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Response of *Oryza sativa* and *Phragmites australis* plants to potentially toxic elements stress in phytoremediation strategies

Respuesta de *Oryza sativa* y *Phragmites australis* al estrés por elementos potencialmente tóxicos en estrategias de fitorrecuperación

Dña. María José Álvarez Robles
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María José Álvarez Robles
Murcia, 2022

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Dña. **María Pilar Bernal Calderón**, Doctora en Química y Profesora de Investigación del CSIC, y D. **Rafael Clemente Carrillo**, Doctor en Química y Científico Titular del CSIC, ambos con destino en el Centro de Edafología y Biología Aplicada del Segura (CEBAS), autorizan:

La presentación de la Tesis Doctoral titulada "**Response of *Oryza sativa* and *Phragmites australi*s to potentially toxic elements stress in phytoremediation strategies**", realizada por Dña. **María José Álvarez Robles** bajo nuestra dirección y supervisión en el Departamento de Conservación de Suelos y Agua y Manejo de Residuos Orgánicos del CEBAS-CSIC, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

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RESUMEN

La contaminación de suelos por elementos potencialmente tóxicos (metales pesados y metaloides) o PTE (por sus siglas en inglés) procedentes de actividades mineras supone un grave problema medioambiental y numerosos riesgos para los seres vivos que habitan en ecosistemas próximos. La sobreexplotación y el uso insostenible de los recursos han dado lugar a suelos con altas concentraciones de PTE, desfavorables para el desarrollo vegetal y expuestos a la erosión. Las tecnologías de fitorrecuperación, basadas en el uso de enmiendas, plantas y actividades agronómicas, son una opción eficiente y respetuosa con el medio ambiente para la restauración de los suelos contaminados, así como para revalorizar residuos con problemas de gestión. A través de estas técnicas, los suelos degradados pueden recuperar sus propiedades, total o parcialmente, siendo así también más resilientes al cambio climático.

A pesar de que algunos PTE son en realidad micronutrientes beneficiosos o incluso imprescindibles para las plantas en bajas concentraciones, cuando están presentes en el suelo a altas concentraciones son considerados factores de estrés abiótico junto a los elementos que no tienen función biológica conocida. Las plantas son capaces de lidiar con determinadas concentraciones de PTE hasta un valor umbral, a partir del cual son tóxicos y desencadenan alteraciones fisiológicas y metabólicas, llegando a producir daño oxidativo o incluso la muerte de la planta. Las distintas especies de plantas pueden tener diferentes mecanismos de tolerancia y, por tanto, diferir en su respuesta al estrés por PTE. Por ello, conocer estos indicadores de estrés puede permitir mejorar la eficacia de las estrategias de fitorrecuperación, así como monitorizar la evolución del proceso al actuar como biomarcadores.

Por consiguiente, la presente Tesis ha tenido como objetivo identificar los cambios en el estado fisiológico y oxidativo de plántulas de arroz y carrizo para contrarrestar la toxicidad por PTE al crecer en diferentes suelos mineros contaminados o en disolución hidropónica con distintas concentraciones de arseniato (As(V)). Además, se ha tenido siempre en consideración la relevancia del conocimiento de estos cambios en las plantas de cara a su posible aplicación en estrategias de fitorrecuperación. Para ello, se han llevado a cabo cuatro experimentos, de los que derivan las tres publicaciones que forman parte del compendio y el trabajo incluido en al Anexo I. Se seleccionó carrizo (*Phragmites australis* (Cav.) Trin. ex Steud.) como una de las plantas de estudio por diversos motivos: al ser una planta macrófita, está adaptada a las condiciones hídricas fluctuantes, ha mostrado previamente una alta tolerancia a altas concentraciones de PTE (incluido arsénico), reteniéndolo principalmente en las raíces, y por tanto su adecuación en procesos de fitoestabilización y rizofiltración, y además crece de manera espontánea en la Sierra Minera de Cartagena- La Unión (la zona de estudio de esta tesis doctoral), por lo que también estaría adaptada a las condiciones climáticas de la zona. Por el contrario, el arroz (*Oryza sativa* L.) es considerada una planta modelo para estudios sobre la acumulación y tolerancia a arsénico (As), a consecuencia de su cultivo frecuente en suelos con altas concentraciones de este elemento altamente tóxico. Sin embargo, la respuesta de plantas de arroz a arsenito (As(III)) ha sido el principal objeto de estudio, dadas las condiciones predominantemente anaeróbicas de los arrozales, mientras que la toxicidad de As(V) (especie dominante en el agua de poro y en los propios suelos mineros contaminados y oxidados) ha sido escasamente estudiada antes de esta tesis doctoral. Por tanto, conocer la respuesta de arroz, como planta modelo, a su exposición a concentraciones elevadas de As(V) resulta de interés y podría ayudar a mejorar la efectividad de la fitorrecuperación de áreas contaminadas con As, incluyendo suelos y aguas.

El estudio del comportamiento de arroz y carrizo en suelos contaminados con elevadas concentraciones de PTE se realizó mediante un cultivo en macetas en cámara de crecimiento controlado, con tres suelos mineros procedentes de la Sierra Minera de Cartagena-La Unión (Murcia). Estos suelos difieren entre sí en algunas de sus características fisicoquímicas, además de en las concentraciones totales y biodisponibles de los distintos PTE, pero son, en líneas generales, ligeramente ácidos, con alta conductividad eléctrica y con bajas concentraciones en carbono y nitrógeno. Para garantizar la fertilidad del suelo y el crecimiento de las plantas, se les añadió un compost maduro de alperujo como enmienda orgánica. Debido a las interacciones que se producen entre las sustancias húmicas del compost y los PTE del suelo, en los experimentos posteriores en cultivo hidropónico se complementó la disolución nutritiva con un extracto acuoso de compost de alperujo (misma proporción de carbono), para así reproducir con mayor precisión los posibles resultados que se obtendrían en suelo. A causa de las condiciones semiáridas donde se localizan los suelos del estudio, se utilizaron en el experimento dos capacidades de retención hídrica (al 70% y en condiciones de encharcamiento), ya que los drásticos cambios que se pueden producir en el contenido hídrico del suelo pueden alterar la solubilidad y disponibilidad de los PTE.

En este primer experimento se observó una ligera tendencia del As a estar más biodisponible (fácilmente extraíble) en los suelos en condiciones de encharcamiento, mientras que para los metales pesados esto ocurrió en los suelos húmedos, en concordancia con el ligero aumento del pH que se observó en condiciones de encharcamiento. Los elementos que se encontraron en mayores concentraciones biodisponibles fueron zinc (Zn) y plomo (Pb), siendo además los únicos PTE detectados en el agua de poro extraída de los suelos de las distintas macetas. Asimismo, el cadmio (Cd) fue el único metal que se encontró en formas intercambiables (extraíbles con $CaCl_2$) en la extracción secuencial de metales en todos los suelos y tratamientos, lo que refleja su alta solubilidad y su potencial fitotoxicidad. Se encontró que estas altas concentraciones biodisponibles de Zn y, especialmente Cd, junto con la elevada salinidad impidieron la supervivencia de las plántulas de arroz en uno de los suelos de estudio (el recogido en Llano del Beal (LB)). No obstante, esta misma especie presentó relativa tolerancia a As y Pb, ya que acumuló estos elementos principalmente en las raíces, protegiendo así los tejidos de la parte aérea de las plantas. Las plantas de carrizo mostraron una tolerancia todavía mayor a los distintos PTE, mostrándose como una especie adecuada para su uso en fitoinmovilización de suelos contaminados con PTE, al restringir su translocación a la parte área. El contenido de H_2O_2 mostró ser en general un buen indicador de la acumulación o toxicidad de TE, mientras que la concentración de prolina y la peroxidación lipídica se vieron directamente afectadas por la acumulación de Zn en las plantas de arroz, y por la acumulación de As en el caso del carrizo.

Para conocer la tolerancia a As(V) de las especies seleccionadas y su influencia en los parámetros de estrés, y poder estimar las concentraciones umbrales de toxicidad de este elemento para estas plantas, se llevaron a cabo dos experimentos en hidropónico en los que se aplicó un gradiente creciente de concentración de As(V) (0-10 mg L^{-1} As(V)) en el medio de cultivo. Previamente se realizó un experimento preliminar en el que se comprobó el comportamiento de las plántulas de arroz expuestas a 1 mg L^{-1} de As en disolución nutritiva bajo distintas condiciones de cultivo (diferentes valores de pH y concentraciones de carbono orgánico disuelto (DOC)). En este primer experimento no se encontraron diferencias significativas ni en el rendimiento ni en el contenido de nutrientes de las plantas. Del mismo modo, se realizó un modelo de especiación para los nutrientes y As en la disolución nutritiva bajo las distintas condiciones de pH y DOC estudiadas con el software Visual MINTEQ, y las predicciones proporcionadas fueron muy similares entre sí para todas las combinaciones testadas. A la vista de estos resultados se seleccionaron las condiciones consideradas intermedias (pH 6 y 100 mg L^{-1} DOC) para el desarrollo de los experimentos principales posteriores con las dos especies de plantas. En el caso del arroz, los efectos tóxicos de As(V) en las plantas comenzaron a aparecer a concentraciones superiores a 2,5 mg L^{-1} de As(V) en la disolución nutritiva, a partir de las cuales se redujo el peso y el crecimiento de las plantas, y se observaron alteraciones en el balance de algunos de los nutrientes, como fósforo (P), magnesio (Mg) o potasio (K). Como cabría esperar, la acumulación de As total en las plantas aumentó al aumentar la concentración de As(V), llegando a alcanzar 600 mg kg^{-1} (peso seco) tanto en las hojas como en las raíces. Dicha acumulación, junto con la de las especies identificadas de As en planta (As(III) y As(V)), indujeron la peroxidación de lípidos, pudiendo ésta por tanto considerarse un buen indicador del daño causado por el As en las plantas.

En cuanto al carrizo, curiosamente cuando las plantas fueron sometidas a los mismos tratamientos que las de arroz, éstas no mostraron signos de toxicidad. Paralelamente a las evidencias visuales, no hubo diferencias en la longitud, el peso, o el contenido en pigmentos fotosintéticos entre las plantas de carrizo del tratamiento control y las expuestas a 10 mg L^{-1} As. Asimismo, el carrizo llegó a acumular hasta 2500 mg kg^{-1} As en sus raíces, principalmente en forma de As(III), lo que concuerda con el principal mecanismo de detoxificación de As descrito en la literatura: la reducción de As(V) a As(III), su quelatación con grupos tiol y su secuestro en las vacuolas, reduciendo la translocación del mismo a la parte aérea y su posible posterior daño al aparato fotosintético. A pesar de que los resultados indicaron que el As per se no afectó al estado oxidativo de las plantas de carrizo, las tendencias obtenidas en el análisis de componentes principales relacionan tanto la peroxidación de lípidos como la acumulación de prolina en las plantas con el déficit de determinados nutrientes, que sí podría ser considerado una consecuencia directa de la acumulación de As y el uso compartido de los transportadores celulares. La tolerancia observada de las plantas de carrizo a altas concentraciones de As en el medio de cultivo confirmaría nuevamente su idoneidad para su uso en rizofiltración y fitoestabilización, donde se pretende la inmovilización en la raíz o la rizosfera de este elemento.

Por último, se estudió el efecto del "priming" con ácido ascórbico (AsA) sobre distintos parámetros de estrés en plantas de arroz expuestas a As(V). Como se sabe, mediante la técnica de "priming" se busca inducir una respuesta de defensa de las plantas más rápida o robusta, a través de la adición de distintos compuestos químicos en diferentes estadíos de crecimiento. Dado que los síntomas de toxicidad en el experimento previo comenzaron a ser evidentes a partir de 2,5 mg L^{-1} As, se fijó la concentración de As(V) para este experimento en torno a 3,75 mg L^{-1} As (o 50 µM As), para que se generara una toxicidad moderada en el medio de cultivo que provocara realmente una respuesta de las plantas, pero que, a su vez, permitiese la obtención de suficiente biomasa para llevar a cabo los análisis pertinentes.

La adición de AsA a la disolución nutritiva redujo significativamente la acumulación de As en las raíces de las plantas de arroz; sin embargo, no fue suficiente para evitar mermas en su peso o longitud. No obstante, los resultados sugieren también que la adición de AsA limitó la translocación de As(III) y As(V) a la parte aérea de las plantas. Por lo tanto, el priming con AsA podría considerarse una estrategia prometedora para pre-acondicionar las plantas y reducir la acumulación de As en las plantas de arroz cultivado en suelos o aguas ricas en As. En relación a los indicadores de estrés, la adición de AsA aumentó la acumulación de prolina y proantocianidinas, tanto individualmente como junto con As, así como la formación de tioles. Además, se obtuvieron correlaciones positivas entre las actividades antioxidantes (FRAP y DPPH) y las concentraciones de compuestos fenólicos (TPC), prolina y proantocianidinas (subfamilia de flavonoides, compuestos fenólicos), de modo que se deduce un fuerte control de la homeostasis oxidativa por parte de estos compuestos. En concreto, la prolina fue uno de los pocos parámetros que mostró una respuesta significativa (mayor concentración) a la presencia de As, y que, posteriormente, se mitigó con la adición de AsA, por lo que podría actuar como un marcador temprano de la toxicidad del As en arroz. El análisis de componentes principales también secundó estos resultados, proporcionando una clara separación entre el tratamiento de sólo As y los restantes. El análisis de componentes principales también relacionó, en las raíces de arroz, la acumulación de As(V) con la peroxidación lipídica y otros antioxidantes, y la de As(III) con la formación de fitoquelatinas (PC). No obstante, cabe destacar que se utilizaron dos tratamientos de priming con AsA, en donde el As era añadido a la vez o 24 horas después del AsA a la disolución nutritiva. Sin embargo, las diferencias entre estos dos tratamientos no fueron lo suficientemente significativas para determinar que el pretratamiento con AsA fuese beneficioso para las plantas de arroz, ni si lo fue su adición con el As.

De acuerdo con los resultados descritos, se puede considerar que se ha demostrado la idoneidad del carrizo para la fitorrecuperación de PTE y,

especialmente de As, en aguas y suelos contaminados. Las raíces de esta especie muestran una alta capacidad para lidiar con niveles tóxicos de metales o metaloides, junto con la protección de los tejidos de la parte aérea. Además, puesto que las raíces de las plantas son el primer órgano en enfrentarse, adaptarse y responder al estrés de los PTE, proveer a las células que lo componen de la maquinaria de defensa pertinente sería clave para reducir los efectos perjudiciales de dichos elementos tóxicos. Igualmente, el contenido en prolina o en proantocianidinas en las raíces de las plantas de arroz reflejó el daño inducido por As. Mediante la ingeniería genética de plantas, se abrirían nuevas líneas de investigación enfocadas a mejorar la adaptación al estrés abiótico, siendo la prolina y otros compuestos fenólicos las dianas de actuación. No obstante, la técnica de "priming" ofrece infinitas posibilidades para conducir a un mayor rendimiento de la fitorrecuperación, tal como se ha aplicado durante décadas con otros fines y dando resultados exitosos o prometedores. En este sentido, el uso de prolina u otros compuestos fenólicos, bien a través del "priming" de semillas, aplicación foliar o adición exógena (como la realizada en nuestro experimento), ofrece numerosas posibilidades para ajustar mejor la técnica de pre-acondicionado de las plantas, y así obtener una mayor eficacia en la técnica de fitorrecuperación seleccionada.

INTRODUCTION

1.1. Potentially Toxic Elements (PTE) impact in the ecosystems

Pollution by potentially toxic elements (PTE) has become a primary environmental concern and a serious threat to the (agro-)ecosystems (Sarwar et al., 2017; Nieder et al., 2018). PTE include heavy metals and metalloids, which, unlike organic contaminants, cannot be destroyed by degradation and persist for a long time after their release into the environment (Wuana & Okieimen, 2011; Nieder et al., 2018; Sumiahadi & Acar, 2018). These toxic heavy metals and metalloids are also indistinctly called "trace elements (TEs)" because they are present at low concentrations (mg kg^{-1} or less) in agroecosystems (He et al., 2005). Some of these PTE, such as copper (Cu), nickel (Ni) and zinc (Zn), are essential in small amounts to living organism for biological functions, also known as micronutrients (Nieder et al., 2018). However, present in excessive concentrations, micronutrients may be as toxic as the non-essential PTE (like cadmium (Cd), arsenic (As) and lead (Pb)) (Ali et al., 2013; Nieder et al., 2018).

Soils are the major sink for PTE entrance into the ecosystems (Wuana & Okieimen, 2011). PTE can be naturally inherited from the parent material from which they are originated because of weathering of minerals, erosion and volcanic activity (Sarwar et al., 2017; Sumiahadi & Acar, 2018). Nevertheless, anthropogenic sources, including the use of pesticides and phosphate fertilizers, sewage sludge application, wastewater irrigation or mining activities, have caused an additional accumulation of toxic elements (Ali et al., 2013; Nieder et al., 2018). The disturbance of the natural biogeochemical cycles and the increasing industrialization are growing worldwide PTE-pollution at an alarming rate (Ali et al., 2013).

The intensified accumulation of PTE in soils and water resources poses significant risks to human health (Ali et al., 2013). The hazards of PTE in human beings rely on the direct contact with contaminated soils, their entry into the food chain (soil-plant-(animal)-human) and drinking contaminated groundwater (Wuana & Okieimen, 2011). These toxic elements can be highly dangerous since they are mutagenic, teratogenic, carcinogenic and endocrine disruptors (Ali et al., 2013; Sarwar et al., 2019). Thus, the presence of these trace elements in agricultural lands and water sources must be maintained at healthy levels (Muthusaravanan et al., 2018).

Currently, 60-70 % of soils in the EU have been estimated to be unhealthy as a result of unsustainable management, overexploitation and pollutants emissions (Rodríguez- Espinosa et al., 2021, EC, 2021b). Since soils play a central role in the achievement of the ambitions and goals of the European Green Deal, different measures to preserve soil quality and limit soil contamination need to be implemented (EC, 2021b). In this sense, the European Community has built on first the Thematic Strategy for Soil Protection (2006/2293(INI)), which was then revised in the Resolution on soil protection 2021/2548(RSP) and has ended in the EU soil strategy for 2030 (EC, 2021b), a framework with concrete measures to protect and restore soils, and ensure that they are used sustainably. The main objectives included related to soil contamination consisted of: reducing soil pollution to levels no longer considered harmful to human health and natural ecosystems by 2030 (EC, 2021a); and a significant advance in the remediation of contaminated sites by 2050 (EC, 2020). Among them, a 50 % reduction of pesticides and nutrients excess, implementing organic farming at 25 % of agricultural lands and a 30 % increase of land-protected areas and wetlands restoration have been also proposed to be reached in a climate-neutral EU by 2050 (Montanarella & Panagos, 2021). The Spanish regulation Law 7/2022 regarding wastes and contaminated soils for a circular economy (BOE, 2022) has been recently enacted to reflect the European directives. The Law builds the legal framework to establish the potentially contaminant activities, the declarations of contaminated soils and the decontamination and remediation actions.

Nevertheless, the lack of a protective environmental legislation until very recently is considered the main reason of the alarming situation of our soils, together with the absence of the implementation of effective restoration activities (Conesa & Schulin, 2010; EC, 2021b). The mining area Sierra Minera de Cartagena-La Unión (SE Spain) (Figure 1.1) is a clear example of such soil degradation (Visconti et al., 2020). The mining and metal melting activities carried out for centuries has led to the dispersion of contaminants from abandoned mine ponds and tailings to the surrounding environments (Conesa & Schulin, 2010). The most relevant PTE present in this area with a significant ecotoxicological risk have been reported to be: As (200-6000 mg kg^{-1}), Cd (up to 35 mg kg^{-1}), Cu (150-350 mg kg^{-1}), Pb (4000-19000 mg kg^{-1}) and Zn (2000-15000 mg kg^{-1}) (Pérez-Sirvent et al., 2018; Bernal et al., 2019; Visconti et al., 2020). These concentrations largely exceed the threshold values for non-contaminated soils (5, 1, 100, 60, 200 mg kg^{-1}, respectively) (Tóth et al., 2016).

