# UNIVERSITY OF PARMA

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# BIOTECHNOLOGY OF ARSENIC UPTAKE AND TRANSLOCATION IN PLANTS (*Solanum lycopersicum* L.)


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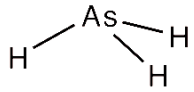
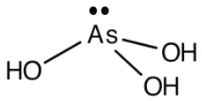
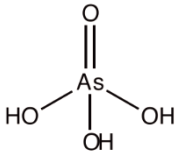
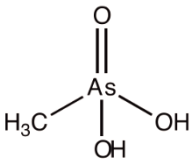
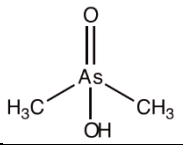
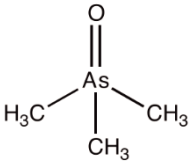
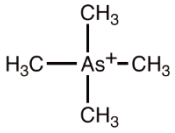
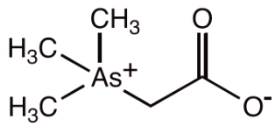
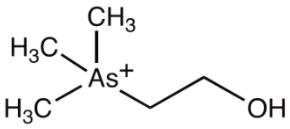
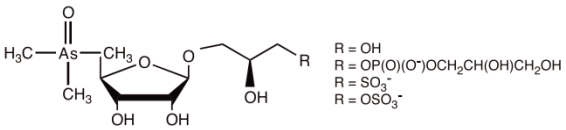
# 1. General introduction

## 1.1 Arsenic: chemical characteristics and distribution in the environment

Arsenic (As), is a metalloid belonging to the V group of the periodic table between nitrogen and phosphorous; it has an excess of electrons and unfilled orbitals that stabilize formal oxidation states from +5 to -3. At the elemental state is a solid and is not soluble in water but in the environment it could be found in some different chemical forms, including organic (e.g. mono or dimethylarsonic acid (MMA, DMA)) and inorganic (e.g. arsenate and arsenite) forms (Tab.1).

**Tab.1 Summary of the most important organic and inorganic As species in the environment (adapted from O'day, 2006).**

| | |
|---|---|
| **Inorganic Arsenic** | |
| Arsine |  |
| Arsenate |  |
| Arsenite |  |
| **Methylated Arsenic Compounds** | |
| Monomethylarsonic acid (MMA) |  |
| Dimethylarsinic acid (DMA) |  |
| Trimethylarsine oxide (TMAO) |  |
| Tetramethylarsonium ion (TETRA) |  |

| **Organoarsenic compounds** | |
|---|---|
| Arsenobetaine |  |
| Arsenocholine |  |
| Organoarsenic lipids |  |

Arsenic represents a non-essential and toxic element for the majority of the living organisms. It could be present in the environment for natural or antropic occurrences. The most important natural occurrences of As are pedogenesis, volcanic activity and biological activity. Inorganic species could be methylated by bacteria and fungi and methylation is thought to be a detoxification mechanism but is also important for the transfer of arsenic from sediments to water or atmosphere. Biotransformation of arsenic can produce highly volatile compounds like arsine, dimethylarsine or trimethylarsine (Kumaresan and Riyazuddin, 2001). Occurrences of antropic origins regards mainly coal combustion, mining, industrial and agricultural activities. In fact As could be found in sulphide ores containing copper, lead or gold and mine tailings derived from active or abandoned mining or ore processes, usually containing high concentrations of As. Arsenic has been used in agriculture and was dispersed on soils as a pesticide and consequently may enter in the food chain. The most important arsenic compounds utilized in pesticides are: Monosodium methane arsenate (MSMA), Disodium methane arsenate (DSMA), Dimethylarsinic acid (cacodylic acid) and Arsenic acid. Other uses of arsenic and arsenic compounds includes wood preservatives, glass manufacture, alloys, electronics, catalysts, feed additives and veterinary chemicals (Kumaresan and Riyazuddin, 2001).

### 1.1.1 As in soil

In the earth crust As is the 20th element in abundance and is present mainly in minerals containing Cd, Pb, Ag, Au, Sb, P, W, and Mo (O'day, 2006). Realgar (AsS), Orpiment ($As_2S_3$) and arsenopyrite (FeAsS) are the most common minerals among the over 300 As minerals identified (Table 2). Its average content in earth crust was estimated to be as high as 1.8 mg $kg^{-1}$ but this value could vary between the different types of rocks with higher values in sedimentary rocks than igneous rocks (Baroni et al., 2004). Since As could accumulates during weathering and translocation in colloid fractions, its concentration is usually higher in soil than in parent rocks (Baroni et al., 2004).

**Tab.2 Major As minerals occurring in nature (Smedley and Kinninburgh, 2002).**

| Mineral | Composition | Occurrence |
|---|---|---|
| Native arsenic | As | Hydrothermal veins |
| Niccolite | NiAs | Vein deposits and norites |
| Realgar | AsS | Vein deposits, often associated with orpiment, clays and limestones, also deposits from hot springs |
| Orpiment | $As_2S_3$ | Hydrothermal veins, hot springs, volcanic sublimation products |
| Cobaltite | CoAsS | High-temperature deposits, metamorphic rocks |
| Arsenopyrite | FeAsS | The most abundant As mineral, dominantly in mineral veins |
| Tennantite | $(Cu,Fe)_{12}As_4S_{13}$ | Hydrothermal veins |
| Enargite | $Cu_3AsS_4$ | Hydrothermal veins |
| Arsenolite | $As_2O_3$ | Secondary mineral formed by oxidation of arsenopyrite, native arsenic and other As minerals |
| Claudetite | $As_2O_3$ | Secondary mineral formed by oxidation of realgar, arsenopyrite and other As minerals |
| Scorodite | $FeAsO_4.2H_2O$ | Secondary mineral |
| Annabergite | $(Ni,Co)_3(AsO_4)_2.8H_2O$ | Secondary mineral |
| Hoernesite | $Mg_3(AsO_4)_2.8H_2O$ | Secondary mineral, smelter wastes |
| Haematolite | $(Mn,Mg)_4Al(AsO_4)(OH)_8$ | |
| Conichalcite | $CaCu(AsO_4)(OH)$ | Secondary mineral |
| Pharmacosiderite | $Fe_3(AsO_4)_2(OH)_3.5H_2O$ | Oxidation product of arsenopyrite and other As minerals |

In soil the bioavailability of this metalloid depends on the chemical and physical characteristics of the soil, especially pH, redox conditions, biological activity, organic matter content, presence of other minerals and soil texture (Han et al., 2003). Arsenic concentration range in non-contaminated soils is from 1 to 40 mg $kg^{-1}$, but it can reach thousands of mg $kg^{-1}$ in the surface horizons of soil contaminated by different sources (Cances et al., 2008). In soil arsenic exists mainly as pentavalent arsenate or trivalent arsenite but these different anions presents different mobilization rate depending on the soil conditions. In general arsenate anion is rather easily chemisorbed by soil colloids and, adsorbs most effectively at low pH; therefore arsenate mobility is quite low in acidic soils, especially where high contents of clays or metal oxides are

involved (Branco, 2007). The element has a rather long residence time in soils (from 1000 to 3000 years) and tends to be enriched into top horizons by cycling trough vegetation, atmospheric deposition and sorption by soil organic matter (Branco, 2007). Iron oxides are one of the most common phases found in soil and sediments, either as discrete particles or as coatings on other mineral solids. They have been identified as one of the most important arsenic adsorbents in natural systems, followed by Al oxides that are structural analogous to hydrous Fe oxides. Sorption processes to the surfaces of Fe, Al and Mn oxides or clay phases, plays an important role in controlling As distribution and mobility in the environment.

As (V) could form inner-sphere surface complexes on both Al and Fe oxides, while As (III) forms inner and outer sphere surface complexes on Fe oxides and outer sphere surface complexes on Al oxide (Wang and Mullligan, 2006). Manganese oxides have shown the capacity to adsorb arsenic and to oxidize As (III) into As (V) (Wang and Mulligan, 2006).

### 1.1.2 As in water

Arsenic was not on the list of constituents in drinking water routinely analysed by national laboratories, water utilities and non-governmental organizations (NGOs); thus the information about its distribution in drinking water is not as well-known as for many other drinking-water constituents (Smedley and Kinninburgh, 2002). Arsenic speciation and solubility in water is controlled basically from redox potential and pH (Smedley and Kinninburgh, 2002); furthermore the processes that control As mobility in water are mainly: (i) adsorption and desorption reactions and (ii) solid phase precipitation and dissolution reactions (Khan et al., 2009).

Arsenic can occur in natural waters mostly in the inorganic form as oxyanions of trivalent arsenite [As(III)] or pentavalent arsenate [As(V)] (Smedley and Kinninburgh, 2002). Metalloid concentration in fresh water can vary greatly, depending on different factors including source of As, amount available and local geochemical environment (Smedley and Kinninburgh, 2002). Without antropic contribution, the highest value of concentrations were found in groundwater, as a result of the strong influence of water-rock interactions and the greater tendency in these aquifers, for the physical and geochemical conditions, to be favorable for As mobilization and accumulation (Smedley and Kinninburgh, 2002). Average As concentration in river is very low, in

the range of 0.1-0.8 mg $l^{-1}$ but can reach 2 mg $l^{-1}$ depending on the geochemical characteristics of the environment. In general As concentrations in rivers are not so high than that found in groundwater because of oxidation and absorption of As species onto the river sediments and dilution effects due to surface recharge and runoff. In lake waters there have been found As concentrations very close or lower than those found in river waters (Smedley and Kinninburgh, 2002). There is also much evidence for stratification of As concentrations in some lake waters as a result of varying redox conditions (Aggett and Roberts, 1986). Average As concentrations in open seawater usually show little variations and are typically around 1.5 µg $l^{-1}$ (Smedley and Kinninburgh, 2002). Finally, average As concentrations in groundwater are in most countries less than 10 mg $l^{-1}$ ; however values quoted in the literature show a very large range from <0.5 to 5000 mg $l^{-1}$ (Smedley and Kinninburgh, 2002).

In Italy elevated concentrations of this metalloid were found in the groundwater of some municipalities in Emilia Romagna, Lombardia, Veneto, Campania and Lazio since the '90s (INAIL, 2010). Among the areas with the most contaminated water in Italy there are Scarlino plain in Tuscany, near Grosseto, where a combination of natural and antropic occurrences determined an accumulation of As that reach, in some areas a concentration of 1000 mg $kg^{-1}$, the mining area of Furtei in Sardinia with arsenic concentration of about 5 mg $kg^{-1}$ and the mining area of Valle Anzasca in Piemonte (INAIL, 2010). In Emilia Romagna arsenic in groundwater can be found at different concentrations depending on the considered area but the concentration range is 10-50 µg $l^{-1}$ (INAIL, 2010); this signified that also in the water of our region the international threshold of 10 µg $l^{-1}$ can be reached and exceeded.

### 1.1.3 As in atmosphere

In the atmosphere arsenic could be found due to different type of inputs, including wind erosion, volcanic emissions, low-temperature volatilisation from soils, marine aerosols and pollution, and could return to the earth's surface by wet and dry depositions. The most important arsenic species in atmosphere is As (III), in the form of dust particles (Smedley and Kinninburgh, 2002). Anthropogenic sources contributes to around 70% of the global atmospheric As flux (Nriagu and Pacyna, 1988).

Concentrations amounting to around $10^{-5}$–$10^{-3}$ mg $m^{-3}$ recorded in unpolluted areas, increasing to 0.003–0.18 mg $m^{-3}$ in urban areas and greater than 1 mg $m^{-3}$ close to industrial plants (WHO, 2001).

### 1.2 Arsenic toxicity for human

As is toxic to most living organisms; in humans, its ingestion has been associated with a whole series of pathologies, leading to its classification as a carcinogenic agent (Goyer, 1995). In the past it was utilized for humans both as a poison (especially arsenic trioxide that is a tasteless, odourless, white powder) and a curative (in the traditional medicine but also in the advanced medicine for chemotherapy). Arsenic toxicity for humans, as for animal and plants, depends on a large part on the considered chemical species; in general arsenite is more toxic than arsenate and these inorganic forms are more toxic than the methylated species DMA and MMA, while Arsenobetaine and Arsenocholine are virtually non-toxic (Kumaresan and Riyazuddin, 2001). Toxicity of As also depends on available exposure routes, frequency of exposure, biological species, age, gender, individual susceptibilities, genetics, and nutritional sources (Khan et al., 2009). Another important factor to consider is the nutritional status of a country; a number of studies have found that people in Bangladesh with poor nutritional status are more susceptible to arsenicosis compared with people with better nutritional status (Hadi and Parveen, 2004; Hasnat, 2005 ; Ahmad et al., 2007). Poor nutritional status in combination with high level of As in water and large amounts of daily water intake can significantly increase the risk of adverse health effects from As exposure (Islam, 2004). Usually As level in urine, hairs and nails are utilized as biomarkers for short term internal dose (1 year), as found by Chen et al. (2005), and the effects of long term internal dose were skin hyperpigmentation and palmoplantar hyperkeratosis. There are many different sources of arsenic contamination for humans, including inhalation, smoking, water or food, but the most important are ingestion of contaminated drinking water or contaminated food. Chronic exposure to inorganic arsenic species has serious health effects, including gastrointestinal damage, hyperkeratosis, pigmentation changes, hypertension, diabetes mellitus, cardiac damages and vascular, respiratory, neurological, liver and kidney disorders (Revanasiddappa et al., 2007; Carbonell-Barrachina et al., 2009). Furthermore inorganic arsenic is a well-documented human carcinogen, causing cancer in skin, lungs, urinary bladder, kidney, and liver (WHO, 2001).

Another concern regards prenatal exposure to the metalloid that could result in serious short and long term toxicities; both inorganic arsenic and methylated metabolites can cross the placenta and impair fetal growth, even fetal loss, post-birth mortality, development of certain malignancies (Vahter, 2008).

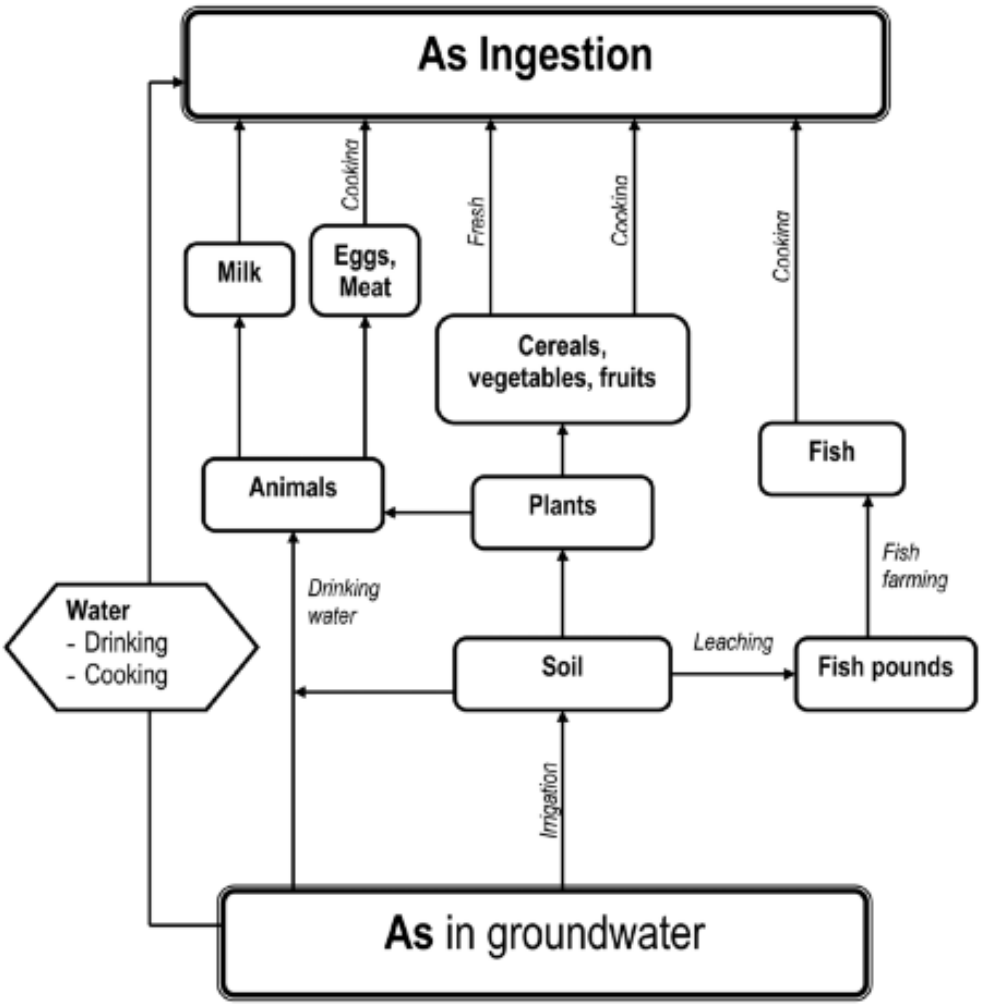



**Fig.2 Possibile pathways of arsenic ingestion by humans (Carbonell-Barrachina et al., 2009).**

World Health Organization recommended a Provisional Tolerable Weekly Intake (PTWI) of 15 µg of inorganic arsenic per week per kg of body weight; this value could be converted in the TDI (Total Daily Intake) = PTWI/7 days= 127 µg of inorganic arsenic per day for adult, assuming a mean body weight of 58 kg (Carbonell-Barrachina et al., 2009). Kile et al. (2007) studied the dietary As exposure in Bangladesh. Median daily total As intake was 48 µg per day from food and 4 µg per day from drinking water (mean concentration of 1.6 µg $l^{-1}$). On average, 82% of the As found in their food samples were in the inorganic forms. This study was a clear

example of how important food becomes in studying the dietary intake of As when As concentration in drinking water is below the 10 μg $l^{-1}$ drinking water standard of WHO (as in this case). Again is highlighted the importance of food as a source of As, and also inorganic As, and that this source should never be forgotten in populations depending heavily on vegetables (mainly rice) for their diets. It is highly recommended that the intake of As should be evaluated on the basis of the product as ingested by the consumer and not from raw products (Carbonell-Barrachina et al., 2009). Arsenic is also a potent antitumor agent (Douer and Tallman, 2005; Sanz et al., 2009; Tallman, 2002); $As_2O_3$ has been approved by Food and Drug Administration in USA for the treatment of patients affected by Acute Promyelocytic Leukemia (APL) that are refractory to other treatments. Although $As_2O_3$ is very effective in the treatment of APL, there are associated toxicities with its use, and appropriate precautions should be in place during its administration (Platanias, 2009). Furthermore $As_2O_3$ demonstrated potent effects *in vitro* against other malignant cells, suggesting a substantial interest in its potential clinical development for the treatment of other hematologic malignancies (Schiller et al., 2006; Lunghi et al., 2008).

### 1.2.1 Contaminations in the world

Among the countries with the highest daily intake of total arsenic (inorganic and organic species) there are Spain, Japan, India, France and Chile (Carbonell-Barrachina et al., 2009) but there are some important differences concerning the type of contamination of these countries. In particular in Spain and Japan seafood is the main source of As in the diet, containing organic arsenic, theoretically non-toxic (arsenobetaine and arsenocholine), whereas in Chile drinking water is the main source and contains inorganic arsenic that is the most toxic form; finally in India (especially West Bengal) cooked rice is the most important source of arsenic, together with water, and both sources contain mainly toxic inorganic arsenic forms (Carbonell-Barrachina et al., 2009). For cooked food it is important to highlight that cooking processes may alter arsenic concentration in food because of the arsenic content in water utilized to cook; this last consideration suggests that also the effect of processing and cooking must be considered in arsenic risk assessment, together with the main arsenic species present in the environment (Carbonell-Barrachina et al., 2009).

