UNIVERSITÀ DEGLI STUDI DI PADOVA

Dipartimento di Territorio e Sistemi Agro-Forestali (TESAF)

Second Cycle Degree (MSc) in
FOOD & HEALTH

Physiological and morphological effects of the perfluoroalkyl compound GenX in plants

Effetti fisiologici e morfologici del composto perfluoroalchilico GenX

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ACADEMIC YEAR 2022/2023

Acknowledgments

My gratitude goes to DAFNAE department, prof. Antonio Masi for the opportunity given to me, Anna Rita Trentin, Laura Ravazzolo and especially my co-supervisor, Ilaria Battisti, for leading me with expertise, passion and especially patience.

I sincerely thank my parents, Orietta and Angelo, for always believing in me and their infinite support.

To my sister, Monica, for listening and talking me up when I was feeling hopeless.

To the friends made in these two years, you made me rethink my beliefs and live experiences and emotions I would have never imagined.

To myself, with the hope for the future to learn to believe more in what I'm capable of.

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1. ABSTRACT

Per- and polyfluoroalkyl substances, PFAS, are a large family of synthetic chemicals largely used in the industry, but persistent in the environment and hazardous for the health. GenX is an ether-PFAS with short chain produced in 2010 as a “safer” substitute for PFOA (perfluorooctanoic acid), one of the most studied PFAS. The shorter chain was thought to be less persistent in the environment and less toxic, being expelled more promptly by the body. As recent concerns raised after finding GenX in the environment, studies on toxicity investigated its behaviour, but many questions remain unanswered to date. Up to now, there is a gap in knowledge especially on GenX potential bioaccumulation and effects in plants, as exposure to the chemical through the food chain is a possible reality.

With this work, we wanted to investigate the potential effect of GenX on germination and plant growth, as well as some physiology-related aspects such as nutrient solution uptake and chlorophyll content in leaves. This was done by conducting three experiments with four different plant species. Cress (*Lepidium sativum* L.) and tomato (*Lycopersicum esculentum* Mill.) were employed to test germination and seedling growth in filter paper soaked in varying GenX concentrations, ranging from 0.5 mg/L to 20 mg/L. *Arabidopsis thaliana* was grown in a GenX-contaminated agar medium (0.5 up to 20 mg/L) to test germination and root development. Maize (*Zea mays* L.) seedlings were tested for possible GenX effects at 20 mg/L on growth in hydroponics. Findings showed that in cress, tomato, and *Arabidopsis*, germination was not significantly different between controls and treatments. Cress seedlings growth was not reduced by GenX increasing levels, even at high GenX concentrations, and with the 5 mg/L group resulting in significantly longer seedlings than control. Tomato showed a slightly decreased tolerance at increasing GenX concentration, but results were not statistically significant. *Arabidopsis* root length over time showed normal length in all concentrations except for 1 mg/L where roots were shorter. Significant results of possible GenX toxic effect were observed in maize, where the exposure to 20 mg/L affected significantly root biomass and architecture. In leaves, a slight but statistically significant reduced chlorophyll content resulted in treated plants, in comparison to controls.

2. INTRODUCTION

PFAS, per- and polyfluoroalkyl substances, are a big group of compounds largely used in the industry for their peculiar properties and stability. Their persistence in the environment, especially in water, has been causing many concerns for the community as trials on animals showed disturbing effects on the health, especially on the liver, kidneys, and the immune system, leading also to cancer.

After a better understanding of the toxicity of some of these compounds, especially PFOA (perfluorooctanoic acid) and PFOS (perfluorooctane sulfonate), restrictions were imposed, and alternative chemicals were studied and introduced in the market. Among these, GenX, trade name for HFPO-DA (hexafluoropropylene oxide dimer acid), has been proposed as an apparently safe alternative for PFOA. Recent studies highlighted the possibility that this new PFAS can be persistent in the environment and showed some toxic effects on in vivo studies.

Human exposure to this compound, as well as to other legacy PFAS, can happen through many pathways. Water, especially that used for irrigation purposes or in the sludge used for cultivation could lead to the occurrence of GenX in food: from the contaminated water and soil, GenX can move into the vegetables we consume but also in the feed for livestock.

However, GenX toxicity has still many gaps of knowledge as its technology was introduced quite recently, in 2010.

The aim of this work is to better understand the impact that GenX has on germination and growth in some model plants in agriculture.

2.1. The "Forever chemicals": chemical and physical properties of PFAS

PFAS, short for per- and polyfluoroalkyl substances, are synthetic chemicals that have been used since the 1940s. PFAS are commonly found in products such as fire retardants, as well as stain, water, and grease-resistant items. DuPont began using these chemicals in 1951, incorporating them into products like Teflon®, well-known for its application in non-stick cookware (DiGiannantonio, 2022).

Per- and polyfluoroalkyl chemicals have been widely used because of the properties of carbon-fluorine bonds, which are among the strongest chemical bonds known to man: chemical stability and thermal resistance make them highly resistant to degradation.

Due to their extreme persistence, and the scientific evidence that links exposure to harmful effects on wildlife and human health, PFAS pose an “unacceptable” risk according to REACH (article 68.1) and threaten current and future generations.

For this reason, in February 2023, ECHA (European Chemicals Agency) published a restriction proposal, submitted by five national authorities (the Netherlands, Germany, Sweden, Norway, and Denmark), which is tailored to address the manufacture, market, and use of PFAS. The EU’s adoption of the restriction proposal is anticipated in 2025 (Cockcroft et al., 2023).

PFAS are a diverse class of chemicals with a common aliphatic carbon backbone in which hydrogen atoms are fully (perfluorinated) or partially (polyfluorinated) replaced by fluorine.

The strong, chemically inert carbon-fluorine bonds make PFAS highly stable, persistent, and recalcitrant chemicals, thereby making it difficult to manage their occurrence in various environmental media such as soil, water, and air (Kurwadkar et al., 2022).

PFOS ($C_8F_{17}SO_3H$) and PFOA ($C_7F_{15}COOH$) are 8-carbon PFAS; they are referred to as “legacy” PFAS as they are among the most commonly used and frequently detected PFAS in the environment (Meegoda et al., 2022).

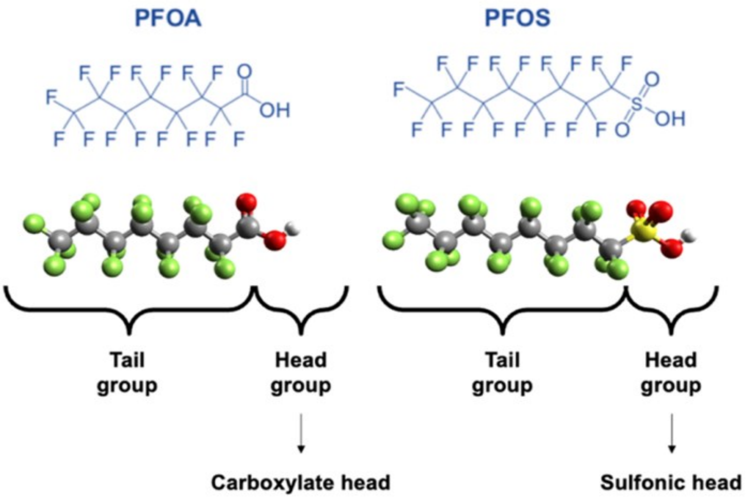


Figure 1 - PFOA and PFOS chemical structures (Meegoda et al., 2022)

The PFAS family can be broken down into two categories: long-chain and short-chain. Short-chained PFAS are classified by carbon atoms in the chain, with less than 8 carbons for PFCAs (Perfluoroalkyl carboxylates) and less than 6 for PFSAs (Perfuoroalkane sulfonates) (Interstate Technology & Regulatory Council, 2022) .

Long-chain PFAS, such as PFOS and PFOA, have a longer half-life and take more time to break down. In fact, in 2009, PFOS and PFOA were listed under the Stockholm Convention on ‘Persistent Organic Pollutants’ due to their demonstrated toxicity, bioaccumulation, persistence in the environment, and ability to travel long distances from the point of release or application (ECHA, 2022).

However, it is important not to generalize PFAS behaviour based only on chain length, as recent research suggests that other factors besides chain length may affect the bioaccumulation potential of PFAS (Interstate Technology & Regulatory Council, 2022). Some manufacturers have moved to using short-chain compounds, like GenX, with the hopes of reducing environmental accumulation, but short-chain PFAS can still accumulate in the body and environment over time with prolonged exposure. Because short-chain PFAS are a recent-use compound group, not as much research has been conducted about their health and environmental impact (North Carolina Coastal Federation, 2020).

A fundamental distinction is to divide PFAS into 2 primary classes: non-polymers and polymers. Each class may contain many subclasses, groups, and subgroups (Figure 2).

Precise criteria for distinguishing polymers from non-polymers have been established, for instance, under the European Union REACH legislation and reported by ITRC (Interstate Technology & Regulatory Council, 2022).

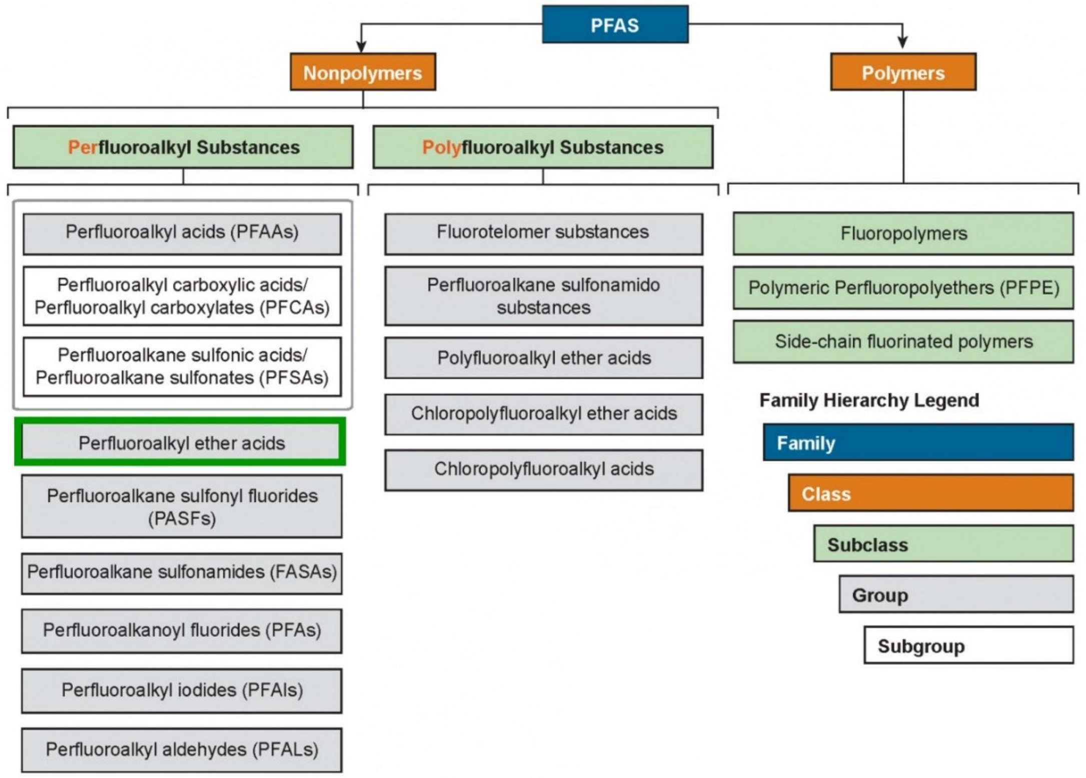


Figure 2 - PFAS family classification; (ITRC (Interstate Technology & Regulatory Council), 2022)

Nonpolymeric PFAS encompass two major subclasses: perfluoroalkyl substances and polyfluoroalkyl substances, which include many groups and subgroups of chemicals.
Perfluoroalkyl substances are fully fluorinated alkane molecules that include perfluoroalkyl acids (PFAAs), to which PFOA (PFCAs, perfluoroalkyl carboxylates subgroup) and PFOS (PFSAs, perfluoroalkane sulfonates subgroup) belong to. They are the most commonly detected PFAS in humans, biota, and other environmental media (Interstate Technology & Regulatory Council, 2022).

