Wrack as a strategy to increase *Hordeum vulgare* L. tolerance to copper-contaminated soils

Filipa Rodrigues de Sousa
Mestrado em Biologia Funcional e Biotecnologia de Plantas
Departamento de Biologia
2020

Orientador
Fernanda Fidalgo, Professora Associada com Agregação, FCUP

Coorientador
Ruth Pereira, Professora Auxiliar com Agregação, FCUP

U.PORTO

FACULDADE DE CIÊNCIAS
UNIVERSIDADE DO PORTO

Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

Porto, _____/_____/_____

For the elaboration of this master thesis, data present in the following scientific article and communications was used:

- Sousa F, Martins M, Sousa B, Soares C, Azenha M, Pereira R, Fidalgo F (2020) Does beach wrack have the potential to be used as a plant biostimulant? Mineral composition, antioxidant properties, and effects against metal-induced stress– article in preparation for submission;
- Sousa F, Martins M, Sousa B, Soares C, Azenha M, Pereira R, Fidalgo F (2020) Insights of the mineral composition and antioxidant properties of wrack from the north Portuguese sandy beaches. Verão com Ciência – Hands on Science for Sustainable AgriFood Production: From the Soil to the Fork;

- Sousa F, Martins M, Sousa B, Soares C, Pereira R, Fidalgo F (2020) Assessment of copper impacts on barley plants grown in a natural agricultural soil. IJUP 20 - Encontro de Jovens Investigadores da Universidade do Porto, Portugal;

I would like to acknowledge the financial support from:

- Research Project No. 029818, co-financed by COMPETE 2020, Portugal 2020 and the EU through the European Regional Development Fund (ERDF), and by Fundação para a Ciência e Tecnologia (FCT) through national funds.

- FCT and GreenUPorto through the summer school scholarship "Verão com Ciência – Hands on Science for Sustainable AgriFood Production: From the Soil to the Fork", promoted by FCT, in collaboration with Direção Geral do Ensino superior (DGES);

ACKNOWLEDGMENTS

Ao fim de um ano de trabalho, chega a hora de agradecer a quem me ajudou a tornar este sonho possível.

À Professora Fernanda, um enorme obrigada pela disponibilidade e simpatia com que me acolheu, pelo voto de confiança que depositou em mim, por toda a ajuda que disponibilizou ao longo deste ano e pelos conselhos e sugestões que tornaram este trabalho mais rico. À Professora Ruth agradeço a disponibilidade e toda a ajuda que me deu ao longo da realização desta dissertação e por ter direcionado muitas vezes o trabalho de forma a que este se tornasse mais robusto e completo. Agradeço ainda ao Professor Manuel Azenha, pela ajuda nas quantificações químicas pela simpatia e disponibilidade com que sempre me recebeu.

Aos meus pais, que sempre me incentivaram a perseguir os meus sonhos e por serem o maior exemplo de perseverança que eu podia ter. Por todo o amor e carinho que sempre tiveram por mim ao longo da minha vida, que me tornou a pessoa que sou hoje. As minhas irmãs, à Silvana, porque sem ela nada disto era possível. Obrigada por me teres permitido realizar o sonho de tirar o curso que sonhei mesmo que para isso tivesses de abdicar de alguns confortos. Sei bem que não há irmãs como tu e tenho muito orgulho no que fizeste por mim. À Mariana por ser a menina mais carinhosa que eu conheço e por estar sempre lá para mim muitas vezes mesmo sem perceber, obrigada. Aos meus avós, que sempre mostraram um orgulho gigante em mim, o que me deu sempre forças para continuar e para fazer melhor.

Um obrigada também aos meus amigos, à Mariana, à Beatriz, ao Miguel e à Gabriela, por terem sempre a certeza que eu ia ser capaz, por me apoiarem em tudo e por serem os melhores amigos que alguém pode ter.

Às minhas companheiras de mestrado, à Sofia, à Margarida e à Mafalda, obrigada por todo o apoio, por estarem sempre lá, tenho a certeza que sem vocês esta caminhada teria sido muito mais difícil.

Por fim, obrigada ao Cris, à Maria e ao Bruno. Mil obrigadas não chegam para agradecer tudo o que fizeram por mim ao longo deste ano. Obrigada por toda a paciência, por todos os ensinamentos e por estarem sempre disponíveis para me ajudar, sem vocês não sei se conseguiria.

Obrigada a todos que de uma forma ou de outra contribuíram para que este trabalho se realizasse da melhor forma possível.

RESUMO

O crescimento da população humana, bem como as acrescidas necessidades alimentares, representam atualmente um desafio para a agricultura, sendo necessário produzir cada vez mais alimentos com menos recursos. Neste sentido, e devido às múltiplas pressões ambientais a que as colheitas estão sujeitas, entre as quais a contaminação dos solos, torna-se necessário o desenvolvimento de estratégias eficientes e sustentáveis que garantam elevados índices de produtividade agrícola. Numa perspetiva de economia circular, assente na valorização do sargaço, um resíduo orgânico de origem natural cuja abundância se prevê que aumente nas próximas décadas, este estudo visa avaliar o seu potencial para fertilizar o solo e aumentar a tolerância de espécies vegetais com interesse económico a solos contaminados com cobre (Cu). Para este efeito usou-se como espécie modelo *Hordeum vulgare* L. (planta de cevada). Primeiramente, procedeu-se à caracterização do perfil químico e bioquímico do sargaço, seguida de uma abordagem *in vitro* na qual se avaliou os efeitos da adição de um extrato aquoso deste material vegetal (0, 1,25; 2,5; 5,0 e 7,5 g L^{-1}) a um meio de cultura contendo, ou não, 7 mg Cu L^{-1}. Os dados obtidos sugeriram que o extrato utilizado tem a capacidade de melhorar o desempenho das plântulas de cevada não só em condições normais (não-stresse), mas também de stresse, sendo que este efeito poderá estar relacionado com a composição química e o perfil bioquímico do sargaço.

De seguida, os estudos incidiram na avaliação dos efeitos da adição do sargaço ao solo. Para tal, amostras de um solo agrícola foram tratadas com diferentes concentrações de sargaço [0; 0,5; 1 e 2 % (m/m)], sendo as suas propriedades químicas avaliadas após dois períodos de estabilização (15 e 30 d). Os resultados mostraram que as aplicações de doses crescentes de sargaço ao solo contribuíram para um ligeiro aumento do pH, bem como da condutividade elétrica e do teor em matéria orgânica. Após um conjunto de testes de otimização, nos quais foram selecionadas as concentrações de Cu e de sargaço a utilizar através de ensaios de fitotoxicidade, foi realizada uma última experiência, onde, depois de 15 d de estabilização, plantas de cevada cresceram num solo contaminado com Cu (219 mg kg^{-1}) e tratado com sargaço 2% (m/m). Após 14 d, a presença de Cu inibiu todos os parâmetros relacionados com o crescimento vegetal (comprimento das raízes e biomassa das folhas e raízes); porém, a aplicação de sargaço ao solo reverteu a maioria destes efeitos deletérios e promoveu uma menor acumulação de Cu nas raízes. A avaliação do estado redox das plantas, obtida pela quantificação das principais espécies reativas de oxigénio (ROS) e da peroxidação lipídica (PL), mostrou um aumento dos níveis de anião superóxido ($O_2^{\bullet-}$) em ambos os órgãos da planta, acompanhado de aumentos no conteúdo de peróxido de hidrogénio (H_2O_2) e de PL nas raízes, causados pelo Cu. Contudo, o co-tratamento com sargaço foi capaz de reverter, pelo menos parcialmente, alguns destes efeitos (diminuindo os níveis de PL e $O_2^{\bullet-}$), principalmente nos tecidos radiculares. Os mecanismos de defesa

utilizados pelas plantas de forma a lidar com o stresse induzido pelo Cu mostraram estar associados, principalmente, a mecanismos da componente não-enzimática do sistema antioxidante (AOX), com aumentos no teor de glutationa (GSH), ascorbato (AsA) e fenóis, principalmente nas raízes, o principal órgão alvo da fitotoxicidade do Cu. Pelo contrário, a acumulação de prolina (Pro) foi inibida, o que poderá ter favorecido o aumento da PL nas raízes. Relativamente à proteção mediada pela aplicação de sargaço contra o Cu, foi possível observar a modulação dos níveis de GSH e do estado redox do AsA, além de um aumento significativo da atividade da peroxidase do ascorbato (APX) nas folhas.

Em suma, os resultados obtidos neste estudo parecem indiciar o potencial do sargaço como mitigador do stresse induzido por Cu em plantas de cevada, não só quando aplicado sob a forma de extrato aquoso, mas também quando incorporado no solo. Adicionalmente, foi possível elucidar que o efeito benéfico do sargaço está provavelmente relacionado com a redução do dano oxidativo induzido pelo Cu, não só pela estimulação de componentes não enzimáticos do sistema AOX da planta, mas também pela adsorção do Cu e limitação da bioacumulação do metal, principalmente nas raízes.

PALAVRAS-CHAVE

Macroalgas; extrato de macroalgas; bioestimulantes; remediação do solo; economia circular; tolerância a stresse abiótico;

ABSTRACT

The rising global demand for food by a growing human population is pushing agriculture besides its boundaries, since more food must be produced with fewer resources. Therefore, and since crops are subjected to distinct environmental pressures, such as metal contamination, which severely compromise crop growth and food safety, new, efficient and sustainable strategies are urgently needed to ensure high agronomic yields. From a circular economy perspective, based on the valorisation of wrack, an organic residue whose abundance is expected to increase over the next decades, this study aims to provide an integrative evaluation of wrack's potential to increase the tolerance of plant species of economic interest to soils contaminated with copper (Cu). For this purpose, the model species *Hordeum vulgare* L. (barley plant) was used. Firstly, a characterisation of wrack's chemical and biochemical profile was conducted, paired with an *in vitro* assay in which different concentrations of an aqueous extract of wrack (0, 1.25, 2.5, 5.0 and 7.5 g L^{-1}) were applied to a growth medium supplemented, or not, with Cu (7 mg L^{-1}). Obtained preliminary data suggested that the ability of this extract to slightly increase seedlings' performance under stress and non-stress conditions, being this effect probably related to wrack's mineral composition and AOX profile.

Afterwards, focus shifted towards the use of wrack as a soil amendment. In this way, an agricultural soil was treated with 0, 0.5, 1 and 2% (m/m) wrack and its chemical properties were evaluated upon two stabilisation periods (15 and 30 d). Results allowed to observe slight increases in pH, electrical conductivity and organic matter upon wrack application. Following several optimisation assays, where Cu and wrack concentrations were selected through phytotoxicity assays, a main experiment was conducted with barley plants grown in a soil contaminated by 219 mg Cu kg^{-1} and amended with 2% (m/m) wrack, left for stabilisation during 15 d. Controls without Cu or wrack were also considered. After 14 d of exposure, Cu impaired all growth-related parameters (root length, and root and leaves biomass), but wrack application reverted most of these negative impacts, being this accompanied by a lower metal accumulation in roots. The evaluation of the redox status, through the quantification of the main reactive oxygen species (ROS) and lipid peroxidation (LP), showed that Cu increased the levels of superoxide anion ($O_2^{\bullet-}$) in both organs, and roots' hydrogen peroxide (H_2O_2) content and LP. Nonetheless, the co-treatment with wrack was able to, at least partially, revert some of these effects (decrease in LP and $O_2^{\bullet-}$ levels), especially in roots. The defence mechanisms employed to counteract Cu-induced stress were mostly related to the non-enzymatic antioxidant (AOX) system, with observed rises in levels of glutathione (GSH), ascorbate (AsA) and phenols, mostly on the roots, as this appears to be the primary target organ of Cu phytotoxicity; contrarily, proline (Pro) accumulation was inhibited, which could favour the increase of root LP. Concerning wrack-

mediated protection against Cu, a modulation of GSH levels and AsA redox state was found, along with an enhancement of ascorbate peroxidase (APX) in leaves.

Overall, the results herein obtained seem to indicate the potential of wrack to alleviate Cu-induced stress in barley plants, when applied either as a liquid extract or as soil amendment. From the soil-based assays, it was possible to unravel that wrack ameliorative action probably relies on the reduction of the oxidative stress imposed by Cu, not only by a more efficient activity of the non-enzymatic components of the plant AOX system, but also by limiting Cu absorption and bioaccumulation, especially in roots.

KEYWORDS

Macroalgae, seaweed extract, plant biostimulants, soil remediation, circular economy, abiotic stress tolerance.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS, ACRONYMS AND SYMBOLS

$^{\bullet}OH$ – hydroxyl radical;

$^{1}O_2$ – oxygen singlet;

Abs – absorbance;

$AlCl_3$ – aluminium chloride;

AOX – antioxidant;

APX – ascorbate peroxidase;

AsA – ascorbic acid;

ATX – autotaxin;

BIP - 4,4'-bipyridyl

BSA – bovine serum albumine;

C – carbon;

Ca – calcium;

Car – carotenoid;

CAT – catalase;

CC – climate change;

CCS – Cu chaperone for SOD;

Cd – cadmium;

Chl a – chlorophyll a;

Chl b – chlorophyll b;

Chl c - chlorophyll c;

CO_2 – carbon dioxide;

COX – cyclooxygenase;

Cr – chromium;

Cu – copper;

$CuSO_4.5H_2O$ - copper (II) sulphate pentahydrate;

ddH2O – deionised water;

DHA – oxidised ascorbate;

DHAR – dehydroascorbate reductase;

Dm – dry mass;

DPPH - 2,2-diphenyl-1-picrylhydrazyl;

DTNB - 2-nitrobenzic acid;

DTT - dithiothreitol;

EC – electrical conductivity;

EDTA - ethylenediaminetetraacetic acid;

FAAS – flame atomic absorption spectroscopy;

Fe – iron;

$FeCl_3$ - iron (III) chloride;

Fm – fresh mass;

GFAAS - graphite furnace atomic absorption spectroscopy;

GPOX – guaiacol peroxidase;

GR – glutathione reductase;

GS – glutamine synthetase;

GSSG - oxidised GSH;

GST – glutathione S-transferase;

GSH – glutathione;

H_2O – water;

H_2O_2 – hydrogen peroxide;

H_3PO_4 – phosphoric acid;

HNO_3 – nitric acid;

H_2S - hydrogen sulphide;

H_2SO_4 – sulphuric acid;

K – potassium;

KCH_3COO – potassium acetate;

LP – lipid peroxidation;

MAPK – mitogen-activated protein kinases;

MDA – malondialdehyde;

MDHAR – monodehydroascorbate reductase;

Mg – magnesium;

MS – Murashige and Skoog;

N – nitrogen;

Na_2CO_3 – sodium carbonate;

Na_2HPO_4 – sodium phosphate;

NADPH - nicotinamide adenine dinucleotide phosphate;

NaN_3 – sodium azide;

NBT – nitroblue tetrazolium;
NEM - N-ethylmaleimide;
$(NH_4)_2MoO_4$ – ammonium molybdate;
Ni – nickel;
NR – nitrate reductase;
O_2 – oxygen
$O_2^{\bullet-}$ - superoxide anion;
OECD - Organisation for Economic Co-operation and Development;
OM – organic matter;
P – phosphorus;
PB – plant biostimulants;
Pb – lead;
PCD – programmed cell death;
PK – potassium phosphate;
PMSF – phenylmethylsulphonyl fluoride;
Pro – proline;
PS I – photosystem I;
PS II – photosystem II;
PTE - potentially toxic elements;
PVPP – polyvinylpolypyrrolidone;
ROS – reactive oxygen species;
RT – room temperature;
SOD – superoxide dismutase;
SN – supernatant;
SWE – seaweed extracts;
TAC – total antioxidant capacity;
TBA – thiobarbituric acid;
TCA – trichloroacetic acid;
TFC – total flavonoid content;
TPC – total phenols content;
WHC – water holding capacity;
WHC_{max} – maximum water holding capacity;
YSL – yellow stipe like family;
ZIP – zinc transporter proteins;
Zn – zinc.

1. INTRODUCTION

1.1. Climate change (CC) and wrack production

Throughout Earth's history, the climate has considerably changed due to natural processes, including cycles of glacial advance and retraction. However, over the past 50-100 years, these changes have been much larger and faster than anticipated (https://climate.nasa.gov/evidence/). According to the United Nations, climate change (CC) is currently "the issue of our time" and if no major actions are taken now, the adaptation to CC-mediated impacts will be each day more difficult and costly (https://www.un.org/en/sections/issues-depth/climate-change/). Scientific evidences have been unequivocally showing that CC is mainly driven by the increased abundance of greenhouse gases in the atmosphere, as a result of many anthropogenic activities, such as fossil fuel combustion, breeding cattle and agricultural practices (https://climate.nasa.gov/causes/). Although greenhouse gases, by trapping solar heat, have allowed the development of life on Earth, their unbalanced increase can, paradoxically, lead to long-term environmental and health effects, besides affecting the normal functioning of marine and terrestrial ecosystems (Li et al., 2018). Particularly in the ocean, CC is already evoking multiple alarming incidents, which include, among others, the increase of sea temperatures and acidification, along with changes in the timing and volume of freshwater runoffs in coastal marine waters, leading to some unpredictable events, such as algal blooms (Moore et al., 2008). These macroalgae blooms occur as a consequence of nutrient enrichment of the water, namely with high levels of nitrogen (N) and phosphorous (P), caused by their large transport from land to the sea. The impacts of this transport also have consequences for other marine and brackish water species, thus leading to changes in trophic chains and loss of ecosystem functions and services. Additionally, this accumulation of macroalgae and other macrophytes, results in high amounts of decomposing biomass along the coastline (Ansell et al., 1998; Franzén et al., 2019), leading to a rise in wrack production and accumulation (Macreadie et al., 2011; Alobwede et al., 2019).

1.2. Wrack: ecological role, hazards and mitigation actions

Wrack is the result of the accumulation of macroalgae organic debris (mainly *Laminaria, Fucus, Saccorhiza, Sargassum, Codium, Palmaria, Chondrus,* and *Gelidium*) along the coastline. In fact, macroalgae growing on continental shelves can be detached by the action of the tides, reaching coastal areas, such as beaches, where they accumulate and

are considered a natural and essential component of these ecosystems providing food and shelter to intertidal species and playing an important role in reducing coastal erosion (Macreadie et al., 2011; Alobwede et al., 2019; Martins et al., 2020). However, the species constituting wrack can significantly vary, since they are determined by a wide range of factors such as the marine environment, the composition of the original algae community, and the wind and current directions. Additionally, the spatial and temporal location of wrack can also change depending on the abundance and composition of its constituent species, the physical characteristics of the beach and its mobility due to wind and waves. All these factors prompt the formation of mosaics of empty and filled wrack zones on the beach sand, having impact on the abundance, composition, and population structure of the primary consumers/detritivores that are part of local food chains (Martins et al., 2020). When wrack accumulates in the upper part of the intertidal zone, it is protected from the action of tides. However, it may be exposed to a variety of abiotic and biotic conditions and may undergo several processes, such as consumption by invertebrates, dryness, and microbial degradation, which overall influence wrack's nutritional composition. In terms of its degradation, the process depends, once again, on a variety of factors, such as abundance and the morphological, physicochemical and nutritional characteristics of the wrack-forming macroalgae (Martins et al., 2020). Moreover, the decomposition of wrack plays a central role in providing producers with nutrients, such as N, in the form of nitrate, nitrite, and ammonia, and P that are released during this process (Macreadie et al., 2017).

As mentioned before, wrack is considered an essential component of coastal ecosystems, since it has a key role in providing biomass for decomposers and detritivores, as well as providing habitat, shelter and food for meiofauna, marine and terrestrial macrofauna, birds, and fish, while granting protection to coastal dunes (Duong, 2008). However, excessive amounts of this residue can also be problematic for the environment. In fact, the overaccumulation of this organic waste causes eutrophication of coastal areas, disturbing the habitat of some species, restricting oxygen (O_2) exchange and causing toxic effects due to the release of hydrogen sulphide (H_2S), which decreases soil pH and increases greenhouse effect (Colombini et al., 2003). Additionally, due to the ability of macroalgae to accumulate fecal bacteria, excessive accumulation of wrack may increase the incidence of these microorganisms on beaches, posing a public health threat (Martins et al., 2020). At the same time, large amounts of this residue can negatively impact tourism activities, by affecting the amenity value of beaches, requiring its removal from these coastal areas every year. Once removed, this organic residue is mainly wasted, generating environmental and economic costs (Colombini et al., 2003;

Villares et al., 2016; Macreadie et al., 2017). In this context, and bearing in mind the recent Circular Economy Action Plan of the European Commission (European Commission, 2019), that aims to reduce waste by increasing the life-cycle of organic material, as well as the urgent need of incorporating and sequestrating carbon (C) in soil, removing it from the atmosphere, the exploitation of valorisation approaches of wrack is a very important topic of research (Alobwede et al., 2019). In fact, recent studies point out to a wide range of wrack's potential utilisations (Villares et al., 2016; Macreadie et al., 2017). For instance, it is already known that wrack contains several essential nutrients for plants, showing a great fertilising potential. Actually, wrack has been used in Portugal since the middle ages to the mid-20th century as a way to fertilise the agricultural fields (Sousa et al., 2020). Furthermore, the utilisation of wrack in soil remediation, as an adsorbent for water treatment, animal feed additive, insulating material and even as biochar, was already reported (Villares et al., 2016; Macreadie et al., 2017).

In this way, and considering that the abundance of wrack is expected to increase over the following years (Macreadie et al., 2011; Alobwede et al., 2019), wrack's reuse and valorisation is important and should be promoted, preventing the adjacent risks to its accumulation and preventing it from being removed and placed in landfills without any kind of valorisation.

1.3. Macroalgae as biostimulants and soil enhancers

Nowadays, it is a general aim to reduce the use of inorganic fertilisers in agricultural practices, as these formulations are harmful for the environment as part of them are not used by plants being lost through leaching or runoffs (Bulgari et al., 2019). In the last three decades, several technological innovations have been proposed to increase the sustainability of agricultural production systems (Rouphael and Colla, 2020), not only to ensure crop performance and resilience to environmental stress, but also to reduce the use of pesticides and fertilisers (Chiaiese et al., 2018; Rouphael and Colla, 2020).

Plant biostimulants (PB) attract interest in modern agriculture, representing a promising and environmentally friendly strategy to complement traditional synthetic agrochemicals in order to reduce its use. The definition of PB has changed over time, having been intensively discussed by several researchers in the past decade (references in Rouphael and Colla, 2020). According to the recent Regulation (EU) 2019/1009 a plant biostimulant is a "*fertilising product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving*

one or more of the following characteristics of the plant or the plant rhizosphere: i) nutrient use efficiency, ii) tolerance to abiotic stress, iii) quality traits, or iv) availability of confined nutrients in the soil or rhizosphere" (EU, 2019). Based on this definition, PB include: animal and vegetal protein hydrolysates, humic and fulvic acids, chitosan, beneficial microorganisms (arbuscular mycorrhizal fungi and N-fixing bacteria), inorganic compounds and macroalgae/seaweeds extracts (Rouphael and Colla, 2020).

Algal-derived biostimulants, typically referred as seaweed extracts (SWE), have been used in modern agriculture for the past 60 years, as extracts from several macroalgae species such as *Ascophyllum nodosum* L., *Fucus*, *Laminaria*, *Sargassum*, and *Turbinaria*. In fact, it is known that the use of seaweed-based products brings some benefits to plants, contributing for a better germination rate, a higher root nodulation and fruit quality, while simultaneously improving crop resilience to different abiotic and biotic stressors (Sharma et al., 2013; Arioli et al., 2015) (Figure 1). Thus, the use of macroalgae residues (i.e. wrack) as biostimulant and its main benefits are already a proven matter (Nabti et al., 2016).

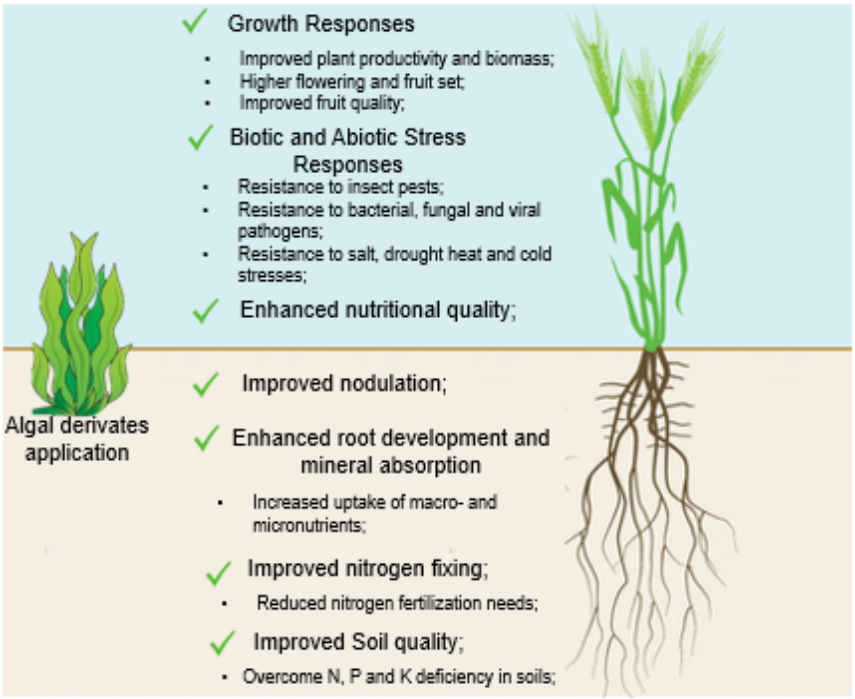

Figure 1: A summary of the effects of the action of algae extracts in plants. Adapted from Khan et al., (2009); Adrees et al., (2015); Arioli et al., (2015) and Battacharyya et al., (2015).

In this way, although some of the effects of growth-promoting SWE have been reported on several species, it is yet unclear what mechanisms are behind those beneficial effects (Van Oosten et al., 2017). Indeed, it is recognised that the biomass of macroalgae contains bioactive molecules capable of interacting at different stages of plant growth. Ranging from mineral nutrients and phytohormones, to antioxidants (AOX) and

macromolecules (e.g. carbohydrates and proteins), macroalgae extracts/composts can be an important source of biologically active compounds for sustainable agriculture (Sharma et al., 2013). However, among all components, the most important for plant growth are phytohormones, namely cytokinins and auxins (indole acetic acid), auxin-like compounds, and abscisic acid (Zhang and Ervin, 2004; Mancuso et al., 2006; Khan et al., 2009; Nabti et al., 2016; Mukherjee and Patel, 2019). In parallel to the phytohormones, seaweeds also contain polysaccharides along with phlorotannins which are capable of triggering signalling cascades in order to activate plants' defence against biotic and abiotic stressors, for instance protecting plant cells from oxidative stress and injury. Furthermore, these organisms are also a source of polysaccharides like alginates fucoidans, carrageenans and laminarins, which present not only water-binding capacity, but also possess chelating properties, being able to bind cations of trace elements (namely micronutrients), thus favouring, on one hand, nutrients uptake and, on the other hand, limiting the mobility and uptake of toxic metals when used in remediation programs in metal-contaminated areas (Abdel-Raouf et al., 2012; Sharma et al., 2013; Arioli et al., 2015; Battacharyya et al., 2015). Additionally, each type of macroalgae - brown, green and red - contain distinct molecules that act as plant elicitors, such as laminarins and fucans, ulvans, and carrageenans, respectively. As a diverse taxonomic group, macroalgae detain different chemical compositions and metabolites, providing distinct effects on plants growth (Sharma et al., 2013). For this reason, the use of SWE should be plant-specific and additional research regarding the use of different macroalgae species must be carried out, since the majority of the studies and products available only use the same species, *A. nodosum* (Table 1).

To date, and although the chemical identity of the responsible bioactive compounds is still unknown, the implication of phytohormones, such as cytokinins, provided by seaweeds, and the ability of some seaweed compounds to improve the activity of several AOX enzymes has been suggested as a possible mechanism to induce plant stress tolerance to a variety of stress factors. Additionally, seaweed extracts have also been proven to contain high levels of non-enzymatic AOX (Khan et al., 2009; O'sullivan et al., 2011; Fan et al., 2013; Van Oosten et al., 2017; Mukherjee and Patel, 2019). Moreover, many studies have reported the potential of seaweed application as a biofertiliser, due to its ability to overcome N, P and potassium (K) deficiency in soils and to improve plant productivity and biomass (Nabti et al., 2016).

Despite of that, important details about the use of biostimulants (e.g. timing and mode of application) are not clear, thus requiring further research in order to increase

the effectiveness of these substances in enhancing plant's tolerance to stress. For instance, these compounds can be applied as liquid extracts or as powder, being added before, after or during the stress episode (Bulgari et al., 2019). Thus, studies concerning these aspects are important, since the period and the optimal mode and dose of application are topics of great interest in order to guarantee the highest positive effect, high production incomes and prevent unexpected consequences on crops and environment (Bulgari et al., 2019; Mukherjee and Patel, 2019). Accordingly, knowing that algae-based biostimulants are biodegradable, non-toxic, non-polluting and non-hazardous to humans (Nabti et al., 2016), it would be important to study the potential of macroalgae extracts or composts, obtained either by freshly collected seaweeds or wrack, to be used in agriculture and evaluate how it can protect and or stimulate the development of plants under stressful and non-stressful conditions. In the following subsections, a general overview of the probable role of macroalgae as biofertilisers, stress ameliorators and soil quality enhancers will be briefly described.

1.3.1. Effects of SWE on soil properties and plant nutrition

SWE contain alginic acid, which has the ability to improve soils' physical characteristics and to chelate metal ions, forming a polymer with increased molecular weight, resulting in a crumb structure. The presence of this polymer enhances soil water holding capacity (WHC) leading to a better aeration of the soil and to a better capillarity, contributing to a stimulation of the root system, an increase of the soil bacteria activity and a better nutrient uptake (Battacharyya et al., 2015). In fact, the activity of the soil bacteria in the presence of SWE displays enhanced features such as: i) the secretion of substances that contribute to soil amelioration and ii) the ability to increase the N content of the soil. Concerning the first feature, the substances secreted to the soil by bacteria include polyuronides, compounds that are similar to alginic acid, which may have a direct effect on the soil structure and its capacity to maintain water content. Parallelly, the capacity of soil bacteria to degrade soil-added organic matter such as seaweed biomass, leads to an increase in the total N available and, consequently, to a higher N availability to plants, which positively affects their growth and productivity (Stephenson and Booth, 1968). Additionally, a previous study showed that the application of *A. nodosum* extracts to alfalfa (*Medicago sativa* L.) plants resulted in an increased number of N-fixing nodules in the roots, due to the activation of the expression of the bacteria gene *NodC* (Khan et al., 2012). Other studies reported that the application of similar extracts also upregulated the expression of *NRT1.1*, a gene coding for a nitrate transporter responsible for increasing the auxin transport and to act as a nitrate sensor, inducing a higher growth of

lateral roots and subsequently better N assimilation rates (Battacharyya et al., 2015). Besides the effects on bacterial activity, SWE also seem to be capable of inducing soil colonisation by beneficial mycorrhizal fungi, leading to an improvement in plant nutrition, namely on the metabolism of P (Stephenson and Booth, 1968; Khan et al., 2012; Battacharyya et al., 2015).

Lastly, plants are capable of absorbing nutrients either through roots or from the leaf surface. As so, the foliar application of SWE can also boost plant mineral status, promoting the uptake of nutrients and other beneficial elements through the leaf surface. In fact, minerals present in SWE, when applied by foliar treatment, are effectively absorbed by the leaves through stomata and cuticle pores (Sharma et al., 2013). Therefore, when considering this approach, one must also take into consideration the effects of different environmental factors, such as light intensity, temperature and humidity since these parameters affect the leaf status (Sharma et al., 2013; Arioli et al., 2015; Battacharyya et al., 2015).

1.3.2.Effects of SWE on plant stress tolerance

1.3.2.1.Abiotic stress

It is known that the productivity and quality of the agricultural crops are affected by unfavourable practices and climate-related constraints, such as drought, salinity and extreme temperatures (Raza et al., 2019). However, strategies to overcome these agronomic impacts are urgently needed and remain scarce. SWE have shown to portray a positive role in the mitigation of the above referred stresses in a large number of plant species and, although their mechanisms of action are not fully understood, some hypothesis have been suggested (Sharma et al., 2013). For example, the improvement of the chlorophyll content in leaves of some species, namely *Phaseolus vulgaris* L., *Hordeum vulgare* L., *Zea mays* L. and *Triticum durum* Desf., which led to a higher photosynthetic rate, has been associated with the presence of betaines in SWE, a compatible solute that not only improves photosynthesis, but also alleviates osmotic stresses (salinity and drought) (Sharma et al., 2013). Additionally, seaweeds' beneficial properties have also been linked to cytokinins, probably by their capacity to scavenge and prevent the formation of reactive oxygen species (ROS). Moreover, SWE ability to improve K^+ uptake and to enhance the performance of some AOX enzymes such superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11) and glutathione reductase (GR, EC 1.6.4.2) have been suggested as mechanisms to mitigate stresses such as extreme temperatures, drought and salinity (Khan et al., 2009;

Sharma et al., 2013). The beneficial effects of SWE application to improve plant abiotic stress tolerance have been described in several studies and are summarised in Table 1.

Table 1*:* A summary of some of the existent studies on the use of seaweeds as abiotic stress mitigators on different plant species.

Abiotic Stress	Seaweed species	Mode of application	Plant species	Beneficial effects	Reference
Cold	*Ascophyllum nodosum* L.	Foliar treatment; Soil drench	*Arabidopsis thaliana* L. *Cynodon dactylon* (L.) Pers. *Zea mays* L.	Enhanced root formation and development; Increased shoot growth and branching; Improved nutrient uptake.	(Munshaw et al., 2006; Rayirath et al., 2009; Nair et al., 2012; Van Oosten et al., 2017)
Drought	*Ascophyllum nodosum* L.	Foliar treatment; Soil drench	*Agrostis stolonifera* L. *Picea glauca* (Moench) Voss. *Pittosporum eugenioides* A. Cunn. *Spiraea nipponica* Maxim. *Spinacia oleracea* L.	Increased leaf area and number; Increased vegetative growth and physiological performance; Improved plant water status, stomatal conductance and photosynthetic rate.	(Zhang and Ervin, 2004; Xu and Leskovar, 2015; Elansary et al., 2016)
Salinity	*Ascophyllum nodosum* L. *Ecklonia maxima* (Osbeck) Papenfuss	Foliar treatment	*Cucurbita pepo* L. *Vitis vinifera* L. *Poa pratensis* L.	Increased plant growth; Enhanced macronutrient accumulation.	(Mancuso et al., 2006; Nabati et al., 2008; Rouphael et al., 2017)
Heat	*Ascophyllum nodosum* L.	Foliar treatment	*Agrostis stolonifera* L.	Increased photochemical efficiency and root viability.	(Zhang and Ervin, 2008)

By the analysis of Table 1, besides SWE capacity to mitigate a variety of stresses, it becomes clear that most research was conducted using few algae species and mainly explored the potential of macroalgae to alleviate climate-related stresses (e.g. temperature, salinity, drought). In this sense, the focus needs to be extended to the potential of different algal resources in alleviating the phytotoxicity of emerging anthropogenic pollutants, such as metals since no related studies have been conducted up to the date of the current study.