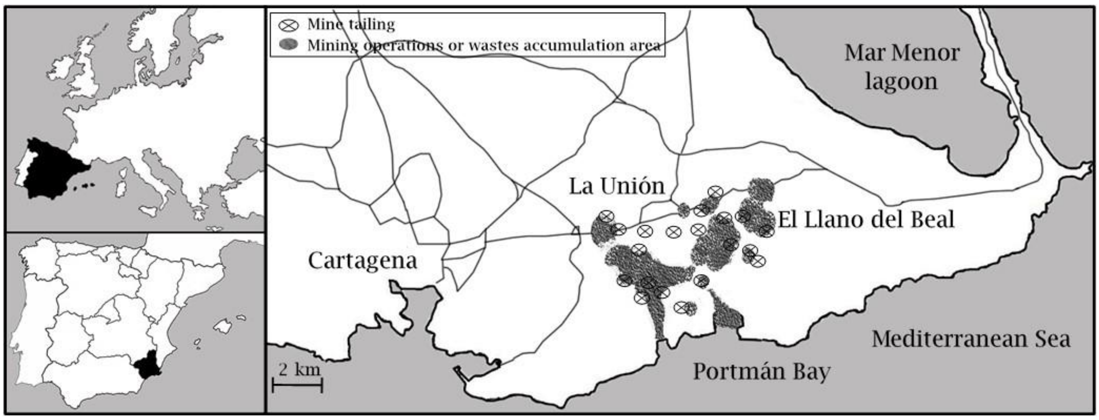

Figure 1.1. Location of the mining district Sierra Minera de Cartagena-La Unión. Adapted from Conesa and Schulin (2010) and Álvarez-Robles et al. (2022).

Approximately 2.8 million sites are potentially contaminated in the EU, of which 390,000 are expected to require remediation and where solely 17 % of them were reported to have already been remediated in 2018 (EC, 2021a). Therefore, the clean-up of the remaining PTE-contaminated soils is utmost necessary in order to minimize their impact on the ecosystems (Ali et al., 2013). A large number of physical, chemical and biological techniques can be used to alleviate PTE toxicity in polluted soils (Sarwar et al., 2019). Nevertheless, most of the traditional physico-chemical remediation methods are usually costly and complex, disturb soil native microflora and, principally, may result in secondary pollution problems (Muthusaravanan et al., 2018). Fortunately, different biological remediation processes (known as bioremediation methods) have emerged in the last decades to overcome soil toxicity, which provide sustainable environmental remediation options. Within these techniques, phytoremediation is a cost-effective, efficient and eco-friendly ‘green’ technology based on the use of plants to decontaminate PTE contaminated soils and for site restoration (Sumiahadi & Acar, 2018; Patra et al., 2020). Phytoremediation techniques (Figure 1.2) include (Wenzel et al., 1999; Clemente et al., 2015):

- Phytoextraction, which refers to the processes where the pollutants are eliminated by accumulation in the aboveground tissues of the plants and harvested. Species suitable for phytoextraction must have fast growth, high biomass, high tolerance towards PTE effects, high bioacummulation factor, ease cultivation and harvest, and being non-edible to avoid the entry of the contaminants into the food chain. Soil properties, PTE bioavailability and speciation also condition the efficiency of this technique.
- Phytostabilization or phytoimmmobilization is based on the use of certain plants to reduce the bioavailability of the pollutants in the environment, preventing their dispersion through wind erosion and/or leaching to ground water or to agricultural lands. Precisely, phytostabilization consists of the physical retention of the contaminants by plant roots, while in phytoimmobilization the contaminants are immobilized in the rhizosphere by adsorption, precipitation or complexation.
- Phytodegradation is the degradation of organic pollutants by plants to simpler less toxic forms through metabolic processes or enzymatic reactions, and also by the rhizosphere-associated microorganisms.
- Phytovolatilization: roots uptake the pollutants, translocate them to the leaves and are finally released in less toxic forms to the atmosphere. This technique can be applied particularly for the alleviation of organic contaminants and some PTE like As, Hg and Se that are able to form volatile compounds. PTE are not completely removed, they are transferred from one media to another, from where it could be redeposited.

- Rhizofiltration is used for the elimination of PTE from aquatic ecosystems by adsorption/absorption or precipitation on the roots surface. The metabolic activities of the microorganisms present in the rhizosphere may help to enhance the degradation of the pollutants.

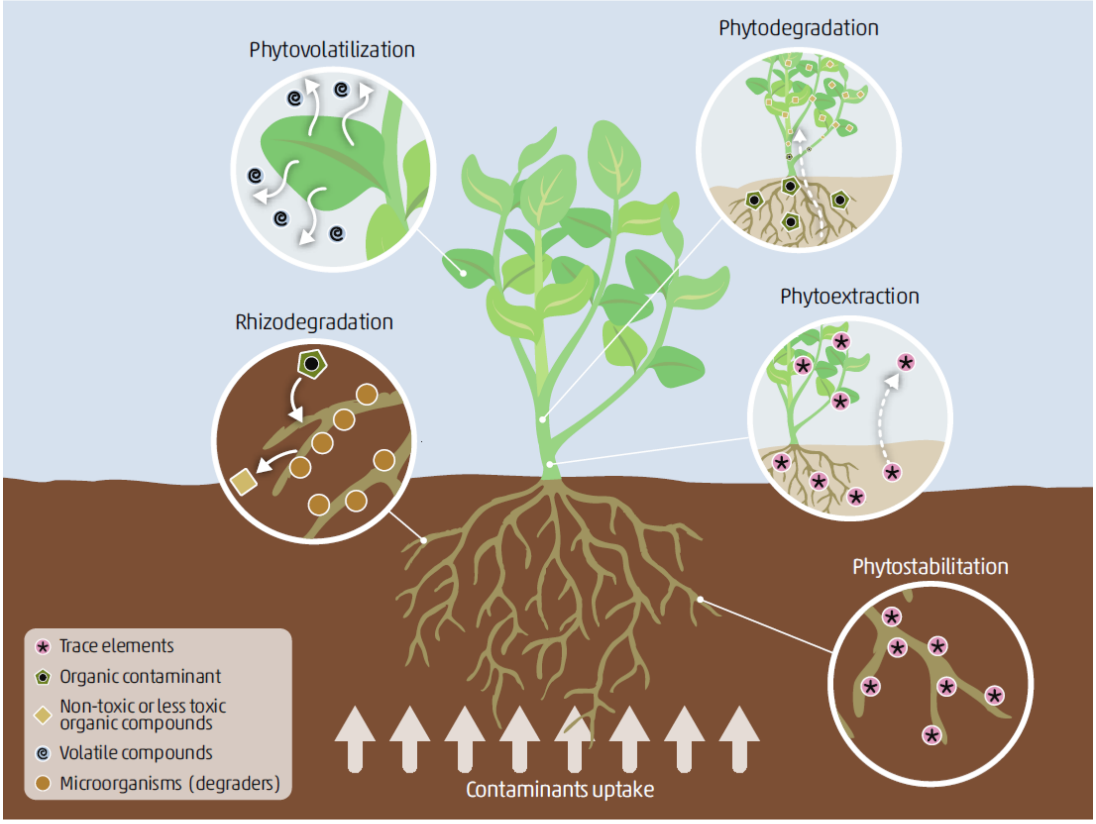

Figure 1.2. Phytoremediation processes (from FAO & UNEP, 2021).

Plant selection plays a key role in phytoremediation processes (Sumiahadi & Acar, 2018). Native and/or local species are preferred because these plants are already adapted to the local climatic conditions and usually spontaneously colonize the remediation-needed area (Párraga-Aguado et al., 2014). In addition, exotic plants might be invasive and endanger the harmony of the ecosystem (Sumiahadi & Acar, 2018). The remediation potential of the selected species is mainly defined by its ability to deal with the PTE (Fig. 1.3). Hyperaccumulator plants are capable to bioconcentrate 100-fold greater levels of PTE in the aerial part than non-hyperaccumulator plants (without producing any severe toxic symptoms when growing in PTE-enriched habitats (Sarwar et al., 2019; Patra et al., 2020). Thus, plant species that are considered as hyperaccumulator are those able to concentrate in their above-ground biomass more than 100 mg kg^{-1} Cd, 1000 mg kg^{-1} As, Co, Cr, Cu, Pb and Ni, and 10000 mg kg^{-1} Mn and Zn (on a dry weight basis) under natural conditions (Baker & Brooks, 1989; Wuana & Okieimen, 2011). However, the main disadvantage of hyperaccumulators is the fact that these species usually have a slow growth rate, a shallow root system and low aboveground biomass production (Salt et al., 1998; Saifullah et al., 2009). Excluder plants restrict PTE entry into cell roots or limit the transportation of the absorbed elements to the shoots, whilst indicator species reflect the concentration levels of the PTE in the soil in their aerial parts (Patra et al., 2020). Thus, hyperaccumulator species are adequate for phytoextraction, and indicators or, preferably, excluders for phytoimmobilization or phytostabilization processes. Nevertheless, if the threshold concentration for tolerance of the excluder or indicator is surpassed, the uptake mechanism may become uncontrolled, and the element is taken up, translocated and accumulated in the aerial plant tissue without control, which leads to plant death (Baker, 1981). Novel phytoextraction approaches focus on the improvement of the potential of non-hyperaccumulator plants through the use of chelators, organic amendments and microbes, which influence key soil physicochemical properties for bioavailability (Patra et al., 2020). In phytostabilization, the establishment of a sustainable plant cover that protects against erosion and contaminant dispersion normally requires the use of different soil-conditioning methods (Clemente et al., 2015).

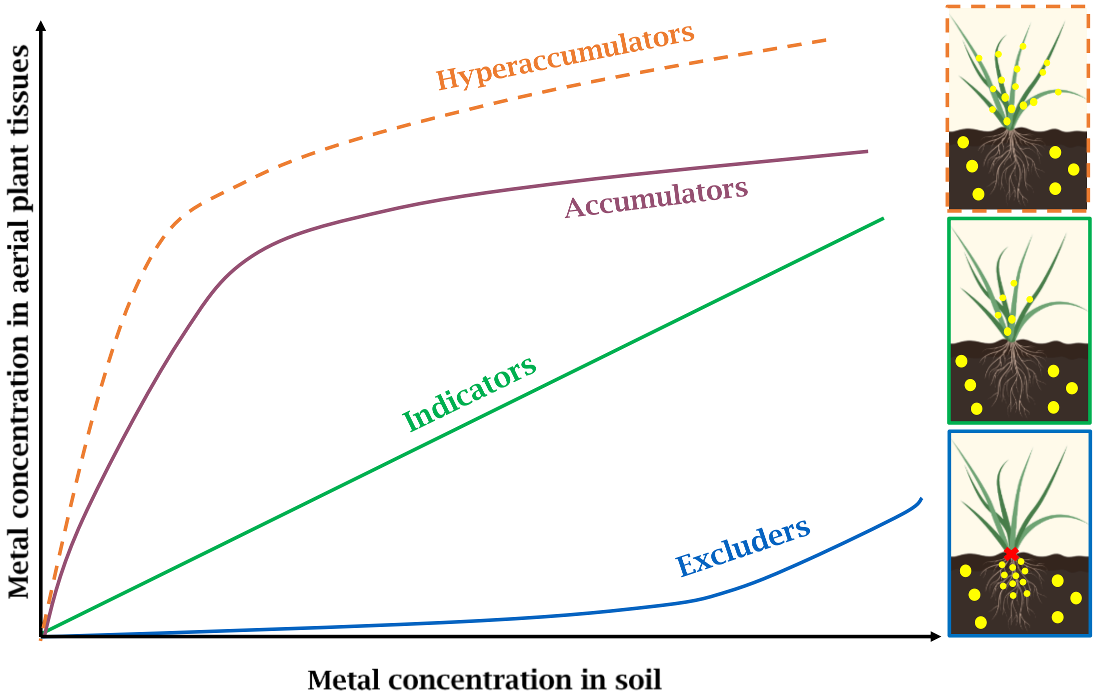

Figure 1.3. Plant response strategies to increasing PTE concentration in the soil (axes do not indicate a real scale). Modified from Adriano (2001) and Hunt et al. (2014).

Several factors affect the mobility, bioavailability and uptake of the nutrients and PTE in the soils such as pH, electrical conductivity (EC), redox potential (Eh), cation exchange capacity (CEC), organic matter (OM) content or the presence of root exudates. These properties can be influenced by temperature and precipitation and, therefore by the moisture content of the soil (Karmakar et al., 2016). Low pH values may favor the mobility/increase the availability of cationic metal elements like Cd, Cu, Pb and Zn, while the opposite is true for elements occurring mostly in anionic forms (like As). High redox potential may facilitate PTE mobilization in the form of soluble sulfates and high EC values may reduce the storage capacity of PTE in the soil. Similarly, low CEC and OM may limit PTE retention capacity of the soils. The physico-chemical characteristics of the soils will, therefore, play a principal role in the potential toxicity of PTE in the soil.

1.2. PTE phytotoxicity

Toxicity caused by PTE is considered one of the major abiotic stresses that plants can find in soils, and may result in physiological and biochemical anomalies in the plants. PTE toxicity in the plants varies with the species, specific element, concentration, chemical form and soil composition. Phytotoxicity symptoms differ among the PTE, since they have different mechanisms of toxicity. In particular, microelements impact on cell metabolism is expected to be completely different to that of the PTE that have no biological function (Gill, 2014). Generally, plants can withstand PTE accumulation until they reach the toxic threshold level in their tissues, which leads to toxicity or cellular damage (Figure 1.4) (Cuypers et al., 2013).

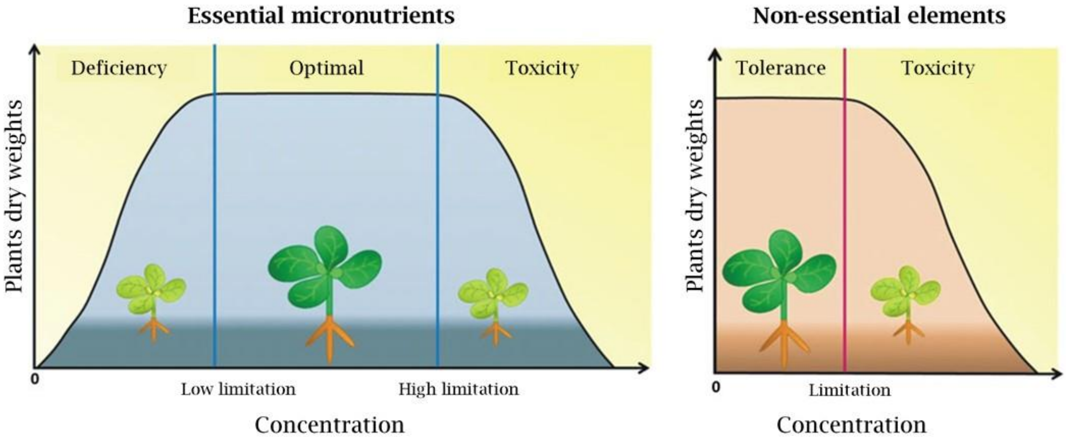

Figure 1.4. Dose-response curves of plants to essential micronutrients and non-essential elements. Axes do not indicate a real scale (modified from Lin & Aarts, 2012; Alloway 2013).

Deleterious effects due to PTE phytotoxicity can be mostly grouped, from a biochemical viewpoint, in: i) PTE-induced oxidative stress and damage; ii) inactivation of key enzymes through cation replacement; and iii) proteins dysfunctionality due to sulfhydryl groups binding (Cuypers et al., 2013). However, it has been stated that most of the observed physiological disturbances in PTE-exposed plants may be ultimately related to their photosynthetic performance (Cuypers et al., 2013).

Cadmium is a non-essential metal, toxic for certain metabolic processes, which is effectively absorbed by plant roots and whose toxicity lies on the disturbance of enzyme activities (Kabata-Pendias, 2011). Cadmium hampers the uptake and transport of several elements (Ca, Mg, P and K) and disrupts the water balance. The visible symptoms of Cd injury are reflected as chlorosis, growth inhibition, browning of root tips and plant death (Kabata-Pendias, 2011). Cadmium alters as well the functionality of membranes (inducing lipid peroxidation) and chloroplast metabolism through the inhibition of chlorophyll biosynthesis and the reduction of the activity of CO_2 fixation enzymes (Gill, 2014). Cadmium toxicity also results in disturbed stomatal conductance and electron transport flow (Kabata-Pendias, 2011).

Lead is also a non-essential element that can cause adverse effects on morphology, seed germination, seedling growth, photosynthesis, water content, mineral nutrition and enzymatic activities on all plant species (Gjorgieva Ackova, 2018). The primary subcellular effect of Pb is the disturbance of the electron transfer reaction, thus inhibiting respiration and photosynthesis (Kabata-Pendias, 2011). In addition, elevated Pb concentrations induce oxidative stress by increasing the production of reactive oxygen species (ROS) in plants (Gill, 2014).

Copper is an essential metal in plants that acts as a co-factor of several key enzymes and proteins (Kabata-Pendias, 2011). Excess Cu has been reported to damage root growth by altering membrane permeability and nutrient (K and P) and water uptake (Shabbir et al., 2020). High levels of Cu diminish the content of photosynthetic pigments and electron carriers, thereby hampering photosynthetic rate (Kumar et al., 2021). As a redox active metal, excess Cu exposure also triggers ROS overproduction and DNA damage in plant cells (Kabata-Pendias, 2011).

Zinc plays an essential role in metabolic processes of plants, and most plant species have great tolerance to elevated concentrations of Zn (Kabata-Pendias, 2011). The most significant activity is as component of a variety of enzymes, such as dehydrogenases, proteinases, peptidases and phosphohydrolases, it being involved in carbohydrate, nucleic acid and lipid metabolisms (Kabata-Pendias, 2011). However, chlorosis, depressed plant growth, imbalanced mineral nutrition, decreased photosynthetic and respiratory rate and enhanced generation of ROS are possible symptoms of Zn toxicity (Kabata-Pendias, 2011; Kaur & Garg, 2021). Moreover, excess Zn can produce Cu and Mn deficiencies in plant shoots, owing to limited transport of these elements from root to shoot, leading to root accumulation of Fe and Mn (Martínez-Alcalá & Bernal, 2020).

1.3. Arsenic occurrence in plants and soils

Arsenic is a naturally occurring metalloid, considered one of the most toxic PTE and known to cause toxicity in plants, animals and humans (Bali & Sidhu, 2021). In fact, the Agency for Toxic Substances and Disease Registry (ATSDR) has ranked arsenic to the first position among the 20 priority hazardous substances (Sodhi et al., 2019). Arsenic is present in more than 200 minerals in the Earth’s crust, in the form of sulfides, oxides, arsenates, arsenites, arsenides and as elemental As. The background As concentration in the soil ranges from 5 to 10 mg kg^{-1}, with an average As concentration of 7 mg kg^{-1} reported in European topsoil (Kabata-Pendias, 2011; Abbas et al., 2018). The World Health Organization (WHO) recommends a safe threshold limit of arsenic in drinking waters of 10 µg L^{-1} (WHO, 2017). Nevertheless, As presence in groundwater has been observed in many countries, showing concentrations within a wide range (from 0.5 to 5000 µg L^{-1}), and more than 200 million people worldwide are at risk of As poisoning, especially in South Asia (Bali & Sidhu, 2021; Nabi et al., 2021).

The speciation of As, that is the different chemical forms, oxidation states and mineral phases in which it can be found in the nature, is often more important than its total concentration regarding its mobility, bioavailability and toxicity (Abbas et al., 2018). The main oxidation states of As in nature are -3 (arsine), 0 (elemental), +3 (arsenite) and +5 (arsenate), with As(III) and As(V) as the most abundant (Kabata-Pendias, 2011; Moreno-Jiménez et al., 2012; Bhattacharya et al., 2021). Arsenic in soil can also occur as organic species, like trimethylarsine oxide (TMAO), monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Bhumbla & Keefer, 1994; Zhang & Selim, 2008). However, these organic forms are not the major As species in the soil, since they appear mainly as a consequence of the utilization of arsenical pesticides (Zhang & Selim, 2008; Bali & Sidhu, 2021). In addition, inorganic species of As (As(III) and As(V)) are more toxic and mobile than the organic As species (Abbas et al., 2018). The abundance of the different As inorganic forms in the soil depends on the pH and redox potential of the surrounding environment (Nabi et al., 2021). Arsenic solubility in the soil generally increases with a rise in soil pH and a decrease in soil redox potential, due to As desorption from Al, Fe or Mn oxides and oxyhydroxides (Bhumbla & Keefer, 1994; Moreno-Jimenez et al., 2012). However, in the usual range of soil pH (4-9), the predominant As species found in reducing environments is H_3AsO_3 (As(III)), while under oxidizing or aerobic conditions the prevalent

As(V) species are $H_2AsO_4^-$ and $HAsO_4^{2-}$ (Figure 1.5) (Smedley & Kinninburgh, 2002).