The focus of this research work is the compound HFPO-DA, referred to as GenX, primarily used in the industry to substitute PFOA. GenX belongs to the same subclass of the "legacy" PFAS, falling into the perfluoroalkyl ether acids group (Interstate Technology & Regulatory Council, 2022).

2.1.1. Occurrence and consequences of PFAS

The amphiphilic properties of PFAS makes them useful in a wide range of applications, including non-stick cookware, waterproof clothing, and especially firefighting foam (AFFFs), where they have been widely used for many years.

However, such properties also make PFAS highly mobile in the environment and difficult to remove once released. PFAS stability makes them difficult to break down and can remain in the environment for decades: they have been detected in water, air, soil, and biota worldwide, and have been found to accumulate in human and animal tissues (Pérez et al., 2013). Current research suggests that water is likely the largest exposure pathway (Herkert et al., 2020).

The industrial fluorination process is complex and costly and requires specific know-how and facilities. Large companies are those mostly involved in PFAS manufacturing and selling to thousands of downstream users (Le Monde, 2023).

The lack of maintenance and effective filtering systems, coupled with carelessness by these industries gave rise to important contaminations of the surrounding areas, mainly through wastewaters (Figure 3).

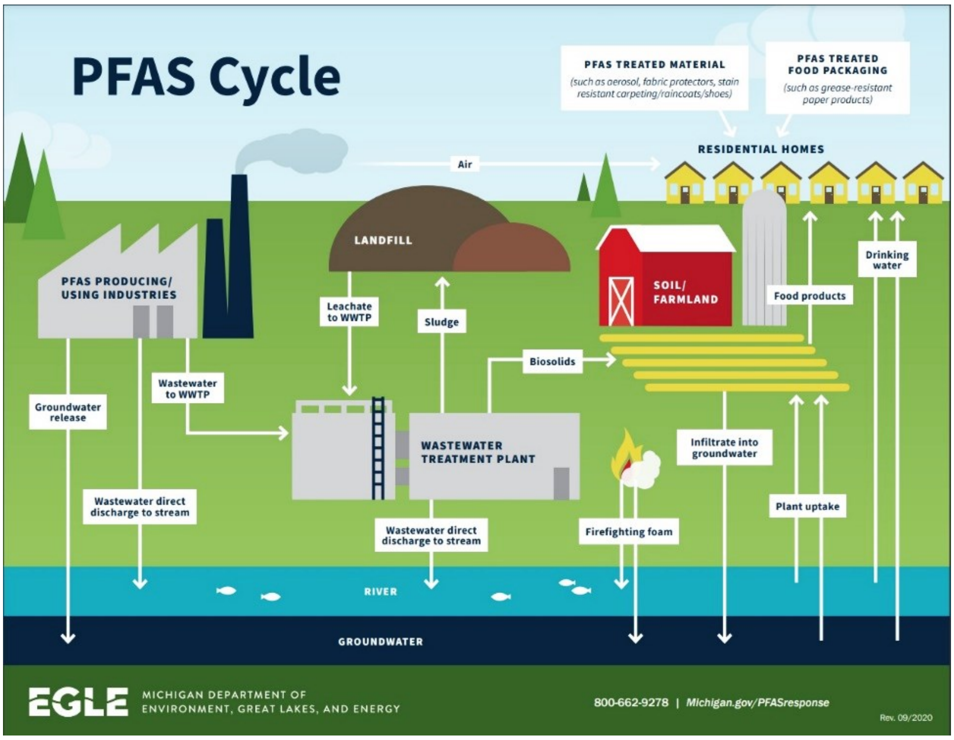


Figure 3 - PFAS and environmental cycle (Michigan PFAS Action Response Team, 2022).

One of the most well-known cases of PFAS pollution from contaminated wastewaters that shed light on many other similar situations is that of Parkersburg, West Virginia in the US, where the DuPont chemical company site, since the early 2000s, released PFAS into the air and water supply, contaminating the surrounding area (Right Livelihood, 2017).

PFOA has been released in the Ohio River for many years, and, thanks to the lawyer Robert Billot, the community struggle was brought to light in 2001. In 2004, DuPont paid $70 million in a class-action lawsuit, and furthermore, was obliged to install filtering systems in the six districts involved and to finance epidemiological studies to evaluate the cause-effect correlation between PFOA exposure and diseases. In 2012, the scientific panel appointed by the Wood County Circuit Court released a report documenting a probable link between C8 PFAS and testicular cancer, kidney cancer, thyroid disease, ulcerative colitis, pregnancy-induced hypertension, and high cholesterol that reflected on the workers and the community (C8 Science Panel, 2012).

From 2002 to 2006, the PERFORCE European project investigated perfluoro derivatives in major European rivers. The Po River in Europe, notably, had the highest concentrations of perfluorooctanoic acid (PFOA), reaching up to 200 ng/L (Polesello et al., 2013).

These findings prompted action in Europe, where the European Food Safety Authority (EFSA) set initial limits for PFAS concentrations in water (PFOS < 300 ng/L; other PFAS < 3000 ng/L). In 2009, PFOS was added to the Stockholm Convention on Persistent Organic Pollutants (Tromba, 2017).

In Italy, where the Po River was contaminated, a study conducted by the CNR (National Research Council) and the Ministry of the Environment in 2013 traced some perfluoroalkylic substances in groundwater, surface water, and drinking water (Polesello & Valsecchi, 2013). Two major contamination sites were identified: Trissino (Veneto), where Miteni S.p.A. produced perfluorinated substances until 2018 before bankruptcy, and Spinetta Marengo (Piedmont), an active contamination site managed by Solvay Specialty Polymers Italy S.p.A. (Commissione Parlamentare di Inchiesta, 2022).

The Veneto region in Italy has faced severe PFAS pollution from various industrial sites. The contamination traces back to 1965 when the RiMar site, formerly owned by Marzotto (a textile company), used PFOA for textile research, imported from the American company 3M (Tromba, 2017). The contamination was discovered in 2013, first by CNR and later by ARPA (Regional Agency for Environmental Protection) Veneto, stemming from industrial waste from Miteni Spa in Trissino (VI). Such contamination, persisting for half a century before its discovery, polluted groundwater across a 180 km^2 area, affecting the water supply and raising health concerns for residents in the provinces of Vicenza, Verona, and Padua (Commissione Parlamentare di Inchiesta, 2022). Groundwater monitoring also identified two other perfluoroalkyl

compounds, HFPO-DA (GenX) and cC6O4 (perfluoro([5-methoxy-1,3-dioxolan-4-yl]xy) acetic acid), produced by the Miteni company since 2013.
In Piedmont, the Solvay Solexis plant, acquired from Ausimont in 2002, had been using PFAS since the 1990s. In 2008, NOE (Ecological Operational Unit) investigations led to the charge of the top management from Ausimont and Solvay Specialty Polymers for intentional water contamination. The neglected maintenance of the plant's water network caused significant leakage, leading to pollution of the underground aquifer and widespread contamination beyond the industrial area (Ministero dell’Ambiente e della Sicurezza Energetica, 2021). PFAS pollution from the Solvay plant extended through both groundwater and surface water for hundreds of kilometres outside the Alexandria area. ARPA reported that Solvay’s wastewater had peaks of 120 µg/L, discharging PFOA into the Bormida River, a tributary of the Po River (Fazzini, 2023). Concerns about various health issues, including cancer, neurological diseases, endocrine, and metabolic disorders, prompted the regional "STOP Solvay" committee to call for population testing. In 2020, Liege University conducted blood tests, revealing alarmingly high PFOA levels, reaching 10.20 µg/L compared to the 1.9 µg/L limit, among Spinetta residents. This is particularly concerning as PFOA was banned in 2009 (Bocchio, 2022). Following the ban on PFOA production in 2013, Solvay replaced it with cC6O4, a "novel" PFAS, which was found in substantial quantities in the industrial site's wastewater (Gasparini, 2023).

Another big source of global contamination is found in military sites where Aqueous Film Forming Foams (AFFFs) were used. PFAS take advantage of the unparalleled aqueous surface tension–lowering properties used to extinguish fires involving highly flammable liquids and for this reason were used in particular for AFFFs (Kissa, 2001). AFFFs pollution of groundwater is a major source of drinking water contamination and has been identified as a nationally significant challenge in countries such as the United States and Sweden (Sunderland et al., 2019). A famous case of PFAS contamination by AFFFs happened in the US, at the Wurtsmith Air Force Base (AFB), Michigan. The Environmental Protection Agency (EPA) found a level of nearly 80 µg/L of total PFAS in the drinking water on Wurtsmith AFB in 2018, which is well above the permissible limit of 70 ng/L (Michigan PFAS Action Response Team, 2022).

The properties that made PFAS into an industrial success also led to their persistence and bioaccumulation. The health symptoms in the inhabitants near the plants alarmed the communities and scientific research on the potential toxicity of those compounds was carried out.

2.1.2. Routes of exposure and health consequences

In humans, perfluoroalkyl substances last for extremely long periods, with a mean half-life estimated of almost 3.4 years for PFOS and 2.7 years for PFOA (Y. Li et al., 2018). According to the European Environment Agency (EEA) and its briefing no.12/2019, PFAS sources of contamination include drinking water, food, consumer products and dust. Regarding food contamination, fish species at the top of the food chain and shellfish are among the most significant sources (EEA, 2019).

Livestock raised on contaminated land can accumulate PFAS in their meat, milk, and eggs. Among these, meat and its derivatives contribute significantly to PFOS exposure whereas milk and eggs to a lower extent (German Federal Institute for Risk Assessment, 2021). As elevated PFAS concentrations in livestock have also been reported, PFAS uptake from plant-based feed is likely a source (Sunderland et al., 2019).

Regarding human exposure through plant-based foods, more studies are needed as the available data cannot assess the overall human exposure (German Federal Institute for Risk Assessment, 2021).

PFAS can potentially generate a wide range of adverse health effects depending on the circumstances of exposure and individual factors such as age. The health effects of PFAS exposure have led to increased anxiety and stress among affected communities, giving rise to various protesting associations “STOP Solvay” and “Mamme no PFAS” based in northern Italy.

The concern is real as multiple studies showed potential long-term health effects of exposure to these chemicals, including cancer, developmental delays, and other health problems (Mastrantonio et al., 2018; National Toxicology Program, 2020; Yao et al., 2023).

A comprehensive review published in 2020 by the "Environmental Toxicology and Chemistry" journal highlighted the association between exposure to specific PFAS and a range of adverse health effects (National Toxicology Program, 2020).

These effects include altered immune system function, disruptions in thyroid hormone regulation, liver disease, disturbances in lipid and insulin regulation, kidney disease, adverse reproductive and developmental outcomes, and cancer. Many studies have focused on legacy PFAS, especially PFOA and PFOS, while hundreds of other PFAS in commercial use lack toxicity data and need further investigation. Regarding the immune system, both animal and human epidemiological observations confirm that PFAS exposure, particularly in children, can lead to immunosuppression (National Toxicology Program, 2020). This is associated with a higher incidence of inflammation in the lower respiratory tract and atopic dermatitis. PFAS exposure has also been linked to the modification of the C-reactive protein response, a marker for systemic inflammation and a major risk factor for cardiovascular disease (Feng et al., 2022). In terms of thyroid function, PFAS have been shown to affect thyroid hormones in a sex-specific manner and may contribute to thyroid autoimmunity (National Toxicology Program, 2020). Elevated thyroid-stimulating hormone (TSH) levels during early pregnancy have raised concerns about potential adverse maternal and foetal outcomes. The liver is a primary target for the accumulation of long-chain PFAS, and evidence of toxicity includes hepatocyte fat infiltration, specific P450 cytochrome pathway induction, apoptosis, and development of hepatocellular adenomas and carcinomas (K. Li et al., 2017). PFAS exposure has been associated with non-alcoholic fatty liver disease (NAFLD), and supportive data suggests a link to advanced liver disease, including liver cancer in humans (National Toxicology Program, 2020).