1.4. Copper (Cu) in the environment – from essential micronutrient to toxic metal

Due to their sessile nature, plants are almost permanently exposed to multiple environmental pressures, including high concentrations of metals in soils, whose main sources are anthropogenic activities, such as industrial emissions, mining, disposal of metal-rich residues and application of fertilizers and pesticides (Rehman et al., 2019).

Copper (Cu; atomic weight: 63.5 g mol^{-1}; density: 8.96 g cm^{-3}) is a structural component of the Earth's crust, that can exist under different complex forms, such as Cu sulphides, sulfosalts and carbonates, and as Cu (I) or Cu (II) oxides depending on its oxidation state. Being a naturally occurring metal, Cu can derive from parent material, rocks' disintegration, minerals' dissolution, and volcanic eruptions. However, anthropogenic sources, especially livestock production, industrial activities and intensive agriculture practices are the main sources of this metal to the environment (Figure 2) (Adrees et al., 2015).

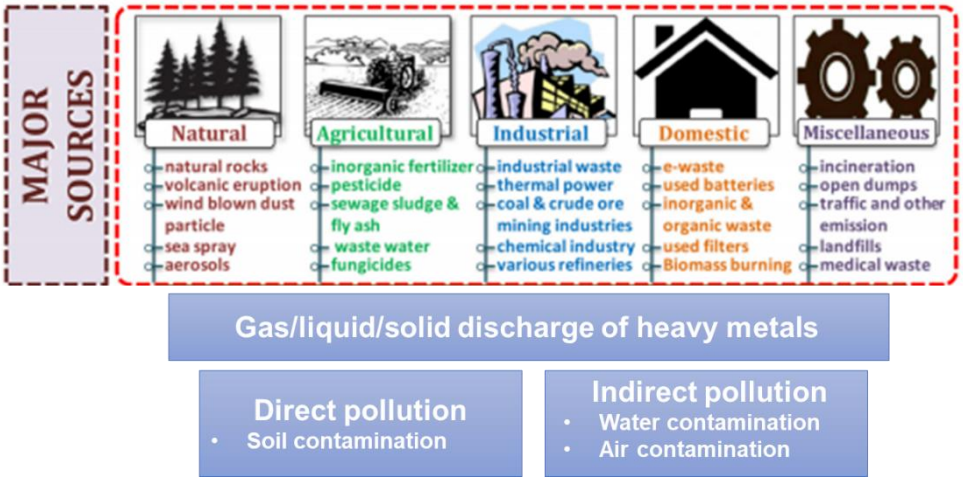

Figure 2: Main sources of copper accumulation in the environment. Adapted from Srivastava et al., (2017)

This element has been used in agriculture since 1880s with the discovery of the "Bordeaux mixture" used to combat mildew in grapevines (Rehman et al., 2019). Since that time, Cu has been used in agriculture as a fungicide and bactericide especially in organic farming since most methods of disease management rely almost exclusively on its usage (La Torre et al., 2018; La Torre et al., 2019). In this way, and since organic farming is expected to grow (http://www.rederural.gov.pt/centro-de-recursos/send/13-producao-sustentavel/1052-organic-farming-in-the-eu-a-fast-growing-sector), thus increasing Cu usage, the European Commission has recently established a reduction to a maximum amount of 4 kg per hectare per year of Cu until 2025 (SANTE 2018). In

addition to those efforts, researchers are working intensively on finding innovative alternative strategies to further reduce Cu use in organic agriculture, as this practice contributes to its accumulation in the environment up to concentrations above the legal thresholds (https://organic-farmknowledge.org/news-events/news/detail/organic-farming-copper-re-approved-in-europe) (Adrees et al., 2015).

In fact, when present in concentrations above background values, Cu can become toxic, disrupting nutrient cycling, inhibiting the mineralisation of essential nutrients and affecting the quality of soils (Adrees et al., 2015). Cu behaviour and bioavailability in soils depends on different variables, including soil pH and organic matter content (Adrees et al., 2015). The bioavailability of Cu in soils increases in acidic soils, since, at low pH, Cu is less adsorbed from the soil components and organic matter, increasing the amount of dissolved Cu and, consequently, the free ion activity. Additionally, plant roots, as well as the soil microbiome, by modulating soil properties, can also influence Cu mobility and bioavailability (Adrees et al., 2015; Rehman et al., 2019).

In non-contaminated areas, the levels of Cu in agricultural soils range from 5 to 30 mg kg^{-1} (Rehman et al., 2019), with only 1-20% being bioavailable (Adrees et al., 2015). However, with its increasing utilisation due to its presence in pesticides, concentrations of Cu in soils have reached 200, 495 and 1508 mg kg^{-1} in Australia, in the United States of America and in the United Kingdom, respectively (Yruela, 2005; Martins, 2014; Adrees et al., 2015; Merrington, 2018). Thus, the excessive, injudicious and unregulated use of Cu-based fungicides, bactericides and pesticides to control plant diseases and pests has resulted in the accumulation of this metal in the top layers of agricultural soils, which turned into a real problematic nowadays (Adrees et al., 2015; Khan et al., 2015). Moreover, as metals are not biodegradable, plants grown under Cu-contaminated soils can accumulate toxic levels of this metal in their tissues, posing a threat to humans, whose diet relies mainly on crops. Indeed, vegetables account for about 90% of human metal intake (Waqas et al., 2015). Nevertheless, and although several countries and international organisations have defined the recommended levels of metals in plants, there are numerous records reporting excessive Cu levels in different plant species, namely lettuce, rice, potato, spinach, tomato, pudina and radish (Khan et al., 2015), highlighting the importance of prioritising food safety assessment.

1.4.1. Cu as a micronutrient

Despite its recognised toxicity, an adequate amount of Cu is vital for the maintenance of the normal plant growth and for a balanced crop nutrition, since it is an essential micronutrient (Adrees et al., 2015; Rehman et al., 2019). As other mineral nutrients, the range of concentrations in which Cu can benefit plant growth is species-specific. Nonetheless, Cu plays an active role in various physiological processes of plants, including photosynthesis, cellular respiration and AOX metabolism, and it can be found in several subcellular components, such as endoplasmic reticulum, mitochondria, cytosol, chloroplasts, thylakoid and apoplast (Yruela, 2005, 2009). Moreover, Cu acts as a structural element of regulatory proteins, mediates oxidative stress responses and participates in cell wall metabolism, being also involved in signalling pathways. In addition, Cu serves as a cofactor of several important enzymes, such as Cu/Zn SOD and plastocyanin. Furthermore, due to its ability to bind to small molecules, Cu is also a structural component of a large range of oxidases (Yruela, 2005, 2009).

The root system of plants is responsible for Cu uptake from the soil pore water. This mechanism begins with Cu dissociation from its complex forms, followed by its adsorption on the root surface, allowing its uptake by the plant. After being absorbed by the root system, Cu can be translocated to shoots through both xylem and phloem. However, Cu is poorly translocated in plants, so roots usually contain the highest Cu concentration when compared to the aerial parts of the plant. Thus, Cu tolerance is dependent on plants' ability to accumulate this metal in their roots (Adrees et al., 2015; Rehman et al., 2019). Besides that, in attempt to avoid high accumulation of Cu in their tissues, and to ensure the maintenance of cellular metabolism, plants possess different homeostasis mechanisms, which allow the translocation of this metal through different plant tissues and organs (Yruela, 2005, 2009; Shahid et al., 2014). The mechanisms of Cu transport are linked to the regulation of the expression of genes encoding metal transporters in higher plants, such as the COPT family - with high affinity to Cu^{+}; zinc (Zn) transporter proteins (ZIP) with a role in divalent cation (Cu^{2+}) transport and P1B-ATPase and yellow stipe like family (YSL), as well as several chaperones belonging to autotaxin (ATX), cyclooxygenase (COX) and Cu chaperone for SOD (CCS) (Yruela, 2005, 2009; Shahid et al., 2014). It is also known that, in other organisms, these metal transporters are regulated at a transcriptional level via metal-sensing transcriptional factor proteins. Accordingly, plants also probably display metal sensors capable of detecting fluctuations in metal concentrations, triggering signalling pathways that activate specific responses which in turn result in the activation or inactivation of

transcription factors. For example, Jonak et al. (2004) reported the activation of mitogen-activated protein kinases (MAPK) pathways in *M. sativa* as a response to the exposure to an excess of Cu. Additionally, studies carried out with *Arabidopsis thaliana* L. suggested the transcription of miRNA activated by Cu-deficiency, thus downregulating the expression of chloroplastidial Cu/Zn SOD, in order to enhance the availability of Cu for other essential functions, such as photosynthesis.

As a micronutrient, both deficiency and excess of Cu inhibit plant growth by dysregulating important biochemical and molecular processes. Indeed, when compared with other potentially toxic essential elements [e.g. nickel (Ni), Zn], Cu has a higher phytotoxic potential (Adrees et al., 2015). Deficiency of Cu in plants occurs at levels below 5 μg g^{-1} dry mass (dm), and can retard plant growth, by compromising its development, reducing fruit production, modifying root and leaf architecture, and impairing the proper water cell transport (Rehman et al., 2019). Cu deficiency is manifested by symptoms, which include chlorotic and necrotic spots, as well as a significant reduction in photosynthetic pigments and changes in gene expression (Yruela, 2005, 2009; Adrees et al., 2015). As already mentioned, since Cu is a co-factor of several proteins, its deficiency can disturb many vital enzymes, such as SOD, nitrate reductase (NR; EC: 1.7.1.2) and glutamine synthetase (GS; EC: 6.3.1.2), as well as some physiological events (Hristozkova et al., 2006). In fact, it is known that both photosystem I (PSI) and II (PSII) activities are reduced under Cu deficiency, which clearly compromises photosynthesis, due to a decrease in plastocyanin levels and to a disintegration of the thylakoid membranes, respectively. Moreover, given that the electron transporter plastocyanin is one of the most abundant Cu-proteins in green tissues, it is not surprising that Cu deficiency reduces the photosynthetic electron transport (Yruela, 2009). However, excess levels of this metal, similar to its deficiency, can cause the inhibition of plants' growth and the reduction of photosynthetic yield, because Cu toxicity inhibits photosynthetic apparatus, resulting in the degradation of stromal lamellae and loss of grana stacking (Yruela, 2005). Thus, high Cu concentrations in plants cause negative consequences at both macro- and microscopic levels (Figure 3).

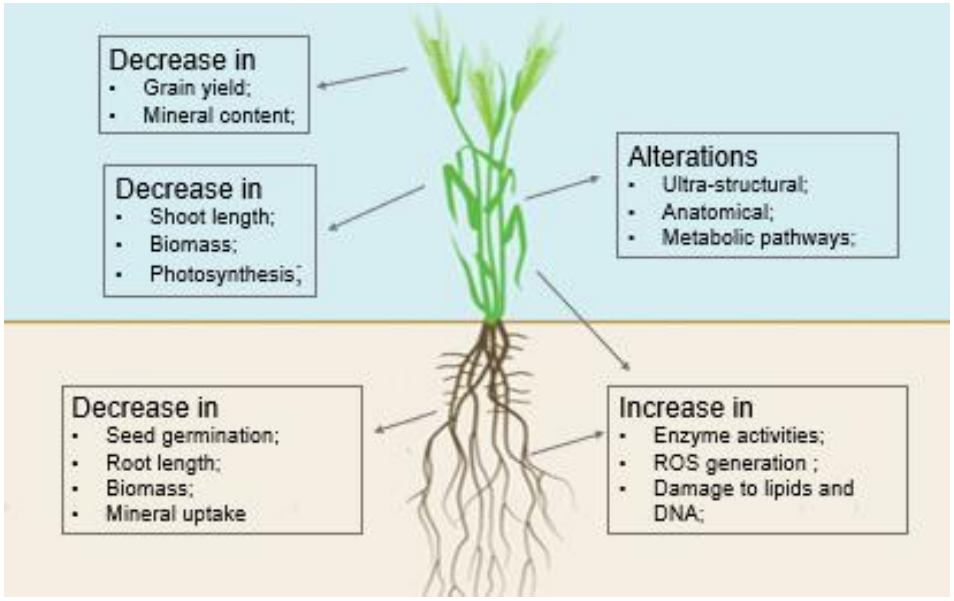

Figure 3: Toxic effects of Cu-excess in plants. Adapted from Adrees et al., (2015)

The excess of Cu may also affect the cell ultrastructure, causing the appearance of smaller, rounded shaped, numerous chloroplasts without starch granules, and the presence of larger vacuoles in the cytoplasm. These ultrastructural alterations may reflect in macroscopic effects, namely yield reduction, poor germination and anatomic alterations in leaves and roots such as destroyed epidermis and increased diameter of xylem vessels (Yruela, 2009). In addition, the biosynthesis of the photosynthetic machinery is affected, causing alterations in the protein and pigment composition due to a reduction of chlorophyll content (Yruela, 2009).

The role of Cu excess on disrupting the uptake of other mineral nutrients is well documented (Marschner, 2012). This phenomenon can be explained by the fact that similar cations can compete for plant uptake since the homeostasis mechanisms are not able to avoid or select the entrance of high concentrations of non-essential ions. As a matter of fact, several studies report that the excessive concentration of Cu in soil results in a decrease of the uptake and accumulation of certain minerals [calcium (Ca), K, iron (Fe), N, P], disturbing the normal nutrient balance in different plant organs (Adrees et al., 2015). Additionally, besides the competition for plants' uptake, these ions also compete for binding sites, causing the removal of some essential metal ions from the active centers of different biomolecules. For example, when Cu is above the average levels in plants (10 µg g^{-1} dm), it competes for the binding site of magnesium (Mg) in the molecules of chlorophyll, leading to a lack of function of the chlorophyll-complexes (Yruela 2009). In addition, when compared to other nutrients, high levels of Cu particularly impact Fe content, causing its deficiency, since Fe and Cu compete in ion uptake (Adrees et al.,

2015). Accordingly, in the majority of higher plants, Cu and Fe present an antagonistic relationship which may result in negative impacts in photosynthesis, since low Fe levels induce changes in the structure and function of the whole photosynthetic apparatus and a decrease in photosynthetic pigments (Yruela, 2005, 2009; Adrees et al., 2015).

Ultimately, the presence of Cu in concentrations above the normal leads to an overproduction of ROS (Adrees et al., 2015; Rehman et al., 2019).

1.5. Oxidative stress and antioxidant (AOX) response

ROS are oxygen-derived molecules and/or free radicals, continuously produced as a consequence of the aerobic cell metabolism (Scandalios, 2005). The main ROS include two radical forms – superoxide anion ($O_2^{\bullet-}$) and hydroxyl radical ($^{\bullet}OH$) – and two molecules – hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). In plants, as in other organisms, ROS play a dual role depending on their concentration in cells: at low levels, they can act as intracellular signalling agents, inducing a positive response of the AOX system, while at high levels, ROS become toxic, disturbing the cellular homeostasis. As a typical response to all types of stress, an increase in ROS production or inactivation of the AOX machinery can disrupt the balance between the production and elimination of ROS by the plants, leading to oxidative stress (Soares et al., 2019). In this way, maintaining the above described balance is considered as one of the most complex processes occurring within the cell (Soares et al., 2019). The oxidative damages caused by excessive levels of ROS include peroxidation of lipids and oxidation of proteins, inhibition of enzymatic activity, DNA damages and, ultimately, activation of programmed cell death (PCD) (Adrees et al., 2015; Soares et al., 2019).

1.5.1. Main ROS – characteristics, sites of production and biological effects

Singlet oxygen ($^1O_2$) is described as an unusual ROS, since its production is not related to electron transfer to O_2, but rather it is linked to the energy dissipation of the chlorophyll triplet state, which can relocate its electrons to O_2, leading to the production of $^1O_2$ (Gill and Tuteja, 2010). Since the occurrence of this ROS is related to strong light conditions and/or low carbon dioxide (CO_2) assimilation rate, studies indicated that this molecule can damage PS I and II (Gill et al., 2010; Soares et al., 2019). However, as different environmental stresses affect stomatal conductance, contributing for a consequent loss of CO_2 availability, this ROS can also be formed upon exposure to other unfavourable conditions, such as salinity and drought (Sharma et al., 2012). $^1O_2$ is a powerful oxidant, with a short lifetime between 1-4 μs, that reacts directly and rapidly with macromolecules

in close proximity to its production location, the chloroplast. The short lifetime of $^1O_2$ makes it an unlikely candidate to diffuse over any distances within the cell (Foyer, 2018; Soares et al., 2019).

In contrast, the $O_2^{\bullet-}$, the most typical ROS and the first to be produced, results from the monovalent reduction of O_2 during electron transport across electron transport chains of photosynthesis and respiration, and it is produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and cell wall peroxidases (Sewelam et al., 2016). The reactivity of $O_2^{\bullet-}$ is moderate when compared to most biological molecules and is rapidly converted to H_2O_2 by SOD. Additionally, this ROS possesses low mobility, as its negative charge and its extremely short half-life, of around 1-4 μs, prevent it to cross biological membranes (Gill et al., 2010; Mittler, 2017). Despite its lower toxic effects when compared to other ROS, due to its inability to interact directly with organic macromolecules, this ROS has a powerful reducing ability, being capable of reducing transition metals, like Fe and Cu, which can later interact with H_2O_2 and increase the production of $^{\bullet}OH$, a strong oxidant agent, which plays a key role in oxidative stress. This process is globally known as the Haber-Weiss reaction with the last step, where Fe^{2+} or Cu^{+} interact with H_2O_2 to form $^{\bullet}OH$, being referred to as the Fenton's reaction (Demidchik, 2015).

Along with $O_2^{\bullet-}$, H_2O_2 is considered a primary ROS (Soares et al., 2019) and it is highly stable due to the absence of impaired electrons. Paired with this characteristic, H_2O_2 possesses a relatively long half-life (1 ms), being able to cross biological membranes and to diffuse across long distances, thus increasing its possible sites of action. These features contribute to boost the toxicity of this molecule, which can cause significant cellular damages, leading to the inhibition of the activity of some enzymes, such as those involved in the Calvin cycle, Cu/Zn and Fe-SOD (Soares et al., 2019), ultimately causing PCD (Quan et al., 2008). The selective reactivity, stability, and diffusion ability of H_2O_2, make it fit for signalling (Sewelam et al., 2016) in many cellular events, increasing plant tolerance to different kinds of abiotic and biotic stress (Quan et al., 2008; Gupta et al., 2011). It is also known that H_2O_2 plays an important function in several physiological processes, like senescence, cell cycle, photosynthesis, photorespiration, stomatal movement, growth and development (Quan et al., 2008; Gill and Tuteja, 2010). Additionally, the role of H_2O_2 as a signalling molecule in metal stress tolerance has been suggested, since this ROS can mediate the activities of protein kinases, protein phosphatases and transcription factors associated to Cu homeostasis maintenance in plants (Shahid et al., 2014).

Lastly, the $^{\bullet}OH$ is the most reactive and toxic ROS and mainly arises from the Haber-Weiss reaction, from the interaction of $O_2^{\bullet-}$ with H_2O_2. Although this ROS has an extremely short lifetime (1 ns), it has a strongly positive redox potential and the capacity of negatively interacting with all organic molecules, which explains its high reactivity and toxicity, leading to strong oxidative damage that can be even higher due to the lack of enzymatic mechanisms responsible for its degradation and metabolism (Sharma et al., 2012).

1.5.2. ROS induced oxidative stress

If a disturbance in the equilibrium of ROS formation and scavenging occurs, severe damage can be caused to several biomolecules such as lipids, DNA and proteins, ultimately leading to cell death (Sharma et al., 2012). One of the first and most severe results of oxidative stress is lipid peroxidation (LP), a series of biochemical reactions as a consequence of ROS action on unsaturated fatty acids of cellular and subcellular membranes (Soares et al., 2019). Lipid-derived radicals are formed as a result of LP and aggravate the oxidative stress, since these products may react with proteins and DNA, causing damages to these molecules. Additionally, LP causes increased leakiness of the membrane to substances that do not normally cross membranes other than via specific channels, the decrease of membrane fluidity, and induce serious damage to membrane proteins that inactivate receptors, enzymes, and ion channels (Shahid et al., 2014). The presence of ROS in levels above their threshold may also cause a variety of modifications in proteins being the most common: nitrosylation, carbonylation, disulphide bond formation, and glutathionylation, triggering the modulation of proteins' activity. Proteins can also be modified indirectly by conjugation with breakdown products of fatty acid peroxidation (Sharma et al., 2012). DNA damage as an effect of ROS accumulation involves not only the nuclear DNA, but also the mitochondrial and chloroplast DNA and the damages include deletions and modifications, strand breaks or pyrimidine dimers, resulting in the inhibition of protein synthesis, destroying the cell membrane and affecting plant development (Gill and Tuteja, 2010; Sharma et al., 2012).

1.5.3. AOX system – main players and functions

In order to maintain the cell redox homeostasis and to avoid possible oxidative damages, plants developed an AOX system that is composed by enzymatic and non-enzymatic mechanisms, which are involved in sensing, detoxification, elimination and/or neutralisation of ROS (Gill et al., 2010). The non-enzymatic AOX system comprises diverse molecules that allow the management and sensing of ROS homeostasis. These

molecules are low mass metabolites like glutathione (GSH), ascorbic acid (AsA), phenolic compounds, proline, carotenoids and sugars (Soares et al., 2019).

Two of the most studied non-enzymatic AOX are AsA and GSH, as they control the redox status of cells, with AsA being the most abundant AOX metabolite in plants. AsA, commonly known as vitamin C or ascorbate, can directly neutralize the toxic effects of $^1O_2$, $O_2^{\bullet-}$ and $^{\bullet}OH$, while concomitantly acting as an electron donor in an enzymatic reaction that leads to the elimination of H_2O_2. Similarly to AsA, GSH also plays an important AOX role, as a reductant ROS, directly eliminating $^1O_2$, $O_2^{\bullet-}$ and $^{\bullet}OH$ and being considered an efficient radical scavenger. Furthermore, this non-protein thiol is also capable of reacting with H_2O_2 and acts as substrate for certain peroxidases. In addition, these two metabolites play a central role in the AsA-GSH cycle that will be explored in the further sections (Gill and Tuteja, 2010; Sharma et al., 2012; Soares et al., 2019).

Proline (Pro), a compatible solute, occupies a prominent place among the AOX metabolites, since it can eliminate $^{\bullet}OH$ radicals, in a reaction where Pro converts into γ-aminobutyric acid. Actually, Pro possesses a dual role on oxidative stress control as it is efficient not only in the scavenging of ROS, but also in avoiding their production (Gill and Tuteja, 2010; Sharma et al., 2012; Foyer, 2018). In fact, as NADPH is consumed in order to synthetise Pro, this amino acid plays a role as an electron sink preventing the generation of ROS (Soares et al., 2019). Additionally, Pro acts as an osmolyte and seems to be important in the prevention of LP, due to an increase in its levels under stress conditions, probably in order to obtain osmotic adjustments, leading to the protection of membrane integrity (Jain et al., 2001).In fact, it is known that Pro levels increase as a way to balance possible osmotic unbalances caused by membrane injuries while also interacts with membrane phospholipids promoting its stabilisation (Hossain et al., 2019).

Phenolic compounds are a complex group of secondary metabolites, being abundant in plant tissues. Based on their structural chemistry, phenolics display a high potential for ROS scavenging, being even more efficient than other AOX, like AsA (Arora et al., 2000). Flavonoids, in particular, are a sub-class of phenolic compounds, which tend to accumulate in the vacuoles of plant cells. Among different functions in plant's development, flavonoids can efficiently scavenge ROS, directly interacting with $^1O_2$ and H_2O_2 and acting as a substrate for different peroxidases (Arora et al., 2000; Michalak, 2006).

Carotenoids, besides acting as accessory pigments, are a class of lipophilic compounds, like β-carotene, zeaxanthin and tocopherols, which are also fundamental in the photooxidative stress tolerance, due to its capacity of protection of the photosynthetic machinery and reduction of LP, as they inhibit the production of $^1O_2$ (Soares et al., 2019). Additionally, sugars like glucose, sucrose and its derived water-soluble carbohydrates are also important components of the AOX system, since, apart from directly interact with some ROS, like $^{\bullet}OH$ and H_2O_2, they also induce the expression of genes responsible for the production of other AOX compounds like Pro (Soares et al., 2019).

The enzymatic component of the AOX system is composed by a diverse group of enzymes such as SOD, catalase (CAT, EC 1.11.1.6), guaiacol peroxidase (GPOX; EC 1.11.1.9), glutathione S-transferase (GST; EC 2.5.1.18), and a set of enzymes from the AsA-GSH cycle, which includes APX, GR, monodehydroascorbate reductase (MDHAR; EC 1.6.5.4) and dehydroascorbate reductase (DHAR; EC 1.8.5.1) (Sharma et al., 2012). Considering the role of enzymatic components as ROS scavengers, plants under biotic and abiotic stresses may present variations in the activity and/or transcript accumulation of these enzymes, thus indicating the occurrence of stress in plants (Gill et al., 2010).

Being considered the first defence line against ROS, SOD is a ubiquitous metalloenzyme responsible for the dismutation of $O_2^{\bullet-}$ to H_2O_2 and O_2. By controlling the levels of the $O_2^{\bullet-}$, SOD is also efficient in the prevention of the consequent formation of $^{\bullet}OH$, through the Haber-Weiss reaction (Gill and Tuteja, 2010). This enzyme has three main subclasses based on the metal present in its active site - Fe, Cu/Zn, and Mn. In plants, SOD can be present in all organelles where the $O_2^{\bullet-}$ is formed. As so, Fe-SOD is present in chloroplasts, Mn-SOD in mitochondria and Cu/Zn-SOD in the cytosol, chloroplast, peroxisome and mitochondria - being the most abundant isoform of SOD in plants. The different isoforms of SOD are all encoded in the nucleus, with the different location sites being determined by a terminal amino acid targeting tag (Soares et al., 2019). Several studies report an increase of SOD activity in plants under stressful conditions, like salinity, drought and metal-induced stress (Soares et al., 2019), since the upregulation of SOD transcripts or enzyme activity can occur directly through the action of metal ions on SOD or indirectly through an increase in $O_2^{\bullet-}$ content. In this way, the overexpression of SOD is often related to an enhanced tolerance to oxidative stress in plants (Shahid et al., 2014).

Being present in the mitochondria and peroxisomes, CAT is responsible for decomposing H_2O_2 to H_2O and O_2. CAT possesses 3 isoenzymes based on the organs they are expressed in. Thus, class I CAT enzyme is preferentially expressed in the

photosynthetic organs in a light-dependent manner, class II CAT enzyme is typically located in vascular tissues, and class III CAT enzyme is commonly found in young plants. Although CAT does not have a great affinity to H_2O_2, acting as peroxidase when H_2O_2 levels are high, CAT shows a high turnover rate, since one enzyme molecule can eliminate 6 million molecules of H_2O_2 per minute. In addition, contrarily to other H_2O_2-detoxifying enzymes, CAT does not require reducing power, highlighting its importance in the redox homeostasis (Gill et al., 2010; Soares et al., 2019). CAT activity is affected by several types of stress, depending on several features, such as its nature, intensity, or duration, which can change the response of this enzyme (enhancement or inhibition). Indeed, even when exposed to the same conditions, CAT activity can be up or downregulated, functioning either as a sign of tolerance or susceptibility, respectively (Sharma et al., 2012)

In parallel with CAT, APX plays a leading role in the protection of plant cells against oxidative stress, being responsible for the dismutation of H_2O_2 to H_2O and O_2 (Sharma et al., 2012). This enzyme is present in most organelles where the production of H_2O_2 takes place – cytosol, chloroplasts, mitochondria and peroxisomes – and, unlike CAT, APX has high affinity to H_2O_2, revealing its high relevance even when the concentrations of this ROS are low. In this way, it is assumed that APX is more involved in signalling processes, while CAT is much likely implicated in ROS-induced damage mitigation (Hertwig et al., 1992; Shigeoka et al., 2002). In addition, and in parallel to what is described for SOD and CAT, the literature suggests that a higher activity of APX is often correlated to a higher tolerance to stressful conditions. In fact, overexpression of APX genes in different plant species, like *A thaliana*, *Nicotiana tabacum* L. and *Solanum lycopersicum* L., increased their tolerance to oxidative stress (Shigeoka et al., 2002; Wang et al., 2005). In order to reduce H_2O_2 to water, APX uses two molecules of AsA, with the subsequent production of two molecules of monodehydroascorbate (MDHA). In order to ensure AsA's AOX role and APX activity, the cellular levels of reduced AsA must be tightly controlled. To guarantee this homeostasis, MDHA radicals can either be reduced back to AsA by MDHAR with the oxidation of NADPH or react with another MDHA molecule, arising the production of one molecule of AsA and one molecule of dehydroascorbate (DHA). DHA is then reduced by DHAR, using GSH as the reducing agent, thus regenerating the reduced form of AsA and oxidised GSH (GSSG). However, while the equilibrium of reduced/oxidised AsA is maintained, this process causes an imbalance of the GSH/GSSG ratio. To overcome this disequilibrium, GSH must be regenerated. Hence, the enzyme GR performs the NADPH-dependent reaction of disulphide bond of GSSG guaranteeing this regeneration (Ahmad et al., 2008; Gill et al.,

2010; Soares et al., 2019) This cycle of H_2O_2 detoxification, consisting of consecutive oxidations and reductions of AsA and GSH, is named AsA-GSH or Halliwell-Asada cycle and appears to be one of the most important metabolic cycles occurring in plant cells (Soares et al., 2019).

1.5.4. Cu-mediated oxidative imbalances in plants

As above mentioned, although Cu is an essential micronutrient, high concentrations of this metal are toxic for the organisms (Rehman et al., 2019). In fact, at high concentrations Cu is correlated to the formation of $^{\bullet}OH$ from H_2O_2 through Haber–Weiss and Fenton reactions, thus provoking LP.

Several authors reported that metal-induced stress increases ROS production, consequently affecting the normal functioning of the AOX system (Shahid et al., 2014). Specifically referring to Cu, the excess of this metal directly induces oxidative stress in plant cells caused by the increased concentration of ROS which react with proteins, lipids and DNA, causing oxidative damage and impairing the normal cell functions (Adrees et al., 2015). Accordingly, some studies reported the accumulation of ROS, specifically H_2O_2 and $O_2^{\bullet-}$, probably due to their negative impacts on membrane stability, in plants exposed to Cu excess (Demidchik, 2015). Additionally, an increased performance of the AOX system was also reported (Table 2). However, the formation of ROS and the AOX response is dependent on plant species, and also on the severity, type and duration of Cu exposure. Altogether, the studies presented in the Table 2 point out an increase in ROS generation, as well as the increase of AOX enzyme activities and metabolite levels in response to Cu. However, in some plants, this occurs only until a certain Cu threshold, after which these parameters begin to be negatively affected, possibly due to the severity of Cu-induced stress in plants (Adrees et al., 2015).

Table 2*:* A summary of some of the existent studies about Cu alterations on the oxidative status of plant cells.

Reference	Crop	[Cu]	Mode of exposure	Alterations on ROS and AOX system
Gajewska and Skłodowska (2010)	*Triticum aestivum* L.	75 µM	Hydroponics	Increased LP, GST and thiols levels;
Posmyk et al. (2009)	*Brassica oleracea* L.	2500 µM	Hydroponics	Increased LP degree and SOD, CAT and APX activity; Decreased GR activity;

Dresler et al. (2014)	*Zea mays* L.	50 and 100 µM	Hydroponics	Increased LP degree;
Sánchez-Pardo et al. (2014)	*Lupinus albus* L. and *Glycine max* (L.) Merr.	192 µM	Semi-hydroponics	Increased LP degree;
Lukatkin et al. (2014)	*Raphanus sativus* L.	10, 100 and 1000 µM	Hydroponics	Increased $O_2^{\cdot-}$ levels and CAT and APX activity; Decrease SOD activity;
Meng et al. (2007)	*Allium sativum* L.	10, 100 and1000 µM	Hydroponics	Increased SOD activity;
Liu et al. (2014)	*Zea mays* L.	1000 mM	Hydroponics	Increased CAT activity;
Azooz et al. (2012)	*Oryza sativa* L.	2, 10, 20, 40, 80 and 100 mM	Semi-hydroponics	Increased SOD, CAT and APX activity and proline levels;
Sharma and Singh (2013)	*Cicer arietinum* L.	70, 140, 210, 280 and 350 µM	Hydroponics	Increased LP degree and SOD, CAT and APX activity;
Kumar et al. (2014)	*Cicer arietinum* L.	1.26 mol kg^{-1}	Soil application	Increased proline levels and SOD activity;
Thounaojam et al. (2012)	*Oryza sativa* L.	100 µM	Hydroponics	Increased LP, H_2O_2, ASH and GSH levels and SOD, GPX APX and GR activity;
İşeri et al. (2011)	*Solanum lycopersicum* L. and *Cucumis sativus* L.	22 and 120 µM	Hydroponics	Increased LP and H_2O_2 levels and CAT and APX activity;
Mei et al. (2015)	*Gossypium hirsutum* L.	100 µM	Semi-hydroponics	Increased SOD activity;

1.6. Barley as a model species for stress physiology studies

According to FAO, cereals are among the most produced crops worldwide (FAOSTAT 2017). *Hordeum vulgare* L., commonly known as barley plant, is one of the world's most important crops, being at the top four of the most produced cereals and having its production increased in Portugal in the last years (FAOSTAT 2017). Beyond being an economically important species, due to its use for food and animal feeding (Katerji et al., 2006), *H. vulgare* also has several characteristics, which turn it in a good model species for plant science and agronomic studies. These characteristics comprise aspects such as being a fast-growing monocotyledonous species, and being capable of growing in a

long spectrum of environments (Katerji et al., 2006). For these reasons, barley was used as a model in the current project to achieve the designed goals and objectives.

1.7. Aims

Given the predicted increase of wrack production and accumulation on sandy beaches by CC-related phenomena and coastal water's enrichment, new strategies need to be developed to overcome the environmental degradation caused by the excessive amount of this organic residue. Two possible ways to add value to wrack, and therefore reduce waste, may be related to its use as a biostimulant, and as a soil quality enhancer since macroalgae are known to improve plant tolerance to abiotic stress and to play a role in soil remediation (Sharma et al., 2013). However, the metabolic and physiological adjustments induced by treatments with macroalgae-based products in stressed plants are still unknown, requiring further research. Thus, based on a circular economy perspective and integrated in the working plan of Val-WRACK project (POCI-01-0145-FEDER-029818), this MSc's dissertation aims to explore the potential of wrack as a soil amendment to improve plant abiotic stress tolerance, using barley (*Hordeum vulgare* L.) as a model species, and thus for improving soils production function.

