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EFFECTS OF SPELT HUSK EXTRACTS ON GROWTH AND STRESS-RELATED BIOCHEMICAL AND PHYSIOLOGICAL PARAMETERS IN SALT-STRESSED MAIZE (*Zea mays* L.)

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1. GENERAL INTRODUCTION

1.1 PLANT STRESSES

As sessile organisms, plants cannot avoid adverse environmental conditions and contact with other living organisms, which can induce stress conditions and negatively influence their growth, productivity, reproductive capacity and survival (Mosa et al., 2017). Stress factors are therefore broadly divided into two main categories: abiotic or environmental stress factors, and biotic or biological stress factors. These latter are represented by organisms such as parasites and pathogens (viruses, bacteria, fungi, nematodes or insects), which can damage or cause diseases to the plants. Conversely, the abiotic stress factors that most commonly influence plant performance include deficiencies or excesses of water, extremes of irradiance, excessively low or high temperature, imbalance in several nutrients, including macro- and microelements, high salinity, and extreme pH values of soil. In particular, elevated levels of salts in the soil cause loss of water (osmotic/drought stress) leading to plant desiccation, as well as accumulation in plant tissues of potentially toxic ions (ion toxicity).

Abiotic stresses may also include mechanical stresses (e.g. wind, hail, mechanical impedance of root growth in compacted soils, and wounding), and stresses associated with toxic, man-made pollutants, such as gaseous pollutants (sulfur dioxide, nitrogen oxides, ozone), heavy metals and xenobiotics (e.g. herbicides) (Mantri et al., 2014).

1.1.1 Salt stress

Owing to limited rainfall and high evapotranspiration demand, coupled with poor soil and water management practices, salinity has become a serious threat to crop production, especially in arid and semi-arid regions of the world (Flowers and Yeo, 1995; Munns, 2002). Although the general perception is that salinization only occurs in the regions mentioned above, no climatic zone is free from this problem (Rengasamy, 2006). More than 800 million hectares worldwide are currently affected by salt and with global climate change, drought and salinity are expected to increase even further, affecting more than 50% of all arable lands by the year 2050 (Zhu, 2002; Wang et al., 2003). Given that most of the important crops (maize, wheat, rice) are glycophytes (*i.e.*, salt-sensitive), this global environmental condition is closely linked to the issue of food security for a growing world population.

The term salinity indicates accumulation of salts, such as sodium, chloride, magnesium, calcium, sulphates, carbonates and others elements that derive from several processes (Munns and Tester, 2008; Parihar et al., 2015):

1) weathering, which breaks down the rocks with the consequent release of soluble salts, or deposition of oceanic salt, carried in wind and rain (natural or primary salinity);
2) land cleaning, changes in the hydrological balance of the soil (water applied *vs*. crop water use efficiency), the use of annual crops instead of perennial vegetation, the use of irrigation schemes based on saline water, and insufficient drainage (secondary or human-induced salinity).

Soils are classified as saline when the electrical conductivity of the saturated paste extract (ECe) is ≥ 4 dS m^{-1} at 25 °C. This is equivalent to approximately 40 mM NaCl and generates an osmotic pressure of roughly 0.2 MPa (Munns and Tester, 2008).

In plants, salt stress, depending on duration, timing, and severity of exposure, affects various essential processes, leading to perturbation of metabolism and, consequently, altered growth and development. A fundamental and critical phase that allows the establishment of plants and the consequent success of agricultural production is germination. The beginning and the rate of this process are affected by salinity, which reduces the osmotic potential leading to enzyme and protein metabolism dysregulation and to hormonal and nutritional imbalance (Parihar et al., 2015).

As reported above, salt stress consists of two components, *i.e*., osmotic stress and ion toxicity. The osmotic or water-deficit effect takes place when the salt concentration around the roots increases to a threshold level with the consequent reduction of the soil water potential, and decrease in water uptake by the plants. A decrease in leaf expansion, number of new leaves and a general slower growth of the plants are typical phenomena that characterize this phase because of reduction in cell elongation and cell division. Moreover, even though there is no mechanistic explanation, shoot growth is generally more sensitive than root growth (Farooq et al., 2015). A reduction in leaf area that leads to a lower use of water by the plants, with consequent prevention of the increase in the concentration of salt in the soil, could account for this behaviour (Munns and Tester, 2008). Conversely, enhanced root growth (elongation or branching) or modified root gravitropism (known as halotropism) allows the plant to explore zones where salt is less concentrated, resulting in an “avoidance” mechanism (Sun et al., 2008).

The ion-specific phase occurs when the concentration of salt (especially Na^+ and Cl^- ions) within the plants reaches a toxic level resulting in the death of old leaves that are not able to dilute the salt as the younger leaves do. Furthermore, if vacuolar compartmentalization is not effective, ion accumulation in the cytosolic compartment or in the cell walls leads to inhibition of enzymatic activity and/or cell dehydration, respectively.

The difference between these phases is that the osmotic one is faster and more effective than the ion-specific one (Parihar et al., 2015).

The main site where Na exerts its toxic effect is the leaf blade, due to the partial inability of most plants to recirculate this ion from the shoots to the roots (Munns and Tester, 2008). Photosynthesis represents the main biochemical pathway through which green plants convert solar energy into chemical energy, in the form of organic compounds necessary for their growth. Salinity modifies the rate of photosynthesis through multiple factors (Chaves et al., 2009; Qu et al., 2012; Farooq et al., 2015):

- it determines a reduction of the water potential and a consequent accumulation of Na^+ and Cl^-, whose high concentrations influence the bioenergetics processes of photosynthesis;
- it induces closure of the stomata, mediated by shoot- and root-generated hormones, leading to the reduction of both CO_2 diffusion into the chloroplast and its transport through the mesophyll cells. Furthermore, it can affects leaf photochemistry and carbon metabolism;
- it decreases the efficiency of photosystem II and of the electron transport chain when the salt stress is prolonged.

The availability/solubility of nutrients in the soil depends on the pH and the oxidation-reduction potential of the soil solution. It is known that salinity also induces a nutritional imbalance that can negatively affect crop performance. Excessive build-up of Na^+ and Cl^- in the rhizosphere strongly interferes with the uptake of essential mineral elements. In particular it reduces the uptake of nitrogen, potassium, calcium, magnesium and iron (Karimi et al., 2005; Tavakkoli et al., 2010; Turan et al., 2010; Yasmeen et al., 2013).

Potassium (K) is an essential element necessary for plant growth and a decrease in its physiological concentration can lead to osmotic imbalance and an altered function of the stomata and of some enzymes (Zeinolabedin, 2012). Owing to physicochemical similarities between Na^+ and K^+, the former could compete with K^+ for major binding sites in key metabolic processes, including control of enzymatic activity and both low- (e.g. non-selective cation channels) and high-affinity (e.g. High-affinity K^+ transporter) transporters. Therefore, the ability of a plant to maintain the cellular K^+ content above a certain threshold and a high cytosolic K^+/Na^+ ratio (by retaining K^+ or preventing Na^+ accumulation in the leaves) is essential for plant growth and salt tolerance (Wang et al., 2013; Shabala and Pottosin, 2014).

As reported above, one of the main short-term responses of plants to salinity is stomatal closure and the consequent inhibition of atmospheric CO_2 fixation. The resulting exposure of chloroplasts to excessive excitation energy leads in turn to the over-production of reactive oxygen species (ROS). Thus, as a consequence of salt stress, plants also experience a condition of oxidative stress due to an altered equilibrium between production and scavenging of ROS. Although at low concentrations ROS behave as second messengers in the intracellular signaling cascades that mediate several plant

responses (stomatal closure, programmed cell death, tolerance to biotic and abiotic stresses), at high concentrations they are responsible for lipid peroxidation, protein oxidation, enzyme inactivation, and membrane and DNA damages (Ahanger et al., 2017).

Among the ROS-induced alterations, membrane damage is the main cause of cell toxicity. Indeed, modification of the intrinsic properties of the membrane such as fluidity and ion transport capability results in loss of enzymatic activity, inhibition of protein synthesis and DNA damage, ultimately resulting in cell death (Sharma et al., 2012). Another dramatic effect of ROS is lipid peroxidation with the production of lipid-derived radicals that exacerbate the oxidative stress (Xie et al., 2019).

Plants have developed in the course of their evolution efficient strategies to avoid, tolerate, or adapt to different types of stress situations. The diverse stress factors that plants have to face often activate similar cell signaling pathways and cellular responses, such as the production of stress proteins, up-regulation of the antioxidant machinery, and accumulation of compatible solutes (Fraire-Velázquez et al., 2011; Verma et al., 2013)

To grow appropriately in the presence of high salinity, in particular when exposed to the most soluble and widespread salt NaCl, plants have developed multiple structural, physiological and biochemical mechanisms aimed at regulating its accumulation and at maintaining appropriate levels of other nutrients. These mechanisms include:

a) changes in the anatomical organization of roots and leaves. An increase in palisade parenchyma and intracellular spaces, and a decrease in spongy parenchyma to facilitate the diffusion of CO_2 in a situation of reduced stomatal aperture. The increase in root to shoot ratio, common in presence of salinity, can favour the retention of toxic ions in this organ, controlling their translocation to the aerial parts (Rewald et al., 2013; Acosta-Motos et al., 2017);
b) "exclusion" by the root system of toxic ions, *via* limited uptake or negative halotropism, in order to prevent their accumulation at toxic levels inside the shoots (Munns and Tester, 2008);
c) vacuolar compartmentalization and sequestration in older tissues (which eventually are sacrified) of toxic ions. The transport of Na^+ from the cytoplasm to the vacuoles is done by a salt-inducible enzyme Na^+/H^+ antiporter: the vacuolar type H^+-ATPase. This pump, under normal growth conditions, plays an important role in maintaining solute homeostasis, energizing secondary transport and facilitating vesicle fusion, playing a crucial role for stressed-plant survival (Dietz et al., 2001). Moreover, Na^+ efflux at the cellular level and its transport from roots to shoots are regulated by the Salt Overly Sensitive (SOS) stress-signaling pathway. It consist of three major proteins one of which is a plasma membrane Na^+/H^+ antiporter (Gupta and Huang, 2014). In fact, Na^+ exclusion from uptake by roots is mediated by the Na^+/H^+ exchanger located at the plasma

membrane of epidermal root cells, encoded by the *SOS1* (*Salt Overly Sensitive1*) gene (Ji et al., 2013);

d) cytoplasmic accumulation of low-molecular-mass compounds known as osmo-protectants or compatible solutes, which do not interfere with normal biochemical reactions, to increase the plants hyperosmotic tolerance. Based on their chemical nature these molecules can be categorized in: betaines and related compounds, amino acids, polyols, non-reducing sugars, and polyamines (Parida and Das, 2005; Kumar et al., 2018);

e) production of antioxidant enzymes (e.g. superoxide dismutase, catalase, ascorbate peroxidase, glutathione reductase, etc.) and small non-enzymatic molecules (e.g., ascorbate, flavonoids, polyphenolic compounds, etc.) that act as ROS scavengers. The harmonized activity of these enzymes achieve a balance between the rate of formation and removal of ROS, and sustain H_2O_2 at an adequate level for cell signaling (Türkan and Demiral, 2009; Ahanger et al., 2017).

1.2 BIOSTIMULANTS

Prevention of damage caused by biotic and abiotic stresses to field crop requires the identification of strategies that protect plants and simultaneously allow to increase their productivity, guaranteeing safe and high-quality agricultural products. The application of biostimulants has been proposed as an innovative agronomic practice, not only to ensure optimal nutrient uptake, crop yield, and quality (Chen et al., 2003; Schiavon et al., 2008, 2010; Ertani et al., 2009; Khan et al., 2009), but also to mitigate the detrimental effects of environmental stresses, including high salinity (Feitosa de Vasconcelos et al., 2009; Povero et al., 2016; Desoky et al., 2018).