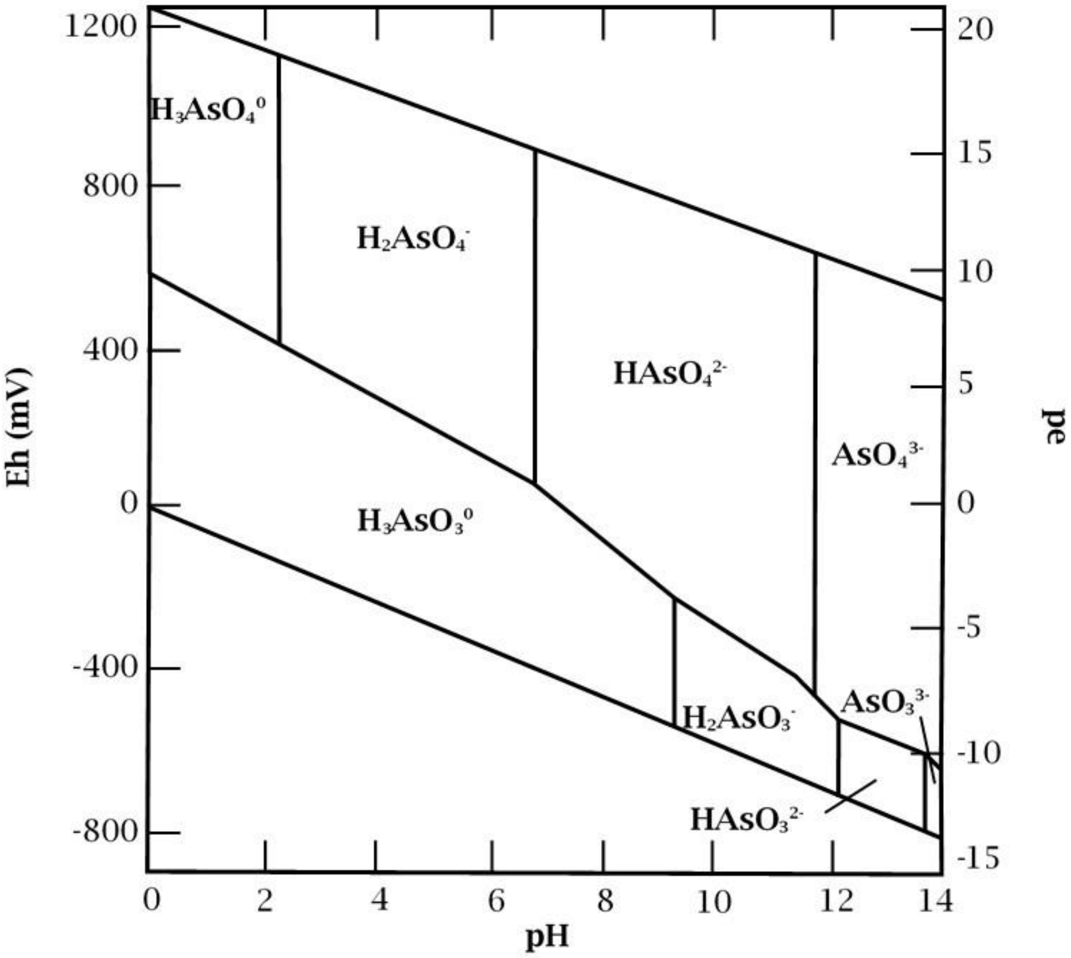

Figure 1.5. Eh-pH diagram for aqueous As species in the system As-O_2-H_2O at 25 ˚C and 1 bar total pressure (Smedley & Kinninburgh, 2002).

Soil microorganisms are able to alter As chemical speciation, affecting As adsorption/desorption, solubility, bioavailability, mobility, and soil-plant transfer (Zhang & Selim, 2008; Mishra et al., 2017). Microbes can interconvert As(III) and As(V), biomethylate inorganic As species to organic forms of As and demethylate them as well (Sodhi et al., 2021), thus being capable of either solubilizing or immobilizing As in the soil-plant system (Abbas et al., 2018). Microorganism species and their mechanisms involved in As biotransformation are now being highlighted to reach effective bioremediation strategies (Bhattacharya et al., 2021). In addition, the bioavailability of As is also greatly influenced by soil organic matter, although inconsistent effects have been reported (Nabi et al., 2021). Organic matter has been characterized both by the capacity to retain As and by its competition with As for soil retention positions (Arco-Lázaro et al., 2016). There is a complex relationship between soil arsenic and organic matter that depends on multiple factors including the ratio of soluble organic carbon, the fractions of insoluble and stable humus, and the concentrations of Fe, Al, and Mn present in the organic matter (Moreno-Jiménez et al., 2012).

1.3.1. Arsenic uptake and transport in plants

Arsenic is considered non-essential for living organisms and the production of reactive oxygen species (ROS) is its most dangerous biochemical effect at subcellular level (Abbas et al., 2018). Plants normally take up As through root absorption in its inorganic forms, although organic forms can be also taken up via different transporters and pathways (Bali & Sidhu, 2021). The uptake rate of methylated As species is lower than the inorganic species, even though the latter are more noxious (Abbas et al., 2018; Bali & Sidhu, 2021).

Arsenic(V), the predominant species under oxidizing soil conditions, enters root cells through phosphate transporters (PHTs) (Figure 1.6), predominantly high affinity PHT1-type transporters, due to their structural analogy with phosphate (Pi) (Finnegan & Chen, 2012; Abedi & Mojiri, 2020). Under Pi sufficient environments, the overexpression and downregulation of PHTs promote plant tolerance to withstand As-enriched conditions (Bali & Sidhu, 2021). Several phosphate transporters have been identified for the uptake of As(V) in different plants, such as rice (*Oryza sativa* L.), *Arabidopsis* *thaliana* and the As-hyperaccumulator *Pteris vittata* (Zhao et al., 2010; Awasthi et al., 2017). Also, As(III) and methylated As species are transported through different aquaglyceroporins (Figure 1.6), predominantly via nodulin 26-like intrinsic protein (NIP) subclass of aquaporin channels (Awasthi et al., 2017). NIPs are sub-divided into three main groups, namely NIP-I, NIP-II and NIP-III, which mediate the transport of a range of small molecules including ammonia, urea, boron or silicic acid. There are at least 15 NIP transporters identified in rice and *Arabidopsis* (Zhao et al., 2010; Abedi & Mojiri, 2020). Nevertheless, the major silicon transporters are: Lsi1 (NIP2;1), which is involved in As(III) uptake as well as MMA and DMA uptake; and Lsi2 (NIP3;2), which mediates As(III) efflux and xylem loading (Awasthi et al., 2017; Bali & Sidhu, 2021). Besides NIP transporters, plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs), two different aquaporin subfamilies, are involved in As(III) uptake (Awasthi et al., 2017).

Once As enters into the plants, it exists mostly in its reduced form, what means that As(V) is rapidly reduced to As(III) by HAC1 (high arsenic concentration 1 arsenate reductase) (Figure 1.6). HAC1 has been found in the outer cell layer of the root (epidermis), facilitating the efflux of arsenite back into the soil, thus limiting its accumulation in the root. In addition, HAC1 in the inner layer adjacent to the xylem (pericycle) may play a role in reducing arsenic loading into the xylem and transport to the shoot (Chao et al., 2014). Arsenic(III) detoxification is accomplished by the formation of thiol-rich peptides complexes, such as those formed with glutathione (GSH) and phytochelatins (PCs), and their subsequent storage in vacuoles (Figure 1.6) (Kumar et al., 2015). High accumulation of As(V), higher translocation of As(III) and lower tendency for As(III)-thiol complexes formation have been found in As-hyperaccumulating plant species compared to non-As-hyperaccumulators within tolerance strategies (Abbas et al., 2018). Translocation of As from the root to the shoot and its further distribution among different plant tissues takes place through the xylem, in which methylated As species mobility is faster (Kumar et al., 2015). Arsenic-thiol complexes were not detected in the xylem sap of sunflower and castor bean, suggesting that thiol complexation restricts As(III) movement (Awasthi et al., 2017). In addition, the speciation of As in the phloem is crucial, since it determines the distribution of As within plant tissues, organic arsenic species being more transportable too (Abbas et al., 2018). In this way, As(III) is transported to rice grains principally through the phloem, whilst DMA is translocated to rice grains through both the xylem and phloem pathways (Abedi & Mojiri, 2020).

1.3.2. Arsenic phytotoxicity

Arsenic has no known biological function in plants and may cause toxicity through morphological, physiological and metabolic disturbances (Bali & Sidhu, 2021; Nabi et al., 2021). Arsenic exposure has been shown to cause a reduction in both shoot and root growth, fresh and dry weight, number of leaves and in the leaf area of plants; As triggers wilting, curling and necrosis of leaves (Nabi et al., 2021). Cellular membranes are susceptible to As damage due to an imbalanced nutrient uptake and reduced stomatal conductance, disturbing the transpiration process (Figure 1.7) (Abbas et al., 2018; Bali & Sidhu, 2021).

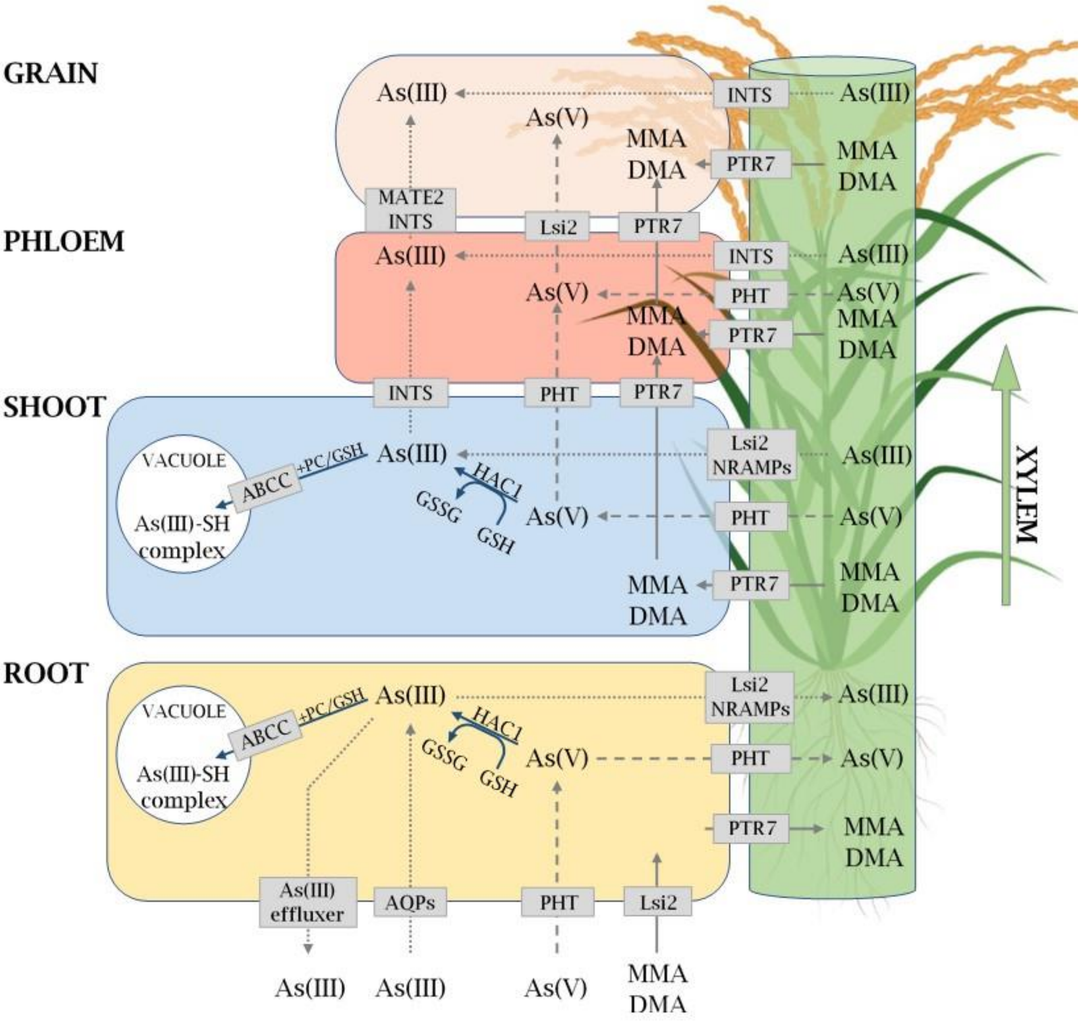

Figure 1.6. Arsenic uptake and distribution transporters in plant tissues. PHT: phosphate transporters; AQPs: aquaporins, including NIPs, PIPs and TIPs; Lsi1: low silicon transporter (NIP2;1); PTR7: nitrate transporter 1/peptide transporter; Lsi2: low silicon transporter (NIP3;2); NRAMPs: Natural Resistance-Associated Macrophage Protein 1; ABCC: c-type ATP binding cassette transporters (vacuolar storage); INTS: inositol transporter; MATE2: multidrug and toxic compound extrusion transporters; HAC1: high arsenic concentration 1 arsenate reductase; As(III) effluxer: undefined transporter. Modified from Bali & Sidhu (2021) and Thounaojam et al. (2021).

Photosynthetic rate has been widely reported to be altered in As exposed plants (Abbas et al., 2018). The light harvesting system is affected by As with the reduction in chlorophyll content and photosystem II (PS II) efficiency (Figure 1.7) (Finnegan & Chen, 2012). Arsenic may compete and replace Mg ions in the chlorophyll molecule, disrupting the photosynthetic activity (Bali & Sidhu, 2021), and may also cause injuries in chloroplast membrane (Abbas et al., 2018). In addition, enzymes involved in CO_2 fixation might be hampered by As, resulting in a reduction of its rate (Nabi et al., 2021). Carbohydrates metabolism is deleteriously affected in As-stressed plants (Figure 1.7) (Abbas et al., 2018) and soluble sugars may accumulate in the plants. Also, a large percentage of recently fixed carbon is stored as starch as a result of the inhibition of starch degrading enzymes (Finnegan & Chen, 2012; Nabi et al., 2021).

Owing to the structural analogy, As(V) toxicity in plants occurs mainly due to the replacement of Pi in key biochemical reactions (Mishra et al., 2019). Arsenic(V)-sensitive processes include glycolysis, phospholipid and nucleic acids metabolism, and protein (de)phosphorylation of cellular signaling cascades (Figure 1.7) (Finnegan & Chen, 2012). Arsenic(V) can interfere in the ATP production by forming ADP-As(V) adducts and disrupting the energy status of the cell as well (Figure 1.7) (Abbas et al., 2018). The toxicity of As(III) is also a result of its affinity to bind up thiol groups. Arsenic(III) can bind to transcription factors, signal transduction and structural proteins, or to metabolic enzymes (Finnegan & Chen, 2012), this leading to protein misfolding and functionality loss (Figure 1.7).

Besides the mentioned direct effect of As on plants normal functioning, As is well demonstrated to enhance the production of ROS (Figure 1.7), including superoxide ($O_2^{\cdot-}$) and hydroxyl ($^{\cdot}OH$) free radicals, and non-radical singlet oxygen ($^1O_2$) and H_2O_2, resulting in a cascade of irreversible injuries in plants (Sharma et al., 2012). ROS are the byproducts of the aerobic metabolism pathways placed in chloroplasts, mitochondria and peroxisomes (Mishra et al., 2019). Nevertheless, an imbalance between ROS production and scavenging is caused under As stress in addition to under other PTE stressors (Abbas et al., 2018). Generally, electron transport chain (ETC) in PSI and PSII in chloroplasts, and complexes I, II and III in mitochondria, are the main source of ROS in these organelles because of the lack of electron acceptors. However, enzymatic reactions are also responsible of ROS in mitochondria and in peroxisomes (Sharma et al., 2012). The As-induced burst of ROS may overwhelm the defense mechanisms leading to cellular damage, and ultimately, cell death. The electron leakage during As(V) reduction to As(III) and the induction of PCs synthesis are supposed to play an important role in ROS overproduction because of As stress (Meharg & Hartley-Whitaker, 2002; Finnegan & Chen, 2012).

The excessive ROS production damages amino acids and proteins, lipids, carbohydrates, and DNA (Figure 1.7) (Finnegan & Chen, 2012). Cellular and organellar membranes alteration is mainly a consequence of lipids oxidation in response to As stress. Enhanced lipid peroxidation takes place when ROS level is above a permissible threshold (Abbas et al., 2018). Under stressful conditions, an increase in the extent of lipid peroxidation has been settled as a ROS-mediated membranes damage indicator (Mishra et al., 2019). The polyunsaturated fatty acids (PUFAs) in the phospholipids of membranes are notably sensitive to ROS attack (Sharma et al., 2012). Lipid peroxidation reactive byproducts include alkoxyl radicals and aldehydes, such as malondialdehyde (MDA), some of which can conjugate with both DNA and proteins (Abbas et al., 2018). The direct ROS-induced modifications in proteins activity likely come from carbonylation, disulfide bond formation and nitrosylation (Sharma et al., 2012). When the amino acids Arg, Cys, His, Lys, Met, Pro, Thr and Trp of any protein become oxidized, free carbonyl groups, disulfide bridges or sulfoxide derivatives are formed, which may inhibit or alter the protein activities. These proteins subsequently become more susceptible to proteolysis (Abbas et al., 2012). Furthermore, both the sugar and base moieties of DNA are vulnerable to ROS oxidation, yielding mismatches between strands and genotoxicity (Sharma et al., 2012).

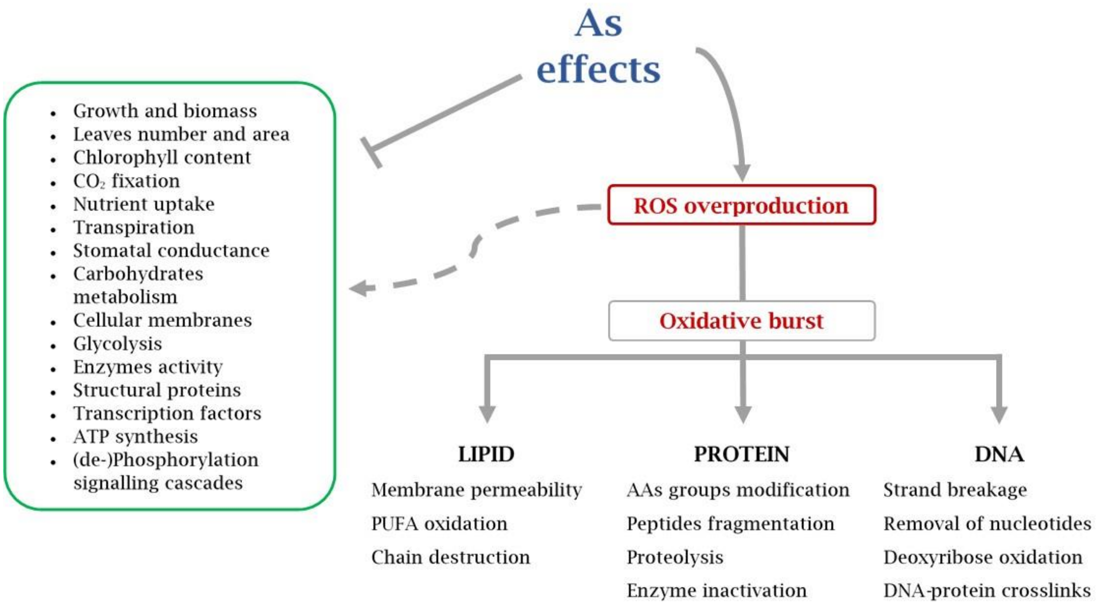

Figure 1.7. Direct phytotoxic effects of arsenic on plant functions and induced effects due to ROS overproduction as a response to As stress. From Sharma et al. (2012) and Abbas et al. (2018).

The majority of these ROS effects on biomacromolecules and cell functions are common to different abiotic stressors. Nevertheless, ROS are not only toxic by-products of metabolic processes, ROS primarily function at basal levels is as secondary messengers to increase tolerance against diverse environmental stresses (Mittler, 2017; Hasanuzzaman et al., 2020).

1.3.3. Future perspectives in As-stress amelioration

Different strategies can be adopted by the plants to cope with As-stress, whether the aim is to reduce As accumulation in crop plants (such as rice, to avoid As entering in food chain) or to enhance As levels in shoots, like in hyperaccumulators. The methodology applied in recent As-research is related to priming, either seed priming, foliar sprayed or exogenous application, and omics technologies. Priming had been generally used to trigger a faster or robust defense response in plants under biotic and abiotic stress, including alleviating As toxicity. Reduced As uptake or translocation along with diminished oxidative damage had been achieved by priming (Saboor et al., 2019). However, priming technology was also applied to increase the phytoextraction efficiency, by increasing plant biomass and enhancing PTE removal, in contaminated soils (Chen et al., 2022) and, similarly, Grifoni et al. (2021) found that cytokinin was able to enhance As translocation into the aerial parts of *Cannabis sativa*. Phytohormones are the most conventional priming agents, in addition to ascorbic acid or melatonin, among others (Ibrahim, 2019). Nevertheless, priming possibilities are as broad as the type and concentration of the compound and the application mode can be, which means that there is still a wide range of possible research approaches in this regard that need to be studied.