To achieve this, the following specific objectives were pursued:

- Assess the chemical composition and antioxidant potential of wrack;
- Establish a protocol for wrack application (dose and mode) to soil;
- Evaluate if the application of wrack results in a higher tolerance of barley to Cu exposure;
- Unravel the mechanisms behind wrack-mediated alleviation of Cu in barley plants, focusing on the AOX metabolism.

2. MATERIAL AND METHODS

2.1. Wrack collection and processing

Samples of wrack were collected from a beach of Viana do Castelo (Praia Norte) (between 41°43′0.3″N and 41°41′36.36″N; 8°51′10.52″W) during the winter, and immediately transported to the *Plant Stress* lab (FCUP/GreenUPorto). At the lab, species that composed wrack were identified, and the litter removed after washing the wrack samples with tap water. Then, all samples were pooled, dried at 60 ºC, reduced to powder using a miller, and stored in dark conditions at room temperature (RT) until further utilisation.

2.2. Chemical and biochemical profile of wrack

2.2.1. Quantification of inorganic elements

For the quantification of inorganic elements (micro and macro nutrients and trace elements), a dried sample of wrack was crushed with an ultracentrifuge mill at 8000 rpm (ZM 200, Retsch) and then, three sub-samples (0.3-0.5 g) were obtained. Each sub-sample plus two analytical blanks were digested in a microwave oven with 4 mL of concentrated nitric acid (HNO_3) and 2 mL 30% (m/v) H_2O_2. The digestion proceeded at 800 W during 10 min, followed by 5 min at 1000 W and a cooling period of 15 min. Each clear solution obtained was quantitatively transferred to 50 mL volumetric flasks.

The analysis was performed by flame or graphite furnace atomic absorption spectroscopy (FAAS or GFAAS, respectively) depending on the levels of the elements.

Na, K, Mg, Ca, and Zn were determined by FAAS, operated at the optical and flame parameters recommended for the instrument used (Perkin Elmer, AAnalyst 200). Calibration was performed with external standards (in 0.5% HNO_3) in the following ranges: Na (1-10 mg L^{-1}), K (5-25 mg L^{-1}), Mg (1-10 mg L^{-1}), Ca (0.5-4 mg L^{-1}), Zn (0.05-1 mg L^{-1}).

Manganese (Mn), lead (Pb), nickel (Ni), cadmium (Cd), Cu, and chromium (Cr) were determined by GFAAS at the recommended optical parameters for the instrument used (Perkin Elmer, PinAAcle 900Z). In all cases, a drop of 20 µL was introduced into the furnace. Apart from Ni, to all other elements a matrix modifier [1% $Mg(NO_3)_2$] was applied (5 µL modifier + 15 µL sample or standard). The furnace temperature followed a common scheme:

Table 3:**:** Combination of temperature, ramp and hold times, and argon flow applied into the furnace.

Step	Temp / ºC	Ramp time/ s	Hold Time/ s	Argon Flow/ (mL min^{-1})
1	110	1	30	250
2	130	15	30	250
3	Pyrolysis	10	20	250
4	Atomisation	0	5	0
5	2450	1	3	250

The pyrolysis and atomisation temperatures were adjusted according to the different elements:

Table 4: Applied temperatures of pyrolysis and atomisation for each element in steps 3 and 4, respectively, of the furnace.

Element	Pyrolysis Temp / ºC	Atomisation Temp / ºC
Mn	1300	1900
Pb	850	1600
Ni	1100	2300
Cd	500	1500
Cu	1200	2000
Cr	1500	2300

Calibration was performed with external standards (in 0.5% HNO_3) in the following ranges: Mn (3-22.5 µg L^{-1}), Pb (7.5-75 µg L^{-1}), Ni (10-100 µg L^{-1}), Cd (4-15 µg L^{-1}), Cu (15-52.5 µg L^{-1}), Cr (1-6 µg L^{-1}). Results were expressed in mg g^{-1} dry mass (dm).

2.2.2.Extraction and quantification of photosynthetic pigments

The evaluation of chlorophyll *a* (Chl *a)* and *c* (Chl *c*) and carotenoids (Car) was performed based on the protocol of Lichtenthaler (1987) and Ritchie (2008). Samples of 200 mg of wrack were homogenised in 80% (v/v) acetone and centrifuged for 10 min at 1400 *g*. After collecting the supernatant (SN), the absorbance (Abs) at 470, 630, 647, 664 and 691 nm was recorded and the concentrations of Chl *a*, Chl *c* and Car were calculated according to the following equations:

$$\text{Chl } a \text{ (mg L}^{-1}) = (Abs_{630} \times 0.3319) - (Abs_{647} \times 1.7485) + (Abs_{664} \times 11.9442) - (Abs_{691} \times 1.4306)$$

$$\text{Chl } c \text{ (mg L}^{-1}) = (Abs_{630} \times 23.5902) - (Abs_{647} \times 7.8516) - (Abs_{664} \times 1.5214) - (Abs_{691} \times 1.7443)$$

$$\text{Car (mg L}^{-1}) = ((Abs470 \times 1000) - (1.82 \times \text{Chl a}) - (85.2 \times [(Abs647 \times 21.5) - Abs664 \times 5.1])) /198$$

The results were expressed in mg g^{-1} dm.

2.2.3. Evaluation of wrack's AOX potential

2.2.3.1. Preparation of methanolic extracts

Wrack samples (ca. 15 mg) were homogenised, on ice, in a mortar containing quartz sand and 1.5 mL of 80% (v/v) methanol. Then, samples were centrifuged for 10 min at 2 500 *g* and the SN collected to new tubes. The methanolic extracts were stored at -20 ºC until use for the estimation of the total flavonoid and total phenolic contents, as well as the evaluation of AOX potential (total antioxidant capacity and free radical scavenging potential).

2.2.3.2. Total phenols content (TPC)

The determination of TPC was performed according to Zafar et al. (2016), after properly diluting (1:2) the methanolic extracts. Forty µL of diluted samples were added to 180 µL of Folin-Ciocalteu reagent. A blank tube was prepared by replacing the extract with 80% (v/v) methanol. The mixture was incubated for 5 min at RT and, after that time, 180 µL of 7.5% (m/v, in water) sodium carbonate (Na_2CO_3) were carefully added to all tubes. All samples were incubated at RT, in dark conditions, for 1 h, and the Abs read at 725 nm. TPC was calculated using a calibration curve, prepared with known concentrations (0-200 µg mL^{-1}) of gallic acid solutions, and the results were expressed in mg of gallic acid g^{-1} dm.

2.2.3.3. Total flavonoids content (TFC)

The quantification of TFC was assessed based on Zafar et al. (2016). For this purpose, 20 µL of 10% (m/v) aluminium chloride ($AlCl_3$), 20 µL of 1 M potassium acetate (KCH_3COO) and 120 µL of distilled and deionised water (ddH_2O) were added to 40 µL of the previously obtained methanolic extract, appropriately diluted (1:2). In parallel, a blank tube was prepared by replacing the extract by 80% (v/v) methanol. Then, mixtures were incubated for 30 min, in dark conditions at RT, and the Abs at 415 nm was read. A calibration curve, obtained with solutions of known concentrations (0-200 µg mL^{-1}) of quercetin, was used to calculate the total flavonoid content. The results were expressed in µg of quercetin g^{-1} dm.

2.2.3.4. Total antioxidant capacity (TAC)

The TAC of wrack was determined based on the method of Zafar et al. (2016). Briefly, 100 µL of methanolic extract (previously diluted - 1:10) were mixed with 900 µL of a reaction solution [0.6 M sulphuric acid (H_2SO_4), 4 mM ammonium molybdate

[$(NH_4)_2MoO_4$] and 28 mM sodium phosphate (Na_2HPO_4)], and incubated at 95 ºC for 90 min. Hereafter, reaction mixture was cooled on ice and the Abs was read at 695 nm. In parallel, a calibration curve was prepared with solutions of known concentrations of AsA (0-200 µg mL^{-1}) and the results were expressed in µg AsA equivalents g^{-1} dm.

2.2.3.5. Free radical scavenging potential

The determination of free radical scavenging potential was performed according to Zafar et al. (2016). Multiple dilutions of the extract (2-20 µL methanolic extract in a final volume of 20 µL) were added to 380 µL of 0.004% (m/v) 2,2-diphenyl-1-picrylhydrazyl (DPPH). In parallel, a blank tube was prepared by replacing the extract with 20 µL 80% (v/v) methanol. The reaction mixture was incubated for 30 min, in dark conditions. After this period, the Abs of each sample was read at 515 nm. The percentage of scavenging was calculated by the following formula:

$$\% \text{ scavenging} = [(Abs_{blank} - Abs_{sample}) / Abs_{blank}] \times 100$$

The concentration of extract required to cause an inhibition of 50% (IC_{50}) was calculated through linear regression and the results expressed in IC_{50} g^{-1} dm.

2.2.3.6. Proline quantification

The quantification of proline (Pro) was performed following the protocol described by Bates et al. (1973). For this purpose, samples of 200 mg of wrack were homogenised in 1.5 mL of 3% (m/v) sulfosalicylic acid and centrifuged for 10 min at 500 *g*. Afterwards, 200 µL of SN were mixed with 200 µL of glacial acetic acid and 200 µL of acid ninhydrin and incubated for 1 h at 96 ºC. After cooling on ice, 1 mL of toluene was added to each tube, followed by vigorous mixing in a vortex, for 15 s. In parallel a blank tube, containing toluene, was prepared. After the separation of the red upper phase from the whiteish lower phase, the upper one was collected, and its Abs read at 520 nm. Pro content was estimated through a calibration curve, obtained with different solutions of known concentration of Pro, and expressed in mg g^{-1} dm.

2.2.3.7. Determination of total sugars and total amino acids

The quantification of total sugars and total amino acids was done according to Irigoyen et al. (1992) and Lee and Takahashi (1966), respectively. In both situations, samples of 15 mg of wrack were homogenised in 1.5 mL of 80% (v/v) ethanol. Then the extracts were incubated for 15 min at 50 ºC and centrifugated at 2000 *g* during 20 min. For the

quantification of total sugars 100 µL of the SN was diluted 1:5 and mixed with 1500 µL of anthrone prepared in concentrated H_2SO_4. After incubation of the tubes at 100 ºC for 10 min, followed by cooling on ice, the Abs of the mixture was read at 625 nm. In parallel, a calibration curve was obtained using known concentration of glycose. The results were expressed in mg glucose equivalents g^{-1} dm. Concerning the amino acids quantification, the extracts were incubated for 15 min at 50 ºC and centrifugated at 2000 *g* during 20 min. Then, 75 µL of the SN were added to 1430 µL of a reaction solution containing 1% (m/v) ninhydrin, 99% (v/v) glycerol and 0.5 M (pH 5.5) sodium citrate buffer in a 5:12:3 proportion. The tubes containing the mixture were incubated at 100 ºC, for 15 min and, after cooling on ice the Abs of the mixture was read at 570 nm. To determine total amino acids content a calibration curve was prepared with known concentrations of glycine. The results were expressed in mg glycine equivalents g^{-1} dm.

2.2.4.Effects of wrack on alleviating Cu stress in seedling development

2.2.4.1. Preparation of wrack liquid extracts

The preparation of the extract was conducted according to the protocol described by Anisimov et al. (2013), with few modifications. Briefly, 5 g of dried wrack were added to 500 mL deionized water [1:100 (m/v)] and heated at 60 ºC for 45 min. Afterwards, the suspension was centrifuged at 2220 *g* during 10 min and the extracts were filtered with 1.2 and 0.2 µm filters. Finally, and to avoid any contamination, the extract was stored at -80 ºC until further utilisation. For plant growth assays, the extract was successively diluted with deionized water, resulting in different concentrations: 0,1.25, 2.5, 5 and 7.5 g L^{-1}.

2.2.4.2. Petri dishes assay

2.2.4.2.1. Plant material and growth conditions

Seeds of *H. vulgare* L., obtained from a local supplier, were individually observed to discard the damaged ones and, then, surface desinfected with 70% (v/v) ethanol for 10 min and 20% (v/v) commercial bleach for 7 min, followed by a series of washing with deionized water. Then, seeds were left to germinate and grown for 8 d at 24 ºC in a growth chamber, in the dark for the first 2 d, and at a photoperiod of 16 h light/8 h dark and photosynthetic active radiation (120 µmol m^{-2} s^{-1}). For each treatment, three experimental replicates were considered, with 10 seeds each.

2.2.4.2.2. Selection of Cu concentrations

The selection of Cu concentration was conducted prior to the germination assay with wrack-based extracts. For that purpose, after disinfection, barley seeds were distributed in Petri dishes containing 0.5x Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium solution solidified with 0.625% (m/v) agar and supplemented with increasing concentrations of Cu [0 (control), 2.5, 5, 10, 12.5, 15 and 17.5 mg L^{-1}], applied in the form of copper (II) sulphate pentahydrate ($CuSO_4.5H_2O$; Merk). The growth conditions were the same as previously described in section 2.2.4.2.1. After 8 d, radicle and leaves length were measured. In order to select the Cu concentration to be used in the further assays, the obtained results were used to determine the metal concentration causing a 50% reduction (EC_{50}) of the above-described parameters and the lowest EC, for the endpoints measured, was chosen.

2.2.4.2.3. Effect of wrack extract on Cu-induced phytotoxicity – preliminary screening

The effect of different concentrations of a wrack extract on the germination and seedling growth of *H. vulgare* grown in a Cu-contaminated medium was assessed. For this purpose, and following a bifactorial experimental design, barley seeds were distributed on Petri dishes containing 0.5x MS medium solution solidified with 0.625% (m/v) agar, supplemented with different concentrations of wrack extract (0, 1.25, 2.5, 5 and 7.5 g L^{-1}) with or without 7 mg Cu L^{-1} (estimated as described in section 2.2.4.2.2). In parallel, a negative control was prepared without wrack and Cu. The growth conditions were the same as previously described in section 2.2.4.2.1. After 8 d, radicle and leaves length and total fresh biomass were measured.

2.3. Collection and characterisation of the agricultural soil

2.3.1. Soil collection and processing

An agricultural soil from Vairão (Porto, Portugal; 41°19’35.1” N 8°40’27.7” W), maintained in a fallow state for over 40 years with no historical application of plant protection products or any soil treatment was used. The litter layer of the soil was removed, and samples were randomly collected in the area (approximately 20000 m^2) from the top 0 – 20 cm layer. Soil samples were allowed to air-dry and then part of this soil was sieved through a 2 mm mesh and used for the evaluation of several physicochemical parameters. The remaining soil was sieved through a 4 mm mesh and stored until being used for plants’ growth.

2.3.2. Soil characterisation

2.3.2.1. Determination of soil pH

The pH of the soil was assessed following Dewis and Freitas (1970) and ISO (1998). Approximately 10 g of soil were placed into plastic beakers and mechanically shaken for 30 min with 50 mL of deionised water (soil:water ratio of 1:5). After 30 min resting, the pH of soil suspensions was measured using a pH meter (accument® AE150 Ficher Scientific).

2.3.2.2. Determination of soil electrical conductivity (EC)

Soil electrical conductivity (EC) was measured according to Dewis and Freitas (1970) and Beck et al. (2000). Approximately 10 g of soil were placed into plastic beakers and mechanically shaken for 30 min with 20 mL of deionized water (soil:water ratio of 1:2). This mixture was allowed to stand overnight to allow the settling of the soil bulk, and the solution EC was then measured using an EC meter (CDM210 MeterLAb).

2.3.2.3. Determination of soil organic matter (OM)

Organic matter content was determined by the loss on ignition at 450 ºC, for 8 h according to Beck et al. (2000). A dry soil sample of 10 g was placed into a pre-weighted porcelain crucible, previously identified in the bottom, and placed in an oven at 105 ºC overnight. After this period soil dry weight (SDW) was recorded and the crucibles were placed in a muffle furnace at 450 ºC for 8 h. The procedure was made in triplicates. Then, the weight of the ignited soil (SIW) was recorded and OM content was determined according to the following expression:

$$\% \, OM = [(SDW-SIW) / (SDW)] \times 100$$

2.3.2.4. Determination of soil maximum water holding capacity (WHCmax)

Soil WHC_{max} was assessed according to ISO/DIS 11268-2.2 (1998). For this purpose, the bottom of plastic flasks was removed and replaced with filter paper and the flask, the lid and the filter paper were weighted (FW). Thereafter, the flasks were filled with oven dried soil, without compressing it and the flasks closed with the lid. The flasks were placed within a tray with water, allowing only the bottom to become submerged in the first minutes and then the tray was filled with water up to the lid of the flasks. After 3 h, the flasks were removed from the water and placed in absorbent paper for 2 h, changed whenever necessary. After this period, flasks were weighted, to record de value of

saturated soil (SSW+FW) and placed at 105 ºC overnight. Finally, the flasks with dried soil were weighted (SDW+FW) and the WHC was determined according to the following expression:

$$\%WHC = ([(SSW) - (SDW)] / SDW) \times 100$$

2.4. Experimental setup

2.4.1. Ecotoxicological assessment of copper (Cu)

2.4.1.1. Tested concentrations and treatments

In all assays performed, Cu was supplied as copper sulphate ($CuSO_4 \cdot 5H_2O$) at concentrations ranging from 0 to 344 mg Cu kg^{-1} with a dilution factor of 1.5, giving rise to the following concentrations: 30, 45, 68, 102, 153, 229 and 344 mg kg^{-1}, which were tested against a Cu-free control. The concentrations were selected to represent a real environmental contamination scenario.

2.4.1.2. Plant growth assays

The ecotoxicological assays of plant growth, performed according to the OECD (Organisation for Economic Co-operation and Development) protocol for terrestrial plants (Aktar et al., 2009) were carried out in plastic pots containing 200 g of the test soil to which the solutions with the desired Cu concentrations [0 (control), 30, 45, 68, 102, 153, 229 and 344 mg kg^{-1}] were added. The exact volume of ddH_2O needed to adjust WHC_{max} to 40% was used as a carrier to prepare $CuSO_4.5H_2O$ solution to obtain the set of concentrations in soil above described. In order to guarantee the maintenance of soil moisture, a cup filled with ddH_2O water was placed at the base of the soil pots, and a cotton rope was used to ensure the capillarity rise of the water during the assays. Twenty barley seeds were placed in each pot and kindly covered with the soil. For each experimental condition, 4 replicates (pots) were considered. The assay started after 50% of the control seeds germinate and lasted 14 d. Only the 7 first germinated seeds were left in each pot to avoid intraspecific competition between organisms. Plants were maintained in a greenhouse, with controlled conditions of temperature (21 ºC), photoperiod (16 h light/8 h dark) and photosynthetic active radiation (120 µmol m^{-2} s^{-1}). The water content of each pot was adjusted when necessary. At the end of the experiment, (i.e., after 14 d post CTL validation), plants were collected and washed in tap water and plant material was used for the evaluation of the standard endpoints (root length and fresh and dry biomass). After roots and leaves separation, root length was measured, and the fresh masses of roots and leaves were registered. Then, plant

material was left to dry in an oven at 60 ºC, until reaching a stable weight for analysis of the dry biomass. An EC_{50} was estimated for the biometric parameters above described, and this threshold was used in the final growth assay (for more details please see section 2.4.3).

2.4.2. Optimisation of wrack application: concentration *vs* stabilisation time

In order to select the best wrack concentration to be used in the final growth trial, a Cu-contaminated (concentration selected based on the EC_{50}) and non-contaminated soil were mixed with wrack at three different levels [0, 0.5, 1 and 2 % (w/w)] and left for stabilisation for two periods, 15 d and 1 month. At the end of each stabilisation period soil chemical properties were once again assessed according to section 2.3.2 with the exception of WHC_{max}. Subsequently, a plant growth assay was performed as described above (2.3.1). After the 14 d assay seedlings growth period, the same biometrical parameters were evaluated in the barley plants.

2.4.3. Final growth trial

Aimed in understanding the possible effects of wrack in increasing plant tolerance to Cu, after optimising Cu concentration and wrack application (dose and stabilisation time), a bifactorial experimental trial was performed and barley plants were grown for 14d as previously described (2.4.1.2). For this purpose, the different experimental conditions considered in the final trial were:

CTL: control, where no wrack nor Cu was added to the soil;

Wrack: soil previously treated with the previous optimised wrack dose;

Cu: soil previously treated with the optimised Cu concentration;

Cu + wrack: soil previously treated with the optimised Cu concentration and wrack dose.

For each condition, 8 experimental replicates were prepared. After the growth period, barley plants from each treatment were harvested and separated into roots and leaves, for the evaluation of biometrical parameters (root length and roots and leaves' fresh mass). Then, part of the plant material was immediately processed for biochemical analysis, and the remaining frozen in liquid nitrogen (N_2) and stored at -80 °C to be used for biochemical techniques.

2.5. Biochemical parameters

2.5.1. Extraction and quantification of photosynthetic pigments

The evaluation of Chl *a,* Chlorophyll *b* (Chl *b*) and Car was performed based on the protocol of Lichtenthaler (1987). For this purpose, frozen aliquots of leaves (c.a. 200 mg) were homogenised in 80% (v/v) acetone and centrifuged at 1400 *g* for 10 min. After collecting the SN, Abs at 470, 647 and 663 nm was recorded and the concentrations of Chl *a,* Chl *b* and Car were calculated according to the following equations (Lichtenthaler, 1987):

$$\text{Chl } a \text{ (mg L}^{-1}) = (\text{Abs}_{663} \times 12.25) - (\text{Abs}_{647} \times 2.79)$$

$$\text{Chl } b \text{ (mg L}^{-1}) = (\text{Abs}_{647} \times 21.5000) - (\text{Abs}_{663} \times 5.10)$$

$$\text{Car (mg L}^{-1}) = ((\text{Abs}470 \times 1000) - (1.82 \times \text{Chl } a) - (85.2 \times \text{Chl } b)) / 198$$

The results were expressed in mg g^{-1} fresh mass (fm).

2.5.2. Soluble protein quantification

The quantification of soluble proteins was performed based on Bradford protocol (Bradford, 1976). Aliquots of 200 mg of frozen tissue were homogenised, using 2 mL of a potassium phosphate buffer (100 mM, pH 7.3) with 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 mM L-ascorbic acid, 8% glycerol and 1% (m/v) polyvinylpolypyrrolidone (PVPP). The homogenates were then centrifuged at 4 ºC for 25 min at 16 000 *g* and the SNs were collected for soluble protein quantification. A 1:4 dilution of each SN was prepared and 75 µL of this dilution were mixed with 750 µL of Bradford solution. Afterwards, this mixture was left to incubate in the dark at RT for 15 min and Abs were read at 595 nm. A standard curve utilising known doses of bovine serum albumin (BSA) was determined, to establish a link between obtained Abs and protein content. Results were expressed in mg g^{-1} fm.

2.5.3. Cu quantification

The quantification of Cu in roots and leaves of barley plants was assessed through the same methodology described in 2.2.1. The samples were obtained by oven-drying, at 60 ºC, both organs of plants obtained in 2.4.3 until constant mass was recorded.

2.5.4. Lipid peroxidation

The membrane injury was evaluated in terms of lipid peroxidation (LP), by the quantification of malondialdehyde (MDA) in accordance to Heath and Packer (1968). Frozen samples of leaves and roots with approximately 200 mg were homogenised in 1.5 mL of 0.1 % (m/v) trichloroacetic acid (TCA) with quartz sand, and centrifuged for 5 min at 10 000 *g*. Then 1 mL of 0.5 % (m/v) thiobarbituric acid (TBA) in 20 % (m/v) TCA was mixed with 250 µL of SN. In parallel, a blank tube was prepared using 0.1 % TCA instead of SN. After incubation at 95 ºC for 30 min, the mixture stayed cooling on ice for approximately 15 min.

A final centrifugation (10 000 *g*; 7 min) was conducted and Abs values of each sample were read at 532 and 600 nm. The Abs values of 532 nm were subtracted from those obtained at 600 nm to eliminate the effects of non-specific turbidity. The MDA content was calculated by applying the extinction coefficient (ε) of 155 mM^{-1} cm^{-1} and the results were expressed as nmol g^{-1} fm.

2.5.5. ROS quantification

2.5.5.1. H_2O_2 content

The quantification of H_2O_2 levels in barley plants was performed according to de Sousa et al. (2013). For this purpose, frozen aliquots (around 200 mg) of barley roots and leaves were homogenised in 1.2 mL of potassium phosphate (PK) buffer (50 mM, pH 6.5), centrifuged at 4 ºC for 25 min at 6000 *g* and then the SN was collected. Afterwards, 500 µL of SN were mixed with a reaction mixture containing 500 µL of 0.1% (m/v) $TiSO_4$ in 20% (m/v) H_2SO_4. In parallel, a blank tube was prepared using 500 µL of PK buffer instead of SN. All the tubes were vortexed for 15 s and centrifuged at 4 ºC for 15 min at 6 000 *g*. Abs were recorded at 410 nm and the levels of H_2O_2, expressed in nmol g^{-1} fm, were calculated using 0.28 μM^{-1} cm^{-1} as the extinction coefficient.

2.5.5.2. $O_2^{\bullet-}$ content

$O_2^{\bullet-}$ levels were quantified by the reduction of the nitroblue tetrazolium (NBT) reagent, according to Gajewska and Skłodowska (2007), using fresh samples of barley leaves and roots. Briefly, using a scalpel, samples were cut into equal and small pieces, amounting to a total of around 200 mg, and immersed in a 3 mL of a reaction solution consisting of 10 mM sodium azide (NaN_3) and 0.05% (m/v) NBT in sodium phosphate buffer (0.01 M, pH 7.8) and left in dark conditions, with constant agitation for 1 h. Posteriorly, 1.5 mL of this mixture was collected and incubated for 15 min at 85 ºC. A

blank tube consisting of 1.5 mL of the reaction solution was subjected to the same conditions. Upon cooling the samples on ice, the tubes were vortexed and centrifuged (maximum speed; 15 s), and Abs of each SN was read at 580 nm. The content of $O_2^{\bullet-}$ was expressed as $Abs_{580\ nm}\ h^{-1}\ g^{-1}$ fm.

2.5.6. Extraction and quantification of AOX metabolites

2.5.6.1. Proline levels

The quantification of proline levels in roots and leaves of barley plants was assessed through the same methodology described in 2.2.3.6, using frozen aliquots of 200 mg of both roots and leaves of plants obtained in 2.4.3.

2.5.6.2. Total glutathione (GSH) quantification

Total glutathione was quantified in both leaves and roots of barley plants. For this purpose, samples of 200 mg of plant material were homogenised in 3% (m/v) sulfosalicylic acid and incubated for 10 min, at 2–8 ºC, followed by a 10 000 *g* centrifugation, for 10 min, at 4 ºC. SNs were collected to new tubes and kept on ice, and then GSH levels were determined by quantifying the amount of DTNB [5,5'- dithiobis (2-nitrobenzic acid)], a reaction product of the GSH reduction. Abs was read at 412 nm and the levels of GSH were calculated based on a standard curve of GSH. The results were expressed in nmol GSH g^{-1} fm.

2.5.6.3. Ascorbate - reduced (AsA) and oxidised (DHA) forms

The quantification of AsA was performed following the protocol of Gillespie and Ainsworth (2007). Frozen aliquots of plant material were homogenised in 6% (m/v) TCA followed by a 15 min centrifugation at 13 000 *g* at at 4 ºC, and the SN was collected. Then, 50 µL of 75 mM PK buffer (pH 7.0) was added to 100 µL of SN. For the total ascorbate reaction, 50 µL of 10 mM dithiothreitol (DTT) were added to the tubes and after a 10 min incubation at RT, 50 µL of 0.5 % (m/v) N-ethylmaleimide (NEM) were added to each tube following by an incubation of 30 s at RT. In parallel, a blank tube was prepared by replacing the SN for 6% (m/v) TCA. For the quantification of the reduced ascorbate (AsA), 100 µL of distilled H_2O were added to the tubes substituting DTT and NEM. For both total (AsA + DHA) and AsA content quantification, 250 µL of 10% (m/v) of TCA, 200 µL of 43 % (v/v) phosphoric acid (H_3PO_4), 200 µL of 4 % (m/v) 4,4'-bipyridyl (BIP) and 100 µL of 3 % (m/v) iron (III) chloride ($FeCl_3$) were added to each tube. Finally, all samples were incubated at 37 ºC for 1 h and the Abs was read at 525 nm. The concentration of total and reduced ascorbate was obtained from a calibration curve,

prepared with AsA solutions of known concentrations. Results were expressed in µmol ascorbate g^{-1} fm. The levels of DHA were calculated by subtracting the levels of its reduced form from the total

2.5.6.4. TPC

The quantification TPC in roots and leaves of barley plants was assessed through the same methodology described in 2.2.3.2, using frozen aliquots of 200 mg of both roots and leaves of plants obtained in 2.4.3.

2.5.6.5. TFC

The quantification of TFC in roots and leaves of barley plants was assessed through the same methodology described in 2.2.3.3, using frozen aliquots of 200 mg of both roots and leaves of plants obtained in 2.4.3.

2.5.7. Extraction and activity of AOX enzymes

2.5.7.1. Extraction procedure

Samples of plant material (ca. 0.2 g of roots and leaves), stored at -80 ºC, were homogenised with 2 mL of extraction buffer, containing 100 mM PK buffer (pH 7.3), 8% (v/v) glycerol, 1 mM PMSF, 1 mM EDTA, 5 mM L-ascorbic acid and 1% (m/v) PVPP. The extracts were centrifuged, at 4 ºC, for 25 min at 16 000 *g* and the resulting SN were divided into aliquots, used for protein quantification (see 2.5.2) and enzyme activity assays. Regarding specifically SOD activity assay, SN aliquots were combined with NaN_3 and glycerol to final concentrations of 10 µM and 40% (v/v), respectively.

2.5.7.2. Activity of SOD (E.C. 1.15.1.1)

The total activity of SOD was spectrophotometrically assayed in both roots and leaves according to Donahue et al. (1997), by measuring the inhibition of the photochemical reduction of NBT at 560 nm. For each sample, an appropriate volume of extract (50 µg of protein) was added to a reaction mixture containing 50 mM phosphate buffer (pH 7.8), 0.093 mM EDTA, 12.05 mM L-methionine, 0.0695 mM NBT and 0.0067 mM riboflavin in a final volume of 3 mL. The reaction was started by the addition of riboflavin and the tubes were immediately placed under 6 fluorescent 8 W lamps for 10 min. After this period the light source was removed in order to stop the reaction. In addition, for each sample, a blank tube was prepared by substituting the protein extract by the 50 mM phosphate buffer (pH 7.8). Abs of each sample was measured at 560 nm and the results

were expressed as units of SOD mg^{-1} protein, with one SOD unit being defined as the amount of enzyme that inhibits by 50% the photochemical reduction of NBT at 560 nm.

2.5.7.3. Activity of APX (E.C. 1.11.1.11)

APX activity was quantified according to Nakano et al. (1981), with slight modifications. As in CAT, APX assay was performed in a UV-microplate and in a final volume of 200 µL. Basically, in each well, 170 µL of 50 mM PK buffer (pH 7.0), containing 0.6 mM AsA, were mixed with 20 µL of protein extract and 10 µL of 254 mM H_2O_2. The same procedure was followed to prepare a blank, in which the protein extract was replaced by the extraction buffer. Afterwards, the UV-microplate was shaken for 5 s and the AsA oxidation, at 290 nm, was monitored in intervals of 5 s over 70 s. The total activity of APX was calculated using the AsA extinction coefficient of 0.49 mM^{-1} cm^{-1} and expressed as µmol DHA min^{-1} mg^{-1} of protein

2.5.7.4. Activity of CAT (E.C. 1.11.1.6)

The quantification of CAT activity was performed in roots and leaves, following the protocol of Aebi (1984), with slight modifications. The procedure was executed in a UV-microplate in a final volume of 200 µL. Briefly, 160 µL of PK buffer (pH 7.0) were combined with 20 µL of sample and 20 µL of 100 mM H_2O_2. Concomitantly, a blank was prepared, in which the protein extract was replaced by the extraction buffer. After shaking the UV-microplate for 5 s, the rate of H_2O_2 degradation was followed over 70 s, in 5-s intervals, at 240 nm for. Based on the Lambert-Beer law, and knowing that H_2O_2 extinction coefficient is 39.4 mM^{-1} cm^{-1}, CAT activity was determined and expressed as nmol H_2O_2 min^{-1} mg^{-1} of protein.

2.6. Statistical analysis

All measurements were, at least, carried out in triplicate for each experimental condition ($n \geq 3$) and the results expressed as mean ± standard deviation of the mean (STDEV).

The calculation of the EC_{50} (effective concentration for a 50% of effect) values, and the corresponding 95% confidence limits, was performed by fitting the nonlinear least squares regression model to data in Statistica software (version 12). Prior to any statistical analyses, the homogeneity of variances by using the Levene's test was assessed.

In the experiments aimed at optimizing the Cu concentration (Petri dish and soil-based assays), a one-way ANOVA was performed, followed by a Dunnet post-hoc test, when significant differences were recorded ($p \leq 0.05$). For the screening of the effects of wrack on Cu-induced phytotoxicity (Petri dish and soil-based assays), a two-way ANOVA was conducted, with the fixed factors “Cu concentration” and “Wrack dose”. When significant differences were recorded ($p \leq 0.05$) for each variable, Tukey’s post-hoc test was performed to discriminate differences among groups. Regarding the final growth trial, where plants were exposed to Cu and/or wrack, after checking variance homogeneity and performing a one-way ANOVA, Tukey’s multiple range tests were used for determining significant differences among groups, whenever $p \leq 0.05$. All statistical data was generated by GraphPad® Prism 8 (GraphPad Software Inc.,USA)

3. RESULTS

Data regarding the statistical analyses, detailing the results of the ANOVAs performed for each parameter, are presented in Tables 1 - 7, found in Annex.

3.1 Biochemical and elemental analysis of wrack

The analysis of species composition of samples of wrack collected on a sandy beach (Praia Norte) on the North coast of Portugal showed that wrack was predominately composed of the fucoids *Fucus* spp., *Ascophyllum nodosum* L. and *Pelvetia canaliculata* (L.) Decne. & Thur.

Results obtained with the study of several biochemical parameters of wrack, focusing on the analysis of AOX metabolites, as well as its elemental composition, are presented in Tables 5 and 6, respectively.

Table 5: Levels of several biochemical parameters, including AOX metabolites, of wrack samples collected in the North coast of Portugal. Data are expressed as average ± standard deviation (STDEV) (n = 3).