Biostimulants are biologically active, ecological and safe substances that, in contrast to bioregulators and hormones, enhance the metabolic processes of plants without changing their natural pathways (Posmyk and Szafrańska, 2016). Unfortunately, the precise mechanisms activated by biostimulants are often unknown and difficult to identify, because they mainly derive from complex sources containing multiple bioactive components that, together, contribute to specific responses in plants (Bulgari et al., 2015).

1.2.1 Classification

Due to the heterogeneous chemical composition of biostimulants, their highly variable biological activity and the absence of a legal and normative definition, there is no official classification of them. Nevertheless, biostimulants have been classified in some main categories, based on the origin of the

products used for their manufacturing (Ertani et al., 2013, 2016; du Jardin, 2015). These categories are widely recognized by scientists, regulatory authorities and stakeholders. They are:

a. Microorganisms

Fungi, bacteria and their metabolites are broadly used as biostimulants. Fungi (such as *Trichoderma atroviride*, *Candida* spp., *Saccharomyces cerevisiae*, etc) may establish different interactions with plants, in particular with their root system, such as:

- mutualistic symbioses that take place when two organisms live in direct contact with each other, with mutual benefit;
- commensalism in which an organism takes advantage without causing harm to the other;
- parasitism that leads to the damage of one organism while the other benefits.

Similarly, bacteria (such as *Pseudomonas fluorescens*, *Streptomyces* spp., *Lactobacillus* spp., etc.) interact with plants through mutualism and parasitism in a transient or permanent association. Moreover, they have the ability to move from the soil to the root cells and to be transmitted vertically until reaching the seeds (du Jardin, 2015).

Several beneficial effects are induced in plants by these microorganisms when used as biostimulants. They determine an increase in nutritional efficiency by raising the availability of macro- and micro-nutrients in the soil and their uptake (Miransari, 2013). They raise soil fertility and, at the same time, reduce fungal diseases and pathogen infections (Rashid et al., 2016). Photosynthesis, hormonal status, water balance, antioxidant defence and stress resistance are other processes enhanced by the use of microorganisms (Ilangumaran and Smith, 2017).

b. Seaweed extracts

The use of these components as a source of fertilizers and organic matter in agriculture is well established and consolidated for centuries, but their use as source of biostimulators has only recently been considered. Seaweed, based on their pigmentation, are divided into brown (*Ascophyllum nodosum, Ecklonia maxima, Sargassum* spp., etc.), red (*Corallina mediterranea, Petrocladia piumata*, etc.) and green algae (*Cladophora dalmatica, Ulva lactuca*, etc.). Among these, the former are the most widely used in the production of biostimulants.

Algae are characterized by multiple components such as macro- and micro-nutrients, vitamins, amino acids, polysaccharides, N-containing compounds like betaines, and phytohormones (cytokinins, auxins, abscisic acid, gibberellins) (Khan et al., 2009). The synergistic activity of these components allows seaweed extracts to exert positive effects both on soils and on plants. In soils, they promote the growth of beneficial bacteria and pathogen antagonists and induce water retention and better soil aeration, which leads to the growth and expansion of the plant root system and

optimization of nutrient absorption (du Jardin, 2015). In plants, algal extracts or purified compounds increase resistance to frost, insect and pathogen attack, diseases, parasites, and abiotic stress (Tuhy et al., 2013).

c. Plant and animal materials

The use of crop residues and agro-industrial by-products such as bark, straw, rice husk, etc. (Fritsch et al., 2017) and animal waste products (e.g. epithelial and connective tissues, collagen, elastin) for the production of biostimulants has been taken into consideration (Xu and Geelen, 2018).

Within this category, protein hydrolysates are the most studied and known biostimulants. They are mainly composed of a mixture of peptides and amino acids of plant or animal origin, whose levels vary according to the type of hydrolytic process used (chemical, enzymatic or thermal), but they may also contain non-protein components such as fats, carbohydrates, and polyphenols (Ertani et al., 2009, 2013; de Lucia and Vecchietti, 2012).

Due to the heterogeneous composition of this class of biostimulants, their ameliorative effects on plant performance are different. Some amino acids, such as proline, display a chelating property against heavy metals and ameliorate the availability and absorption of nutrients (Kaur and Asthir, 2015). Proline and betaine increase the plant defence responses and resistance to different abiotic stresses, including salinity, drought, high temperature, freezing and oxidation (Ashraf and Fooland, 2007; Sidhu Murmu et al., 2017). These biostimulants can also enhance both the metabolism and the uptake of nitrogen and carbon, and promote growth and activity of beneficial microbes in the soil, as well as soil fertility and aeration (Calvo et al., 2014; Colla et al., 2014). All these positive effects result in the promotion of plant growth, health, and productivity.

Plant-derived biostimulants (PDBs) are naturally present, in varying concentrations and combinations, in the roots or leaves of higher plants. PDBs can be directly extracted from the different plant organs (Hanafy et al., 2012; Abbas and Akladious, 2013; Pardo-García et al., 2014; Rady et al., 2019) or from agro-industrial residues (Abou Chehade et al., 2018; Xu and Geelen, 2018). They can positively affect the growth and productivity of several crops by regulating the photosynthetic process and nitrogen and carbon metabolism (Lucini et al., 2015; Kumar et al., 2019) as well as trigger stress tolerance by activation of the antioxidant system, the delay of photo-inhibition and the increase in sterol contents, which regulate membrane stability (Posmyk and Szafrańska, 2016; Zulfiqar et al., 2019).

d. Humate-based raw materials

Humic substances (HS) are naturally occurring components in the organic matter of soil, resulting from the decomposition and degradation of plant, animal and microbial residues, and from the metabolic activity of soil microbes that use these substrates. Based on their molecular weight and solubility, these substances are classified into humic acids, fulvic acids and humins. Humic acids are characterized by a high molecular weight and are soluble in basic media; fulvic acids have a low molecular weight and are soluble in both alkali and acid media, and the latter are commonly described as humic-containing substances, since they are aggregates of humic and non-humic materials. Additionally, all these compounds have the ability to form supra-molecular colloids when interacting with exudates released from the root system of the plant (Theng, 2012).

The effects of HS on plants are very variable, due to their heterogeneous composition, and depending on their source of origin, dose, and method of application (Rose et al., 2014). The main effects observed in plants when they are used as biostimulants is a marked development of the root system, that leads to a higher degree of macro- and micro-nutrient absorption and to a more efficient translocation from root to shoot of elements having a direct effect on plant metabolism (Ertani et al., 2011; Jindo et al., 2012). Among HS, the small molecular size of fulvic acids allow them to pass across the micropores of the biological membrane system and persist in soil solutions in the presence of higher concentration of salt and a wide range of pH. These features allow them to interact with the root system in a more lasting way (Calvo et al., 2014).

As biostimulants, HS also offer protection from stress, by stimulating the activity of key enzymes involved in different metabolic pathways. For example, humic acids activate the enzymes of the phenylpropanoid pathway, which is essential for the production of phenolic compounds, endowed with an antioxidant capacity (Schiavon et al., 2010).

1.2.2 Technologies of production, methods and time of application

As previously mentioned, biostimulants are made up of multiple compounds; for this reason, an extensive comprehension of their chemical and biological features is essential to identify, characterize and preserve bioactive molecules (Povero et al., 2016). In this regard, the process used for the production of biostimulants plays an important role and the technologies employed are various and include cultivation, fermentation, extraction, hydrolysis, processing and purification, enzyme digestion, and high-pressure cell rupture. Nevertheless, in some cases the manufacturing process can generate compounds that were not present in the original material and whose formation cannot be predicted (Yakhin et al., 2017). Other important parameters that must be taken into consideration when using raw materials (such as plants and algae) for producing biostimulants are the season in

which they are collected and their stage of development. It is known that variation of these two factors also varies the composition of the raw materials in terms of both quality and quantity (Apostolidis et al., 2011).

In order to guarantee its effectiveness, it is essential to evaluate the best method of application of the biostimulant. Apart from the classic application in the soil, another possibility is adding it in the nutrient medium used for hydroponic cultivation, a plant production method belonging to the Soilless Culture Systems (SCSs). These systems are based on the absence of soil as a root medium and on the addition of inorganic macro- and micro-nutrients in the irrigation water, thus creating a nutrient solution. In this model, the root system can grow either in the presence of porous substrates or without any solid phase. In the first case, the root support, which is irrigated with the nutritive solution, can be inorganic, e.g., sand, rock wool, perlite, vermiculite, expanded polystyrene, pumice and others, or organic e.g., wood residues, peat, bark, and so on. In this case, the roots are partially or totally in contact with the solution (Hussain et al., 2014; Putra and Yuliando, 2015).

The foliar spray technique represents another option for conveying biostimulants. It is based on the principle that leaves can absorb the nutrients present in the rainwater through a natural process that depends on the thickness of the cuticle and the number of its pores, as well as on the distribution of trichomes and stomata on their surface (Smoleń, 2012). Generally, the technique is used for delivering nutrients in case of deficiency. However, due to the high rate of absorption that characterizes this procedure, it can be employed to mitigate the negative effects caused by various stresses (including abiotic stress) through the spraying of substances with biostimulant activities. These compounds can be applied in combination with the spray nutrient solution or separately (Alshaal and El-Ramady, 2017).

Another factor that influences the effectiveness of a biostimulant is the timing of its application. Plants are known to have crucial developmental stages such as germination, seedling emergence, flowering and fruit formation. Among these, germination represents one of the most limiting process, which affects the occurrence of the subsequent stages of development; thus, a good final yield depends on the quality and health of the seeds (Posmyk and Szafrańska, 2016). To guarantee these characteristics, the seed priming process has been introduced into agricultural practices. Priming is a water-based technique that consists in the imbibition of seeds, allowing it to go through the first reversible stage of germination, but not allowing radicle emergence through the seed coat (Paparella et al., 2015). This treatment leads to a physiological state ("primed state") that enables seeds to germinate more efficiently. The seed priming method is characterized by the stimulation of metabolic processes, which normally occur in the initial phase of germination, such as the *de-novo* synthesis of nucleic acids and proteins, the increase in the activity of numerous enzymes (phosphatases,

peroxidases, synthases and others), the accumulation of sterols and phospholipids, the activation of antioxidant mechanisms, etc (Jisha et al., 2013). This precocious activation of these processes leads to several benefits: an increase in the rate and uniformity of germination, an enhanced, rapid and homogeneous emergence of sprouts and seedlings, better harvests and greater resistance to biotic/abiotic stresses, pests and diseases (Ibrahim, 2016; Lutts et al., 2016). Among the multiple priming methods (hydropriming, osmopriming, hormonal, chemical and biological priming, etc.), priming with biostimulants represents a novel technique that strengthens the previously mentioned benefits through the addition of these compounds in the aqueous medium.

1.3 PHENOLIC COMPOUNDS

Polyphenols are secondary metabolites widespread in the plant kingdom. They are naturally present in vegetables, fruits, seeds, nuts, flowers and bark. Based on the number of phenolic rings (Fig. 1) and the type of structural elements through which these rings are connected, the phenolic compounds can be classified into four groups (Manach et al., 2004; El Gharras, 2009):

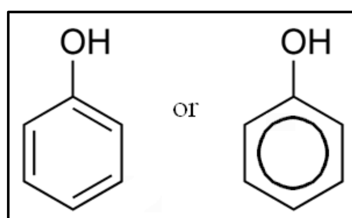

Fig.1 *Phenol structure*

Flavonoids

They are low molecular weight polyphenols with the flavan nucleus (Fig. 2) as the basic structure. Based on the presence of different substituents on this structure, they can be further divided into flavonols, flavones, isoflavones, flavanones, anthocyanins, and flavan-3-ols (Cook and Samman, 1996; Panche et al., 2016).

