{DW}$).

The concentration of Fe, Ca, Zn and Mn in roots, leaves, and fruits was measured in order to understand the distribution pattern of these essential elements in wild strawberry plants under Cd and glufosinate treatments. For all elements, the concentration was higher in roots followed by leaves and finally by fruits (Fig. 4). Zn concentration in roots was in the range of 70–156 mg kg^{-1}, decreased at leaves level reaching the range concentration of 7–9 mg kg^{-1} in leaves and 3.8–8.5 mg kg^{-1} in wild strawberries where a higher Zn concentration was measured in the control plants compared to the other treatments (Fig. 4c). Roots manifest a reduction of Zn microelement when expose to Cd or a Cd + glufosinate (P = 0.024 and P = 0.0046, *t-test*) (Fig. 4d). Cd had a significant negative effect on Fe uptake in leaves when applied alone or in a mix with glufosinate (P = 0.0079 and P = 0.0102, *t-test*) and similarly in Ca uptake (P = 0.0068 and P = 0.0023, *t-test*) (Fig. 4a, b). Mn uptake and distribution followed the order: leaves ≥ roots > strawberries (Fig. 4c)

Table 1 Fruit number (n plant^{-1}), fruit, leaves and roots fresh weight (g plant^{-1}). Chl $a+b$ (µg g^{-1} FW) and leaves MDA (nmol g^{-1} FW^{-1}). *Fragaria vesca* L. cv. Annabelle after 28 days of exposure to control (0 mg L^{-1} Cd, 0 mg L^{-1} glufosinate ammonium), 1 mg L^{-1} of Cd, 10 µg L^{-1} of glufosinate ammonium, 1 mg L^{-1} of Cd and 10 µg L^{-1} of glufosinate ammonium. Statistical significance was determined after two-way ANOVA followed by Bonferroni test (n=5). Data represent mean±SD. Different letters indicate significant difference among treatments

	Treatments				Two way ANOVA		
	Control	Cd	Glufosinate	Gluf.+Cd	*Glufosinate*	*Cd*	*Gluf.x Cd*
Fruits (n. plant^{-1})	5.6±3.78	3.2±2.59	5.2±4.09	5.8±6.26	0.361	0.594	0.361
Fruits FW (g plant^{-1})	34.5±16.45	10.2±9.36	25.3±22.55	21.0±23.29	0.515	0.003	0.172
Leaves FW (g plant^{-1})	47.3±25.08	49.2±16.65	43.3±12.32	54.3±16.88	0.946	0.462	0.602
Roots FW (g plant^{-1})	4.0±1.84	4.1±2.11	5.3±2.23	5.2±2.97	0.657	0.078	0.658
Chl a+b (µg g^{-1} FW)	1340±269 a	1013±336 ab	875±284 c	1172±277 bc	0.237	0.763	0.002
Leaves MDA (nmol g^{-1} FW)	5.37±1.12	6.48±1.54	7.47±1.94	6.10±1.17	0.211	0.843	0.079
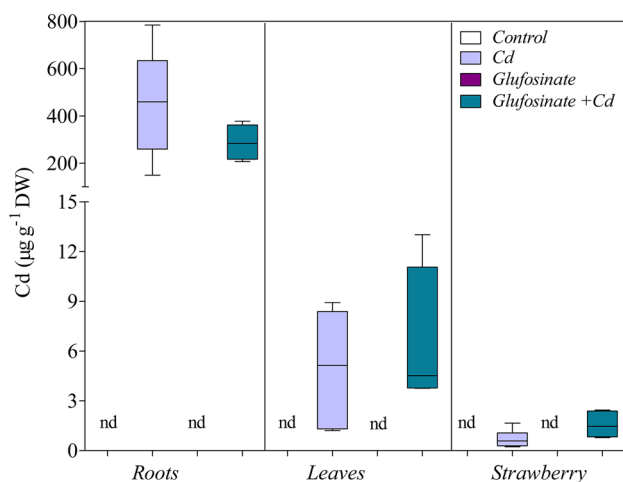
Fig. 2 Box-and-whiskers representation (n=5) of Cd concentration in *Fragaria vesca* L. cv. Annabelle after 28 days of exposure to control (0 mg L^{-1} Cd, 0 mg L^{-1} glufosinate ammonium), 1 mg L^{-1} of Cd, 10 µg L^{-1} of glufosinate ammonium, 1 mg L^{-1} of Cd and 10 µg L^{-1} of glufosinate ammonium. nd = not detected
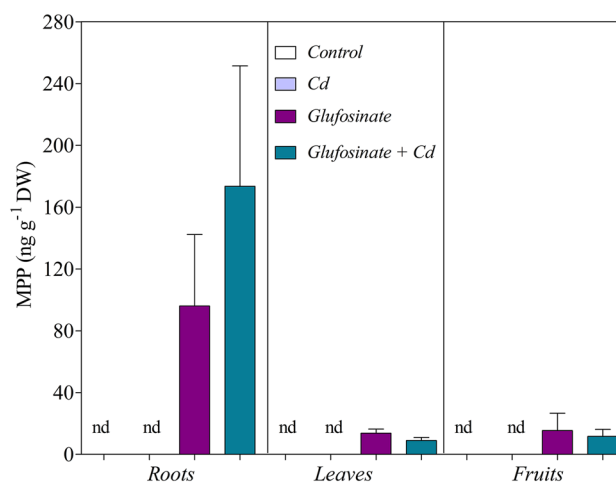