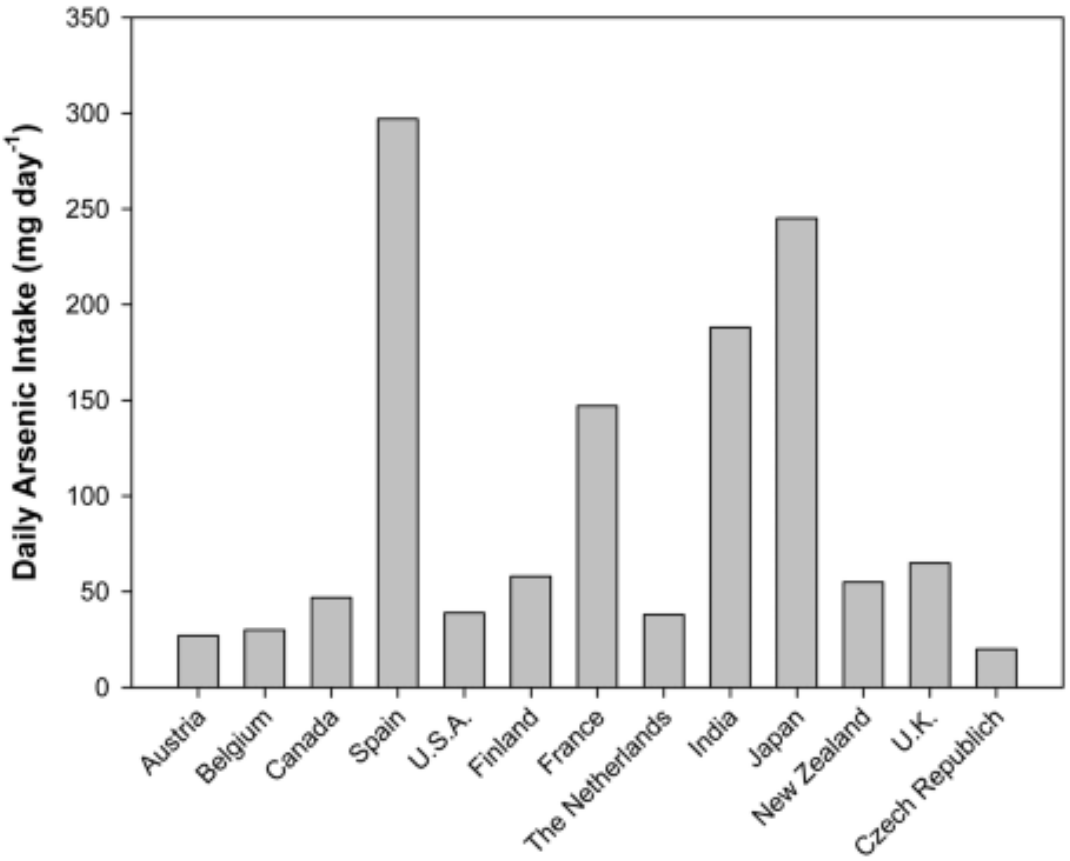



**Fig. 1 Daily As intake around the world (Carbonell-Barrachina et al., 2009).**

| Country | Mean daily total As intake µg/person/day | References |
|---|---|---|
| Bangladesh | 214 (males) 120 (females) | Watanabe et al. (2004) |
| Belgium | 12 | Buchet et al. (1983) |
| Canada | 59.2 | Dabeka et al. (1993) |
| Canada | 16.7 | Dabeka et al. (1987) |
| Croatia | 11.7 | Sapunar-Postruznik et al. (1996) |
| Japan | 182 | Mohri et al. (1990) |
| Japan | 160–280 | Tsuda et al. (1995) |
| Mexico | 394 | Del Razo et al. (2002) |
| The Netherlands | 38 | Dokkum et al. (2007) |
| Spain | 223.6 | Llobet et al. (2003) |
| UK | 65–67 | Ministry of Agriculture, Fisheries, and Food (MAFF) (1999) |
| USA | 61.5 | Gartrell et al. (1985) |
| USA | 88 | Gunderson (1995) |
| USA | 3.2 (children[a]) | Yost et al. (2004) |
| West Bengal, India | 60.3–102 | Roychowdhury et al. (2003) |

**Tab.3 Worldwide variation of mean daily total As intake trough food. (*children from 1 to 6 years) (Khan et al., 2009).**

## 1.3 Arsenic in plants

An excessive soil content of As is prejudicial to plant growth. Its availability is dependent on a range of chemical and physical factors (Bissen and Frimmel, 2003) but when is taken up by the plant it could interfere with metabolic processes and inhibit plant growth, sometimes leading death (Carbonell-Barrachina et al., 2009). The uptake is controlled by a number of factors including As species, concentration, pH, Eh and draining conditions, amount of organic matter, seasonal effects, plant species and chemical factors operating on the soil, fertilizer addition and physiological state of the plant (Carbonell-Barrachina et al., 2009; Coddington, 1986). Kabata-Pendias and Pendias (1984) reported that the As background for terrestrial plants growing on uncontaminated soils ranges from 0.009 to 1.5 mg $kg^{-1}$ on a dry weight basis. In general it could be said that the most important arsenic accumulation in plants occurs in roots (especially in potatoes, carrots, radishes, turnips), followed by vegetative top growth (e.g. spinach and grasses) and seeds and fruits with the lowest As content (Carbonell-Barrachina et al., 2009). An important topic in the context of food safety is to understand how plants uptake, transport, metabolize and tolerate As (Ali et al., 2009). In terrestrial plants, the capacity to take up As appears to be quite species-specific (Baroni et al., 2004). In general a higher accumulation in the roots system is correlated to a higher tolerance to this metalloid as it happens for tomato and turnip (Carbonell-Barrachina et al., 1997 ; Carbonell-Barrachina et al., 2009). Higher upward transportation is correlated to sensitive plants as in the case of beans (Carbonell-Barrachina et al., 2009). Most of the As encountered by the roots is in the inorganic form, and uptake and translocation mechanisms are different depending on the As chemical form. In general As (V), thanks to its chemical similarity with phosphorous, utilizes the same transport system through the plant (Tripathi et al., 2007). Once inside the plants As (V) can interferes with metabolic processes like ATP synthesis and oxidative phosphorylation (Tripathi et al., 2007). As (III) could enter in the roots in the form of arsenous acid ($As(OH)_3$), through aquaporins, specifically aquaglyceroporins of the NIP family (noduline 26-like intrinsic proteins), that are implicated in the silicic acid uptake (Ma et al., 2008). Once inside the plants cells As (III) can be coordinated to sulphur ligands and transported as As (III) – *tris* – glutathione complex (Pickering et al., 2000). Inside the plant cell, inorganic arsenic strongly induce Phytochelatin synthesis, with an important role in detoxification (Schmöger et al., 2000).

Together phytochelatins and glutathione contributes to As sequestration in the vacuole (Sneller et al., 1999; Pickering et al., 2000; Schmöger et al., 2000; Raab et al., 2005) and this is considered the main mechanism of As detoxification in plants, although in the As hyperaccumulator *Pteris vittata* most of the arsenite is uncomplexed (Webb et al., 2003; Zhao et al., 2003).

As (V), after its uptake in the root cells can be actively reduced to As (III) by arsenate reductase; this enzyme was recently characterized from different plant species including *Holcus lanatus, Arabidopsis thaliana* (Bleeker et al., 2006), *Pteris vittata* (Ellis et al., 2006) and rice (Duan et al., 2007). The reduction of arsenate to arsenite mediated by arsenate reductase involves glutathione as electron donor and is followed by the formation of the disulphide form of glutathione (Pickering et al., 2000).

Our current understanding of how As it is taken up and translocated (and the interaction between silicon (Si) and As uptake) is represented in Fig.3.

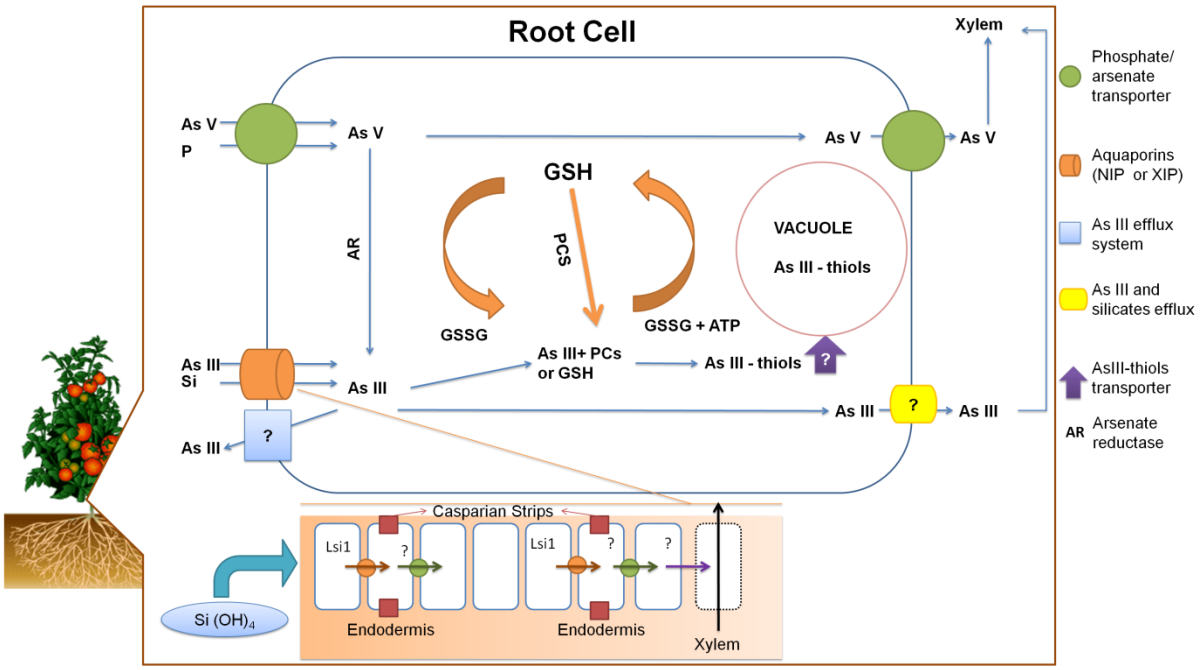



**Fig.3. A schematic illustration of the mechanism of As uptake and translocation, adapted from Ali et al. (Ali et al., 2009) and Ma et al. (2006). Arsenate enters the root via phosphate transporters where it behaves as a P toxic analogue (Ali et al., 2009). Most of the arsenate taken up is rapidly reduced to arsenite by the action of arsenate reductase (AR) using glutathione (GSH) as a reductant. In most plant species, arsenite is chelated by phytochelatins (PCs) and deposited in the root cell vacuoles (Ali et al., 2009) . Arsenite is taken up via the same channels as Si (Ma et al., 2008).**

## 1.4 Tomato cultivation

Tomato (*Solanum lycopersicum* L., Solanaceae family) is a major horticultural crop in both Europe and the US; FAOSTAT (Food and Agriculture Organization Statistics) reported in 2010 a tomato market of about 22 million tonnes/day in Europe and 13 million tonnes/day in USA (FAOSTAT, 2010). This cultivation was introduced in Europe from Central and Southern America at the beginning of the 16$^{th}$ century and first cultivated only as an ornamental plant, but in the 17$^{th}$ century it became popular that fruits of the tomato plant are edible and therefore their cultivation spread rapidly throughout the world. In Europe tomato plants are successfully cultivated in Mediterranean countries, especially Italy and Spain (Soressi, 1969). In these countries tomato found a secondary centre for diversification which resulted in a wide array of variations including round, obovoid, long, heart, rectangular, and even bell-pepper shaped fruit (Bailey et al., 1960) that are still present among tomato landraces used for fresh consumption (Mazzucato et al., 2008). Actually there are about 350 varieties in the National Register of Horticultural Species in Italy and 70% of those are constituted by hybrids. Every year about 25 new varieties were signed in this register. The most commercial varieties of tomato for industrial transformation are F1 hybrid (Savo Sardaro et al., 2013) and in Italy there were produced in 2010 more than 0.6 million tons of tomato variety for processing and 0.06 million tons of salad tomatoes were harvested.

Tomato was classified as a tolerant plant for arsenic, characterized by a generally high root uptake but very low translocation to the aerial parts (Carbonell-Barrachina et al., 1997). Xu et al. (2007) found that arsenate added to the nutrient solution was rapidly reduced to arsenite by tomato plants; more than 95% of arsenate was reduced to arsenite within one day. In this study they have also demonstrated that arsenate reduction was not mediated by microbes or root exudates; thus roots must be responsible for most of the reduction of arsenate in the external medium and this was supported by the fact that most of the As present in tomato roots was in the form of arsenite. Arsenite was also the main form of As transported in the xylem sap of tomato and some of the arsenite was likely to be complexed with thiols as has been demonstrated in other plant species (Xu et al., 2007).Due to the historical use of inorganic arsenicals (such as sodium arsenite) in agriculture, there is a legacy of contaminated orchard soils. In Spain, soils where sodium arsenite was widely applied

are now frequently used for tomato and bean growing (Carbonell-Barrachina et al., 1997).

Arsenic may be toxic to tomato plants and also may accumulate in the plant with the possibility of entering in human food chain trough the fruits (Burló et al., 1999) and this is the reason why we decided to study uptake and translocation mechanisms of arsenic in this plant, in order to provide insights about human risk of contamination trough tomato consumption.

# 2. The effect of silicon on the uptake and translocation of inorganic arsenic in tomato

## 2.1 Introduction

### 2.1.1 Silicon in plants

Si represents the second most abundant element in the earth's crust (Exley, 1998). Si-rich compounds in the soil structure are inert quartz or crystalline silicates but the physically and chemically active silicon substances in the soil include: soluble monosilicic acids, polysilicic acids ad organosilicon compounds (Balakhnina et al., 2012). Si compound absorbed by plants and microorganism is only monosilicic acid (Balakhnina et al., 2012). Plants silicon concentrations vary sensibly in the aboveground parts, ranging from 0.1% to 100% of Si in the dry weight (Ma, 2004). Silicon uptake and transport systems are different according to the plant species, with a typical distribution of Si accumulation among plants of different families: in higher plant, only Graminaceae and Cyperaceae show high Si accumulation, Cucurbitales, Urticales and Commelinaceae show intermediate silicon accumulation, while most other plants species show low accumulation. The differences in Si accumulation are probably due to the ability of roots to take up silicon (Mitani and Ma, 2005). There have been proposed three Si uptake models for plants: active, passive and rejective uptake (Takahashi et al., 1990). Uptake system of tomato was studied in comparison with rice and cucumber by Mitani and Ma (2005) and it was found that tomato have a passive Si uptake model with absence or very low presence of silicon transporters but this last aspect must be well elucidated. Since is not considered an essential element for the majority of plant species, silicon is a beneficial element for plant growth, because it can help to overcome multiple stresses including biotic and abiotic stresses (Ma, 2004 ; Ma and Yamaji, 2006 ; Balakhnina et al., 2012). In particular Si can enhance resistance of plants to diseases caused by both fungi and bacteria in different plant species and it can also suppress insect pests (Ma and Yamaji, 2006). This beneficial effect is due to the physical barrier that silicon constitutes beneath the cuticle to form a cuticle-Si double layer (Ma and Yamaji, 2006).

But silicon demonstrates beneficial effects also in attenuating the effects of some abiotic stresses due to different mechanisms: a decreased cuticular water loss by transpiration, thanks to the deposition of Si beneath the cuticle, a decreased uptake of toxic minerals due to the deposition of silicon in the roots, chelation with toxic metals, silicon enhanced strength of the stem (Ma and Yamaji, 2006), formation of Si-enzymes complexes that act as protectors and photosynthesis regulators as well as influencing other enzymatic activities (Toresano-Sánchez et al., 2012). Applying of Si to tomato crops has been used to alleviate drought and salinity stress (Toresano-Sánchez et al., 2012), but to date no attempt has been made to correlate Si supplementation with As uptake and its translocation to the aerial part of the plant. Here, we show that Si treatment can indeed influence As uptake in tomato.

### 2.1.2 Detection of As and Si in plants

There are many different types of As detection analysis that can vary with the aim of the study; for example, speciation analysis has the aim to discriminate among As chemical species, inorganic arsenic analysis is for the determination only of the inorganic species while organic arsenic analysis is for the determination only for the organic species. Furthermore it could be necessary to make a qualitative analysis or a quantitative analysis and for them it will be necessary dedicated methods. For each type of analysis, different sample pre-treatments and different instrumentations are required; therefore it is important to establish a correct experimental design based on the aims of the study. This study had the first aim of detection of inorganic arsenic concentrations in different parts of the plant and then determines inorganic arsenic tissue distribution in plants. For the analysis of arsenic concentrations in plants it was chosen atomic absorption spectroscopy (AAS) because it is a specific, sensitive and economic technique and it's relatively easy to do. It involves the absorption of optical radiation by free analyte atoms in gaseous state for detecting their concentration in a sample; in fact this absorption is, in a linear range, proportional to the concentration of analyte atoms present in the optical path. Atomic absorption spectroscopy analysis for the detection of arsenic concentration, as for other elements like Sn, Sb or Se, is usually coupled with hydride generation technique (HG-AAS) in order to increase analysis sensitivity. In this last technique arsenic hydrides are generated through the reaction with sodium borohydride and hydrochloric acid and then they are transported,

thanks to an inert gas flow, to the optical cell of the spectrophotometer and atomized with the conventional techniques: flame atomization based on a flame heat achieved by different mix of gases like air-acetylene, air- hydrogen, nitrous oxide-acetylene, or electrotermal atomization as in the graphite furnace where graphite tubes are heated through their ohmic resistance using a low-voltage high-current power supply.

For arsenic tissue distribution in plants it was employed a scanning electron microscope (SEM) coupled with energy-dispersive X-ray detector (SEM/EDX). Scanning electron microscopes employs a focused electron beam to scan a sample and produce its image. Samples hit by the electron beam produce various signals that can be detected and provide important informations about sample characteristics, including: secondary electron emitted from the sample, characteristics X-rays, back scattered electrons. Analyzed sample are thin sections of an organ or a tissue or sometimes cell suspensions, laid on a glass slide or a special specimen holder (stub). Detection of emitted secondary electrons could be useful to reconstruct the topography of the sample and produce an image because the number of secondary electrons is a function of the angle between the beam and the sample surface. X-rays emission is utilized for the microanalysis of elements concentration in the sample because each element had its characteristic X-rays emission spectrum.

#### 2.1.2.1 Simple Sequence Repeats

Morphological descriptors do not always allow the quantification of genotypic differences, because quantitative characters can be altered by environmental factors (Cooke et al., 1995). In contrast, molecular markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), single nucleotide polymorphism (SNP), and simple sequence repeats (SSR) can provide an effective tool for variety identification as they are independent of environmental effects (Lee and Henry, 2001; Sim et al., 2009). Among the different available marker systems, SSR markers have become important for variety identification because of their property of genetic codominance, high reproducibility, and multiallelic variation (Powell et al., 1996).

The work of Smulders et al. (1997), Bredemeijer et al. (2002), He et al. (2003), Frary et al. (2005), Garcia-Martinez et al. (2006), Song et al. (2006), Kwon et al. (2009), Turci et al. (2010), and Caramante et al. (2011) confirmed the utility of DNA molecular markers for studying genetic diversity and variability in the genus Solanum and for selecting tomato cultivars. SSRs are better performing for identification of varieties because they are codominant markers, while SNP, AFLP, RAPD, and other methodologies are only able to highlight the dominant alleles. In comparison to the other codominant technique RFLP, SSR experiments are faster to perform and the results are clearer cut. Thus it was decided to: genotype the cultivars and assess the phylogenetic distances between them using Simple Sequence Repeats (SSR) as a molecular marker.

## 2.2 Materials and methods

### 2.2.1 Plant material utilized for soil experiments and germination tests

Eight commercial processing tomato cultivars were used in the experiments, namely Aragon, Axel, Frigio, Gladis, Podium, Rapidus, Ruphus and Wally-Red; six of these produce round berry and two of them produce plum berry. The seeds were provided by ESASEM s.p.a., Casaleone, Verona, Italy. The cultivars are all subject to plant breeders' rights, and pedigree informations are not publicly available.

**Tab.1. Tomato varieties used in this study**

| Variety | Origin | Maturity[1] | Fruit shape[2] | Use |
|---|---|---|---|---|
| Aragon | Hybrid | EE | P | Industrial Transf. |
| Axel | Hybrid | M | R | Industrial Transf. |
| Frigio | Hybrid | M | R | Industrial Transf. |
| Gladis | Hybrid | ML | R | Industrial Transf. |
| Podium | Hybrid | ML | R | Industrial Transf. |
| Rapidus | Hybrid | EE | P | Industrial Transf. |
| Ruphus | Hybrid | M | R | Industrial Transf. |
| Wally Red | Hybrid | M | R | Industrial Transf. |

[1]Maturity: EE=extra early; M=medium; ML=medium late
[2]Fruit Shape: P=Plum; R=Round

### 2.2.2 Germination and seedlings growth on culture plates

The seeds of each cultivar were rinsed in deionized water to remove any fungicidal coating, surface-sterilized by immersion in 5% (v/v) sodium hypochlorite and plated on Murashige-Skoog medium (Duchefa Biochemie, Haarlem, The Netherlands) containing 10 g $l^{-1}$ sucrose (AppliChem GmbH, Darmstadt, Germany) and 0.8% (w/v) agar (AppliChem GmbH, Darmstadt, Germany).

**Tab.2 Chemical composition of MS medium utilized for these experiments.**

| **Compound** | **Concentration (mg $l^{-1}$)** |
|---|---|
| Ammonium Nitrate ($NH_4NO_3$) | 1,650 |
| Boric Acid ($H_3BO_3$) | 6.2 |
| Calcium Chloride ($CaCl_2 \cdot 2H_2O$) | 440 |
| Magnesium Sulphate ($MgSO_4 \cdot 7H_2O$) | 370 |
| Copper Sulphate ($CuSO_4 \cdot 5H_2O$) | 0.025 |
| Potassium Phosphate ($KH_2PO_4$) | 170 |
| Ferrous Sulphate ($FeSO_4 \cdot 7H_2O$) | 27.8 |
| Potassium Nitrate ($KNO_3$) | 1,900 |
| Manganese Sulphate ($MnSO_4 \cdot 4H_2O$) | 22.3 |
| Potassium Iodide (KI) | 0.83 |
| Sodium Molybdate ($Na_2MoO_4 \cdot 2H_2O$) | 0.25 |
| Zinc Sulphate ($ZnSO_4 \cdot 7H_2O$) | 8.6 |
| $Na_2EDTA \cdot 2H_2O$ | 37.2 |

The medium was supplemented with either 0.2 mM or 0.5 mM of $NaAsO_2$ or $Na_2HAsO_4 \cdot 7H_2O$ (Sigma-Aldrich, St. Louis, MO, USA), with and without further supplementation with 0.025 mM $CaSiO_3$ (Sigma-Aldrich, St. Louis, MO, USA). Other experiments involved non-treated substrate or substrate with added Si. Five replicates were performed per treatment per cultivar.