Studies have also shown that PFAS can increase total and LDL (low-density lipoprotein) cholesterol levels in both children and adults. Dysregulation of lipogenesis may contribute to insulin resistance, although the relationship with diabetes risk is inconsistent (Andersen et al., 2021).

Long-chain PFAS have extended human half-lives due to active renal tubular reabsorption, leading to their concentration in renal tissues and evidence of nephrotoxicity. Exposure to these contaminants has been associated with diminished glomerular filtration and chronic kidney disease in adults and children, as well as increased uric acid levels, a biomarker of risk (Conway et al., 2018). A study in 2018 found a significant increase in kidney cancer mortality in PFAS-contaminated areas (Mastrantonio et al., 2018). Furthermore, PFAS have been linked to reproductive and developmental effects, including deleterious impacts on conception, pregnancy, and infant development (Szilagyi et al., 2020). Interestingly, fertility rates and birth weight improved following water filtration in a PFAS-contaminated community (Mastrantonio et al., 2018). One concerning aspect is that serum PFAS levels in young children often exceed maternal serum concentrations, as PFAS can cross the placenta and enter breast milk, potentially prolonging contamination during early life stages (Yao et al., 2023).

These findings emphasize the need for continued research and regulatory measures to address the potential risks associated with PFAS exposure and safeguard public health.

2.1.3. Restrictions, regulations and limits

The discovery of PFAS toxicity led to increased regulatory attention on these chemicals. In some cases, regulatory action has been taken to limit exposure to PFAS, such as restricting their use in certain products or setting limits on allowable levels in drinking water.

The global situation regarding PFAS regulation is varied, but the Stockholm Convention on Persistent Organic Pollutants, starting in 2004, has the overarching objective to globally protect human health and the environment from persistent organic pollutants (POPs), including PFAS (United Nations, 2022). PFOS and PFOA, two of the most prominent long-chain compounds, have been restricted under the EU’s Persistent Organic Pollutants (POPs) Regulation since 2010 and 2020, respectively. PFOS, PFOA, and their related compounds have been listed since 2019 under Annex A of the Stockholm Convention, implying that the 185 parties to the Convention should eliminate the production and use of such chemicals. The Norwegian Environment Agency acted in 2019 by proposing to list PFHxS (perfluorohexanesulfonic acid) and related compounds in the Stockholm Convention on POPs to address the associated risks. More recently, in 2022, the Conference of the Parties listed PFHxS and its salts (also used to replace PFOS) in Annex A, with other chemical variants expected to follow (Secretariat of the Stockholm Convention, 2022).

Aside from the EU’s Persistent Organic Pollutants (POPs) Regulation, the European Union stated a regulation to address the production and use of chemical substances in Europe called REACH, "Registration, Evaluation, Authorization, and Restriction of Chemicals" (EC No 1907/2006). REACH requires companies to register and provide information about the chemicals they manufacture or import into the EU, assess the risks of these chemicals, and take measures to manage and reduce any potential risks to human health and the environment. Certain PFAS substances, such as PFOA and PFOS, are subjected to authorization or restriction under REACH. (Polesello et al., 2013)

ECHA, the European Chemicals Agency, is responsible for implementing and enforcing REACH, regulates chemicals in the European Union (EU) and maintains updated the Candidate List of Substances of Very High Concern (SVHC). As of September 2021, ECHA has identified several PFAS substances as SVHC, including PFOA, PFOS, and their salts. Furthermore, in January 2023, ECHA added also PFHpA (Perfluoro-n-heptanoic acid), PFBS (Perfluorobutanesulfonic acid), and GenX. This decision was based on the risk profile of persistence, bioaccumulation, and toxicity, keeping attention on these chemicals' mobility (Schiavone & Portesi, 2023).

In response to advancements in detection technologies and increasing contamination levels, the European Commission proposed a ban on all PFASs in firefighting foams across the European Union in February 2022, aimed to combat soil and groundwater contamination (Schiavone & Portesi, 2023).

In a collective effort against PFAS contamination, Denmark, Germany, the Netherlands, Norway, and Sweden submitted a comprehensive proposal to ECHA to prohibit PFAS under REACH. They identified risks related to the manufacturing, marketing, and use of these contaminants, which required better regulation and control within the EU and the EEA. ECHA published this detailed proposal, one of the most extensive in EU history, in February 2023. The proposal does not specifically define sectors or product categories but is proposing a “blanket” ban for all PFAS. The committees within ECHA were scheduled to evaluate the proposal in March 2023, followed by a six-month consultation period (ECHA, 2023).

Since drinking water and food, of both plant and animal origin, could be contaminated by PFAS exposure, in 2008 the European Food Safety Authority (EFSA) issued a scientific opinion focusing on PFOA and PFOS, pointing out concerns about their contribution to environmental contamination. Then, in 2018, EFSA conducted a risk assessment of PFOS and PFOA in food, revealing that these compounds were detected in blood samples from nearly all individuals tested, indicating widespread exposure. This finding highlighted the urgency of strengthening legislative measures (Schiavone & Portesi, 2023).

In the United States, both state legislatures and the government have been proactive in addressing environmental and public health concerns.

The Safe Drinking Water Act (SDWA) authorizes the U.S. Environmental Protection Agency (EPA) to develop drinking water Health Advisories (HAs): national, non-enforceable advisories on maximum water concentration levels of a specific contaminant intended to provide information to the local governments. As a result, states have adopted a patchwork of regulations and guidance standards that present significant compliance challenges to impacted industries (Schiavone & Portesi, 2023).

In 2016, US EPA issued the “Drinking Water Health Advisory” with HA of 70 ng/L for PFOA and PFOS, individually or combined. Then, in 2019, a formal PFAS Action Plan “Interim Recommendations to Address Groundwater Contaminated with PFOA and PFOS” was introduced by the EPA, which was further updated in February 2020. This plan includes applying a screening level of 40 ng/L for states and local water utilities, designating PFOA and PFOS as hazardous substances under the Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA), and advancing innovative techniques for identifying contaminants in drinking water, soil, and groundwater (EPA, 2020).

In 2022, US EPA released final HAs for 4 PFAS, including GenX chemicals and PFBS for the first time, based on EPA's 2021 final toxicity assessments. The final health advisories for GenX chemicals and PFBS were 10 ng/L and 2 μg/L, respectively (EPA, 2022). To date, however, even for the well-known PFOA and PFOS, there are still discrepancies in the values that different states have adopted.

On March 14, 2023, EPA announced the proposed National Primary Drinking Water Regulation (NPDWR) to establish legally enforceable levels, called Maximum Contaminant Levels (MCLs), for six PFAS: PFOA, PFOS, PFNA (Perfluoronanoic acid), GenX compounds, PFHxS, and PFBS. EPA anticipates finalizing the regulation by the end of 2023 (EPA, 2023; Schiavone & Portesi, 2023).

The global scenario of PFAS contamination laws was also put under scrutiny by the World Health Organization (WHO) updates Guidelines for Drinking-Water Quality (GDWQ) which released the draft “PFOS and PFOA in Drinking Water” in September 2022 for public comment recommending limits for these chemicals. Some criticism was moved to the limits they proposed for PFOA, PFOS, and other PFAS, as the limits suggested by WHO were “much higher” than recent limits set, for example, by Denmark or proposed by Canada (Southerland & Birnbaum, 2023).

2.2. GenX: what do we know about the novel PFAS?

Considering evidence of health hazards, in 2006 EPA initiated a stewardship program with the goal of eliminating chemical emissions of PFOA and related compounds by 2015. To overcome PFOA stoppage, companies sought other chemical compounds that could be able to have the same technical results but without the safety hazards (Ahearn, 2019).

GenX is a trademark name introduced by the Chemours chemical company (a DuPont’s spin-off) to indicate the synthetic ammonium salt of hexafluoropropylene oxide dimer acid (HFPO-DA), a short-chain organofluorine chemical compound, and was then introduced to the market in 2012 to produce fluoropolymers (Beekman et al., 2016). The name GenX can also be used more informally to refer to the group of related fluorochemicals associated with the same processing aid technology (Beekman et al., 2016).

Transition to GenX processing aid technology began in 2009 as part of DuPont company’s commitment under the 2010/2015 PFOA Stewardship Program and with a legal agreement with EPA under the federal Toxic Substances Control Act. GenX was considered a “sustainable replacement” for PFOA as DuPont pointed out GenX in a 2010 marketing brochure. That document describes GenX as having “a favourable toxicological profile” and claims it was eliminated rapidly from the bodies of laboratory animals (DuPont, 2010). There was a thought that these smaller chemicals would be less environmentally or, ecologically dangerous, but they are more difficult to remove as they cannot be easily filtered out (Todd, 2023).

2.2.1. Chemistry of GenX

From a chemical point of view, GenX compounds are perfluoroalkyl ether carboxylic acids (PFECAs), characterized by containing ether groups in perfluoroalkyl carboxylic acid backbones. The length of the carbon chain can vary, with most GenX compounds having between six and eight carbons.

As reported in the PubChem database, the main compound to which, also in this paper, we refer talking about GenX is 2,3,3,3-tetrafluoro-2-(1,1,2,2,3,3,3-heptafluoropropoxy) propanoic acid (IUPAC) with CAS number 13252-13-6, also referred to as HFPO-DA (hexafluoropropylene oxide dimer acid) (Figure 4). Its molecular weight is 330.05 g/mol (National Center for Biotechnology Information, 2023).

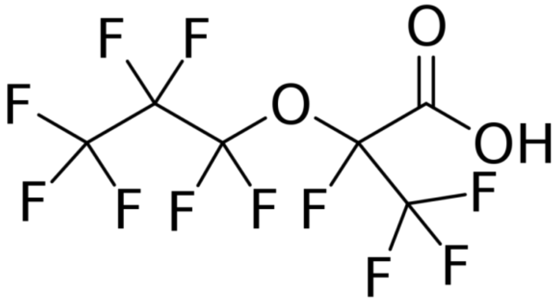


Figure 4 - GenX chemical structure (NC State University, 2017)

GenX is a liquid whereas its ammonium salt is a solid at room temperature. Both are highly soluble in water (Figure 5Figure 5 - HFPO-DA and its ammonium salt (United States Environmental Protection Agency, 2021). Except in very acidic solvents (pH less than 3), the acid will dissolve and be present as the acid anion with a -1 charge. Both compounds can volatilize from water to air, where they will dissolve in aerosolized water droplets or bind to suspended particulate matter. In soils, they will migrate with the aqueous phase or bind to the soil particle surfaces with areas of positive charge (US EPA, 2021).

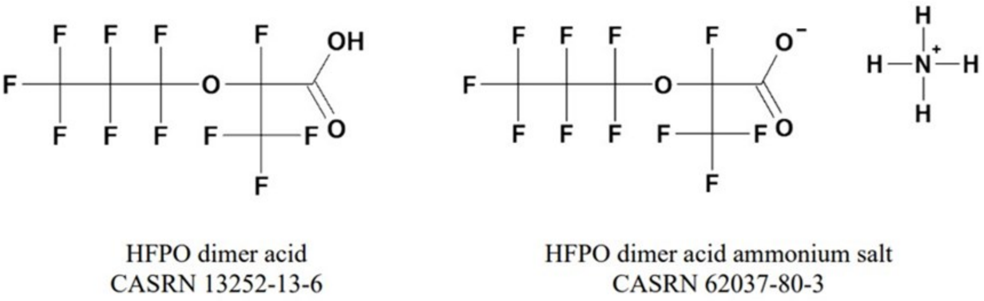


Figure 5 - HFPO-DA and its ammonium salt (United States Environmental Protection Agency, 2021)

According to ECHA, GenX is a substance of very high concern (SVHC) and it is included in the candidate list for restriction authorisation due to its environmental and health effects, particularly its persistence and potential for increasing pollution (ECHA, 2021).