Identification and modulation of the expression of As responsive genes and transporters have helped in understanding the As-tolerance mechanisms displayed by plants (Kumar et al., 2015). Transporters or proteins involved in the uptake and translocation of As, chelation and compartmentalization to vacuole, efflux from the roots and As-species interconversion are the crucial players determining As concentrations in various tissues, thus their encoding genes have been an important target to engineer plants (Kumari et al., 2018). Arsenic exposure leads to the upregulation of certain defense and stress-responsive transporters and proteins like heat shock proteins (hsp), metallothioniens (MTs), glutathione S-transferase (GST), or MATE and NRAMP transporters, whereas certain genes encoding phosphate transporter or peroxidases are downregulated (Gautam et al., 2020). Namely, overexpression of aquaporins of PIP group and *OsPHT1;8* in rice plants increased As tolerance of transgenic plants as compared to wild type. Similarly, the overexpression of *OsHAC1;1* and *OsHAC1;2* in rice resulted in higher As(V) reduction and As(III) efflux, whilst As was accumulated when these genes were knocked out (Shi et al., 2016). In *Arabidopsis thaliana*, the knockout of vacuolar sequestration ABCC transporters led to As hypersensitivity as well as increased As tolerance and accumulation through the overexpression of PCs synthase (PCS) (Li et al., 2004; Song et al., 2010).

However, As(V) and As(III) make use of the same transport system of essential elements like phosphate or boron; therefore, the modulation of the expression of these transporters needs to be meticulous to avoid blocking (Kumari et al., 2018). In addition, the genetic and biochemical mechanisms underlying hyperaccumulators response to As exposure may serve as a tool in designing strategies for both mitigation and phytoextraction purposes. Modulation of genes related to defense and transport of As is common to other environmental stresses, thus there is a need of detailed studies to identify specific pathways involved in As stress (Kumar et al., 2015). For that reason, advanced biotechnological approaches would open up possibilities for producing ambition transgenic plants with increased or decreased As accumulation capacity.

1.4. Plant response to PTE stress

Plant responses to PTE stress are the combined results of cellular transport mechanisms and the activation of signal transduction pathways (Lin

& Aarts, 2012). These defense processes are dependent on the PTE type, stress level, plant species and cell type (Choudhury et al., 2017).

1.4.1. Antioxidant defense system

The main components of the defense machinery of plants are low molecular weight antioxidant metabolites and antioxidant enzymes. Enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT), and the enzymes involved in the ascorbate-glutathione cycle (AsA-GSH; Foyer–Halliwell-Asada pathway), which are briefly described in Figure 1.8. Plants protect themselves from the oxidative stress of free radicals increasing those antioxidant enzyme activities (Abbas et al., 2018). GSH, AsA, NAD(P)H, proline, carotenoids, tocopherols and phenolics are the primary non-enzymatic antioxidants.

Ascorbate is the most abundant and powerful ROS scavenger, capable of donating electrons in both several enzymatic and non-enzymatic reactions (Sharma et al., 2012). AsA can be found in all plant tissues and nearly all cell compartments, although it mostly remains in a reduced form in leaves and chloroplasts under normal conditions (Hasanuzzaman et al., 2020). It protects membranes by directly scavenging $O_2^{\cdot -}$ and $OH^{\cdot}$ and by regenerating α-tocopherol from its radical. AsA also plays an important role in cell division, hormone biosynthesis and preserves the activities of metal-binding enzymes (Das & Rouychoudhury, 2014). Glutathione is a thiol tripeptide (γ-glutamyl-cysteinyl-glycine; GSH) abundantly found in almost all cellular compartments, which plays a central role in antioxidant defense due to its high reductive potential (Hasanuzzaman et al., 2020). It is involved in a wide range of biological processes, including cell growth and senescence, regulation of sulfate transport, detoxification of xenobiotics, conjugation of metabolites, regulation of enzymatic activity, signal transduction and the expression of the stress responsive genes (Sharma et al., 2012). GSH is also precursor of phytochelatins, thus minimizing another potential source of ROS formation by chelating PTE in plants (Das & Rouychoudhury, 2014). GSH directly scavenges H_2O_2, $^1O_2$, $OH^{\cdot}$, and $O_2^{\cdot -}$ and is crucial in the regeneration of AsA (Sharma et al., 2012).

Proline (Pro) is a well-known osmoprotectant, which is largely accumulated in response to environmental stresses. Pro protects plants against ROS-mediated damages as an efficient scavenger of $OH^{\cdot}$ and $^1O_2$, and inhibiting lipid peroxidation-related damages (Das & Rouychoudhury, 2014; Abbas et al., 2018). In cytoplasm, Pro protects and stabilizes proteins acting as a molecular chaperone in addition to act as a metabolic signaling molecule and influence cell growth and cell death (Szabados & Savouré, 2010; Hayat et al., 2012).

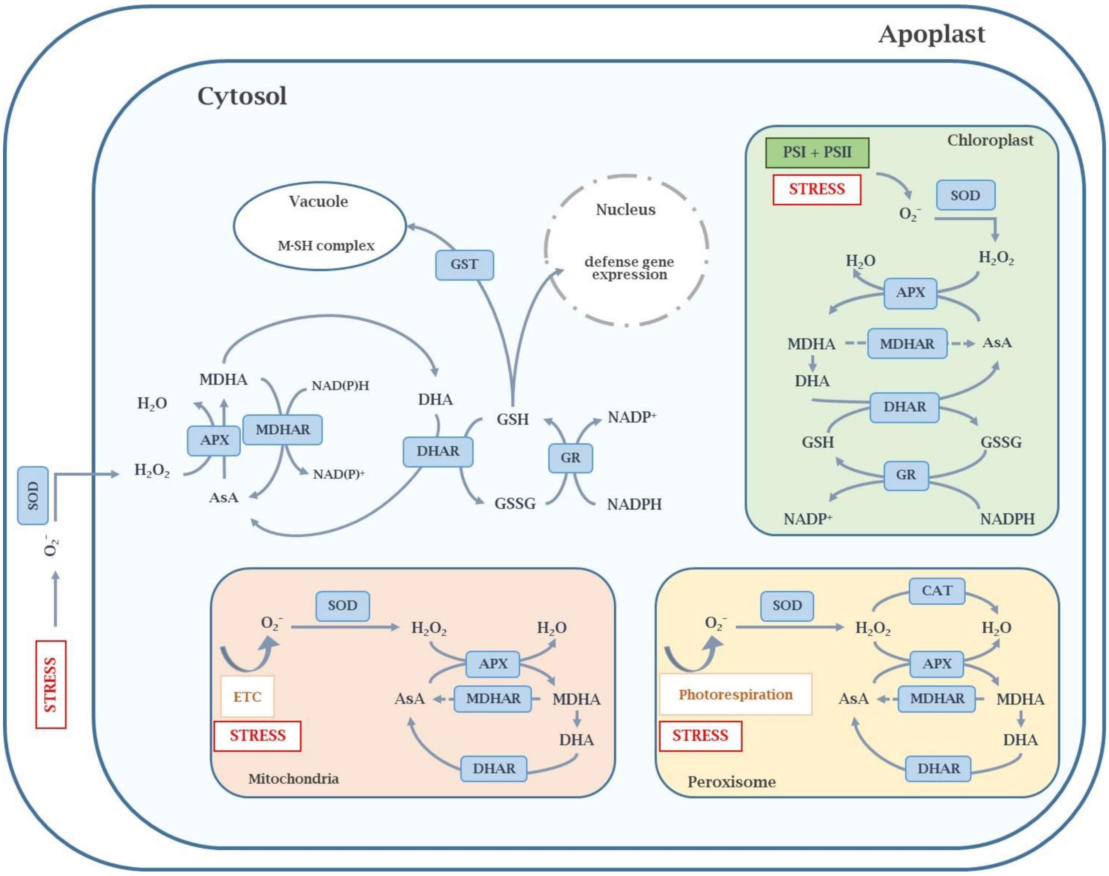

Figure 1.8. ROS formation and enzymatic scavenging in the cytosol, chloroplasts, mitochondria, and peroxisomes. SOD, superoxide dismutase; CAT, catalase; APX, ascorbate peroxidase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GST: glutathione S-transferase; M: metal(loid); AsA, ascorbate; GSH, reduced glutathione; GSSG, oxidized glutathione; PSI/II, photosystem I/II; ETC, respiratory electron transport chain. Modified from Ding et al. (2020).

Carotenoids are part of the lipophilic antioxidants and are capable of detoxifying several forms of ROS (Das & Rouychoudhury, 2014). Carotenoids have three major roles: i) absorb light wavelengths between 450 and 550 nm and transfer the captured energy to the chlorophyll molecule; ii) prevent the formation of $^1O_2$ to protect the photosynthetic apparatus; and iii) dissipate the excess excitation energy as heat. Carotenoids also serve as precursors to signaling molecules that affect plant development and biotic or abiotic stress responses (Sharma et al., 2012). Tocopherols also belong to the lipophilic antioxidants family, which efficiently scavenge ROS and lipid radicals. Tocopherols are synthesized only by photosynthetic organisms and thus are solely present in green parts of plants (Das & Rouychoudhury, 2014). Tocopherols protect lipids and other membrane components of chloroplasts by quenching O_2, thus protecting both the structure and function of PSII. Among the four tocopherol isomers (α-, β-, γ-, δ-), α-tocopherol has the highest antioxidant capability (Sharma et al., 2012). Phenolic compounds constitute a diverse group of secondary metabolites, with an appropriate chemical structure that confers them antioxidant properties. Phenolics can directly scavenge ROS and lipid radicals or chelate metal ions. Phenolic metabolism has been reported to be induced in plants as a response to different stresses (Sharma et al., 2012).

1.4.2. Metal-binding peptides mediated detoxification

The principal method of PTE detoxification in plants is their chelation and sequestration by metal-binding peptides. The best-characterized chelating ligands in plant cells are the phytochelatins (PCs) and metallothioneins (MTs). PCs are enzymatically synthesized peptides, whereas MTs are gene-encoded polypeptides (Cobbett & Goldsbrough, 2002).

MTs are cysteine-rich polypeptides with high affinity for both essential and non-essential metals, which is significant for the maintenance of homeostasis of some essential elements such as Zn and Cu ions, in addition to the protection mechanism (Anjum et al., 2015).

PCs are the principal metal-detoxifying thiolate peptides, synthesized by PC synthase (PCS) from GSH, with the general structure of $(\gamma\text{-Glu-Cys})_n\text{-Gly}$ (n = 2-11), PC2 and PC3 being the most common forms (Mitra et al., 2018). The synthesis of PCs is rapidly induced in the presence of metals such as Cd, Cu, Zn, Ag, Au, Hg and Pb, which depends not only on the concentration but also on the existing form of the element. PCs bind metals to their sulfhydryl groups, immobilizing them. Thus, PCs are considered the major mechanism of metal detoxification in higher plants, as immobilized ions are less toxic than free ions (Liu et al., 2015). PCs production increase has been largely reported upon As exposure. Furthermore, it is widely reviewed that As in plants is mainly complexed with PCs and stored in the vacuole, where they remain stable and allow the accumulation of high As concentrations, without overly affecting normal cellular functioning (Awasthi et al., 2017; Mitra et al., 2018; Bali & Sidhu, 2021).

1.5. Relevance of stress related parameters in PTE phytoremediation approaches

Through the evaluation of certain physiological parameters, a better understanding of plants response to PTE contamination, including As, can be reached, and allow the selection of the most suitable species for each particular phytoremediation process (phytoextraction, phytoimmobilization, etc.). In this sense, the screening of plant species in order to increase the number of suitable species that could be potentially used at the different contaminated scenarios has been often carried out (Becerra-Castro et al. 2018). Thus, the knowledge of the stress response to PTE presence in the soil of different plant species can be considered to be a capital issue for the improvement of (phyto)remediation technologies as well as for the monitoring of the evolution of the restoration processes. Usually, under PTE stress, the biosynthesis pathways of antioxidant compounds and metal-chelators peptides are activated along with antioxidant enzymes activity and the expression of its regulating genes. For instance, Cd induced H_2O_2 generation and lipid peroxidation in rice seedlings (Mostofa et al., 2015; Srivastava et al., 2015), Pb toxicity causing a similar response in *Zygophyllum fabago* seedlings (López-Orenes et al., 2014). Also, aubergine seedlings increased H_2O_2 accumulation and protein oxidation in response to As(V) (Alamri et al., 2020), and Ni toxicity at different levels provoked similar lipid damage and H_2O_2 accumulation in pea (*Pisum sativum)* and in rice seedlings (El-Amier et al. 2019; Hasanuzzaman et al., 2019). Plant species with different tolerance mechanisms differ in their response to PTE stress and their relationships with stress indicators. In fact, comparing the response of an excluder (*Lupinus albus*) with a hyperaccumulator (*Noccaea caerulescens*), Martínez-Alcalá et al (2013) found a stronger lipid peroxidation and Cys and phenolics concentrations where PTE were accumulated (in *L. albus* roots and in *N. caerulescens* aerial parts). However, it is important to consider that the response to the presence of contaminants likely can vary greatly with the duration of stress exposure, tolerance level and even within varieties from the same plant species (Kidd et al., 2015). In this sense, two different varieties of quinoa were assessed for the phytostabilization of As contaminated saline soils, and the more tolerant variety showed lower As accumulation and membrane peroxidation besides higher antioxidant enzyme activities and chlorophylls levels (Parvez et al., 2020).

The exposure to different PTE-stresses triggers proline accumulation and accelerates the development of phytochelatins, thus increasing the metal-chelating capacity of the plants (either metal-Pro and metal-PC complexes) (Ghosh et al., 2021). Phytochelatins synthesis and accumulation has been reported to be widely enhanced under PTE stress, and therefore it can be used as a biomarker for the early detection of PTE stress in plants (Ahmad et al., 2019). The expression of phytochelatin synthase gene (*PCS1*) was monitored in tomato plants and higher expression was related with the highest doses of PTE (50 ppm Cu, Pb or Cd) (Kisa, 2019). Proline concentration has been reported to increase in Ni-stressed rice and Cu-/Zn-stressed tomato plants (Hasanuzzaman et al., 2019; Badiaa et al., 2020). Proline pretreatment of different plants (wheat and Indian mustard) displayed tolerance to certain PTE through, for example, lesser degradation of chlorophylls and accumulation of MDA and H_2O_2 (Rasheed et al., 2014; Noreen et al., 2018; Wang et al., 2022).

Besides the direct quenching of ROS by proline, it up-regulates antioxidant enzymes (Ghosh et al., 2021). To sustaining the cellular redox status, different plants subjected to As or Cd stress exhibited increases in SOD, CAT and APX activities and diminished MDHAR, DHAR and GR activities, leading to the synthesis of antioxidants (AsA, GSH and NADPH) (Jung et al., 2019; Parvez et al., 2020; Jung et al., 2021). However, photosynthesis sustains plants survival so that the different evaluable photosynthetic machinery could easily advance the capacity of plants to deal with PTE stresses.

Despite the above mentioned findings regarding stress response to PTE toxicity are primarily studied under hydroponic conditions, similar results had been observed in contaminated soil experiments (Moreno-Jiménez et al., 2010). The clear insights provided by hydroponic studies often reflect the behavior that takes place in the complex plant-soil system interactions. For example, *Salix triandra* plants grown in As and Pb contaminated soil exhibited increased levels of proanthocyanidins and phenolic compounds, and of antioxidant activity (through DPPH radical scavenging and GST and SOD enzymatic activities) (Lebrun et al., 2020). Also, protein and lipid oxidation was greater in *Silybum marianum* plants grown in a high-PTE-contaminated mining soil (Clemente et al., 2019). However, the PTE-induced oxidative stress response is largely diminished with the use of soil amendments. Shahid (2021) carried out a meta-analysis regarding the effects of soil amendments on trace-element-induced oxidative stress, which shed light on an overall decrease in stress parameters (ROS and lipid peroxidation) in addition to an increase in antioxidants and photosynthetic pigment contents. In any case, the general trends in plant response also vary for different kinds of soil amendments, PTE, plant species and organs, and exposure cultures, so that specific responses still can be of great interest for further research.

1.6. Context of study site

The Sierra Minera of Cartagena-La Unión (Murcia, SE Spain) has been intensely mined until the last decade of the twentieth century to obtain metals like Ag, Fe, Pb and Zn. The mining activities resulted in an anthropogenically impacted landscape and an important degradation of the natural environment in this area, that still holds numerous abandoned mine tailings accumulation ponds and heaps of highly unstable waste materials. The soils that remain bare in the area are highly enriched with PTE, usually in soluble and phytoavailable forms (Conesa & Schulin, 2010; Pardo et al., 2017), which, together with their low OM and nutrients content and their poor physical structure, hinder plant growth and leave soils exposed to wind and water erosion (Martínez-Fernández et al., 2014). The dissemination of contaminants from these tailings, such as As, Cd or Pb, represents a threat for the surrounding ecosystem and for human health and, therefore, requires remediation to reduce the environmental risk they pose. Numerous efforts and scientific research have been performed in the last two decades to alleviate and remediate PTE toxicity in these soils (e.g., Párraga-Aguado et al., 2014; Arco-Lázaro et al., 2017; Pérez-Sirvent et al., 2018; Clemente et al., 2019; Visconti et al., 2020).

Due to the semi-arid climatic conditions of the studied area, these soils suffer long drought periods, but may get rapidly flooded during heavy rainfalls events, which can alter soil redox conditions and PTEs biogeochemistry. The resulting changes provoked in the PTEs speciation may directly affect their mobility and phytoavailability, frequently increasing their toxicity. Wetland macrophytes have been considered appropriate plant species for the phytoremediation of the mining soils from Sierra Minera of Cartagena-La Unión (Ait Ali et al., 2004; Pardo et al., 2016). Macrophytes are well adapted to fluctuating water conditions and tolerate large PTEs concentrations and may act as biofilters for PTEs removal or as biomonitors. Common reed (*Phragmites australis* (Cav.) Trin. ex Steud.) is a macrophyte species broadly proposed as a suitable option for the phytostabilization of PTEs-contaminated soil and rhizofiltration in water environments. High biomass production, well-developed roots and high tolerance to PTEs characterize common reed and make it a good candidate for the accumulation and sequestration of these pollutants. In addition, common reed has been found growing spontaneously in the contaminated soils of the Sierra Minera and is therefore already adapted to the semi-arid climatic conditions of the area (Conesa & Schulin, 2010; Pérez-Sirvent et al., 2017).

Rice (*Oryza sativa* L.) is one of the most important staple crops worldwide. The frequent cultivation of rice in As-enriched land and its small genome size has given rise to extended environmental research using this species. As a consequence, rice has become a model plant for studies regarding As accumulation and tolerance in plants. Arsenic(III) is the predominant species of As in soil solution under reducing conditions in paddy soils, where rice toxicity has been extensively studied (Khan et al., 2010; Syu et al., 2015). Contrarily, As(V) has been found to be the dominating species in the soil solution of strongly oxidized contaminated mine soils (Beesley et al., 2014). Therefore, novel approaches regarding the study of rice behavior under As(V) toxicity would increase the knowledge not only about the physiological and stress response of the plants, but also on the effectiveness of different remediation strategies for As-contaminated areas.

OBJECTIVES

The aim of the present Thesis was to identify and examine the adjustments on plant physiological and oxidative homeostasis to counteract PTEs toxicity in two contrasting plant species, rice and common reed. To achieve the main aim, four partial objectives are addressed:

I. To study the tolerance and oxidative response to PTEs under different water saturation conditions of common reed and rice plants cultivated in different mine contaminated soils. The water regimes were expected to differently influence soil physico-chemical conditions, varying available PTEs in soil solution and within soil fractions, and likely causing different stress reactions.

II. To elucidate the influence of As(V) toxicity in the plants under controlled hydroponics conditions, specifically:
    - To evaluate the effects of increasing As(V) concentrations on lipid and protein oxidative damage, and their relation with major As chemical forms in rice plants.
    - To elucidate As accumulation and tolerance in common reed by exposing plants to distinct As(V) concentrations, through the analysis of different nutritional and oxidative stress related parameters and associated to the concentrations of the major As species in the different plant compartments.
    - To analyze the effectiveness of AsA priming in reducing the phytotoxicity of As(V) on the growth, photosynthetic activity, antioxidant compounds and oxidative stress response in rice plants.

The experimental design is summarized in figure 2.1, in which three mining soils and two plant species were used to accomplish the expected aims.