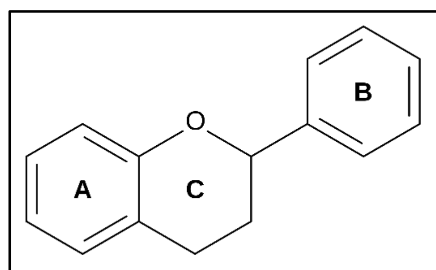

Fig.2 *Chemical structure of the flavan nucleus*

Their distribution in plants varies in both type and quantity according to the stage of growth or maturity, the phylum they belong to and the conditions of growth.

Flavonols represent the ubiquitous flavonoids in food and their presence is prevalent in onions, broccoli, leeks, blueberries, red wine and tea. Kaempferol and quercetin are the main representative compounds of this class.

Flavones are mainly glycosides of luteolin and apigenin, and are present in parsley, celery and cereals such as millet and wheat.

Isoflavones are exclusive to plants of the Fabaceae family and the main source is soybean and its derived products.

Flavanones are characterized by glycosylation with a disaccharide in position 7 of the flavan nucleus structure. They are present in considerable concentration in citrus fruits and at a lower level in tomatoes and in aromatic plants such as mint.

Anthocyanins are pigments responsible for the colour of leaves, flowers, and fruits. Cyanidin, malvidin, delphinidin, peonidin and pelargonidin are the most studied and mainly occur in the epidermis of various red fruits.

Flavan-3-ols are present both in monomeric and polymeric form. Monomers are called catechins, and comprise (+) catechin and (-) epicatechin, present in several fruits, and gallocatechin, epigallocatechin and epigallocatechin gallate, present in grapes and tea. In the polymeric form, flavan-3-ols are called proanthocyanidins or condensed tannins and are the predominant polyphenols of grapes.

Phenolic acids

They can be divided in two classes: benzoic acid- or cinnamic acid- derivatives (Fig. 3). Among the former there are salicylic, *p*-hydroxybenzoic, gentisic, protocatechuic, gallic, vanillic and syringic acids. The latter include *p*-coumaric, caffeic, ferulic, synaptic and chlorogenic acids and are more common than are the hydroxybenzoic acids. Caffeic acid is the most abundant hydroxycinnamic acid in fruits, while ferulic acid in cereal grains (Chandrasekara, 2019).

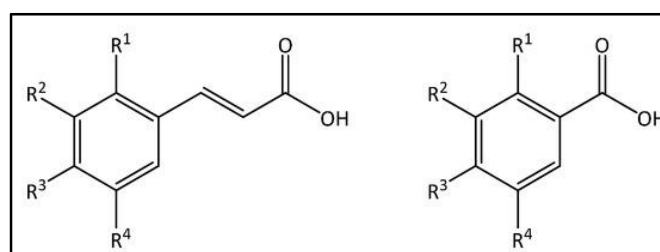

Fig. 3 *Chemical structure of cinnamic and benzoic acids*

Both soluble and insoluble phenolic acids are detectable in the cell. Soluble phenolics are localized and trapped in the vacuoles, while insoluble phenolics are localized in the cell wall where they are covalently bound to structural components such as hemicellulose, cellulose, pectins, lignin, and structural proteins through ester, ether and C-C bonds (Acosta-Estrada, 2014).

Lignans

These compounds derive from the condensation of two phenylpropane units (Fig. 4).

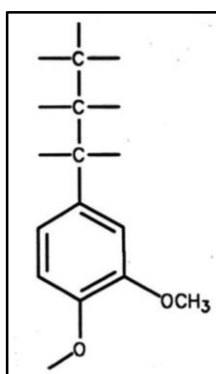

Fig.4 *Chemical structure of phenylpropane unit*

They are present in low concentrations in algae, leguminous plants, cereals, vegetables such as garlic, asparagus and carrots, and fruits like prunes and pears. Oilseeds are the richest source of such compounds, whose highest concentrations are observed in linseeds (Smeds et al., 2007).

Stilbenes

They are a small group of phenylpropanoids, characterized by a backbone of diphenylethylene (Fig. 5). These phenolic compounds are also referred as "phytoalexins", low molecular weight compounds which are produced by the plant in response to a pathogen attack. They are common in the bark, roots, leaves and fruits of Angiosperms and Gymnosperms. Resveratrol represents the most studied stilbene due to its health-promoting effects (Morales et al., 2000).

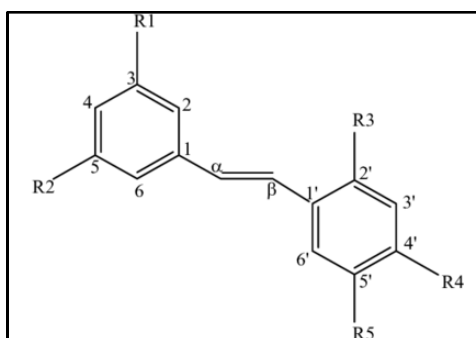

Fig.5 *Chemical structure of diphenylethylene*

1.3.1 Function of polyphenols in plants

Environmental cues stimulate plants to produce a wide range of metabolites (natural products). In fact, conditions such as drought, salinity, heat, heavy metals, nutritional deficiency and pathogens modify the metabolic homeostasis in plants, negatively influencing their development. In these situations, plants are able to change their metabolism with the aim of producing *de novo* or increasing the concentration of substances able to cope with or mitigate the stress (Yang et al., 2018). Among these, polyphenols play an important protective role and, in particular, flavonoids and phenolic acids are the most important group of molecules with this protective function. The main benefical effect of polyphenols is attributed to their antioxidant activity, related to their redox properties (Halliwell, 2008; Ghasemzadeh and Ghasemzadeh, 2011), through which they can act as:

- scavengers of reactive oxygen and nitrogen species (ROS and RNS) and/or inhibitors of their formation;
- chelators of metals implicated in the production of free radicals;
- regulators or protectors of antioxidant systems by stimulating the activity/expression of specific enzymes.

The structure of polyphenols plays a key role in determining these activities. For both phenolic acids and flavonoids, the antioxidant activity is based on the number and the position of hydroxyl groups in the rings that characterize their chemical structure (Balasundram et al., 2006). Among phenolic acids, hydroxycinnamic acids display a higher activity compared to hydroxybenzoic acids, due to the presence of a different side chain (Fig. 3) which guarantees a better hydrogen-donating capability and allows radical stabilization. Instead, for the flavonoids, the structure/activity relationships are more complicated since both B and C rings (Fig. 2) contribute to determine their reactivity.

Polyphenols are also responsible for other processes.

- Anthocyanins, flavonols and flavones determine both the color and the flavour of leaves, flowers and fruits, allowing the plant to attract pollinators and animals that, by eating the fruits, promote seed dispersion. For example, chalcones and aurones are resposible for the yellow pigmentation of the flowers of several plants, whereas vanillin, eugenol and other volatile polyphenols are strong odorants (El Gharras, 2009);
- Polyphenols also prevent the oviposition of insects and growth and development of larvae. They can alter the presence of inorganic and organic nutrients in the soil by acting on decomposer communities, with consequent alteration of the decomposition process and of nutrient cycling. For example, flavonoids, ferulic and gallic acids can promote or inhibit the development of

saprophytic fungi. Furthermore, by forming complexes with proteins, phenolics can modify the availability of nitrogen (Lattanzio et al., 2006; Hilker and Fatouros, 2015);

- *p*-hydroxybenzoic and *p*-coumaric acids, quercetin and others possess allelopathic properties (Li et al., 2010);
- Polyphenols act as physiological regulators or chemical messengers in plant growth processes. An example is the lignification process, in which wall-bound polyphenols, such as ferulic and *p*-coumaric acids, represent a reservoir of phenylpropanoid units for lignin biosynthesis and the beginning of lignification. In another instance, polyphenols, present in seed coats and embryos, influence the processes of seed germination and dormancy. Moreover, phenolics may exert a gibberellin-like activity (Lattanzio et al., 2006; Inácio et al., 2013; Savy et al., 2017);
- They also protect plants from UV-B solar radiations, which lead to the production of ROS. Phenolics perform this function by acting both as a barrier in the epidermal cell layer, thus preventing UV penetration, and by regulating and stimulating the cell's antioxidant system (Stapleton and Walbot, 1994; Schmitz-Hoerner and Weissenböck, 2003).

1.4 SPELT

Triticum spp. or dressed wheat, is one of the most ancient cereal species cultured in the world. Three different species belong to this genus: small spelt or *Triticum monococcum* L., medium spelt or *Triticum dicoccum* L. and big spelt or *Triticum spelta* L. Spelt presents a high nutritional value being rich in carbohydrates, proteins and minerals, and poor in fats. In addition, it contains nutraceutical compounds, such as carotenoids and polyphenols (Čurná and Lacko-Bartošová, 2017; Dhanavath and Prasada Rao, 2017).

Its cultivation in Italy is estimated at around 2,000 hectares (Fatichenti, 2012) but this value is destined to grow. This increasing interest for spelt is linked to different reasons: consumers seeking alternative and more healthy diets, the rediscovery of typical foods, greater interest in biological and eco-sustainable agriculture, measures of agrarian policy aimed at diversifying the productive directions and at the recovery of marginal and disadvantaged areas, sensitivity towards the conservation of agricultural species at risk of extinction or genetic erosion, new opportunities linked to the recovery of traditions and historical-cultural values. The main production area is that of central Italy, with most of the production in Tuscany, Umbria, and Marche.

1.5 MAIZE

Maize (*Zea mays* L.) is a main food and economical crop and with wheat and rice represents one of the most important crops throughout the world. In 2017, over 110 million tons of maize were produced in Europe and of this amount about 6 million tons were produced in Italy (FAOSTAT, 2012). It is widely used in both human and animal nutrition. Kernels can be submitted to different processsing procedures (drying, frying, roasting, fermentation, etc.), which allow to use them for the preparation of breads, porridges, cakes and alcoholic beverages or as food thickeners, sweeteners or for the production of oil. Moreover, maize is also used for the manufacturing of non food products, such as clothing, adhesives, papers, fuels, cosmetics, batteries, shopping bags, and so on (Ranum et al., 2014).

1.5.1 Morphology

Maize is a tall, monecious annual grass belonging to the Poaceae family (Tab. 1, Fig. 6). It is characterized by a finely branched root system. Most of these plants initially develop a primary root which is subsequently replaced by a permanent root system. However, in some cultivars, this transition does not take place and the initial root remains functional for the entire life of the plant. The permanent root system is composed of two different roots, the adventitious and the prop. Adventitious roots develop into a crown of roots from nodes below the soil surface. By contrast, prop roots, also called brace roots, originate from the first two, three aerial nodes and have the role of supporting the stem of the plant. These roots are pigmented, thick and covered with a waxy substance. However, if they penetrate the soil, they acquire both the aspect and function of normal underground roots. Furthermore, the young plants present plentiful root hairs that increase the surface area of the root epidermis, leading to a greater absorption rate of water and nutrients (Feldman, 1994; Hochholdinger, 2009).

Tab. 1 *Botanical classification of maize*

Kingdom	Plantae
Subkingdom	Tracheobionta (vascular plant)
Class	Monocotyledon (Liliopsida)
Subclass	Glumiflorae
Order	Poales
Family	Poaceae (grass family)
Subfamily	Pinicoideae
Genus	*Zea*
Species	*mays*

The maize leaves (from 8 to 20 in an adult plant) are typical grass leaves and consist of sheath, ligules, auricles and a blade. They present a pronounced midrib whit function of support. The leaf blade is long, narrow, ondulating and tapers towards the tip. Stomata occur in rows along the entire of the leaf surface but their number is higher in the underside. On the upper surface motor cells, responsible of leaf turgidity, are present. The upper leaves, due to their greater exposition to the light, are the main contributors of the photosynthetic process of the plant (Freeling and Lane, 1994).