Fig. 3 Glufosinate concentration (n=3) in *Fragaria vesca* L. cv. Annabelle after 28 days of exposure to control (0 mg L^{-1} Cd, 0 mg L^{-1} glufosinate ammonium), 1 mg L^{-1} of Cd, 10 µg L^{-1} of glufosinate ammonium, 1 mg L^{-1} of Cd and 10 µg L^{-1} of glufosinate ammonium. nd = not detected

and a significant Cd induced stress on Mn concentration in strawberries was observed (P=0.0395). The effect on Mn and Zn uptake under Cd stress was observed in fruits (Fig. 4c, d).

A glufosinate induced stress on Fe amount was recorded in fruits (P=0.0334) and a significant reduction of fruits Ca uptake has been observed under glufosinate exposure (−35%) compared to control.

Fruit brix content, total polyphenols and ethylene production

To evaluate if Cd and glufosinate ammonium alone or in combination could affect wild strawberries, the Brix content, the total polyphenols and the ethylene production were monitored after four weeks of treatments (Fig. 5). Brix data reached the higher value under glufosinate treatment (13.6

°Brix) and the lower under control condition. Similarly the higher total polyphenols value was registered under glufosinate treatment (1.14 mg acid gallic equivalent). Ethylene production increase in all treatments compared to control but not significant statistical differences were recorded (Fig. 5). All these parameters did not show any significant difference among the four treatments.

As concern the estimated daily intake –EDI– data (Table 2), we reached value 3.74×10^{-6} mg kg^{-1} per body weight per day for wild strawberry grown under Cd and 9.04×10^{-6} mg kg^{-1} per body weight per day for wild strawberry grown under Cd and glufosinate mix considering the Cd average value.