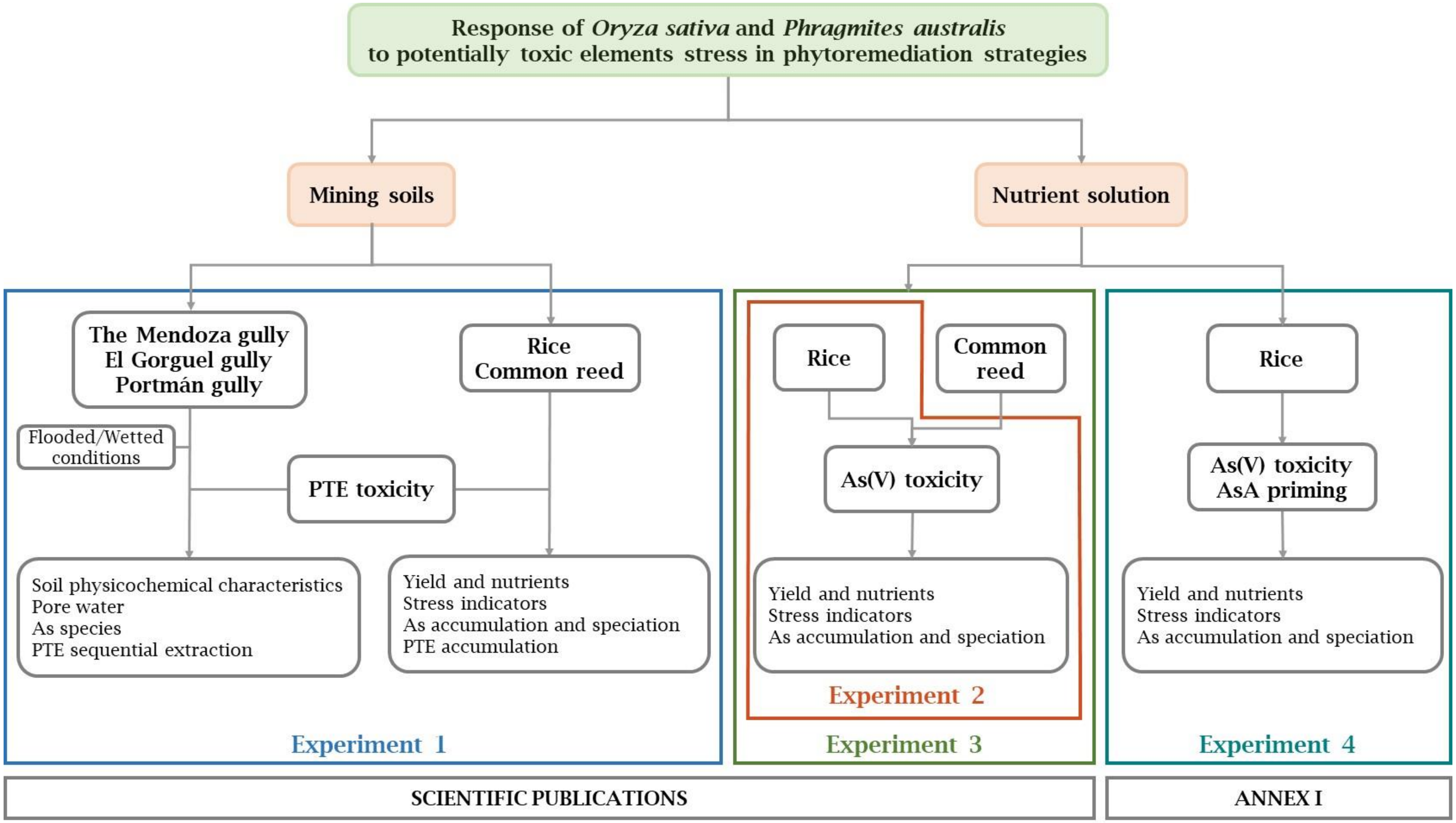

Figure 2.1. Schematic overview of the experiments carried out in the present thesis.

SCIENTIFIC PUBLICATIONS

Differential response of *Oryza sativa* L. and *Phragmites australis* L. plants in trace elements contaminated soils under flooded and unflooded conditions

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M.J. Álvarez-Robles contributed to conception and design, acquisition of data, analysis and interpretation of data, drafting the article, approval of the version to be submitted for publication.

Abstract

Drastic changes in the water regime of trace elements (TEs) contaminated soils under semiarid conditions, from completely dry to flooding situations, may alter the solubility of the contaminants and, therefore, their potential mobility and availability to plants. Certain macrophyte species have shown a promising suitability for their use in the phytoremediation of TEs contaminated soils under fluctuating flooded–unflooded conditions, as a consequence of their high resistance and tolerance to contamination. Similarly, different water conditions occur during rice *(Oryza sativa*) cultivation, a species often used as a model plant for TEs toxicity studies. The aim of this work was to study the tolerance and oxidative response to TEs of common reed (*Phragmites australis*) and rice grown in contaminated mining soils, when exposed to different water saturation conditions. Both species (common reed and rice) were cultivated in three different contaminated soils from the Sierra Minera of La Unión-Cartagena (SE-Spain) under contrasting water saturation conditions (flooded and unflooded) in a pot experiment. Soil EC and elevated metal (mainly Cd and Zn) soluble concentrations conditioned the survival of the plants. Whereas, As accumulation in the aerial part of both species influenced the most oxidative stress homeostasis. Common reed showed to be a good candidate for its use in the phytostabilization of TEs contaminated soils under both flooded and unflooded conditions.

Major As species, lipid peroxidation and protein carbonylation in rice plants exposed to increasing As (V) concentrations.

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M. J. Álvarez-Robles conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; wrote the paper.

Abstract

Arsenic (As) uptake by plants is mainly carried out as arsenate (As(V)), whose chemical analogy with phosphate is largely responsible for its elevated toxicity. Arsenate is known to stimulate reactive oxygen species (ROS) formation in plants that provoke oxidative stress. This manuscript reports the results of a hydroponics study using rice (*Oryza sativa* L.) seedlings as a test plant, where the effects of increasing arsenate concentrations (0–10 mg L^{-1}) on both lipid and protein oxidation, as well as As accumulation and speciation in plant roots and shoots were examined. Plant yield was negatively affected by increasing As concentration. Accumulation in plant roots was higher than in shoots at low arsenate doses (0.5–2.5 mg L^{-1}), while root to shoot transport was drastically enhanced at the highest doses (5 and 10 mg L^{-1}). Moreover, As(V) was the dominating species in the shoots and As(III) in the roots. Rice leaves in the 10 mg As L^{-1} treatment showed the highest lipid peroxidation damage (malondialdehyde concentration), whilst protein oxidation was not remarkably influenced by As dose. Lipid peroxidation seems to be therefore conditioned by As accumulation in rice plants, particularly by the presence of high As(V) concentrations in the aerial part of the plants as a consequence of unregulated translocation from roots to shoots above a threshold concentration (1.25–2.5 mg L^{-1}) in the growing media. These results provide relevant information regarding As(V) toxic concentrations for rice plants, highlight the importance of major As species analysis in plant tissues regarding As toxicity and contribute to better understand plants response to elevated As concentrations in the growing media.

Response of *Phragmites australis* to increasing As(V) concentrations: accumulation and speciation of As, and plant oxidative stress

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Abstract

The use of macrophytes has been proposed recently as a suitable option for the phytostabilization or rhizofiltration of soils or waters contaminated by trace elements. As one of the most representative species of this type of plant, common red (*Phragmites australis* (Cav.) Trin. ex Steud.) has shown tolerance to high concentrations of potentially hazardous elements, as is the case of arsenic. However, a deeper knowledge of how these plants deal with this toxicity, including their oxidative response, is needed for the optimum utilization of this species in phytoremediation procedures. In fact, little is known about how common reed plants react to As toxicity or the tolerance limits and accumulation potential of this species. In this work, common reed plants were exposed to a range of As(V) concentrations (0.5-10 mg L^{-1}) in a hydroponic experiment, and the performance of the plants (growth, photosynthetic pigments, and oxidative stress related parameters) was evaluated and related to the major As species present in the different parts of the plants. The plants did not show any apparent symptom of toxicity and no significant effects were found for any of the different plant parameters analyzed. Arsenic was mostly accumulated as As(III) in the roots of the plants, and almost no translocation to the aerial part of the plants was observed for any of the As species analyzed. Common reed has shown a high capacity for As accumulation in its roots with no signs of toxicity, despite small nutrient imbalances. Thus, it can be considered to be a good candidate for use in the rhizofiltration and phytostabilization of As contaminated waters and soils, respectively.

GENERAL DISCUSSION

4.1. Arsenic accumulation, compartmentalization and speciation in rice and common reed plants

Soil degradation is considered one of the major environmental problems that occur in mining areas, which usually present not only high levels of PTE (potentially toxic elements) but also a hostile habitat for plant growth due to poor soil structure, organic matter and nutrients. A multitude of phytoremediation technologies has been developed for the cleanup of PTE-polluted soils, within which the improvement of the biological and physico-chemical properties of the soil has a key role. Nevertheless, an important issue regarding the distribution of PTE in contaminated soils is whether they are able or not to interact with plants (i.e., present in phytoavailable forms), although their toxicity and accumulation will depend upon the particular plant species involved and the concrete stress response developed (Clemente et al., 2015). In this sense, rice plants from the pot experiment described in experiment 1 were not able to survive in two of the studied mining soils (LB and GR), which agrees with elevated concentrations of available Zn and, especially, Cd found in the PTE-sequential extraction carried out in those soils. A drastic decrease in plant weight and biomass upon Cd exposure has been previously reported in different crop plants like wheat (Agami & Mohamed, 2013), maize (Ekmekçi et al., 2008), mustard (Mohamed et al., 2012) and rice (Aina et al., 2007), as well as plant death in coriander (de Godoi Pereira et al., 2018). In contrast, common reed plants showed higher adaptability and were capable to cope with the same elevated PTE concentrations in our experiment. Ait Ali et al. (2004) also found common reed plants as tolerant to relatively high concentrations of Cd (≤8.8 µM) in the growing media, although the plants showed certain sensitivity to the combined effect of Cd:Cu:Zn presence in nutrient solution. In any case, both plant species showed excluder behavior, retaining the PTE mostly in their roots. Owing to this high-tolerant PTE-dealing ability of common reed, it has been largely reported as an excellent option for phytostabilization of highly contaminated soils (Ghassemzadeh et al. 2008; Rocha et al., 2014; Bello et al., 2018; Bonnano et al., 2018). High tolerance of common reed to PTE includes As, notably present in the studied mining soils (Pardo et al., 2016), but with particular interest due to its different biogeochemistry and behavior in plant as a metalloid.

Arsenic(V) was the only As species found in the soil solution of the highly oxidized mine soils used in the pot experiment. As a consequence, this was the As form used in the next experiments, where rice and common reed plants were exposed to a gradient of As(V) concentrations under controlled conditions (nutrient solution) in order to evaluate plant's tolerance and oxidative response (experiments 2 and 3). The accumulation of As in both rice and common reed plants increased with increasing As dose in nutrient solution, which was therefore highest in the greatest dose used (10 mg L^{-1} As). Common reed bioaccumulation ability resulted in the accumulation of As preferentially in the roots (ca. 2500 mg kg^{-1} in the highest As(V) dose) and mainly as As(III), with very little translocation to the above ground tissues of the plants (< 100 mg kg^{-1}; experiment 3) and very low translocation factors (<< 1), in agreement with previous results for common reed plants in PTE contaminated soils (Castaldi et al., 2018). Contrastingly, the results of the hydroponic experiment (experiment 2) showed elevated total-As accumulation (ca. 600 mg kg^{-1}) in both rice roots (as As(III) and As(V)) and shoots (predominantly as As(V)) in the highest As(V) dose treatment. At lower As(V) doses, much lower As concentrations were found in the aerial part than in the roots, which agrees with the results previously reported by Srivastava et al. (2019) for rice plants grown under 25 µM As(V) in nutrient solution.

In *Arabidopsis thaliana,* the loss of function of the enzyme arsenate reductase HAC1 led to a c. 50-fold increase in As accumulation in the shoots during a short-term As(V) feeding hydroponics experiment (Wang et al 2018). HAC1 is known to confer As(V) tolerance through the reduction of As(V) to As(III), allowing the latter to be extruded to the external medium or sequestered in the vacuole as a phytochelatin(PC)–As(III) complex, thus decreasing the cellular As content in the roots available for xylem loading (Fischer et al., 2021). In this sense, the different predominance of the identified inorganic As species in the studied plants might suggest HAC1 saturation or down regulation in rice plants. The prevalence of As(V) in rice roots enable its translocation and accumulation in shoots, whilst As(III) majority in the roots enhanced As tolerance of common reed. In agreement with the present results, Ye et al. (2010) found that the proportion of As(III) in roots decreased progressively with increasing As(V) exposure in castor bean. Moreover, these authors evidenced that more As(V) was loaded into xylem when the As(V) reduction capacity in roots was exceeded in the treatments with high As(V) concentrations. Ye et al. (2010) also concluded that As(V) could be reduced to As(III) in leaves since As(III) proportion was higher in leaves than in xylem exudates. Despite plants response to As in hydroponics system may vary from their response when cultivated on As-contaminated soils, common reed plants have manifested once again their high capacity for storing contaminants in roots, thus converting this species in a promising plant for its use in phytostabilization.

4.2. Oxidative stress response of the plants to As toxicity and the priming effect of ascorbic acid

Environmental stresses trigger a wide range of physiological and metabolic alterations besides ROS overproduction. Plants antioxidant machinery primarily deals with ROS imbalance, although oxidative damage and cell death is occasionally unavoidable (Hasanuzzaman et al., 2020). The extent of lipid peroxidation and protein carbonylation are used as indicators to scale ROS threats to plant cells. Accumulation of H_2O_2 and the increase in the oxidation of lipids and proteins have been largely reported under As-stress in different plants such as rice (Pandey & Gupta, 2015), lettuce (Silveira et al., 2015), sunflower (Saidi et al., 2017), Artemisia (Kumari et al., 2018) and tomato fruits (Marmiroli et al., 2017), as well as under other abiotic stresses like salinity and drought (Hasanuzzaman et al., 2020), and Cd (Gupta et al.,2017) and Pb (López-Orenes et al., 2018) presence. However, those stress markers were scarcely affected and did not seem to reflect the extent of oxidative damage in rice and common reed plants assessed in the present work, neither in the pot experiment (experiment 1) nor in the hydroponics ones (experiments 2 and 3). Efficient defense mechanisms may have been activated to avoid the injury to membranes and proteins caused by As/PTE-induced ROS (Alfadul and Al-Fredan, 2013), in agreement with the overall lack of visible toxicity symptoms. In this way, photosynthetic pigments, which are very sensitive and easily disrupted under PTE stress (Finnegan & Chen, 2012), remained unaffected in common reed leaves exposed to increasing As(V) in nutrient solution. The maintenance of chlorophyll levels is vital for plant growth and agreed with the plant health status. Nevertheless, AsA priming (experiment 4) also enhanced photosynthetic pigments content in rice plants.

Nonetheless, considering that PTE toxicity usually interferes negatively at all the stages of plant growth, defense priming has recently emerged as an effective and practical method to face land degradation threats and boost resistance to biotic and abiotic stresses, especially in crops (Saboor et al., 2019; Beckers & Conrath, 2007). Plants can be primed for a faster and robust activation of defense mechanisms. They biosynthesize different compounds, such as phytohormones and secondary metabolites, and induce the expression of stress-related genes, in order to adapt themselves to adverse environments and develop immunity and stress tolerance (Conrath, 2011; Sako et al., 2020). Priming effects have been studied mainly for abiotic stimuli like heat, cold, drought, salinity, light, and certain chemical compounds (Llorens et al., 2020). Owing to the greater production of reactive oxygen, nitrogen and sulfur species in response to abiotic stress, low concentrations of these species are widely used as priming agents (Sako et al., 2020). For instance, exogenous application of nitric oxide (NO) and H_2O_2 were capable to alleviate PTE toxicity in different plants (Xiong et al., 2011; Khan et al., 2018a). In addition, ascorbic acid effectiveness as a priming agent has been proven in many studies. It can mitigate the negative effects of cold in wheat (Shah et al., 2019), drought in sunflower (Madany & Khalil, 2017), salinity in pumpkin (Fazlali et al., 2013), heat in tomato (Alayafi, 2020) and Pb toxicity in rice (Khan et al., 2018b). Moreover, rice plants have been shown to be able to counteract As toxicity when they were seed-primed or exogenously treated with salicylic acid (SA), gibberellins (He et al., 2022), methyl jasmonate (MeJA) (Verma et al., 2020), melatonin (Samanta et al., 2021), Si (Khan & Gupta, 2018) and Se (Moulick et al., 2016). Priming technology successfully confers protection against environmental stresses, including mitigation of As-induced phytotoxicity and its accumulation in rice grains (Saboor et al., 2019). In view of this information, ascorbic acid was selected as a priming agent (experiment 4) to assess the possible beneficial effects that this compound may incite in rice plants under As(V) exposure.

Ascorbic acid (AsA), either through simultaneous addition or as a priming agent (pre-addition to the growing media), resulted in a drastic decrease in As accumulation in rice roots. Additionally, As(III) and As(V) concentrations in the aerial part of the plants were reduced with AsA priming, despite no significant differences were found for total-As concentration in rice shoots. These differences in As accumulation may arise from AsA activation of As detoxifaction mechanisms, such as vacuolar sequestration. In agreement with these results, priming with phytohormones resulted in a decrease in As accumulation in both shoots and roots of rice seedlings exposed to 10 µM As(V) (He et al., 2022).

Classically, thiol-containing compounds, especially glutathione (GSH) and phytochelatins (PCs), are thought to be the major chelators of PTE that facilitate their sequestration in the vacuole (Ahammed & Yang, 2022). An increase in non-protein thiols (NPT) and PCs in the roots of As treated rice plants (experiment 4) stands out their role to counteract As toxicity. Nevertheless, polyphenols, the largest groups of secondary metabolites in plants, have manifested their capability to cope with environmental constraints. Under PTE excess, the increase in phenylpropanoids biosynthesis, resulting in the accumulation of phenolic compounds such as specific flavonoids, has been previously reported (Sharma et al., 2019). Flavonoids not only can directly scavenge ROS, but also they act as metal chelators and are crucial as electron donors in the phenolic/ascorbate-peroxidase cycle (Michalak, 2006). Proanthocyanidins (PA), a subfamily of flavonoids, showed increased concentration in the roots of rice plants after As exposure in the final hydroponic experiment (experiment 4), and their concentration was even further increased with AsA addition or priming. As expected, positive correlations were obtained between PA and the antioxidant activities assessed (FRAP and DPPH), which are partially responsible of the antioxidant properties to withstand As toxicity (Agati et al., 2012). Also, the concentration of total phenols, which are considered a good indicator to predict the extent of abiotic stress tolerance in plants (Sharma et al., 2019), solely showed increased accumulation in rice roots. The response of rice plants followed a similar trend in this experiment (experiment 4) to that observed in the previous ones (experiments 1-3), where the roots are the main barrier to face As stress and they trigger the first protection response. In agreement with these results, seed priming with castasterone (a brassinosteroid) ameliorated Cd toxicity in *Brassica juncea* plants and increased flavonoids concentration in the leaves of the plants (Kaur et al., 2017). Similarly, As(III) and As(V) provoked an increase in total phenolics concentration in rice seedlings, which was diminished with an effective supplementation of Se through seed priming (Moulick et al., 2016).

Notwithstanding, proline response to PTE toxicity displayed an optimistic approach regarding phytoremediation and rice crops improvement. Proline noticeably accumulates in plants in response to a variety of adverse environmental constraints, including different PTE. Depending on the stress episode, proline concentration can reach up to 80 % of its background (Kaur & Asthir, 2015). For instance, proline content significantly raised under Cu or Zn toxicity in tomato plants (Badiaa et al., 2020), whereas its concentration was equally enhanced in primed and Cd-stressed *Brassica juncea* plants (Kaur et al., 2017). Similarly, even though AsA priming did not provoke any significant effect in the proline levels in As-treated rice roots, it diminished significantly proline concentration in plant shoots to control levels (experiment 4). Along with osmoregulation, stabilization of membranes and triggering crucial gene expression for defense and recovery from abiotic stresses, proline imparts stress tolerance as metal chelator and free radicals scavenger (Ghosh et al., 2021). Nevertheless, proline is a resource of energy in the recovery process upon relief from stress, since its catabolism can provide 30 ATP equivalents to the demanding cells (Kaur & Asthir, 2015).

4.3. Relationships among the different parameters studied and concluding remarks

The partial conclusions obtained from the present work were supported by the general tendencies observed in the PCAs performed. In experiment 1, EC and PTE conditioned plant growth and stress related parameters (MDA, proline and H_2O_2). Increasing As(V) concentrations in nutrient solution principally caused certain nutrient deficiencies, leading indirectly to the accumulation of MDA in rice plants and proline in common reed (experiments 2 and 3, respectively). High As accumulation observed in the highest As dose treatment was clearly differentiated from photosynthetic pigments and oxidative stress parameters in common reed (no evident interaction; experiment 3), which reinforces the tolerance capability of common reed to cope with As toxicity. Proline was accumulated in rice shoots in response to As presence, whilst As(III) accumulation induced the formation of NPT and PC in the roots (experiment 4). Arsenic(V) was as well responsible of GSH depletion and antioxidant activity boost in rice roots (experiment 4).

Soils deserve the utmost attention to become resilient to climate change and reversing degradation. New soil contamination should be prevented and resorting to management practices that sustain and enhance soil health and biodiversity; in which protection, sustainable use and restoration of soil becomes the norm (EC, 2021b). Common reed suitability for the phytoremediation of PTE and especially As from water and soil environments has been demonstrated in the present work. Roots machinery of this plant shows a high ability to handle toxic levels of harmful elements alongside to the protection of the photosynthetic apparatus in the aboveground tissues. In addition to the possible advantages of its use in the phytomanagement of PTE contaminated environments, its biomass has recently received increasing attention for the production of value-added by-products such as bioenergy (Bernal et al., 2021). Nevertheless, the exploitation management of its advantages should prevent their outspread to untargeted areas due to its invasive properties (Prabakarana et al., 2019).