**Tab.3 Summary of the treatments performed *in vitro*.**

| Treatment | Concentration |
|---|---|
| Control | MS and sucrose 10 g $l^{-1}$ |
| Silicon | MS + sucrose + $CaSiO_3$ 0.025 mM |
| Arsenite | MS + sucrose + $NaAsO_2$ 0,2 mM |
| | MS + sucrose + $NaAsO_2$ 0,5 mM |
| Arsenate | MS + sucrose + $Na_2HAsO_4 \cdot 7H_2O$ 0,2 mM |
| | MS + sucrose + $Na_2HAsO_4 \cdot 7H_2O$ 0,5 mM |
| Arsenite + Silicon | MS + sucrose + $NaAsO_2$ 0,2 mM + $CaSiO_3$ 0.025 mM |
| | MS + sucrose + $NaAsO_2$ 0,5 mM + $CaSiO_3$ 0.025 mM |
| Arsenate + Silicon | MS + sucrose + $Na_2HAsO_4 \cdot 7H_2O$ 0,2 mM + $CaSiO_3$ 0.025 mM |
| | MS + sucrose + $Na_2HAsO_4 \cdot 7H_2O$ 0,5 mM + $CaSiO_3$ 0.025 mM |

The plates were housed in an incubator (Innova 4230, New Brunswick Scientific, Edison, New Jersey, USA) held at 25°C in the dark, and germination was scored after 48 h. The seedlings were then provided with 16 h per day of 300 µmol $m^{-2}$ $s^{-1}$ light (supplied by metal halide lamps) for two weeks, and shoot length was monitored every two days. After 15 days shoots lengths for each sample were measured and it was calculated the average shoots length of each cultivar in the different treatment conditions. The results were normalized referred to the average shoots length of the control.

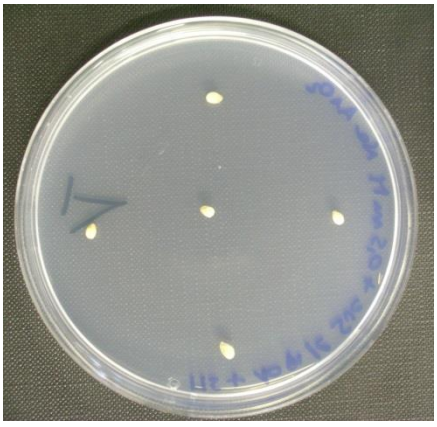


**Fig.1 Example of Petri dish with five seeds of tomato in MS medium supplemented with As.**

### 2.2.3 Pot trials

#### 2.2.3.1 Experiment #1

In an initial series of pot trials, 3 L pots were filled with garden soil (Gebr. Brill Substrate GmbH and Co. KG, Germany) in each of which a single plant was grown. The soil composition was white peat (40%), black peat (20%) and wood fibre (20%), pH ca. 6.0, range nitrogen content 180-300 mg $l^{-1}$, mean phosphorus content 190-310 mg $l^{-1}$ and mean potassium content 240-400 mg $l^{-1}$. The plants were watered as necessary.

**Fig.2 Tomato plants growing in garden soil at greenhouse conditions.**

After three months, either 5 mg $l^{-1}$ of $NaAsO_2$ or $Na_2HAsO_4 \cdot 7H_2O$ was added, either with or without 2 mg $l^{-1}$ of $CaSiO_3$. Control treatments involved either no additive or the provision of only $CaSiO_3$. The experiment comprised three replicates per treatment per cultivar. The temperature was maintained at 25°C, the relative humidity at 50%, and the photoperiod at 16 h, with the light provided by metal halide lamps supplying a photon flux density of 300 µmol $m^{-2}$ $s^{-1}$. Two weeks after the addition of As and Si, the plants were harvested, washed with deionized water, and separated into root, stem and leaf material.

**Tab.4 Summary of the treatments performed *in vivo*.**

| Treatments | Concentration |
|---|---|
| Control | Garden soil |
| Silicon | Garden soil + $CaSiO_3$ 2 mg $l^{-1}$ |
| Arsenite | Garden soil + $NaAsO_2$ 5 mg $l^{-1}$ |
| Arsenate | Garden soil + $Na_2HAsO_4 \cdot 7H_2O$ 5 mg $l^{-1}$ |
| Arsenite + Silicon | Garden soil + $NaAsO_2$ 5 mg $l^{-1}$ + $CaSiO_3$ 2 mg $l^{-1}$ |
| Arsenate + Silicon | Garden soil + $Na_2HAsO_4 \cdot 7H_2O$ 5 mg $l^{-1}$ + $CaSiO_3$ 2 mg $l^{-1}$ |

#### 2.2.3.1.1 SEM/EDX elemental localization

The distribution of As and Si in the various tissues was evaluated by SEM/EDX: a scanning electron microscope (Jeol 6400, Osaka, Japan) combined with an energy dispersive X-ray analyser (SEM/EDX) and LINK ISIS software (Oxford Instruments, Oxford, UK). For this analysis, dry cross sections of roots and stems were positioned on a glass slide and then covered with a graphite layer; for each organ/cultivar/treatment at least three sections were analysed. In some instances samples were badly compromised by treatments and drying, thus it was not possible to obtain observable cross sections, however the majority of the specimens were analysed to gain a general understanding of As and Si distribution in roots and shoots. The SEM/EDX operating parameters were: energy at 20 KeV, working distance of 15 mm, death time of spectra acquisition of 15-20%, enlargement varying according to the dimension of the cross sections. Dot maps of element of interest, macronutrients, micronutrients, arsenic and silicon were acquired utilizing the mapping program available within LINK ISIS software; the characteristics of maps acquisition are described in Marmiroli *et al.*, 2011.

#### 2.2.3.1.2 Chemichal mesaurements of As with HGAAS

The concentrations of As in roots, stems and leaves were measured with Hydride Generation Atomic Absorption Spectrometry (HG-AAS, Varian- Agilent Technologies, Santa Clara, CA, USA). Methods for sample mineralization and the measurement of absorbance, along with setting of the instrument's parameters followed the device's user guide and Marmiroli et al. (2011).

For the analysis of plant material, roots, stems and leaves were dried in an oven at 70°C for 72 hours, then ground and homogenized. For each acid digestion were used 0.2 g of sample plus 10 ml of concentrated nitric acid (65 % v/v, Panreac Quìmica, Barcelona, Spain) in 250 ml glass tubes (Velp Scientifica s.r.l., Usmate, Monza-Brienza). Each glass tube was put onto a 20 holes heating plate (VELP Scientifica s.r.l., Usmate, Monza-Brienza) and heated at 200°C for 2 hours. Samples were allowed to cool and then filtered (filters of 0.45 µm, Sarstedt, Verona, Italy) after which 1 ml of potassium iodide (10% w/v, SIGMA Aldrich, Milano, Italy) was added. The addition of potassium iodide was necessary to obtain the desired As oxidation state for its determination by hydride generation. The solution was then diluted with distilled water up to a volume of 40 ml. All measurements were performed on a Varian AA240FS spectrometer using a Vapour Generator Accessory VGA77, SpectrAA240FS 5.1 PRO software and SpectrAA arsenic hollow cathode lamp (Varian- Agilent Technologies, Santa Clara, CA, USA). The recommended instrument parameters were used on the spectrometer. Absorbance was measured at 189 nm wavelength and the calibration curve was made within the concentration range of 0-100 µg $l^{-1}$. An AAS standard nitric acid solution (Varian AAS Standard) of 10000 ppm of As was used to prepare a working stock solution of 10 ppm As. Final standard solutions were made up daily from the working stock.

#### 2.2.3.2 Experiment #2

The second experiment concentrated on the four cultivars (Aragon, Axel, Frigio and Gladis) which took up the most As in the first experiment. The plants were grown in 25 L pots, using the same soil and growing conditions described above. Plants were watered with tap water as in experiment 1 using temporized drip irrigation. The treatments were initiated in this case at the onset of fruiting (four months after sowing). A month after the beginning of the treatment, stem, leaf, root and fruit tissues were separately processed for HG-AAS analysis, following the acid digestion protocol given by Marmiroli et al. (2011) and described in paragraph 2.2.3.1.

**Fig.3 Examples of tomato plants growing in garden soil at greenhouse conditions for the experiments of the translocation to the fruits.**

### 2.2.4 Statistical analysis

For the germination/seedling growth experiment, statistically significant differences in germination between the non-treated and treated samples were inferred from a conventional analysis of variance, applying the Bonferroni post hoc test applied to the number of seeds germinated per dish. For the analysis of seedling shoot elongation, the Student's two tail t-test was applied. In the pot trial experiment #1, the HG-AAS data were also analyzed using the Student's two tail t-test, while in the Experiment #2, the data were subjected to the non-parametric Kruskall–Wallis test, since the data set was not normally distributed. The software package SPSS v19 (IBM website) was used for all statistical procedures.

### 2.2.5 Microsatellyte genotyping

DNA was isolated from 100 mg of frozen young leaves grounded to fine powder with liquid nitrogen, using a GenElute Plant Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, USA). In order to obtain statistically suitable data, three samples representative of different plants, for each variety, were analysed. DNA concentration and purity were determined using a Cary 50 Spectrophotometer (Varian Inc., Torino, Italy) and electrophoresis on 2 % agarose gel. PCR amplicons were generated from 25 μl reactions containing 1 U of Go Taq DNA Polymerase (Sigma-Aldrich, St. Louis, MO, USA), 0.5 μl 10 mm dNTP mix, 0.25 μmol $L^{-1}$ of each primer, forward and reverse

primer (Sigma-Aldrich, St. Louis, MO, USA), 1 X Go Taq polymerase reaction buffer (Sigma-Aldrich, St. Louis, MO, USA) and 30 ng of genomic DNA. Amplification reactions were run under the following conditions: DNA denaturation at 94°C for 1 min, followed by 40 cycles with: 45 s at 94°C; 45 s according to the Tm of the primer; 45 s of extension at 72°C and finally a primer thermal extension at 72°C for 10 min. PCR products were separated by 3% agarose gel electrophoresis, run with TBE 1X at 80 V (89 mmol $L^{-1}$ Tris borate, 89 mmol $L^{-1}$ boric acid, 2 mmol $L^{-1}$ EDTA) (Sambrook et al., 1989). The gels were stained with 1000 X Gel Red (Biotium, Hayward, California , USA), visualization and acquisition of digital images using Bio-Rad Gel Doc 2000 instrument with Proprietary Software (Bio-Rad, Hercules, California, USA). Eleven most informative SSR loci (He et al., 2003) were chosen and used for the assay (Tab. S1). Allele calling was performed by a CEQ 2000 gene analysis system and amplicons sizes were estimated from internal size standards using a CEQ DNA 400 device (Beckman Coulter, High Wycombe, United Kingdom). For each SSR locus, the number of alleles and the polymorphic information content (equivalent to the expected heterozygosis, He) were calculated as follow:

$$PIC = He = 1-\sum p_i^2$$

where $p_i$ is the frequency of the ith allele at a specific locus (Hartl and Clarck, 1997). Calculations were performed using the GenAlEx 6.2 software (Peakall and Smouse, 2006). For cluster analysis, monomorphic SSR loci were excluded. The estimation of the genetic similarity between all the varieties was calculated according to Nei and Li (1979) and the corresponding trees were drawn with the Unweight Pair Group Method using Arithmetic Average (UPGMA) clustering method (Sneath et al., 1973) using the GDA software Version 1.0 (d16c) (Genetic Data Analysis) (Lewis ans Zaykin, 2001).

**Tab.5 Experimental data related to the SSR primers used for genotyping.**

| SSR name | Chr. location | Core Motif | Sequence (5'-3') | References | Observed size range (bp) | Allele n° | PIC | Tm (°C) |
|---|---|---|---|---|---|---|---|---|
| LEtat002 | - | $(tat)_{12}$ | FW acgcttggctgcctcgga<br>REV actttattattgccacgtagtcatga | He *et al.* (2003) | 195-221 | 3 | 0.23 | 60 |
| LEaat002 | - | $(aat)_{12}$ | FW caacagcatagtggaggagg<br>REV tacatttctctctctcccatgag | He *et al.* (2003) | 99-104 | 2 | 0.22 | 63 |
| LEat002 | - | $(at)_{9}$ | FW actgcatttcaggtacatactctc<br>REV taaactcgtagaccataccctc | He *et al.* (2003) | 201-205 | 2 | 0.51 | 59 |
| LEga003 | - | $(ga)_{20}$ | FW ttcggtttattctgccaacc<br>REV gcctgtaggattttcgccta | He et al. (2003) | 231-235 | 2 | 0.48 | 59 |
| LEaat007 | - | $(aat)_{12}$ | FWcgaagaagatgagtctagagcatag<br>REV ctctctcccatgagttctcctcttc | He *et al.* (2003) | 93-99 | 2 | 0.22 | 59 |
| SSR47 | 6 | $(at)_{19}$ | FW tcctcaagaaatgaagctctga<br>REV ccttggagataacaaccacaa | Solanaceae Genomics Network ww.sgn.cornell.edu | 189-201 | 3 | 0.65 | 56 |
| SSR70 | 9 | $(at)_{13}$ | FW tttagggtgtctgtgggtcc<br>REV ggagtgcgcagaggatagag | Solanaceae Genomics Network ww.sgn.cornell.edu | 115-121 | 2 | 0.31 | 59 |
| SSR248 | 10 | $(ta)_{21}$ | FW gcattcgctgtagctcgttt<br>REV gggagcttcatcatagtaagc | Solanaceae Genomics Network ww.sgn.cornell.edu | 241-252 | 5 | 0.79 | 57 |
| SSR 603 | 4 | $(gaa)_{8}$ | FW gaagggacaattcacagagtttg<br>REV ccttcaacttcaccaccacc | Solanaceae Genomics Network ww.sgn.cornell.edu | 235-254 | 3 | 0.68 | 58 |
| TOM210 | 4 | $(ata)_{15}$ | FWcgttggattactgagaggttta<br>REVACaaaaattcacccacatcg | Suliman-Pollatschek *et al.* (2002) | 218-224 | 2 | 0.12 | 56 |
| TOM236 | 9 | $(at)_{16}$ | FW gttttttcaacatcaaagagct<br>REV ggataggtttcgttagtgaact | Suliman-Pollatschek *et al.* (2002) | 156-211 | 4 | 0.57 | 56 |

## 2.3 Results

### 2.3.1 Germination tests

The only treatment which had any significant effect on germination was the presence of 0.5 mM $NaH_2AsO_4 \cdot 7H_2O$, which reduced germination by between 20% and 40%, depending on cultivar. The inhibitory effect was mitigated by the addition of $CaSiO_3$. However, the presence of both $NaAsO_2$ and $NaH_2AsO_4 \cdot 7H_2O$ had a drastic negative effect on seedling shoot elongation, which was not mitigated by the presence of $CaSiO_3$ (Table 6).

**Tab.6 Elongation of the seedling shoots 15 days after in vitro germination in the presence of As, both with or without Si supplementation. Shoot lengths have been normalized to the control, and are shown as mean values ± SE, based on three replicates. % inhibition of shoot elongation shown in parentheses.**

| Cultivar | Treatment | Control | | As (III) 0.2 mM | As (III) 0.5 mM | As (V) 0.2 mM | As (V) 0.5 mM |
|---|---|---|---|---|---|---|---|
| Aragon | As | 100±1.558 | | 9.069±0.216 (90.931%) | 2.621±0.056 (97.379%) | 13.011±0.272 (86.989%) | 20.265±0.561 (79.735%) |
| | As+Si | Clean soil | Si 0.025 mM | 7.959±0.384 (92.041%) | 6.386±0.060 (93.614%) | 36.015±0.722 (63.985%) | 16.265±0.460 (83.735%) |
| | | 100±1.620 | 87.152±1.142 | | | | |
| Axel | As | 100±0.711 | | 9.562±0.332 (90.438%) | 6.492±0.175 (93.508%) | 22.325±0.545 (77.675%) | 22.911±0.321 (77.089%) |
| | As+Si | Clean soil | Si 0.025 mM | 9.317±0.380 (90.683%) | 6.380±0.337 (93.62%) | 38.281±0.711 (61.719%) | 17.378±0.204 (82.622%) |
| | | 100±0.527 | 112.857±1.662 | | | | |
| Frigio | As | 100±0.507 | | 9.788±0.207 (90.212%) | 3.289±0.052 (96.711%) | 34.675±0.168 (65.325%) | 18.970±0.161 (81.03%) |
| | As+Si | Clean soil | Si 0.025 mM | 11.443±0.159 (88.557%) | 7.438±0.234 (92.562%) | 44.848±0,650 (55.152%) | 22.302±0,170 (77.698%) |
| | | 100±2.963 | 102.102±1,237 | | | | |
| Gladis | As | 100±1.669 | | 7.935±0.191 (92.065%) | 0.000[a] | 38.782±0.279 (61.218%) | 20.135±0.484 (79.865%) |
| | As+Si | Clean soil | Si 0.025 mM | 3.305±0.186 (96.695%) | 3.513±0.247 (96.487%) | 21.768±0.727 (78.232%) | 19.696±0.141 (80.304%) |
| | | 100±0.821 | 77.107±2.366 | | | | |
| Podium | As | 100±1.649 | | 12.671±0.072 (87.329%) | 5.193±0.120 (94.807%) | 54.998±0.715 (45.002%) | 32.394±0.588 (67.606%) |
| | As+Si | Clean soil | Si 0.025 mM | 10.072±0.907 (89.928%) | 6.463±0.804 (93.537%) | 70.571±1.260 (29.429%) | 36.951±1.036 (63.049%) |
| | | 100±1.775 | 107.050±4.949 | | | | |
| Rapidus | As | 100±1.248 | | 12.767±0.095 (87.233%) | 4.813±0.072 (95.187%) | 30.408±0.717 (69.592%) | 21.289±0.299 (78.711%) |
| | As+Si | Clean soil | Si 0.025 mM | 6.694±0.490 (93.306%) | 2.301±0.211 (97.699%) | 31.656±1.399 (68.344%) | 16.676±0.730 (83.324%) |
| | | 100±1.502 | 87.813±1.270 | | | | |
| Ruphus | As | 100±0.182 | | 6.640±0.229 (93.36%) | 2.340±0.036 (97.66%) | 28.165±0.292 (71.835%) | 25.846±0.169 (74.154%) |
| | As+Si | Clean soil | Si 0.025 mM | 4.836±0.868 (95.164%) | 1.142±0.185 (98.858%) | 31.861±1.466 (68.139%) | 24.111±0.915 (75.889%) |
| | | 100±3.778 | 83.571±4.643 | | | | |
| Wally Red | As | 100±1.589 | | 2.469±0.098 (97.531%) | 3.228±0.056 (96.772%) | 32.905±0.660 (67.095%) | 20.825±0.567 (79.175%) |
| | As+Si | Clean soil | Si 0.025 mM | 12.716±0.635 (87.284%) | 3.806±0.338 (96.194%) | 34.935±1.820 (65.065%) | 23.909±0.442 (76.091%) |
| | | 100±3.093 | 86.883±2.266 | | | | |

### 2.3.2 Pot trials

The effect of the treatments on plant morphology is illustrated in Fig. 4. Damage due to the presence of As was visible within 15 days of the initiation of the treatment, and the provision of $CaSiO_3$ was significantly ameliorative. Marked wilting, along with leaf chlorosis and necrosis, were shown by cv. Axel, while cv. Gladis was only mildly affected.