The chemical industry claimed that short-chain PFAS were safer, primarily because they tend to leave our bodies more quickly, and companies took advantage of the structural differences to bypass the regulatory pressure put on long-chain PFAS (Temkin, 2021). In fact, the physical properties of GenX chemicals are similar to those of other PFAS compounds. They are highly stable and do not break down easily in the environment. They have a low surface tension, which makes them excellent surfactants, frequently used to manufacture water-repellent coatings. They are also resistant to heat, acids, and bases. HFPO-DA and its ammonium salt can resist photolysis, hydrolysis, and biodegradation. The degradation data suggest that the substances will be persistent - with a half-life longer than 6 months - in air, water, soil, and sediments. They are not expected to be removed during conventional wastewater treatment or conventional drinking water treatment, as described in a study conducted in the Cape Fear River (Sun et al., 2016).

Carbon filters, commonly used to remove legacy PFAS from water, were found to be less efficient for shorter chain lengths (Herkert et al., 2020). In a 2020 study, PFAS removal efficiency was dependent on chain length, with long-chain PFAS (~60–70% removal) being more efficiently removed than short-chain PFAS (~40% removal); meanwhile, under-sink dual-stage and reverse osmosis filters tested showed near complete removal for all PFAS evaluated (Herkert et al., 2020).

2.2.2. Environmental occurrence and contamination

GenX was commercially introduced by DuPont in 2010 and it has been in production ever since (DuPont, 2010). There are several locations where elevated levels of GenX have been detected, particularly near facilities of the Chemours group where it is manufactured or used.

In the United States, the EPA has been monitoring PFAS in the Cape Fear River, North Carolina, since 2013. The discovery of HFPO-DA and other PFAS in the river was first announced in 2014 and findings were published in 2016, revealing that downstream of the Fayetteville Work site, HFPO-DA was present at a concentration of 631 ng/L, approximately eight times higher than the combined PFCA and PFSA concentrations (79 ng/L) (Sun et al., 2016).

These chemicals were not only found in the river but also in the drinking water, although the Cape Fear Public Utility Authority's robust water treatment. Lower Cape Fear River serves as the drinking water source for Wilmington, a city of around a quarter million people (Ahearn, 2019). The "GenX Exposure study" began in November 2017 in response to drinking water contamination from the Chemours plant in Fayetteville. The study initially measured the occurrence of GenX and other PFAS in blood samples from communities in New Hanover and Brunswick County, downstream from the Chemours facility, which relied on surface water from the river for their public water systems. Although GenX was not detected in the first blood samples, three new PFAS compounds were found. However, these emerging fluorinated products disappeared in subsequent blood samplings. Legacy PFAS were found at significantly higher concentrations in people in Wilmington compared to the U.S. population, indicating once again their persistence (NC State University, 2017).

In Europe, concerns were raised especially in the Netherlands, Dordrecht, were the Dutch Chemours plant is located and has produced GenX since 2013. Elevated levels of GenX have been detected in the groundwater near the Chemours plant. In 2017, the Dutch Institute for Health and Environment (RIVM) established a water quality standard (QS) for GenX at 118 ng/L in surface water, though this was based on assumptions due to limited data (Verbruggen *et al*., 2017). In 2016, Gebbink et al. reported GenX concentrations exceeding the QS in six downstream sampling locations, with concentrations peaking at more than 800 ng/L. In 2017, a concentration of 128 ng/L was detected in a downstream location, and in 2018 a peak concentration of 6800 ng/L was reported in a local waterbody in Helmond near a Fluorochemical production plant, thus unveiling potential risks to human consumption of fish caught in these waters (Gebbink & van Leeuwen, 2020).

2.2.3. Recent studies on GenX toxicity

The persistence of GenX compounds in the environment as in drinkable water and their potential to accumulate in the food chain have raised alarming concerns about their possible health effects.

As previously described, studies have linked exposure to PFAS compounds with various health problems, such as kidney and testicular cancer, immune system dysfunction, and developmental delays in children (National Toxicology Program, 2020). Toxicity of PFAS was generally associated with perfluoro-carbon chain length and the functional group. Hence, “replacements” or alternative compounds to PFOA and PFOS with a shorter chain length (e.g., GenX) were introduced.

The available toxicity studies demonstrate that the liver is particularly sensitive to HFPO dimer acid- and HFPO dimer acid ammonium salt. An EPA's document dated October 2021, assessed toxicity values for HFPO-DA, and included specific findings regarding GenX toxicity (US EPA, Office of Water and Health and Ecological Criteria Division, 2021):

1. Liver Toxicity: GenX has been linked to liver toxicity in animal studies, resulting in apoptosis, necrosis, and increased liver weight;
2. Kidney Toxicity: Studies have shown an increased kidney weight in response to GenX exposure.
3. Immune Effects: GenX has been found to suppress antibody production in animal models.
4. Haematological Effects: Animal studies have reported effects such as low blood cell count, reduced haemoglobin levels, and decreased haematocrit due to GenX exposure.
5. Reproductive and Developmental Effects: GenX exposure has been associated with early delivery, placental lesions, maternal gestational weight gain, and delays in genital development in offspring.
6. Cancer: The EPA has suggested that there is "*Suggestive Evidence of Carcinogenic Potential"* related to oral GenX exposure in humans, based on findings of hepatocellular adenomas and carcinomas in female rats and combined pancreatic acinar adenomas and carcinomas in male rats.

Moreover, the environmental occurrence of GenX and its potential long-term impact have raised additional concerns. While GenX has been found to accumulate less than its predecessor, PFOA, in certain organisms, its environmental concentrations may still increase over time. This could ultimately lead to higher internal concentrations and potentially similar toxicity levels to those associated with PFOA (Satbhai et al., 2022). Furthermore, recent studies have shown that both PFOA and GenX can disrupt hepatic lipid metabolism in mice, with effects entirely mediated by PPARα, a nuclear receptor involved in regulating metabolism. This disruption can lead to hepatomegaly and other hepatic alterations (Attema et al., 2022; Guo et al., 2022; Robarts et al., 2022).

A study conducted by Xu and colleagues revealed similar liver pathology in mice exposed to either PFOA or GenX, including hepatocyte enlargement, cytoplasm loss, nucleus migration, inflammatory cell infiltration, and reduced glycogen storage (Xu et al., 2022). This exposure during gestation induced maternal hepatic alterations through the gut-liver axis (Xu et al., 2022).

Short-chain PFAS have been shown to cross the placental barrier more readily than legacy PFAS, thereby exposing the developing foetus to potentially higher levels of these compounds than maternal exposure (Cai et al., 2020).

In fact, another recent research demonstrated that HFPO-DA (GenX) is a developmental toxicant in rats, causing changes in glucose and lipid metabolism in both mothers and fetuses (Conley et al., 2021). This exposure resulted in neonatal mortality, low birthweight, and hepatomegaly, with similar potency to PFOS in rats based on serum concentration (Conley et al., 2021). Moreover, human placental trophoblasts showed sensitivity to GenX exposure, affecting critical placental genes involved in proper development and function. It has also been found to reduce thyroid cell viability and proliferation in various cell lines (S. Zhang *et al.*, 2021; Blake et al., 2022).

In summary, GenX, as a replacement to legacy PFAS compounds, has been associated with a wide range of adverse health effects, similar to those found with PFOA.

Based on studies on GenX toxicity published up to now, EPA recommended a subchronic RfD (reference dose) of 0.03 µg/kg/day, and a chronic RfD of 0.003 µg/kg/day (US EPA, Office of Water and Health and Ecological Criteria Division, 2021).

2.2.4. Studies on plants

The studies conducted on plants with per- and poly- fluoroalkyl substances showed differences in the uptake of PFAS, depending on various intrinsic and extrinsic variables: different carbon content in the soil, plant species, and the specific PFAS compound. For instance, GenX, being a short-chain PFAS, tends to accumulate more in the aerial parts of plants rather than in the roots.

This last behaviour was described in a recent study by Wang and colleagues, where hydroponically grown lettuce showed a root concentration factor (RCF) of PFOA nearly five times that of GenX, whereas the translocation factor (TF) of GenX from roots to shoots was approximately 66.7% higher than that for PFOA (Wang et al., 2023). Similar results have been reported by other studies, which observed increased GenX translocation to edible parts in certain plants, such as lettuce and cucumber, suggesting potential health risks through vegetable consumption (Gu et al., 2023).

Additionally, a study on *Carex comosa* (long-haired sedge) grown in GenX-contaminated soil showed that the GenX TF was higher with respect to those typical of longer-chain PFAS (Zhang *et al*., 2021a).

While the potential bioaccumulation of PFAS in plants raises concerns, it also presents opportunities for phytoremediation, particularly for short-chain PFAS like GenX and ADONA (3H-perfluoro-3-[(3-methoxy-propoxy) propanoic acid, another “novel” PFAS). Certain plant species, such as cattail (*Typha latifolia*) and urban spontaneous plants like *Phyllanthus urinaria*, *Justicia procumbens*, *Eleusine indica*, and *Aster indicus*, have been studied for their bioaccumulation capacities, with implications for phytoremediation strategies (Zhang et al., 2021b; Zhi *et al.*, 2022).

As previously mentioned, the carbon content of the soil plays a role in GenX uptake, with higher soil compost percentages reducing its bioavailability. This result was described by NC (North Carolina) State University, where the addition of compost to the soil significantly reduced the uptake of both long and short-chain PFAS in lettuce. For instance, GenX uptake decreased by 82.8% in shoots and 87.5% in roots at a compost content of 20% (2.02 TOC, total organic carbon) (Holden et al., 2020).

To better understand the hazard posed by contamination from facilities, a Dutch study conducted near a fluoropolymer factory found the presence of GenX and PFOA in grass and leaves within a 3 km radius from the plant. GenX was detected in all grass and leaf samples collected, with varying levels (Brandsma et al., 2019).

3. AIMS OF THE STUDY

The dietary consumption of plant-based foods, including vegetables and crops, represents a significant pathway for human exposure to PFAS (Wang et al., 2023).
Consequently, GenX may also pose health risks through this same pathway. However, the existing literature on GenX, particularly its uptake by plants especially in edible parts, remains limited, and numerous questions persist regarding the dietary route of this new generation perfluorinated compound.
Given the widespread presence of GenX in water sources, it is imperative to comprehend its absorption and its impact on plant health. This study aims to investigate the potential physiological and morphological effects of GenX on the germination and growth of several species of selected plant species, which are commonly used for basic research in plant sciences but also hold importance in the agri-food sector.
To address these goals, we conducted three experiments to assess the impact of GenX on four distinct plant species.

The initial experiment entailed the assessment of germination rates and subsequent seedling development in tomato (*Lycopersicum esculentum* Mill.) and cress (*Lepidium sativum* L.) seeds grown on filter paper contaminated with GenX.
Tomato is a plant of agricultural significance representing one of the major vegetable species, which contributed to 16% of global vegetable production in 2020 (FAO, 2022).
Cress serves as a model plant for toxicity testing due to its consistent and rapid germination, rendering it a valuable selection for experiments investigating germination processes and abiotic stressors, including pollutants.

Continuing along this line of inquiry, *Arabidopsis thaliana* (L.) seeds were subjected to germination and growth assays in a GenX-contaminated agar medium. This allowed for the daily monitoring of seedling growth and the assessment of concentration-dependent effects, from 0.5 mg/L to 10 mg/L. *Arabidopsis* was chosen as it is one of the most known model plants and has been widely used to study seed germination in angiosperms.