Proline and proanthocyanidins in the roots of rice plants showed solid monitoring of As-induced damage. Several studies have revealed that proline-engineering plants exhibit better adaptation to abiotic stresses, this pointing out a new path for genetic manipulation to ensure agricultural sustainability (Ghosh et al., 2021). In the same way, the use of flavonoids (which comprise a large family of phenolic compounds) enriched plants would strengthen the potential of phytoremediation processes for the restoration of As-polluted soils. Thus, a new field of research, focused on finding more specific phenolic compounds in response to As toxicity, has been identified. Nevertheless, proline and flavonoids priming (either through seed, foliar or exogenous application) may also lead to a greater phytoremediation performance, especially considering that priming has been applied for decades with other aims with successful results.

CONCLUSIONS

From the results obtained in the present Thesis, with regard to the response of rice and common reed plants to potentially toxic elements (PTE) stress grown in contaminated soils and in As(V) contaminated nutrient solution, and their influence in phytoremediation strategies, the following conclusions can be drawn:

1. The water regimen of the PTE-contaminated mining soils (under permanent flooding or under irrigation) slightly altered the soil pH towards higher values in flooded conditions, which increased the easily extractable concentrations of As but reduced metal solubility.

2. Lead and Zn were the only PTE detected in soil solution, and those that presented the highest $CaCl_2$-extractable concentrations in the soils. Furthermore, Cd reflected its elevated solubility and potential phytotoxicity, due to its concentration in easily exchangeable fraction of the sequential extraction ($CaCl_2$-extractable) in all the soils studied and for all the treatments.

3. Rice plants exposed to highly PTE contaminated mining soils did not fully resist to the elevated Cd and Zn available concentrations in the soils and the high electrical conductivity of one of the soils (LB soil). However, rice seedlings showed an excluder behavior when were cultivated in the soil from Portmán (PM), and were able to survive despite the high levels of As and Pb in that soil, and limited the translocation of these PTE form the roots to the shoots of the plants. Proline and lipid peroxidation (MDA) concentrations reflected the vulnerability of rice plants to Zn over-accumulation and toxicity.

4. Common reed cultivation was found to be a suitable option for the phytostabilization of mine soils highly contaminated with PTE, prone to suffer dry and flooded alternative periods. Common reed plants showed high tolerance to PTE, accumulating them mostly in their roots and limiting the translocation

of these toxic elements to the aboveground tissues of the plants. The concentrations of proline and MDA in the plants resulted of particular interest, as they reflected As accumulation and toxicity in the plants.

5. Rice plants showed symptoms of acute toxicity at elevated As(V) concentrations in nutrient solution (> 5 mg L^{-1}), namely reduced growth, very high As accumulation in both roots and shoots and increased lipid peroxidation.

6. Arsenic accumulation in rice plants increased with increasing As(V) dose in the growing media. The translocation of As (mainly As(V)) to the aboveground tissues was unavoidable at 10 mg L^{-1} As, which was likely a consequence of surpassed arsenate reductases. Both total-As and main As inorganic species concentrations in the leaves were significantly (PCA) related to lipid peroxidation.

7. Common reed has demonstrated an elevated tolerance to As(V) exposure, showing an almost absolute absence of toxicity symptoms in the plants, which were assessed through plant growth and biomass determination and the analysis of photosynthetic pigments, oxidative damage and antioxidants and proline concentrations in plant tissues.

8. Common reed plants confirmed their suitability for their use in the rhizofiltration and phytostabilization of As contaminated environments, with high As accumulation, mainly as As(III) in the roots, and scarce translocation to the aerial parts of the plant.

9. Ascorbic acid (AsA) priming diminished total As accumulation in rice plants and, particularly, that of As(III) in the roots, which was found to be directly related to thiolic compounds concentration in the plant roots, this suggesting an effective As(III)-SH sequestration in the vacuole.

10. Proline concentration in the aerial part of rice plants increased upon As(V) exposure, whereas AsA priming induced a reduction in As toxicity in the plants (reduced proline concentrations compared to control (no As) values). Therefore, the analysis of proline concentration in plant tissues could be a useful marker of As presence and toxicity in rice plants.

11. Arsenate exposure caused an increase in the accumulation of proanthocyanidins in rice roots, which was mainly influenced by As(V), and this increase was more prominent after AsA priming. Strong correlations were obtained among the antioxidant compounds (TPC, PAs) and activities (FRAP, DPPH), and those with proline, suggesting an upregulation by AsA priming that provided the plants a robust line of defense to control ROS homeostasis.

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Annex I

Effects of ascorbic acid addition on the oxidative stress response of *Oryza sativa* L. plants to As(V) exposure

I.1. Abstract

Accumulation of noxious elements in edible crops and its impact on food safety is of increasing concern. Rice is one of the major staple food crops worldwide, including arsenic (As)-polluted areas, in which dietary As exposure is becoming a widespread health threat. Plant chemical priming has been shown to be a promising strategy to enhance tolerance to environmental stresses, including metal(loid) exposure. The priming effect of ascorbic acid (AsA) was assessed in rice seedlings exposed to As(V) in a hydroponics experiment. AsA treatment (co-addition to the growing media) prevented an excessive accumulation of As in the roots and enhanced the photosynthetic and the antioxidant activities in the aerial part of the plants. Proline level in both shoots and roots is seen as the most sensitive stress parameter to reflect the AsA-induced reduction of As toxic effects. However, the phytotoxic effects related to As exposure were not fully prevented by priming with AsA, and further research is needed to find alternative priming approaches.

I.2. Introduction

The non-essential metalloid arsenic is becoming a global contaminant that entails serious hazard to human health, plants and animals (Nath et al., 2014). The accumulation of As in agricultural soil seriously increases the risk of As entry into the food chain, which threatens agricultural trade and increases demand of food safety (Bali and Sidhu, 2021). Among crops, rice cultivars (*Oryza sativa* L.) are facing important threats regarding As toxicity, mainly due to the use of As-polluted irrigation groundwater, especially in South Asian countries (Moulick et al., 2016; Nath et al., 2014). Rice has consequently been the subject of extended research, which, together with its small and well-organized genome, has turned this species into a model plant for As toxicity studies (Ahsan et al., 2008).

The mobility and bioavailability of As in soil solution strongly depend on its chemical forms and speciation, with arsenate (As(V)) being the most abundant in aerobic soils, whereas under reducing conditions arsenite (As(III)) is predominant (Bali and Sidhu, 2021; Panda et al., 2010). In fact, arsenate has been found to be the dominating As species in the soil solution of strongly oxidized contaminated mine soils (Álvarez-Robles et al., 2022; Beesley et al., 2014). Both inorganic forms of As (As(V) and As(III)) are highly phytoavailable and more noxious than its major pentavalent methylated species, *i.e.,* monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA) (Bali and Sidhu, 2021; Panda et al., 2010). In plants, As(V) and As(III) are mostly taken up by phosphate transporters and aquaporins, respectively (Tripathi et al., 2007). Inorganic As species are highly phytotoxic: As(V) can replace phosphate groups (Pi) due to their structural analogy, and disrupt energy flows in cells (Tripathi et al., 2007; Nath et al., 2014); and As(III) can interact with thiol groups of proteins altering their structure and functions (Panda et al., 2010; Singh et al., 2015).

Moreover, As exposure can enhance the production of reactive oxygen species (ROS) that can cause a series of damages to cellular structures and metabolic pathways (Finnegan and Chen, 2012; Singh et al., 2015). In plants, ROS homeostasis is controlled through a complex and redundant ROS-metabolizing system, which includes enzymatic and non-enzymatic reactions that remove and keep ROS at basal non-toxic levels (Noctor et al., 2018). The main redox-active metabolites involved in non-enzymatic ROS scavenge pathways are ascorbate, glutathione (GSH) and pyridine nucleotides NAD(P)H (Foyer and Noctor, 2011). Apart of its role in ROS homeostasis, ascorbic acid (AsA) is known to regulate plant growth and development as well as to determine the level of tolerance to several environmental constraints (Gallie, 2013; Akram et al., 2017). In fact, as an effective antioxidant, the exogenous application of AsA has proved to ameliorate As-induced oxidative stress in roots of eggplant (Alamri et al., 2021) and Cd toxicity in wheat (Zhou et al., 2021) and rice (Chao and Kao, 2010).

Also, it is well-established that an early sensing of stress and the induction of an appropriate defense response are vital for the successful adaptation of plants to stress conditions (Jakab et al., 2005). Interestingly, plants show a stronger and faster defense response if they have been previously undergone an acclimation process, a phenomenon known as priming (Conrath et al., 2015). In addition, different natural or synthetic compounds have the potential to act as priming agents, such as phytohormones (López-Orenes et al., 2020; Sytar et al., 2019), reactive oxygen-nitrogen-sulfur species (Antoniou et al., 2016), amino acids (Vijayakumari et al., 2016) and AsA (Akram et al., 2017; Elkelish et al., 2020), among others (Savvides et al., 2016; Siddiqui et al., 2020).

Our starting hypothesis was that priming with AsA would improve As tolerance in rice plants through the modulation of the stress response and the limitation of As accumulation. Therefore, the aim of this work wass to analyze to which extent pretreatment (priming) or simultaneously treatment with AsA affect the response of rice plants to As(V) exposure. To prove this, a hydroponics experiment was carried out to examine the effectiveness of AsA feeding treatments in reducing the phytotoxicity of As on the growth, photosynthetic activity, antioxidant compounds and oxidative stress response in rice plants.

I.3. Materials and methods

I.3.1. Experimental design

Rice seeds (var. J. Sendra; provided by Instituto Valenciano de Investigaciones Agrarias, Moncada, Spain) were wrapped in moistened paper and kept in the darkness during five days for germination. After that, the rice seedlings were shifted to vermiculite for 7 days, and then transferred to hydroponic pots where they were allowed to grow in nutrient solution for 20 days more before the treatments were applied. The nutrient solution used was a modified version of the Hoagland solution (1.50 mM KNO_3, 1.28 mM $Ca(NO_3)_2$; 0.37 mM $MgSO_4$, 0.17 mM KH_2PO_4, 0.15 mM NaCl, 24.71 µM Fe-EDDHA, 16.65 µM H_3BO_3, 2.37 µM $MnSO_4$, 0.92 µM $ZnSO_4$, 0.63 µM $CuSO_4$ and 0.63 µM $(NH_4)_6Mo_7O_{24}$; Álvarez-Robles et al., 2020). The solution was renewed weekly and the pH was adjusted to 5.5 (using either NaOH or HCl) every three days. Aliquots of a water extract (1:10 w/v) of a mature olive-mill waste compost were added as a source of dissolved organic carbon (to a final concentration of 100 mg DOC L^{-1}), as described in Álvarez-Robles et al. (2020). Arsenic was added as $Na_2HAsO_4 \cdot 7H_2O$ (Sigma-Aldrich) in the corresponding As treatments. The different treatments were applied when rice plants (20 per pot) were of uniform size (25 cm height on average, 20 days after transplanting to the pots). The corresponding solutions were renewed three days after the start of the experiment, which lasted one week. The experiment was run in a growth chamber with a 12 h day/night cycle of 25/18 °C temperature and 58/70 % of relative humidity. The five treatments applied were the following: i) control: nutrient solution with no AsA or As added; ii) AsA: nutrient solution + 2 mM AsA; iii) As: nutrient solution + 50 µM As(V); iv) AsA + As t_0: nutrient solution + 2 mM AsA + 50 µM As(V), added simultaneously, and v) AsA + As t_{24}: nutrient solution + 2 mM AsA + 50 µM As(V) added 24 h after ascorbic acid addition. After 7 days of treatment, the plants were harvested and divided into the aerial part and the roots.

I.3.2. Plant measurements and analytical procedures

The plant height was measured at the beginning (day 0) and the end of treatment exposure (day 7). Similarly, roots were marked with a permanent marker 1 cm above the tip at day 0 and the length increase was measured at day 7 of the experiment. Half of the separated plant parts were rapidly frozen in liquid N_2, while the rest of the samples were oven-dried (65 °C) until constant weight and ground to a fine powder in an electric mill (A10 IKA-Labortechnik, Staufen, Germany) for analysis.

Total nitrogen (TN) concentrations were determined in an automatic microanalyser (EuroEA3000, Eurovector, Milan, Italy). Trace element and nutrient concentrations were determined in dried plant materials by ICP-OES (ICP-OES; ICAP 6500DUO ONE FAST, Thermo Scientific, Waltham, MA USA) after microwave assisted acid digestion (UltraClave, Milestone, Shelton, CT USA). The analytical accuracy was checked with a certified reference material (NCS DC 73349). Frozen shoot and root samples were rapidly ground in a mortar with liquid N_2 and individual aliquots (0.2–0.5 g) were extracted in duplicate with 20 ml of phosphate-buffered saline (PBS; 2 mM NaH_2PO_4 and 0.2 mM Na_2-EDTA, pH 6.0) for 1 h under sonication (Ultrasons Medi, JP Selecta, Barcelona, Spain). The extracts were then filtered through 0.45 μm nylon filters before being analyzed for As speciation (determination of major As species: As(III), As(V), MMA and DMA) using high performance liquid chromatography coupled to an atomic fluorescence spectrophotometer (HPLC-AFS, Millennium Excalibur, PSAnalytical, Orpington, UK) as described in Xu et al. (2007).

I.3.3. Physiological, antioxidant and oxidative stress status parameters

The evaluation of the physiological status in rice plants was performed by measuring the content of photosynthetic pigments, total soluble sugars and proteins as previously described (López-Orenes et al., 2018a). In short, shoot and root samples (~ 0.1 g N_2-powdered tissue) were extracted with 80 % ethanol (1 mL) by sonication at 40 °C for 30 min and centrifuged at 15,000 g for 15 min at 4 °C. Shoot ethanolic supernatants were used for chlorophyll a (Chl-a), chlorophyll b (Chl-b) and total carotenoids determinations and their levels were estimated using the extinction coefficients and the equations reported by Lichtenthaler and Wellburn (1983).

The total soluble sugars (TSS) were determined by the anthrone-sulfuric acid method using the ethanolic extracts and glucose (25-500 µg/mL) as standard. The total soluble protein (TSP) concentrations were measured by the Bradford method, using bovine serum albumin (BSA) as the standard.

The total antioxidant capacity was estimated by the FRAP (ferric reducing/antioxidant power) and DPPH (2,2-diphenyl-1-picrylhydrazyl radical scavenging activity) assays using both shoot and root ethanolic extracts as described by Pérez-Tortosa et al. (2012). The reducing power was expressed as µmol Fe(II) per gram (fresh weight) and a standard curve in the range 0-3 mM of $FeSO_4{\cdot}7H_2O$ was used for calibration. DPPH activity was expressed as µmol of gallic acid equivalents (GAE) per gram of fresh weight. The total phenol concentration (TPC) was determined by the Folin-Ciocalteu method using gallic acid (25–2000 µM) as a standard (Everette et al., 2010). The pellets from ethanol extractions, after thoroughly washing with ethanol, were used to estimate the content of cell wall-associated proanthocyanidins (PAs) by measuring the absorbance at 545 nm of the supernatants obtained after an acid attack (butanol-

HCl). Results were expressed as cyanidin equivalents by using an $\varepsilon_{545} = 34.7\ mM^{-1}\ cm^{-1}$ (Vermerris and Nicholson, 2007).

The determination of hydrogen peroxide was carried out by the ferrous ion oxidation–xylenol orange (FOX) method, as described by Cheeseman (2006). Briefly, frozen samples (0.1 g) were homogenized in 1 ml of 6 % trichloroacetic acid (TCA) and centrifuged (15,000 g, 10 min, 4 °C). Then, 50 µl of supernatant were mixed with 200 µL of FOX1 medium (0.25 mM $Fe(NH_4)_2(SO_4)_2$, 25 mM H_2SO_4, 0.1 mM xylenol orange, 100 mM sorbitol, and 1% (v/v) ethanol). After 30 min of incubation in the dark, the concentration of H_2O_2 was determined based on the difference in absorption at 560 nm, using a H_2O_2 standard curve covering the range of 0.1–10 µM.

The concentration of proline (Pro) was determined spectrophotometrically in a sulfosalicylic acid extract, using acid ninhydrin reagent (López-Orenes et al., 2013). Absorbance of the proline-ninhydrin complex was recorded at 518 nm. The proline concentration was determined from a calibration curve with known concentrations of proline in the range 10-1000 µM.

The degree of lipid peroxidation was determined as the concentration of malondialdehyde (MDA) by measuring thiobarbituric acid-reacting substances (TBARS) at 532 nm, with a correction for non-specific absorbance at 440 and 600 nm (Hodges et al., 1999), using the same supernatants as in the FOX1 assay. The concentration of MDA was finally calculated using an extinction coefficient of $155\ mM^{-1}\ cm^{-1}$. Oxidized and reduced forms of ascorbate were measured using the α-α′-bipyridyl method (Gillespie and Ainsworth, 2007).

Oxidized proteins were estimated through the reaction of (2,4-dinitrophenyl)hydrazine (DNPH) with protein carbonyls after inhibition of proteases in plant extracts obtained with cOmplete® (Roche) and phenylmethylsulfonyl fluoride (PMSF) (Levine et al., 1994). Carbonyl proteins were referred to total proteins content calculated using the Bradford method and bovine serum albumin (BSA) as standard.

For ascorbate (AsA), GSH and non-protein thiol (NPT) determinations, about 0.2 g N_2-powdered tissue were homogenized with ice-cold 5% (w/v) metaphosphoric acid. The homogenates were centrifuged at 15,000 g for 15 min at 4 °C, and the supernatants were used for the analysis of AsA, GSH and NPT using the α-α′-bipyridyl method (Gillespie and Ainsworth, 2007), the recycling assay (Queval and Noctor, 2007) and Ellman's reagent (López-Orenes et al., 2018a), respectively. The concentration of total phytochelatins (PCs) was estimated from the difference between NPT and GSH as previously described (Hartley-Whitaker et al., 2001). All the spectrophotometric assays were performed with a microplate UV-Vis spectrophotometer reader (Multiskan GO, Thermo Scientific).

I.3.4. Statistical analysis

The statistical data analysis was performed using IBM SPSS Statistics Version 26.0 software (IBM Corporation, New York, USA). The one-way analysis of variance (ANOVA), followed by Tukey's HSD test, was carried out to assess the significant differences among treatments ($P < 0.05$). Two-way ANOVA was also performed to test significant differences among plant parts data for the different treatments. A Principal Component Analysis (PCA) was run (Varimax rotation) considering all the determined parameters to reveal general tendencies. All determinations were conducted at least in duplicate, and all results are shown as mean ± standard error (SE).

I.4. Results

I.4.1. Effects of AsA feeding treatments on plant growth and nutrient and As (total and major chemical species) accumulation

No significant differences between the yield of the aerial part of the rice plants (fresh weight and shoot height) in the different As treatments as compared to controls were observed (Figure 1). However, the roots of As-treated plants showed a marked reduction in both root biomass (~ 30%) and growth; the addition of AsA to the nutrient solution did not prevent the negative effects observed in the As treatment.

The analysis of macronutrients revealed that As-alone treatment did not provoke significant changes in the accumulation of N, P, K, Ca, and Mg in both shoots and roots (Table 1). However, the addition of AsA to the growing medium resulted in a general decrease of macronutrient concentration (from 22 to 32%) in shoots. In AsA+As-treated plants, the AsA-induced changes were mostly unaffected in shoots, whereas a decrease in K levels (~ 35%) was found in roots.