Biochemical parameter	Concentration
Total phenols	8.35 ± 0.24 mg g^{-1}dm
Flavonoids	3.95 ± 1.22 mg g^{-1}dm
Total amino acids	0.74 ± 0.02 mg glycine equivalents g^{-1}dm
Total sugars	8.17 ± 0.94 µg glucose g^{-1}dm
Total antioxidant capacity (TAC)	22.86 ± 1.28 mg AsA equivalents g^{-1}dm
Free radical scavenging potential	250.51 ± 17.70 IC_{50} mg^{-1}dm
Proline	198.94 ± 0.03 µg g^{-1}dm
Carotenoids	384.31 ± 0.09 µg g^{-1}dm
Chlorophyll *a* + *c*	977.00 ± 0.24 µg g^{-1}dm

Table 6: Major and minor element concentrations of wrack samples collected in the North coast of Portugal. Data are expressed as average ± standard deviation (STDEV) (n = 3). The last columns present the limit values of trace elements/metals allowed in sewage sludge to be used in soils (decree-law 276/2009) and in organic fertilizers (decree-law 103/2015) according to national legislation.

Element	Concentration	Limit values for sewage sludge	Limit values for organic fertilisers
Potassium (K)	17.7 ± 0.7 mg g^{-1} dm	-	-
Calcium (Ca)	12.4 ± 0.4 mg g^{-1} dm	-	-
Magnesium (Mg)	8.3 ± 0.2 mg g^{-1} dm	-	-
Sodium (Na)	12.4 ± 0.9 mg g^{-1} dm	-	-
Manganese (Mn)	10.6 ± 1.3 µg g^{-1} dm	-	-

Cadmium (Cd)	0.6 ± 0.4 µg g^{-1} dm	20 µg g^{-1} dm	1.5 µg g^{-1} dm
Chromium (Cr)	0.7 ± 0.0 µg g^{-1} dm	1000 µg g^{-1} dm	150 µg g^{-1} dm
Copper (Cu)	4.4 ± 0.9 µg g^{-1} dm	1000 µg g^{-1} dm	200 µg g^{-1} dm
Lead (Pb)	1.5 ± 0.3 µg g^{-1} dm	750 µg g^{-1} dm	150 µg g^{-1} dm
Nickel (Ni)	6.2 ± 0.5 µg g^{-1} dm	300 µg g^{-1} dm	100 µg g^{-1} dm
Zinc (Zn)	81.4 ± 4.1 µg g^{-1} dm	2500 mg g^{-1} dm	500 µg g^{-1} dm

According to Table 6, K, Ca and Na were the most abundant elements, followed by Mg. Additionally, as can be observed, all micronutrients and/or trace elements analysed presented values well below the national limit values allowed in organic fertilizers and sewage sludge to be applied into the soils.

3.2 Assessment of a wrack aqueous extract potential to improve barley seedlings development under Cu toxicity

Upon obtaining wrack's phytochemical characterisation (biochemical and elemental analysis), a series of preliminary assays were carried out with an aqueous extract (10 g_{dm} L^{-1}) of wrack, to assess its potential to increase barley seedlings' tolerance to Cu under *in vitro* conditions.

3.2.1 Effects of increasing concentrations of Cu on barley's germination and seedling development – Petri dish assay

In order to select the appropriate concentration of Cu for the subsequent assays, five concentrations (0, 2.5, 4, 10, 15 and 17.5 mg L^{-1}) were tested in a Petri dish assay and its effects on plant biometry were evaluated after 8 d of exposure. As shown in Figure 4, root length was significantly affected by Cu concentrations ≥ 4 mg L^{-1}, causing a decrease up to 87% over the control (0 mg Cu L^{-1}) (Table 1 in Annex I). Leaves' length was also significantly affected; however, the statistical relevance was only observed for the two highest concentrations (up to 51 % of inhibition).

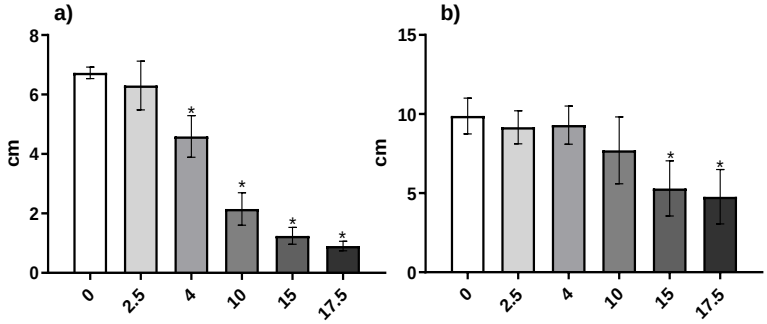

Figure 4: Roots (a) and leaves (b) length of barley seedlings after 8 d of exposure under *in vitro* conditions and exposed to different copper concentrations. Results are expressed as mean ± standard deviation (STDEV). * above bars mean significant differences from the control (0 mg Cu L^{-1}) at $p \leq 0.05$.

Based on this set of results, and by fitting the nonlinear least square regression model, it was estimated the Cu concentrations causing a 50% reduction (EC_{50}) on plants biometry and the results are presented in Table 7.

Table 7: Data obtained for determining EC_{50} values. Concentrations, and corresponding 95% confidence intervals, are expressed as mg L^{-1}.

Biometric parameter	EC_{50}
Root length	7.2 (6.1 – 8.3)
Leaves length	17.4 (13.7 – 21.3)

Based on the most sensitive biometric parameter, which was root length, the Cu concentration of 7 mg L^{-1} (95% confidence levels: 6.1 – 8.3 mg Cu L^{-1}) was selected to be used in the future *in vitro* experiments.

3.2.2 Effects of wrack on alleviating Cu toxicity in barley's seedling development – Petri dish assay

The results regarding the potential of an aqueous extract of wrack (10 g_{dm} L^{-1}, sequentially diluted) to alleviate Cu (7 mg L^{-1}) toxicity in barley seedlings are summarised in Figures 5 and 6. As can be seen (Table 2 in Annex I), significant differences were observed for each tested factor (wrack and Cu), as well as for their interaction in all biometric parameters (root length and root and leaves biomass), with the exception of root length, where no relationship between Cu and wrack factors was observed.

Despite the extract did not influence root length, roots' biomass was 63% higher than control upon the addition of 5.0 g L^{-1} extract (Figure 5). Moreover, supplementing the growth medium with 5.0 and 7.5 g L^{-1} extract positively affected both leaves' length (18 and 15%, respectively) and biomass (45 and 23%, respectively), in comparison with the control (with no Cu nor extract) (Figure 6). Concerning Cu exposure, seedlings grown under 7 mg Cu L^{-1} showed a significant decrease in roots' length (52%) and biomass (57%) (Figure 5), along with a reduction in leaves' length (25%) and biomass (44%), comparing to the control (Figure 6). However, when extract at 7.5 g L^{-1} was added to Cu-containing medium, a recovery of leaves length to values identical to the control was found, leading to an increase of 34% when compared to seedlings only exposed to Cu (Figure 6). Finally, although not significantly, the concentrations of 5.0 and 7.5 g L^{-1} of extract also showed a strong tendency to counteract the negative effects of Cu on leaves' biomass (Figure 6).

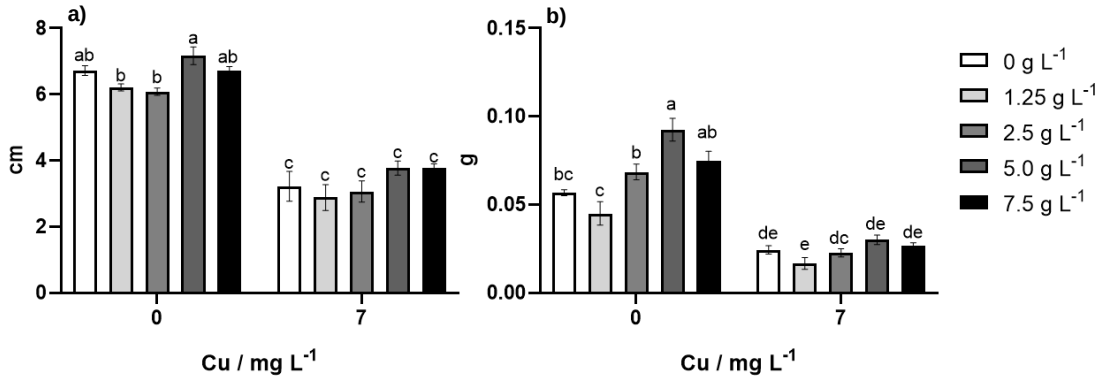

Figure 5: Roots' length (a) and biomass (b) of barley seedlings exposed to increasing concentrations of an aqueous extract of wrack (0, 1.25, 2.5, 5.0 and 7.5 g L^{-1}) and grown in the presence or absence of 7 mg Cu L^{-1}. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

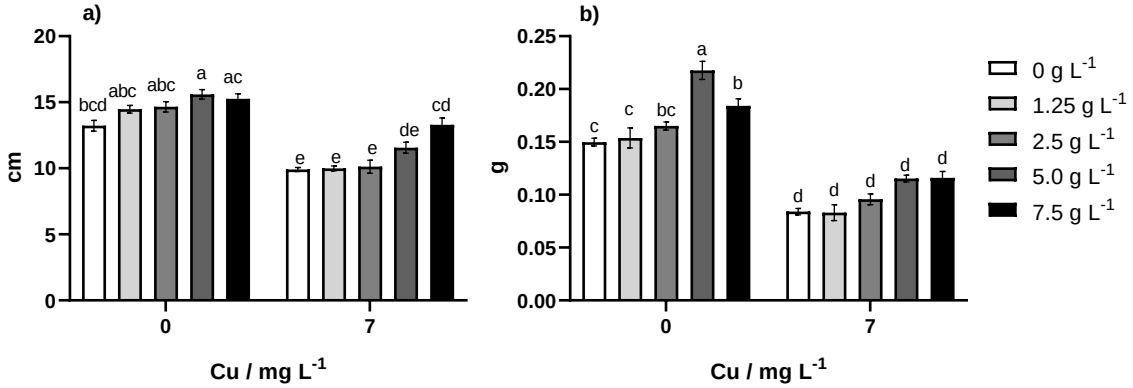

Figure 6: Leaves' length (a) and biomass (b) of barley seedlings exposed to increasing concentration of an aqueous extract of wrack (0, 1.25, 2.5, 5.0 and 7.5 g L^{-1}) and grown in the presence or absence of 7 mg Cu L^{-1}. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

3.3 Effects of soil amendment with wrack on Cu-induced toxicity in barley plants

After the first preliminary screening, where wrack showed an effective potential to alleviate Cu-induced toxicity in seedlings of *H. vulgare*, a set of new experiments was designed to understand if the observed beneficial effects *in vitro* could be transposed to a realistic scenario. For this purpose, an agricultural soil, free of Cu contamination, was collected, amended with wrack [0, 0.5, 1 and 2% (m/m)] and characterised for their chemical properties upon 15 and 30 d of stabilisation. After that, plants were grown under Cu and wrack co-exposure to select the most appropriate wrack dose, to be used in a final growth trial, where the physiological and biochemical basis of wrack-mediated protection against Cu-induced stress was evaluated.

3.3.1 Effects of wrack on soil chemical properties

The impact of the addition of different concentrations of wrack [0, 0.5, 1 and 2% (m/m)] on soil's chemical properties was accessed upon a period of 15 and 30 d of stabilisation and the results are presented in Table 8.

Table 8: Chemical properties of both the non-amended soil and the amended soil batches with different wrack concentrations [0,0.5, 1 and 2% (m/m)] after 15 d and 30 d of stabilisation. Data are presented as mean ± standard deviation (STDEV) (n=3). Different lowercase letters mean significant differences between groups at $p \leq 0.05$

		0% wrack	0.5% wrack	1% wrack	2% wrack
15 d	pH (H_2O)	5.9 ± 0.2^{b}	$6.2 \pm 0.2^{a,b}$	$6.3 \pm 0.2^{a,b}$	6.4 ± 0.2^{a}
	OM (%)	6.0 ± 0.0^{c}	$6.3 \pm 0.5^{b,c}$	$6.8 \pm 0.5^{a,b}$	7.0 ± 0.0^{a}
	EC (dS m^{-1})	0.35 ± 0.02^{b}	0.36 ± 0.02^{b}	$0.41 \pm 0.0^{a,b}$	0.46 ± 0.06^{a}
30 d	pH (H_2O)	6.2 ± 0.0^{d}	6.5 ± 0.0^{c}	6.8 ± 0^{a}	6.6 ± 0.2^{b}
	OM (%)	5.3 ± 0.6^{a}	6.0 ± 1.0^{a}	5.7 ± 0.6^{a}	5.7 ± 0.6^{a}
	EC (dS m^{-1})	0.21 ± 0.00^{d}	0.37 ± 0.01^{c}	0.50 ± 0.01^{b}	0.61 ± 0.03^{a}

As can be observed, after 15 d of stabilisation, only the highest wrack concentration caused a significant increase in soil pH. Regarding OM content and EC, both parameters were significantly higher with the addition of both 1% (m/m) and 2% (m/m) of wrack. On the other hand, after 30d of stabilisation, all wrack concentrations contributed for a significant increase of pH and EC values, but OM content was not altered (Table 3 in Annex II).

3.3.2 Selection of Cu concentration

The effects of the exposure of barley plants to increasing concentrations of Cu (0, 30, 45, 68, 102, 153, 229, 344 mg kg^{-1}) on root length can be observed in Figure 7. After 14 d of growth, the three highest concentrations (153, 229 and 344 mg kg^{-1}) led to a significant decrease in root elongation up to 82% over the control (0 mg Cu kg^{-1}).

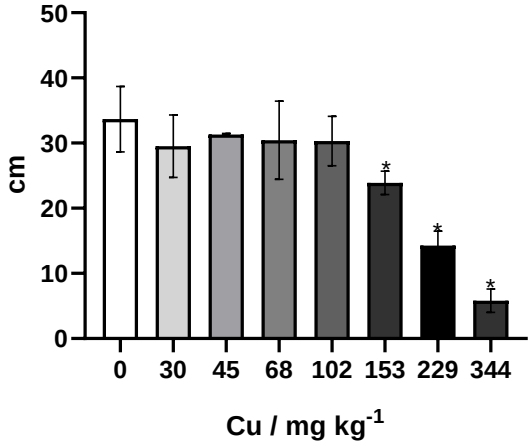

Figure 7: Roots' length of barley plants exposed to increasing Cu concentrations (0-344 mg kg^{-1}) and growth for 14 d in an agricultural soil. Results are expressed as mean ± standard deviation (STDEV). * above bars mean significant differences from the control (0 mg Cu kg^{-1}) at $p \leq 0.05$.

Additionally, a negative effect of the two highest Cu concentrations on root biomass (fresh and dry mass) was also observed (Figure 8), being this effect more pronounced for root fresh biomass with a decrease up to 60% in response to 344 mg Cu kg^{-1} (Figure 8a).

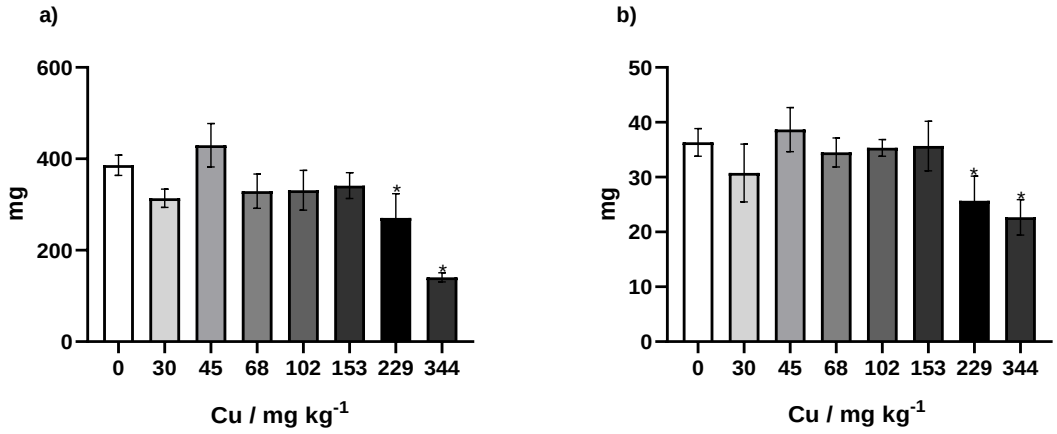

Figure 8: Roots' fresh (a) and dry (b) biomass of barley plants exposed to increasing Cu concentrations (0-344 mg kg^{-1}) and growth for 14 d in an agricultural soil. Results are expressed as mean ± standard deviation (STDEV). * above bars mean significant differences from the control (0 mg Cu kg^{-1}) at $p \leq 0.05$.

As it can be seen in Figure 9, while no statistical differences were found in dry biomass of leaves, the fresh mass of this organ was significantly increased in response to 30 and 45 mg Cu kg^{-1} (Table 4 in Annex II).

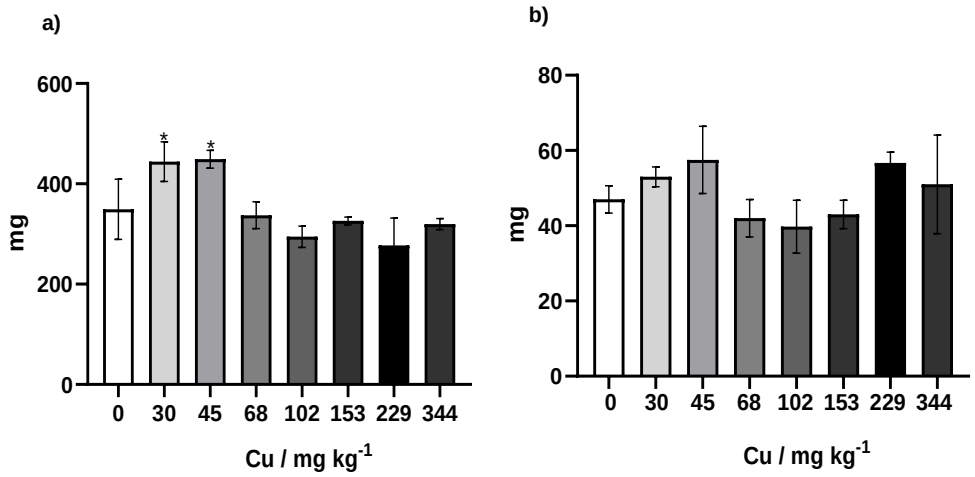

Figure 9: Leaves' fresh (a) and dry (b) biomass of barley plants exposed to increasing Cu concentrations (0-344 mg kg^{-1}) and growth for 14 d in an agricultural soil. Results are expressed as mean ± standard deviation (STDEV). * above bars mean significant differences from the control (0 mg Cu kg^{-1}) at $p \leq 0.05$

Based on these parameters, a nonlinear least square regression model was performed to calculate EC_{50} values (Table 9). As shown, the concentrations corresponding to a 50% of effect were only possible to estimate for root parameters.

Table 9: Summary of data obtained for determining EC_{50} values. Concentrations, and corresponding 95% confidence intervals, are expressed as mg kg^{-1}.

Biometric parameter	EC_{50}
Root length	219.59 (194.32 – 244.86)
Root dry biomass	405.20 (280.94 – 529.46)
Root fresh biomass	304.49 (247.83 – 361.1380)
Leaf length	n.d.
Leaf dry biomass	n.d.
Leaf fresh biomass	n.d.

n.d. denotes situations where it was not possible to fit data to calculate the EC_{50} value

Based on the most sensitive biometric parameter, which was root length, the Cu concentration of 219 mg kg^{-1} (95% confidence levels: 194 - 244 mg Cu kg^{-1}) was selected to be used in the future experiments.

3.3.3 Selection of wrack concentration

In order to select the best wrack concentration to proceed with the studies, the potential effect of wrack on the alleviation of Cu toxic effects was accessed, upon two stabilisation periods (15 and 30 d). As reported in Table 5 (Annex II), all biometric parameters (organ elongation and biomass production) were significantly affected by both factors (Cu and wrack) and by their combination, with the exception of root length, where no relationship between Cu and wrack was found.

As can be seen in Figure 10a, after 15 d of stabilisation, Cu significantly decreased (30%) the root length of barley plants, when compared with the control without Cu. However, the addition of 2% (m/m) of wrack induced an increase of this parameter, reaching values equal to those of the treatment without Cu. No significant changes were observed for plants growing in soils submitted to a period of 30 d of stabilisation among all treatments (Figure 10b).

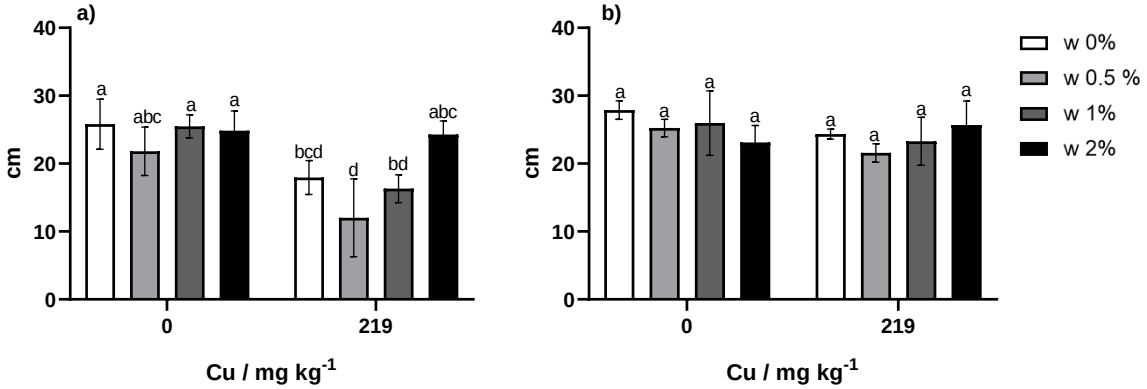

Figure 10: Root's length of barley plants grown for 14 d in an agricultural soil contaminated, or not, by Cu (219 mg kg^{-1}) with the addition of wrack [0, 0.5, 1 and 2% (m/m)] and left for stabilisation for 15 d (a) and 30 d (b). Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

Regarding root biomass production (Figure 11), no significant differences were found with the addition of wrack, except for an increase of 45% under the co-exposure of Cu and the highest level of wrack [2% (m/m)], after 30 d of stabilisation, in comparison with the control (Figure 11b). However, Cu also did not cause a phytotoxic effect in this parameter. No significant changes were observed for leaves biomass (Figure 12).

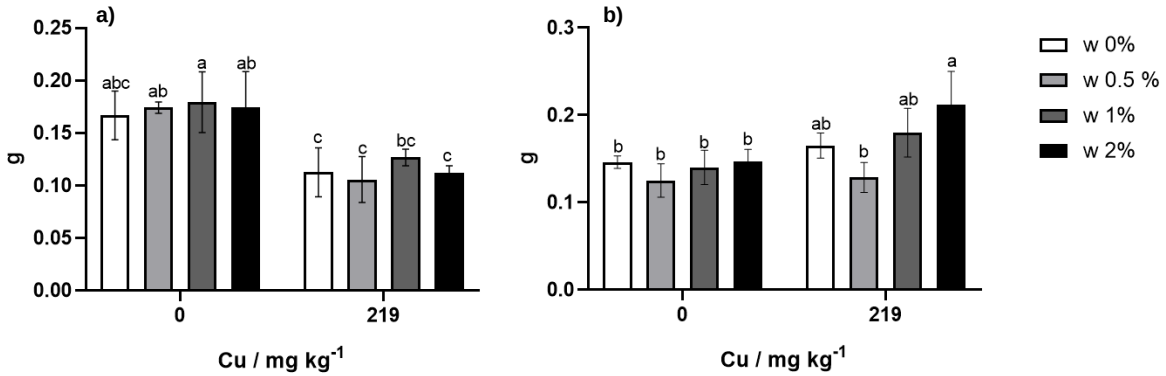

Figure 11: Roots' biomass of barley plants grown for 14 d in an agricultural soil contaminated, or not, by Cu (219 mg kg^{-1}) with the addition of wrack [0, 0.5, 1 and 2% (m/m)] and left for stabilisation for 15 d (a) and 30 d (b). Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

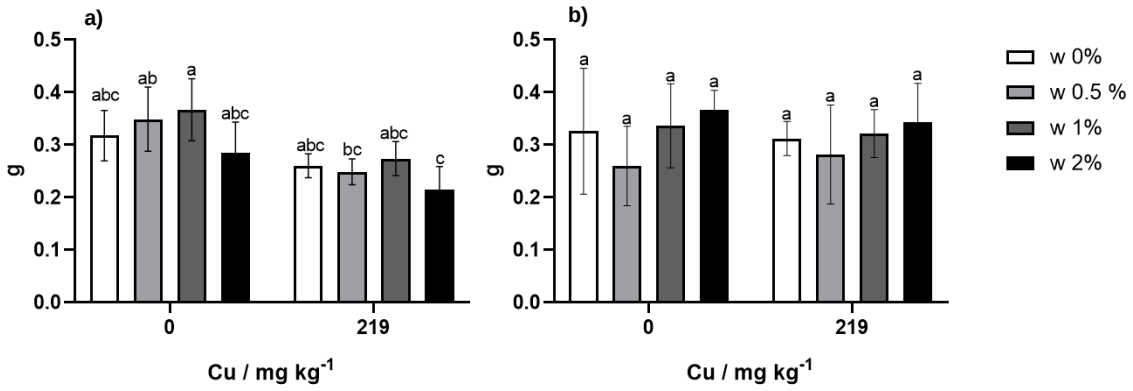

Figure 12: Leaves' biomass of barley plants grown for 14 d in an agricultural soil contaminated, or not, by Cu (219 mg kg^{-1}) with the addition of wrack [0, 0.5, 1 and 2% (m/m)] and left for stabilisation for 15 d (a) and 30 d (b).Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

Comprising these results, along with the data obtained for soil parameters (3.3.1), the wrack concentration selected for the following experiments was set up at 2% (m/m) with a stabilisation period in the soil of 15 d.

3.3.4 Beneficial effects of wrack on Cu-induced toxicity in barley plants – growth performance, physiological status and redox homeostasis

Aiming to get an insight into the mechanisms of wrack-mediated protection against Cu toxicity, barley plants were grown for 14 d under different experimental treatments: **CTL**, where plants grew in an agricultural soil free of Cu contamination and not amended with wrack; **Wrack**, where plants grew in an agricultural soil free of Cu contamination previously amended with 2% (m/m) wrack; **Cu**, where plants grew in an agricultural soil contaminated by 219 mg Cu kg^{-1} not amended with wrack; **Cu + Wrack**, where plants grew in an agricultural soil contaminated by 219 mg Cu kg^{-1} and previously amended with 2% (m/m) wrack.

3.3.4.1 Biometric parameters and biomass production

Results highlighted in Figure 13 show that Cu caused a decrease of 23% in root length, in comparison with the CTL. However, this effect was counteracted by the addition of wrack, since no significant differences were recorded between plants co-exposed to Cu and wrack, and the CTL (Table 5 in Annex III).

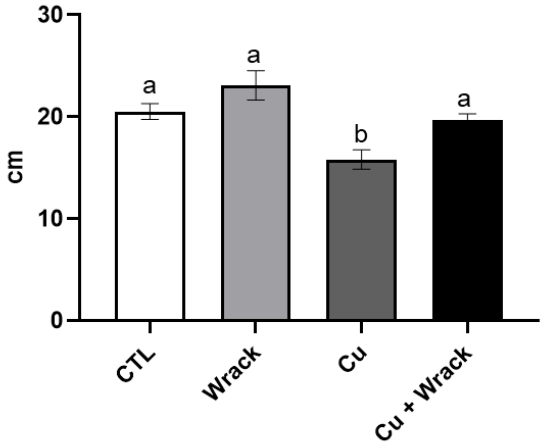

Figure 13: Root length of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

Regarding the biomass production, Cu treatment significantly decreased both roots' and leaves' growth (Figure 14; Tables 5 and 6 in Annex III). Indeed, when compared to the CTL, the fresh weight of Cu-treated plants was 19 and 14% lower in leaves and roots, respectively. Concerning wrack effects, its single application did not differ from the CTL; however, under the co-exposure situation, the fresh biomass of roots was significantly higher than that of plants only exposed to Cu, reaching values identical to the CTL. Regarding leaves, an intermediate response between Cu and CTL plants was found upon the joint application of wrack and Cu.

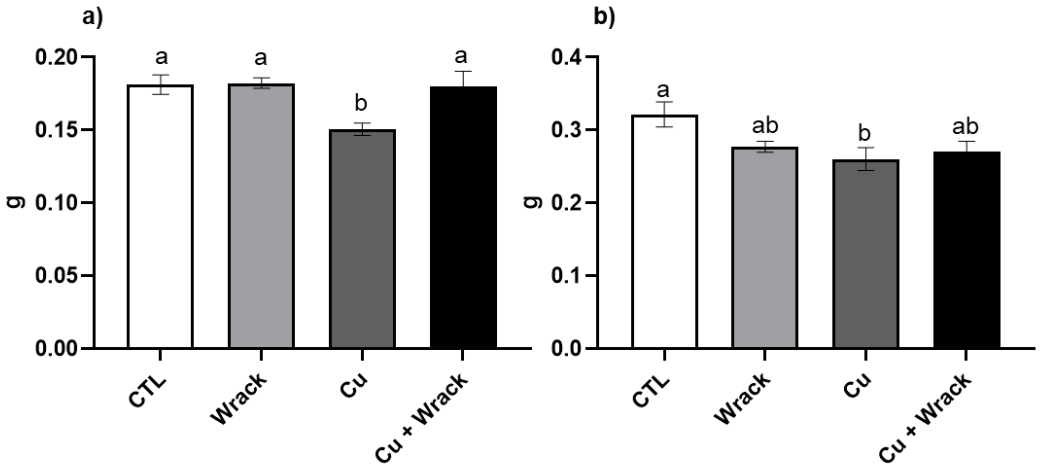

Figure 14: Fresh biomass of roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

3.3.4.2 Physiological status – photosynthetic pigments and total protein

Figures 15 and 16 reflect the quantification of the photosynthetic pigments and soluble protein content, respectively. Regarding both chlorophylls and carotenoids, no significant differences were detected among treatments (Figure 15; Table 7 in Annex III).

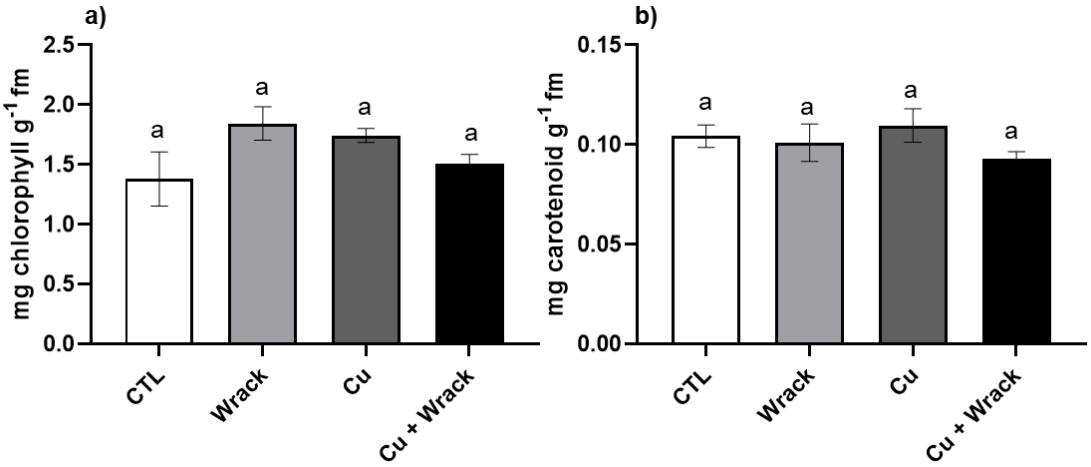

Figure 15: Levels of total chlorophylls (a) and carotenoids (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

As shown in Figure 16, total soluble protein levels were not altered among treatments in roots (Figure 16a). Regarding leaves, wrack caused a 30% decrease of this parameter, regardless of Cu co-exposure, in relation to the CTL (Figure 16b; Tables 6 and 7 in Annex III).

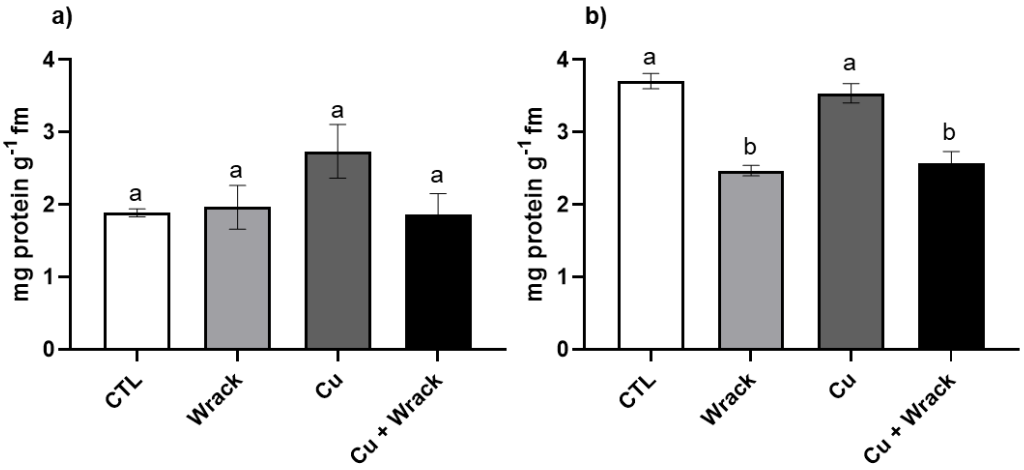

Figure 16: Levels of total soluble protein in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

3.3.5 Cu bioaccumulation

Cu concentration in leaves and roots of barley plants is shown in Figure 17. As highlighted, Cu levels were higher in roots than in leaves. Also, Cu was not detected in leaves of both CTL and wrack groups. Despite of that, soil contamination by Cu increased root concentration of this metal by 20- and 18.5-fold under single and co-exposure with wrack, respectively, and in relation to the CTL. In leaves, the opposite was observed, with plants co-exposed to Cu and wrack presenting the highest values of Cu accumulation. However, although significant, the differences between the two Cu treatments are very slight, with absolute values of Cu concentration ranging from 20.76 µg g^{-1} dm (Cu) to 22.86 µg g^{-1} dm (Cu + wrack) (Tables 6 and 7 in Annex III).