The stem is cylindrical, three to four centimeters thick, solid, and clearly divided into nodes and internodes. Based on the genotype, its height may vary from less than 0.6 to more that 5 meters (Drennan and Evans, 1975).

The male and female inflorescence grow in different parts of the plants. The male one, a loose panicle, originates at the stem apex in the tassel and produces a pairs of spikelets each enclosing both a fertile and a sterile floret. The female inflorescences (cobs or ears) originate from the leaf axils and produce a pairs of spikelets enclosing two fertile florets, one of which ripens into a kernel once fertilized. Flowers are characterized by silk that appear first in the bottom and later in the top part of the ear (Bonnett, 1954; Cheng and Pareddy, 1994).

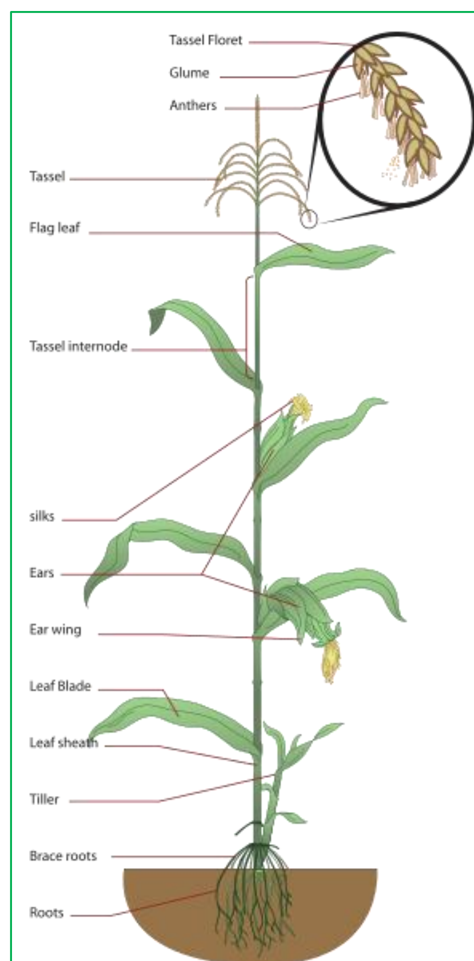

Fig. 6 *Maize plant*

The kernel consists of an endosperm, embryo, pericarp and tip cap. The endosperm constitutes about two-thirds of the kernel volume and contains carbohydrates (among which starch is the predominnat one), fats, and minerals. It comprises a hard (translucent, horny and vitreous) and a soft (opaque, floury and fleshy) portion, both consisting of a protein matrix in which are encapsulated granules of starch. The hard endosperm is composed of densely packed starch granules surrounded by a thick protein matrix, while the soft endosperm has larger, round starch granules and a thinner protein matrix. The germ, or embryo, contains the elements from which a new plant will develop that are embryonic leaves, a radicle and the scutellum, an attached seed leaf (Eckhoff et al., 2003). It is composed of fats, minerals and proteins (Fig. 7).

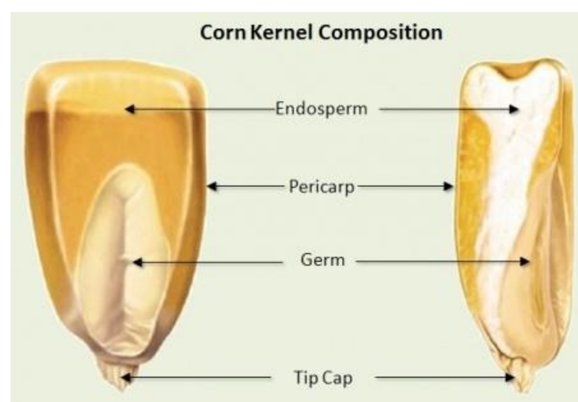

Fig. 7 *Schematic structure of maize kernel*

Based on the size and composition of the endosperm, different types of maize kernels can be recognized (Ranum et al., 2014):

- sweet, caracterized by an elevated content of sugars;
- dent, that is the most widely cultivated, presents a typical dented crown, a central portion composed of soft starch and peripherial areas of hard starch;
- flint type mainly composed of hard starcht;
- flour, one of the oldest types, composed nearly or completely of soft starch and characterized by the white color;
- popcorn, with small dimensions, containing more hard starch than soft .

Starch, located in the endosperm is the main carbohydrate, representing 72% of the dry weight. Sugars (sucarose, maltose, glucose, fructose and raffinose) range from 1% to 3% and are mainly present in the germ, while only in small amount in the endosperm (Watson, 2003).

Fiber is charateristic of the kernel seed coat (representing 7% of total kernel composition), but is also found in smaller amounts in the cell walls of the endosperm and germ. Hemicellulose, cellulose and lignin are the most represented fibers (Ai and Jane, 2016).

The proteins, distribuited between the germ and endosperm, consist of a combination of prolamins, glutelins, albumins and globulins. The amino acid profile of each protein is characteristic and plays a major role in determining their quality. In this regard, levels of prolamin are strongly indicative of overall protein quality in maize, providing half the kernel nitrogen. Prolamin, whose levels increase during kernel maturation, is a low-quality protein due to its low content of lysine and tryptophan, two essential amino acids. Although these amino acids are present in the germ, their total amount is not sufficient to classify maize as a high-quality protein source (Cerletti and Restani, 1985; Prasanna et al., 2001).

Lipids, in the form of oil, are the third macronuntrients in terms of abundance, immediately after starch and proteins. Their main form of storage is triacylglycerol, which accumulates mainly in the germ (Wang and White, 2019).

Provitamin A carotenoids and tocopherols, which are the precursors of vitamin A and E, are the main fat-soluble vitamins found in maize. Among the former, *β*-carotene, *α*-carotene and *β*-cryptoxanthin are the predominant forms, while among the latter, *α*- and γ-tocopherols are the only ones found in significant amounts. The water-soluble vitamins are located in the endosperm, germ and aleurone layer with niacine as the main component (Gwirtz and Garcia-Casal, 2014).

Minerals are mostly located in the germ and only in small quantities in the endosperm. Their levels in the kernel are conditioned by various environmental factors, such as the soil quality. In general, potassium, magnesium, phosphorous (in phytate form) and sulfur (in organic form as component of methionine and cysteine), are the most represented minerals in maize, while manganese, selenium, iodine are scarcely present (Gwirtz and Garcia-Casal, 2014).

In addition to all these nutrients, there are some substances that have anti-nutritional properties. One of these elements, found naturally in maize, is phytic acid (PA), which is essential for the processes of germination and phosphate storage. Because of its negative charges, PA chelates multivalent-cation minerals, such as zinc, calcium and iron, thus affecting their availability for human nutrition. In addition to PA, dietary fibers also contribute to the reduction of the bioavailaility of iron in maize (Nuss and Tanumihardjo, 2010).

1.6 MAIZE AND SALINITY STRESS

As previously reported, osmotic stress, ion toxicity and nutrient imbalance are three possible effects of salinity on crop plants, including maize, with different intensity depending on the degree of stress and the stage of growth. Maize is classified as moderately sensitive to salinity, with wide intraspecific genetic variations in salt tolerance (Katerji et al., 2000). Indeed, a concentration of NaCl greater than 250 mM seriously affects its growth and causes severe damage. In particular, the main toxic ion that leads to deleterious effect on maize is Na^+, due to its marked accumulation in leaves and roots. A direct consequence of the high level of Na^+ is the reduction of K^+ uptake, which leads, in turn, to water loss and necrosis (Sümer et al., 2004). The duration of the salt stress, among other factors, determines the accumulation of Na^+ within the plant. Conversely, short-term exposure to salinity does not allow the plant to reach a toxic level of Na^+ and the observed negative effects would be due only to omotic stress. Furthermore, the presence in the rhizosphere of Na^+ and Cl^- at high concentrations also negatively affect the absorption of other minerals, such as nitrogen, calcium, magnesium and iron, causing marked nutritional imbalance (Turan et al., 2010).

In maize, shoots are more sensitive than roots to salinity, although the root system is the first to encounter salt stress. As a consequence of this sensitivity, the development and expansion of shoots are inhibited, leading to a decrease in the net photosynthetic process rate (Hasan et al., 2018; Sozharajan and Natarajan, 2016). The decrease could be due to the reduction of the levels of photosynthetic pigments, such as chlorophyll *a*, chlorophyll *b* and carotenoids. However, when the exposure to salt is prolonged, other processes, such as ion toxicity, stomatal closure and membrane damage, set in (de Azevedo Neto, 2004; Guo et al., 2019). All of these factors are responsible for the limited capacity of maize to fix carbon under salinity conditions (Omoto et al., 2012). Moreover, the combination of salt stress and K^+ deficiency further exacerbates the inhibition of photosynthesis, intensifying the oxidative damage and decreasing plant growth (Gong et al., 2011).

All plants, including maize, activate various mechanisms at subcellular, cellular and organ level (stomatal regulation, activation of antioxidant defence system, hormonal and osmotic adjustments, ion exclusion, etc.) to contrast salinity. For instance, in order to achieve osmoregulation, maize plants accumulate organic solutes, such as glycine betaine, proline, and organic acids (Mansour et al., 2005; Molazem et al., 2010).

2. AIM OF THE STUDY AND DESCRIPTION OF THE PROJECT

The availability of essential enviromental resources as light, water and nutrients, and their balanced distribution and utilization in the different physiological processes are essential for plant normal growth, development and reproduction. As mentioned above, these processes can be negatively affected by different stress conditions. Biotic and abiotic stresses, in fact, can alter this equilibrium because part of the energy and organic products must be devoted to establish the defence machinary and, consequently, plant metabolism must be modified to make it possible to produce compounds necessary to cope with the stress (Shulaev et al., 2008). The production of defensive secondary compounds is costly to the plant, requiring precursors from primary metabolism, enzymes, and co-factors (such as ATP and NADPH) to drive biochemical reactions (Herms and Mattson, 1992). Plant phenolics, like other natural compounds, provide the plant with specific adaptations to changing environmental conditions and, therefore, they are essential for plant defense mechanisms.

Cereals contain a broad range of polyphenols, including phenolic acids, which occur in the grain both in free and bound forms. Ferulic, caffeic, *p*-coumaric, *p*-hydroxybenzoic, siringic and vanillic acids are typically present in the kernel. In particular, husk, pericarp, testa and aleurone cells, which represent the bran coatings, contain the highest concentration of total phenolic acids, while the remaining part is located in the endosperm (Kähkönen et al., 1999). In the last decades, a rediscovery of spelt (*Triticum dicoccum* L.), a cereal with high nutrient value, occurred in Italy for various reasons, in particular for its use in biological agriculture and for the recovery of marginal and disadvantaged areas. Currently, in Italy, most of the spelt is produced in Tuscany, Umbria and Marche. Most cereals, before being used for the preparation of foodstuffs or non food products, are subjected to the dehulling process and the husk represents a waste material. The husk obtained from the dehulling process of spelt corresponds to about 25% of the weight of the seed and, therefore, can represent an agro-industrial by-product available in large quantities.

In recent years, several studies have investigated the application in agriculture of food by-products enriched in phenols since these secondary metabolites are particularly efficient in stimulating plant growth and improving plant-water relationships (Ertani et al., 2016). Phenolic compounds are involved in plant hallelopaty influencing the accumulation and availability of soil nutrients and rates of nutrient cycling, which both ultimately affect plant growth (Li et al., 2010). In addition, they play an important protective role against oxidative stress (Habermann et al., 2016; Castrica et al., 2019). Based on the assumption that spelt husks contain bioactive polyphenols, in my PhD work the potential of methanolic extracts prepared from spelt husks as plant biostimulants under stress conditions was investigated on maize plants subjected to salt stress. Maize, in fact, is considered as paramount crop in the context of global nutrition, providing about 15% and 20% of the world's

protein and calories, respectively (Nuss and Tanumihardjo, 2010); it is moderately sensitive to salinity (Farooq et al., 2015).