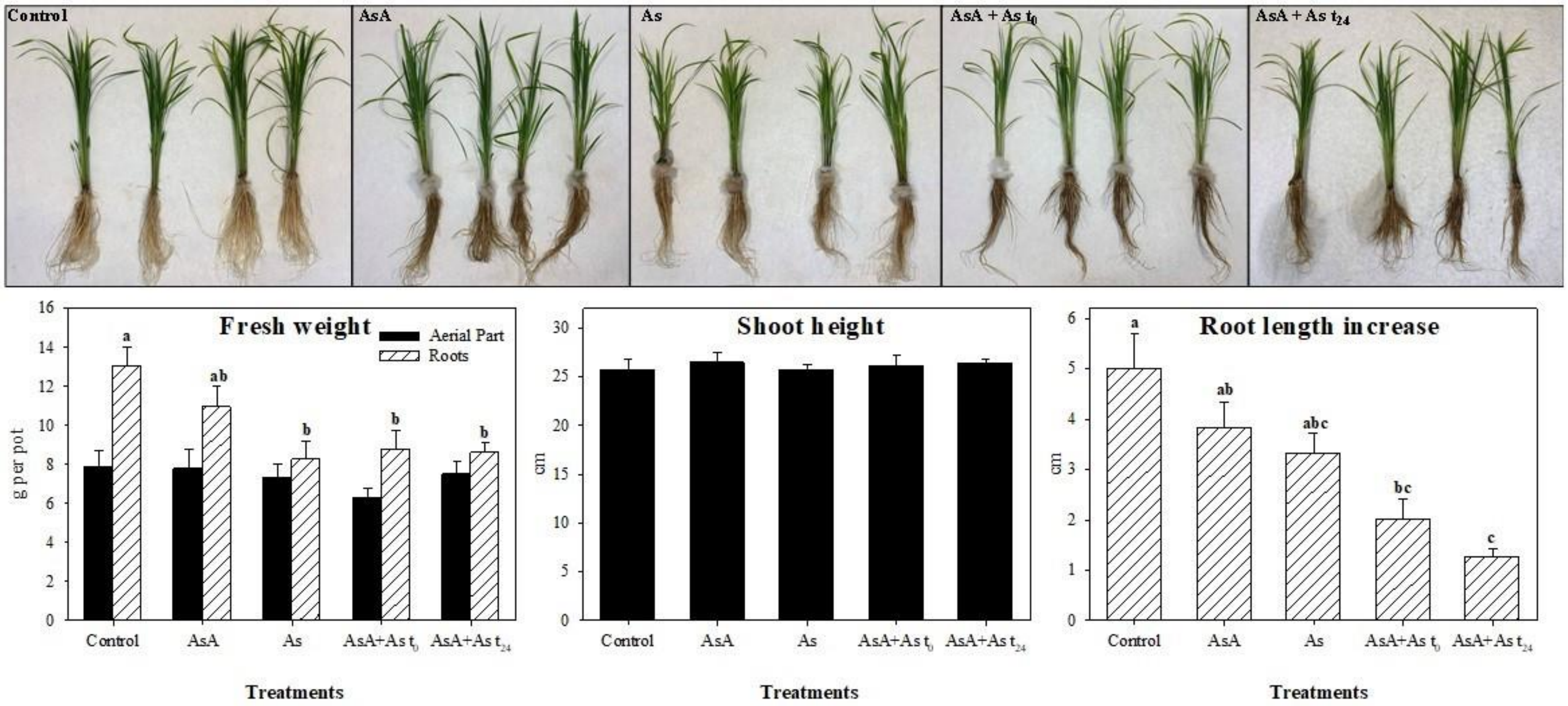

Figure 1. Effect of AsA addition on the biomass production (grams of fresh weight per pot) and growth (shoot height (final) and length increase (days 0-7 after treatment) in the roots, cm) in 20 days old rice plants (N=4) after 7 days of As exposure. Bars marked with the same letter for each parameter do not differ significantly according to Tukey's test at $P < 0.05$.

Table 1. Effect of AsA addition on the macro- and micronutrients concentration (g kg^{-1}) in 20 days old rice plants (N=4) after 7 days of As exposure.

		N	P	K	Ca	Mg	Cu	Fe	Mn	Zn
Aerial part	Control	28.00±1.04 a	4.17±0.15 a	27.85±1.81 a	4.17±0.27 a	3.73±0.15 a	15.54±1.12 a	175±27 a	350±25 a	120±6 a
	AsA	18.89±1.37 b	2.82±0.16 bc	20.46±1.10 b	3.22±0.12 ab	2.62±0.10 b	9.31±0.53 b	130±11 ab	225±18 b	71.5±5.2 b
	As	25.39±1.37 a	3.46±0.27 ab	25.92±1.45 a	3.66±0.18 ab	3.38±0.08 a	9.99±0.69 b	82.4±5.7 b	307±10 ab	74.7±5.3 b
	AsA+As t_0	17.65±2.25 b	2.46±0.16 c	15.10±0.60 b	2.69±0.31 b	2.31±0.13 b	7.00±0.55 b	92.7±8.9 b	243±30 b	55.6±3.4 b
	AsA+As t_{24}	17.13±0.68 b	2.54±0.05 c	16.71±0.38 b	3.03±0.20 b	2.62±0.10 b	8.57±0.62 b	133±22 ab	235±6 b	63.4±7.9 b
	ANOVA	***	***	***	**	**	***	*	**	***
Roots	Control	23.05±0.99	2.11±0.06	21.25±1.29 a	2.02±0.13	5.85±0.92	38.2±3.82	2048±376	94.9±12.0	60.6±9.0
	AsA	26.90±2.13	2.35±0.26	18.27±2.24 ab	2.44±0.11	4.43±0.38	27.8±2.53	1539±224	86.8±12.6	51.8±10.8
	As	25.90±0.68	2.24±0.06	22.69±1.28 a	2.11±0.15	4.71±0.68	29.0±2.47	1603±303	85.9±9.6	46.4±1.3
	AsA+As t_0	26.92±2.76	2.14±0.34	13.84±1.22 b	2.47±0.47	4.40±0.51	26.8±2.18	1548±210	79.4±9.8	49.5±4.9
	AsA+As t_{24}	23.74±1.77	1.82±0.18	13.82±0.88 b	2.59±0.36	4.71±0.66	29.0±2.86	1834±267	84.3±8.3	48.1±7.5
	ANOVA	NS	NS	**	NS	NS	NS	NS	NS	NS
ANOVA	Plant Part	**	***	**	***	***	***	***	***	***
	Treatment	*	***	***	NS	*	***	NS	**	***
	PxT	***	**	NS	**	NS	NS	NS	**	**

NS: not significant. ***, ** and *: significant at $P < 0.001$, 0.01 and 0.05, respectively. Values followed by the same letter in each column for each plant part do not differ significantly according to Tukey's test at $P < 0.05$.

Contrastingly, the analysis of micronutrients showed that As-alone treatment markedly reduced the shoot Fe levels (>50%) and to a lower extent the concentrations of Cu and Zn (~ 40%), whereas in roots the micronutrient concentrations were indistinguishable among the treatments (Table 1). AsA-alone pretreatments also provoked a reduction in the concentrations of Cu, Mn and Zn (~ 40%) (Table 1). Interestingly, the AsA pretreatments (AsA+As t_{24}) alleviated the reduction in shoot micronutrient concentrations, particularly the levels of Fe.

As expected, the concentrations of As in shoot and root tissues were below the detection limit in the non-As treatments (Table 2). In the shoots of all As-treated rice plants, the levels of As found were slightly above the toxicity limits stated for plants (5-20 mg kg^{-1}; Kabata-Pendias, 2011) and were unaffected by AsA exposure. In roots, however, the concentrations of As were much higher (~ 600 mg kg^{-1}), but significantly dropped to ~ 60% in AsA+As treated plants, either with simultaneous application or with AsA priming (Table 2).

When major As chemical species were analyzed in the plants, only As(III) and As(V) were found in detectable amounts. The concentrations of these species were slightly lower than those previously reported for rice plants exposed to similar concentrations of As(V) in the growing medium (Álvarez-Robles et al., 2020). The concentrations of As(III) and As(V) extracted from the aerial parts of the plants were particularly low (~ 8 and 5 mg kg^{-1}, respectively), and the addition of AsA 24 h before As treatment resulted in a significant decrease of their levels (Table 2). Higher As(V) and, especially As(III) concentrations were found in the roots, where AsA feeding treatments provoked a drastic decrease in As(III) concentration (~70%) (Table 2).

Table 2. Effect of AsA addition on the total As and As species concentration (mg kg^{-1} DW) in 20 days old rice plants (N=4) after 7 days of As exposure.

		Total-As	**As(III)**	**As(V)**
Aerial part	**Control**	bdl	bdl	bdl
	AsA	bdl	bdl	bdl
	As	36.2±6.64	7.52±0.89 a	4.70±1.33 a
	AsA+As t_0	33.7±6.41	4.56±0.96 ab	1.80±0.55 ab
	AsA+As t_{24}	46.0±5.85	3.48±0.95 b	1.39±0.14 b
	ANOVA	NS	*	*
Roots	**Control**	bdl	bdl	bdl
	AsA	bdl	bdl	bdl
	As	605±24.7 a	459±106 a	12.8±6.69
	AsA+As t_0	256±40.1 b	135±23.0 b	28.0±5.07
	AsA+As t_{24}	232±3.29 b	158±33.2 b	32.7±3.47
	ANOVA	***	**	NS
ANOVA	**Plant Part**	***	***	***
	Treatment	***	***	**
	PxT	***	***	***

bdl: below detection level (1 mg kg^{-1} DW). NS: not significant. ***, ** and *: significant at $P < 0.001$, 0.01 and 0.05, respectively. Values followed by the same letter in each column for each plant part do not differ significantly according to Tukey's test at $P < 0.05$.

I.4.2. Effects of AsA feeding treatments on physiological and oxidative stress parameters in rice plants under arsenate exposure

The biochemical parameters such as photosynthetic pigments, soluble sugars, and proteins were differently affected by As exposure (Table 3): As-alone treatment led to a reduction in the content of Chl-b (>20%) in the plants compared to AsA treatment, while no major changes occurred in the content of sugars and proteins in shoots.

With respect to oxidative stress, no pronounced changes were observed in the H_2O_2 contents either in shoot or root tissues among the different treatments (Figure 2). In contrast, the accumulation of the stress amino-acid proline showed a marked increase (> 2-fold) in both shoots and roots in As-treated plants compared to control treatment. Interestingly, proline concentration in AsA+As treatments was similar to that in the controls and AsA-alone treatment, although this effect was not observed in plant roots (Figure 2). The concentrations of MDA and carbonyl proteins in both the aerial part and the roots of the plants from the different treatments did not show significant differences (data not shown), in agreement with previous results that did not find these parameters to be good indicators of As induced oxidative stress in rice plants (Álvarez-Robles et al., 2020).

Table 3. Effect of AsA addition on the physiological parameters in 20 days old rice plants (N=4) after 7 days of As exposure.

		Chl-a **(µg Chl-a g^{-1} FW)**	**Chl-b** **(µg Chl-b g^{-1} FW)**	**Carotenoids** **(µg carotenoids g^{-1} FW)**	**TSS** **(mg glucose g^{-1} FW)**	**TSP** **(mg protein g^{-1} FW)**
Aerial part	Control	792±66	353±26 ab	240±22	23.3±1.8	9.14±0.36
	AsA	990±128	450±61 a	291±38	26.3±2.9	9.67±1.30
	As	593±81	271±34 b	184±20	23.6±1.9	9.52±1.25
	AsA+As t_0	702±49	314±21 ab	214±7	26.4±2.9	8.80±0.91
	AsA+As t_{24}	792±80	367±35 ab	233±19	25.8±0.9	7.74±0.39
	ANOVA	NS	*	NS	NS	NS
Roots	Control	-	-	6.53±2.34	4.15±0.57	2.17±0.21
	AsA	-	-	6.30±0.70	3.26±0.29	2.67±0.32
	As	-	-	8.64±1.18	3.01±0.36	2.21±0.10
	AsA+As t_0	-	-	9.16±1.53	3.57±0.54	2.70±0.26
	AsA+As t_{24}	-	-	9.37±1.98	3.38±0.30	2.38±0.10
	ANOVA	-	-	NS	NS	NS
ANOVA	Plant Part	-	-	***	***	***
	Treatment	-	-	**	NS	NS
	PxT	-	-	*	NS	NS

TSS: total soluble sugars. TSP: total soluble proteins. NS: not significant. ***, ** and *: significant at $P < 0.001$, 0.01 and 0.05, respectively. Values followed by the same letter in each column for each plant part do not differ significantly according to Tukey's test at $P < 0.05$.

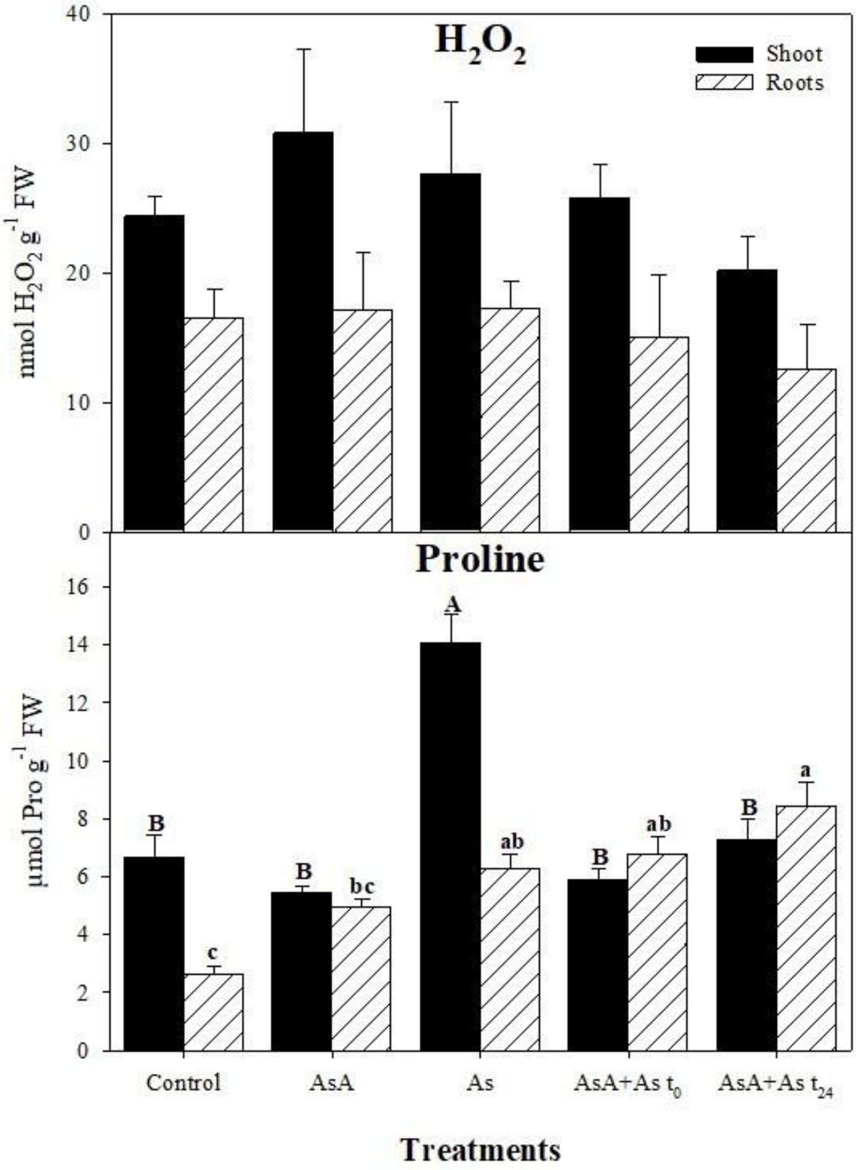

Figure 2. Effect of AsA addition on H_2O_2 (nmol H_2O_2 g^{-1} FW) and proline (µmol Pro g^{-1} FW) concentration in 20 days old rice plants (N=4) after 7 days of As exposure. Bars marked with the same letter (uppercase for shoot and lowercase for roots) for each parameter do not differ significantly according to Tukey's test at $P < 0.05$.

The As-alone treatment did not provoke significant changes in the values of the antioxidant properties FRAP and DPPH concentrations in plant shoots or roots (Figure 3). Only AsA+As t_{24} treatment showed significantly lower FRAP concentrations in plant shoot compared to AsA-alone treatment, and higher FRAP and DPPH ones in plant roots compared to control treatment.

The analysis of total phenolics showed a similar trend as in the FRAP and DPPH tests in both shoot and root tissues, with higher levels in shoots than in roots and no significant differences in the aerial part and higher concentrations in all AsA and As treatments in the roots (Figure 3). Moreover, strong correlations between TPC and FRAP or DPPH values were found (r > 0.7, $P < 0.01$ in shoots and r > 0.9, $P < 0.01$ in roots). Interestingly, As(V) exposure caused an increase in the accumulation of proanthocyanidins in root tissues (~2-fold), and this increase was even more prominent in both AsA feeding treatments (>3-fold) as compared to controls (Figure 3).

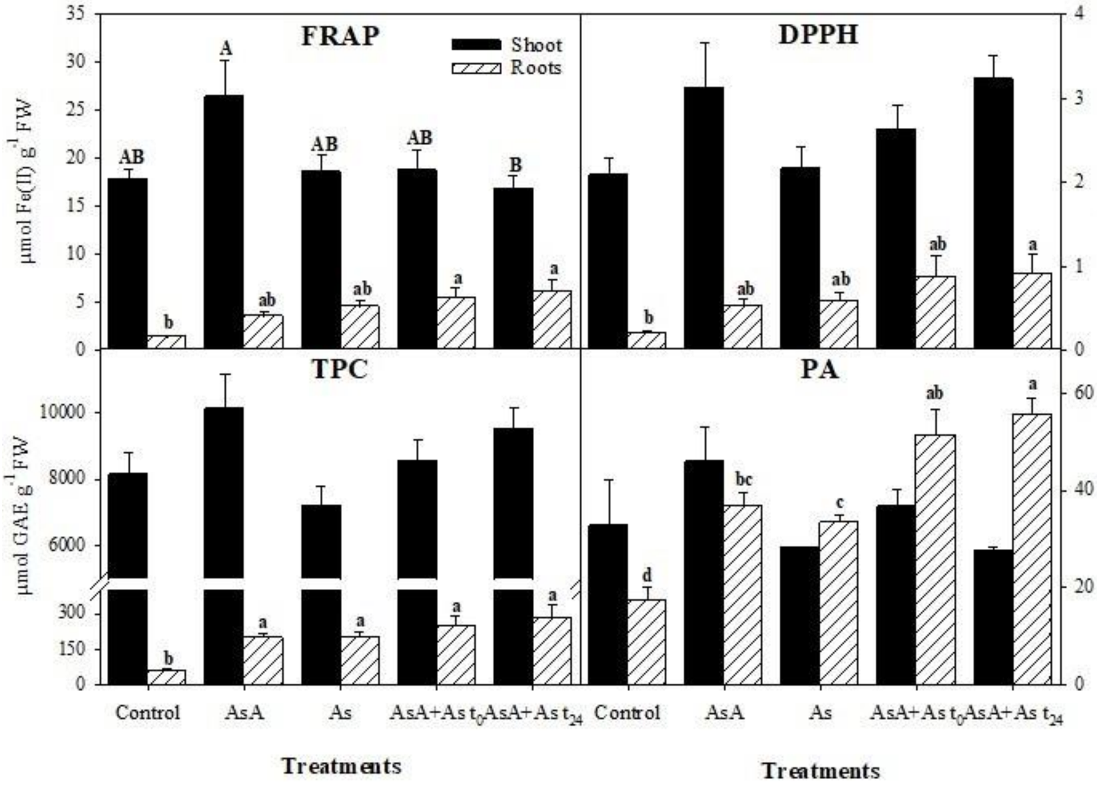

Figure 3. Effect of AsA addition on the total antioxidant activity (FRAP and DPPH assays; µmol Fe(II) g^{-1} FW, µmol GAE g^{-1} FW, respectively), total phenolics (TPC; µmol GAE g^{-1} FW) and proantocyanidins (PA; nmol eq. cyaniding g-1 FW) concentration in 20 days old rice plants (N=4) after 7 days of As exposure. Bars marked with the same letter (uppercase for shoot and lowercase for roots) for each parameter do not differ significantly according to Tukey's test at $P < 0.05$.

The analysis of AsA concentrations in the plants showed that neither As-alone nor AsA treatments provoked any significant effect compared to controls in both shoots and roots (Figure 4). Similarly, no significant differences were observed between GSH, non-protein thiol and phytochelatin concentrations in all assayed conditions in shoot tissues. However, As(V) exposure provoked a marked increase in the content of NPT and PC (~2.8-fold) in the roots, which decreased to normal (control) values in the combined AsA+As treatments. In the roots of AsA-As-exposed plants, the concentrations of GSH were significantly lower than in control and AsA-alone treatments (Figure 4).