Also MPP estimated daily intake –EDI– data (Table 2) was calculated reaching the average value of 7.6×10^{-4} µg kg^{-1} per body weight per day for wild strawberry grown under Cd and 5.7×10^{-4} µg kg^{-1} per body weight per day
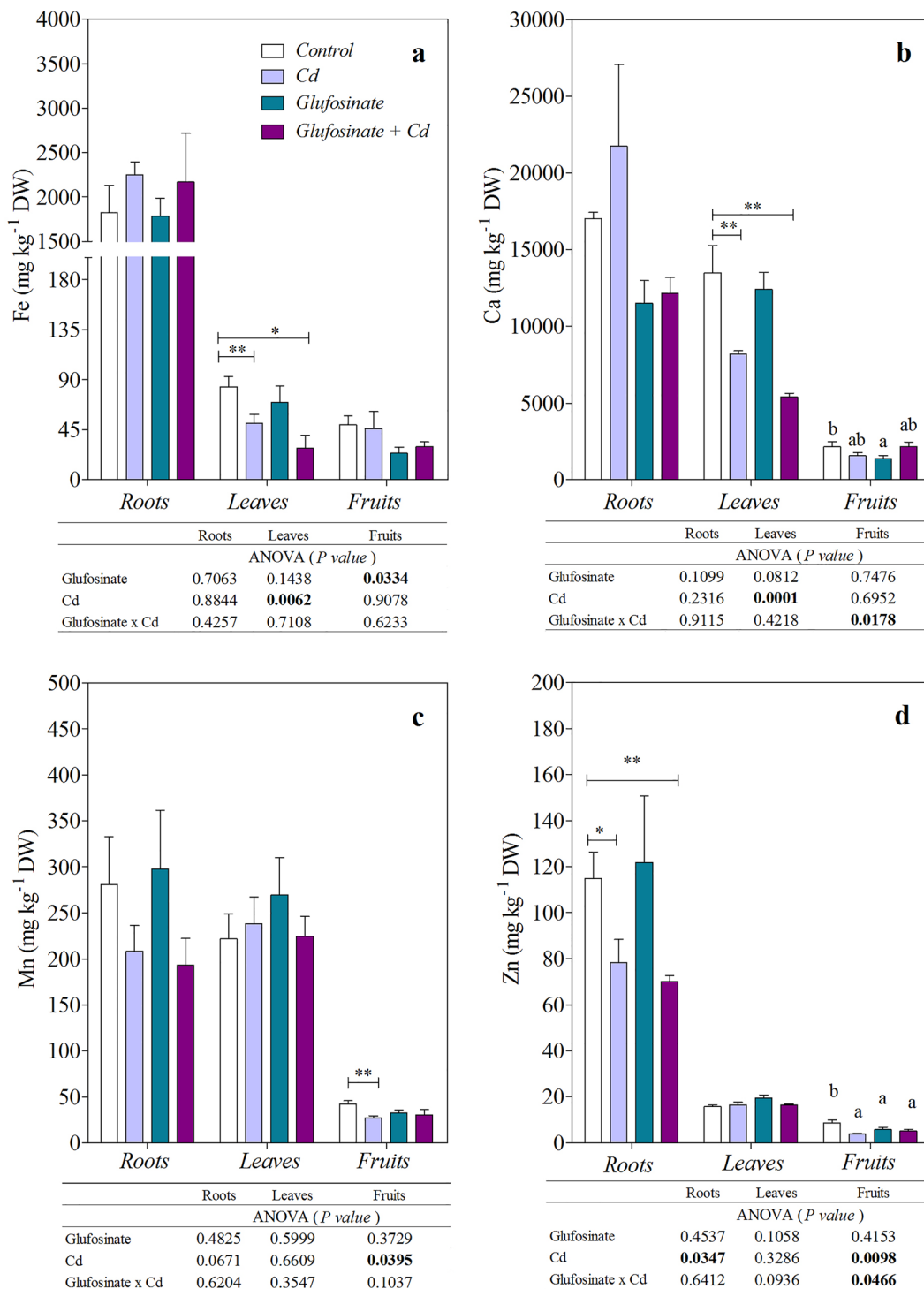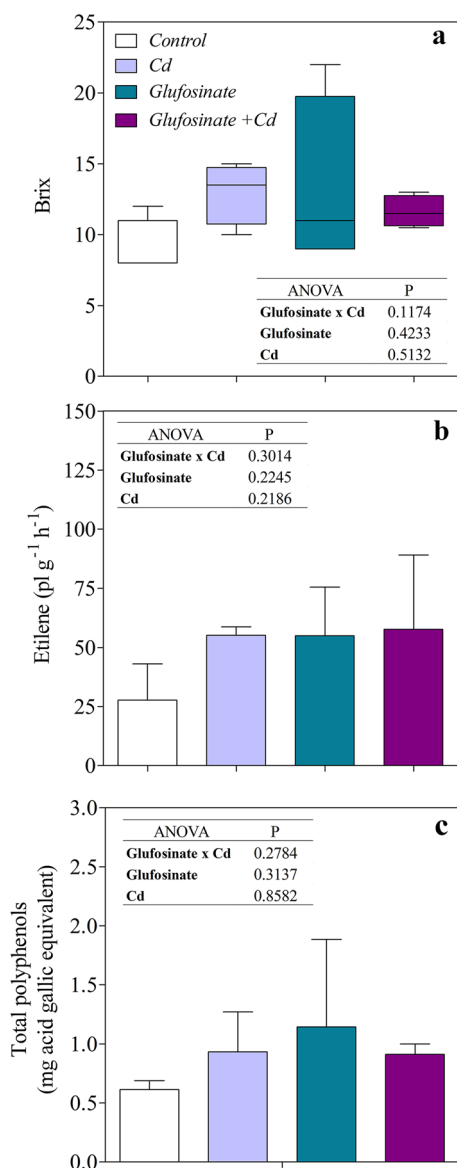
Fig. 4 **a** Iron (Fe), **b** Calcium (Ca), **c** Manganese (Mn) and **d** Zinc (Zn) concentration in wild strawberry plants exposed to Hoagland solution (control), 1 mg L^{-1} of Cd, 10 µg L^{-1} of glufosinate ammonium and a mix with 1 mg L^{-1} of Cd and 10 µg L^{-1} of glufosinate ammonium for 28 days. In each organ, statistical significance was determined after two-way ANOVA followed by Bonferroni test (n = 5). Data represent mean ± SD. Different letters indicate significant difference among treatments