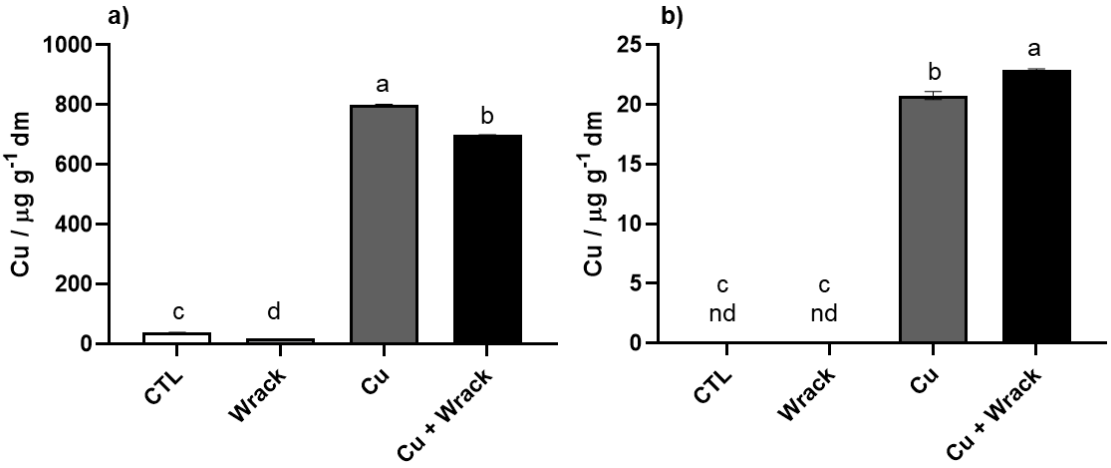

Figure 17*:* Levels of Cu in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

3.3.6 Oxidative stress markers – LP, H_2O_2 and $O_2^{\bullet-}$

Lipid peroxidation degree, determined by the quantification of MDA, one of the final products of polyunsaturated fatty acids peroxidation in the cells, was assessed in roots and leaves of barley plants. As can be seen (Figure 18; Tables 6 and 7 in Annex III), MDA content revealed to be differentially affected depending on the analysed organ: in roots, plants exposed only to Cu showed an increased value (57%) of MDA, being this effect counteracted upon the addition of wrack to values lower than those recorded in the CTL (Figure 18a); in leaves, Cu did not cause significative alterations in the MDA content, however wrack caused a significant decrease in this parameter both under the presence or not of Cu (Figure 18b).

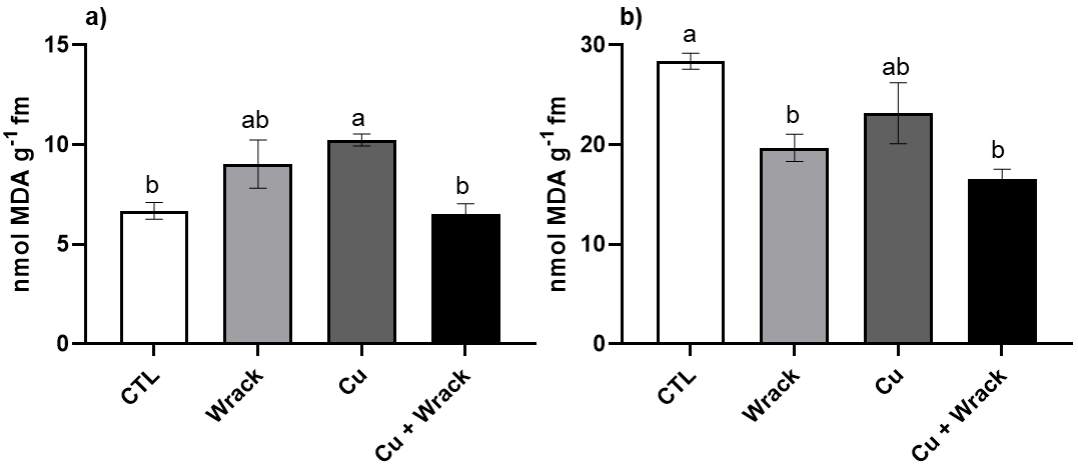

Figure 18: Levels of MDA in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

The effects of Cu and wrack single and co-exposure on H_2O_2 accumulation in *H. vulgare* are represented in Figure 19. As can be observed, the content of this ROS has significantly increased in the roots due to Cu exposure, when compared to the CTL. However, wrack both under single and Cu co-exposure, caused even highest increases in this parameter (rises up to 2.4-fold and 3.3-fold, respectively), in relation to the CTL (Figure 19a). In leaves, the Cu treatment did not affect H_2O_2 content, but the application of wrack increased, once again, the levels of this ROS, especially when applied alone (1-fold in comparison with the CTL; Figure 19b) (Tables 6 and 7 in Annex III).

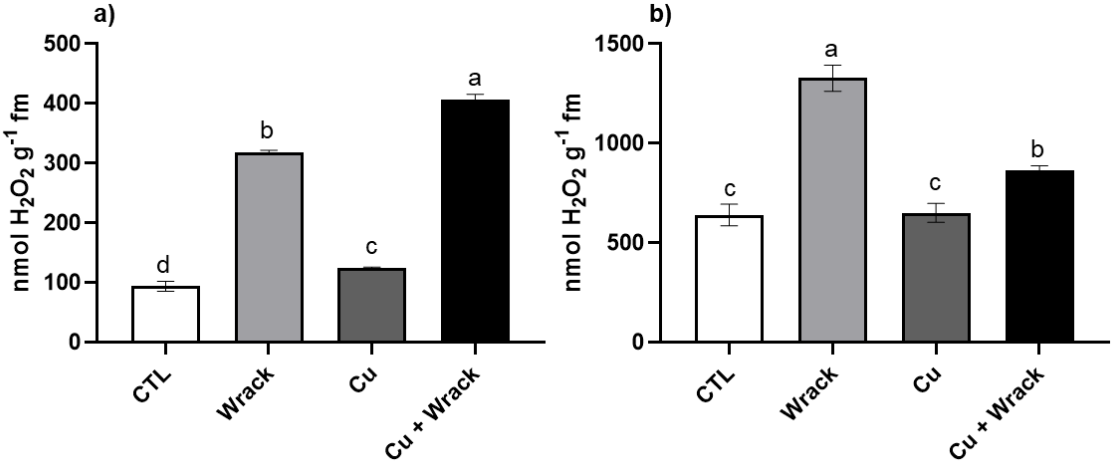

Figure 19: Levels of H_2O_2 in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

Regarding $O_2^{\bullet-}$ quantification (Figure 20; Tables 6 and 7 in Annex III), the exposure to Cu resulted in a significant accumulation of this ROS in both organs (40% and 32% in roots and leaves, respectively), in relation to the CTL. However, the co-application of wrack caused a decrease of $O_2^{\bullet-}$ content at both root and leaves, in comparison with Cu-treated plants (leaves – 47%; roots – 55%) and with the CTL (leaves – 31%; roots – 37%).

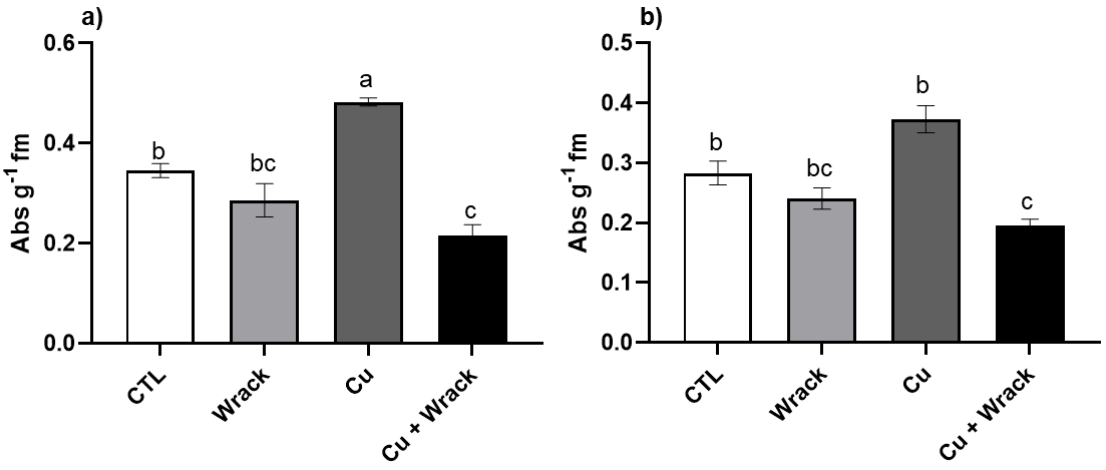

Figure 20: Levels of $O_2^{\bullet-}$ in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

3.3.7 Non-enzymatic AOX system – proline, GSH, AsA, total phenolics and flavonoids

Starting by Pro, results showed that its levels significantly decreased in all treatments in roots, with reductions up to 54% over the CTL (Figure 21a). Concerning leaves, a significant decrease in the accumulation of this AOX was observed under Cu single exposure, however no differences were found between wrack-amended soils and the CTL (Figure 21b) (Tables 6 and 7 in Annex III).

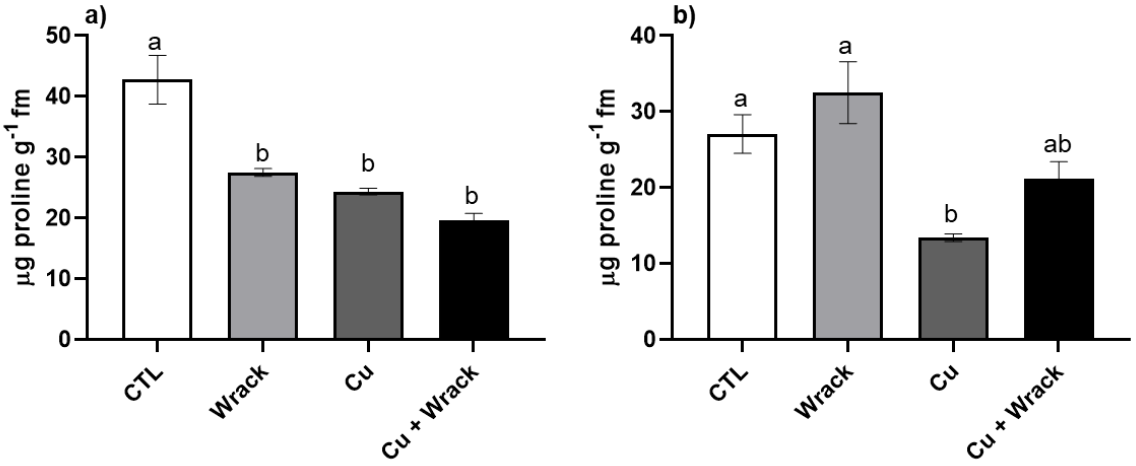

Figure 21: Levels of proline in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

The variation of GSH levels in both plant organs analysed are presented in Figure 22. In roots, Cu exposure significantly increased the accumulation of this AOX by 45%, while a decrease of 45% upon the treatment with 2% (m/m) wrack was observed. Moreover, in response to the co-treatment, values were restored to those found in the CTL (Figure 22a). Concerning leaves, only the co-exposure to Cu and wrack significantly enhanced the levels of GSH, with rises around 70%, in comparison with the CTL group of plants (Figure 22b) (Tables 6 and 7 in Annex III).

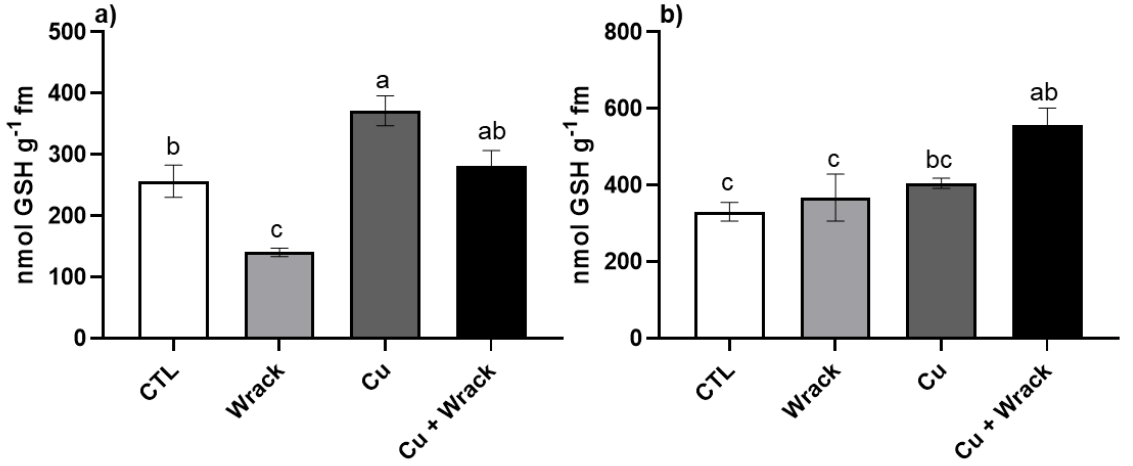

Figure 22: Levels of GSH in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

Results regarding the total content in ascorbate, as well as its reduced (AsA) and oxidised (DHA) forms, are compiled in Table 10. In roots, total AsA significantly increased

upon Cu single (6.4-fold) and wrack co-exposure (4-fold), relatively to the CTL. Concerning AsA and DHA portions, both reduced and oxidised forms were increased in response to Cu (AsA – 4.2-fold; DHA – 8.1-fold), especially under single exposure. In fact, when plants were grown in Cu-contaminated soil amended with wrack, the observed increase in DHA (5.7-fold) was lower than that observed for plants exposed to Cu alone. AsA content did not change in response to the co-exposure treatment in relation to the CTL (Table 10; Tables 6 and 7 in Annex III).

Total AsA levels were not affected in leaves, as no differences were detected among treatments. However, an increase of AsA and a decrease of DHA was found in response to Cu and wrack, both individually or in combination.

Table 10: Levels of total, reduced (AsA) and oxidised (DHA) ascorbate in roots and leaves of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

	ROOTS			LEAVES		
	Total ascorbate	Reduced ascorbate (AsA)	Oxidised ascorbate (DHA)	Total Ascorbate	Reduced Ascorbate (AsA)	Oxidised Ascorbate (DHA)
	(**µ**mol ascorbate g^{-1} fm)					
CTL	0.283±0.032^{c}	0.073±0.021^{b}	0.187±0.005^{b}	0.613±0.087^{a}	0.483±0.078^{b}	0.122±0.022^{a}
Wrack	0.330±0.044^{c}	0.030±0.033^{b}	0.223±0.006^{b}	0.798±0.111^{a}	0.717±0.081^{a}	0.032±0.012^{b}
Cu	2.107±0.309^{a}	0.386±0.056^{a}	1.717±0.308^{a}	0.625±0.052^{a}	0.572±0.033^{a}	0.030±0.010^{b}
Cu + wrack	1.423±0.250^{b}	0.160±0.010^{b}	1.267±0.250^{a}	0.690±0.088^{a}	0.620±0.096^{a}	0.070±0.035^{b}

The ratio between AsA and DHA can be found in Figure 23. As can be observed, in roots, AsA/DHA proportion was 50% lower in response to Cu, being this effect even more evident under the combined exposure with wrack (ca. 70%). Concerning leaves, the AsA/DHA ratio significantly increased upon exposure to wrack (7.3-fold) and Cu (4.3-fold) (Tables 6 and 7 in Annex III).

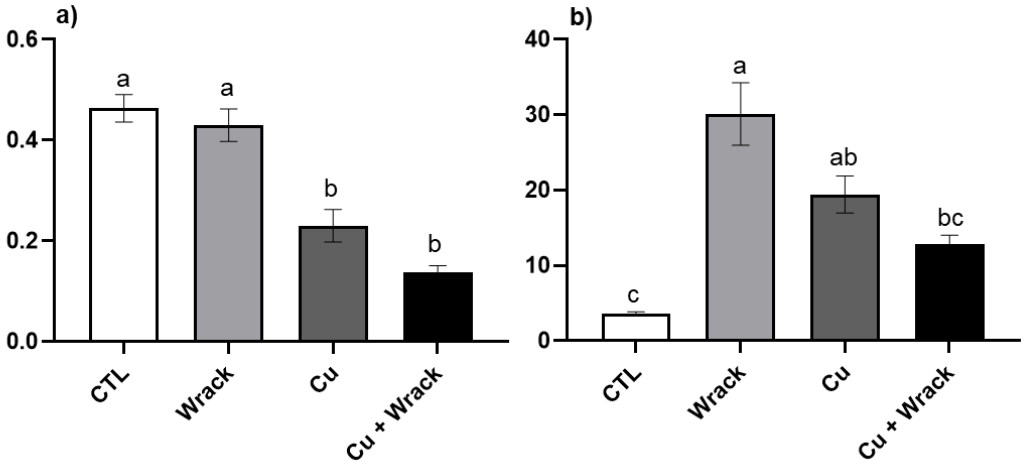

Figure 23: AsA/DHA ratio in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

As can be seen in Figure 24, root phenolic compounds, measured as gallic acid equivalents increased 75% when plants were treated with Cu, in relation to the CTL. However, the co-treatment with wrack reduced the total content of phenolic compounds to a level similar to the CTL. In leaves, all treatments caused a significant enhancement of this parameter, being this effect particularly evident when plants were co-exposed to Cu and wrack (27% increase over the CTL) (Tables 6 and 7 in Annex III).

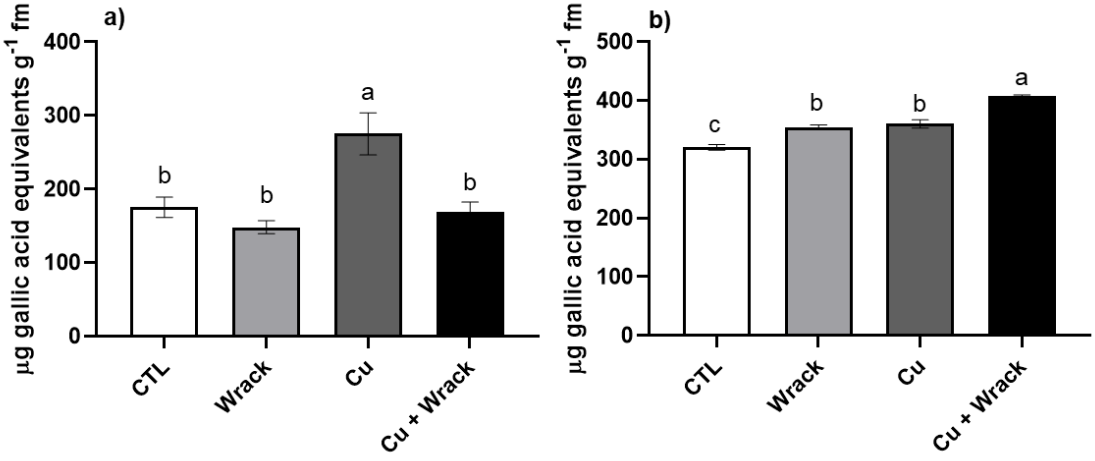

Figure 24: Levels of total phenols measured as gallic acid equivalents in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

As shown in Figure 25, only Cu treatment significantly changed the content of flavonoids measured as quercetin equivalents, in barley roots, with an increase of around 60% in

relation to the CTL. In leaves, although not significant, a strong tendency for reduced levels of flavonoids was noticed in Cu-treated plants. However, plants grown under the presence of both wrack and Cu exhibited significant higher (47%) levels of this parameter in leaves, when compared to plants exposed to Cu alone (Figure 25b) (Tables 6 and 7 in Annex III).

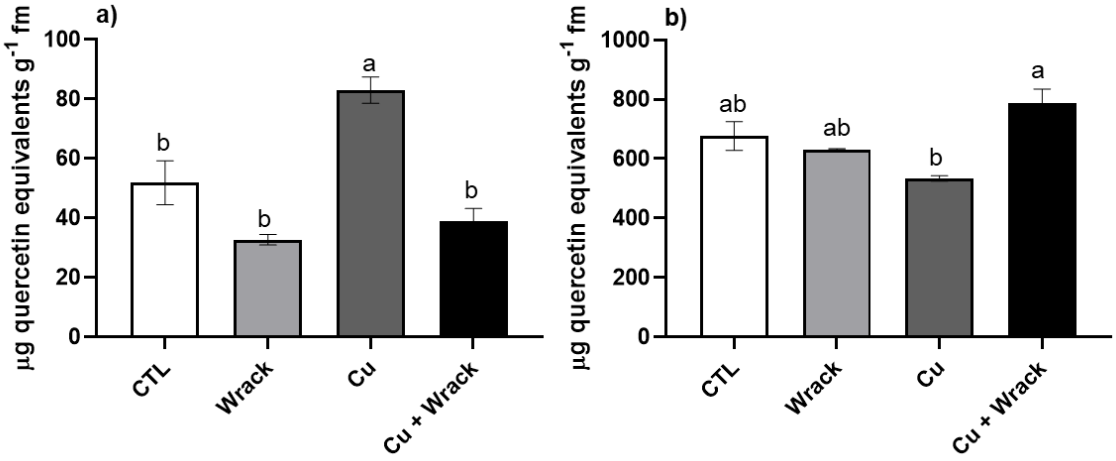

Figure 25: Levels of flavonoids measured as quercetin equivalents in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

3.3.8 Enzymatic AOX system – SOD, APX and CAT

Regarding the enzymatic AOX system, the activities of SOD, CAT and APX were evaluated in both roots and leaves of barley plants. Concerning the total activity of SOD (Figure 26; Tables 6 and 7 in Annex III), no differences among treatments were detected in roots, while in leaves, a significant increase of 41%, in comparison to the CTL, was found in plants treated only with wrack.

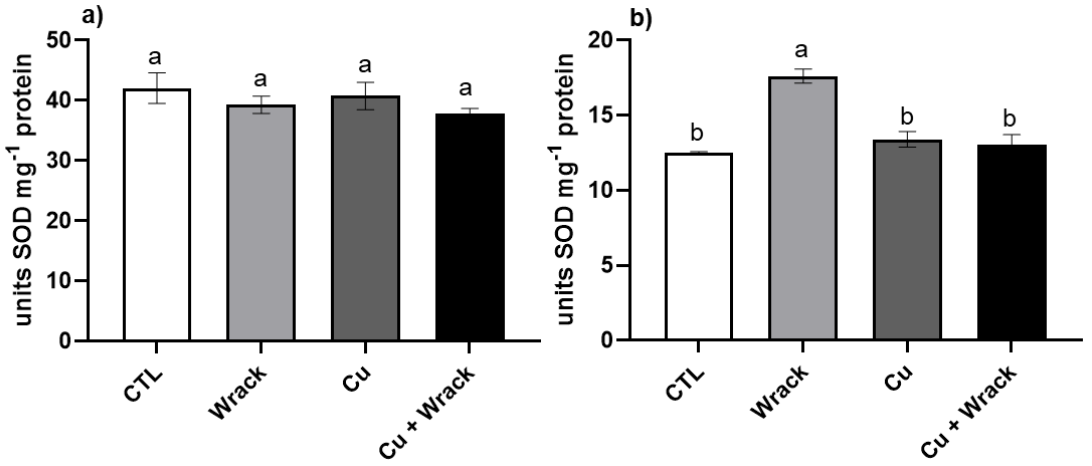

Figure 26: SOD activity in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

Similarly to what was observed for SOD, APX activity (Figure 27; Tables 6 and 7 in Annex III) was not altered in roots. However, in leaves, wrack-treated plants, either under single or Cu-combined exposure, showed higher activity of this enzyme, with increases of about 49% in relation to both CTL and Cu treatment.

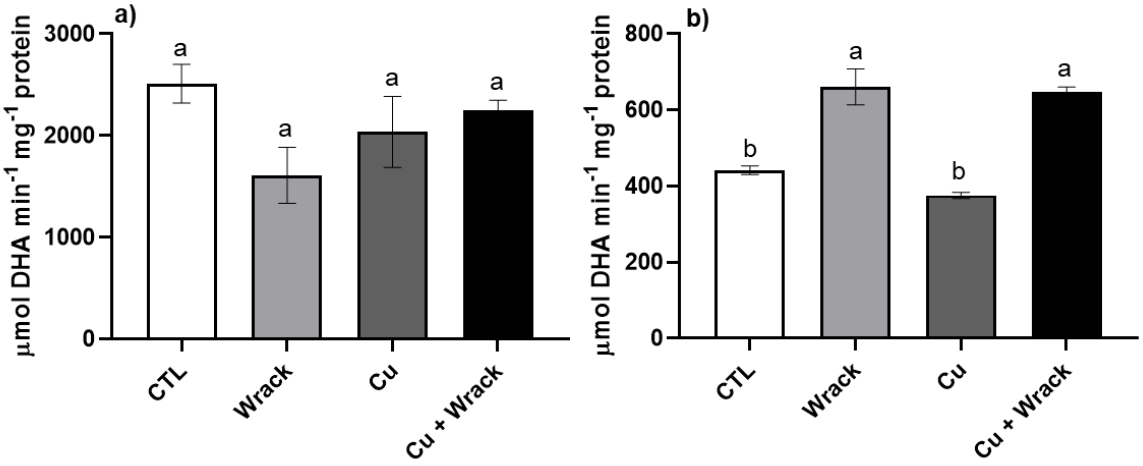

Figure 27: APX activity in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

Lastly the data reported in Figure 28 show that, in the roots, all treatments caused a significant decrease in CAT activity, especially in plants exposed to wrack (alone or together with Cu), where the activity values were 49 % lower than those of CTL; Cu alone reduced CAT activity by 28%. In the leaves, no significant differences were detected among treatments (Tables 6 and 7 in Annex III).

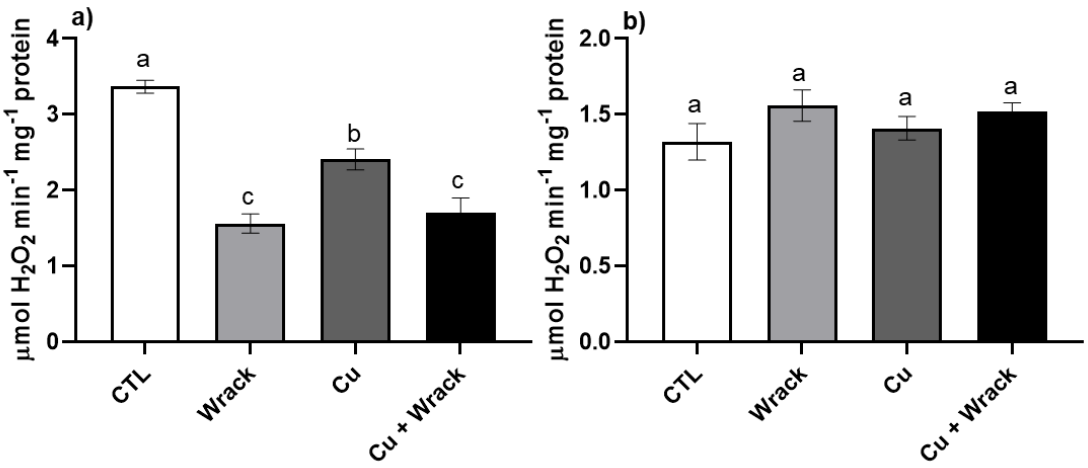

Figure 28: CAT activity in roots (a) and leaves (b) of barley plants grown for 14 d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu. Results are expressed as mean ± standard deviation (STDEV). Different letters above the bars mean significant differences between groups at $p \leq 0.05$.

4. DISCUSSION

4.1 Biochemical and elemental analysis of wrack

The need to develop sustainable alternatives to chemical fertilisers, with less environmental impact, has led to the emergence of different PBs, in which SWE are included (Rouphael and Colla, 2020). Additionally, the chemical composition of seaweeds, besides being seasonal- and species-specific, is also conditioned by environmental conditions such as salinity, light, temperature, and nutrient availability (Nabti et al., 2016). A previous study, which demonstrated the correlation between wrack's composition and its fertiliser potential, highlighted the need to perform a complete characterisation of this residue, due to the capacity of seaweeds to accumulate undesirable substances, such as metals and other contaminants (Villares et al., 2016). Thus, the present study aimed at verifying the chemical (major, minor and/or trace elements) and biochemical profile of wrack samples, collected in a northern Portuguese sandy beach, composed by a mixture of brown seaweeds (*Fucus* spp., *A. nodosum* and *P. canaliculata*). Regarding wrack's elemental analysis, and as expected, K, Ca and Na were the most abundant elements, followed by Mg. The predominance of these elements over the remaining minerals was also found in *A. nodosum*, *Fucus vesiculosus* L. and *Bifurcaria bifurcata* (Lorenzo et al, 2017). Additionally, similar trends in the mineral composition of brown seaweeds were also reported by Kumar et al. (2011) and Rodrigues et al. (2015). Furthermore, in a previous study, which aimed at verifying the chemical composition of wrack collected in two coastal areas of the northwest of Spain, higher levels of K, Ca, and Na were also noticed (Villares et al., 2016). Given the marine origin of wrack, information about Na content is particularly important to assess the suitability of using this residue for agricultural purposes without causing problems of salinisation. In this sense, and when looking at the mineral composition of other organic fertilisers (chicken, sheep, cow and pig manure, and sewage sludge) (Villares et al., 2016 and the references therein) the results herein obtained showed that wrack had similar levels of Na, as well as K, but lower levels of Ca. On the other hand, the quantification of trace elements on wrack is particularly important given the ability of seaweeds for accumulating these substances (Nbati et al., 2015), thus impairing the use of this residue as fertiliser. Among the micronutrients and trace elements, our study showed that Zn was the most abundant (81.4 µg g^{-1} dm), having higher levels than those reported for wrack collected in Spain (42.58 and 54.70 µg g^{-1} dm) (Villares et al., 2016). However, the contents of Cu, Cr and Ni were lower than those reported for the above-mentioned organic fertilisers (Villares et al., 2016 and the references therein). Additionally, since wrack may contain potentially toxic elements (PTE), it is also important to assess and

clarify this aspect in order to validate the safety of its use as a PB and/or soil amendment. The concentration of metals/trace elements in wrack were compared with the respective limit values established by the decree-law regulating the use of sewage sludge in agricultural soils (https://data.dre.pt/eli/dec-lei/276/2009/10/02/p/dre/pt/html) as well as limit values established by the decree-law regulating the use of fertilizers containing organic compounds in agriculture (https://data.dre.pt/eli/dec-lei/103/2015/06/15/p/dre/pt/html). Sewage sludge is a by-product of sewage treatment processes, and its application in land is considered excellent way of recycling both nutrients and organic matter contained in sludge. In this way, these materials are used in agriculture in order to fertilise the lands and also for degraded soils recovery. Given the risk associated with the presence of some metals, legal levels for several PTEs were established to allow an ecologically safe use of this residue as well as for fertilisers containing organic components (Singh and Agrawal, 2008; Waqas et al., 2015). Our results indicate that all the values obtained for the legislated elements are well below the stablished limit. Therefore, no concerns are expected with application of this organic residue in soils. Moreover, this comparison is particularly important since one of the aspects that this study aims to evaluate is the potential use of this residue to recover contaminated soils.

Regarding wrack's biochemical profile, special attention was given to its AOX potential, through the quantification of TAC, free radical scavenging potential, total phenols and flavonoids, total sugars and proline. Our results showed that TAC was 22.86 mg ascorbate equivalents g^{-1} dm and wrack's free radical scavenging potential was 250.51 IC_{50} mg^{-1} dm. In a study conducted by Peinado et al. (2014), that investigated the AOX activity of some brown seaweeds, including *A. nodosum, F. vesiculosus, Fucus spiralis* L. and *P. canaliculata*, it was reported *that A. nodosum* and *Fucus* spp showed a higher TAC than *P. canaliculata* and *Laminaria digitata* (Huds.) J.V.Lamour., which was in line with previous research (Wang et al., 2009). Considering the increased AOX activity reported *for A. nodosum* and *Fucus* spp and knowing that our wrack samples are mainly composed by biomass of these species, its AOX properties might be related to the presence of these seaweeds.

The AOX potential of plants and seaweeds is related to their total phenol and flavonoid contents (Peinado et al., 2014; Nunes et al., 2017). Phenolic compounds, which include, among others, flavonoids, are specialised metabolites that protect cells and their components against abiotic stress conditions (e.g. UV radiation or salinity) by acting as radical scavengers. These AOX compounds have also allelopathic activity,

serving as a defence strategy against herbivores, fungi, and bacteria (Balboa et al., 2013). Brown seaweeds are rich in phenols like phlorotannins and polyphenols such as fucol, fucophlorethol, fucodiphloroethol G, and ergosterol (Holdt and Kraan, 2011; Nunes et al., 2017). Although some authors stated that brown seaweeds did not contain flavonoids (Balboa et al., 2013), others unequivocally showed their presence and identified several flavonoids such as morin, myricetin and quercitrin in those species, with the latter being exclusive of this group of seaweeds (Yoshie-Stark et al., 2003). The total phenolic and flavonoid concentrations of wrack were 8.35 and 3.95 mg g^{-1} dm, respectively. These values are substantially higher than those of other brown seaweeds, such as *Zonaria tournefortii* (J.V.Lamouroux) (2.1 mg gallic acid equivalents g^{-1} dm and 0.156 mg quercetin equivalents g^{-1} dm), collected in Madeira island (Nunes et al., 2017), *Sargassum muticum* (Yendo) Fensholt (0.5 mg catechol equivalents g^{-1} dm) and *Saccorhiza polyschides* (Lightf.) Batt (0.2 mg catechol equivalents g^{-1} dm) (Rodrigues et al., 2015), both collected in the Central West Portuguese Coast. The total phenolic content in brown seaweeds can be highly variable, sometimes within the same species (Rodrigues et al., 2015; Nunes et al., 2017). These discrepancies arise from the influence of geography and some environmental conditions like UV radiation and herbivores predation (van Hees et al., 2017). Considering the conditions in which wrack was collected, where macroalgae were stranded on the beach, starting the decomposition process, the high levels of these AOX were quite surprising and go against those obtained for TAC and total reducing capacity.

Besides their richness in phenolic compounds, brown seaweeds are also known as a good source of other bioactive substances, including proteins and polysaccharides (Vieira et al., 2018). Regarding the protein content of these organisms, it is highly variable, corresponding to 5-15% of brown seaweeds' dm (Rodrigues et al., 2015). Additionally, the essential amino acid levels contribute to almost half of total amino acids composition of these species (Vieira et al., 2018). Being an amino acid with osmoprotective effects, Pro is also a recognised AOX, stabilising the membranes under hyperosmotic conditions, scavenging radicals, and preventing lipid peroxidation (Soares et al., 2019). For the above-described reasons, the total amino acid and Pro contents of wrack were quantified. Concerning the amino acids content, our results (0.74 ± 0.02 mg glycine equivalents g^{-1} dm) were substantially lower than the ones reported in previous studies. For instance, Lorenzo et al. (2017) reported levels of 74.88, 119.4 and 73.2 mg g^{-1} dm for *A. nodosum*, *F. vesiculosus* and *B. bifurcata*. In the same way, Astorga-España et al. (2016) reported that the amino acids content of three brown seaweeds (*Adenocystis*, *Lessonia* and *Macrocystis*) rounded 184.3 mg g^{-1} dm. Additionally, in this

work, Pro levels of wrack corresponded to approximately 26% of the total amount of amino acids. The values of Pro in wrack (198.94 µg Pro g^{-1} dm) were substantially lower than those reported by Lorenzo et al. (2017) for *A. nodosum* (3.9 mg Pro g^{-1} dm), *F. vesiculosus* (5.8 mg Pro g^{-1} dm) and *B. bifurcata* (3.2 mg Pro g^{-1} dm) collected in area of Camariñas (A Coruña, Spain). In accordance, previous research from our group, in which Pro levels of *A. nodosum* and *F. serratus* were quantified (Soares et al.,2018), showed a higher content of this amino acid in comparison with that obtained in the current study. Indeed, Pro levels of these two species, when expressed in a dm basis [assuming that water accounts for ca. 60-70% of the total fresh biomass of *A. nodosum* and *Fucus* spp (Olsson et al., 2020)], were around 3.3 mg g^{-1} dm and the wrack levels were 198.94 µg g^{-1} dm. On the other hand, our wrack samples had a higher content of Pro than that reported by Peinado et al. (2014) for *A. nodosum* (11-14 µg g^{-1} dm), *F. vesiculosus* (17-23 µg g^{-1} dm), *Fucus spiralis* L. (40-58 µg g^{-1} dm) and *P. canaliculata* (10-17 µg g^{-1} dm) collected in the west coast of Scotland. Altogether, these data suggest that Pro levels present a high variability, which might result, at least partially, from distinct geographic areas, the temperature of the water, the season of the year that correspond to different UV radiation, among others.