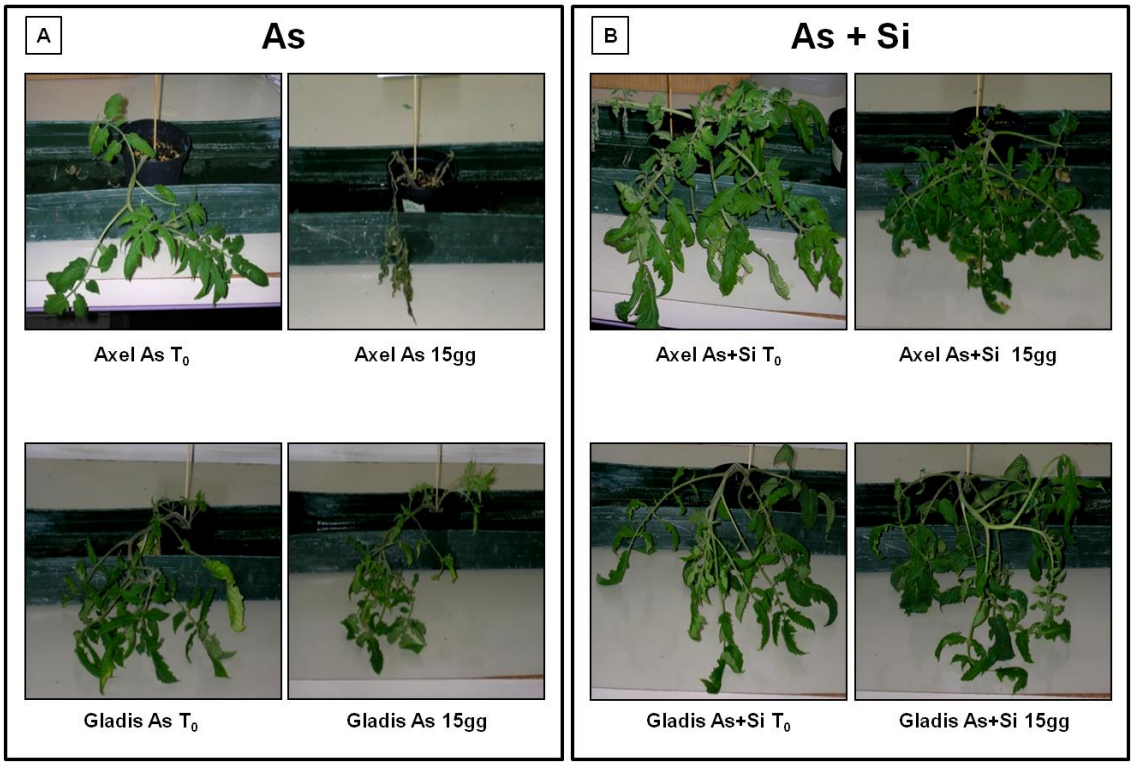



**Fig.4 The appearance of three month old tomato plants (cvs. Axel and Gladis) following exposure to arsenite for 15 days with or without supplementation by $CaSiO_3$.**

**Tab.7 As uptake in the root of plants exposed to As stress, as determined by HGAAS. Data given in µg $g^{-1}$ dry weight ± SE (experiment #1). Comparison between As and As+ Si treatments: * = p 0.1/ ** = p 0.05/ *** = p 0.01; comparison between $NaAsO_2$ and $NaH_2AsO_4 \cdot 7H_2O$ treatments: a = p 0.1 / b = p 0.05 / c = p 0.01.**

| Cultivar | Control | Si 2 mg $l^{-1}$ | As (III) 5 mg $l^{-1}$ | As (III) + Si 5 mg $l^{-1}$+2 mg $l^{-1}$ | As (V) 5 mg $l^{-1}$ | As (V) + Si 5 mg $l^{-1}$+2 mg $l^{-1}$ |
|---|---|---|---|---|---|---|
| **Aragon** | 0.481±0.018 | 0.210±0.001 | 194.263±0.422 (***,c) | 162.684±0.367 (***,c) | 1.73±0.023 (***,c) | 2.33±0.075 (***,c) |
| **Axel** | 0.553±0.021 | 0.372±0.001 | 193.210±0.013 (***) | 1.741±0.144 (***,c) | 1.34±0.127 (***) | 9.94±0.127 (***,c) |
| **Frigio** | 0.557±0.002 | 0.322±0.001 | 204.789±0.208 (***,c) | 1.848±0.115 (***,b) | 3.27±0.069 (***,c) | 4.68±0.110 (***,b) |
| **Gladis** | 0.099±0.002 | 0.135±0.001 | 38.99±0.006 (***,c) | 1.825±0.191 (***,c) | 3.3±0.035 (c) | 2.97±0.144 (c) |
| **Podium** | 0.296±0.001 | n.d. | 1.806±0.013 (***,a) | 1.422±0.008 (***,a) | 0.915±0.121 (a) | 0.940±0.035 (a) |
| **Rapidus** | 0.113±0.001 | 0.015±0.001 | 1.769±0.020 (***,a) | 1.461±0.001 (***,a) | 0.969±0.069 (a) | 0.924±0.023 (a) |
| **Ruphus** | n.d. | n.d. | 1.631±0.058 (***) | 1.545±0.001 (***,c) | 0.956±0.208 | 0.879±0.006 (c) |
| **Wally-Red** | n.d. | 0.308±0.001 | 34.91±0.013 (***,a) | 1.515±0.208 (***,c) | 0.959±0.023 (a) | 0.939±0.012 (c) |

**Tab.8 The As content of the stem and leaves of plants exposed to As stress, as determined by HGAAS. Data given in µg $g^{-1}$ dry weight ± SE (experiment #1). Comparison between As and As+ Si treatments: * = p 0.1/ ** = p 0.05/ *** = p 0.01; comparison between $NaAsO_2$ and $NaH_2AsO_4 \cdot 7H_2O$ treatments: a = p 0.1 / b = p 0.05 / c = p 0.01.**

| Cultivar | Control | Si 2 mg $l^{-1}$ | As (III) 5 mg $l^{-1}$ | As (III) + Si 5 mg $l^{-1}$+2 mg $l^{-1}$ | As (V) 5 mg $l^{-1}$ | As (V) + Si 5 mg $l^{-1}$+2 mg $l^{-1}$ |
|---|---|---|---|---|---|---|
| **Aragon** | 0.259±0.070 | 0.240±0.008 | 0.372±0.013 | 0.306±0.383 | 0.97±0.121 (**) | 0.12±0.002 (**) |
| **Axel** | 0.216±0.001 | 0.265±0,001 | 0.465±0.006 (***,a) | 0.398±0.001 | 1.2±0.081 (***,a) | 0.22±0.058 (***) |
| **Frigio** | n.d. | n.d. | 0.309±0.008 (***) | 0.022±0.001 (***) | 0.170±0.115 | 0.170±0.012 |
| **Gladis** | n.d. | n.d. | 0.094±0.001 (***) | n.d. | 0.31±0.046 (*) | 0.13±0.012 (*) |
| **Podium** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |
| **Rapidus** | n.d. | n.d. | 0.059±0.001 (***,a) | n.d. | n.d. | n.d. |
| **Ruphus** | n.d. | n.d. | 0.013±0.001 (***) | n.d. | n.d. | n.d. |
| **Wally-Red** | n.d. | n.d. | n.d. | n.d. | n.d. | n.d. |

SEM/EDX images of tissue sampled from cv. Aragon are given in Fig. 5. The SEM/EDX analysis highlighted inter-cultivar differences with respect to As uptake and translocation (Figs. 7–10), and these were used to make the selection of the four cultivars chosen for more detailed examination. In Fig. 5 shoots and roots of the cv. Aragon are represented; As is more concentrated in shoots external tissues, epidermis and parenchyma, while in roots is ubiquitous in all tissues. In general, in the accumulating cvs. Si and As competed for the same localization in roots external tissues (Figs. 5 and 7). In the three excluders cv. Frigio, Axel, and Gladis (Figs. 8–9), As is present only in low quantities in the outer parts of the roots. When $CaSiO_3$ supplementation was provided, Si became ubiquitously distributed throughout the root and shoot of cvs. Aragon, Axel and Frigio, and particularly abundantly in the parenchyma and epidermis. Arsenic was taken up by these cultivars whether or not $CaSiO_3$ supplementation was provided, and was then translocated to the aerial part of the plant. In contrast, cvs. Wally-Red, Podium, Rapidus and Ruphus only took up small quantities of As, whether or not $CaSiO_3$ was provided, and As was
inefficiently translocated to the aerial part of the plant (Figs. 7–10,Tables 7 and 8). Finally, cv. Gladis expressed an intermediate level of As uptake and a low rate of translocation (Fig. 8).

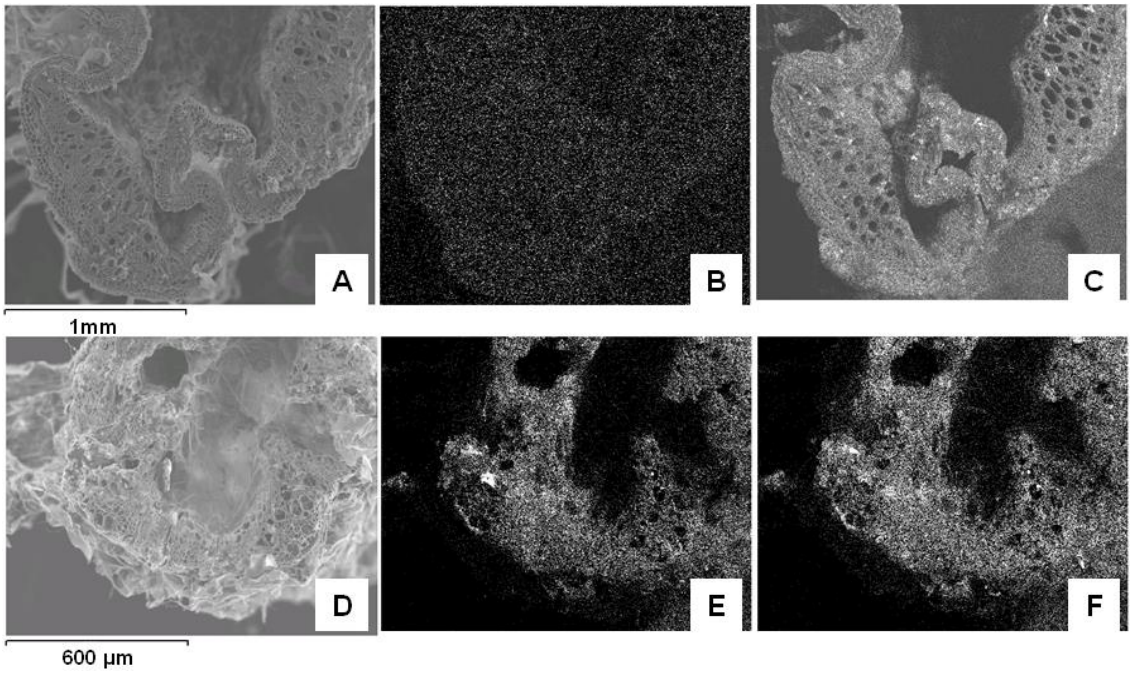



**Fig.5 The distribution of As and Si in As treated tomato cv. Aragon plants. (A-C) Stem sections: (A) SEM/EDX acquired image, (B) dot map showing As distribution, (C) dot map showing Si distribution. (D-F) Root sections: (D,E,F): (D) SEM/EDX acquired image, (E) dot map showing As distribution, (F) dot map showing Si distribution.**

The flux of As into the fruits of cvs. Aragon, Axel, Frigio and Gladis was investigated in the follow-up pot trial. The HG-AAS analysis revealed that $CaSiO_3$ supplementation had a significant effect on As concentration in the fruit (Fig. 6C). In the fruit of cv. Aragon, As was accumulated when the plants were treated with either $NaAsO_2$ or $NaH_2AsO_4 \cdot 7H_2O$, whether or not $CaSiO_3$ supplementation was provided. The other cvs. only accumulated As in the fruit in the treatment $NaAsO_2$ + $CaSiO_3$; the highest accumulator was cv. Gladis (Fig. 6C). The leaf and stem concentration of As was unaffected by the $CaSiO_3$ treatment in cvs. Aragon and Axel, but was affected in cvs. Frigio and Gladis (Fig. 6A and B). In cvs. Axel, Frigio and Gladis, the As content of the fruit in the absence of $CaSiO_3$ supplementation was below the limit of detection (Fig. 6C).

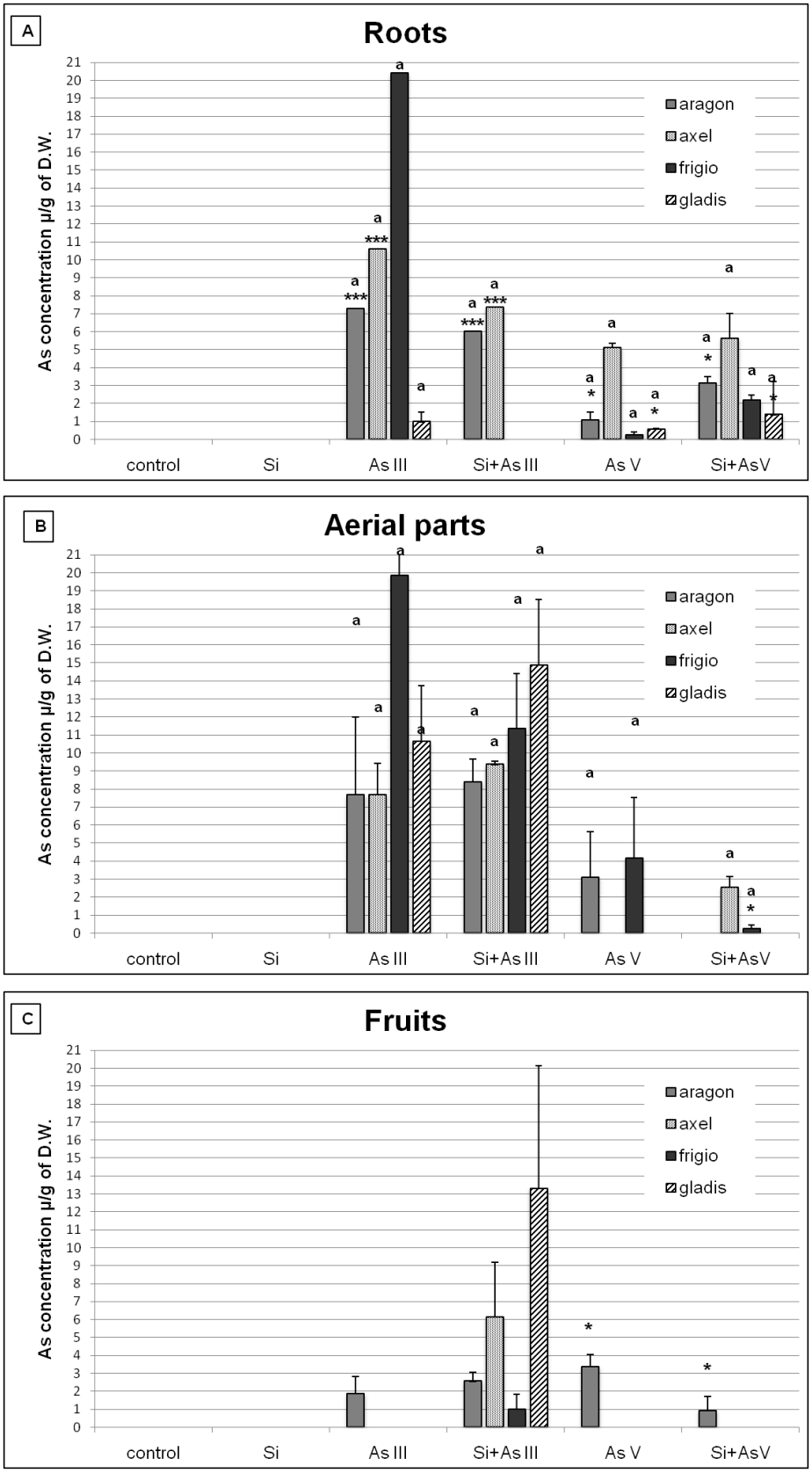



**Fig.6 HGAAS determined As concentration in (A) the root, (B) the stem and leaf, (C) the fruit of tomato plants following their exposure to As with or without supplementation by $CaSiO_3$. Data are given in µg $g^{-1}$ (dry weight basis) ± SE. Statistical analysis performed using a Kruskall-Wallis test. Comparison between As and As+ Si treatments: * = p 0.1/ ** = p 0.05/ *** = p 0.01; comparison between $NaAsO_2$ and $NaH_2AsO_4 \cdot 7H_2O$ treatments: a = p 0.1 / b = p 0.05 / c = p 0.01.**

Among the plants exposed to $NaAsO_2$ supplementation in the absence of $CaSiO_3$, the highest content of As in root tissue occurred in cv. Frigio, and the lowest in cv. Gladis. In the presence of the $CaSiO_3$ supplementation, As uptake was reduced in cvs. Frigio and Gladis. Feeding plants with $NaH_2AsO_4 \cdot 7H_2O$ induced little As uptake, and there was no evidence of either any inter-cultivar variation or any effect of $CaSiO_3$ supplementation.

A set of translocation factors (TFx) were calculated from the ratio between the content of As in different parts of the plant (Table 8). We are considering here two cvs. Aragon and Axel (Table 8) which have properties of accumulators though at different extent.

In Aragon, the translocation roots/aerial parts ($TF_{R/AP}$) has high value in all conditions tested, particularly for As (V), value which is abated by the simultaneous addiction of $CaSiO_3$. The translocation roots/fruits ($TF_{R/F}$) was significantly lower than previously, only a part of As taken up by roots can reach the fruits. The translocation between the aerial parts and fruits ($TF_{AP/F}$) is close to unit, almost all the As translocated into aerial parts can reach the fruits. In Axel the behaviour is different because trough $TF_{AP/F}$ evidenced a good translocation (with an increase for As (V)), the translocation from roots to fruits ($TF_{R/F}$) and from aerial parts to fruits ($TF_{AP/F}$) is nihil with the exception for the simultaneous addition of $CaSiO_3$. For cvs. like Gladis and Frigio the uptake was very small in all conditions (Table 7 and 8) and consequently also the translocation between the different parts of the plants. The positive $R^2$ (correlation coefficient) found for $TF_{R/AP}$, $TF_{R/F}$ and $TF_{AP/F}$ suggests that there is a direct relationship between uptake and translocation to aerial parts and fruits when Si is simultaneously applied. In the other conditions the correlation was not observed ($R^2_{AP/F}$ and $R^2_{AP/F}$ small or negative) (Table 8).

**Tab.8 Translocation factors (TFx): $TF_{R/AP}$ = ratio between As concentration in roots and As concentration in aerial parts, $TF_{R/F}$ = ratio between As concentration in roots and in fruits, $TF_{AP/F}$ = ratio between As concentration in green aerial parts and in fruits. $R^2$x is the correlation coefficients of the cvs. Aragon (A) and Axel (B) in the different conditions.**

| **A** | **Roots / Aerial Parts** | | **Roots / Fruits** | | **Aerial Parts / Fruits** | |
|---|---|---|---|---|---|---|
| **Cultivar Aragon** | **$TF_{R/AP}$** | **$R^2_{R/AP}$** | **$TF_{R/F}$** | **$R^2_{R/F}$** | **$TF_{AP/F}$** | **$R^2_{AP/F}$** |
| **As (III)** | 130.791 | -0.725 | 25.624 | -0,784 | 104,104 | 0.142 |
| **As (III) + Si** | 182.431 | 0.997 | 42.721 | 0,999 | 103.270 | 0.998 |
| **As (V)** | 603.805 | -0.748 | 314.867 | 0,942 | 100.917 | -0.484 |
| **As (V) + Si** | 29.758 | 0 | 29.758 | -0,919 | 100 | 0 |
| **B** | **Roots / Aerial Parts** | | **Roots / Fruits** | | **Aerial Parts / Fruits** | |
| **Cultivar Axel** | **$TF_{R/AP}$** | **$R^2_{R/AP}$** | **$TF_{R/F}$** | **$R^2_{R/F}$** | **$TF_{AP/F}$** | **$R^2_{AP/F}$** |
| **As (III)** | 72.320 | 0.999 | 0 | 0 | 0 | 0 |
| **As (III) + Si** | 211.096 | 0.999 | 83.583 | 0.900 | 101.525 | 0.908 |
| **As (V)** | 0 | 0 | 0 | 0 | 0 | 0 |
| **As (V) + Si** | 45.257 | -0.552 | 0 | -0.552 | 0 | 0 |

## SEM/EDX ANALYSIS

**Fig.7 Stem and root sections of cv. Aragon plants treated with As and Si: SEM/EDX acquired images, As distribution dot map and Si distribution dot map.**

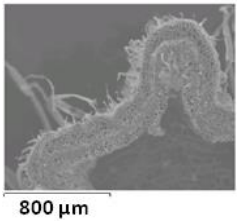
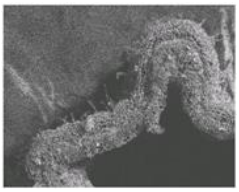
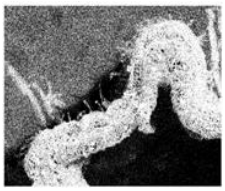
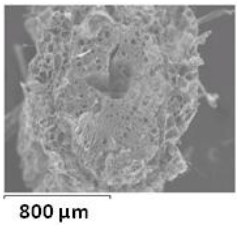
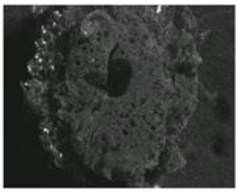
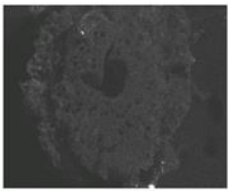
| Aragon | Image | As map | Si map |
|---|---|---|---|
| As + Si Stem |   |  |  |
| As + Si Root |   |  |  |

**Fig.8 Stem and root sections of cv. Frigio plants treated with As both with and without Si supplementation: SEM/EDX acquired images, As distribution dot map and Si distribution dot map.**