Fig. 5 Brix, ethylene production, and total polyphenols and fruit of wild strawberry plants exposed to Hoagland solution (control), 1 mg L^{-1} of Cd, 10 µg L^{-1} of glufosinate ammonium and a mix with 1 mg L^{-1} of Cd and 10 µg L^{-1} of glufosinate ammonium for 28 days. Statistical significance was determined after two-way ANOVA followed by Bonferroni test (n = 5) in each organ. Data represent mean ± SD

for wild strawberry grown under Cd and glufosinate mix. Concerning glufosinate, it is not possible to indicate the EDI of samples since the concentrations were below the detection limits and not references value are actually present in literature.

Discussion

Strawberry (Fragaria spp.) is normally produced in protected cultivation or in open field. Open field may be very susceptible to Cd contamination with damage to plants (Zhang et al. 2020; Elgallal et al. 2016). Among the negative effects of Cd stress there are a reduction on the content of soluble sugar and strawberry yield (Zhang et al. 2020). In our experimental condition, Cd reduced the wild strawberry weight of 50% compared to control wild strawberries. Our data showed that the plants treated with Cd had significant decrease of Chl *a + b* compared to control after 28 days. Cadmium toxicity effects on chlorophylls could be explained because of inhibitory effect of Cd on enzymes involved in pigment biosynthesis (Muradoğlu et al. 2015). Furthermore, Cd induced oxidative damage and usually leads to the degradation of pigments (Chen et al. 2003). Cadmium has been shown to interact with the uptake of numerous elements (Haider et al. 2021). In sugar beet, deficiency of Fe is induced by Cd in roots (Chang et al. 2003). The uptake of Ca, Zn, Mn in pea was strongly inhibited after exposure to Cd (Metwally et al. 2005). In saltbush (*Atriplex halimus* L.), a decreased uptake of Ca was found due to the toxicity of Cd (Kinay 2018). In strawberry Camarosa cultivar (Muradoğlu et al. 2015), increasing Cd affect other mineral elements. Zn concentration was found higher in Camarosa root when Cd concentrations increase. An opposite trend has been observed in our experiment, where Zn decrease in root under Cd exposure. It was also demonstrated that concentration of Fe, Ca, and Zn in strawberry leaves decreased with the increase of Cd concentration (Bi-qing et al. 2007). In our study, the interaction between Cd and glufosinate factors, changed Zn concentration in wild strawberries with a lesser concentration of this element in treated plants compared to control. Glufosinate is considered a contact herbicide due to its fast activity and limited translocation in plants. The risk of leaching of glufosinate to river and groundwater along with its permanent presence in wastewater remains unclear (Masiol et al. 2018; Geng et al., 2018).