Maize (*Zea mays* L.), a prominent model plant in agriculture, represents one of the four primary crops, contributing to 12% of global agricultural production, amounting to 1.2 billion tonnes (FAO, 2022). Notably, maize production has outpaced that of wheat and rice, exhibiting more than a three-fold growth rate over the past two decades. Given this context and the absence of prior studies on the effects of GenX on maize, our third investigation focused on evaluating growth-related outcomes, including biomass, and assessing physiological changes in chlorophyll content. These assessments were conducted in hydroponically cultivated maize plants exposed to 20 mg/L GenX.

4. MATERIALS AND METHODS

4.1. Plant material and chemicals

The seeds used for the experiments are:

- Common cress seeds (*Lepidium sativum* L.)
- Tomato seeds, variety Roma (*Lycopersicum esculentum* Mill.)
- *Arabidopsis thaliana* Col-0 seeds
- Maize seeds (*Zea mays* L.; P0848 hybrid, Pioneer)

GenX (HFPO-DA, CAS number 13252-13-6) was purchased from SynQuest Laboratories (Alachua, FL, USA).

4.2. Tomato and Cress

A germination test was conducted to see the effects of the novel PFAS, GenX, on two different species of seed, cress (*Lepidium sativum*) and tomato (*Lycopersicum esculentum*).

Seeds were sterilized using a 70% v/v ethanol + 0.05% v/v Triton-X100 (Sigma Aldrich, USA) solution for 1 minute followed by absolute ethanol for another 1 minute, then soaked for 2 hours in water. After soaking, the seeds were put in round Petri dishes containing a filter paper sheet each.

In each Petri dish with 10 seeds neatly placed, we added 3 mL of water for control and 3 mL of aqueous GenX solutions at different concentrations to test the effects on germination in both species. Four Petri replicates were arranged for each treatment. Five different concentrations of GenX were tested: 0 mg/L (control), 0.5 mg/L, 1 mg/L, 5 mg/L, 10 mg/L, and 20 mg/L.

The Petri dishes were then closed with breathable adhesive tape and then put in a germination chamber at 25 ± 1 °C for 3 days for cress and 5 days for tomato.

After this period, the non-germinated seeds were counted, and the seedlings' shoot and root lengths were measured using a digital caliber (Garant, Hoffmann Group, Germany).

To calculate the Germination Percentage (GP), Seed Vigour Index (SVI), Phytotoxicity, and Tolerance Index (TI), the respective formulas were used:

- Germination Percentage*, GP = (germinated seeds / total seeds)* × *100*;
- Seed Vigor Index*, SVI = GP (%)* × *SL (mm)*; where SL: Seedling Length (Abdul-Baki & Anderson, 1973)
- Phytotoxicity = ((*RLcontrol – RLtreated) / RLcontrol)* × *100;* where RL: Radicle Length (Nouri & Haddioui, 2021)
- Tolerance Index, *TI = (MeanRL in treated / MeanRL in control)* × *100;* (Nouri & Haddioui, 2021)

4.3. Arabidopsis thaliana

Arabidopsis thaliana Col-0 seeds were surface sterilized in Eppendorf by shaking in 70% v/v ethanol + 0.05% v/v Triton-X100 for 1 min followed by absolute ethanol for another 1 min. The seeds were put in a sterile Petri to make sure all ethanol would evaporate.

Eighteen sterilized seeds were placed under sterile conditions in two rows on each square Petri dish containing sterilized half-strength Murashige and Skoog (½ MS) medium added with 1% w/v sucrose, pH 5.8, 0.8% agar and five different concentrations of GenX were tested: 0 mg/L (control), 0.5 mg/L, 1 mg/L, 5 mg/L, and 10 mg/L.

The GenX concentrations used in this study were guided by Chen et al., (2020) who investigated GenX bioaccumulation in *Arabidopsis thaliana*.

Each treatment was conducted in quadruplicate, with 72 seeds tested per concentration. A total of 20 Petri dishes were used.

The seed-containing plates were sealed with breathable surgery tape and placed at 4°C in the dark for 24 hours to synchronize the seedling germination. The plates were placed vertically on a rack in random order and put in the growth chamber with a 16h/8 h (day/night) photoperiod with a light intensity of 150 µmol m^{-2} s^{-1} at 22 ± 1°C with 70% humidity for 12 days.

To monitor the growth of the seedlings, each Petri dish was photographed with a phone camera (8000 × 6000 pixels resolution) on the fifth day after germination and then each 2-3 days.

The germination percentage was assessed at the end of the experiment using the formula reported in paragraph 4.2.

Raw images were analysed with Fiji v. 9.0 image processing software (Schindelin et al., 2021) to evaluate root length at different time points. Roots regions were manually selected and length values in pixels were converted to mm.

4.4. Maize

Maize (*Zea mays* L.) seeds were surface sterilized first with a solution of 70% v/v ethanol + 0.05% v/v Triton X100 for 1 minute and then in absolute ethanol for another 1 minute. The seeds were then put in distilled water for 24 hours. The next day the imbibed seeds were then put on wet accordion-pleated filter paper in the dark at 25 °C until germination for 3 days.

After germination, 24 uniform seedlings were selected and transferred into half-cut 1.5 mL Eppendorf tubes embedded in the cap of 50 mL tube in aerated distilled water for another 4 days. Each tube contained one seed and the water covered the majority of the radicle surface. The tube was covered with aluminium foil to block direct light to the roots, as shown in Figure 6. After 3 days, the water of the seedlings was replaced by a half-strength modified Hoagland nutrient solution (Table 1).

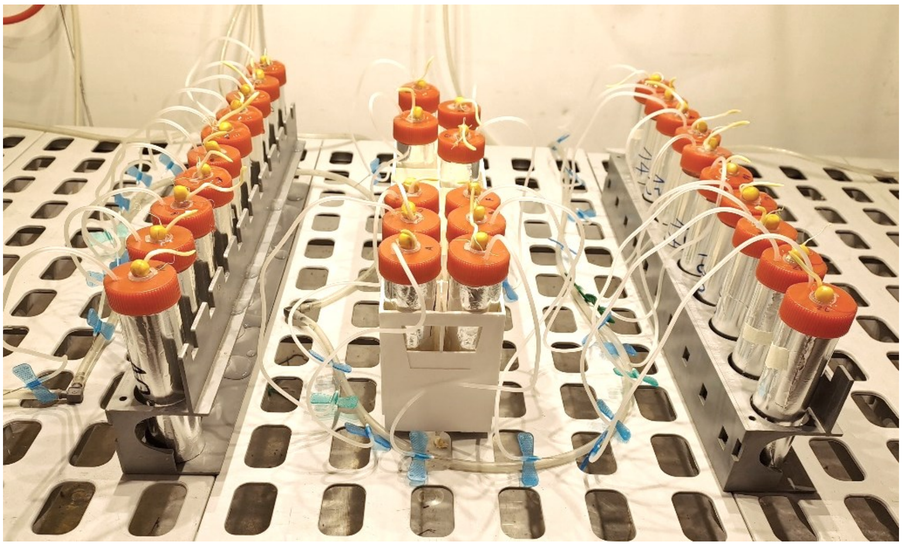


Figure 6 - Experimental setup of hydroponically grown maize.

Modified Hoagland Solution

- KNO3: 1.5 g/L
- Ca $(NO_3)_2 \cdot 4\ H_2O$: 1 g/L
- $MgSO_4 \cdot 7\ H_2O$: 0.5 g/L
- KH_2PO_4: 0.25 g/L
- Fe-Na-EDTA: 2 g/L
- Micronutrients: 0.5 g/L

The micronutrients solution includes: $MnCl_2 \cdot 4\ H_2O$, H_3BO_3, $ZnSO_4 \cdot 7\ H_2O$, $Na_2MoO_4 \cdot 2\ H_2O$, $CuSO_4 \cdot 5\ H_2O$.
For the half-strength solution, the values of grams per litre indicated are halved

Table 1 - Modified Hoagland Solution composition.

After 3 days, the half-strength nutrient solutions were replaced by full-strength modified Hoagland nutrient solutions, untreated (n = 12) or spiked with 20 mg/L GenX (n = 12). Hence, the hydroponic system consisted of 24 tubes carrying one plant and filled with 50 mL of nutrient solution each, either GenX-spiked or not. Four aerated 50 mL tubes without plant, two untreated and two treated, were placed to monitor the evaporation rate.

The plants were maintained in the growth chamber at approximately 22 °C with 70% humidity, a 16 h/8 h (day/night) photoperiod, and 250 µmol m^{-2} s^{-1} light intensity.

The experiment lasted 4 days. Then, roots and shoots were harvested.

Chlorophyll content was measured for each plant five times on different spots of the first non-cotyledonary leaf where possible at the end of the experiment. The measures were taken with a SPAD-502Plus chlorophyll meter (Konica Minolta, Japan) which measures the absorbances of the leaf in the red and near-infrared regions. Using these two absorbances, the instrument calculates a numerical SPAD value which is proportional to the amount of chlorophyll present in the leaf.

Fresh roots and shoots were collected and weighed with a precision scale. Roots were further scanned with an Epson Expression 11000 XL PRO scanning system and images were analysed with WinRHIZO v. 5.0 software.

Then, fresh tissues were frozen in liquid nitrogen and stored at -20 °C for further analysis.

4.5. Statistical analysis

Statistical analyses were carried out with Excel and Minitab software (v. 20.1.1.0), and differences were considered significant at $p \leq 0.05$.

One-way ANOVA (Analysis of Variance) was used to test GenX effects on the germination percentage and seedlings' length (tomato, cress, and *Arabidopsis* at individual timepoints) among the different treatment concentrations. Two-way ANOVA was used to test the impact of GenX concentrations, time, and their interaction on the root length of *Arabidopsis* seedlings.

Student's t-test and Mann-Whitney U-test were used to evaluate the effect of GenX exposure on residual nutrient solution, fresh biomass, root morphology (root length, surface area, volume, and radicles' diameter), and chlorophyll content in maize plants, after checking for normality with Shapiro-Wilk test.

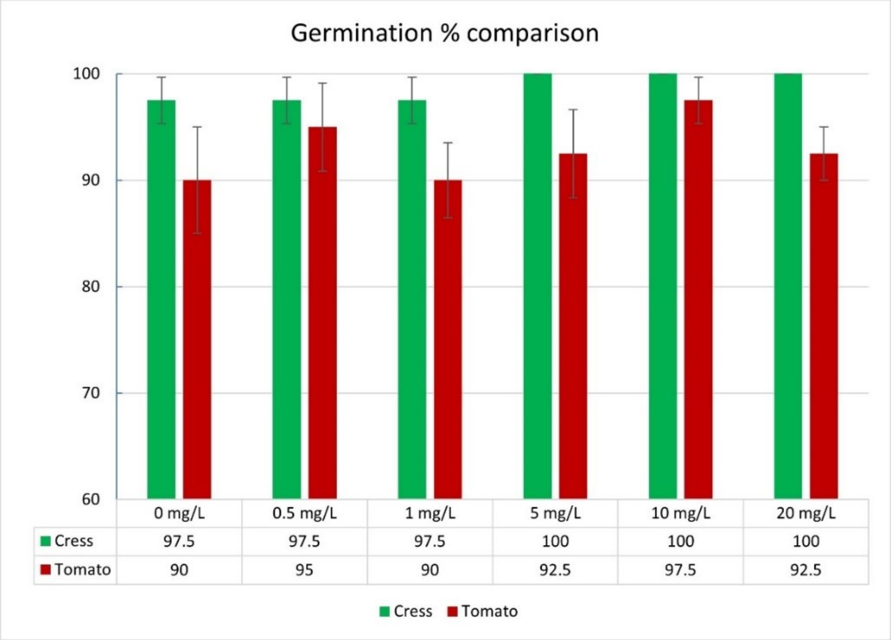
5. RESULTS and DISCUSSION

5.1. Tomato and Cress

Germination percentage and seedling growth of cress and tomato were assessed after 3 and 5 days, respectively.