Polysaccharides are the major compounds of brown seaweeds, accounting for 70% of their total dm. Contrary to plants, where the main cell wall component is cellulose, in brown seaweeds, the most abundant polysaccharides present on the cell wall are alginates, fucoidans, and laminarians (Balboa et al., 2013; Afonso et al., 2019). These compounds provide seaweeds flexibility and strength, maintain the ionic equilibrium, and prevent desiccation (Balboa et al., 2013). In the present work, the sugar content of wrack was evaluated by the amount of total soluble sugars. The concentration of these compounds in wrack (8.17 µg glucose equivalents g^{-1} dm) was much lower when compared to what was reported for other brown seaweeds [when expressed in a dm basis, assuming that water accounts for ca. 70% of the total fresh biomass of brown seaweeds (Olsson et al., 2020)], such as *Sargassum dentifolium* (Turner) C.Agardh (5.13 mg g^{-1} dm), *Padina pavonia* (L.) JV Lamouroux, (2.9 mg g^{-1} dm) and *Dictyota dichotoma* (Hudson) JV Lamouroux (4.83 mg g^{-1} dm) (Emam et al., 2014). Apart from the seasonal variability in sugar content in seaweeds (Schiener et al., 2015; Afonso et al., 2019), those discrepant results might be attributed to the fact that upon the beginning of decomposition process of macroalgae, soluble sugars are one of the first molecules consumed by microorganisms (Sharma et al., 1994). In this way, the lower levels of sugars in wrack may be a result of its consumption by microorganisms.

The biochemical characterisation of wrack also included the quantification of photosynthetic pigments such as chlorophylls *a* and *c*, as well as total carotenoids. Carotenoids are effective thylakoid AOXs, located in close proximity to chlorophylls in the light-harvesting complexes, and are able to neutralize ROS during metabolic processes (Soares et al., 2019). The most abundant carotenoids on brown seaweeds are fucoxanthin and violaxanthin. Indeed, fucoxanthin is exclusive from brown seaweeds, being responsible for their coloration (Afonso et al., 2019). For both photosynthetic pigments, levels lower to the ones reported in different bibliographic reports were observed in the current study. For instance, wrack's total chlorophylls (0.88 mg g^{-1} dm) and carotenoid (384.41 µg g^{-1} dm) contents were lower than those reported in *Z. tournefortii* (chlorophylls: 2.44 mg g^{-1} dm; carotenoids: 2.98 mg g^{-1} dm) according to Nunes et al. (2017).

Upon seaweed arrival to the beach, these organisms are mostly exposed to intense light, UV radiation and desiccation (Ruiz-Delgado et al., 2014), starting to be decomposed by beach meio-, macrofauna and bacteria, resulting in the loss of nutrients and organic elements (Gómez et al., 2013). Thus, the observed lower values of some biochemical compounds (photosynthetic pigments, amino acids and sugars) in wrack, a residue composed by fragments of seaweeds, were expected. On the other hand, and surprisingly, wrack presented higher levels of phenols and flavonoids, when compared to those reported for samples from the fresh specimens. Regarding this matter, it should be not forgotten the important role of phenolic compounds in the protection of seaweeds to a variety of environmental constraints, especially in the habitat of wrack-forming species, the intertidal (Catarino et al., 2017). In this sense, and despite being in decomposition, the high basal levels of these AOX on these species might be responsible for the observed values in wrack.

Overall, by assessing wrack's elemental and biochemical profile, our data points towards the use of wrack as a source of plant nutrients and bioactive compounds, especially phenols and flavonoids. Although some AOX parameters (TAC, reducing potential and Pro) were shown to be lower than those found in extracts prepared with seaweeds, this natural residue, mainly composed of *Fucus* spp., *A. nodosum* and *P. canaliculata*, still presents significant levels of AOX metabolites, which can be an aid for improving plant growth under stressful conditions.

4.2 Assessment of wrack aqueous extract potential to improve barley seedlings development under Cu toxicity

More important than verifying the chemical composition of wrack, it is of extremely importance to understand if the presence of bioactive compounds in this residue can be traduced in an enhanced tolerance of plants to environmental constraints. Indeed, although the benefits of SWE application on plants growth and development (Nabti et al., 2017), as well as in improving plants tolerance to abiotic stresses such as drought, salinity and temperature have been reported (Silva et al., 2019), the role of SWE in mitigating metal-induced stress has not been explored. In this sense, in the current study, barley seeds were germinated on a Cu-contaminated medium (7 mg L^{-1}) supplemented with several dilutions of a wrack aqueous extract.

The role of Cu in plants is highly dependent on its concentration: while it is essential for these organisms at low concentrations, when present at high levels, it becomes phytotoxic (Rehman et al., 2019). In order to select a Cu concentration for the subsequent assays, six concentrations of this metal (0, 2.5, 4, 10, 15, and 17.5 mg L^{-1}) were tested and their effects on barley seedlings' biometry were evaluated upon 8 d. Our results showed that both root and leaves length were negatively affected by Cu. The reduced size of roots and leaves upon metal exposure reflect the phytotoxicity of this metal on barley seedlings, with roots being the most affected organ. Equivalent findings were reported in *Oryza sativa* L. exposed for 5 days to 10, 50 and 100 mM Cu (Thounaojam et al., 2012) and *Solanum nigrum* L. exposed for 28 days to 100 and 200 µmol Cu L^{-1} both studies carried out in hydroponic conditions (Fidalgo et al., 2013). Although in the case of *in vitro* assays the whole plant is in contact with the contaminant, roots are the preferential organ to absorb Cu from the media, thus explaining the higher impact of this metal in this organ. Additionally, the negative effects of Cu on roots are enhanced by the low translocation of this metal to the aerial parts of the plants (Adrees et al., 2015; Shabbir et al., 2020).

The results of this study also suggest that the supplementation of the medium with an aqueous extract of wrack, particularly in the concentrations of 5.0 and 7.5 g L^{-1}, was overall beneficial for seedlings' growth. Indeed, similar findings were reported by other researchers, who observed the stimulation of shoot growth in cabbage (Lola-Luz et al., 2013) and wheat (Kumar and Sahoo, 2011) grown under homeostasis-promoting conditions. However, and in contrast to our results, these authors detected an increase in root growth upon SWE application. Additionally, and although the sugar content of

wrack was lower than observed in previous studies, the improved barley seedlings biomass may result from the additional amount of these compounds in the growth medium, as a result of wrack supplementation. Moreover, as the photosynthetic rate of barley seedlings at this stage of growth is low, an additional sugar input in the growth medium may have helped to improve their biomass. As a matter of fact, plants under *in vitro* conditions are not fully autotrophic and the addition of sugars in the culture media in necessary not only to the maintenance of the osmotic conditions but also to stimulate shoot and root growth (Yaseen et al., 2017).

When added to a Cu-containing medium, wrack led to a better performance of leaf growth, suggesting the potential of the organic extract to counteract the negative effects of Cu on plant growth. Indeed, in the case of leaf length, wrack approximated its values to those of control. A previous study, using maize plants subjected to moderate and severe water stress for 70 days and treated with an extract prepared from *Ulva rigida* C. Agardh *and F. spiralis* showed, not only improved shoot growth, but also an increase in phenolics content (Mansori et al., 2015). Thus, the increase in the growth of barley seedlings exposed to Cu-induced stress, mostly in the aerial part of plants, is suggested to be related to the presence of wrack, possibly associated to its AOX profile and elemental composition.

Overall, the composition of wrack, ranging from its enriched content in some macro- and micro-nutrients to sugars and AOX compounds, may be behind its positive effects on the growth of barley seedlings, thus supporting the use of wrack not only as a promising biofertiliser, but also to alleviate the phytotoxic effects of Cu on the growth and development of barley and possibly other important crops.

4.3 Soil amendment with wrack – effects on soil chemical properties

Natural soils exhibit a remarkable variability as a consequence of two important factors: i) the geological nature of parent materials and, ii) the conditions (biotic and abiotic) under which they were formed. These two factors determine soil characteristics, such as its physicochemical properties, which not only directly impact the bioavailability of different contaminants, but also influence the growth and development of plants (Caetano et al., 2012). Soil organic amendments, including composts, sewage sludges, agricultural wastes and plant residues, are able to modulate soil's physicochemical characteristics, being an efficient and cost-effective approach for environmental remediation (Ferreras et al., 2006; Angelova et al., 2013; Cercioglu and analysis, 2017),

as well as for carbon storage in soils and to enhance their fertility. By increasing the OM content, these amendments can also have positive effects on other soil properties, contributing for a better water holding capacity (WHC), an improved soil aeration and aggregation, and stimulating the soil microbial community (Ferreras et al., 2006; Angelova et al., 2013; Cercioglu and analysis, 2017). Thus, prior to assessing if wrack can significantly modulate barley tolerance to Cu, it is important to understand how this residue affects soil characteristics. Having this in mind, the effects of wrack application on soil's main physicochemical properties were evaluated after a stabilisation period of 15 and 30 d.

Our results pointed out that the addition of wrack at different concentrations [0, 0.5, 1 and 2% (m/m)] after two stabilisation times (15 and 30 d) increased soil pH up to values of 6.8, an observation that has already been reported in other studies (Eyras et al., 2008; Silva et al., 2019; Ahmed et al., 2020). For instance, soil amendment with a compost resultant of a mixture of green, red and brown seaweeds [*Ulva spp*., *Codium vermilara* (Olivi) Delle Chiaje, *Dictyota dichotoma* (Hudson) and *Ceramium rubrum* C.Agardh], was found to enhance soil pH (Eyras et al., 2008). Alongside, the recent findings of Silva et al. (2019) and Ahmed et al. (2020) also confirm this pattern, suggesting that wrack-induced increase of soil pH is a common effect. Despite the observed changes in pH values, soils from all experimental situations presented a slightly acidic pH, ranging from a minimum of 5.9 to a maximum of 6.8. Knowing that the optimum pH for plant growth, where most of the nutrients are bioavailable in the pH range of 5.5 to 6.5 (Taiz et al., 2015), it can be concluded that the application of increased wrack concentrations did not negatively affect this parameter after 15d of stabilisation (where pH values range from 5.9 to 6.4). According to Lopez-Mosquera et al. (1997), the increased pH values in response to seaweed application can be related to an enhanced exchangeable Ca, which replaces H^+ and Al^{3+} from soil colloids increasing the pH (Fanun, 2014). Indeed, a common method for increasing soil pH is to lime soils with calcium carbonate, calcium oxide or calcium hydroxide, which by the mechanism before explained alleviates soil acidity increasing its pH (McCauley et al., 2009). In this way and as discussed above, Ca was detected at high levels in wrack samples, which can further explain this increase in soil pH.

Given the marine origin of wrack residues, one important parameter that should be critically evaluated is the electrical conductivity (EC) of soils, after being amended with SWE, since it provides information on the total amount of soluble salts present in the soil (soil salinity). Upon wrack treatments, the EC values assessed in soils from each

situation [0, 0.5, 1 and 2% wrack (m/m)] ranged from 0.21 to 0.61 dS m^{-1} (Table 8). Although wrack, especially at the highest concentrations [1 and 2% (m/m)] upon 30 d of stabilisation, led to an increased soil EC, the obtained values are typically from soils defined as very little saline, according to de Varennes (2003). Although Ahmed et al. (2020) reported the decrease of EC when *Ulva fasciata* Delile, *Sargassum lacerifolium* (Turner) C. Agardh and a mixture of both species was applied to a soil, rises in this parameters upon seaweed incorporation in soils are well-described in literature (Eyras et al., 2008; Possinger et al., 2016). This is much likely related to the marine origin of these residues, which accounted for an input of salts into the soil. However, this finding may not cause negative impacts in plant growth, as some authors (Nabti et al., 2016; Silva et al., 2019) suggest that slight alteration in EC values caused by macroalgae are not harmful to plants, since seaweeds contain bioactive molecules capable of inducing plants' tolerance to stresses, including those caused by excessive salt levels (Mancuso et al., 2006; Nabati et al., 2008; Rouphael et al., 2017). Moreover, as discussed above, the recorded levels of Na in wrack samples were identical to that usually found in other organic wastes frequently used for soil amendment in agricultural practises. Still, the potential risks of this practices should not be discarded as successive applications of this residue may contribute to soil and groundwater salinisation.

Finally, the levels of OM, the organic fraction of the soil that it is composed by partially decomposed dead organisms, and by humus (Marschner, 2011), were also evaluated. Here, the obtained results showed that, even when not treated, the soil used in this study has a high OM content (Bodenkunde, 1982). However, upon wrack amendment, an increase of total OM upon 15 d of stabilisation was observed. This result pairs with the findings of Ahmed et al. (2020), where a mixture of brown and green macroalgae [2% (m/m)] applied to the soil caused an increase in the OM content. Furthermore, and recalling the main goal of this study, which is increasing barley tolerance to Cu-contaminated soils, this rise in the OM is a promising result, since a high level of OM can decrease Cu bioavailability (Adrees et al., 2015; Rehman et al., 2019). Curiously, upon 30 d of stabilisation, no changes were found between treatments in what regards the OM. Bearing in mind that the increase in pH was more demarked in the 30 d stabilisation period and that the soil acidity alleviation increases microbial activity which accelerates OM decomposition (Yao et al., 2009), the lack of differences may be due to a partial mineralisation of the OM added upon wrack application.

4.4 Soil amendment with wrack – effects of Cu-induced toxicity on barley plants

4.4.1 Selection of Cu concentration

As previously commented, Cu represents nowadays a major contamination concern worldwide, not only in industrial areas, but also in regions with large agricultural fields (Rehman et al., 2019). Given its great efficiency as fungicide, high levels of Cu-containing pesticides are applied even in organic farming systems, ultimately causing Cu accumulation in soils (Adrees et al., 2015; Rehman et al., 2019). Thus, knowledge on the phytotoxic effects of Cu on crops are particularly important, especially using relevant approaches. For this purpose, barley plants were grown for 14 d in a natural agricultural soil, contaminated by Cu at different concentrations, ranging from 30 to 344 mg Cu kg^{-1}. In plant stress-related studies, the analysis of growth-related parameters is a common practice, since growth measurements are important endpoints to evaluate the toxicity of specific compounds, including contaminants such as metals (Adrees et al., 2015). Our data revealed that leaf growth was not hampered by any of the tested Cu concentrations, since no negative effects were recorded at both fresh and dry biomass. Actually, the two lowest applied doses (30 and 45 mg Cu kg^{-1}) even increased the biomass production of that organ. Although quite surprising, this augment is probably related to Cu's role as a micronutrient, where it participates in various physiological processes of plants, including photosynthesis, cellular respiration and AOX metabolism, contributing for a balanced growth (Yruela, 2009). This hypothesis makes sense when looking at the normal concentrations of Cu in soils, which usually vary between 2-50 mg Cu kg^{-1} (Rehman et al., 2019). Therefore, the lowest concentrations herein used can mimic a non-contaminated soil. In agreement with this pattern, root growth was also not negatively affected by Cu at these concentrations.

However, and contrastingly to leaves, root growth performance was severely affected by Cu, especially at high concentrations. Accordingly, root length of Cu-exposed plants was found to be inhibited right after 102 mg kg^{-1}, while root fresh and dry biomass were diminished upon exposure to 229 and 344 mg kg^{-1}. Substantial reductions of root growth are a common response in plants exposed to Cu (Adrees et al., 2015). When studying the phytotoxic effects of Cu (100 and 200 μM) on *Solanum nigrum* L. grown under a semi-hydroponic system, Fidalgo et al. (2013) reported a great decrease in root's biometric parameters. The same pattern has also been described for other species, namely rice and maize (Barbosa et al., 2013; Lin et al., 2013). Moreover, when

integrating all biometric results, it is evident that roots were more severely affected than leaves. Given that roots are the organ directly exposed to the contaminant, this response was not a surprise. Indeed, overall, results pinpoint towards the negative effect of Cu on plant growth, at concentrations above 153 mg Cu kg^{-1} for roots' length and 229 for roots' biomass while no negative consequences were found for leaves' biomass of barley plants in none of the tested concentrations of Cu on barley plants. Equivalent findings were also previously reported by Branco-Neves et al. (2017), where root growth was much more hampered than shoot's in *Solanum lycopersicum* L. exposed to 250 µM Cu. Additionally, similar observation were obtained by Xu et al., (2006) who reported that soil contamination by 300 – 500 mg Cu kg^{-1} caused a demarked higher impact in rice roots than in the straw. Thus, based on the above-described results, and knowing that root growth was more severely affected than that of leaves, the selection of the Cu concentration to be used for subsequent assays (219 mg Cu kg^{-1}) was based on root length, as it was the most sensitive parameter.

4.4.2 Selection of wrack concentration

None of the different wrack concentrations, when applied without Cu, caused significant differences in any tested parameter although the bibliography often describes an improvement of plant biomass as a result of macroalgae fertilizing features [see the review of Khan et al. (2009) and the references therein]. The discrepancy between our results and those reported in the bibliography may occur due to the fact that most of the studies describe the effects of seaweed extracts, instead of soil amendments. In fact, it is known that the mode-of-application, as well as the applied dose and exposure period, can largely change the efficiency of different plant biostimulants (Bulgari et al., 2019). Corroborating this, the obtained data for the wrack aqueous extract proved to enhance plant growth even under non-stressed conditions. Additionally, although it could have been argued that the experiment duration was not enough to allow wrack mineral decomposition, this is unlikely, since other studies, before mentioned, reporting beneficial effects of seaweeds also measured plant growth traits upon 2-3 weeks of growth. Finally, since no negative effects were observed in plants, the amendment of soil with wrack *per se* does not represent a risk to plants' growth and can be considered as a potential strategy to overcome or, at least, mitigate Cu toxicity. For the 15 d stabilisation period, a reduction in root length was observed in response to Cu; however, this was not observed over the 30 d stabilisation period. Here, it can be hypothesised that the reason behind these differences relies on the bioavailability of Cu through the stabilisation periods. In fact, the complexation of Cu with the soil OM is a well-described phenomenon

(Adrees et al., 2015; Rehman et al., 2019). Thus, upon 30 d, it seems that the adsorption of Cu to soil OM may have lowered Cu phytotoxicity, explaining the maintenance of root growth in Cu-exposed plants. Thus, from an experimental point of view, since the objective was to observe the role of wrack in Cu adsorption and in reducing its phytotoxicity, the 15 d stabilisation period was selected. In fact, after this period, when soils were amended with 2% (m/m) wrack, Cu-induced decrease in root length was less prominent, approaching to the levels found in the control plants.

4.5 Beneficial effects of wrack on Cu-induced toxicity in barley plants – growth performance, physiological status and redox homeostasis

The main goal of this study was to assess if the application of wrack to the soil could positively affect barley plants' tolerance to Cu. Thus, after all the optimisations, plants were exposed to 219 mg Cu kg^{-1} in the presence of 2% (m/m) wrack after a 15 d stabilisation period. Aiming to achieve a global approach, after the growth period, different biometric and physiological methodologies were applied in order to concretely understand the biochemical basis of the effects observed in plants after wrack and Cu co-application.

4.5.1 Biometric parameters, Cu accumulation, photosynthetic pigments and total soluble protein

As previously discussed, Cu-induced toxicity can translate into several growth disorders, impacting not only organ elongation but also biomass production [see the review of Rehman et al. (2019) and the references therein]. Indeed, several studies report a decrease in shoot and root length of several crops as a response to Cu toxicity, including monocotyledons, such as rice (Lin et al., 2013), rye grass (Verdejo et al., 2015), maize (Ali et al., 2002; Benimeli et al., 2010; Aly and Mohamed, 2012; Barbosa et al., 2013), and wheat (Gajewska et al., 2010; Gang et al., 2013). Additionally, negative effects of Cu on biomass production have been also reported in some plant species (Xu et al., 2006; Wani et al., 2007; Benimeli et al., 2010; Barbosa et al., 2013; Dresler et al., 2014). Accordingly, our results pointed towards a decrease in the length of roots and in the biomass of both organs in response to Cu. This growth inhibition caused by Cu is positively correlated with its bioaccumulation pattern in roots and leaves of barley plants. Indeed, Cu levels were greatly increased at both studied organs, especially in roots, since this organ represents the first contact point between the plant and the contaminant

(Eid et al., 2012; Galal and Shehata, 2015; Slima et al., 2020). Despite the effects of Cu *per se*, the observed decreases in growth performance of barley can also result of Cu-interference with the absorption and accumulation of other nutrients (Ke et al., 2007; Puig et al., 2007). In a previous work, Vassilev et al. (2002) reported that exposure of barley to Cu resulted in an altered uptake of different nutrients, including K and Fe.

Despite the absence of changes upon single exposure to wrack discussed before, its simultaneous application with Cu completely reverted metal-induced effects on roots' length and biomass and partially reverted those effects in leaves' biomass. The involvement of macroalgae extracts and/or residues in conferring plant tolerance to adverse conditions, by stimulating growth traits, is well-described for different species and stresses (references summarised in Table 1). According to Mancuso et al. (2006), Nabati et al. (2008) and Rouphael et al. (2017), the enhancement of plant growth induced by seaweed application was associated with a higher tolerance to salinity stress. Additionally, Zhang and Ervin (2004), Xu and Leskovar (2015) and Elansary et al. (2016) showed that increased leaf area and number, as well as enhanced vegetative growth under drought stress, were signs of increased tolerance. However, to the best of our knowledge, this is the first report exploring the potential of wrack to enhance crop tolerance to Cu. Therefore, our results are promising and suggest that wrack amendment can represent an efficient tool for reduce Cu-mediated phytotoxicity. Thus, until now, and based on our results, it can be suggested that i) wrack has triggered defence mechanisms that increased plant tolerance to Cu and/or ii) wrack was responsible for reducing Cu bioavailability. In fact, this last hypothesis is corroborated by the Cu accumulation pattern. Indeed, the total levels of Cu were much lower in plants grown under Cu and wrack co-exposure, in comparison with those only exposed to the metal. Furthermore, this difference seems to be tightly related to wrack's potential to decrease Cu uptake by roots, since a significant decrease in Cu accumulation was found in roots of plants under the co-exposure situation. In this way, it is possible that, at least in roots, wrack is reducing the accumulation of Cu, allowing a recovery of the normal growth of the plant. In fact, studies point out that seaweeds possess efficient metal biosorption capabilities (Das et al., 2017; Nasab et al., 2017) due to the existence of active functional groups on the surface of their cell walls (such as polysaccharides, proteins, amino, hydroxyl, carboxyl, and sulphate groups) that act as metal-binding sites (Kang et al., 2012). Thanks to these characteristics, seaweeds have been suggested as one of the most promising types of organic residues to be used as bio-adsorbents for various contaminants, including metals (Das et al., 2017; Nasab et al., 2017). Although the aforementioned studies discussed the removal of metals by seaweeds from polluted

water or aqueous media, a recent study carried out with a mixture of two macroalgae species, *U. fasciculata* and *S. lacerifolium*, showed that this potential can be transposed to soils (Ahmed et al., 2020). The work performed by Ahmed et al. (2020) reported a seaweed-induced decrease in the concentrations of a wide range of metals tested in the soil, including Cu. The authors suggest that mechanisms such as adsorption, electrostatic attraction, ion-exchange complexation, covalent binding, and microprecipitation by seaweed chemical groups are the ones responsible for that phenomenon. In this way, it is suggested that the decrease in the accumulation of Cu in roots' tissues of plants treated with wrack may occur due to the decrease in Cu bioavailability in soils caused by wrack, which translates into a decrease of Cu accumulation and a subsequent decrease in its phytotoxic properties.

While the macroscopic analysis is a valid indicator of Cu toxicity, an intrinsic evaluation of different biochemical endpoints related to Cu phytotoxicity is needed to clearly understand the impacts of Cu at the cellular level, as well as to unravel wrack's-mediated protection. For this, and as a first assessment of the physiological status of barley plants, the quantification of the soluble protein and photosynthetic pigments content was performed. Concerning total soluble protein levels, Cu did not alter this parameter in any of the studied organs. However, in leaves, there was a decrease of total protein levels in the wrack treatment, disagreeing with the bibliography where normally there was an increase of this parameter after exposure of plants to seaweed compounds [e.g. (Manaf, 2016; Ahmed et al., 2020)]. Regarding photosynthetic pigments (total chlorophylls and carotenoids), this biomarker demonstrated not to be a good indicator, as it was not affected by Cu, in opposition to what has been described in the literature (Mocquot et al., 1996; Yruela, 2005; Shahbaz et al., 2010; Ali et al., 2015; Feigl et al., 2015; Branco-Neves et al., 2017). Nevertheless, leaves from Cu-exposed plants showed signs of chlorosis (data not shown), suggesting the occurrence of physiological imbalances. In this way, as an attempt to really assess how the photosynthetic metabolism was affected by Cu, other approaches targeting other mechanisms/endpoints will be considered in the future, namely chlorophyll fluorescence analyses and gas exchange measurements. Indeed, it is known that Cu-induced chlorosis can be related to alterations occurring in the chloroplasts, including disintegration of thylakoids membrane and degradation of grana stacking (Sağlam et al., 2016).

4.5.2 Oxidative stress markers – LP, H_2O_2, and $O_2^{\bullet-}$

It is widely accepted that under different types of stress, including Cu exposure (see table 2), pro-oxidative conditions can take place, due to an exacerbated increase and accumulation of ROS and/or a depletion in AOX system performance (Gill and Tuteja, 2010; Sharma et al., 2012; Gupta et al., 2015). In this way, in the present study, the implications of oxidative stress in plants were assessed. As can be noticed, the exposure of barley plants to Cu-contaminated soils caused an increase of $O_2^{\bullet-}$ levels in both leaves and roots. On the other hand, H_2O_2 presented a tissue-specific response, being only increased in roots. These observations are in accordance with the existing literature, as several studies have reported an overproduction of ROS in plants under Cu stress (Li et al., 2012; Thounaojam et al., 2012; Lukatkin et al., 2014; Branco-Neves et al., 2017). In accordance with the ROS quantification results, which testify an increase in both H_2O_2 and $O_2^{\bullet-}$ in roots, Cu treatment induced an increase of LP in this organ, confirming the occurrence of severe oxidative damage. Similar results in response to Cu stress have been reported in many plant species, such as black nightshade (Fidalgo et al., 2013), cucumber (İşeri et al., 2011), rice (Thounaojam et al., 2012), chick pea (Anshula et al., 2013) , maize (Dresler et al., 2014), lupin and soybean (Sánchez-Pardo et al., 2014). However, in shoots, MDA levels remained unchanged for Cu-treated plants. Given the highest Cu levels observed in roots, the much more pronounced effect on the redox homeostasis makes sense and, once again, reinforces that roots are the major target of Cu toxicity.

In what concerns plants grown in soils amended with wrack, our results showed that, independently of Cu co-exposure, H_2O_2 levels rose in both plant organs. However, $O_2^{\bullet-}$ levels remained unchanged. Together, these observations suggest that the increase of H_2O_2 promoted by wrack may not represent the induction of oxidative stress, being much likely related to the role of this ROS as signalling molecule. Indeed, from all ROS, H_2O_2 is recognised to be involved in various signalling pathways (Mittler, 2017; Soares et al., 2019). Supporting this hypothesis, the content in $O_2^{\bullet-}$ was decreased under the co-exposure treatment, showing a recovery of the Cu-induced effects. Based on this, it can be hypothesised that wrack is inducing defence pathways capable of neutralizing $O_2^{\bullet-}$.

Although studies on the role of seaweeds in modulating the oxidative status of stressed plants are scarce, some reports suggest that the ability of seaweeds to increase plant tolerance is linked to the prevention of oxidative stress conditions. In accordance with our results, where a reduction of $O_2^{\bullet-}$ appeared upon wrack treatment, Mansori et al. (2016) reported that ROS levels were diminished when *Salvia officinalis* L. plants

were treated with *U. rigida* extract and exposed to water deficit stress. More recently Anjos Neto et al. (2020) also showed this phenomenon with spinach submitted to heat stress treated with *A. nodosum* extract.

Not only the quantified levels of $O_2^{\bullet-}$ but also the result of the evaluation of LP degree, especially in the roots, suggested a protective effect of wrack. Indeed, in contrast to the Cu treatment, roots of wrack and Cu co-exposed plants revealed a lower MDA content, thereby confirming the potential of wrack to fully recover Cu toxic effects. Here, we suggest that this reduction may have occurred due to the Cu lower bioaccumulation and/or the presence of important AOX present in wrack.

Several components of the AOX system may have contributing for reverting LP under the co-exposure treatment, such as Pro and phenolic compounds. Actually, it is known that these compounds can act as membrane stabilizers, decreasing the LP degree of stressed plants (Jain et al., 2001; Blokhina et al., 2003). Additionally, seaweeds also contain betaines that are known to stabilize membrane lipids (Rudolph et al., 1986), which may also have taken part in this result. In fact, studies point out benefit effects in exogenous application of betaine and Pro in stressed plants (Ashraf and Foolad, 2007). Moreover, the fact that the wrack treatment did not affect MDA levels in roots and decreased it in leaves, combined with the absence of negative effects on the plant biometry, supports the above-raised hypothesis that the increased H_2O_2 content in this situation is linked to signalling mechanisms and not to a possible situation of oxidative stress.

Taken together, our data point towards the occurrence of oxidative stress in response to Cu, especially in roots, being this effect partially reverted by the soil amendment with the wrack, either by lowering root Cu bioaccumulation and/or stimulating mechanisms that prevent redox imbalance.

4.5.3 Effects on the antioxidant system – enzymatic and non-enzymatic components

Throughout the evolutionary process, and to cope with oxidative stress, plants have developed a complex AOX system, comprising both non-enzymatic and enzymatic mechanisms, in order to maintain or restore cellular homeostasis under stressful conditions. In this sense, under a situation of oxidative imbalance, plants enhance the production of these antioxidants (Bhaduri and Fulekar, 2012), ensuring the equilibrium between ROS generation and removal (Gill & Tuteja, 2010). Aiming to assess if the

prevention of oxidative stress gathered by wrack was due to an upregulation of the AOX metabolism, the main metabolites and enzymes of the plant AOX system were evaluated in barley plants under Cu single and wrack co-exposure.

4.5.3.1 Non-enzymatic component

The accumulation of Pro is a common response of plants to a large range of stress factors, like soil pollution (Hayat et al., 2012). However, in our study, Pro levels decreased in response to the presence of Cu. Although it might seem surprising, this observation may explain the observed increase of LP in roots. Indeed, Pro is recognised as a potent inhibitor of LP, by allowing membrane stabilisation (Hayat et al., 2012). Additionally, and despite many studies reporting an overaccumulation of this amino acid in metal-exposed plants (Öncel et al., 2000; Gajewska and Skłodowska, 2008; Handique and Handique, 2009; Soares et al., 2016) including Cu (Tripathi and Gaur, 2004; Sharmila et al., 2017) , there are also some studies where the same response herein observed was detected for Cd, Pb and Cu (Chen et al., 2004; John et al., 2009). Additionally, and combining MDA and Pro data, a very curious hypothesis can be raised at this point: proline decreases can be due to its complexation with ·OH, arising the production of γ-aminobutyric acid (Soares et al., 2019), in order to prevent LP. Yet, while in leaves, this response appeared to be effective, with no apparent changes in LP, the opposite could have occurred in roots, where MDA levels were still higher, suggesting once again that Cu toxicity is primarily reflected in roots, with much more pronounced effects. Concerning the co-exposure treatment, although in roots Cu-mediated inhibition of Pro accumulation was not reverted, Pro levels in leaves were restored to those found in the CTL, suggesting, once again, the protective role of wrack in maintaining homeostasis within plant cells.

In contrast, wrack treatment alone induced a differential response in both organs: while in roots, proline levels declined, they did not change in the leaves of wrack-exposed plants. Accordingly, the accumulation of Pro in response to algal treatments is variable and does not always follow the same trend. For instance, while Chbani et al. (2015) reported a decrease in Pro levels in roots of *Luffa aegyptica* Mill. upon the application of an extract from *Ulva lactuca* L. in the growth medium, a result that in in line with ours, Butler and Hunter (2006) observed an increase in Pro levels leaves in response to a seaweed-based extract.