The absence of active transporters and physicochemical characteristics that facilitate its translocation are the main reasons of its low translocation rates (Takano et al. 2020a). An efficient uptake of glufosinate depends on several factors, such as, spraying conditions, air temperature, humidity, and the target species. Low temperature and low humidity reduce glufosinate uptake (Takano et al., 2020). Our data demonstrated that glufosinate has been uptaken by wild strawberry and degraded to MPP metabolite. Glufosinate uptake and degradation did not affect plant growth and fruits quality.

The use of glufosinate ammonium causes glutamine synthetase inhibition with ammonium ion accumulation and alteration of photosynthesis. A previous study (Petrovic

Table 2 Cd and MPP minimum and maximum concentration in *Fragaria vesca* L. cv. Annabelle after 28 days of exposure to 1 mg L^{-1} of Cd, and 1 mg L^{-1} of Cd and 10 µg L^{-1} of glufosinate ammonium. The estimated daily intake – EDI - (mg kg^{-1} per body weight per day) calculated for the minimum, average and maximum Cd and MPP concentration are reported. The Cd reference Acceptable Daily Intake – ADI - (mg kg^{-1} per body weight per day) and the reference estimated daily intake (EDI) are also repoterd. (a) Van Paemel et al. 2010; (b) Leclercq et al. 2009

	Cd	Glufosinate + Cd
Cd $_{min}$ (mg kg^{-1} FW)	0.02	0.09
Cd $_{max}$ (mg kg^{-1} FW)	0.20	0.29
Cd - EDI_{min} (mg kg^{-1} per body weight per day)	9.88×10^{-7}	4.45×10^{-6}
Cd - $EDI_{average}$ (mg kg^{-1} per body weight per day)	3.74×10^{-6}	9.04×10^{-6}
Cd - EDI_{max} (mg kg^{-1} per body weight per day)	9.88×10^{-6}	1.43×10^{-5}
Cd reference ADI value (mg kg^{-1} per body weight per day)[a]	1×10^{-4}	
Cd reference EDI value (mg kg^{-1} per body weight per day)[c]	7.4×10^{-6}	
	Glufosinate	Glufosinate + Cd
MPP $_{min}$ (ng g^{-1} FW)	7.83	7.25
MPP $_{max}$ (ng g^{-1} FW)	28.3	16.3
MPP - EDI_{min} (µg kg^{-1} per body weight per day)	3.8×10^{-4}	3.5×10^{-4}
MPP - $EDI_{average}$ (µg kg^{-1} per body weight per day)	7.6×10^{-4}	5.7×10^{-4}
MPP - EDI_{max} (µg kg^{-1} per body weight per day)	14×10^{-4}	8×10^{-4}
MPP reference ADI value (mg kg^{-1} per body weight per day)	*Not available*	
MPP reference EDI value (mg kg^{-1} per body weight per day)	*Not available*	

et al. 2009) investigated the relationship between excess NH_4–N in foliar tissues and interveinal chlorosis in leaves of "Nyoho" strawberry, a cultivar that often suffers leaf-yellowing symptoms. Glufosinate dose of 185 mg L^{-1} was shown to be lethal to "Nyoho", inducing severe interveinal chlorosis that progressed into necrosis and rapid desiccation of the leaf tips. In accordance with this study, we observed a significant reduction in chlorophyll *a + b* concentration in plants treated with glufosinate ammonium.