5.1.1. Germination

Cress seeds did not show significant variation in the germination percentage among the different groups of GenX treatment. Conversely, tomato seed germination showed some variation among the different groups but did not exhibit a constant trend determined by the action of increasing GenX concentrations (Figure 7, Appendix 1).
After analysis, no statistically significant differences in germination percentage were found in response to different GenX levels, both in cress (p = 0.70) and in tomato (p = 0.79).



	0 mg/L	0.5 mg/L	1 mg/L	5 mg/L	10 mg/L	20 mg/L
🟩 Cress	97.5	97.5	97.5	100	100	100
🟥 Tomato	90	95	90	92.5	97.5	92.5

Figure 7 - Germination Percentages of tomato and cress exposed to different levels of GenX.

Evaluating the germination percentage at different concentrations of the same stress is important to understand how a plant responds and tolerates a condition different from normality.

Testing GenX at varying levels on tomato and cress did not show inhibited germination on both species, even at relatively high doses of the chemical. These findings are coherent with GenX's legacy counterpart, PFOA, as it also exhibited no significant effects on germination percentage at 20 mg/L on wheat (*Triticum aestivum* L.) (Zhou et al., 2016).

5.1.2. Seedling growth

To understand whether there is a statistically significant disparity in root and shoot growth among seeds exposed to different concentrations of GenX, a One-Way ANOVA was performed on the length of the two seedling species.

The control group exhibited on average shorter root and shoot lengths compared to the other groups. Indeed, GenX-exposed seedlings were more vigorous (Figure 8, Appendix 2). One-way ANOVA tested a statistically significant difference for both shoot length ($p = 0.0019$) and root length ($p = 0.00075$).
Roots of the control group showed a significantly shorter length with respect to the majority of the treated groups: 0.5 mg/L ($p = 0.004$), 1 mg/L ($p = 0.047$), 5 mg/L ($p = 0.015$) and 20 mg/L ($p < 0.001$). Regarding shoot length, the 5 mg/L group showed a significantly longer shoot with respect to the control group ($p = 0.002$) and the 20 mg/L group ($p = 0.04$).
Overall, the 20 mg/L group displayed longer roots but shorter shoots, particularly when compared to the 5 mg/L group, which demonstrated vigorous growth in both organs. Notably, the control samples exhibited significantly lower growth in both roots and shoots, compared to the treated groups.

Similarly, the same tests were applied to the tomato dataset. Although a preliminary graphical analysis of the length data (Figure 9, Appendix 2), particularly for roots, suggested shorter lengths in seedlings exposed to higher GenX concentrations (10 mg/L and 20 mg/L), the subsequent one-way ANOVA showed no statistical difference for both shoot ($p = 0.438$) and root ($p = 0.107$) lengths.

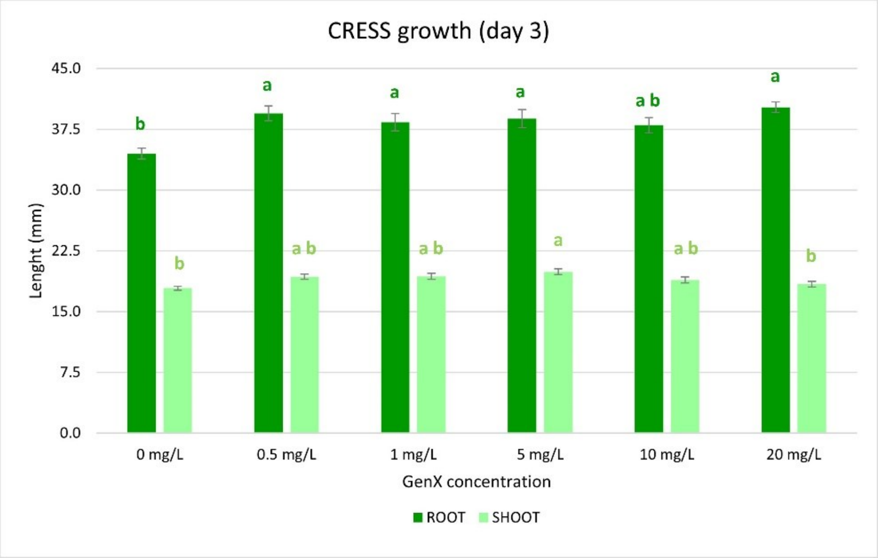


Figure 8 - Cress: root and shoot lengths of seedlings exposed to different GenX concentrations. Means that do not share a letter are statistically different.

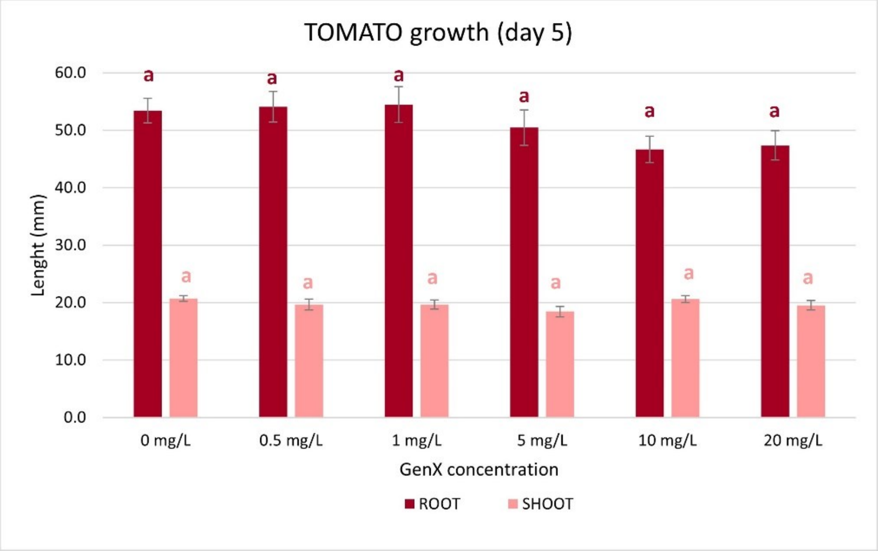


Figure 9 - Tomato: root and shoot lengths of seedlings exposed to different GenX concentrations. Means that do not share a letter are statistically different.

To better assess the behaviour of both species, seed vigour was investigated by calculating Seed Vigour Index (SVI). Coherently with previous results, cress showed a positive trend with the increasing concentrations of GenX, with the control group at the lowest vigour (Figure 10).

Tomato's SVI showed a downward trend with increasing GenX concentrations from 0.5 mg/L, with the minimum value at 20 mg/L (Figure 11, Appendix 3). However, as previously discussed, this difference is too limited, and it is not considered statistically reliable.

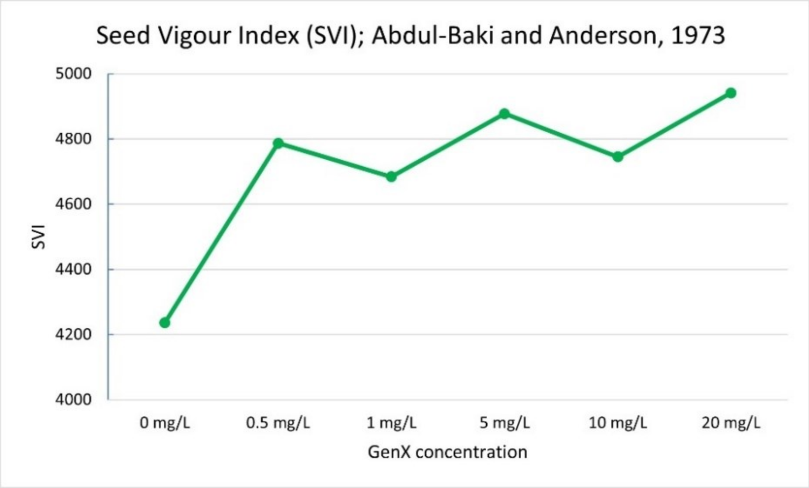


Figure 10 - Cress seed vigour index (SVI)

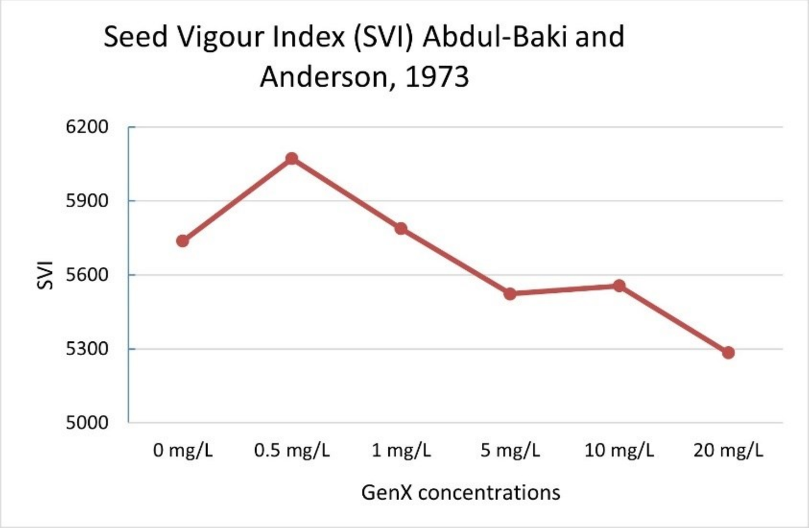


Figure 11 - Tomato seed vigour index (SVI)

Phytotoxicity to GenX was evaluated to estimate adverse effects on plant health (Figure 12, Appendix 3).

In cress, the trend line did not indicate a phytotoxic effect on the seeds treated with different GenX concentrations. Being negative, it indicates the opposite of toxicity, almost a beneficial effect.

In tomato, the results showed a negative effect on the seeds. In particular, from 1 mg/L up to 10 mg/L phytotoxicity increases up to 12.6%.

The tolerance index (TI) is a measure of how well or poorly a particular species or variety of plant can withstand or tolerate adverse conditions, including exposure to phytotoxic substances. TI at 100 indicates the normal condition, lower tolerance means the stressor is acting against normal growth.

Results showed a decreased tolerance for tomato seeds as the GenX concentration increased (Figure 13, Appendix 3). However, these results still need to be thoroughly investigated as statistical analyses did not reveal any significant effect up to 20 mg/L. In the case of cress, the control plants exhibited decreased growth compared to the plants exposed to different GenX concentrations. Possible explanations can be ascribed to natural variation or experimental variability but, to go deeper into this phenomenon, further investigations are needed.