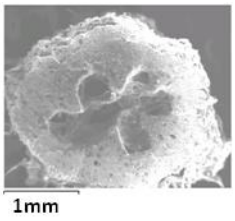
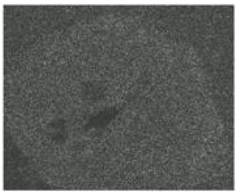
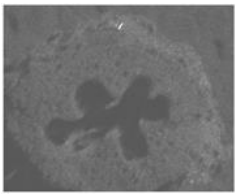
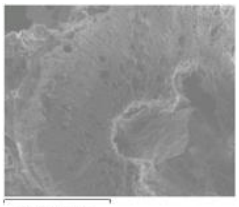
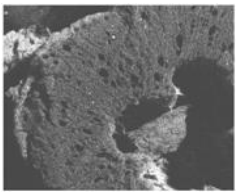
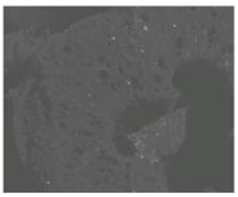
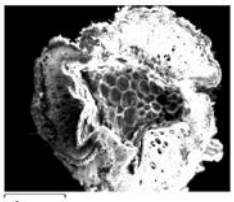
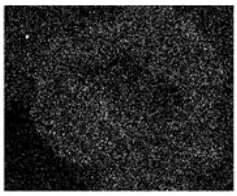
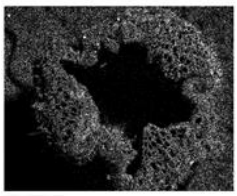
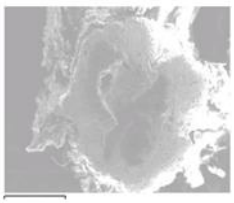
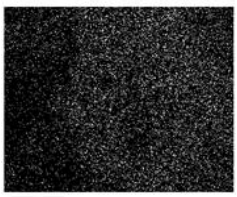
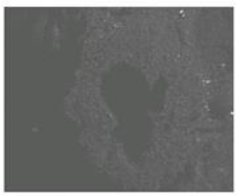
| Frigio | | Image | As map | Si map |
|---|---|---|---|---|
| As | Stem |   |  |  |
| As | Root |   |  |  |
| As + Si | Stem |   |  |  |
| As + Si | Root |   |  |  |

**Fig.9 Stem sections of cv. Gladis plants treated with As: SEM/EDX acquired images, As distribution dot map and Si distribution dot map.**

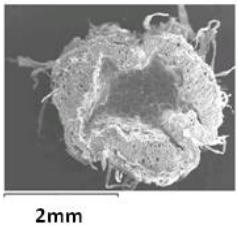
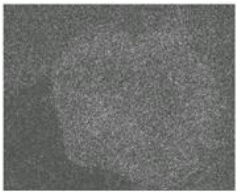
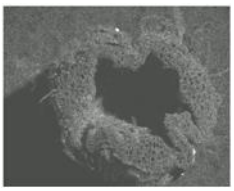
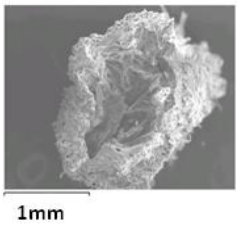
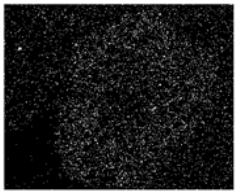
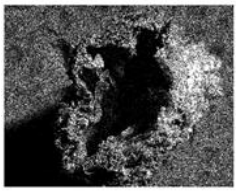
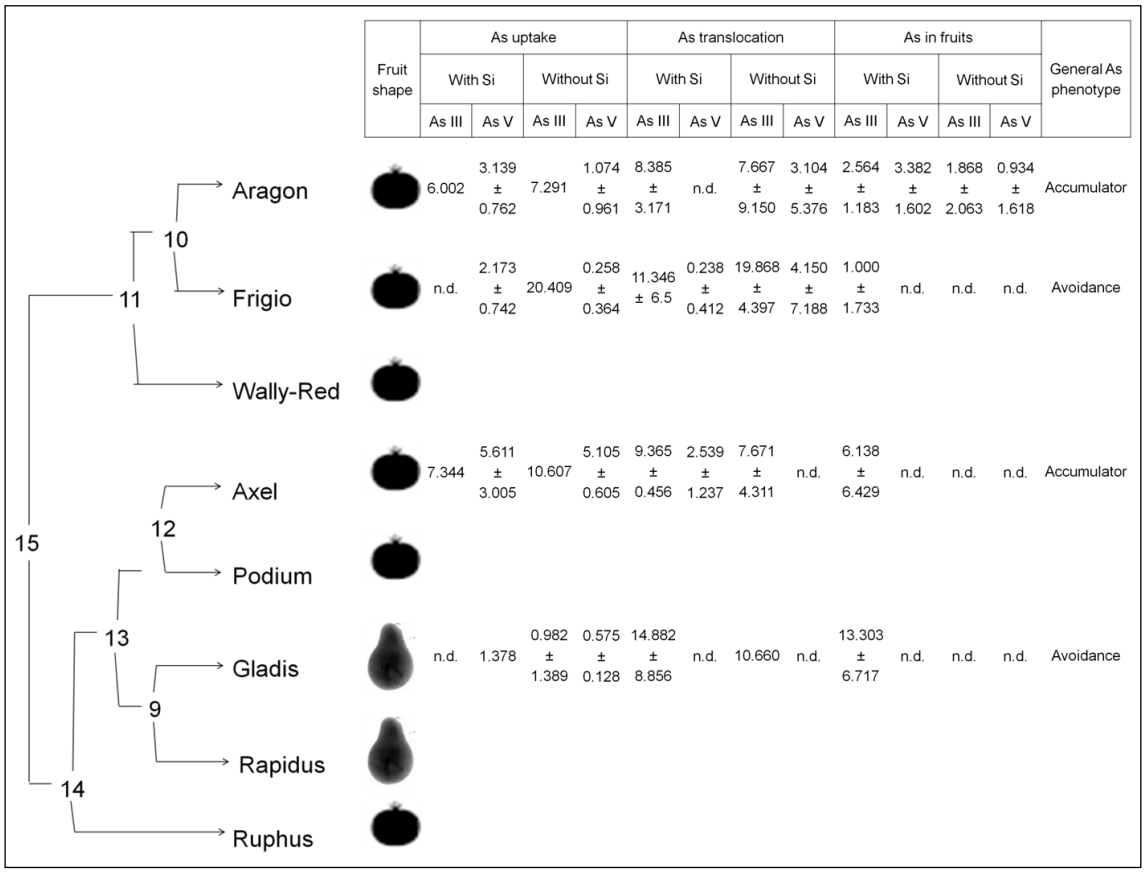
| Gladis | Image | As map | Si map |
|---|---|---|---|
| As Stem |   |  |  |

**Fig.10 Stem sections of cv. Podium plants treated with As: SEM/EDX acquired images, As distribution dot map and Si distribution dot map.**

| Podium | Image | As map | Si map |
|---|---|---|---|
| As Stem |   |  |  |

### 2.3.3 Microsatellyte genotyping

The SSR-based phylogeny of the cultivars suggested that their genetic background was similar. Three of the six round-fruited cultivars were clustered in one clade; of the other three, cvs. Axel and Podium appear to be rather closely related to one another, as did the two plum-fruited cultivars Gladis and Rapidus. The round-fruited cv. Ruphus lay somewhat distant from the rest of the cultivars (Fig.11).




| Cultivar | Fruit shape | As uptake | | | | As translocation | | | | As in fruits | | | | General As phenotype |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | With Si | | Without Si | | With Si | | Without Si | | With Si | | Without Si | | |
| | | As III | As V | As III | As V | As III | As V | As III | As V | As III | As V | As III | As V | |
| Aragon | | 6.002 | 3.139 ± 0.762 | 7.291 | 1.074 ± 0.961 | 8.385 ± 3.171 | n.d. | 7.667 ± 9.150 | 3.104 ± 5.376 | 2.564 ± 1.183 | 3.382 ± 1.602 | 1.868 ± 2.063 | 0.934 ± 1.618 | Accumulator |
| Frigio | | n.d. | 2.173 ± 0.742 | 20.409 | 0.258 ± 0.364 | 11.346 ± 6.5 | 0.238 ± 0.412 | 19.868 ± 4.397 | 4.150 ± 7.188 | 1.000 ± 1.733 | n.d. | n.d. | n.d. | Avoidance |
| Wally-Red | | | | | | | | | | | | | | |
| Axel | | 7.344 | 5.611 ± 3.005 | 10.607 | 5.105 ± 0.605 | 9.365 ± 0.456 | 2.539 ± 1.237 | 7.671 ± 4.311 | n.d. | 6.138 ± 6.429 | n.d. | n.d. | n.d. | Accumulator |
| Podium | | | | | | | | | | | | | | |
| Gladis | | n.d. | 1.378 | 0.982 ± 1.389 | 0.575 ± 0.128 | 14.882 ± 8.856 | n.d. | 10.660 | n.d. | 13.303 ± 6.717 | n.d. | n.d. | n.d. | Avoidance |
| Rapidus | | | | | | | | | | | | | | |
| Ruphus | | | | | | | | | | | | | | |

**Fig.11 Phylogeny of the eight tomato cultivars based on microsatellite alleles. The numbers indicate coefficients of similarity. Fruit shape, the uptake of As in the presence or absence of Si supplementation and the accumulation of As in the fruit are also illustrated.**

## 2.4 Discussion

### 2.4.1 Effects on tomato germination

Although inorganic forms of As are known to be phytotoxic, the presence of 0.2 mM of either arsenite or arsenate had no effect on the in vitro germination of any of the eight tomato cvs. However a higher concentration of arsenate (but not of arsenite) did inhibit germination to a degree. As for the effect of As on seedling growth, arsenite appeared to be more toxic than arsenate (whether or not $CaSiO_3$ was supplied).

The different responses shown at germination and early seedling growth presumably reflects the ways in which the two forms of inorganic As exert their toxicity.

In fact previous studies found that arsenate affects mainly cell wall, primary and secondary metabolism, abscisic acid metabolism and germination of the seedling, while As (III) mainly affects hormonal and signaling process (Sharma, 2012). Arsenite is considered to be more phytotoxic than arsenate (Zhao et al., 2010) but arsenate is an analogue of the macronutrient phosphate and compete with P for uptake in roots and in the cytoplasm where it may replace P in ATP giving rise to an unstable ADP-As (Meharg and Hartley-Whitaker et al., 2001 ). The addition of $CaSiO_3$ had no significant mitigating effect on As toxicity during early seedling growth, but did counteract to a degree the inhibition to germination imposed by the presence of a high concentration of arsenate. Supplementation with soluble Si has been shown to be beneficial for tomato plants subjected to various stresses, such as salinity (Romero-Aranda et al., 2006) and sodic soils with a high boron content (Gunes et al., 2006). Silicic acid fertilization raises the level of fruit set and thereby improves the yield of cherry tomato crops (Toresano-Sánchez et al., 2012). Arsenate is taken up by plants via phosphate transporters but arsenite enters into roots via the nodulin 26 intrinsic proteins (NIPs). Thus Si efflux carrier Lsi2 affects also the As (III) transport to the xylem and As accumulation in shoots (Ma et al., 2008).

### 2.4.2 As uptake and translocation

The set of eight cultivars responded in two distinct ways to As contamination. One group actively excluded As (cvs. Wally-Red, Podium, Rapidus and Ruphus), while the other did not (cvs. Aragon, Axel, Frigio and Gladis). Among the latter group, cvs. Frigio and Gladis exhibited a mild avoidance phenotype, restricting the uptake of As into the root, and translocating it rather inefficiently into the aerial part of the plant. Supplementation with $CaSiO_3$ reduced As uptake and translocation in Gladis, while in Frigio it was only effective when the As was provided as arsenite, since uptake was increased when arsenate was provided. Thus As uptake and translocation are clearly strongly cultivar-dependent, as is the nature of the interaction between As uptake and Si supplementation. This outcome has some significance in the context of food safety, since it is common practice in commercial tomato cropping to plough in residues after harvest (Sainju et al., 2002).

The effect of this practice for cultivars which take up and translocate As is to risk concentrating As in the upper layer of the soil, which should not be allowed to pass the threshold set by the WHO of 20 mg $kg^{-1}$ (WHO website).

The physiological stage during which a plant is exposed to As is an important variable when attempting to characterize its As uptake and translocation (Coddington, 1986). In the more long term pot trial, the four cultivars investigated varied for their tendency to accumulate As in the fruit. The provision of $CaSiO_3$ did not prevent the entry of As into the fruit, in fact in some cases it did the opposite; the treatment reduced As entry into the fruits of cvs. Axel and Gladis, but increased it in cvs. Aragon and Frigio. As a consequence, some caution will need to be exercised in the choice of cultivar in situations where Si fertilization is required to mitigate drought stress (Gunes et al., 2006). In general, arsenite uptake and its translocation into the fruit were higher than for arsenate. No data were derived regarding the prevalent form of As in planta, although the literature suggests that arsenite is the more common whether plants are challenged with arsenite or arsenate (Ali et al., 2009; Burlò et al., 1999; Zhao et al., 2010). In fact arsenate is ready transformed in arsenite after uptake by a process of reduction mediated by GSH and GST (glutathione S-transferase). This process generates ROS (reactive Oxygen Species) together whit electron leakage (Sharma, 2012). The most heavily contaminated fruit were produced by cv. Gladis, reaching a concentration of 13.3 µg $g^{-1}$ As, measured on a dry weight basis (therefore about 0.13 µg $g^{-1}$ fresh weight). A weekly consumption of 1 kg of fresh tomato containing this level of As would therefore involve the intake of about 130 µg As, equivalent to about 2 µg $kg^{-1}$ body weight of an average adult. Against this level, current international guidelines indicate a tolerable weekly intake of 15 µg As $kg^{-1}$ body weight (WHO website).

# 3. Effect of biochar addition to an arsenic contaminated soil on arsenic concentration in pore water and uptake to tomato plants

## 3.1 Introduction

### 3.1.1 What is biochar

In general biochar is defined as a solid material obtained from the anoxic combustion of biomass (NCBI website). This process is called pyrolysis. The type of biomass utilized as starting material could vary widely including wood or crop residues, animal manures, and other plant residues like leaves or green urban wastes. The combustion of these materials is conducted with limited or null oxygen supply and relatively low temperatures (<700°C). In this combustion process not only biochar is produced but also other important substances like Bio-oil or Syngas that can be utilized for energy production. Different type of temperatures and oxygen supply rate could be chosen for the combustion, that is named pyrolysis, in order to favor one or other final product; for instance at the lower temperatures it could be obtained more syngas or bio-oil while at temperatures more than 400°C it could be obtained more biochar than the other products. In fact pyrolysis involves different chemical reactions and the overall process could be divided into four general phases: moisture removal, hemicellulose decomposition, cellulose decomposition, and lignin decomposition. Each of these phases requires a specific temperature range and produces a different mix of products. The question as to what biochar actually is from a chemical point of view is much more difficult to answer due to the wide variety of biomass and charring conditions used (Lehmann and Joseph, 2009). The defining property is that the organic portion of biochar has a high C content, which mainly comprises so-called aromatic compounds characterized by rings of six C atoms linked together without Oxygen or Hydrogen (Lehmann and Joseph, 2009). The most important use of biochar is the application to cultivated soil in order to improve soil properties and soil productivity, carbon storage and the filtration of percolating soil water. This particular use has a very old tradition in some states of Europe but also in U.S.A. and Asia (China in particular) but the interest on biochar characteristics and properties only began in the past few years. The basis for this strong recent interest is mainly correlated to two aspects; the first is the discovery that biochar-type substances are the explanation for high amounts of organic C and sustained fertility in Amazonian Dark Earths locally known as Terra Preta de Indio (Lehmann and Joseph, 2009).

The second aspect is that, over the past five years, unequivocal proofs has become available showing that biochar is not only more stable than any other amendment to soil, and that it increases nutrient availability beyond a fertilizer effect, but that these basic properties of stability and capacity to hold nutrients are fundamentally more effective than those of other organic matter in soil (Lehmann and Joseph, 2009). This means that biochar is not merely another type of compost or manure that improves soil properties, but is much more efficient at enhancing soil quality than any other organic soil amendment and this ability is rooted in specific chemical and physical properties, such as the high charge density, that results in much greater nutrient retention, and its particulate nature in combination with a specific chemical structure, that provides much greater resistance to microbial decay than other soil organic matter (Lehmann and Joseph, 2009). Another advantage correlated to biochar production and use includes a general improvement in the management of wastes because the biomass utilized for biochar production could include animal and crop wastes or green urban wastes. Furthermore appropriate management of wastes can improve indirectly the mitigation of climate change thanks to the decreased methane emissions from landfill, reduced industrial energy use and emissions for recycling and waste reduction, recovering energy from waste, enhanced C sequestration in forests due to the decreased demand of virgin paper and decreased energy use in long-distance transport of wastes (Lehmann and Joseph, 2009). The use of biochar, especially for agricultural aims, requires some attentions and a proper evaluation of biochar characteristics must be performed, in particular when the starting material is composed by wastes. In fact wastes could contain some xenobiotics or heavy metals that can be transferred to soil after biochar application, while pathogens contained in wastes do not constitute a risk because they are eliminated by pyrolysis temperatures. Furthermore biochar application on contaminated soils could influence significantly contaminants bioavailability and mobility and thus there can be a correlated environmental risk. In general biochar has the capacity to complex metal ions on its surface and therefore reduce bioavailability, but trough these mechanism also essential plant nutrients could be immobilized. Adding biochar increased the rate at which the soil solution came to equilibrium (sorption-desorption hysteresis) (Uchimiya et al., 2011) which may increase the rate of sorption of any further contaminants added to the soil matrix; but specific soil parameters influence complexation and competition between elements and consequences of biochar addition to soil chemistry (Beesley et al., 2011).

Concerning arsenic, it is demonstrated the efficacy of biochar on arsenic removal from wastewater (Mohan and Pittman, 2007), but there are some concerns regard its application to arsenic contaminated soils, especially correlated with the possibility of the increase in soil pH an soluble C (Hartley et al., 2010; Beesley and Dickinson, 2011). This last aspect evidenced the importance of considering soil parameters such as pH and C content when considering biochar application on arsenic contaminated soil because mobilization and co-mobilization could be respective consequences of increased pH and soluble C. Beesley and Dickinson (2011) found an increased concentration of arsenic in the pore water of a moderately contaminated urban soil after the amendment with 30% (V/V) of hardwood derived biochar, whilst Beesley and Marmiroli (2011) found a negligible biochar influence on arsenic concentrations in contaminated effluent leached from a multi-element polluted soil. An increase in phosphate- extractable As after biochar addition was observed by Namgay et al. (2010) while it was found by Hartley et al. (2009) that the increase in arsenic pore water could not be translated to an increased foliar concentration of the contaminant. It could be concluded that the effect of biochar on arsenic mobility and availability, and consequently the effect on arsenic uptake and translocation in plants, could be quite different depending on soil characteristics and plant species. Biochar effect on arsenic uptake and translocation in tomato has not yet been investigated; thus it was decided to try to elucidate some aspects of this topic. For this aim one of the tomato cultivar utilized in the previous part of the project was grown in a highly arsenic contaminated soil in the presence or absence of biochar and after that, arsenic concentration in plant tissues and in the pore water of the growth medium were determined as described below.

## 3.2 Materials and methods

Plant material for this part of experiments includes one cultivar, previously utilized in the Si-As experiments, namely Aragon.

### 3.2.1 Germination tests

A simple in-vitro germination assay was performed where 15 seeds were placed onto Petri dishes containing agar, Murashige–Skoog (MS) medium (Murashige and Skoog, 1962) and biochar (1%, 30% and 50% biochar by weight). Controls of 100% biochar and 100% agar were included for comparison. Environmental conditions were identical to those detailed in the Si-As germinations tests. The percentage of the 15 seeds whose shoot elongation was >1 cm after 5 days were classified as germinated.

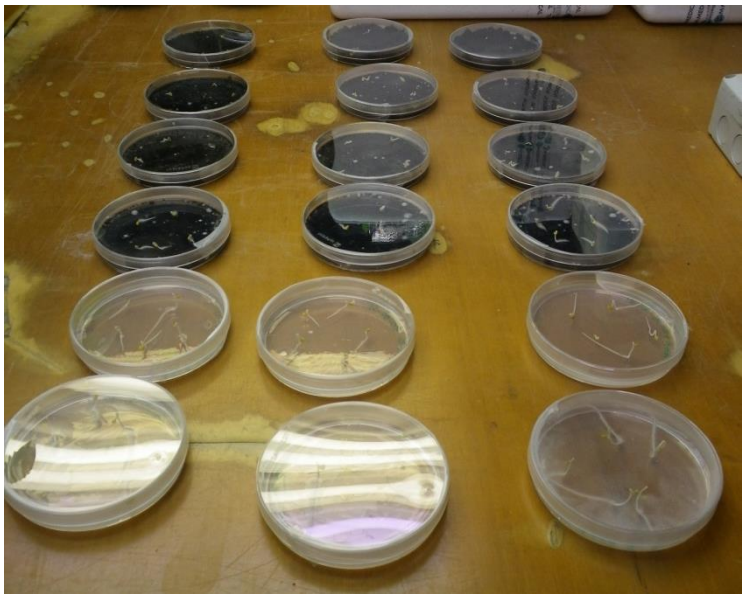


**Fig.1 Examples of tomato seeds grown in Petri dishes with MS or MS + different concentrations of biochar utilized for these experiments.**

### 3.2.2 Growth substrates

Contaminated soil (S) for this investigation was collected from non-vegetated soil within a woody and shrubby area of land impacted by mining activities at Mina Mónica (Madrid, Spain); Lat/Long: 40° 52' 07.06″ N3°43'48.87″ W. From previous studies (Moreno-Jiménez et al., 2011) this soil was known to have high concentrations of As in pseudo-total (≤3003 mg $kg^{-1}$) and pore water (≤2901 $\mu gL^{-1}$) but support spontaneous vegetation cover, so it was selected for this study in preference to the artificial spiking of a non-contaminated soil. Soil pH was ~5 and organic matter content was <2%. Biochar (BC) was produced in a pyrolysis plant in Bagnacavallo (Ravenna, Italy) using orchard prune residues combusted at 500°C and had an organic matter content of ~50% (Fellet et al., 2011). The pH of biochar was determined in a soil/water ratio 1:20 (w/v) following Cheng and Lehmann (2009) after centrifuging at 10,000 rpm for 20 min and was ~pH 10. Contaminated soil (S) was mixed with biochar (BC; 30% vol.) by hand, using an end-over-end method in a sealed drum until a visually homogenous incorporation was achieved. The mixture was left to equilibrate for 48 h in the dark and then placed into triplicated 1 L pots, saturated and allowed to drain down for a further 48 h to reach an approximate field moisture capacity. One of these mixtures received the addition of 10 mL of liquid fertiliser per pot (N, P, K ratio 8:4:6, Cifo s.p.a., Bologna, Italy) applied every two days (S+BC+F) throughout the experiment, whilst the other did not (S+BC). A further treatment, of the mixture without plants was included to give a no-plant reference treatment (S+BC-P).