Most abiotic stress conditions like heavy metal toxicity result in increased production of the plant hormone ethylene (Maksymiec 2007). Ethylene synthesis increased after inhibition of the photosynthetic apparatus by heavy metals, probably resulted from increased activity of enzymes synthesizing this hormone after heavy metal action. In barley plants, Vassilev et al. (2004) showed that increasing Cd concentrations (0, 14, 28, and 42 mg kg^{-1}), significantly increased ethylene production by 29 and 44% at the 14 and 28 mg kg^{-1} and significantly decreased it by 29% at the 42 mg kg^{-1} after 10 days of treatment. Our observation about ethylene did not show any significant difference in the two sampling between Cd and control treatments.

Ethylene evolution is also an indicator of herbicidal action. You and Barker (2002) showed that ethylene evolution rose with increase of glufosinate ammonium concentration to 25 mg L^{-1} in tomato plants. Despite this evidence, the ethylene measurement did not show any significant difference in our experiment. Again, these evidence in our study showed that realistic residual concentrations of glufosinate

(10 µg L^{-1}) in the wastewater have no impact on ethylene production.

Although heavy metals and pesticides are individually toxic, the interaction between the two should be evaluated. This relation can be synergistic or antagonistic. The synergism occurs when the joint toxicity increase (compared to the individual one) while the antagonism happens when the joint toxicity decrease (Alengebawy et al. 2021). A study conducted by Wang et al. (2015) presented the effect of combined toxicity of Cd with five types of insecticides on earthworm. Eleven mixtures had synergistic effects while 5 exhibited antagonistic ones: this evidence showed that synergic effects occurred more than antagonistic one. To date, there is little data on the effects of joint toxicity in plants. Examples about the interaction between heavy metals and pesticides in plants regarded the use of Cd and Acetochlor or Bensulfuron-methyl as pesticides in rice seedlings (*Oryza sativa* L., cv. Jinyou 402). In these experiments, treated plants showed a synergic interaction decreasing soluble protein content and suppressing roots and shoots growth (Huang and Xiong 2009). In our experiments, we observe an antagonistic effect, with a decrease in the joint toxicity for the chlorophyll *a + b* concentration.

As reported in the Regulation (EC) n. 1881/2006 (OJ L 364 2006), human foodstuffs have been regulated for the maximum levels of some heavy metals. As concern Cd, the maximum acceptable concentration in strawberry was set to 0.050 mg kg^{-1} wet weight. The 57% of our analysed wild strawberry grown under Cd exposure exceed the Cd

concentration indicated by EU while all the fruits grown under the mix Cd and glufosinate exceed the Cd concentration indicated by EU. Following the public health literature information about Acceptable Daily Intake of Cd (Melai et al. 2018), our results about EDI calculated on the base of maximum concentration measured of Cd (worst-case) and the more exposed consumers (people from 10 to 18 years) did not exceed the indicated value. It is important to note, however, that the EDI values are based on the assumption of daily consumption of contaminated strawberries, and the actual risk of adverse health effects would depend on the frequency and amount of contaminated strawberries consumed, as well as individual sensitivity to MPP. The public health literature information about Acceptable Daily Intake of MPP are not available, and for this, we can only report that wild strawberry fruits growth under our experimental condition present this molecule.

In conclusion, this work proves that concentrations that are found in waste water of Cd and glufosinate ammonium did not chang the quality of wild strawberry fruits, in terms of Brix and polyphenols. However, it has been demonstrated that in hydroponic conditions Cd and MPP metabolite can be present in the fruits representing a potential risk to human health. Therefore, more studies are needed to assess the potential health effects of MPP exposure through consumption of contaminated strawberries.

The use of wastewater like alternative water source for agriculture fits into the circular economy (CE) concept in which waste should be treated as a secondary raw that can be recycled to be reused. Our data confirmed the importance of understanding how contaminants interact each other and translocate in edible parts like fruit. The investigation of the effects of wastewater will make wastewater use possible and safe for human, plants, and the environment.

AB Sciex LLC, 4210 MP-AES Agilent Technologie, LIO5P digital Bioclass).

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interest The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent for publication All Authors have given approval for publication.

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