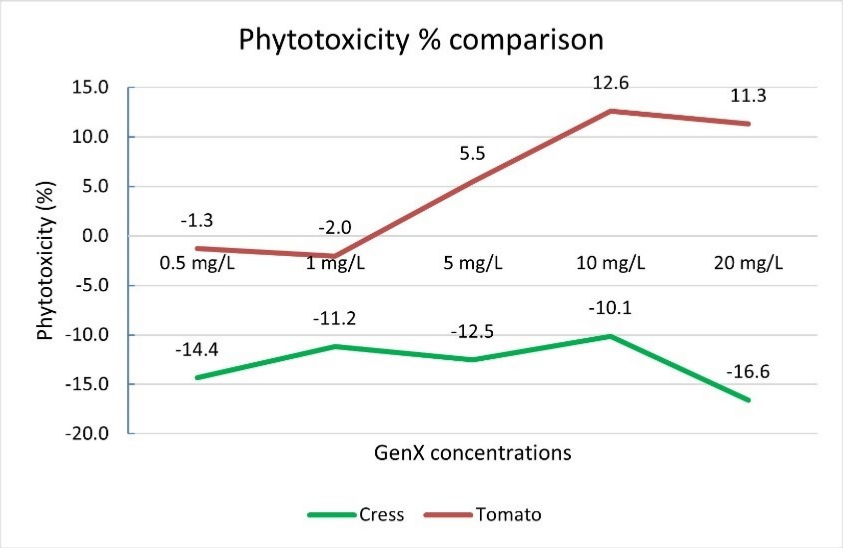


Figure 12 - GenX Phytotoxicity in Cress (green) and Tomato (red)

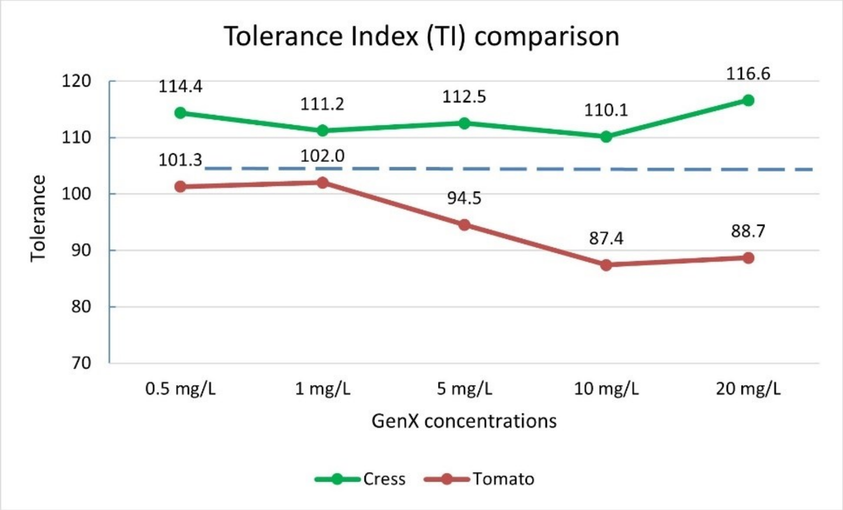


Figure 13 - GenX TI (Tolerance Index) in Cress (green) and Tomato (red)

In the case of tomato, a decreasing trend in growth was observed with increasing chemical stress due to GenX. Although this trend is of interest and aligns with our initial hypotheses, it is important to note that the results did not reach statistical significance (p > 0.05). Albeit these findings were not statistically conclusive, they highlighted the need for more extensive experiments to validate the trend.

5.2. *Arabidopsis thaliana*

5.2.1. Germination

In the context of this scientific thesis, the germination percentage of *Arabidopsis* seeds exposed to different concentrations of GenX was evaluated

Contrary to our initial expectations, the germination percentage did not exhibit statistically significant alterations (p = 0.237) in response to increasing GenX concentrations, indicating *Arabidopsis* resilience against such chemical in the germination process. The lowest recorded germination percentage, as shown in Figure 14 (Appendix 4), was observed at a concentration of 0.5 mg/L, but it was not supported by statistical analysis.

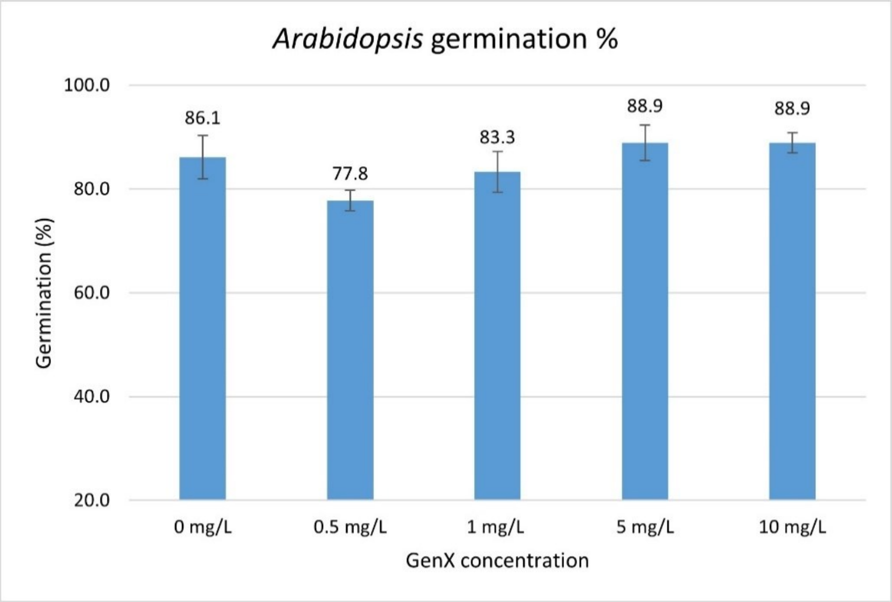


Figure 14 - Arabidopsis germination percentage at different GenX concentrations.

5.2.2. Root growth

Root length was recorded at four timepoints for each treatment condition.

Root length is a critical metric in understanding the growth and development of plants, and it can be influenced by various factors. To make sure whether there were significant differences in root lengths attributed to time and, especially, GenX exposure, we employed the analysis of variance (ANOVA) in two distinct ways.

Initially, we utilized a two-way ANOVA to assess the effects of time, concentration and their interaction on root length. The results unveiled highly significant p-values for both time ($p = 1.19\times10^{-6}$) and concentration ($p < 2\times10^{-16}$), affirming their substantial influence on root length variations, whereas their interaction was not significant ($p = 0.097$).

As root length is, by definition, related to time by the normal plant growth, it was further analysed considering only the effect of GenX concentrations. Consequently, we tested for significance considering individual timepoints using one-way ANOVAs to further dissect the impact of GenX concentration over time.

As reported in Figure 15 (Appendix 4), there was a systematic difference between the 1 mg/L group and the other groups. In fact, plants exposed to 1 mg/L GenX showed a statistically significant reduced length with respect to plants exposed to 5 mg/L at all timepoints (day 5: $p = 0.012$; day 7: $p = 0.006$; day 10: $p = 0.018$; day 12: $p = 0.035$). Moreover, a significantly decreased root length was observed in plants exposed to 5 mg/L with respect to the control plants at day 7 ($p = 0.011$) and day 10 ($p = 0.035$), and to plants exposed to 0.5 mg/L at day 7 ($p = 0.014$).

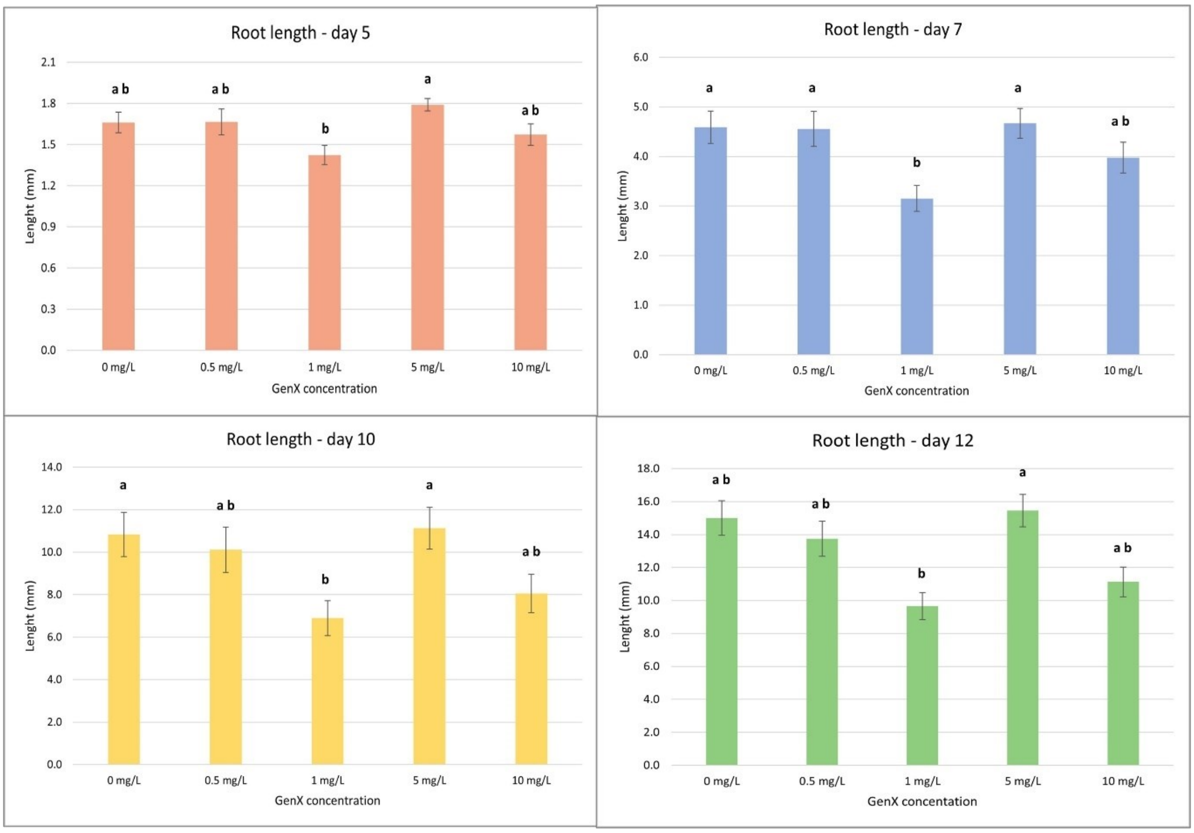


Figure 15 - Arabidopsis thaliana root length (mm) at each timepoint. Means that do not share a letter are statistically different.

Generally, the differences in root length were not statistically different between controls and GenX-exposed plants, with the only exception of the 1 mg/L group, which displayed a noticeable deviation from the pattern, with a reduced average length. In fact, this difference is significant from the first measurement, and it is constant at each timepoint, therefore it does not seem to be affected by longer exposure to GenX. Considering this context, the observed deviation in the 1 mg/L group may be the result of natural biological variability, or possibly due to experimental variability affecting one or more replicates of this group.

Arabidopsis thaliana was previously taken into consideration for GenX toxicity in a remarkable study by Chen and colleagues (2020), where the growth of the seedling exposed to 20 mg/L GenX was visibly lower than control seedlings.

As our result did not show visible differences depending on concentration, the different outcomes could be attributable also to the different exposure times, as the abovementioned study assessed the growth after 21 days. On the other hand, that study also concluded that *Arabidopsis* showed more resilience than other species to the potential toxic effect of GenX, which is consistent with our results. In addition, such study detected GenX accumulation in both shoots and roots of *Arabidopsis*.

These findings may be relevant for the agri-food field as *Arabidopsis* is closely related to several important crops including canola, cabbage, turnips, and broccoli, hence further experimentation can be extended to such crops aiming to assess the impact of GenX and its bioaccumulation.

5.3. Maize

5.3.1. Nutrient solution and Biomass changes

Nutrient Solution (NS) weight was recorded at the beginning and at the end of the experiment, to check for potential differences in nutrient uptake between control and treated plants (Figure 16).

Controls showed a residual NS percentage of 86.8 ± 2.8 % while GenX-exposed plants of 90.1 ± 2.2 %, however, this difference of ~3.7% was not statistically significant ($p = 0.37$).

It can be supposed that the possible effect of GenX on NS consumption might be more prominent with longer exposure time.

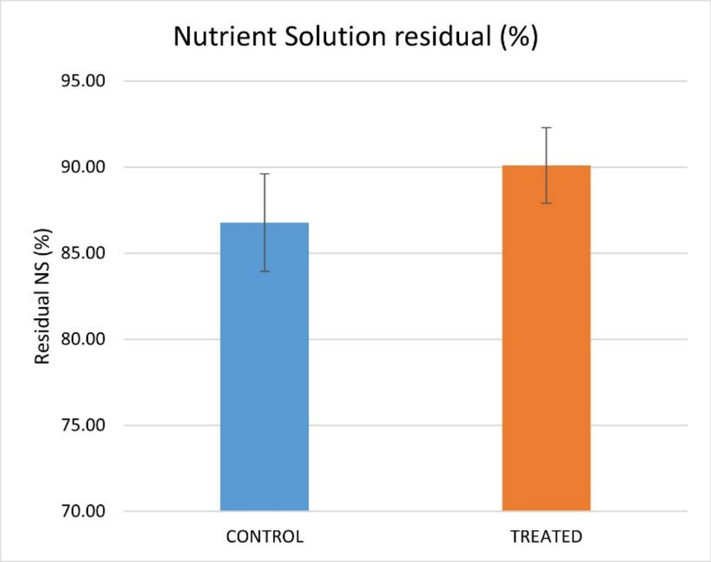


Figure 16 - Percentage of residual NS in the control groups and treated group

Fresh roots and shoots were weighted to determine potential differences in biomass due to GenX exposure.