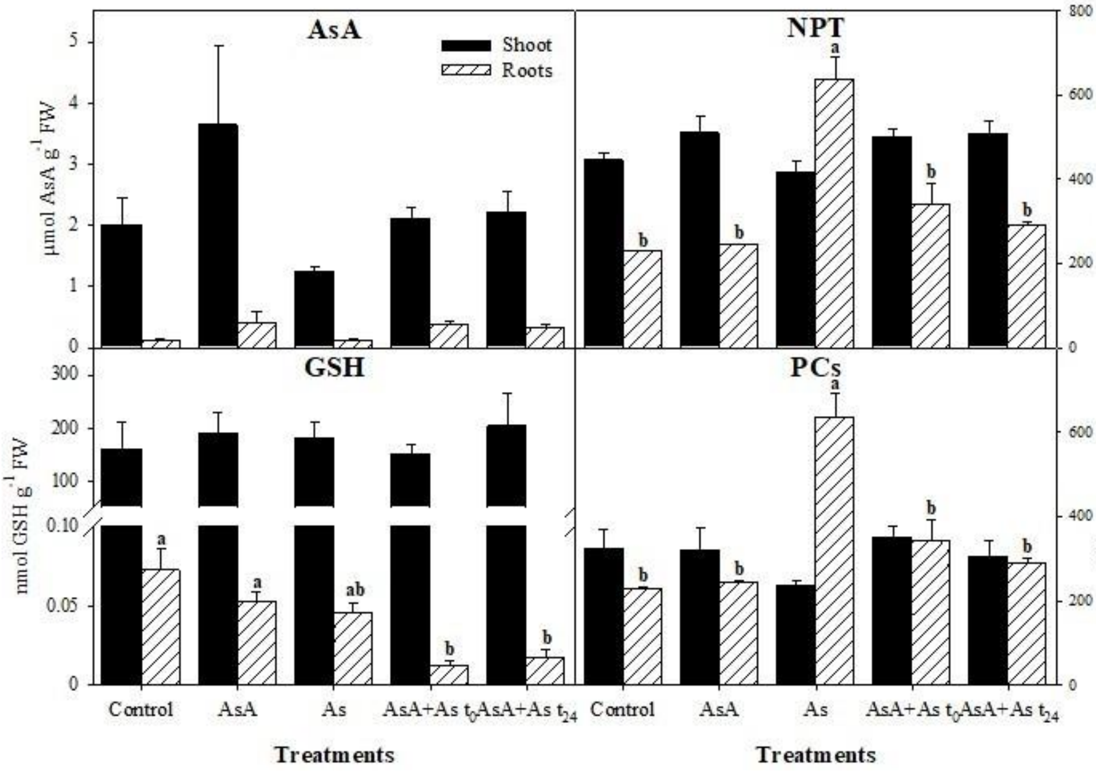

Figure 4. Effect of AsA addition on total ascorbic acid (AsA, μmol g^{-1} FW), total glutathione (GSH, μmol g^{-1} FW), non-protein thiols (NPT, nmol eq. GSH g^{-1} FW) and phytochelatins (PCs, nmol GSH g^{-1} FW) concentrations in 20 days old rice plants (N=4) after 7 days of As exposure. Bars marked with the same letter for each parameter do not differ significantly according to Tukey's test at $P < 0.05$.

I.4.3. Relationships between plant growth and nutritional parameters and antioxidant/oxidative stress markers

Two PCAs were performed, one with data corresponding to the aerial part of the plants and another one with data from the roots, in an attempt to elucidate interrelationships and possible dependencies between the plant stress related parameters and the concentrations of As (total and major species) in the plants. The first PCA (aerial part) resulted in six different components, from which the first three accounted for more than 65% of the variance (Figure 5a, Table S1; SI). The first component grouped together most of the stress and antioxidant activity related parameters and the photosynthetic pigments (Figure 5a). The second component associated the concentrations of the different As forms determined in the plants (total, As(III) and As(V)) positively among them and with proline content, and negatively with Fe concentration in the aerial part of the plants. Proline was one of the few parameters that showed a significant response (increased concentration) to the presence of As in the growing media that was then alleviated by AsA addition. This fact appears to be related to the accumulation of As (both As(III), As(V) and total) in the plants (Abbas et al., 2018). This can be considered to be an interesting finding, as this parameter is determined quite easily in the plants, and may act as an early marker of As toxicity in the plants. The third component related plant nutrients (N, P) concentrations in the plants and the rest of the components did not provide any relevant relationships (Table S1; SI).

The PCA performed with roots data resulted also in 6 different components, the first three again justifying more than 64 % of the variance (Figure 5c, Table S2; SI). The first component associated some of the oxidative stress (MDA) and antioxidant activity (PAs, TPC, FRAP, proline and DPPH) parameters positively with As(V) concentration in the roots, and negatively with roots length, fresh weight and GSH concentrations. This indicates that As(V) accumulation in the roots of the plants may affect their normal growth and increase the antioxidant and stress response of this part of the plants. The second factor related positively total As and As(III) concentrations to phytochelatins and NPT concentrations in the roots. This points out that, in the roots, the formation of NPT and phytochelatins was the response to the presence of As(III) and the accumulation of total As in these tissues, while the addition of AsA to the growing media prevented this from happening. The third component related negatively total proteins and AsA concentrations with carbonyl proteins in plant roots, and the fourth related again N and P concentrations (Table S1; SI).

A clear separation between As only and control treatments was observed in the aerial part PCA, mainly along PC2 (As, proline and Fe concentrations), whilst AsA and combined AsA-As treatments were not so clearly separated among them (Figure 5b). The factors obtained for treatments in the roots PCA (Figure 5d) separated As only from the rest of the treatments along PC2 (As(III), NPT and PCs concentrations), which were not evidently differentiated in any component. This suggests again the mitigation of As toxicity by AsA addition.

Therefore, in the aerial part of the plants, proline was the compound whose concentration significantly increased in As-alone treatments and reflected the positive effect on the plants of AsA addition. A different situation was found in the roots, where As(V) was responsible of the reduced plant growth and the antioxidant response of the plants, while As(III), which was the major form in this part of the plants (Table 3), provoked the formation of NPT and PCs, which were significantly decreased when AsA was added to the growing medium.

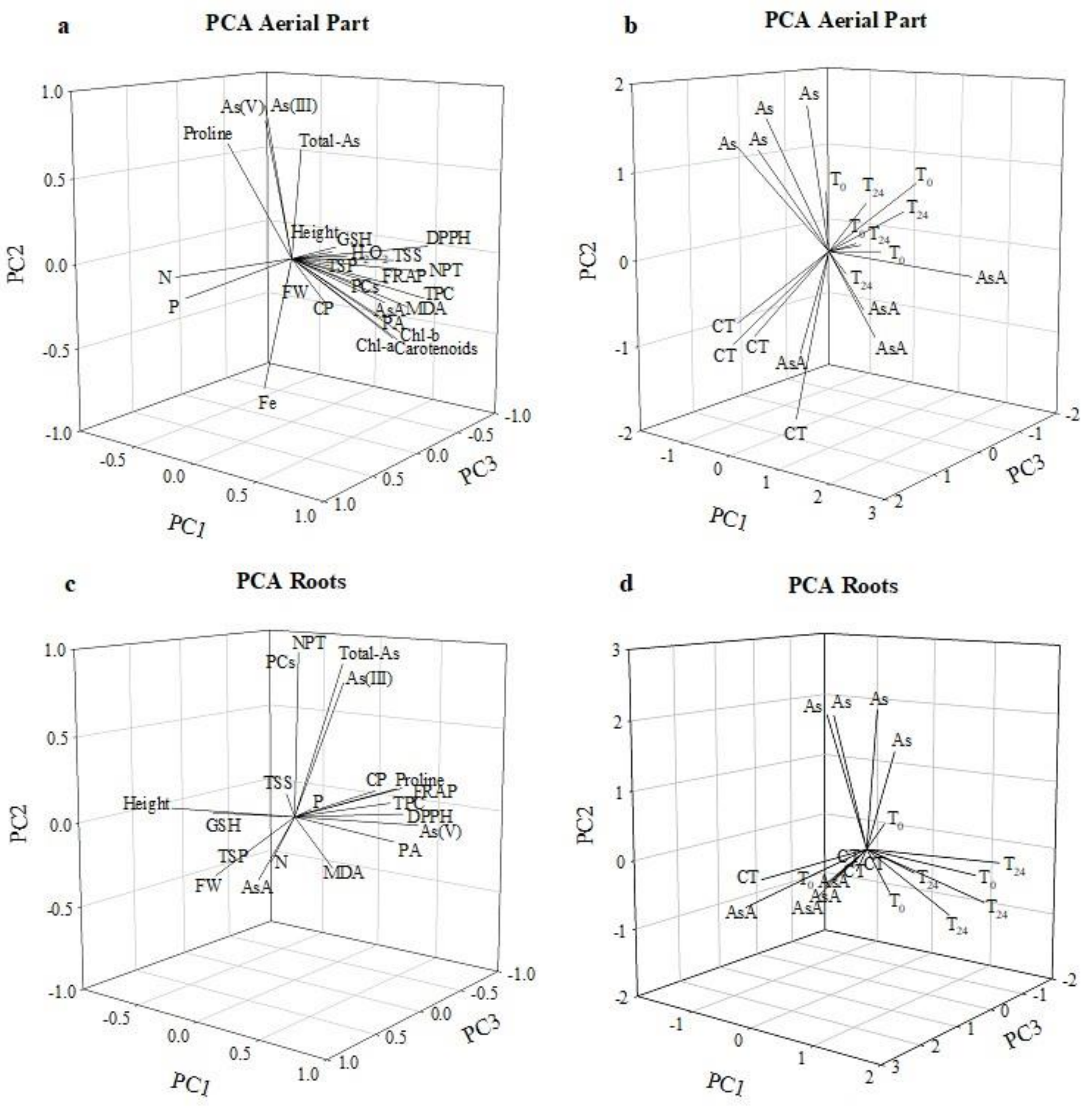

Figure 5. PCA combined plot for rice plant parameters (a,c) and treatments (b,d) in the aerial part and in the roots. CT: Control; T0: AsA+As t_0; T24: AsA+As t_{24}.

I.5. Discussion

I.5.1. AsA feeding alleviation of the As-induced negative effects on rice plants

The As-dependent reduction of root growth was mainly attributed to the higher accumulation of As(III) in these tissues (Table 2), since no differences in macro- and micronutrients were noticed in comparison to untreated root controls (Table 1). It is well-established that As(V) is uptaken by phosphate transporters (Zhao et al., 2009). In the experiment, phosphate concentration in the nutrient solution was 0.17 mM, thus, the high phosphate/As molar ratio can explain that phosphate intake in As-treated plants was the same as did untreated control plants.

Moreover, the addition of AsA to the nutrient media lead to a marked decrease in accumulation of As(III) in both shoots and roots (Table 2). AsA reduction of As(V) uptake has also been reported in eggplant plants (Alamri et al., 2021). Arsenic is well known to be accumulated in the roots mainly as As(III) (Tripathi et al., 2007) and the efflux of As(III) to the nutrient solution due to the intracellular reduction of As(V) in rice plants exposed to arsenate has been also reported (Awasthi et al., 2017; Su et al., 2010; Xu et al., 2007). In fact, the reduction of As(V) to As(III) is considered the first step of As detoxification, and the resulting As(III) can be either extruded outside the cells or complexed with thiol-rich peptides (Bali and Sidhu, 2021). Here, the results suggest that AsA is limiting As accumulation in the roots (mainly As(III)) as well as reducing the translocation of As(III) and As(V) to the aerial part of the plants. Then, AsA feeding appears as a promising strategy to reduce As content in rice from As rich soil/water areas.

AsA feeding treatments also alleviated the As-induced reduction of Fe in shoots (Table 1). These results are in accordance with those reported by (Ghorbani et al., 2021) where the inoculation of As-stressed rice plants with the endophytic fungus *Piriformospora indica* increased both Fe translocation to shoots by upregulating the expression of Fe transporters and AsA concentration in shoots. Taken together, these results suggest that As exposure provoked a downregulation of nutrient transporter genes and AsA reduced As toxicity by enhancing Fe uptake. In fact, AsA is known to play a role in the chemical reduction and transport of Fe(II) in plants (Grillet et al., 2014).

The photosynthetic pigment levels showed a (non-significant) trend to increase in AsA-As-exposed plants compared to As-alone ones (Table 3). In addition, AsA feeding treatments had a positive effect on root performance under As exposure. Roots are the first line of defense against metal(loid) toxicity as well as the first organ to respond and to adapt to metal(loid) stress (Kul et al., 2021; Hasanuzzaman et al., 2018). Thus, the high content of soluble sugars in roots could contribute to an adequate root performance and to tackle As phytotoxicity (positive significant correlations were found between TSS concentration in the roots and fresh and dry root weight; $r = 0.484$ and 0.640, $P < 0.05$ and 0.01, respectively). In fact, the low root biomass found in AsA+As treatments seemed to point towards a trade-off between root growth and As stress acclimation.

No significant effects on the content of soluble sugars and proteins were found in shoots upon As exposure, although the content of photosynthetic pigments tended to decrease, particularly the levels of carotenoids. Similar results have been described in rice plants exposed to 25 μM As(III) (Chauhan et al., 2017) and in fava beans exposed to 5 μM As(V) (Siddiqui et al., 2020), which seemed to indicate that photosynthetic pigments are very sensitive to As stress even at low doses (Finnegan and Chen, 2012). Here, the beneficial effects of AsA supplementation upon As stress in shoots could be attributed mainly to the enhancement of carotenoids levels, as regard the results of the two-way ANOVA (Plant part $P < 0.001$; Treatment $P < 0.01$; PxT $P < 0.05$; Table 3). Carotenoids are known to play a key role in the protection of the photosynthetic apparatus from oxidative stress generated by ROS (Niyogi, 2000; Song et al., 2006). These results confirmed that the maintenance of photosynthesis is vital for plant growth and survival under stress conditions (Allakhverdiev, 2020).

I.5.2. AsA feeding effects on oxidative stress and antioxidant parameters in the plants

A common hallmark of environmental stress factors is the generation of ROS in plant cells (Gill and Tuteja, 2010). The perception of the stress stimulus is followed by an increase of diverse signaling molecules such as free Ca^{+2}, reactive nitrogen species (RNS) and ROS within the first minutes. These signaling molecules, in turn, trigger the appropriate defense response through transcriptomic and metabolic changes (Garcia-Brugger et al., 2006). Here, H_2O_2 levels were determined in plants exposed to As(V) during 1 week, and no major changes were noticed either in shoot or root tissues (Figure 2). These results suggest that rice plants have induced acclimation mechanisms to withstand As(V) stress. In fact, the results clearly revealed that As exposure provoked a sharp rise in proline contents in both shoot and root tissues in As-challenged rice plants (Figure 2). AsA feeding treatments provoked an even further increased proline levels in roots, whereas in shoots proline content remained unaffected, compared to control treatment, upon AsA+As(V) exposure. Accumulation of proline in plants subjected to both abiotic and biotic stresses is a known response, widely reported in the literature (Szabados and Savoure, 2010; Verslues and Sharma, 2010), including As exposure (Siddiqui et al., 2020). Proline is known to act as an osmoprotectant, as well as to protect and stabilize macromolecules and to maintain cellular ROS balance (Szabados and Savoure, 2010; Verslues and Sharma, 2010).

Moreover, several studies have also demonstrated a link between increased proline content and the biosynthesis of phenylpropanoids (Caretto et al., 2015; Shetty, 2004; Youssef et al., 2022). The present results also suggest an association between proline and phenol compounds under As exposure, and their upregulation particularly in roots by AsA feeding treatments (a positive correlation between proline and TPC in plant roots was found; $r = 0.903$, $P < 0.001$). Phenol compounds, particularly flavonoids, are known to possess metal-chelation, ROS-scavenging and antioxidant properties (Agati et al., 2012; Rice-Evans et al., 1997). In fact, phenolics are considered to act as key mediators of plant defense response to environmental constraints (Cheynier et al., 2013) including metal stress (López-Orenes et al., 2018a, 2018b; Michalak, 2006) and As exposure (Chauhan et al., 2017). Proanthocyanidins (the polymeric condensation products of flavan-3-ols) have been also reported to be effective scavengers of ROS, peroxyl radicals and the powerful oxidant peroxynitrite ($ONOO^-$) (Gould et al., 2002). The antioxidant properties of PAs have been related to the presence of a catechol group on the B ring in their structure (Pannala et al., 2001). In the present study, strong correlations between TPC and total antioxidant activities as well as between PAs and FRAP (shoots and roots) and DPPH (roots) were found ($r > 0.6$, $P < 0.01$), indicating that these compounds could provide a robust line of defense to control ROS homeostasis. Genes involved in PAs production have also been reported to be up-regulated upon As treatment in *Salix purpurea* plants (Yanitch et al., 2017).

Taken together, these results revealed that both proline and phenolic compounds were relevant in the acclimation response to counteract As toxicity in rice plants. The beneficial effects of AsA application can be related to the higher induction of PAs and proline in roots that boost the tolerance of rice plants challenged with As(V).

As mentioned above, As(III) in roots can be detoxified by either efflux outside the cells or chelation with thiol(SH)-rich compounds (Bali and Sidhu, 2021). Phytochelatins are considered the main chelators of As(III) in plants (Bali and Sidhu, 2021). Here, a marked increase in PCs and NPTs was observed in roots of As-treated plants (Figure 4). These results are also in line with previous results reported in the same plant species (Zhang et al., 2011), confirming the role of these thiolic ligands to withstand As toxicity in rice. However, PC and NPT contents hardly increased in the roots of AsA-As-treated plants. The biosynthesis of PCs represents a high energy-cost to the cell associated with sulfate reduction, GSH metabolism and PC biosynthesis itself (Cobbett and Goldsbrough, 2002). The reduction in the accumulation of PCs in roots of AsA-As-treated plants can be explained by the lower As(III) accumulation found in these tissues. Given the energy cost associated with the synthesis of PCs, another beneficial effects of AsA application can be related to the use of alternative defense mechanisms that can be more sustainable and effective under chronic As exposure (Maestri et al., 2010).

Reduced glutathione is the precursor for the synthesis of PCs (Cobbett and Goldsbrough, 2002), and also serves as an electron donor for the reduction of As(V) to As(III) (Duan et al., 2005). Here, a reduction of GSH levels was found in roots of AsA-As-treated plants in comparison to control and AsA-alone treated plants (Figure 4). AsA and GSH are the main redox buffer systems present in plant cells, and there is a close relationship between these multifaceted molecules (Foyer and Noctor, 2011); changes in their levels in response to developmental and environmental stimuli are well reported in the literature (Ferrer et al., 2018; Foyer and Noctor, 2011; Gill and Tuteja, 2010; Hasanuzzaman et al., 2019).

I.6. Conclusions

Rice plants exposed to moderate As concentrations in the growing media did not show any major or evident sign of toxicity. However, certain oxidative related parameters, mainly proline concentration in the aerial part of the plants and NPT and PCs concentrations in the roots, were significantly increased by the presence of As in the nutrient solution. These effects disappeared when ascorbic acid was added to the growing media, either concomitantly or as a pretreatment. The concentration of As in the roots of the plants, retained mostly as As(III), was also significantly lowered in the presence of AsA, which accounted for the alleviation of the toxic effects that appeared in the plants according to the PCAs performed. These findings reinforce the previously observed compartmentalization of As as As(III) in the roots of rice plants and points out to proline concentration in the shoots, which can be easily determined, as a useful marker of As presence and toxicity in rice plants. This may be of relevance for the use of rice as a test plant in future As toxicity experiments or even as a parameter to be determined in cultivated rice as an indicator of As toxicity. The priming effect of AsA on As toxicity was also found to be useful in alleviating As toxicity to rice plants, although it may have to be further evaluated in plants suffering from more severe toxic symptoms.

I.7. References

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I.8. Supplementary Information

Table S1 (SI). Shoots PCA.

	1	**2**	**3**	**4**	**5**	**6**
TPC	0.886					
MDA	0.866					
FRAP	0.851					
AsA	0.842					
Carotenoids	0.833					
DPPH	0.826					
Chl-b	0.803					
NPT	0.780					
Chl-a	0.759					
PA	0.682					
TSS	0.599					
As(III)		0.884				
As(V)		0.809				
Fe		-0.747				
Proline		0.694				
Total As		0.582				
N			0.929			
P			0.897			
TSP				0.840		
H_2O_2	0.505			0.769		
Carbonyl proteins				-0.747		
GSH					0.928	
PCs					-0.834	
Fresh weight						0.882
Plant height						0.876
% Variance	38.1	14.7	12.8	9.6	6.3	4.3

TPC: total soluble phenolic compounds. PA: proanthocyanidins. TSS: total soluble sugars. TSP: total soluble proteins. FRAP, DPPH: antioxidant capacity. AsA: Total ascorbic acid. GSH: Total glutathione. NPT: Non-protein thiols. MDA: Malondialdehyde. PCs: phytochelatins.

Table S2 (SI). Roots PCA.

	1	**2**	**3**	**4**	**5**
Proline	0.899				
TPC	0.897				
FRAP	0.896				
PA	0.894				
DPPH	0.882				
As(V)	0.843				
Length increase	-0.801				
GHS	-0.743				-0.510
Fresh weight	-0.685				
MDA	0.606				
NPT		0.971			
PCs		0.971			
Total-As		0.910			
As(III)		0.790			
Carbonyl proteins			-0.896		
TSP			0.707		
AsA			0.677		
P				0.919	
N				0.893	
TSS					-0.737
% Variance	36.01	16.87	11.30	9.32	6.67

TPC: total soluble phenolic compounds. PA: proanthocyanidins. TSP: total soluble proteins. TSS: total soluble sugars; FRAP, DPPH: antioxidant capacity. AsA: Total ascorbic acid. GSH: Total glutathione. NPT: Non-protein thiols. MDA: Malondialdehyde. PCs: phytochelatins.