The study was divided into three parts. The first part concerned the preparation of extracts from spelt husks and their characterization as regards the content of phenolic compounds. The results of these analyses have been published in Plant Physiology and Biochemistry (Ceccarini et al., 2019; section 3 in this thesys). In the second part, the effect of salt on the growth of maize seedlings grown in semi-hydroponic conditions and the ability of the extracts to modulate salt stress, by analysing different biochemical and physiological parameters, was investigated. The data obtained have been in part published in Plant Physiology and Biochemistry (Ceccarini et al., 2019; section 3 in this thesys) and in part reported in section 4 (unpublished results). Finally, the effect of husk extracts on seed germination under saline conditions was checked by means of seed priming experiments (section 4, unpublished results).

The experiments concerning the effect of salt on chlorophyll content index, stomatal conductivity, water and osmotic potentials and abscisic acid content as well as those on seed priming were carried out at the Department of Plant and Environmental Sciences of the University of Copenhagen (DK).

3. Polyphenol-enriched spelt husk extracts improve growth and stress-related biochemical parameters under moderate salt stress in maize plants

Data published in
Ceccarini et al. (2019) Plant Physiol. Biochem. 141, 95-104

Polyphenol-enriched spelt husk extracts improve growth and stress-related biochemical parameters under moderate salt stress in maize plants

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Abstract

Biostimulants improve yield, quality, and stress acclimation in crops. In this work, we tested the possibility of using phenolics-rich extracts from spelt (*Triticum dicoccum* L.) husks to attenuate the effects of salt stress (100-200 mM NaCl) in maize. Two methanolic extracts were prepared from the soluble-conjugated (SC), and the insoluble-bound (IB) phenolic acid fractions of the spelt husk, and their effects were investigated on several stress-associated biochemical parameters, such as proline, lipid peroxidation, H_2O_2, GSH levels, and ion content. Results show that SC and IB fractions of husk extracts behaved very differently, no doubt due to their greatly divergent chemical composition, as revealed by both GC-MS and HPLC analyses. The efficacy of treatments in mitigating salt stress was also dose- and timing-dependent. IB, even at the lower concentration tested, was able to recover the performance of stressed plants in terms of growth, photosynthetic pigments content, and levels of salt stress markers. Recovery of shoot growth to control levels and reduction of stress-induced proline accumulation occurred regardless of whether plants were pre-treated or post-treated with IB, whereas only pre-treatment with the higher dose of IB was effective in mitigating oxidative stress. Although in some cases SC and even methanol alone exerted some positive effects, they could also be deleterious whereas IB never was. Overall, results indicate that a polyphenol-containing extract obtained from spelt by-products can behave as biostimulant in maize plants and can mitigate their response to salt stress, by acting on different biochemical targets.

Keywords: agricultural by-products, biostimulant, oxidative stress, phenolic acids, salt stress, *Triticum dicoccum*, *Zea mays*

Introduction

Salinity is one of the major abiotic stresses that affects plant growth and biomass production as well as crop yield. More than 800 million hectares worldwide are currently affected by salt and with global climate change, drought and salinity are expected to increase even further affecting more than 50% of all arable lands by the year 2050 (Zhu, 2002; Wang et al., 2003). High concentrations of salt decrease water potential, thereby restricting water and nutrient uptake by roots (Ashraf, 2004); they are also responsible for nutritional and hormonal imbalance as well as oxidative stress (Basile et al., 2011), and they reduce chlorophyll content and RuBisCO activity (Rady, 2013; Kahrizi et al., 2012) leading to impaired plant growth and productivity.

The application of biostimulants has been proposed as an innovative agronomic practice, not only to ensure optimal nutrient uptake, crop yield, and quality (Chen et al., 2003; Schiavon et al., 2008, 2010;

Ertani et al., 2009; Khan et al., 2009), but also to mitigate the detrimental effects of environmental stresses, including high salinity (Feitosa de Vasconcelos et al., 2009; Povero et al., 2016; Desoky et al., 2018). The nature and activity of biostimulants is heterogeneous, since they include humic acids, protein hydrolysates, extracts from seaweeds and higher plant species, as well as beneficial microbes, such as plant growth-promoting bacteria, and mycorrhizal fungi (Ertani et al., 2013, 2016; du Jardin, 2015). For this reason, their chemical composition is also highly variable, consisting of a broad range of bioactive compounds, characterized by a huge chemical complexity. An interesting source of biostimulants are agro-industrial residues, such as bark, straw, rice husks, etc. (Ertani et al., 2011a), which often contain bioactive molecules, including antioxidants (Schieber et al., 2001; Balasundram et al., 2005), able to exert a protective effect under a variety of stress conditions.

Phenolic compounds are a very large group of secondary metabolites, ubiquitous in all plant tissues, where they play an important role by providing plants with specific adaptations to changing environmental conditions and by eliciting defense mechanisms (Caretto et al., 2015 and references therein). Indeed, activation of stress acclimation in plants is often accompanied by the synthesis of secondary metabolites (Mazid et al., 2011; Ramakrishna and Ravishankar, 2011). In particular, the role of phenolic compounds in mediating stress responses is due to their capacity to scavenge free radicals produced in stress-induced oxidative reactions (Cook and Samman, 1996; Halliwell, 2008; Sharma et al., 2012), thus protecting cell components from damage (Bulgari et al., 2017). Phenolic compounds, amongst which phenolic acids and flavonoids (El Gharras, 2009), are, in fact, reported to possess powerful antioxidant activity *in vitro* (Kähkönen et al., 1999). In recent years, several studies have investigated the application in agriculture of phenol-enriched products derived from food residues, since these secondary metabolites are particularly efficient in stimulating plant growth. In fact, it has been reported that phenolic molecules may exert a gibberellin-like activity (Savy et al., 2017) and also interact with several other plant hormones, including auxins (Ertani et al., 2016). Moreover, they improve plant-water relationships, stomatal function, rate of photosynthesis, and respiration (Ertani et al., 2016).

Maize (*Zea mays* L.), being rich in nutrients, such as starch (72%), protein (10%), fiber (8.5%), and edible oil (4.8%), is one of the major sources of food, sugar, cooking oil, and animal feed (Okoruwa, 1997). It is classified as a salt-sensitive crop (Maas & Hoffman, 1977). Antioxidant defense mechanisms have been demonstrated to play a fundamental role in mitigating salt stress (Zhu, 2001; Sairam and Tyagi, 2004) and salt stress tolerance in some maize genotypes was explained by the maintenance and/or increased activity of antioxidant enzymes (De Azevedo Neto et al., 2006). The effects of biostimulants on growth and stress tolerance in maize have been investigated. Maize seedlings treated with plant extracts derived from red grape, blueberry fruits, and hawthorn leaves

showed increased root and leaf biomass, chlorophyll and sugar content, as well as phenolic acids compared to untreated plants (Ertani et al., 2016). Recently, a positive effect against drought stress was also observed in maize treated with an aqueous extract of sorghum leaves (Maqbool and Sadiq, 2017).

By-products from spelt (*Triticum dicoccum* L.) processing (husks, glumes and stems) are employed for energy production (electricity or heating) as they present a number of advantages, both in the renewable energy sector and in agriculture and environmental protection (Kiš et al., 2017). Based on the assumption that this by-product may still contain bioactive polyphenols, we assessed the potential of this material as plant biostimulant under stress conditions. To this purpose, a pot trial was conducted to test the performance of methanolic extracts of spelt husks in mitigating the negative impact of salt stress in maize. Since methanol was used as solvent and previous reports demonstrate its important role in plant growth and development (Dorokhov et al., 2018) as well as in mitigating the effects of drought and salt stresses (Mirakori et al., 2009; Wei et al., 2015), the effect of methanol alone was investigated in parallel.

Materials and methods

Spelt husk extract preparation

Two phenolic acid-enriched extracts were prepared from husks of *Triticum dicoccum* L. (kindly provided by Terra Bio Soc. Coop, Schieti di Urbino, PU, Italy) according to the protocol described in Antognoni et al. (2017). Husk samples (1.5 g) were extracted with 30 mL acetone/methanol/water mixture (7:7:6, v/v/v) in an ultrasound bath at 30 °C for 30 min. The homogenate was centrifuged at 1,500 x *g* for 20 min and the pellet was re-extracted once with the same procedure. Pooled supernatants and pellets were used to prepare the soluble-conjugated (SC) and the insoluble-bound (IB) phenolic acid fractions, respectively. An aliquot (8 mL) of supernatant was mixed with 2 mL 10 M NaOH and hydrolyzed under nitrogen flow and constant stirring for 1 h. The solution was then acidified to pH 2 with 12 M HCl and extracted three times with an equal volume of a diethyl ether/ethyl acetate (1/1, v/v) mixture. The organic extracts were merged, brought to dryness in a rotary evaporator, and re-dissolved with 2 mL of methanol. A 0.5 g aliquot of the pellet was mixed with 40 mL of 2 M NaOH and hydrolyzed under nitrogen flow and constant stirring for 1 h. The sample was then centrifuged for 20 min at 1.500 x *g*; the supernatant was acidified to pH 2 and then subjected to the extraction procedure already described for the SC fraction.

Gas chromatography-mass spectrometry (GC-MS) analysis of extracts

An aliquot (*ca.* 1 mg) of each dry extract was derivatized with 200 µL of bis-(trimethyl) trifluoroacetamide (BSTFA) including 1% trimethylchlorosilane (TMCS) (Sigma-Aldrich, Milan, Italy) and 200 µL of pyridine (Sigma-Aldrich, Milan, Italy) for 2 h at room temperature (RT). The silylating reagent was removed under a nitrogen stream and the sample re-dissolved in 1 mL of *n*-heptane. The GC-MS analysis of the trimethylsilyl derivatives was carried out using a Trace GC Ultra coupled to an ion-trap mass spectrometer detector Polaris Q and equipped with a split/splitless injector (Thermo Scientific, Italy). The column was a Supelco SLB-5ms, 30 x 0.25 mm, 0.1 µm film thickness (Supelco, Sigma-Aldrich, Milan, Italy). Column oven temperature was programmed as follows: 80 °C held for 2 min, up to 280 °C at 10 °C min^{-1}, held for 10 min, up to 300 °C at 10 °C min^{-1} and held for 10 min. Helium was used as carrier gas at a flow rate of 1 mL min^{-1}. The injector, transfer line, and ion source were held at 280, 280 and 200 °C, respectively. Split ratio was 1:10 and injection volume 1 µL. The mass spectra were recorded under electron ionization (EI) conditions at 70 eV electron energy with a mass range from *m/z* 50 to 650. Relative percentage amount of each identified compound was expressed as percent peak area relative to total ion current GC-MS peak area without using the correction factor.

HPLC analysis of extracts

Twenty µL of each extract were injected into an HPLC system (Jasco, Tokyo, Japan; PU-4180 pump, MD-4015 PDA detector, AS-4050 autosampler). The stationary phase was an Agilent (Santa Clara, CA, USA) Zorbax Eclipse Plus C18 reversed-phase column (100 mm x 3 mm I.D., particle size 3.5 µm).The chromatographic method for the analysis of phenolic acids was adapted from Mattila et al. (2005). Gradient elution was carried out with a mixture of acidic phosphate buffer and acetonitrile flowing at 0.7 mL min^{-1}. The signals at 254, 280, and 329 nm were used for analyte quantitation. Identification and quantification were performed based on standard compounds (gallic, *p*-hydroxybenzoic, syringic, ferulic, *p*-coumaric, cinnamic, and caffeic acids). The recovery values in spiked samples ranged from 78.8 to 92.2% (RSD < 9.8%, n = 6).