The tripeptide GSH is listed as one of the main components of the non-enzymatic AOX system (Ahmad et al., 2008; Gill and Tuteja, 2010). Furthermore, this molecule has the capacity to reduce metal toxicity in plants (Hasanuzzaman et al., 2017). In fact, and

although GSH levels did not suffer significant alteration in leaves, probably because Cu levels in this organ were much lower, there was a significant induction of the levels of this AOX in roots of Cu-treated barley plants. The increase of this AOX is well documented in literature as a common response of plants to a large range of metals, including Cu [see review of Anjum et al. (2012) and the references therein]. This observation may occur due to GSH ability to protect plants' cells from metal induced stress in three ways: i) by direct scavenging of metal-induced ROS (H_2O_2, $O_2^{\bullet-}$ and $^{\bullet}OH$); ii) by the conjugation with metals; and iii) by acting as a precursor for phytochelatins synthesis – oligomers of GSH that act as metal chelators (Hasanuzzaman et al., 2017). Although the methodology performed in this study cannot confirm or deny if the two last possible ways of action of GSH are active, the first one makes sense as GSH may be produced in order to scavenge the H_2O_2 and $O_2^{\bullet-}$ formed upon Cu treatments in roots. In addition, wrack treatment did not cause difference in GSH levels in leaves but caused a significant decrease in roots. Lastly, and once again, co-exposure treatment reverted this symptom in roots, bringing the augmented levels caused by Cu to levels of the control. Although this might seem strange, the protocol herein used only quantifies the free GSH pool, so this apparent reduction of GSH levels can be related to its higher AOX role in response to wrack. Probably, roots from plants co-exposed to Cu and wrack are investing on metal conjugation with GSH, or GSH derivates, which explains not only the decreased values of this AOX, but also the higher tolerance observed for biometric and redox parameters. Moreover, it cannot be completely excluded that, under the co-exposure, roots are translocating GSH to shoots, since its levels were increased in response to the co-treatment, but not in plants exposed to Cu alone. In fact, it is known that this molecule can be translocated from organ to organ in plants and be transported in the phloem for long distances (Zeng et al., 2017)

AsA, one of the most powerful antioxidants (Soares et al., 2019), was also quantified. Under normal physiological conditions, AsA mostly exists in its reduced state (Soares et al., 2019). However, under pro-oxidative conditions and/or as a consequence of the activity of certain enzymes, AsA can be oxidised, being converted into DHA and losing its AOX properties. Our results showed that, upon Cu treatment, roots presented an increase in total AsA, as well as in its reduced and especially in its oxidised forms. The same observation has been reported in many species upon metal-induced stress, including Cu-excess [see the review Bielen et al. (2013) and the references therein]. Furthermore, the ratio between AsA/DHA decreased in this treatment, giving us the information that DHA levels are higher than AsA. This observation shows that the plant's cells are probably under oxidative stress and/or that AsA is being used as an AOX

defence. Actually, AsA could be involved in the antioxidant defence of the plants in two possible ways: i) by directly eliminating ROS ($_1O^2$, $O_2^{\bullet-}$ and $^{\bullet}OH$) or ii) by serving as the substrate for the action of APX, in reducing H_2O_2. Since no alterations were found in the activity of APX in roots in Cu treatment (discussed later), we suggest that this metabolite is being synthesised in order to directly deal with the increase in the $O_2^{\bullet-}$ levels and $^{\bullet}OH$ in roots, since an increase LP in this organ indicates the accumulation of this ROS. However, given that $O_2^{\bullet-}$ levels remained higher and the LP in roots stays increased, in Cu treated plants, the observed changes in AsA/DHA ratio are probably related to redox imbalance. On the contrary, the observed decrease of AsA in relation to DHA in roots of plants co-treated with wrack suggest the opposite. Here, and taking into account the lower values of $O_2^{\bullet-}$ and MDA, the decrease of AsA reduced state is probably indicative of a higher AOX potential, favouring the elimination of $O_2^{\bullet-}$ excess. Concerning leaves, all treatments showed the same pattern: the maintenance of the total AsA levels, an increase in reduced AsA and a decrease in DHA. The observation of the increase in AsA in plants caused by seaweeds is well-documented (Ramya et al., 2010) as well as its increase caused by Cu [see the review Bielen et al. (2013) and the references therein].

Besides the classical AOX players, phenolics and flavonoids are also important metabolites for plant abiotic stress tolerance (Soares et al., 2019). Concerning total phenols, an increase was observed in both roots and leaves of barley plants grown in the presence of Cu. In fact, this is a common response of plants exposed to metals (Ali et al., 2006; Khatun et al., 2008), including Cu (Santiago et al., 2000; Ali et al., 2006; Vinod, 2012). This stimulation of phenolic compounds may rely on the fact that these compounds are not only capable of eliminating ROS (Rastgoo and Alemzadeh, 2011) but also have the ability to chelate metals (Lavid et al., 2001). In addition, Rice-Evans et al. (1997) suggested that phenolic compounds are able to inhibit Fenton-Reaction due to its capacity to chelate transition metal ions. This reaction results in the production $^{\bullet}OH$, the most toxic and most reactive ROS that induces LP. Apparently, the observed increase of phenolics by Cu could limit the occurrence of oxidative damage in leaves, where LP did not change, but not in roots. Indeed, as discussed earlier, roots underwent a much more prominent state of oxidative stress, probably related to the higher Cu accumulation in this organ. As a result of the co-exposure, root levels of phenols did not change in relation to the CTL but increased in leaves. This stimulation has also been reported in other studies (Krajnc et al., 2012; Chernane et al., 2015; Vasantharaja et al., 2019) and is much likely related to the high abundance of these metabolites in seaweeds, which are species rich in polyphenols (Khan et al., 2009). Additionally, by protecting roots, lowering Cu bioaccumulation, wrack may be allowing plants to invest in the

production of these compounds in leaves to protect this organ. This increase may also be related to the decrease in LP in the same treatment before discussed, since phenols have been pointed out as membrane stabilizers (Blokhina et al., 2003).

Flavonoids are a class of secondary metabolites whose AOX capacity is due their capacity to interact directly with ROS ($_1O^2$ and H_2O_2), but also due to their ability to serve as substrate for different peroxidases (Soares et al., 2019). As described for the other AOX, increases in these compounds are correlated with a higher stress tolerance [see review Di Ferdinando et al. (2012) and the references therein], including to Cu-excess (Babu et al., 2003; Ali et al., 2006). In general, Cu-specific responses were detected between roots and leaves of barley plants. While flavonoid levels were increased in roots, their levels tended to decrease in leaves. Based on this, one can hypothesise that these metabolites are being translocated from leaves to roots, the most affected organ by Cu toxicity. In fact, it is proved that this AOX is able to move from its synthesis location to other cells and tissues (Saslowsky and Winkel-Shirley, 2001; Buer and Muday, 2004; Buer et al., 2007). Additionally, while the increase of the phenol content in roots are linked with the increase of flavonoids, the same is not true for leaves, thus indicating that in this organ other phenolic compounds rather than flavonoids are being produced, showing a differential, organ-dependent, response of the phenolic metabolism. This situation also applied to wrack-containing treatments, since flavonoids did not change in any of the tested organs, either alone or in combination with Cu, probably due to the fact that flavonoids are minor constituents of the phenolic pool of brown seaweeds (Balboa et al., 2013; Nunes et al. 2017). In this way the augment of phenolic levels is probably linked to an increase of other phenolic compounds.

4.5.3.2 Enzymatic component

Besides the non-enzymatic component, the enzymes of the AOX system were also evaluated in order to obtain a better insight about the performance of the AOX system and the activity of three key enzymes of plants' AOX system were assessed: SOD, responsible for the dismutation of $O_2^{\bullet-}$ to H_2O_2 and molecular oxygen, CAT and APX, that are both related to the control of H_2O_2 levels (Soares et al., 2019). Concerning Cu treatment, it was possible to observe that no differences were found for any of the AOX enzymes, except for CAT, which suffered a significant decrease in roots. Stress-mediated inhibition of CAT activity, including by the exposure to metals, is a common feature found for several studies (Balestrasse et al., 2001; Iannelli et al., 2002; León et al., 2002; Cho and Seo, 2005; Zhang et al., 2007; Zaimoglu et al., 2011; Branco-Neves et al., 2017; Branco-Neves et al., 2017), including some reports regarding Cu toxicity

(Gallego et al., 1996; Sgherri et al., 2001; Khatun et al., 2008). According to different works, this response can be the result of CAT's auto reduction (De Vos et al., 1992; Gallego et al., 1996; Weckx and Clijsters, 1996; Mazhoudi et al., 1997; Yamamoto et al., 1997) or a consequence of Fenton reactions that may cause oxidative injury, inhibiting the activity of the AOX defence enzymes (Schutzendubel and Polle, 2002). The modulation of the plant enzymatic AOX system by Cu is well-documented in the literature [see the review of Yruela (2009) and the references there in]. However, not always a common response is found, with works reporting either increases or decreases in the activity of these important cellular players. As an example, the absence of alterations on APX activity in response to Cu was already reported (Mazhoudi et al., 1997; Branco-Neves et al., 2017). Since no alterations were found in the H_2O_2 levels in response to Cu, at least in leaves, APX activity may not have been stimulated. Here, it appears that, under Cu stress, barley plants preferentially invested on non-enzymatic mechanisms, such as GSH, AsA, phenols and flavonoids.

Concerning wrack co-exposure, in general, no major changes were found either. As in Cu-treated plants, CAT activity also decreased in roots. However, SOD activity increased in leaves of plants only exposed to wrack. In this way, the stimulation of SOD by wrack may help to understand the decreased levels $O_2^{\bullet-}$, and the increased content of H_2O_2 since these two observations combined point out the role of SOD in the dismutation of $O_2^{\bullet-}$ (Soares et al., 2019). However, in the co-exposure treatment this behaviour has not been observed. Accordingly, Fike et al. (2001) showed an increase in SOD activity in unstressed turf grasses treated with an extract of *A. nodosum*. Other study, carried by Zhang (1997) showed that tall fescue treated with an extract from same species exhibited an enhanced SOD activity of approximately 30%. Moreover, here, APX increased in the aerial organs of barley exposed to wrack, independently of Cu presence. This finding reinforces the idea that the observed rises in H_2O_2 under plants exposed to wrack probably reflect its role as a signalling agent. Indeed, differently from CAT, APX is primarily responsible for the modulation of H_2O_2 levels necessary for signalling events (Shigeoka et al., 2002; Soares et al., 2019). Yet, given the few available records on the positive effects of seaweeds on plant stress tolerance, a more evident upregulation of the studied enzymes was expected. Nevertheless, one cannot exclude that other players, especially those found in large amounts in wrack, such as sugars, can also be mediating the intracellular response against Cu, contributing for a better metal cellular homeostasis.

Overall, differential cellular responses between Cu and Cu + wrack groups of plants can be observed, suggesting the existence of distinct activated mechanisms. Altogether, the results regarding the AOX system show that the non-enzymatic component seems to the one enhanced in order to respond to oxidative stress caused by Cu. Furthermore, wrack was shown to stimulate both some enzymatic and non-enzymatic players of the AOX system, being efficient at reverting symptoms of Cu toxicity, presenting itself as a possible mitigator in what regards Cu-induced stress.

5. CONCLUDING REMARKS

5.1 Biochemical and elemental analysis of wrack

- The biochemical analysis of wrack, with focus on AOX metabolites showed the presence of phenols, flavonoids, Pro, and carotenoids, although in levels not as high as in freshly collected seaweeds, as evidenced by the lower TAC and reducing potential;
- Wrack presented a range of macro and micronutrients in its composition, being mostly rich in K, Ca, Na and Mg. Additionally, levels of trace elements are below the national limit values allowed in sewage sludge to be applied into the soils, guaranteeing the safe use of this residue.

5.2 Wrack aqueous extract potential to improve barley seedlings development under Cu toxicity

- The application of an aqueous extract of wrack showed potential to improve barley seedlings' growth and biomass production under *in vitro* conditions, either alone or in combination with Cu;

5.3 Soil amendment with wrack

5.3.1 Effects on soil chemical parameters

- The amendment of the soil with wrack [0.5, 1 and 2% (m/m)] caused an increase of soil pH, OM and EC values; however, these alterations did not negatively affect the quality of the soil for plant growth and development.
- The soil characterisation point towards the use of 2% (m/m) wrack after a 15 d stabilisation period. However, the stabilisation period of 15d was selected based on experimental reasons.

5.3.2 Beneficial effects on Cu-induced toxicity

- Cu impaired barley plants' biometry (root length, and root and leaves biomass); nevertheless, wrack co-exposure was capable to counteract these negative effects, especially in roots, which were the most impaired organ;
- Wrack co-application contributed for a decrease in Cu accumulation on plant tissues, mostly on roots, although the bioaccumulation of Cu was still recorded;

- Cu clearly induced oxidative stress in barley plants, being this effect more prominent in roots, where ROS levels and LP were increased. Wrack application was capable to recover some symptoms of oxidative stress caused by Cu toxicity. Indeed, a recovery of LP and $O_2^{\bullet-}$ levels was observed in barley plants' roots grown under the co-exposure situation;
- Upon Cu single exposure, barley plants preferably induced the non-enzymatic component of the AOX system, as metabolites like GSH, AsA, phenols and flavonoids were increased, mainly in roots; however, in response to the combined exposure, the levels of GSH, phenols and flavonoids were restored to those found in the CTL, suggesting wrack's ameliorative features;
- Altogether, the results obtained in this study seem to validate the potential of wrack to alleviate Cu-induced stress in barley plants, when applied either as a liquid extract or as soil amendment. From the soil-based assays, it was possible to unravel that wrack ameliorative action probably relies on the reduction of the oxidative stress imposed by Cu, not only by a more efficient activity of the plant AOX system, but also by limiting Cu bioaccumulation, especially in roots

6. FUTURE PERSPECTIVES

This work provided new insights regarding the utilisation of wrack as a tool to reduce the effects of soil contamination by Cu. It was possible to observe that soil amendment with this residue reverted some of the phytotoxic effects caused by the metal. Nevertheless, in the future it would be interesting to focus in:

- Study the possible role of wrack in modulating plant's mineral nutrition by analysing possible benefit impacts in soil's physical properties such as its structure and WHC, as well as in the activity of soil microbial community;
- Study the possible role of wrack in modulating plant's mineral nutrition, not only by quantifying the main macro and micronutrients, but also by exploring the regulation of the N metabolism through the evaluation of key-enzymes, such as NR and GS;
- Evaluate the response of other AOX enzymes, such as MDHAR, DHAR and GR, to clearly understand the influence of wrack on the enzymatic AOX response against Cu toxicity;
- Explore other strategies to valorise wrack in plant stress management, namely by testing other modes-of-application, such as by foliar spray or by irrigation with SWE extracts, as well as its potential to alleviate other abiotic stress factors.

7. BIBLIOGRAPHY

Abdel-Raouf N, Al-Homaidan A, Ibraheem I (2012) Agricultural importance of algae. African Journal of Biotechnology **11:** 11648-11658

Adrees M, Ali S, Rizwan M, Ibrahim M, Abbas F, Farid M, Zia-Ur-Rehman M, Irshad MK, Bharwana SA (2015) The effect of excess copper on growth and physiology of important food crops: a review. Environmental Science and Pollution Research **22:** 8148-8162

Aebi H (1984) Catalase in vitro. Methods in enzymology **105**: 121-126

Afonso NC, Catarino MD, Silva A, Cardoso S (2019) Brown macroalgae as valuable food ingredients. Antoxidants **8:** 365

Ahmad P, Sarwat M, Sharma S (2008) Reactive oxygen species, antioxidants and signaling in plants. Journal of Plant Biology **51:** 167-173

Ahmed D, Gheda SF, Ismail G, (2020) Efficacy of two seaweeds dry mass in bioremediation of heavy metal polluted soil and growth of radish (*Raphanus sativus* L.) plant. Environmental Science and Pollution Research **1:** 1-16

Aktar W, Sengupta D, Chowdhury AJ (2009) Impact of pesticides use in agriculture: their benefits and hazards. Interdisciplinary toxicology **2:** 1-12

Ali MB, Singh N, Shohael AM, Hahn EJ, Paek K (2006) Phenolics metabolism and lignin synthesis in root suspension cultures of Panax ginseng in response to copper stress. Plant Science **171:** 147-154

Ali NA, Bernal MP, Ater MJP, Soil (2002) Tolerance and bioaccumulation of copper in *Phragmites australis* and *Zea mays*. Plant and Soil **239:** 103-111

Ali S, Shahbaz M, Shahzad AN, Khan HAA, Anees M, Haider MS, Fatima AJP (2015) Impact of copper toxicity on stone-head cabbage (*Brassica oleracea* var. capitata) in hydroponics. PeerJ **3:** e1119

Alobwede E, Leake JR, Pandhal J (2019) Circular economy fertilization: Testing micro and macro algal species as soil improvers and nutrient sources for crop production in greenhouse and field conditions. Geoderma **334:** 113-123

Aly AA, Mohamed A (2012) The impact of copper ion on growth, thiol compounds and lipid peroxidation in two maize cultivars (*Zea mays* L.) grown'in vitro'. Australian Journal of Crop Science **6:** 541

Angelova V, Akova V, Artinova N, Ivanov K (2013) The effect of organic amendments on soil chemical characteristics. Bulgarian Journal of Agricultural Science **19:** 958-971

Anisimov M, Chaikina E, Klykov A, Rasskazov V (2013) Effect of seaweeds extracts on the growth of seedling roots of buckwheat (*Fagopyrum esculentum* Moench) is depended on the season of algae collection. Agriculture Science Developments **2:** 67-75

Anjos Neto APd, Oliveira GRF, Mello SdC, Silva MSd, Gomes-Junior FG, Novembre ADdLC, Azevedo RA (2020) Seed priming with seaweed extract mitigate heat stress in spinach: effect on germination, seedling growth and antioxidant capacity. Bragantia

Anjum NA, Ahmad I, Mohmood I, Pacheco M, Duarte AC, Pereira E, Umar S, Ahmad A, Khan NA, Iqbal MJE (2012) Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids—a review. Journal of Experimental Botany **75:** 307-324

Ansell A, Gibson R, Barnes M (1998) Ecological impact of green macroalgal blooms. Oceanography and Marine Biology: an annual review **36:** 97-125

Anshula S, Gurpreet S (2013) Studies on the effect of Cu (II) ions on the antioxidant enzymes in chickpea (*Cicer arietinum* L.) cultivars. Journal of Stress Physiology and Biochemistry **9**

Arioli T, Mattner SW, Winberg PC (2015) Applications of seaweed extracts in Australian agriculture: past, present and future. Jorurnal of Applied Phycology **27:** 2007-2015

Arora A, Byrem TM, Nair MG, Strasburg GM (2000) Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. Archives of Biochemistry and Biophysics **373:** 102-109

Arora A, Byrem TM, Nair MG, Strasburg G (2000) Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. Archives of Biochemistry and Biophysics **373:** 102-109

Ashraf M, and Foolad M (2007). Roles of glycine betaine and proline in improving plant abiotic stress resistance. Environmental and Experimental Botany, **59:** 206-216

Astorga-España M, Rodríguez-Galdón B, Rodríguez-Rodríguez E, Díaz-Romero C (2016). Amino acid content in seaweeds from the Magellan Straits (Chile). Journal of Food Composition and Analysis **53:** 77-84.

Azooz MM, Abou-Elhamd MF, Al-Fredan MA (2012) Biphasic effect of copper on growth, proline, lipid peroxidation and antioxidant enzyme activities of wheat (*Triticum aestivum* cv. Hasaawi) at early growing stage. Australian Journal of Crop Science **6:** 688

Babu TS, Akhtar TA, Lampi MA, Tripuranthakam S, Dixon DG, Greenberg B (2003) Similar stress responses are elicited by copper and ultraviolet radiation in the aquatic plant *Lemna gibba*: implication of reactive oxygen species as common signals. Plant and Cell Phisiology **44:** 1320-1329

Balboa EM, Conde E, Moure A, Falqué E, Domínguez H (2013) In vitro antioxidant properties of crude extracts and compounds from brown algae. Food Chemistry **138:** 1764-1785

Balestrasse KB, Gardey L, Gallego SM, Tomaro M (2001) Response of antioxidant defence system in soybean nodules and roots subjected to cadmium stress. Functional Plant Biology **28:** 497-504

Barbosa RH, Tabaldi LA, Miyazaki FR, Pilecco M, Kassab SO, Bigaton D (2013) Foliar copper uptake by maize plants: effects on growth and yield. Jornal de Ciência Rural **43:** 1561-1568

Bates LS, Waldren RP, Teare I (1973) Rapid determination of free proline for water-stress studies. Plant and Soil **39:** 205-207

Battacharyya D, Babgohari MZ, Rathor P, Prithiviraj B (2015) Seaweed extracts as biostimulants in horticulture. Scientia Horticulturae **196:** 39-48

Beck R, Kalra Y, Vaughan B, Wolf A (2000) Soil analysis handbook of reference methods. Plant and Soil Analysis Council, 4th edition. CRC Press LLC, New York, USA**:** 109-115

Benimeli CS, Medina A, Navarro CM, Medina RB, Amoroso MJ, Gómez M (2010) Bioaccumulation of copper by *Zea mays*: impact on root, shoot and leaf growth. Water, Air and Soil Pollution **210:** 365-370

Bhaduri AM, Fulekar M (2012) Assessment of arbuscular mycorrhizal fungi on the phytoremediation potential of Ipomoea aquatica on cadmium uptake. Biothec **2:** 193-198

Bielen A, Remans T, Vangronsveld J, Cuypers A (2013) The influence of metal stress on the availability and redox state of ascorbate, and possible interference with its cellular functions. Journal of Molecular Sciences **14:** 6382-6413

Blokhina O, Virolainen E, Fagerstedt K (2003) Antioxidants, oxidative damage and oxygen deprivation stress: a review. Annals of Botany **91:** 179-194

Bodenkunde AG (1982). Bodenkundliche Kartieranleitung. Aufl., *Hannover*.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical biochemistry **72:** 248-254

Branco-Neves S, Soares C, de Sousa A, Martins V, Azenha M, Gerós H, Fidalgo F (2017) An efficient antioxidant system and heavy metal exclusion from leaves make *Solanum cheesmaniae* more tolerant to Cu than its cultivated counterpart. Food and Energy Security **6:** 123-133

Buer CS, Muday GK, Djordjevic MA (2007) Flavonoids are differentially taken up and transported long distances in *Arabidopsis*. Journal of Plant Physiology **145:** 478-490

Buer CS, Muday G (2004) The transparent testa4 mutation prevents flavonoid synthesis and alters auxin transport and the response of *Arabidopsis* roots to gravity and light. The Plant Cell **16:** 1191-1205

Bulgari R, Franzoni G, Ferrante A (2019) Biostimulants application in horticultural crops under abiotic stress conditions. Agronomy **9:** 306

Butler T, Hunter A (2006) Impact of seaweed extract on turfgrass growth and nutrition on a Golf green to USGA specification. XXVII International Horticultural Congress-IHC2006: International Symposium on Horticultural Plants in Urban and Peri-Urban **762**: 81-90

Caetano A, Gonçalves F, Sousa J, Cachada A, Pereira E, Duarte A, da Silva EF, Pereira R (2012) Characterization and validation of a Portuguese natural reference soil to be used as substrate for ecotoxicological purposes. Journal of Enviornmental Monitoring **14:** 925-936

Catarino M, Silva A, Cardoso S (2017). Fucaceae: A source of bioactive phlorotannins. International journal of molecular sciences **18:** 1327.

Cercioglu MJC (2017) The role of organic soil amendments on soil physical properties and yield of maize (*Zea mays* L.). Communications in Soil Science and plant Analysis **48:** 683-691

Chbani A, Majed S, Mawlawi H, Kammoun M (2015) The use of seaweed as a bio-fertilizer: Does it influence Proline and Chlorophyll concentration in plants treated? Arabian Journal of Medicinal Aromatic Plants **1:** 67-77

Chen C-T, Chen T-H, Lo K-F, Chiu C (2004) Effects of proline on copper transport in rice seedlings under excess copper stress. Plant Science **166:** 103-111

Chernane H, Latique S, Mansori M, El Kaoua M (2015) Salt stress tolerance and antioxidative mechanisms in wheat plants (*Triticum durum* L.) by seaweed extracts application. IOSR Journal of Agriculture and Veterinary Science **8:** 36-44

Chiaiese P, Corrado G, Colla G, Kyriacou MC, Rouphael Y (2018) Renewable sources of plant biostimulation: microalgae as a sustainable means to improve crop performance. Frontiers in Plant Science **9:** 1782

Cho U-H, Seo N (2005) Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. Plant Science **168:** 113-120

Colombini I, Chelazzi L, Gibson R, Atkinson R (2003) Influence of marine allochthonous input on sandy beach communities. Oceanography and Marine Biology: An Annual Review **41:** 115-159

Das C, Naseera K, Ram A, Meena RM, Ramaiah NJ (2017) Bioremediation of tannery wastewater by a salt-tolerant strain of *Chlorella vulgaris*. Journal of Applied Phycology **29:** 235-243

de Sousa A, Teixeira J, Regueiras MT, Azenha M, Silva F, Fidalgo F (2013) Metalaxyl-induced changes in the antioxidant metabolism of *Solanum nigrum* L. suspension cells. Pesticide Biochemistry and Physiology **107:** 235-243

de Varennes A (2003) Produtividade dos solos e ambiente. Escolar Editora

De Vos CR, Vonk MJ, Vooijs R, Schat H (1992) Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene cucubalus*. Journal of Plant Physiology **98:** 853-858

Demidchik V (2015) Mechanisms of oxidative stress in plants: from classical chemistry to cell biology. Environmental and Experimental Botany **109:** 212-228

Dewis J, Freitas F (1970) Physical and chemical methods of soil and water analysis. FAO Soils Bulletin

Di Ferdinando M, Brunetti C, Fini A, Tattini M (2012) Flavonoids as antioxidants in plants under abiotic stresses. Abiotic stress responses in plants 159-179

Donahue JL, Okpodu CM, Cramer CL, Grabau EA, Alscher RG (1997) Responses of antioxidants to paraquat in pea leaves (relationships to resistance). Plant physiology **113:** 249-257

Dresler S, Hanaka A, Bednarek W, Maksymiec W (2014) Accumulation of low-molecular-weight organic acids in roots and leaf segments of *Zea mays* plants treated with cadmium and copper. Acta Physiologiae Plantarum **36:** 1565-1575

Duong HLS (2008) Investigating the ecological implications of wrack removal on South Australian sandy beaches. Flinders University, School of Biological Sciences.

Eid EM, Shaltout KH, El-Sheikh MA, Asaeda T (2012) Seasonal courses of nutrients and heavy metals in water, sediment and above-and below-ground Typha domingensis biomass in Lake Burullus (Egypt): perspectives for phytoremediation. Flora-Morphology, Distribution, Functional Ecology of Plants **207:** 783-794

Elansary HO, Skalicka-Woźniak K, King IW (2016) Enhancing stress growth traits as well as phytochemical and antioxidant contents of *Spiraea* and *Pittosporum* under seaweed extract treatments. Plant Physiology and Biochemistry **105:** 310-320

Emam M, Mansour H, Shaaban A, Mostafa N (2014). Biochemical Constituents and Antioxidant Capacity of Some Seaweeds from Red and Mediterranean Coasts of Egypt. Egyptian Journal of Botany **54:** 333-346.

European Union (2019). Regulation of the european parliament and of the council laying down rules on the making available on the market of EU fertilising products and amending Regulations (EC) No 1069/2009 and (EC) No 1107/2009 and repealing Regulation (EC) No 2003/2003. https://eur-lex.europa.eu/legalcontent/ EN/TXT/?uri=OJ:L:2019:170:TOC.

European Commission (2019) Circular Economy Action Plan of the European Comission. Volume 2019.

Eyras MC, Defosse G, Dellatorre FJ (2008) Seaweed compost as an amendment for horticultural soils in Patagonia, Argentina. Compost Science and Utilisation **16:** 119-124

Fan D, Hodges DM, Critchley AT, Prithiviraj B (2013) A commercial extract of brown macroalga (*Ascophyllum nodosum*) affects yield and the nutritional quality of spinach in vitro. Communications in Soil Science and Plant Analysis **44:** 1873-1884

FAOSTAT (2017) Food and Agriculture Organization of the United Nations.

Fanun M (2014) The role of colloidal systems in environmental protection. Elsvier

Feigl G, Kumar D, Lehotai N, Pető A, Molnár Á, Rácz É, Ördög A, Erdei L, Kolbert Z, Laskay G (2015) Comparing the effects of excess copper in the leaves of *Brassica juncea* (L. Czern) and *Brassica napus* (L.) seedlings: growth inhibition, oxidative stress and photosynthetic damage. Acta Biologica Hungarica **66:** 205-221

Ferreras L, Gómez E, Toresani S, Firpo I, Rotondo R (2006) Effect of organic amendments on some physical, chemical and biological properties in a horticultural soil. Bioresource technology **97:** 635-640

Fidalgo F, Azenha M, Silva AF, de Sousa A, Santiago A, Ferraz P, Teixeira J (2013) Copper-induced stress in *Solanum nigrum* L. and antioxidant defense system responses. Food and Energy Security **2:** 70-80

Fike J, Allen V, Schmidt R, Zhang X, Fontenot J, Bagley C, Ivy R, Evans R, Coelho R, Wester D (2001) Tasco-Forage: I. Influence of a seaweed extract on antioxidant activity in tall fescue and in ruminants. Journal of Animal Science **79:** 1011-1021

Foyer CH (2018) Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. Enviornmental and Experimental Botany **154:** 134-142

Franzén D, Infantes E, Gröndahl F (2019) Beach-cast as biofertiliser in the Baltic Sea region-potential limitations due to cadmium-content. Ocean and Coastal management **169:** 20-26

Gajewska E, Skłodowska M (2007) Effect of nickel on ROS content and antioxidative enzyme activities in wheat leaves. Biometals **20:** 27-36

Gajewska E, SkŁodowska M (2010) Differential effect of equal copper, cadmium and nickel concentration on biochemical reactions in wheat seedlings. Ecotoxicology and Environmental Safety **73:** 996-1003

Gajewska E, Skłodowska M (2008) Differential biochemical responses of wheat shoots and roots to nickel stress: antioxidative reactions and proline accumulation. Plant Growth Regulation **54:** 179-188

Galal TM, Shehata H (2015) Bioaccumulation and translocation of heavy metals by *Plantago major* L. grown in contaminated soils under the effect of traffic pollution. Ecological Indicators **48:** 244-251

Gallego SM, Benavides MP, Tomaro M (1996) Effect of heavy metal ion excess on sunflower leaves: evidence for involvement of oxidative stress. Plant Science **121:** 151-159

Gang A, Vyas A, Vyas H (2013) Toxic effect of heavy metals on germination and seedling growth of wheat. Journal of Environmental Research and Development **8:** 206-213

Gill SS, Tuteja N (2010) Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiology and Biochemistry **48:** 909-930

Gillespie KM, Ainsworth EA (2007) Measurement of reduced, oxidized and total ascorbate content in plants. Nature Protocols **2:** 871

Gómez M, Barreiro F, López J, Lastra M, de la Huz R (2013). Deposition patterns of algal wrack species on estuarine beaches. Aquatic botany **105:** 25-33

Gupta KJ, Igamberdiev AU, Manjunatha G, Segu S, Moran JF, Neelawarne B, Bauwe H, Kaiser W (2011) The emerging roles of nitric oxide (NO) in plant mitochondria. Plant Science **181:** 520-526

Handique G, Handique A (2009) Proline accumulation in lemongrass (*Cymbopogon flexuosus* Stapf.) due to heavy metal stress. Journal of Environmental Biology **30:** 299-302

Hasanuzzaman M, Nahar K, Anee TI, Fujita M (2017) Glutathione in plants: biosynthesis and physiological role in environmental stress tolerance. Physiology and Molecular Biology of Plants **23:** 249-268

Hayat S, Hayat Q, Alyemeni MN, Wani AS, Pichtel J, Ahmad A (2012) Role of proline under changing environments: a review. Plant Science and Behavior **7:** 1456-1466

Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplasts: I. Kinetics and stoichiometry of fatty acid peroxidation. Archives of Biochemistry and Biophysics **125:** 189-198

Hertwig B, Streb P, Feierabend J (1992) Light dependence of catalase synthesis and degradation in leaves and the influence of interfering stress conditions. Plant Physiology **100:** 1547-1553

Holdt SL, Kraan S (2011) Bioactive compounds in seaweed: functional food applications and legislation. Journal of Applied Phycology **23:** 543-597

Hristozkova M, Geneva M, Stancheva I (2006) Response of pea plants (*Pisum sativum* L.) to reduced supply with molybdenum and copper. International Journal of Agriculture and Biology **8:** 218-220

Iannelli MA, Pietrini F, Fiore L, Petrilli L, Massacci A (2002) Antioxidant response to cadmium in *Phragmites australis* plants. Plant Physiology and Biochemistry **40:** 977-982

Irigoyen J, Einerich D, Sánchez-Díaz M (1992) Water stress induced changes in concentrations of proline and total soluble sugars in nodulated alfalfa (*Medicago sativa*) plants. Physiologia plantarum **84:** 55-60

İşeri ÖD, Körpe DA, Yurtcu E, Sahin FI, Haberal M (2011) Copper-induced oxidative damage, antioxidant response and genotoxicity in *Lycopersicum esculentum* Mill. and *Cucumis sativus* L. Plant Cell Reports **30:** 1713

ISO (2008) Soil quality: effects of pollutants on earthworms (*Eisenia fetida*). Part 2. Determination of effects on reproduction. Annex C. ISO/Dis 11268-2.2. International Organization for Standardization, Geneva, Switzerland.