Further reference replicates of a non-contaminated soil (RS) and biochar without soil (BC) were included for comparison. In total there were 18 pots, giving 6 triplicated treatments, S, S+BC, S+BC+F, S+BC-P, RS and BC.

| Treatments | Symbol |
|---|---|
| Clean soil | RS |
| Contaminated soil | S |
| Biochar 100% | BC |
| Mixed soil | S+BC |
| Mixed soil fertilized | S+BC+F |
| Mixed soil without plants | S+BC-P |

**Tab.1 Summary of the treatments performed in this experiment.**

### 3.2.3 Plant material and pore water analysis

Tomato plantlets (*Solanum lycopersicum* L.), cultivar Aragon, germinated and grown for 4 weeks in separate pots (250 mL) of reference soil (RS) in a controlled environment of 23 °C, 33% relative humidity and a 16 h per day photoperiod, were transplanted to each of the 1 L treatment pots described previously . Thereafter plants were maintained in the same ambient conditions and watered (tap water) with 50 mL per pot daily. Leachate was allowed to drain away to maintain oxic conditions within the pots. During the subsequent growth and maturation period the stems were lightly bound to inserted stays to prevent damage or breakage. Plants were maintained in these conditions until fruiting of the reference (RS) which was deemed to be the conditions most likely to have promoted the latest fruiting (lack of nutrient deficiency, negligible toxicity, minimal stress etc.). Into each of the 18 pots, one rhizon pore water sampler (Eijkelkamp Agrisearch Equipment, The Netherlands) was carefully inserted following a pre-bored pilot hole. Samples were filtered in-situ by means of an incorporated 0.45 μm PTFE membrane. As these devices are designed to sample pore water from the rhizosphere zone of soils, they were inserted at 45° in the pots to traverse the rooting one and to ensure that only the applied vacuum, rather than gravity, evacuated the samplers. The relatively small volume of the pots used in the experiment meant that the whole soil mass was occupied by roots and the rhizon samplers would be within the rhizosphere at all times.

One, 2 and 4 weeks hence, pore water samples were collected from all 18 pots using removable syringes connected via a luer-lock system to the samplers. Immediately following their collection the pH of the sample was measured. Evacuated sample volumes ranged from 1 to 10 ml and were briefly refrigerated (5 °C) before analysis by ICP-OES (SPECTRO CirOS Vision EOP, SPECTRO Analytical Instruments GmbH KG, Kleve, Germany). A certified reference material (ERM-CC020, JRCIRMM, Belgium) was used to maintain quality control.

**Fig.2 Experimental set-up shortly after transplantation of tomato plantlets showing rhizon samplers inserted into pots and, inset, and collection of pore water in progress.**

### 3.2.4 Chemical analysis

Soils and plants were destructively sampled following the experimental period described previously. Fruits were removed and retained for As analysis and soil and root mass was separated from the pots and first shaken, to remove strongly adhered soil from roots, before thorough double washing with de-ionised water. Roots were separated from the aerial parts and fresh biomass determined before being dried (60 °C) and re-weighed for dry biomass. Soils were dried at 40–60 °C and sieved to 2 mm prior to analysis. Soils, roots and stem/leaves were digested in 20 ml of $HNO_3$ (65%) for 2 h at 120 °C in tubes positioned in a heating block (DK20, VELP Scientifica, Milan, Italy), the remaining digestate was collected and transferred to falcon tubes with deionised water addition to reach 10 ml and analysed by ICP-OES, alongside certified reference materials (ERM-CD281 and BRC-402, JRC-IRMM, Belgium).

A sample of dried and milled tomato fruit (0.2 g) was left in 10 ml 1% $HNO_3$ overnight. The following day 3 mL $HNO_3$ (65%) and 1 mL $H_2O_2$ (33%) were added to 6 mL of the 1% $HNO_3$ extract and a microwave assisted extraction was performed (0–200 °C over 15 min, hold for 5min, 200–210 °C over 15 min, hold for 5 min, 210–220 °C over 15 min and hold for 40 min). Samples were filtered (Filterlab 1238) and made up to 15 mL. Arsenic was determined by HG-AFS (PS Analytical 10.055, Millennium Excalibur system). Only a sufficient mass of fruit was obtained for analysis from the treatment of soil plus biochar (S+BC) to provide replicated results, whilst other treatments provided only enough fruit for one analysis (S+BC+F), or no analyses (S).

### 3.2.5 Statistical analysis

For the germination/seedling growth experiment, statistically significant differences in germination between the non-treated and treated samples were inferred from a conventional analysis of variance, applying the Bonferroni post hoc test applied to the number of seeds germinated per dish. For the analysis of seedling shoot elongation, the Student's two tail t-test was applied. In the pot trial Experiment #1, the HG-AAS data were also analysed using the Student's two tail t-test, while in the Experiment #2, the data were subjected to the non-parametric Kruskall-Wallis test, since the data set was not normally distributed. For biochar analysis differences between soil treatments were analysed by ANOVA using Tukey's test for means comparison (Pb0.05). Data were checked for normality and homogeneity of variances prior to statistical analysis, if necessary data were log transformed. The software package SPSS v19 (IBM website) was used for all statistical procedures.

## 3.3 Results and discussion

### 3.3.1 Effects on pore water concentrations

Although soil pseudo-total arsenic concentration was not significantly influenced by biochar addition (Tab.2), within one week from application, biochar significantly increased As concentration in pore water above that of the control (S=~430 $\mu gL^{-1}$) ~5 fold with plants (S+BC=~2000 $\mu gL^{-1}$ and S+BC+F=~2200 $\mu gL^{-1}$) and~9 fold without plants (S+BC-P=~3500 $\mu gL^{-1}$; Fig. 3). The magnitude of change varied with time with a mean reduction in As concentration between the first week and weeks 2 and 4 (Fig. 3). In the soil and biochar references (RS and BC) concentrations of As in pore water were generally <100 $\mu gL^{-1}$, which is at the upper end of the range reported by Moreno-Jimenez et al. (2011) in a survey of field pore water sampling of various contaminated and non-contaminated soils.

Circumneutral pH was measured for both reference and contaminated soil pore water alike in the present study but in the case of all additions of biochar to soil, pore water pH was significantly (Fig. 4; $P<0.05$) increased above that of the control contaminated and clean soils. In the case of the biochar reference (BC) pore water pH was ~1 unit above that of any other control or treatment (Fig. 4), probably due to the high pH of the biochar itself. These results are in general agreement with previous studies that found variable magnitudes of As solubilisation after biochar addition to soils, one of the most recent reporting a sharp increase in As concentration in pore water (Zheng et al., 2012), citing mechanisms such as a pH increase and competition of As for binding sites with P as explanations. Phosphorous concentrations in pore water in the present study were more than 14 times greater in the biochar reference (BC) than in the contaminated soil (Table 3); P is an analogue of As, so its addition to soils can displace retained As (Moreno-Jiménez et al., 2012) which may explain the apparent mobilization of As by biochar. Biochar's physical structure may also influence the behavior of As. Macro, micro and nanoporous structures throughout biochar's matrix could harbor conditions aiding the reduction of Fe (Lin et al., 2012) and Mn species (Joseph et al., 2010), both indicating negative redox potential. In such reduced conditions As would be adsorbed less readily to soils, and would be more mobile (Moreno-Jiménez et al., 2012), explaining an increase in its concentration in pore water after biochar addition. Pore water Fe and Mn concentrations displayed rather an opposing trend in the present study; compared to the control (S), Fe concentrations were increased, up to ~20 fold by the addition of biochar, whereas Mn concentrations were reduced to the same magnitude (S+BC; Table 3). Generally, under reducing conditions, Fe and Mn oxides are solubilized (Moreno-Jimenez et al., 2012), so their concentrations would both be expected to increase in pore water if redox potential became negative. In the present study, similarly to P, pore water concentrations of Fe from biochar alone (BC) were greater than those from soil (S), whereas the opposite was true for Mn (Table 3). This tend suggests that biochar was a source of Fe in solution, rather than its increased concentration in pore water after biochar addition to soils being a result of reducing conditions. In a study where field-aged biochar was recovered from a maize planted ferrosol (iron-rich soil), Lin et al. (2012) measured increased biochar surface contents of Fe suggesting some biochars could retain Fe from soils. The effects may be soil and biochar specific, and also a function of biochar ageing in soils.

| Treatment | Soil | Roots | Shoots | Fruits (µg $kg^{-1}$) |
|---|---|---|---|---|
| S | 6230 (429) a | 2040 (685) b | 8.63 (1.3) c | NM[2] |
| S + BC | 5759 (1076) a | 662 (80) a | 1.69 (0.08) a | 2.5 (0.6) |
| S + BC + F | 5521 (1247) a | 1534 (583) b | 4.3 (1.5) b | 1.19 (NR) |
| S + BC – P | 6585 (981) a | NA | NA | NA |
| RS | 8.8 (0.9) | 14.1 (4) | 3.01 (0.9) | NM[1] |
| BC | ND | 1.9 (0.08) | 0.11 (0.09) | NM[1] |

**Tab.2 Pseudo-total concentration (mg $kg^{-1}$) of arsenic in soil, roots, shoots, fruits (µg $kg^{-1}$) in S=contaminated soil; S + BC= mix; S + BC + F= mix plus NPK fertilizer; S + BC –P=mix withouth plants; RS= reference soil; BC= biochar only (mean n=3 ± s.e.m.). different letters correspond to significant differences between tratments (p<0-05). NA= not applicable; ND= below the detection limit; NM[1]= not determined; NM[2]= not determined due to insufficient fruit mass; NR= no replicates.**

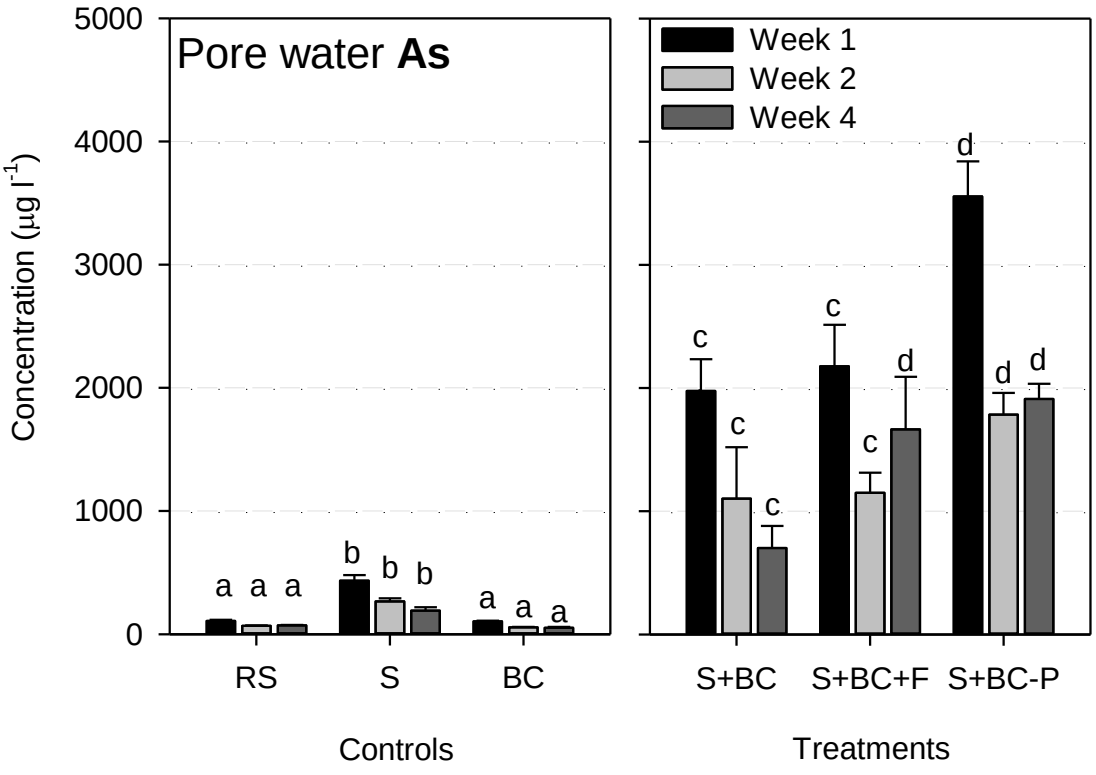



**Figure 3. Concentrations of arsenic in pore water from S= Contaminated soil, treatments (S+BC=Mix; S+BC+F=Mix plus NPK fertiliser) and references (RS= Reference soil; BC=Biochar only; S+BC-P= mix without plants) sampled at successive periods during 1 month (mean n=3, ± S.E). Different letters correspond to significant differences between treatments for each sampling time (*P < 0.05*).**

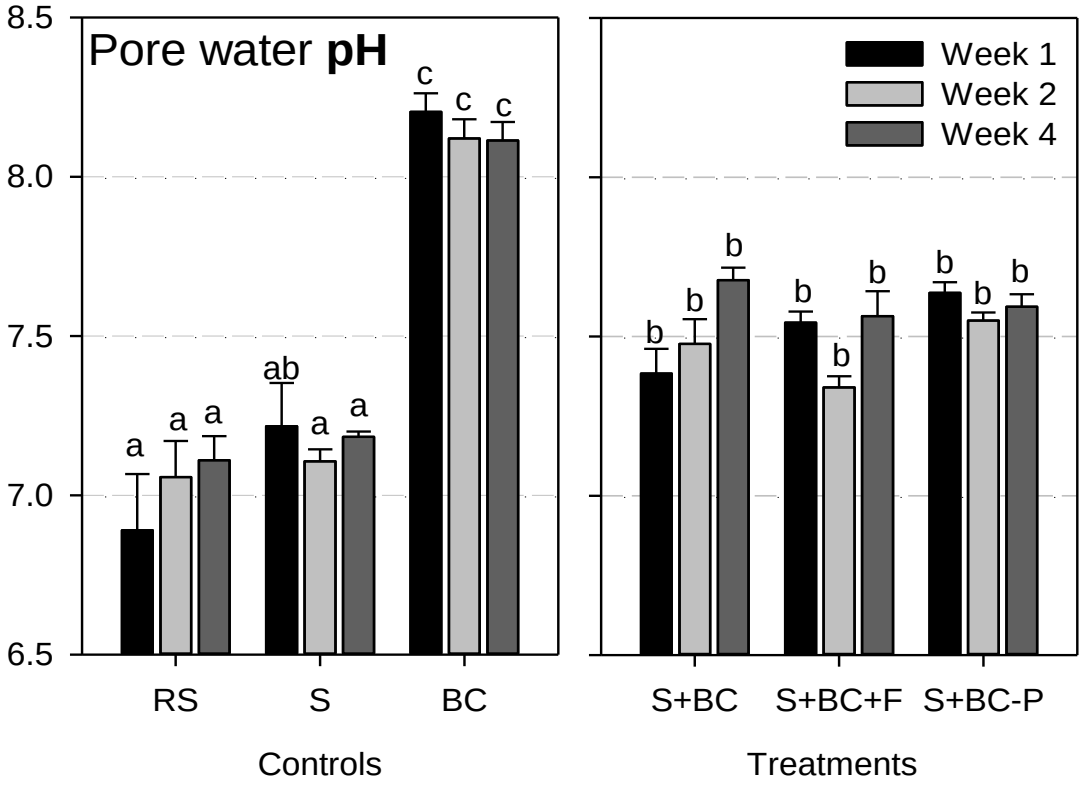



**Figure 4. Pore water pH of S= Contaminated soil, treatments (S+BC=Mix; S+BC+F=Mix plus N,P,K fertiliser) and references (RS= Reference soil; BC=Biochar only; S+BC-P= mix without plants) sampled at successive periods during 1 month (mean n=3, ± S.E). Different letters correspond to significant differences between treatments for each sampling time (*P < 0.05*).**

| Treatment | Fe | Mn | P |
|---|---|---|---|
| S | 27 (4) | 128 (79) | 1744 (464) |
| S + BC | 613 (185) | 6.1 (1.9) | 2325 (300) |
| S + BC + F | 280 (422) | 51 (23) | 3854 (422) |
| S + BC – P | 4.7 (0.2) | 16 (5.4) | 2362 (290) |
| RS | 1194 (192) | 61 (13) | 28000 (7405) |
| BC | 3261 (21) | 18 (2.5) | 23557 (2910) |

**Table 3. Pore water total concentrations (µg $l^{-1}$) of Fe, Mn and P at week 1 in S= Contaminated soil, treatments (S+BC=Mix; S+BC+F=Mix plus NPK fertiliser) and references (RS= Reference soil; S+BC-P= mix without plants) (mean n = 3; ± s.e.m).**

### 3.3.2 General plant health and germination success

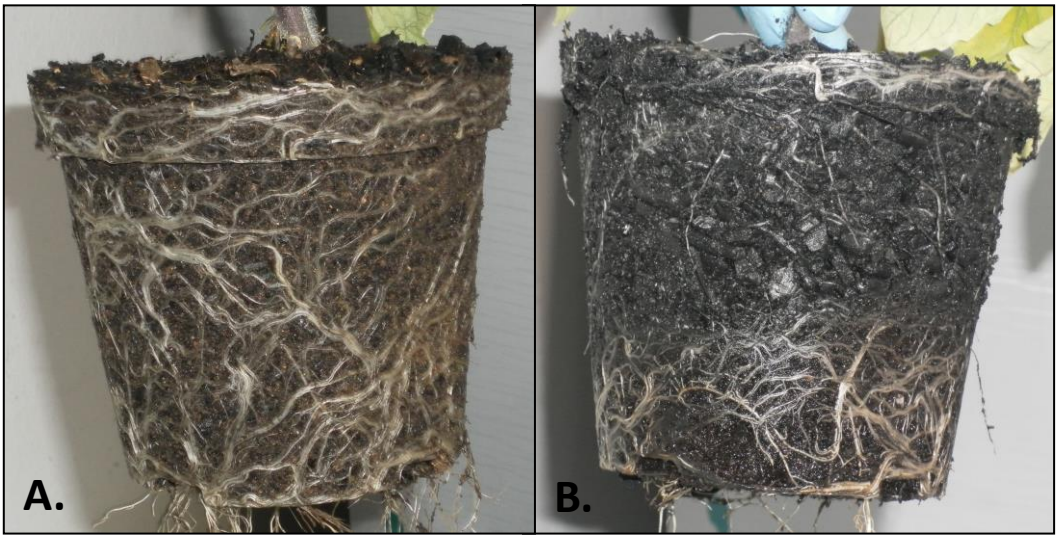



**Figure 5. Demonstration of rooting preferences between soil only (A.) and soil with an upper amendment of biochar (B.).**

A significantly lower fresh and dry biomass was measured in plants grown only in biochar (BC), even compared to the contaminated soil (S) (Table 4). Fertilization after mixing soil and biochar was able to significantly increase biomass yields compared to the control (Table 4) but according to the germination test, higher proportions of biochar added to nutrient (MS) medium adversely affected germination (0, 1, 30, 50 and 100% biochar equalled 100, 80, 60, 60 and 0% germination respectively, with the agar only control showing 100% germination). Solaiman et al. (2012) observed mixed factoral effects of rate of application and biochar type on germination and root length of wheat (*Triticum aestivum* L.) in a petri-dish investigation. However, poor biomass and germination combined in the present study suggest that this biochar is lacking in a balanced provision of nutrient capital. Other authors have noted either no significant increases in biomass of plants grown in biochar amended soils (Namgay et al., 2010), variously significant and non-significant increases depending on species (Gartler et al., in press) significant increases only after the addition of fertilizer together with biochar (Van Zwieten et al., 2010) or further increases in biomass following subsequent fertilization of biochar amended soils (Hossain et al., 2010). There have been previous suggestions that in non-nutrient limiting conditions, reduced phyto-toxic metal concentrations have promoted seed germination in biochar amended contaminated soil (Beesley et al., 2011) and reduced metal stress in plants (Buss et al., 2012). Given that arsenic is not essential for plants (Fitz and Wenzel, 2002) the balance of mineral fortification versus toxicity need not be sought as plant health is not affected by reducing As uptake, as is

the case for some metals. But it must be noted that, in the present study, the inhibitory effects of As on plant growth should also be accounted for. For example, chlorosis was evident by a yellowing of some leaf tips; in leaves chlorophyll absorbance was lower in soil with biochar than control soil, and lowest in plants grown in only biochar (data not shown), perhaps reflecting low nutrient status of biochar. Cell respiration however was higher in biochar amended soil and similarly low in control soil and biochar only (data not shown) indicating that plant functionality was not inhibited by biochar; these results suggest that nutrients are the master limiting factor after biochar addition to soils.

| Treatment | Fresh | Dry |
|---|---|---|
| S | 85 (2.3) b | 11.6 (0.4) b |
| S+BC | 80 (4.4) b | 11.2 (0.5) b |
| S+BC+F | 122 (5.6) c | 12.2 (1.5) bc |
| RS | 138 (4.4) c | 14.6 (1.6) c |
| BC | 24 (11.5) a | 1.9 (1.0) a |

**Table 4. Fresh and dry biomass (g) of tomato plants grown in S= Contaminated soil, treatments (S+BC=Mix; S+BC+F=Mix plus NPK fertilizer) and references (RS= Reference soil; BC=Biochar only) (mean n=3, ± S.E). Different letters correspond to significant differences between treatments (*P < 0.05*).**

| Treatment | % germination |
|---|---|
| Agar only | 100 |
| Biochar only | 0 |
| **MS+0% biochar** | **100** |
| **MS+1% biochar** | **80** |
| **MS+30% biochar** | **60** |
| **MS+50% biochar** | **60** |

**Table 5. Percentage of tomato seeds with root elongation > 1cm (classified germinated), according to proportion of biochar added to nutrient solution (MS).**

### 3.3.3 Roots, shoots and fruits arsenic concentrations

Biochar universally reduced concentrations of As in tomato plant organs and tissues compared to the control of contaminated soil; for fruits there was insufficient mass to analyze those of the control (S) but those of the mixtures had very low As concentrations (<3 $\mu g\ kg^{-1}$).