The sum of all individual phenolic acid concentrations was calculated and used to express the total phenolics acid index (TPAI) for each extract.

Plant material

Seeds of *Zea mays* L. (var. FAO 700, kindly provided by Società Italiana Sementi, San Lazzaro di Savena, Bologna, Italy) were washed under running tap water for 30 min and then placed on wet filter paper in Petri dishes. They were germinated for two days in the dark at 25 °C. Seedlings were then transferred to plastic pots (12 x 8 x 7 cm) with drainage holes and containing Perlite soaked in

Hoagland's nutrient solution (Hoagland and Arnon, 1950). The pots were placed on trays to which half-strength Hoagland's solution was added when necessary in order to keep the Perlite fully wet. Plants were grown in a growth chamber with a photoperiod of 16/8 h day/night (400 µmol m^{-2} s^{-1} flux density supplied by fluorescent TL/D Aquarelle Philips lamps) at a temperature of 24 ± 2 °C. During the day, an additional irradiation (180 m^{-2} s^{-1} flux density from Lumatek HPS Grow lamps) was supplied for 5 h.

Salt treatment and husk extract application

After 8-10 days, the pots (four per treatment and each containing five plants) were transferred to trays containing Hoagland's solution added with either 0 (control), 50, 100, or 200 mM NaCl. The solution in the trays was changed every two days to maintain the same salt concentration. Plants were harvested at 5, 12, and 19 days after the start of salt treatment.

Husk extracts (HEs) were added to the nutrient solution at two different concentrations, 0.1 or 1.0 mL L^{-1}, in the trays before (pre-treatment) or after (post-treatment) the addition of NaCl as follows:

a) pre-treatment (PRE): IB, SC or methanol (solvent control) were added for two days before the beginning of salt treatment. Plants were then transferred to trays containing Hoagland's solution added with 0, 100 or 200 mM NaCl.
b) post-treatment (POST): IB, SC or methanol (solvent control) were added two days after the start of salt treatment; plants were grown in the presence of extract or methanol plus NaCl until sampling.

Plants were harvested at 12 (100 mM NaCl) or 8 days (200 mM NaCl) from the start of salt treatment. Shoots and roots were weighed separately and then ground to a powder in liquid nitrogen; some samples were freeze-dried, while others were left frozen and kept at -80 °C until use. For dry weight (DW) determination, shoots and roots were dried in an oven at 80 °C for two days.

Photosynthetic pigment determination

Freeze-dried shoot samples (50 mg) were extracted in a chilled mortar with 80% (v/v) cold acetone (1:5 ratio) in dim light. The homogenate was centrifuged at 10,000 x *g* for 10 min at 4 °C and the extraction repeated once. The supernatants were pooled and absorbance determined spectrophotometrically (V-530 Jasco, Jasco Corporation, Tokyo, Japan) at 663 nm (chlorophyll *a*), 647 nm (chlorophyll *b*), and 470 nm (carotenoids and xanthophylls). Pigment concentrations were estimated based on specific absorbance coefficients (Lichtenthaler, 1987) and are expressed as mg g^{-1} DW.

Lipid peroxidation assay

The level of lipid peroxidation was measured in terms of malondialdehyde (MDA) production by the thiobarbituric acid (TBA) reaction method (Velikova et al., 2000). Absorbance of the supernatant was read at 532 nm. After subtracting the value for non-specific absorption at 600 nm, the concentration of the MDA-TBA complex was calculated from the extinction coefficient 155 mM^{-1} cm^{-1}.

Hydrogen peroxide determination

Hydrogen peroxide (H_2O_2) levels were determined according to Velikova et al. (2000). Frozen shoot and root tissue (500 mg) was extracted on an ice-bath with 5 mL 0.1% (w/v) trichloroacetic acid. The homogenate was centrifuged at 12,000 x *g* for 15 min. Then, an aliquot (0.5 mL) of the supernatant was added to a mixture of 0.5 mL potassium phosphate buffer (10 mM, pH 7.0) and 1 mL 1 M KI. After 10 min at RT, the absorbance of the samples was spectrophotometrically determined at 390 nm. The concentration of H_2O_2 was calculated from a standard curve.

Proline determination

Shoot and root proline concentrations were estimated following the method of Bates et al. (1973) with slight modifications. About 20 mg of freeze-dried plant material was crushed in 1.2 mL 3% sulphosalicylic acid and the homogenate centrifuged at 16,000 x *g* at RT for 20 min. A 0.5 mL aliquot of the supernatant was made up to 1 mL with water and to this 1 mL glacial acetic acid and 1 mL ninhydrin reagent [2.5% ninhydrin in glacial acetic acid-distilled water-85% orthophosphoric acid (6:3:1)] were added. The reaction mixtures were kept in a water bath at 90 °C for 1 h to develop the colour. Test tubes were then cooled in an ice-bath, and 3 mL toluene added to separate the chromophore. Absorbance of the toluene phase was read in a spectrophotometer at 546 nm, and proline concentration calculated by comparing sample absorbance with the standard proline curve.

Glutathione (GSH) measurement

GSH was determined as previously described by Brundu et al. (2016). Approximately 20-25 mg of freeze-dried shoots or roots were transferred to microcentrifuge tubes containing 200 μL of precipitating solution (1.67 g glacial meta-phosphoric acid, 0.2 g disodium EDTA, and 30 g NaCl in 100 mL). Samples were homogenized on ice with a mortar and pestle, kept on ice for 10 min and then centrifuged at 12,000 × *g* for 10 min at 4 °C. Fifteen μL 0.3 M Na_2HPO_4 were added to 60 μL of extract followed immediately by 45 μL 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) prepared as follows: 20 mg DTNB in 100 mL 1% (w/v) sodium citrate solution. The mixture was stirred for 1

min at RT, then left at RT for another 5 min and finally used for GSH determination by Reverse Phase-HPLC (Jasco LG-980-02, Jasco Europe S.R.L., Cremella, LC, Italy).

Na^+ and K^+ content determination

Freeze-dried samples were mineralized (MDS 2000, CEM, Italy) with a mixture of concentrated HNO_3/H_2O_2 (8+2 mL). All chemicals used in sample treatment were suprapure grade (HNO_3 65%, H_2O_2 30%, Merck Suprapur, Darmstadt, Germany). Na^+ and K^+ concentrations were measured by inductively coupled plasma-optical emission spectrometry using a Perkin Elmer Optima 8000 ICP-OES Spectrometer (PerkinElmer Inc., Shelton, CT, USA). Ultrapure water (Milli-Q system, Millipore Corporation, USA) was used for all solutions. Standard solutions were prepared from stock solutions of ultrapure grade metals (PerkinElmer Pure Plus, 100 mg L^{-1}). The error of metal determinations, based on variation in replicate analyses (n=2) on the same samples, was 10% or lower.

Results

Phytochemical characterization of HEs

The phytochemical composition of both IB and SC was investigated through HPLC-DAD and GC-MS analyses. As shown in Table 1, five phenolic acids were detected by HPLC-DAD in both extracts, i.e., two simple phenolics (*p*-hydroxybenzoic, and syringic acids) and three hydroxycinnamic acids (ferulic, *p*-coumaric, and caffeic). The most abundant were ferulic and *p*-coumaric acids, which together represented *ca*. 75% and 97% of total phenolic acids in SC and IB, respectively. In absolute terms, IB contained about 25-fold higher ferulic and *p*-coumaric acids compared to SC and more than six-fold higher caffeic acid; syringic acid was about two-fold higher in SC than IB (Table 1). In both extracts, *p*-hydroxybenzoic acid was detected at a similar concentration (Table 1). Total phenolic acid index was 17.71 µg mL^{-1} for SC and 713.82 µg mL^{-1} for IB (Table 1).

Phenolic acid	SC		IB	
	µg mL^{-1}	µg g DW^{-1}	µg mL^{-1}	µg g DW^{-1}
p-OH benzoic acid	1.7 ± 0.7	16.1 ± 6.6	2.8 ± 0.4	13.2 ±1.9
Syringic acid	1.6 ± 0.7	15.4 ± 6.6	1.8 ± 0.3	8.8 ± 1.4
p-Coumaric acid	7.2 ± 0.9	67.7 ± 8.4	343.4 ±12.1	1635.1 ± 57.6
Caffeic acid	0.4 ± 0.1	3.6 ± 0.5	5.1 ± 0.2	24.3 ± 0.9
Ferulic acid	6.8 ± 0.4	63.4 ± 3.8	360.7 ± 19.4	1717.8 ± 92.4
Total phenolic acid index	17.7 ±1.3	166.3 ± 13.1	713.8 ±12.1	3399.2 ±108.9

Table 1. HPLC-DAD analysis of phenolic acid composition of spelt husk extracts. Data are the means ± S.E. of three independent extractions.

In addition, GC-MS analysis of IB revealed the presence of vanillin (0.7%), vanillic acid (1.6%) and a relatively higher percentage of *m*-coumaric (10.5%) and isoferulic acids (8.4%) (Table 2). GC-MS analysis of SC showed the presence of malic (14.2%), palmitic (27.6%), linoleic (16.8%), oleic (25.6%), and stearic (3.2%) acids accompanied by lesser amounts of 2-hydroxyglutaric acid (0.2%), *m*-anisic acid methyl ester (0.7%), campesterol (0.2%), stigmasterol (0.3%), and β-sitosterol (0.4%) (Table 2). Fatty acids, absent in IB, represented about 73% of all compounds identified in SC. Among phenolic acids, ferulic and *p*-coumaric acids were the major constituents in both extracts representing *ca.* 75% and 98% in SC and IB, respectively, in agreement with the results of the HPLC-DAD analysis. Vanillin and isoferulic acid were absent in SC.

	SC		IB	
Compound	**Mean (%)[a]**	**RSD[b] (%)**	**Mean (%)[a]**	**RSD[b] (%)**
Malic acid	14.2 ± 0.4	2.8	-	-
Vanillin	-	-	0.7 ± 0.01	3.2
2-Hydroxyglutaric acid	0.2 ± 0.01	2.0	-	-
p-Hydroxybenzoic acid	0.5 ± 0.01	3.6	0.2 ± 0.01	3.8
m-Anisic acid methyl ester	0.7 ± 0.01	1.2	-	-
Vanillic acid	1.8 ± 0.01	1.8	1.6 ± 0.1	4.4
m-Coumaric acid	0.7 ± 0.01	0.4	10.5 ± 0.1	0.7
Syringic acid	1.4 ± 0.1	4.0	0.4 ± 0.01	6.4
Isoferulic acid	-	-	8.4 ± 0.2	2.0
p-Coumaric acid	3.3 ± 0.1	1.7	35.2 ± 0.4	1.0
Palmitic acid	27.6 ± 1.2	4.2	-	-
Ferulic acid	3.0 ± 0.01	1.0	42.1 ± 0.1	0.3
Caffeic acid	0.1 ± 0.01	4.6	0.9 ± 0.1	5.9
Linoleic acid	16.8 ± 1.1	6.5	-	-
Oleic acid	25.6 ± 0.4	1.5	-	-
Stearic acid	3.2 ± 0.1	1.9	-	-
Campesterol	0.2 ± 0.01	1.6	-	-
Stigmasterol	0.3 ± 0.01	2.1	-	-
β-Sitosterol	0.4 ± 0.01	1.3	-	-

Table 2. GC-MS analysis of spelt husk extracts.
[a]Percent peak area relative to total ion current GC-MS peak area ± SD (n = 3).
[b]Relative standard deviation.