Jain M, Mathur G, Koul S, Sarin N (2001) Ameliorative effects of proline on salt stress-induced lipid peroxidation in cell lines of groundnut (*Arachis hypogaea* L.). Plant Cell Reports **20:** 463-468

John R, Ahmad P, Gadgil K, Sharma S (2009) Heavy metal toxicity: Effect on plant growth, biochemical parameters and metal accumulation by *Brassica juncea* L.. International Journal of plant Science **3**: 66-75

Jonak C, Nakagami H, Hirt H (2004) Heavy metal stress. Activation of distinct mitogen-activated protein kinase pathways by copper and cadmium. Plant Physiology **136:** 3276-3283

Kang OL, Ramli N, Ahmad M (2012) Cadmium (II) Biosorption onto Seaweed (*Kappaphycus alvarezii* and *Eucheuma ddenticulatum*) waste Biomass: Equilibrium and Mechanism Studies–East. Middle-East Journal of Scientific Research **11:** 867-872

Katerji N, Van Hoorn J, Hamdy A, Mastrorilli M, Fares C, Ceccarelli S, Grando S, Oweis T (2006) Classification and salt tolerance analysis of barley varieties. Agricultural Water Management **85:** 184-192

Ke W, Xiong Z-T, Chen S, Chen J (2007) Effects of copper and mineral nutrition on growth, copper accumulation and mineral element uptake in two *Rumex japonicus* populations from a copper mine and an uncontaminated field sites. Environmental and Experimental Botany **59:** 59-67

Khan A, Khan S, Khan MA, Qamar Z, Waqas M (2015) The uptake and bioaccumulation of heavy metals by food plants, their effects on plants nutrients, and associated health risk: a review. Environmental Science and Pollution Research **22:** 13772-13799

Khan AS, Ahmad B, Jaskani MJ, Ahmad R, Malik AU (2012) Foliar application of mixture of amino acids and seaweed (*Ascophylum nodosum*) extract improve growth and

physicochemical properties of grapes. International Journal of Agriculture and Biology **14:** 383-388

Khan W, Rayirath UP, Subramanian S, Jithesh MN, Rayorath P, Hodges DM, Critchley AT, Craigie JS, Norrie J, Prithiviraj B (2009) Seaweed extracts as biostimulants of plant growth and development. Journal of Plant Growth Regulation **28:** 386-399

Khatun S, Ali MB, Hahn E-J, Paek K (2008) Copper toxicity in Withania somnifera: Growth and antioxidant enzymes responses of in vitro grown plants. Enviornmental and Experimental Botany **64:** 279-285

Krajnc AU, Ivanus A, Kristl J, Susek A (2012) Seaweed extract elicits the metabolic responses in leaves and enhances growth of *Pelargonium cuttings.* European Journal of Horticulrural Science **77:** 170-181

Kumar G, Sahoo D (2011) Effect of seaweed liquid extract on growth and yield of *Triticum aestivum* var. Pusa Gold. Journal of Applied Phycology **23:** 251-255

Kumar M, Kumari P, Reddy C, Jha B (2014) Salinity and desiccation induced oxidative stress acclimation in seaweeds. Advances in Botanical Research **71:** 91-123

Kumar S, Kumar S, Prakash P, Singh M (2014) Antioxidant defense mechanisms in chickpea (*Cicer arietinum* L.) under copper and arsenic toxicity. International Journal of Plant Physiology and Biochemistry **6:** 40-43

Kumar S, Sahoo D, Levine I (2015) Assessment of nutritional value in a brown seaweed *Sargassum wightii* and their seasonal variations. Algal Research **9:** 117-125

La Torre A, Iovino V, Caradonia F(2018) Copper in plant protection: current situation and prospects. Phytopathologia Mediterranea **57:** 201-236

La Torre A, Righi L, Iovino V, Battaglia V (2019) Control of late blight in organic farming with low copper dosages or natural products as alternatives to copper. European Journal of Plant Pathology **155:** 769-778

Lavid N, Schwartz A, Yarden O (2001) The involvement of polyphenols and peroxidase activities in heavy-metal accumulation by epidermal glands of the waterlily (Nymphaeaceae). Planta **212:** 323-331

Lee YP, Takahashi T (1966) An improved colorimetric determination of amino acids with the use of ninhydrin. Analytical Biochemistry **14:** 71-77

León AM, Palma JM, Corpas FJ, Gómez M, Romero-Puertas MC, Chatterjee D, Mateos RM, Luis A, Sandalio L (2002) Antioxidative enzymes in cultivars of pepper plants with different sensitivity to cadmium. Plant Physiology and Biochemistry **40:** 813-820

Li D, Wu S, Liu L, Zhang Y, Li S (2018) Vulnerability of the global terrestrial ecosystems to climate change. Global Change Biology **24:** 4095-4106

Li X, Ma H, Jia P, Wang J, Jia L, Zhang T, Yang Y, Chen H, Wei X2012) Responses of seedling growth and antioxidant activity to excess iron and copper in *Triticum aestivum* L. Ecotoxicology and Enviornmental Safety **86:** 47-53

Lichtenthaler HK (1987) Chlorophylls and carotenoids: pigments of photosynthetic biomembranes. Methods in Enzymology **148:** 350-382

Lin C-Y, Trinh NN, Fu S-F, Hsiung Y-C, Chia L-C, Lin C-W, Huang H 2013) Comparison of early transcriptome responses to copper and cadmium in rice roots. Plant Molecular Biology **81:** 507-522

Liu JJ, Wei Z, Li JH (2014) Effects of copper on leaf membrane structure and root activity of maize seedling. Botanical Studies **55:** 47

Lola-Luz T, Hennequart F, Gaffney MJA, (2013) Enhancement of phenolic and flavonoid compounds in cabbage (*Brassica oleraceae*) following application of commercial seaweed extracts of the brown seaweed,(*Ascophyllum nodosum*). Agricultural and Food Science **22:** 288-295

Lopez-Mosquera M, Pazos P (1997) Effects of seaweed on potato yields and soil chemistry. Biological Agricultural and Horticulture **14:** 199-205

Lorenzo JM, Agregán R, Munekata PE, Franco D, Carballo J, Şahin S, Lacomba R, Barba F (2017) Proximate composition and nutritional value of three macroalgae: *Ascophyllum nodosum*, *Fucus vesiculosus* and *Bifurcaria bifurcata*. Marine Drugs **15:** 360

Lukatkin A, Egorova I, Michailova I, Malec P, Strzałka K (2014) Effect of copper on pro-and antioxidative reactions in radish (*Raphanus sativu*s L.) in vitro and in vivo. Journal of Trace Elements in Medicine and Biology **28:** 80-86

Macreadie PI, Bishop MJ, Booth DJ (2011) Implications of climate change for macrophytic rafts and their hitchhikers. Marine Ecology Progress Series **443:** 285-292

Macreadie PI, Trevathan-Tackett SM, Baldock JA, Kelleway JJ (2017) Converting beach-cast seagrass wrack into biochar: A climate-friendly solution to a coastal problem. Science of the Total Tnvironment **574:** 90-94

Manaf H (2016) Beneficial effects of exogenous selenium, glycine betaine and seaweed extract on salt stressed cowpea plant. Annals of Agricultural Science **61:** 41-48

Mancuso S, Briand X, Mugnai S, Azzarello E (2006) Marine Bioactive Substances (IPA Extract) Improve Foliar Ion Uptake and Water Stress Tolerance in Potted *Vitis vinifera* Plants. Advances in Horticultural Science **20:** 1000-1006

Mansori M, Chernane H, Latique S, Benaliat A, Hsissou D, El Kaoua M (2015) Seaweed extract effect on water deficit and antioxidative mechanisms in bean plants (*Phaseolus vulgaris* L.) Journal of Applied Phycology **27:** 1689-1698

Marschner H (2011) Marschner's mineral nutrition of higher plants. Academic press

Martins MJ, Soares C, Pereira R, Rubal M, Fidalgo F (2020) Sargaço–Importância ecológica, riscos e potenciais aplicações. Revista Captar: Ciência e Ambiente para Todos

Martins VMV (2014) Copper impacts in grapevine (*Vitis vinifera* L.): molecular, biochemical and biotechnological approaches. PhD thesis. University of Minho, Braga, Portugal

Mazhoudi S, Chaoui A, Ghorbal MH, El Ferjani E (1997) Response of antioxidant enzymes to excess copper in tomato *(Lycopersicon esculentum* Mill.) Plant Science **127:** 129-137

McCauley A, Jones C and Jacobsen J (2009). Soil pH and organic matter. Nutrient Management Module, **8:** 1-12.

Mei L, Daud M, Ullah N, Ali S, Khan M, Malik Z, Zhu S (2015) Pretreatment with salicylic acid and ascorbic acid significantly mitigate oxidative stress induced by copper in cotton genotypes. Environmental Science and Pollution Research **22:** 9922-9931

Meng Q, Zou J, Zou J, Jiang W, Liu D (2007) Effect of Cu^{2+} concentration on growth, antioxidant enzyme activity and malondialdehyde content in garlic (*Allium sativum* L.). Acta Biologica Cracoviencsia. Series Botanica **49:** 95-101

Merrington G (2018). The good, the bad and the ugly: Copper and arsenic in soils. Soil Health: The Foundation of Sustainable Agriculture; Wollongbar Agricultural Institute: Wollongbar, Australia.

Michalak A (2006) Phenolic compounds and their antioxidant activity in plants growing under heavy metal stress. Polish Journal of Enviornmental Studies **15**

Mittler R (2017) ROS are good. Trends in Plant Science **22:** 11-19

Mocquot B, Vangronsveld J, Clijsters H, Mench M (1996) Copper toxicity in young maize (Zea mays L.) plants: effects on growth, mineral and chlorophyll contents, and enzyme activities. Plant and Soil **182:** 287-300

Moore SK, Trainer VL, Mantua NJ, Parker MS, Laws EA, Backer LC, Fleming LE (2008) Impacts of climate variability and future climate change on harmful algal blooms and human health. Environmental Health **7 Suppl 2:** S4

Mukherjee A, Patel J (2019) Seaweed extract: biostimulator of plant defense and plant productivity. International Journal of Environmental Science and Technology**:** 1-6

Munshaw G, Ervin E, Shang C, Askew S, Zhang X, Lemus R (2006) Influence of late-season iron, nitrogen, and seaweed extract on fall color retention and cold tolerance of four bermudagrass cultivars. Crop science **46:** 273-283

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologua plantarum **15:** 473-497

Nabati D, Schmidt E, Khaleghi E, Parrish D (2008) The growth of Kentucky bluegrass (*Poa pratensis* cv. Plush) as affected by plant growth regulators and Iron (Fe), grown under limited soil moisture Regimes. Asian Journal of Plant Sciences **7:**1119-1134

Nabti E, Jha B, Hartmann A (2016) Impact of seaweeds on agricultural crop production as biofertilizer. International Journal of Environmental Science and Technology **14:** 1119-1134

Nair P, Kandasamy S, Zhang J, Ji X, Kirby C, Benkel B, Hodges MD, Critchley AT, Hiltz D, Prithiviraj B (2012) Transcriptional and metabolomic analysis of *Ascophyllum nodosum* mediated freezing tolerance in *Arabidopsis thaliana*. BMC genomics **13:** 643

Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. Plant Cell Physiology **22:** 867-880

Nasab SMH, Naji A, Yousefzadi M (2017) Kinetic and equilibrium studies on biosorption of cadmium (II) from aqueous solution by *Gracilaria corticata* and agar extraction algal waste. Journal of applied Phycology **29:** 2107-2116

Nunes N, Ferraz S, Valente S, Barreto MC, De Carvalho M (2017) Biochemical composition, nutritional value, and antioxidant properties of seven seaweed species from the Madeira Archipelago. Journal of applied Phycology **29:** 2427-2437

O'sullivan A, O'Callaghan Y, O'grady M, Queguineur B, Hanniffy D, Troy D, Kerry J, O'Brien N (2011) In vitro and cellular antioxidant activities of seaweed extracts prepared from five brown seaweeds harvested in spring from the west coast of Ireland. Food Chemistry **126:** 1064-1070

Olsson J, Toth GB, Albers E (2020) Biochemical composition of red, green and brown seaweeds on the Swedish west coast. Journal of Applied Phycology **32:**3305-3317

Olteanu Z, Truta E, Oprica L, Zamfirache MM, Rosu CM, Vochita G (2013) Copper-induced changes in antioxidative response and soluble protein level in *Triticum aestivum* cv. beti seedlings. Romanian Agricultural Research **30:** 2012-2190

Öncel I, Keleş Y, Üstün A (2000) Interactive effects of temperature and heavy metal stress on the growth and some biochemical compounds in wheat seedlings. Enviornmental Pollution **107:** 315-320

Peinado I, Girón J, Koutsidis G, Ames J (2014) Chemical composition, antioxidant activity and sensory evaluation of five different species of brown edible seaweeds. Food Research Internaional **66:** 36-44

Posmyk M, Kontek R, Janas K (2009) Antioxidant enzymes activity and phenolic compounds content in red cabbage seedlings exposed to copper stress. Ecotoxicology and Environmental Safety **72:** 596-602

Possinger AR, Amador J (2016) Preliminary evaluation of seaweed application effects on soil quality and yield of sweet corn (*Zea may*s L.) Comunications in Soil Science and Plant Analysis **47:** 121-135

Puig S, Andrés N, García A, Penarrubia L (2007) Copper and iron homeostasis in Arabidopsis: responses to metal deficiencies, interactions and biotechnological applications. Plant Cell and Enviornment **30:** 271-290

Quan LJ, Zhang B, Shi WW, Li H (2008) Hydrogen peroxide in plants: a versatile molecule of the reactive oxygen species network. Journal of Integrative Plant Biology **50:** 2-18

Raghunandan B, Vyas R, Patel H, Jhala Y (2019) Perspectives of seaweed as organic fertilizer in agriculture. Soil Fertility Management for Sustainable Development. 267-289

Ramya SS, Nagaraj S, Vijayanand N (2010) Biofertilizing Efficiency of Brown and Green Algae on Growth, Biochemical and Yield Parameters of *Cyamopsis tetragonolaba* (L.) Taub. Recent Research in Science and Technology. **5:** 45-52

Rastgoo L, Alemzadeh A (2011) Biochemical responses of Gouan (*Aeluropus littoralis*) to heavy metals stress. Australian Journal of Plant Science **5:** 375

Rayirath P, Benkel B, Mark Hodges D, Allan-Wojtas P, Mackinnon S, Critchley AT, Prithiviraj B (2009) Lipophilic components of the brown seaweed, Ascophyllum nodosum, enhance freezing tolerance in *Arabidopsis thaliana*. Planta **230:** 135-147

Raza A, Razzaq A, Mehmood SS, Zou X, Zhang X, Lv Y, Xu J (2019) Impact of climate change on crops adaptation and strategies to tackle its outcome: A review. Plants **8:** 34

Rehman M, Liu L, Wang Q, Saleem MH, Bashir S, Ullah S, Peng D (2019) Copper environmental toxicology, recent advances, and future outlook: a review. Environmental Science and Pollution Research **26:** 18003-18016

Rice-Evans C, Miller N, Paganga G (1997) Antioxidant properties of phenolic compounds. Trends in Plant Science **2:** 152-159

Ritchie RJP (2008) Universal chlorophyll equations for estimating chlorophylls a, b, c, and d and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents. Photosynthetica **46:** 115-126

Rodrigues D, Freitas AC, Pereira L, Rocha-Santos TA, Vasconcelos MW, Roriz M, Rodríguez-Alcalá LM, Gomes AM, Duarte A (2015) Chemical composition of red, brown and green macroalgae from Buarcos bay in Central West Coast of Portugal. Food Chemistry **183:** 197-207

Rouphael Y, Colla G (2020) Biostimulants in Agriculture. Frontiers in Plant Science **11:** 40-47

Rouphael Y, De Micco V, Arena C, Raimondi G, Colla G, De Pascale S (2017) Effect of Ecklonia maxima seaweed extract on yield, mineral composition, gas exchange, and leaf anatomy of zucchini squash grown under saline conditions. Journal of Applied Phycology **29:** 459-470

Rudolph AS, Crowe JH, Crowe L (1986) Effects of three stabilizing agents—proline, betaine, and trehalose—on membrane phospholipids. Archives of Biochemistry and Biophysics **245:** 134-143

Ruiz-Delgado M, Reyes-Martínez M, Sánchez-Moyano J, López-Pérez J, García-García J (2015). Distribution patterns of supralittoral arthropods: wrack deposits as a source of food and refuge on exposed sandy beaches (SW Spain). Hydrobiologia **742:** 205-219.

Sağlam A, Yetişsin F, Demiralay M, Terzi R (2016) Copper Stress and Responses in Plants Plant Metal Interaction. 21-40

Sánchez-Pardo B, Fernández-Pascual M, Zornoza P (2014) Copper microlocalisation and changes in leaf morphology, chloroplast ultrastructure and antioxidative response in white lupin and soybean grown in copper excess. Journal of Plant Research **127:** 119-129

SANTE (2018) Final Renewal report for the active substances Copper compounds. SANTE/10506/2018 Rev. 5. 27 November 2018. European Commission. Health and Consumers Directorate-General.

Santiago LJM, Louro RP, De Oliveira D (2000) Compartmentation of phenolic compounds and phenylalanine ammonia-lyase in leaves of Phyllanthus tenellus Roxb. and their induction by copper sulphate. Annals of Botany **86:** 1023-1032

Saslowsky D, Winkel-Shirley B (2001) Localization of flavonoid enzymes in Arabidopsis roots. The Plant Journal **27:** 37-48

Scandalios J (2005) Oxidative stress: molecular perception and transduction of signals triggering antioxidant gene defenses. Brazilian Journal of Medical and Biological Research **38:** 995-1014

Schiener P, Black KD, Stanley MS, Green D (2015) The seasonal variation in the chemical composition of the kelp species *Laminaria digitata*, *Laminaria hyperborea*, *Saccharina latissima* and *Alaria esculenta*.Journal od Applied Phycology **27:** 363-373

Schutzendubel A, Polle A (2002) Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. Journal of Experimental Botany **53:** 1351-1365

Sewelam N, Kazan K, Schenk P (2016) Global plant stress signaling: reactive oxygen species at the cross-road. Frontiers in Plant Science **7:** 187

Sgherri C, Milone MTA, Clijsters H, Navari-Izzo F (2001) Antioxidative enzymes in two wheat cultivars, differently sensitive to drought and subjected to subsymptomatic copper doses. Journal of Plant Physiology **58:** 1439-1447

Shabbir Z, Sardar A, Shabbir A, Abbas G, Shamshad S, Khalid S, Natasha N, Murtaza G, Dumat C, Shahid M (2020) Copper uptake, essentiality, toxicity, detoxification and risk assessment in soil-plant environment. Chemosphere 127436

Shahbaz M, Tseng MH, Stuiver CEE, Koralewska A, Posthumus FS, Venema JH, Parmar S, Schat H, Hawkesford MJ, De Kok L (2010) Copper exposure interferes with the regulation of the uptake, distribution and metabolism of sulfate in Chinese cabbage. Journal of Plant Physiology **167:** 438-446

Shahbazi F, Nejad MS, Salimi A, Gilani A (2015) Effect of seaweed extracts on the growth and biochemical constituents of wheat. International Journal of Agriculture and Crop Sciences **8:** 283-287

Shahid M, Pourrut B, Dumat C, Nadeem M, Aslam M, Pinelli E (2014) Heavy-metal-induced reactive oxygen species: phytotoxicity and physicochemical changes in plants. Reviews of Environmental Contamination and Toxicology **232:** 1-44

Sharma, Singh (2013) Studies on the effect of Cu (II) ions on the antioxidant enzymes in chickpea (*Cicer arietinum* L.) cultivars. Journal of Stress Physiology and Biochemistry **9:** 5-13

Sharma HSS, Fleming C, Selby C, Rao JR, Martin T (2013) Plant biostimulants: a review on the processing of macroalgae and use of extracts for crop management to reduce abiotic and biotic stresses. Journal of Applied Phycology **26:** 465-490

Sharma P, Jha AB, Dubey RS, Pessarakli M (2012) Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. Journal of Botany **2012:** 1-26

Sharma S, Raghukumar C, Raghukumar S, Sathe-Pathak V, Chandramohan D (1994). Thraustochytrid and fungal component of marine detritus II. Laboratory studies on decomposition of the brown alga *Sargassum cinereum*. Journal of experimental marine biology and ecology **175:** 227-242.

Sharmila P, Kumari PK, Singh K, Prasad N, Pardha-Saradhi P (2017) Cadmium toxicity-induced proline accumulation is coupled to iron depletion. Protoplasma **254:** 763-770

Shigeoka S, Ishikawa T, Tamoi M, Miyagawa Y, Takeda T, Yabuta Y, Yoshimura K (2002) Regulation and function of ascorbate peroxidase isoenzymes.Journal of Experimental Botany **53:** 1305-1319

Silva LD, Bahcevandziev K, Pereira L (2019) Production of bio-fertilizer from *Ascophyllum nodosum* and *Sargassum muticum* (Phaeophyceae). Journal of Oceanology and Limnology **37:** 918-927

Singh R, Agrawal M (2008) Potential benefits and risks of land application of sewage sludge. Waste management **28:** 347-358

Slima DF, Ahmed D (2020) Trace metals accumulated in pea plant (*Pisum sativum* L.) as a result of irrigation with wastewater. Journal of Soil Science and Plant Nutrition. **20:** 2749–2760

Soares C, Carvalho ME, Azevedo RA, Fidalgo F (2019) Plants facing oxidative challenges—A little help from the antioxidant networks. Environmental and Experimental Botany **161:** 4-25

Soares C, de Sousa A, Pinto A, Azenha M, Teixeira J, Azevedo RA, Fidalgo F (2016) Effect of 24-epibrassinolide on ROS content, antioxidant system, lipid peroxidation and Ni uptake in *Solanum nigrum* L. under Ni stress. Enviornmental and Experimental Botany **122:** 115-125

Soares, C, Torres, C, Sousa Pinto, I, Veiga, Puri, Rubal, M, Fidalgo, F (2018). Effects of environmental constraints on the oxidative status of *Ascophylum nodosum* and *Fucus serratus.* Frontiers in Marine Science **5**

Sousa T, Cotas J, Bahcevandziev K, Pereira L (2020). Effects of “sargaço” extraction residues on seed germination. Millenium-Journal of Education, Technologies, and Health **13:** 29-37

Srivastava V, Sarkar A, Singh S, Singh P, de Araujo AS, Singh RP (2017) Agroecological responses of heavy metal pollution with special emphasis on soil health and plant performances. Frontiers in Environmental Science **5:** 64

Stephenson WA, Booth E (1968) Seaweed in agriculture and horticulture. Faber & Faber London

Taiz L, Zeiger E, Møller IM, Murphy A (2015) Plant Physiology and Development, Sixth ed. Sinauer Associates, Inc., Sunderland, U.S.A

Thounaojam TC, Panda P, Mazumdar P, Kumar D, Sharma G, Sahoo L, Sanjib P (2012) Excess copper induced oxidative stress and response of antioxidants in rice. Plant Physiology and Biochemistry **53:** 33-39

Tripathi BN, Gaur J (2004) Relationship between copper-and zinc-induced oxidative stress and proline accumulation in *Scenedesmus* sp. Planta **219:** 397-404

Tuhy Ł, Samoraj M, Baśladyńska S, Chojnacka K (2015) New Micronutrient Fertilizer Biocomponents Based on Seaweed Biomass. Polish Journal of Environmental Studies **24:** 2213–2221

van Hees DH, Olsen YS, Wernberg T, Van Alstyne KL, Kendrick G (2017) Phenolic concentrations of brown seaweeds and relationships to nearshore environmental gradients in Western Australia. Marine Biology **164:** 74-87

Van Oosten MJ, Pepe O, De Pascale S, Silletti S, Maggio A (2017) The role of biostimulants and bioeffectors as alleviators of abiotic stress in crop plants. Chemical and Biological Technologies in Agriculture **4:** 5-17

Vasantharaja R, Abraham LS, Inbakandan D, Thirugnanasambandam R, Senthilvelan T, Jabeen SA, Prakash P (2019) Influence of seaweed extracts on growth, phytochemical contents and antioxidant capacity of cowpea *(Vigna unguiculata* L. Walp). Biocatalysis and Agricultural Biotechnology **17:** 589-594

Vassilev A, Lidon FC, Matos MdC, Ramalho JC, Yordanov I (2002) Photosynthetic performance and content of some nutrients in cadmium-and copper-treated barley plants. Journal of Plant Nutrition **25:** 2343-2360

Verdejo J, Ginocchio R, Sauvé S, Salgado E, Neaman A (2015) Thresholds of copper phytotoxicity in field-collected agricultural soils exposed to copper mining activities in Chile. Ecotoxicology and environmental safety **122:** 171-177

Vieira E, Soares C, Machado S, Correia M, Ramalhosa M, Oliva-Teles M, Morais S (2018). Seaweeds from the Portuguese coast as a source of proteinaceous material: Total and free amino acid composition profile. Food chemistry, **269:** 264-275.

Villares R, Fernández-Lema E, López-Mosquera ME (2016) Evaluation of Beach Wrack for Use as an Organic Fertilizer: Temporal Survey in Different Areas. Thalassas: An International Journal of Marine Sciences **32:** 19-36

Vinod K, Awasthi G, Chauhan PK (2012) Cu and Zn tolerance and responses of the biochemical and physiochemical system of wheat. Journal of Stress Physiology and Biochemistry **53:** 1227-1236

Wang T, Jonsdottir R, Ólafsdóttir G (2009) Total phenolic compounds, radical scavenging and metal chelation of extracts from Icelandic seaweeds. Foof Chemistry **116:** 240-248

Wang Y, Wisniewski M, Meilan R, Cui M, Webb R, Fuchigami L (2005) Overexpression of cytosolic ascorbate peroxidase in tomato confers tolerance to chilling and salt stress. Journal of the American Society for Horticultural Science **130:** 167-173

Wani PA, Khan MS, Zaidi A (2007) Cadmium, chromium and copper in greengram plants. Agronomy for Sustainable Development **27:** 145-153

Waqas M, Li G, Khan S, Shamshad I, Reid BJ, Qamar Z, Chao C (2015) Application of sewage sludge and sewage sludge biochar to reduce polycyclic aromatic hydrocarbons (PAH) and potentially toxic elements (PTE) accumulation in tomato. Environmental Science and Pollution Research **22:** 12114-12123

Weckx JE, Clijsters H (1996) Oxidative damage and defense mechanisms in primary leaves of *Phaseolus vulgaris* as a result of root assimilation of toxic amounts of copper. Physiologia Plantarum **96:** 506-512

Xu C, Leskovar DI (2015) Effects of *A. nodosum* seaweed extracts on spinach growth, physiology and nutrition value under drought stress. Scientia Horticulturae **183:** 39-47

Xu J, Yang L, Wang Z, Dong G, Huang J, Wang YJ(2006) Toxicity of copper on rice growth and accumulation of copper in rice grain in copper contaminated soil. Chemosphere **62:** 602-607

Yamamoto Y, Hachiya A, Matsumoto H (1997) Oxidative damage to membranes by a combination of aluminum and iron in suspension-cultured tobacco cells. Plant and Cell Physiology **38:** 1333-1339

Yao H, Bowman D, Rufty T, and Shi W (2009). Interactions between N fertilization, grass clipping addition and pH in turf ecosystems: implications for soil enzyme activities and organic matter decomposition. Soil Biology and Biochemistry **41:** 1425-1432.

Yaseen M, Ahmad T, Sablok, G (2013) Review: role of carbon sources for in vitro plant growth and development. Mololecular Biology Reports **40:** 2837–2849

Yoshie-Stark Y, Hsieh Y-P, Suzuki T (2003) Distribution of flavonoids and related compounds from seaweeds in Japan. Journal-Tokyo University of Fisheries **89:** 1-6

Yruela I (2005) Copper in plants. Brazilian Journal of Plant Physiology **17:** 145-156

Yruela I (2009) Copper in plants: acquisition, transport and interactions. Functional Plant Biology **36:** 409-430

Zafar H, Ali A, Ali JS, Haq IU, Zia M (2016) Effect of ZnO nanoparticles on *Brassica nigra* seedlings and stem explants: growth dynamics and antioxidative response. Frontiers in Plant Science **7:** 535

Zaimoglu Z, Koksal N, Basci N, Kesici M, Gulen H, Budak F (2011) Antioxidative enzyme activities in *Brassica juncea* L. and *Brassica oleracea* L. plants under chromium stress. Journal of Food, Agriculture and Environment **9:** 676-679

Zhang FQ, Wang YS, Lou ZP, Dong JD (2007) Effect of heavy metal stress on antioxidative enzymes and lipid peroxidation in leaves and roots of two mangrove plant seedlings (*Kandelia candel* and *Bruguiera gymnorrhiza*). Chemosphere **67:** 44-50

Zhang X (1997) Influence of plant growth regulators on turfgrass growth, antioxidant status, and drought tolerance. PhD thesis. Virginia Tech.

Zhang X, Ervin EH (2004) Cytokinin-Containing Seaweed and Humic Acid Extracts Associated with Creeping Bentgrass Leaf Cytokinins and Drought Resistance. Crop Science **44:** 1737-1745

Zhang X, Ervin EH (2008) Impact of Seaweed Extract-Based Cytokinins and Zeatin Riboside on Creeping Bentgrass Heat Tolerance. Crop Science **48:** 364-370

Zeng X, Qiu D, Hu R, and Zhang M (2017). Glutathione Transporters in Plants. In Glutathione in Plant Growth, Development, and Stress Tolerance 359-372 Springer, Cham.

Zou P, Lu X, Zhao H, Yuan Y, Meng L, Zhang C, Li Y (2019) Polysaccharides derived from the brown algae *Lessonia nigrescens* enhance salt stress tolerance to wheat seedlings by enhancing the antioxidant system and modulating intracellular ion concentration. Frontiers in Plant Science **10:** 48

ANNEX

I. Effects of increasing concentrations of Cu on barley's germination and seedling development – Petri dish assays

Table 1: Summary of ANOVA statistical data obtained for the effects of barley plants exposed to increased concentrations of Cu in petri dishes assay.

Parameter	F (DFn, DFd)	P value
Root length	F (5, 12) = 73.96	$p \leq 0.001$
Leaves length	F (5, 12) = 5.985	$p \leq 0.05$

Table 2: Summary of ANOVA statistical data obtained for the effects of increasing concentrations of an aqueous extract of wrack (0, 1.25, 2.5, 5.0 and 7.5 g L^{-1}) on barley seedlings grown in the presence or absence of 7 mg Cu L^{-1}.

Parameter	Factors					
	Wrack application		Cu application		Interaction	
	F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value
Root length	F (4, 42) = 6.115	$p \leq 0.001$	F (1, 42) = 493.6	$p \leq 0.001$	F (4, 42) = 0.5700	$p \geq 0.05$
Root biomass	F (4, 24) = 12.67	$p \leq 0.001$	F (1, 24) = 239.1	$p \leq 0.001$	F (4, 24) = 4.634	$p \leq 0.05$
Leaves' length	F (4, 42) = 11.39	$p \leq 0.001$	F (1, 42) = 157.5	$p \leq 0.001$	F (4, 42) = 2.639	$p \leq 0.05$
Leaves biomass	F (4, 24) = 21.70	$p \leq 0.001$	F (1, 24) = 347.5	$p \leq 0.001$	F (4, 24) = 2.644	$p \leq 0.05$

II. Effects of soil amendment with wrack on Cu-induced toxicity in barley plants

Table 3: Summary of ANOVA statistical data obtained for physical and chemical properties of both non-amended soil and the one amended with different wrack concentrations [0,0.5, 1 and 2% (m/m)] after 15 d and 30 d of stabilisation.

	Parameter	F (DFn, DFd)	P value
15 d	**pH**	F (3, 12) = 3.757	$p \leq 0.05$
	OM	F (3, 12) = 6.667	$p \leq 0.05$
	EC	F (3, 7) = 26.92	$p \leq 0.05$
30 d	**pH**	F (3, 8) = 209.8	$p \leq 0.001$
	OM	F (3, 7) = 0.2121	$p \geq 0.05$
	EC	F (3, 7) = 271.9	$p \leq 0.001$

Table 4: Summary of ANOVA statistical data obtained for obtained for the effects of wrack addition [0, 0.5, 1 and 2% (m/m)] to an agricultural soil contaminated, or not, by Cu (219 mg kg^{-1}) and left for stabilisation for 15 d and 30 d.

Parameter	F (DFn, DFd)	P value
Root length	F (7, 22) = 27.18	$p \leq 0.001$
Root dm	F (7, 18) = 6.744	$p \leq 0.05$
Root fm	F (7, 19) = 19.91	$p \leq 0.001$
Leaves dm	F (7, 20) = 3.799	$p \leq 0.05$
Leaves fm	F (7, 18) = 10,39	$p \leq 0.001$

Table 5: Summary of ANOVA statistical data obtained for obtained for the effects of wrack addition [0, 0.5, 1 and 2% (m/m)] to an agricultural soil contaminated, or not, by Cu (219 mg kg^{-1}) and left for stabilisation for 15 d and 30 d.

	Parameter	Factors					
		Wrack application		Cu application		Interaction	
		F (DFn, DFd)	P value	F (DFn, DFd)	P value	F (DFn, DFd)	P value
15 d	**Root length**	F (3, 21) = 6.185	$p \leq 0.05$	F (1, 21) = 31.61	$p \leq 0.001$	F (3, 21) = 2.592	$p \geq 0.05$
	Root biomass	F (3, 20) = 0.6620	$p \geq 0.05$	F (1, 20) = 54.20	$p \leq 0.001$	F (3, 20) = 0.2064	$p \geq 0.05$
	Leaves biomass	F (3, 21) = 2.979	$p \leq 0.05$	F (1, 21) = 23.11	$p \leq 0.001$	F (3, 21) = 0.3524	$p \geq 0.05$
30 d	**Root length**	F (3, 21) = 1.106	$p \geq 0.05$	F (1, 21) = 3.111	$p \geq 0,05$	F (3, 21) = 1.940	$p \geq 0.05$
	Root biomass	F (3, 19) = 6.537	$p \leq 0.05$	F (1, 19) = 14.61	$p \leq 0.001$	F (3, 19) = 2.551	$p \geq 0.05$
	Leaves biomass	F (3, 21) = 1.854	$p \geq 0.05$	F (1, 21) = 0.078	$p \geq 0,05$	F (3, 21) = 0.1485	$p \geq 0.05$

III. Beneficial effects of wrack on Cu-induced toxicity in barley plants

Table 6: Summary of ANOVA statistical data obtained for roots of barley plants cultivated for 14 days in a d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu.

Parameter	F (DFn, DFd)	P value
Root length	F (3, 22) = 9.939	$p \leq 0.001$
Root biomass	F (3, 16) = 7.466	$p \leq 0.05$
Soluble protein	F (3, 9) = 2.549	$p \geq 0.05$
Cu content	F (3, 8) = 167.38	$p \leq 0.001$
LP	F (3, 11) = 9.453	$p \leq 0.05$
H_2O_2	F (3, 8) = 52.44	$p \leq 0.001$
$O_2^{\bullet-}$	F (3, 10) = 28.50	$p \leq 0.001$
Pro	F (3, 9) = 25.35	$p \leq 0.001$
GSH	F (3, 7) = 17.28	$p \leq 0.05$
Total AsA	F (3, 8) = 58.63	$p \leq 0.001$
Reduced AsA	F (3, 9) = 67.21	$p \leq 0.001$
DHA	F (3, 8) = 44.74	$p \leq 0.001$
AsA/DHA	F (3, 9) = 39.76	$p \leq 0.001$
Phenols	F (3, 11) = 10.97	$p \leq 0.05$
Flavonoids	F (3, 9) = 15.11	$p \leq 0.001$

SOD	F (3, 8) = 0.9305	$p \geq 0.05$
CAT	F (3, 9) = 31.15	$p \leq 0.001$
APX	F (3, 8) = 2.384	$p \geq 0.05$

Table 7: Summary of ANOVA statistical data obtained for leaves of barley plants cultivated for 14 days in a d in an agricultural soil i) only moistened with water (CTL), ii) amended with 2% (m/m) wrack, iii) contaminated by Cu (219 mg kg^{-1}) or iv) amended with wrack and contaminated by Cu.

Parameter	F (DFn, DFd)	P value
Leaves biomass	F (3, 23) = 3.661	$p \leq 0.05$
Chlorophyll	F (3, 6) = 1.689	$p \geq 0.05$
Carotenoid	F (3, 8) = 0.9842	$p \geq 0.05$
Soluble protein	F (3, 13) = 29.69	$p \leq 0.001$
Cu content	F (3, 8) = 4947	$p \leq 0.001$
LP	F (3, 9) = 8.005	$p \leq 0.05$
H_2O_2	F (3, 10) = 40.36	$p \leq 0.001$
$O_2^{\bullet-}$	F (3, 8) = 17.14	$p \leq 0.008$
Pro	F (3, 8) = 9.430	$p \leq 0.05$
GSH	F (3, 9) = 6.642	$p \leq 0.05$
Total AsA	F (3, 11) = 3.547	$p \leq 0.05$
Reduced AsA	F (3, 10) = 5.206	$p \leq 0.05$
DHA	F (3, 11) = 13.53	$p \leq 0.001$
AsA/DHA	F (3, 8) = 20.07	$p \leq 0.001$
Phenols	F (3, 9) = 29.01	$p \leq 0.001$
Flavonoids	F (3, 8) = 9.125	$p \leq 0.05$
SOD	F (3, 8) = 23.49	$p \leq 0.001$
CAT	F (3, 12) = 1.380	$p \geq 0.05$
APX	F (3, 8) = 32.40	$p \leq 0.001$