In normal daily dietary intake these concentrations would not lead to an excessive dose of As so a human health risk cannot be implied; World Health Organisation (WHO, 2010) suggest a tolerable daily As intake level of 3 μg per kg body weight. Root As concentrations in the none-fertilized mixture (S+BC) were reduced by ~68% and the fertilized mixture (S+BC+F) by>25%; the latter was none-significant compared to the control (Table 2). In the case of shoot concentrations, As was reduced by 80% and 50% (S+BC and S+BC+F respectively) compared to the control, both of which constituted a statistically significant decrease (Table 2). Arsenic is generally largely retained at root level, as found in several field crops grown in arsenic- and metal-contaminated pyrite wastes, showing very minimal translocation to the shoot (~4%) (Vamerali et al., 2011). Compartmentalization of As in the roots of tomato has been identified in As (III) spiked soils as root cell damage above toxic As thresholds can reduce transport of As upwards in the plant (Carbonell-Barrachina et al., 1997). This could explain why relative root-shoot As translocation was greater in the reference, non-contaminated soil than the contaminated soil in the present study, related to a lack of soil toxicity and plant perturbation in this clean soil (Table 2). Interestingly, although As concentration of biochar itself was below the detection limits both root and shoot concentrations were detectable (Table 2), possibly due to the poor retention of any inherent As by biochar during leaching and its subsequent transfer to plant parts. Fertilization by the addition of P may exclude As from plant uptake and favor P, as As and P compete for binding sites on soils (Moreno-Jiménez et al., 2012), after which a reduced As uptake to plants could be expected. However biochar can also be a source of, or enhance available P in soils (Cui et al., 2011; Parvage et al., 2013) which may mean that, when added to soils, As is mobilized. This may change with time as P has been associated with the ash content in biochar (Wang et al., 2012) and may be leached out in acid conditions leaving sites for As retention available on biochar surfaces. Such mechanisms could explain the reduced As concentration in pore water between the initial sampling and subsequent ones (Fig. 3), although several other mechanisms could be at play, such as dissolved organic carbon (DOC)-arsenic co-mobilization (Beesley et al., 2011). As discussed previously, the increase in Fe concentration in pore water following biochar addition is unlikely to be related to reducing conditions. However, in biochar–plant systems, roots could enter and block biochar pores as plants grow, stimulating reducing conditions and subsequently enhanced mobility of elements either solute from soil or biochar itself; root hairs can enter water-filled macropores of biochar and bond to surfaces (Joseph et al., 2010). Given the relatively low

eventual soil to root mass ratio in the present experiment (pots were only 1 L), compared to a large scale field plot for example, this is hypothetically a distinct possibility. This may be supported by the rather low Fe concentration in pore water from the un-planted mixture (S+BC-P; Table 3), suggesting fully aerobic conditions, yet concentrations of As in pore water from this treatment were the highest recorded (Fig. 3), somewhat immediately disproving the theory that plant roots induced reduction here. The great difference between Fe in pore water in the planted and non-planted mixtures, assuming oxic conditions, may therefore indicate that iron plaques have formed in the rhizosphere in an attempt to exclude soluble As from the plant (Moreno-Jiménez et al., 2012); peak pore water Fe concentration in the mixtures (S+BC; Table 3) corresponded with the lowest root As concentration (Table 2). Zheng et al. (2012) noted significant increases in Fe plaque formation on rice roots following a 5% biochar addition, but evidence in other species is lacking to confirm this effect. In general theories related to whether plant roots and biochar interact assume roots attach to biochar but plants may give preference to nutrient richer soil, even if contaminated, and actively avoid nutrient poor biochar. To investigate this we conducted a simple experiment for illustrative purposes only as follows. Tomato plantlets were transplanted into pots as described in the Materials and methods section, of only reference non-contaminated soil (1 L; RS) and the same soil with biochar. In this case, the lower 0.5 L of the pot contained the soil and the upper 0.5 L contained only the biochar. These were maintained in identical ambient conditions to those described above. Upon sampling and removal from the pots it is clear to see that plant roots avoided biochar and massed around the bulk soil (Fig. 5B), in contrast to the soil only (Fig. 5A). The interactions of plant roots with biochar and the subsequent effects on element mobility and availability require onward investigation and elucidation.

### 3.4 Conclusion

Clearly, in the soil–biochar–plant system presented here high concentrations of As were mobilized from soil by biochar; since plant uptake of As was reduced however, and fruit As accumulation low, it can be supposed that the main risks of biochar to land application surround leaching of this metalloid to proximal waters. The soil used here was an acidic mine soil, so the aim of biochar addition in the field would be towards liming, restoration and phyto-remediation. In this respect the results are mixed.

The advantages are lower plant As uptake which is safer, especially for widespread application in case of food chain transfer, whilst insignificant biomass changes are less important than in agricultural contexts. However the substantial increases in As in pore water indicate that a degree of planning is required before applying biochar to catchment areas. Furthermore, as balanced nutrient capital of biochar is uncertain, and success of remediation from seed is questionable, then the quantity, quality and complementarity of biochar, together with additional inputs (fertilizers, composts etc.) would need to be prior evaluated.

# 4. Expression analysis with Real-Time PCR of some genes involved in As stress response

## 4.1 Introduction

### 4.1.1 Arsenic stress response

The Arsenic stress response has been widely studied and understood in microorganism but little is know up to now in plants where it remains an undiscovered field, especially regarding the specific mechanism implicated in the stress response induced by this metalloid and not in the generic stress response of the plant. From the few studies available for plants it was found that the major mechanisms involved in arsenic stress response are: reduction of metalloid uptake, defense mechanisms against oxidative stress damages, metalloid chelation, compartmentalization and repair of stress damaged proteins (Panda et al., 2010; Clemens, 2001; Clemens, 2006). Arsenic could be an important oxidative stress agent, thus one of the most important group of proteins involved in arsenic stress response are those involved in the protection against ROS (Reactive Oxygen Species) and free radicals damages like Glutathione Reductase and Glutathione Peroxidase. Other important members of this group are Heat Shock Proteins (Hsp) that are the first discovered group of stress proteins. As described before in the general introduction arsenic could also be chelated by different molecules in order to translocate and compartmentalize it in the vacuole or other storage sites (e.g. lignin), and this represents an important mechanism for detoxification in plants. Arsenic chelating molecules belonging to the metallothioneins and phytochelatins families.

#### 4.1.1.1 Glutathione Reductases

Glutathione is a molecule that has some important roles in the As detoxification mechanisms, including the protection against oxidative stress through the maintenance of the redox conditions of the cell, and the synthesis of phytochelatins (other important molecules implicated in As stress response, as described well before). In oxidative stress conditions, reduced glutathione (GSH) is converted to oxidized glutathione (GSSG) and glutathione biosynthesis is stimulated. In fact arsenate, once inside the cell, is frequently reduced to arsenite, thanks to an arsenate reductase and this is indicated as a detoxification mechanism because arsenite can bind with phytochelatins; but arsenate reduction is accompanied with NADPH oxidation through the reduction of GSSG and the production of GSH that represent the electron donor for the arsenate reductase. Furthermore GSH has the important capacity to bind ROS and thus protect the cell against the oxidative stress damage.

Glutathione reductase is a key enzyme in the oxidative stress response mechanism because it could maintain glutathione levels necessary for the most important functions in the cell; it is localized in mitochondria, cytosol and plastids. Glutathione reductase (GR) activities increase as the glutathione pool increase through a multi-level control mechanism, which includes coordinate activation of genes encoding glutathione biosynthetic enzymes and GR (Xiang and Oliver, 1998) . Shri et al. (2009) founded that arsenic induce oxidative stress and thus an elevated requirement of glutathione (GSH) in rice seedling that is executed by the stimulation of GR. Some studies conducted on arsenic stress response evidenced that glutathione reductase expression could occur at different rate in the different parts of the plant with some species with an enhanced GR activity in roots (*Pteris vittata, Pteris ensiformis, Nephrolepsis exaltata*), while an enhanced activity in fronds and rhizomes were observed in *Pteris ensiformis* and *Nephrolepsis exaltata* but not in *Pteris vittata* (Srivastava et al., 2005).

#### 4.1.1.2 Heat shock proteins

Heat Shock Proteins (Hsps) represent an ubiquitous group of proteins that copes with stress-induced denaturation of other proteins. Their function in response to different kind of biotic and abiotic stress has been well studied in eukaryote as in prokaryote organisms and now Hsps action at the molecular and cellular level is becoming well understood. In particular previous studies evidenced that: (i) expression of Hsps can occur in nature, (ii) all species have Hsp genes but they vary in the patterns of their expression, (iii) Hsp expression can be correlated with resistance to stress, and (iiii) specie's thresholds for Hsp expression are correlated with levels of stress that they naturally undergo (Feder, 1999). Hsps are subdivided in family depending on their molecular weight, the most important are: *hsp110*, *hsp100, hsp90, hsp70, hsp60*, *hsp40, hsp10,* and small *hsp.*

In general these proteins act as oligomers, if not as complexes of several different chaperones, co-chaperones, and/or nucleotide exchange factors and interaction with chaperones is variously responsible for: (i) maintaining Hsp's partner proteins in a folding-competent, folded, or unfolded state; (ii) organellar localization, import, and/or export; (iii) minimizing the aggregation of non-native proteins; and (iiii) targeting non-native or aggregated proteins for degradation and removal from the cell (Feder, 1999).

For the different type of environmental stresses, probably the most important function of Hsps interactions are the last two cited before. Hsps are not all stress inducible but those that are stress inducible could respond to a variety of stresses, including temperature changes, cellular energy depletion, extreme concentration of ions, osmolytes gases or toxic substances (e.g. xenobiotics). All known stresses, if sufficiently intense, induce Hsps expression (Feder, 1999) and for this reason Hsps are indicated as stress proteins and their expression is correlated to stress response. In many cases, Hsps are especially useful biomarkers because their induction is much more sensitive to stress than traditional indices such as growth inhibition (Feder, 1999). Stress sensitivity of plants varies during different stages of life cycle; most of the species show different and characteristic Hsps expression during different stages of their growth (Efeoğlu, 2007). Furthermore distribution of Hsps differs among tissue, organ and genetic variability of plant (Efeoğlu, 2007). Hsp90 defines a family of molecular chaperones that are highly conserved from prokaryotes to eukaryotes and they are abundantly expressed in higher eukaryotes where it has been shown to be necessary for viability (Brown et al., 2007). Their major function is to manage protein folding but they also play a role in signal transduction networks, cell cycle control, protein degradation and protein trafficking (Efeoğlu, 2007). They require ATP to function and they represents one of the most abundant protein groups in cells: 1-2% of total cellular protein. Hsps90 expression is closely correlated to stress response, more than the expression of other Hsps family, although some of these proteins could be constitutively expressed (Efeoğlu, 2007)

#### 4.1.1.3 Metallothioneins and phytochelatins

Metallothioneins (MTs) are a group of low-molecular weight proteins characterized by a typical cysteine-rich sequence that can bind metal ions and appear to be ubiquitous; the specific cluster of the sequence are Cys-Cys and Cys-x-Cys where x is an amino acid other than cysteine. These proteins were discovered for the first time in animals whereas the first purification of plant metal-binding peptides did not reveal a gene-encoded plant MT but the molecules were synthetized enzymatically from glutathione with the general structure $(\gamma\text{-glutamyl-cysteinyl})_n$glycine where n=2 (Grill et al., 1985). All plant species tested synthetize $(\gamma\text{-EC})_n$G peptides and these were called phytochelatins (PCs) and later termed class III MTs (Gekeler, 1989). The first discovering of a plant Metallothioneins then classified as class II MT was in 1987 in wheat (Lane et al., 1987) and now many plant MT-like genes have been identified in different plant species including maize (De Framond, 1991), barley (Kille et al., 1991; Okumura et al., 1991), soybean (Kawashima et al., 1991), *Arabidopsis thaliana* (Zhou

and Goldsbrough, 1994), rice (Sasaki et al., 1994), wheat (Snowden and Gardner, 1993), tobacco (Robinson et al., 1992), alfalfa (Robinson et al., 1992), castor bean (Weig and Komor, 1992), *Brassica napus* (Buchanan-Wollaston, 1994) and *Sambuca nigra* (Coupe et al., 1995). Discovered proteins were subdivided in two categories based on the arrangement of cysteine residues: type I have Cys-x-Cys clusters exclusively, whereas type II molecules have a Cys-Cys and a Cys-x-x-Cys within the N-terminal domain (Robinson et al., 1993). In general all these studies demonstrate that type I MT-like genes appear constitutively expressed in the roots whilst transcripts of genes in the type II category are present predominantly in leaves/aerial tissues (Whitelaw et al., 1997). Whitelaw et al. (1997) characterized two tomato metallothioneins, $LeMT_A$ and $LeMT_B$. The analysis of the genomic upstream sequences reveals a putative metal regulatory element (MRE) suggesting the possibility of metal-regulated transcription (Whitelaw et al., 1997).

Giritch et al. (1998) have demonstrated that the genes coding for metallothionein-like proteins in tomato represent a small multigene family and that there are at least five different MT-like genes in the tomato genome, but the entire family is most likely bigger. It was also found that members of tomato MT-like gene family differ greatly in their expression pattern in different plant organs; Foley and Singh (1994) suggested that type I genes are predominantly expressed in roots whereas type II genes are preferentially expressed in aerial plant organs. However this last statement is not always confirmed as Giritch et al. (1998) founded something contradictory: LEMT3 gene clearly belongs to the type II MT-like proteins but is not preferentially expressed in aerial tissue but mainly restricted to roots. The highly root specific LEMT2 gene can neither be classified as type I nor type II MT-like protein and might represent a new structural pattern not found before.

Organ specificity and developmental dependence have been reported for MT-like gene in other plant species. In all cases, the reason for the differential expression is not known precisely. Nevertheless, it strongly suggests a functional differentiation between the proteins, encoded by distinct members of the gene family (Giritch et al., 1998).

Phytochelatins are the other important chelating molecules and, as previously mentioned, they are synthesized post translationally from glutathione through the action of phytochelatin synthase (PCS). The expression is induced by the presence of metalloid or heavy metal ions. Arsenite is complexed with phytochelatins in a range of terrestrial plant species as demonstrated by different recent studies (Sneller et al., 1999; Schmöger et al., 2000; Hartley-Whitaker et al., 2001) and thus they play an important role for the

detoxification of As in plants. Schulz et al. (2008) founded that shorter chains of phytochelatins dominates in tolerant plants and not the longer chains of phytochelatins and they concluded that the phytochelatins production can be used as a method to analyze the degree of arsenic sensitivity in plants. It remain not well understood where the As-PC complexes are localized inside the cell; in this regard is important to note that As-PC complexes are stable at acidic condition that are present in the vacuole and this implies that in this site As-PC complexes might remain stable allowing accumulation of high concentration of As, especially in resistant plants.

## 4.2 Materials and methods

Transcription profiling related to four genes associated with the abiotic stress response was carried out for cvs. Aragon and Gladis. Chosen genes are: Gluthatione Reductase gene (LeGR, XM_004247804), Heat Shock Protein gene (Hsp90-1, AY368906), Phytochelatin Synthase gene (Phyt, XM_004247469) and Metallothioneine gene (LeMT, L77966) because in previous studies they were demonstrated to be involved in As stress response (Goupil et al., 2009; Del Razo et al., 2001; Panda et al., 2010).

### 4.2.1 Growth conditions

Seeds of the two cvs. were germinated on sterile blotting paper soaked with deionized water and Murashige and Skoog salts (4.3 g $l^{-1}$, Duchefa Biochemie, Haarlem, The Netherlands) in dark conditions at 25°C. After germination the seedling were grown for 15 days in sterile vermiculite soaked with the same solution seen before, in a greenhouse at 25°C, relative humidity at 50%, and photoperiod at 16 h, with the light provided by metal halide lamps supplying a photon flux density of 300 µmol $m^{-2}$ $s^{-1}$. Fifteen plants for each cultivar were then transferred to a hydroponic solution with deionize water and Murashige and Skoog salts (2.15 g $l^{-1}$, Duchefa Biochemie, Haarlem, The Netherlands), according to Goupil et al. (2009). Seven days later, the hydroponic solution of five of the fifteen plants was supplemented with 0.5 mM $NaAsO_2$, (As (III)), other five with 0.5 mM $NaAsO_2$, (As (III)) and 0.15 mM $CaSiO_3$ and five were left in hydroponic solution withouth any treatment as control.

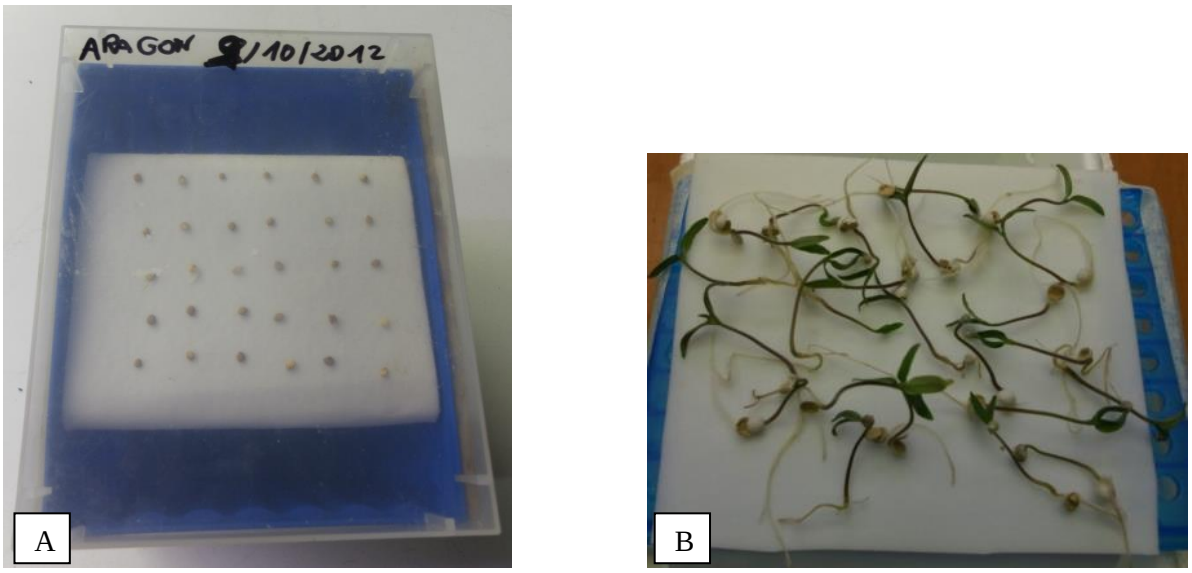



**Fig.1 Images of the germination on the blotting paper: (A) seeds of cv Aragon; (B) seedling of the same cv.**

**Fig.2 Images of the plantlets growing on vermiculite with water and MS medium.**


**Fig.3 Tomato plants grown in hydroponic solution at the moment of transfer from vermiculite medium (A) and at the moment of the sampling, after 24h (B); control plants on the left and plants treated with As (III) 0.5mM on the right.**

### 4.2.2 RNA extraction and Reverse Trascription

After 24 h, the material was harvested and separated into root, stem and leaf tissue, from which RNA was extracted using the Spectrum Plant total RNA reagent (Sigma-Aldrich, St. Louis, MO, USA). Extracted RNA was dissolved in RNase-free water and stored at -80°C. RNA integrity was verified on a 2% agarose gel; three bands corresponding to ribosomal RNA (28S, 18S and 5S) were apparent. RNA concentration was determined with a Cary 50 Spectrophotometer (Varian Inc., Torino, Italy).