Effects of salt treatment on maize plants

In order to characterize the response to salinity of the maize genotype used in this study, a time-course analysis of growth and stress-related biochemical parameters was performed with three different concentrations of NaCl. Shoot fresh weight (FW) and dry weight (DW) were negatively affected by 50, 100 and 200 mM NaCl starting on day 12, with the strongest inhibition in FW (58% relative to controls) at the highest concentration and longest exposure time (19 days; Table 3). Root FW was less affected by all three salt concentrations, with a maximum decline (*ca*. 30-40%) with 200 mM NaCl after 12 and 19 days; root DW was not significantly affected (Table 3).

		Shoots				Roots			
Time (days)	NaCl (mM)	FW (g plant^{-1})	DW (g plant^{-1})	RFW	RDW	FW (g plant^{-1})	DW (g plant^{-1})	RFW	RDW
5	0	0.689±0.208^{a}	0.055±0.017^{a}	100	100	0.572±0.154^{a}	0.03±0.017^{a}	100	100
	50	0.945±0.227^{a}	0.072±0.017^{a}	137.2	130.9	0.655±0.029^{a}	0.038±0.017^{a}	114.5	115.2
	100	0.905±0.216^{a}	0.076±0.016^{a}	131.3	138.2	0.709±0.089^{a}	0.036±0.016^{a}	124.0	109.1
	200	0.907±0.282^{a}	0.083±0.026^{a}	131.6	150.9	0.628±0.178^{a}	0.044±0.026^{a}	109.8	133.3
12	0	2.620±0.263^{a}	0.206±0.017^{a}	100	100	1.338±0.252^{a}	0.077±0.014^{a}	100	100
	50	1.968±0.078^{b}	0.160±0.022^{b}	75.1	77.7	1.144±0.131^{b}	0.061±0.016^{a}	85.5	79.2
	100	1.923±0.220^{b}	0.158±0.034^{b}	73.4	76.7	1.100±0.137^{b}	0.079±0.014^{a}	82.2	102.3
	200	1.229±0.183^{c}	0.137±0.022^{b}	46.9	66.5	0.823±0.126^{c}	0.065±0.006^{a}	61.5	84.4
19	0	4.350±0.391^{a}	0.350±0.026^{a}	100	100	1.596±0.252^{a}	0.100±0.019^{a}	100	100
	50	4.220±1.101^{a}	0.366±0.061^{a}	97.0	105.7	1.560±0.340^{a}	0.099±0.049^{a}	97.7	99.0
	100	2.910±0.555^{b}	0.281±0.058^{ab}	66.9	80.0	1.400±0.291^{ab}	0.097±0.017^{a}	87.7	97.0
	200	1.820±0.448^{b}	0.221±0.050^{b}	41.8	62.9	1.130±0.074^{b}	0.090±0.010^{a}	70.8	90.0

Table 3. Fresh weight, dry weight, relative fresh weight (RFW), and relative dry weight (RDW) of maize plants after exposure for 5, 12, and 19 days to 0, 50, 100 or 200 mM NaCl. Data are means ± S.D. (n=8). Different letters within a column and for each time point indicate significant differences ($p<0.05$).

At day 5, a small but significant reduction in Chl*a* was observed with 200 mM NaCl, while at days 12 and 19, all pigments showed a significant and dose-dependent reduction (Fig. 1). A similar pattern was observed for total carotenoids content (Fig.1).

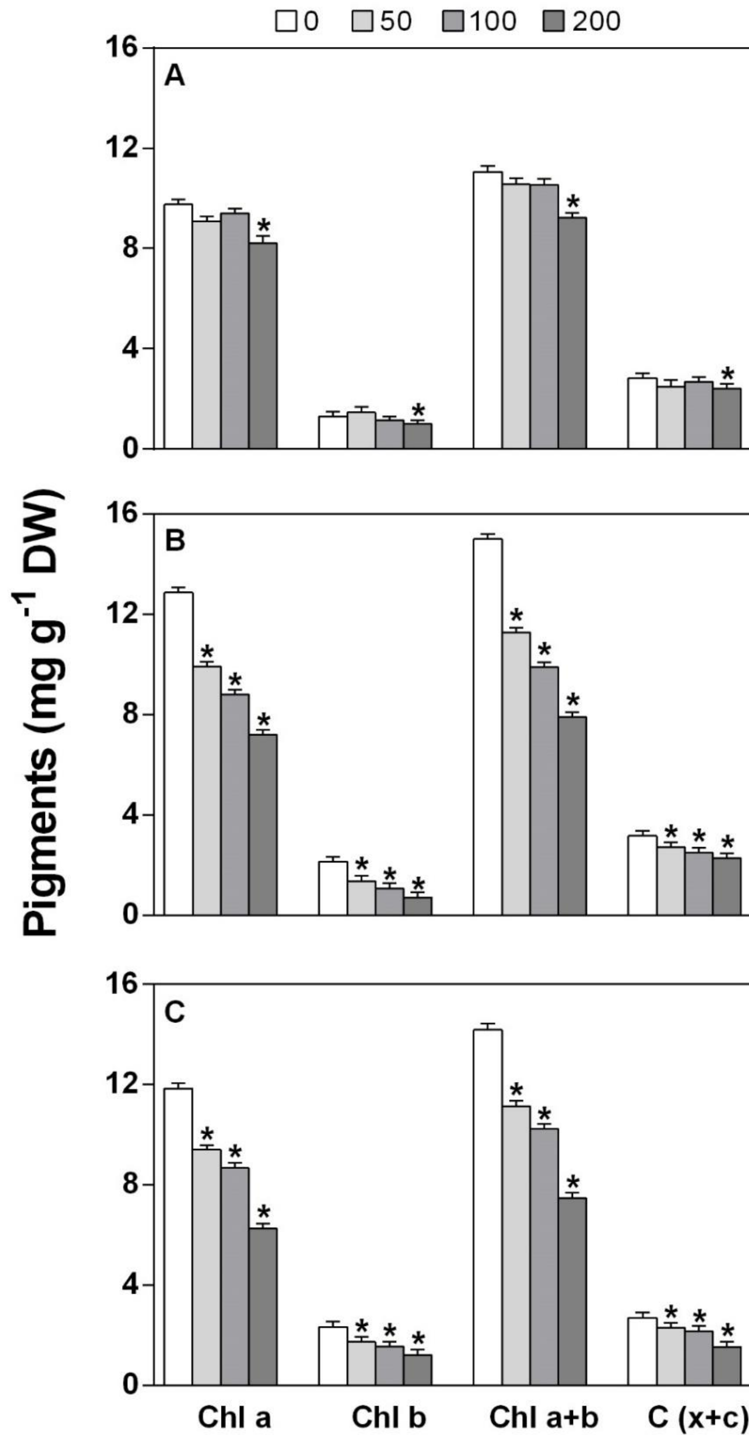

Fig. 1. Concentration of photosynthetic pigments after 5 (A), 12 (B), and 19 (C) days of exposure to 0, 50, 100 or 200 mM NaCl. Data are means ± S.E. (n=3). Asterisks indicate significant differences (P<0.05) relative to controls (0 mM NaCl).

In both shoots and roots of salt-treated plants, a significant increase in proline occurred starting from day 5, but only with 200 mM NaCl. On day 12, the increase was significant relative to controls also with 100 mM NaCl and by day 19 the increase in shoots was *ca.* 6-fold and 26-fold with 100 and 200 mM NaCl, respectively; salt-induced proline accumulation in roots was equally dramatic (Fig. 2).

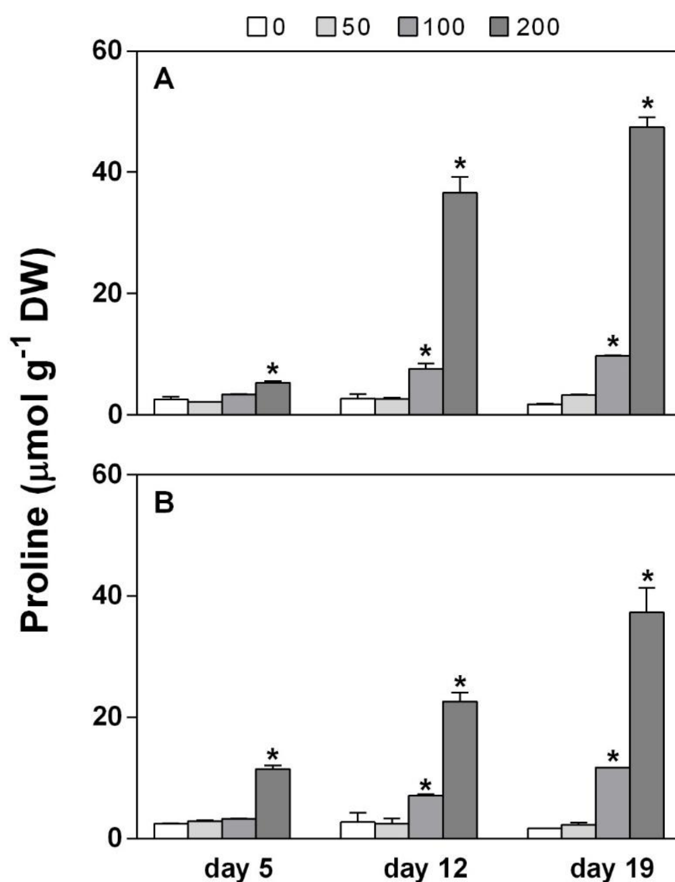

Fig. 2. Proline concentration in shoots (A) and roots (B) after 5, 12, and 19 days of exposure to 0, 50, 100 or 200 mM NaCl. Data are means ± S.E. (n=3). Asterisks indicate significant differences (P<0.05) relative to controls (0 mM NaCl) for each sampling time.

Salt-induced oxidative stress was evaluated by measuring lipid peroxidation (MDA assay) and H_2O_2 production. In shoots, MDA production was significantly enhanced by all NaCl treatments starting from day 5; on day 19, it was *ca*. 40% and 70% higher than in controls with 100 and 200 mM NaCl, respectively (Fig. 3A). In roots, only the highest NaCl concentration significantly enhanced MDA levels, while other treatments had no effect (Fig. 3B). H_2O_2 levels were higher in all NaCl-treated shoots relative to controls at day 5; on day 19, the increase remained significant only for the two higher doses of salt (Fig. 3C). Roots exhibited a similar early response to all salt concentrations, whereas by day 19 only 200 mM NaCl still exerted an effect (Fig. 3D). GSH concentrations in shoots and roots likewise revealed a salt-induced response. At day 5, the lowest concentration of NaCl had

no effect, while at day 12 it caused an increase of shoot GSH content that became significant at day 19; both 100 and 200 mM NaCl significantly enhanced the shoot concentration of GSH as compared with controls at all sampling times (Fig. 3E). In roots, 50 mM NaCl also had no effect, while the most significant increases in GSH levels where measured at 5 and 12 days with 100 and 200 mM NaCl; at day 19, only 200 mM NaCl still exerted a significant effect (Fig. 3F).