As shown in Figure 17 (Appendix 5), the average root fresh weight of GenX-exposed plants (0.31 ± 0.02 g) was statistically reduced compared to controls (0.44 ± 0.04 g) ($p = 0.017$). A similar decreasing effect in fresh biomass was observed for shoots, however, this difference was not significant ($p = 0.23$).

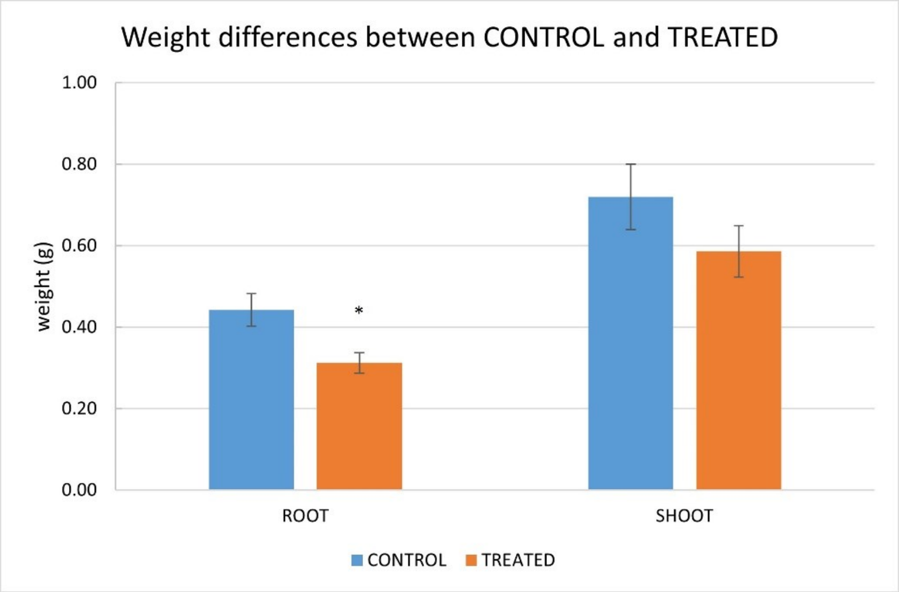


Figure 17 - Maize root and shoot weight differences in control and treated plants. (indicates $p \leq 0.05$)*

These results demonstrated a significant effect of GenX exposure on fresh maize root biomass with treated plants showing a weight variation of -29.4% with respect to controls.

Recent studies on lettuce and other plant species of agricultural interest suggested a high translocation factor for GenX as a result of higher bioaccumulation in the aerial part rather than roots, differently from its legacy counterpart, PFOA (Gu et al., 2023).

In the present study, shoot fresh weight did not show statistical difference between the groups, but more in-depth analysis, such as the determination of GenX content in roots and shoots by targeted LC-MS/MS (liquid chromatography coupled to mass spectrometry) analyses, could clarify the partitioning of this compound among plant tissues.

5.3.2. Root morphological analysis

Roots of control and treated plants were scanned, and data were analysed using WinRHIZO software. Root total length, surface area, volume, and radicles' diameter were considered in our analysis (Appendix 6).

Root surface area was significantly decreased in GenX-exposed plants (17.3 ± 1.2 cm^2) with respect to control plants (25.4 ± 2.4 cm^2) ($p = 0.009$).

Similarly, root volume was significantly reduced in treated plants (0.18 ± 0.01 cm^3) compared to controls (0.30 ± 0.03 cm^3) ($p = 0.004$).

In root length and radicles' diameter there was not statistically significant difference between the two groups.

These results, together with the reduced root fresh weight observed in treated plants, suggest that GenX seems to negatively affect the root system extension and its spatial distribution, similar to what was observed in maize plantlets exposed to a mixture of 11 PFAS (Ebinezer et al., 2022).

5.3.3. Chlorophyll content analysis

Chlorophyll content was indirectly estimated by measuring the SPAD value of a subset of plants, (10 replicates per group), as some leaves were too small, and measurements could not be accurate. Control plants showed a significantly higher chlorophyll content compared to GenX-exposed plants ($p = 0.028$) (Figure 18, Appendix 5).

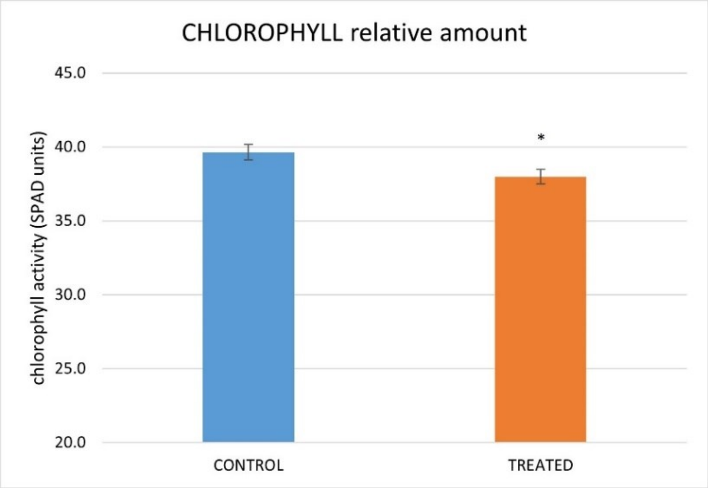


Figure 18 - Maize chlorophyll content in control and treated plants. (indicates p≤0.05)*

Chlorophyll is a crucial pigment for photosynthesis and reflects, not only the plant photosynthetic activity but also, indirectly gives information about the nutritional state of the plant, including nitrogen and magnesium levels.

The results obtained in this analysis provide insights into a physiological effect due to exposure to 20 mg/L GenX in maize.

However, even if it is supported by statistical significance, a reduction of 4.2% in the SPAD value may not imply a remarkable impact on the overall plant health. Moreover, considering that plants were exposed to relatively high GenX concentrations (much higher than those found in the environment) the observed effect is quite negligible.

6. CONCLUSIONS

Research on GenX is still in the early stages, and investigations on its toxicity are a few, especially regarding plant uptake and possible human exposure through contaminated plant-based food. With this thesis work, we investigated the potential effects of GenX on germination and growth of four different plant species of scientific and agricultural interest: Cress, Tomato, *Arabidopsis,* and Maize.

According to our experimental results, GenX exposure did not show significant toxic effects on tomato, cress and *Arabidopsis* germination. The potential concentration-dependent effect of GenX on seedling length was tested but with minor, non-significant effects on tomato. In cress, increasing GenX concentration did not affect seedling growth, on the contrary, it was significantly lower in control. *Arabidopsis* root length over time demonstrated from the start a decrease in length at 1 mg/L, possibly not due to longer GenX exposure but to experimental variability.

Morphological and physiological analyses on maize, on the other hand, unveiled some important outcomes: root biomass was significantly lower in the group with 20 mg/L GenX. An in-depth root architecture analysis showed significantly reduced root volume and surface area in the GenX-exposed plants, compared to controls. Chlorophyll content, even if with a minor effect but statistically significant, was reduced in plants exposed to GenX compared to control ones.

The results gather some insight into possible toxic effects in plants, as studies regarding the topic are still lacking for this novel PFAS. For this reason, urgent research is needed, given the ongoing production of GenX and the absence of comprehensive studies, particularly regarding phenomena such as plant bioaccumulation and its consequent uptake through the food chain.

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8. APPENDIX

Cress germination %		**Tomato germination %**	
0 mg/L	97.5 ± 2	0 mg/L	90 ± 5
0.5 mg/L	97.5 ± 2	0.5 mg/L	95 ± 4
1 mg/L	97.5 ± 2	1 mg/L	90 ± 3
5 mg/L	100 ± 0	5 mg/L	92.5 ± 4
10 mg/L	100 ± 0	10 mg/L	97.5 ± 2
20 mg/L	100 ± 0	20 mg/L	92.5 ± 3

Appendix 1 - Cress and Tomato germination data. Values are expressed as mean ± standard error (n=40).

Cress length (mm)			**Tomato length (mm)**		
GenX conc.	Root	Shoot	**GenX conc.**	Root	Shoot
0 mg/L	34.5 ± 0.7	17.9 ± 0.3	*0 mg/L*	53.4 ± 2.1	20.7 ± 0.5
0.5 mg/L	39.5 ± 0.9	19.3 ± 0.3	*0.5 mg/L*	54.1 ± 2.6	19.6 ± 0.9
1 mg/L	38.4 ± 1.1	19.3 ± 0.4	*1 mg/L*	54.5 ± 3.1	19.7 ± 0.8
5 mg/L	38.8 ± 1.1	19.9 ± 0.3	*5 mg/L*	50.5 ± 3.1	18.5 ± 0.9
10 mg/L	38.0 ± 0.9	18.9 ± 0.4	*10 mg/L*	46.7 ± 2.3	20.6 ± 0.6
20 mg/L	40.2 ± 0.6	18.4 ± 0.3	*20 mg/L*	47.4 ± 2.6	19.5 ± 0.8

Appendix 2 - Cress and Tomato growth data. Values are expressed as mean ± standard error (n=40).

GenX conc.	**CRESS**			**TOMATO**		
	SVI	**P (%)**	**TI**	**SVI**	**P (%)**	**TI**
0 mg/L	4236.5	-	-	5737.9	-	-
0.5 mg/L	4787.1	-14.4	114.4	6071.8	-1.3	101.3
1 mg/L	4683.7	-11.2	111.2	5787.4	-2.0	102.0
5 mg/L	4877.2	-12.5	112.5	5522.9	5.5	94.5
10 mg/L	4744.8	-10.1	110.1	5555.9	12.6	87.4
20 mg/L	4941.3	-16.6	116.6	5284.8	11.3	88.7

Appendix 3 - Cress and Tomato Seed vigour index (SVI), Phytotoxicity (P, %) and tolerance index (TI).

GenX conc.	***G*ermination %**	**Root length (mm) over time**			
		Day 5	**Day 7**	**Day 10**	**Day 12**
0 mg/L	86.1 ± 4.2	1.7 ± 0.1	4.6 ± 0.3	10.8 ± 1.0	15.0 ± 1.5
0.5 mg/L	77.8 ± 2.0	1.7 ± 0.1	4.6 ± 0.4	10.1 ± 1.1	13.7 ± 1.6
1 mg/L	83.3 ± 3.9	1.4 ± 0.1	3.2 ±0.3	6.9 ± 0.8	9.7 ± 1.2
5 mg/L	88.9 ± 3.4	1.8 ± 0	4.7 ± 0.3	11.1 ± 1.0	15.5 ± 1.4
10 mg/L	88.9 ± 2.0	1.6 ± 0.1	4.0 ± 0.3	8.1 ± 0.9	11.1 ± 1.4

Appendix 4 - Germination percentage, average root length of Arabidopsis seedlings measurement at different timepoints. Values are expressed as mean ± standard error (n=72).

GenX conc.	**SPAD values**	**Fresh weight (g)**	
		Root	Shoot
0 mg/L	39.6 ± 0.5	0.44 ± 0.04	0.72 ± 0.08
20 mg/L	38.0 ± 0.5	0.31 ± 0.02	0.59 ± 0.06

Appendix 5 - SPAD values (n=10), roots and shoots biomass (n=12), expressed as mean ± standard error.

GenX conc.	**Length (cm)**	**Surf. Area (cm2)**	**Diameter (mm)**	**Volume (cm3)**
0 mg/L	117 ± 19	25.4 ± 2.4	0.47 ± 0.02	0.30 ± 0.03
20 mg/L	136 ± 15	17.3 ± 1.2	0.43 ± 0.02	0.18 ± 0.01

Appendix 6 - WinRHIZO maize root measurements, expressed as mean ± standard error (n=12).