**Tab.1 Quantification of the extracted RNA from the different sample made with Cary 50 Spectrophotometer.**

| Cultivar | Treatment | Part | µg/µl | R |
|---|---|---|---|---|
| Aragon | As 0.5 mM | aerial part | 0.850 | 2.077 |
| | | root | 0.430 | 2.241 |
| | As 0.5mM + Si 0.15 mM | aerial part | 0.509 | 2.062 |
| | | root | 0.482 | 2.294 |
| | control | aerial part | 0.929 | 1.700 |
| | | root | 0.584 | 2.106 |
| Gladis | As 0.5 mM | aerial part | 0.768 | 2.034 |
| | | root | 0.360 | 2.111 |
| | As 0.5mM + Si 0.15 mM | aerial part | 0.739 | 2.139 |
| | | root | 0.275 | 2.158 |
| | control | aerial part | 0.547 | 2.072 |
| | | root | 0.320 | 2.299 |

The resulting RNA was converted to single-stranded cDNA using a Quantitect Reverse Transcription kit (Qiagen, Venlo, The Netherlands) according to the manufacturer protocol; 1 µg of RNA was treated with one unit of Qiagen Genomic DNA Wipeout Buffer, incubated at 42°C for 2 minutes and then stored at 4°C before the Reverse Transcription (RT) Reaction. RT was performed in a final volume of 20 µl containing 1 µl of Reverse Transcription Master Mix (Reverse Transcriptase and RNase inhibitor), 4 µl of RT Buffer (include $Mg^{2+}$ and dNTPs), 1 µl of RT Primer Mix and the RNA purified in the previous reaction. Reverse Transcription was performed in a thermal cycler (Applied Biosystems, Carlsbad, CA, USA) using the following program: 42°C for 15 minutes, 95°C for 3 minutes and 4°C as a final hold. Tubes were stored at -20°C until use.

### 4.2.3 Primer design and optimization of Quantitative Real-Time PCR

As previously mentioned, four target genes were chosen for this study: Glutathione Reductase gene (LeGR), Heat Shock Protein gene (HSP 90-1), Phytochelatin Synthase gene (Phyt) and Metallothioneine type II gene (LeMT). The quantitative RT-qPCR (qPCR) primer sequences (Tab.1) for LeGR was obtained from Goupil et al. 2009; Primer Express v3.0 software (Applied Biosystems, Carlsbad, CA,USA) was used to design primer targeting Phyt, HSP90-1 and LeMT according to tomato genes sequences available on the NCBI database (NCBI website). Each pair of primer frame a relatively short sequence that is suitable for qPCR, contain a GC percentage near 60% and a Tm close to 60°C.

As internal control gene a previous work (Exposito-Rodriguez et al., 2008) was adopted between some best performing reference genes indicated, three where selected for our studies including: a chlartin adaptor complexes gene (CAC, SGN-U314153), a TIP41-like family protein gene (TIP41, SGN-U321250) and an expressed sequence (exp, SGN-U346908). These three genes, together with the internal control gene utilized in the study of Goupil et al. (2009), an Actin protein-like gene (LeAct, EU884309) were tested for the suitability for the present study. For this aim a quantitative real time PCR was performed with tomato cDNA generated in the first step, utilizing primers of these control genes (sequences for the primers were taken from the literature). For quantification analysis, specific regions of targeted genes were amplified using Power SYBR GREEN PCR Master Mix (Applied Biosystems, Carlsbad, Ca, USA) and real-time PCR was performed with an Applied Biosystems 7000 sequence detection system (Applied Biosystems, Carlsbad, Ca, USA). PCR conditions consisted of a 95°C for 10 min, 40 cycles of 95 °C for 7 s, 60°C for 15 s, 72°C for 10 s and a melting analysis of 52 to 95°C with an increasing temperature 0.5°C $min^{-1}$. Each primer pairs was tested in the amplification of cDNA obtained from RNA of tomato leaf and cDNA obtained from RNA of tomato roots in order to evaluate expression stability among different part of the plant of these control genes.Each amplification was performed in triplicate in order to evaluate the reproducibility of the results. After this test the most stably expressed gene was the expressed sequence (exp) and was therefore chosen as the effective control gene for the subsequent expression analysis.

**Tab.1 Sequences of qPCR primers targeting stress-related genes.**

| Gene target | Forward primer (5'-3') | Reverse primer (5'-3') | References | Expected amplicons lenght (bp) | Tm (°C) | Description |
|---|---|---|---|---|---|---|
| exp | gctaagaacgctggacctaatg | tgggtgtgcctttctgaatg | Exposito-Rodriguez *et al.* 2008 | 183 | 76 | Expressed sequence |
| LeMT | atttgttggctgatcccttg | atccaacccttgcttgtcac | Primer Express v3.0 software (Applied Biosystems, Carlsbad, CA,USA) | 197 | 60 | Lycopersicin esculentum MT2-like protein gene |
| LeHSP90-1 | gcagagacgtttgcatttca | ggtcttgtcagggacgatgt | Primer Express v3.0 software (Applied Biosystems, Carlsbad, CA,USA | 207 | 60 | *Lycopersicon esculentum* molecular chaperone mRNA |
| LeGR | tcccatcggctctgaagttagtggg | tctttgcatcctccagttctggccc | Goupil *et al.* 2009 | 119 | 66 | Predicted *Solanum lycopersicum* cytosolic glutathione reductase, transcript variant, mRNA |
| Phyt | ggtctggtttgtcggataggtt | ttcccgtagctctgtagcatctg | Primer Express v3.0 software (Applied Biosystems, Carlsbad, CA,USA) | 66 | 62 | Predicted *Solanum lycopersicum* glutathione gamma-glutamylcysteinyltransferase 1-like, mRNA |

**Tab.2 Homology of the qPCR amplified fragments to sequences in the databases**

| PCR amplicon | Length (Bp) | Accession number | Homology | BLAST score |
|---|---|---|---|---|
| exp | 183 | XM_004242916.1 | PREDICTED: Solanum lycopersicum uncharacterized LOC101263039 (LOC101263039), mRNA | 6e-70 |
| LeMT | 197 | L77966.1 | Lycopersicon esculentum metallothionein II-like protein (MTB) gene, complete cds | 5e-76 |
| LeHSP90-1 | 207 | NM_001247507.1 | Solanum lycopersicum molecular chaperone Hsp90-1 (Hsp90-1), mRNA | 4e-103 |
| LeGR | 119 | XM_004247804.1 | PREDICTED: Solanum lycopersicum cytosolic glutathione reductase, transcript variant 2 (LOC100301935), mRNA | 2e-54 |
| Phyt | 66 | XM_004247469.1 | PREDICTED: Solanum lycopersicum glutathione gamma-glutamylcysteinyltransferase 1-like (LOC101251125), mRNA | 2e-25 |

Prior the quantification analysis, the interested genes were amplified with a conventional PCR (Applied Biosystems, Carlsbad, Ca, USA), checked on 2% agarose gel and then sequenced for confirmation. Amplified products were purified with an Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Europe GmbH, Freiburg, Germany), according to the manufacturer protocol. Gene products were sequenced by BMR Genomics Sequencing Service (BMR Genomics, Padova, Italy). A BLAST search on NCBI (http://www.ncbi.nlm.nih.gov/) was performed to identify sequence homology (Tab.2).

### 4.2.4 Expression analysis

qPCRs was done by using SYBR Green master mix (Applied Biosystems, Carlsbad, Ca, USA) using an Applied Biosystem 7000 sequence detection system, with reaction conditions following those described before in paragraph

The relative abundance of each transcript was calculated from the average $C_t$ of each amplification according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). In this method the relative quantity of each transcript is determined by:

$RQ= 2^{-\Delta\Delta Ct}$.

Where: $\Delta Ct=Ct_{(exp)}-Ct_{(stress\ related\ gene)}$,

$\Delta\Delta Ct= \Delta Ct_{(treatment)}- \Delta Ct_{(control).}$

Statistically significant differences among transcript abundance of the stress related genes in the different treatments were evidenced with Student's two tail t-test. The software package SPSS v19 (IBM website) was used for all statistical procedures.

## 4.3 Results

In Fig.4 it is shown the Relative Quantity of the transcript of Glutathione Reductase (GR) gene in the two considered cultivars, Aragon and Gladis, in the presence of only arsenite or with arsenite and silicon, as clearly visible; Glutathione Reductase is upregulated following arsenite treatment in both considered cultivars but the up-regulation is higher in cv. Gladis than in cv. Aragon; ≈2 fold higher in the first cv. There is also a significant difference between the expression of GR in the different part of the plants with an up-regulation ≈10 fold higher in roots than in the aerial parts. What resulted not significant in this case is the difference in gene expression among arsenic treatment and arsenic + silicon treatment.

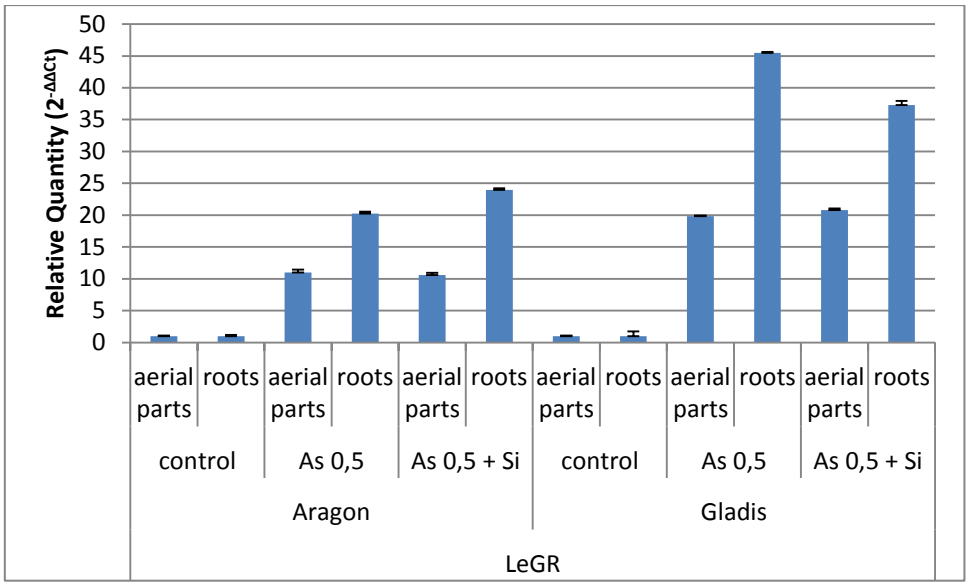



**Fig.4 Transcript abundance of LeGR gene as assessed by qPCR. The data have been normalized to the abundance of the reference expressed sequence (exp) utilized as internal control.**

Considering the expression of HSP90-1 gene (Fig.5), results indicate an up-regulation of the gene in both cultivars but, as previously observed for GR, the up-regultaion is higher in cv. Gladis than in cv. Aragon; especially in roots of Gladis it was observed an up- regulation that is ≈15 fold higher than in the roots of Aragon. In this case the differences in expression among the different part of the plants are significant only in cv. Gladis where the up-regulation of HSP90-1 is higher in roots than in aerial parts; again there were observed differences between roots and aerial parts of about 15 fold the relative quantity. No significant differences among arsenic treatment and arsenic + silicon treatment were observed for both cultivars.

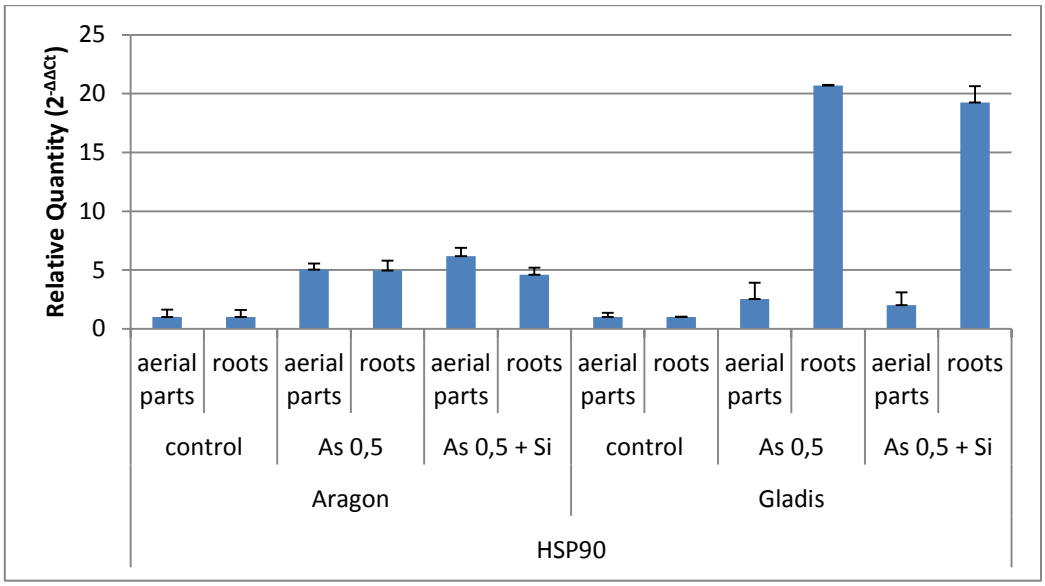



**Fig.5 Transcript abundance of HSP90-1 gene as assessed by qPCR. The data have been normalized to the abundance of the reference expressed sequence (exp) utilized as internal control.**

The expression of Metalothioneine gene (LeMT) (Fig.6) seems to follow a similar pattern of for Glutathione Reductase with an up-regulation of the gene that is ≈2 fold higher in cv. Gladis than in cv. Aragon and furthermore for both cv. the up-regulation is higher in roots than in aerial parts.

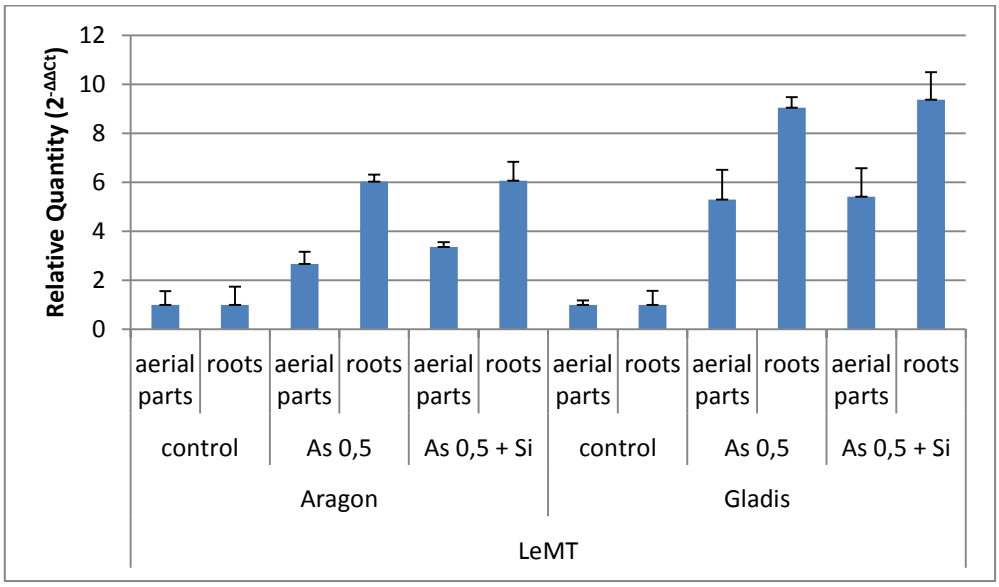



**Fig.6 Transcript abundance of LeMT gene as assessed by qPCR. The data have been normalized to the abundance of the reference expressed sequence (exp) utilized as internal control.**

Finally an up-regultation occurred also for Phytochelatin Synthase gene (Fig.7) but only in cv. Gladis and not for cv. Aragon where the expression of the gene in the treated plants was not significantly different from the control. An up-regulation higher in aerial parts than in

roots in cv. Gladis it was also observed , moreover no signifcant differences were observed in the presence or absence of silicon in the growth medium.

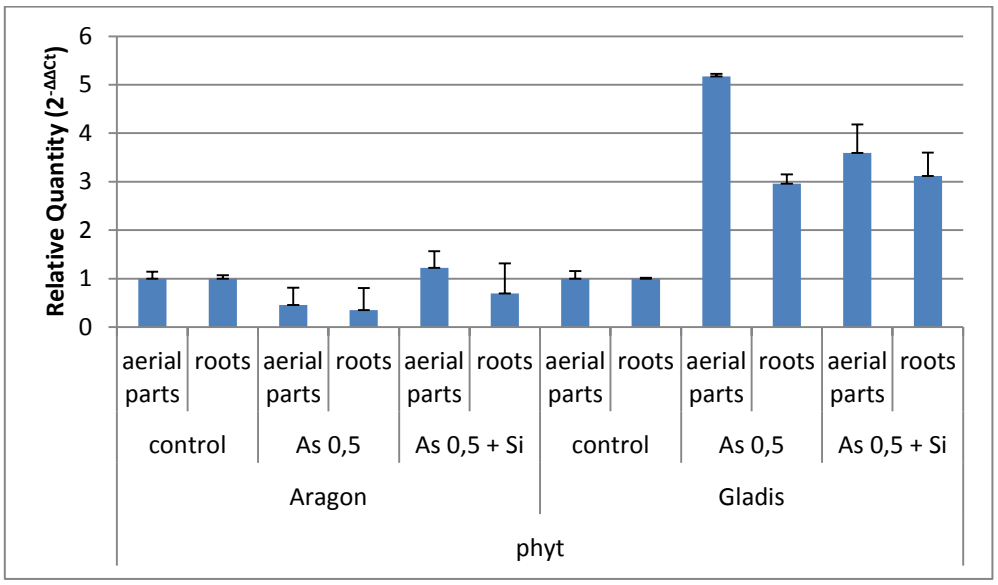



**Fig.7 Transcript abundance of Phyt gene as assessed by qPCR. The data have been normalized to the abundance of the reference expressed sequence (exp) utilized as internal control.**

## 4.4 Discussion

Plants possess both non enzymatic (GSH, ascorbate, carotenoids) and enzymatic (superoxide dismutase, catalase and peroxydase) antioxydant mechanisms. In a comparative proteomic study previously conducted on rice roots subjected to As stress, a total of 23 As regulated proteins were identified (Ahsan et al., 2008) including predicted and novel. It was also shown that the level of total GSH increased as the level of Glutathione Reductase (GR) among others. A particularly strong up-regulation of LeGR was observed in $NaAsO_2$ treated tomato plants, suggesting that plants sensed the stress induced by As and responded by activating their generic stress response. This could be said also for the Metallothionein gene LeMT and for Heat Schock Protein HSP90-1;a strong up-regulation induced by the presence of arsenite indicating the activation of stress mechanisms response is reported in literature (Clemens, 2001; Clemens, 2006; Panda et al., 2010; Goupil et al., 2009).

For the majority of analyzed genes we observed differences in transcript abundance between roots and aerial parts; these can be due to the fact that the first stress response we observed in plant is in general in root tissue (where uptake takes place) and this was in general higher for both the cultivars analyze. In aerial part we observed lower stress response and higher differences between the two cultivars probably because As translocation didn't occur at the

same rate in Aragon and in Gladis (as shown in the previous experiment on arsenic uptake and translocation, paragraph 2.3.2).

Again the evidence that different cultivars show significant differences in the explotitation of defence mechanisms , reinforce the idea that the choice of the cultivar for tomato cultivation, in soils at risk of As contamination, is important and must consider physiological, chemical and molecular aspects correlated to As response

## 4.5 Conclusions and remarks

Considering the results obtained from the different part of this research project, a more detailed picture of the problem of arsenic contamination for tomato cultivation, it is now available; this can help help farmers to develop more safely choice in the field of agriculture, in order to avoid the entry of this contaminant in the food chain. In fact, from the study about the impact of Silicon on uptake and translocation of arsenic it was evidenced that silicon could have a beneficial effect on attenuating arsenic toxic effects on tomato plants, while the effect on uptake and translocation of the metalloid is strongly cultivar-dependent and it could also vary in the different physiological state of the plants. This suggested that before the application of silicon salts to tomato cultivation to confer stress resistance, it will be a good practice the evaluation of cultivar response forthe presence of this element in the growth medium, possibly through a greenhouse experiment. The evaluation of the impact of the application of biochar on tomato plant growing in arsenic contaminated soil shows us that biochar, which is usually utilized as an amendment for the soil, could have a dangerous impact on arsenic availability for the plant and especially on arsenic solubilization in the pore water, thus representing and important risk of leaching and contamination of growndwater sinks. In this regard, an appropriate evaluation of chemical and physical characteristics of the soil and also of the chemical and physical characteristics of biochar, must be performed before any application of this material in the field, to avoid all these mentioned risks. Finally the analysis of stress response at a molecular level demonstrated that tomato plants respond to the presence of arsenic in the growth medium activating the mechanisms that were reported in literature for the generic stress response to metalloid in plant but the differences evidenced among cultivars in the entity of response confirmed a different behavior of them at the molecular level as yet demonstrated at the physiological level in the first part of the research.

In this part it was also confirmed the characteristic of tomato as a tolerant species for arsenic (Carbonell-Barrachina et al., 1997) since for the majority of the analyzed stress related genes a more evident up-regulation was observed in roots, where tolerant plants accumulates the contaminant, than in the aerial parts, where usually there is only a little translocation of the contaminant.

This research project has combined different type of approaches including physiological, biochemical and molecular analysis and the results obtained suggested that, when we consider a complex environmental problem like arsenic contamination in tomato cultivation, one single approach is not sufficient to predict the plant response to the contaminant and that, for reasons of food safety, it will be of great importance in the future to evaluate in detail the phenotypic responses of the different cultivars utilizing field trials/laboratory studies combined together taking into account:

- Chemical and physical characteristics of cultivated soil,
- Agricultural practices performed on these soil;
- Genetic, molecular and physiological characteristics of cultivated cultivars.

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