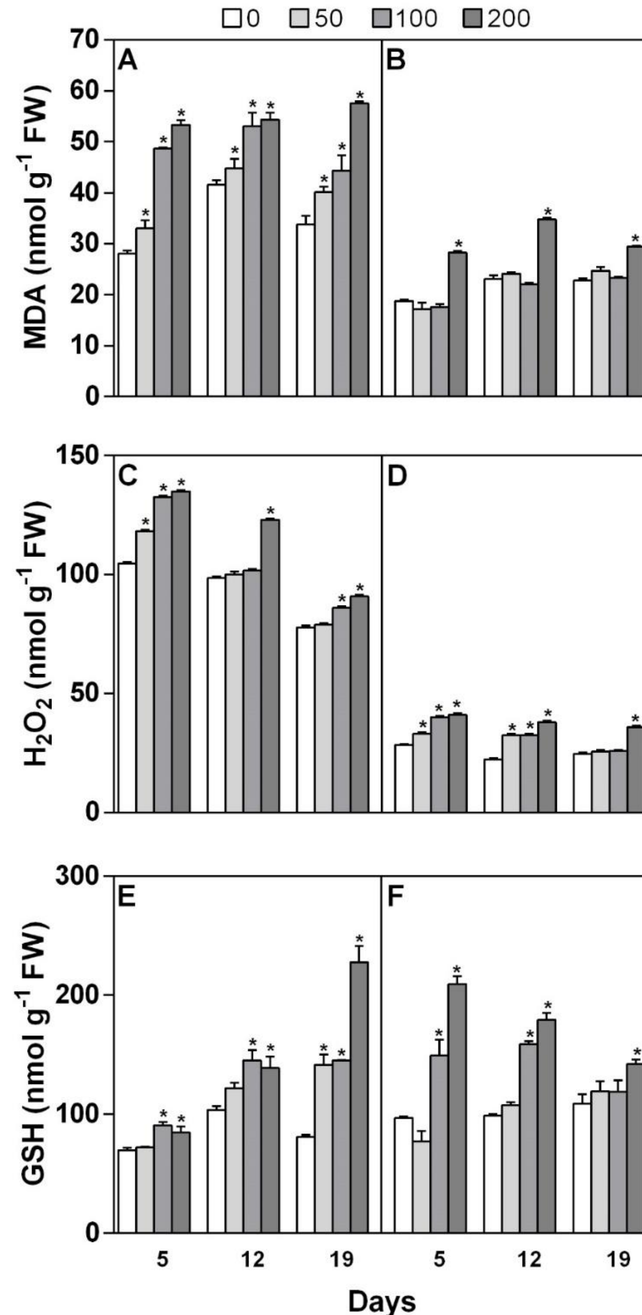

Fig. 3. Malondialdehyde (A, B), H_2O_2 (C, D) and GSH (E, F) concentrations in shoots (A, C, E) and roots (B, D, F) after 5, 12, and 19 days of exposure to 0, 50, 100 or 200 mM NaCl. Data are means ± S.E. (n=3). Asterisks indicate significant differences ($P<0.05$) relative to controls (0 mM NaCl) for each sampling time.

Effects of treatments with 100 mM NaCl and HEs

Pre-treatment with either 0.1 or 1.0 mL L^{-1} IB led to a significant shoot growth recovery, similar for both concentrations, that went *ca*. 22-25% beyond non-saline controls and that was 73.6% (FW) and 77.0% (DW) higher than with NaCl alone (Fig. 4A, C). Conversely, plants pre-treated with SC or methanol exhibited the same reduction in shoot FW and DW as those treated with NaCl alone, regardless of the dose applied. Roots also responded positively to pre-treatment with 1.0 mL L^{-1} IB (PRE-IB 1.0) in terms of FW, but not DW (Fig. 4B, D). Improved shoot growth (FW and DW) relative to both control and saline conditions was also registered after post-treatment with IB (POST-IB) irrespective of IB concentration (Fig. 4A, C); a significant (46.2%) growth recovery, but only in terms of DW, was also observed after post-treatment with 1.0 mL L^{-1} SC (POST-SC 1.0) (Fig. 4C). Root growth (FW and DW) in the presence of 100 mM NaCl was likewise improved, even beyond control levels, by POST-IB 1.0 and, as regards DW, also by POST-SC 1.0 (Fig. 4B, D). Methanol had no effect on the growth performance of maize plants.

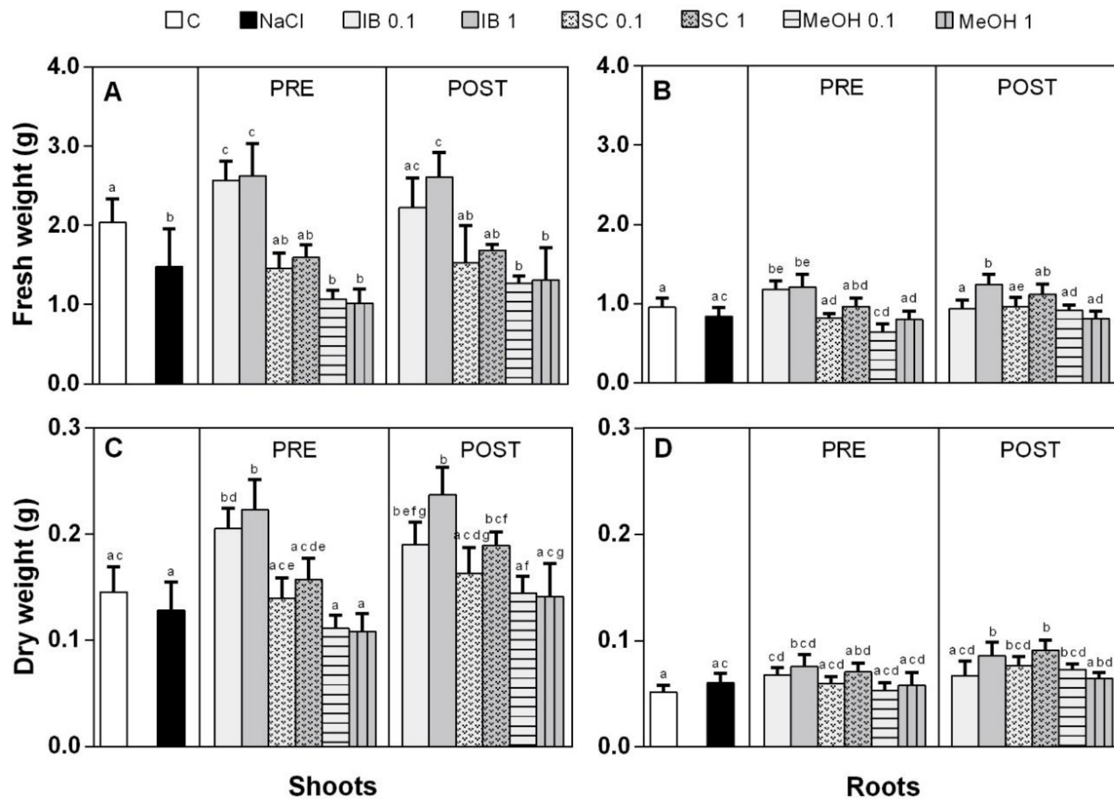

Fig. 4. Shoot (A, C) and root (B, D) fresh and dry weights after 12 days of exposure to 100 mM NaCl in the presence of 0.1 or 1 ml L^{-1} IB, SC, or methanol (MeOH) added two days before (PRE) or two days after (POST) the start of salt treatment. Data are means ± S.E. (n=10). Different letters indicate significant differences (P<0.05).

Under saline conditions, both shoots and roots accumulated Na^+, although the increase, relative to controls, was higher in the former (23-fold) than in the latter (*ca*. 10-fold). By contrast, K^+ levels declined in both organs (25% and 47% in shoots and roots, respectively; Fig. 5A, B). Na^+ accumulation in shoots was not affected by IB treatments, but it was reduced to the same extent by PRE-SC 1.0 and PRE-MeOH 1.0 (Fig. 5A). Na^+ uptake in roots was most effectively reduced by PRE-MeOH 0.1 and POST-IB 1.0; conversely, it was enhanced by both doses of PRE-IB and POST-SC (Fig. 5B). The decline in shoot K^+ levels in salt-treated *vs* control plants was slightly, but significantly ($p < 0.05$), reverted by PRE-IB 0.1 (Fig. 5A). In roots, all treatments with IB as well as PRE-MeOH 1.0 and POST-SC (both doses) increased K^+ levels as compared with salt treatment alone (Fig. 5B). Salt stress caused a strong decline in K^+/Na^+ ratios in both shoots (from *ca*. 45 in controls to 1.5 with NaCl) and roots (from 11.5 to *ca*. 0.7 in control *vs* salt-treated plants). Treatment with HEs or MeOH had no ameliorative effect, except POST-IB 1.0, which doubled the ratio in roots, relative to salt alone, by increasing K^+ without increasing Na^+ levels.

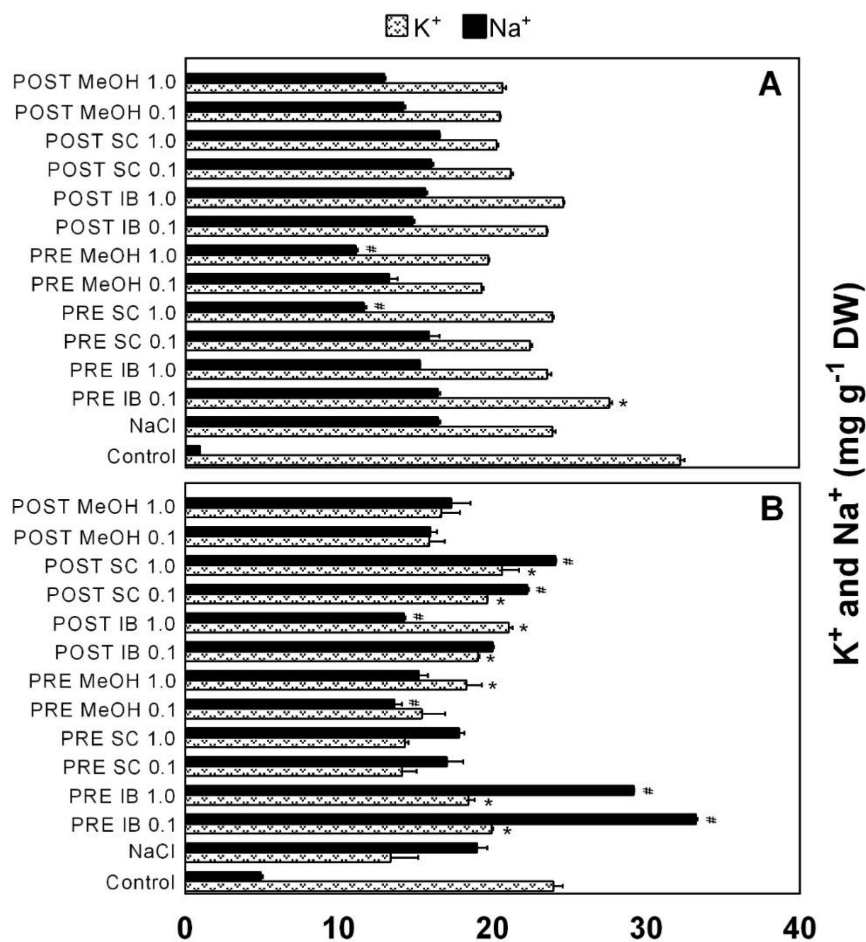

Fig. 5. Shoot (A) and root (B) sodium (Na^+) and potassium (K^+) concentrations after 12 days of exposure to 100 mM NaCl in the presence of 0.1 or 1 ml L^{-1} IB, SC, or methanol (MeOH) added two days before (PRE) or two days after (POST) the start of salt treatment. Data are means ± S.E. (n=3).

The salt-induced decline in Chl*a*, Chl*b* and their sum as well as that in total carotenoids was reverted only by pre-treatment with 0.1 or 1.0 mL L^{-1} IB (Fig. 6A). Post-treatment with IB, however, did not produce the same positive effects (Fig. 6B). Both pre- and post-treatments with SC and MeOH had no effect or even exacerbated the negative response in terms of pigment concentrations (Fig. 6). PRE-IB (both doses) also decreased the Chl*a*/Chl*b* ratio, while other treatments either had no effect or increased it. Finally, the Σcarotenoid/Σchlorophyll ratio ranged from 0.14 to 0.18 and did not change notably in relation to the treatments (data not shown).

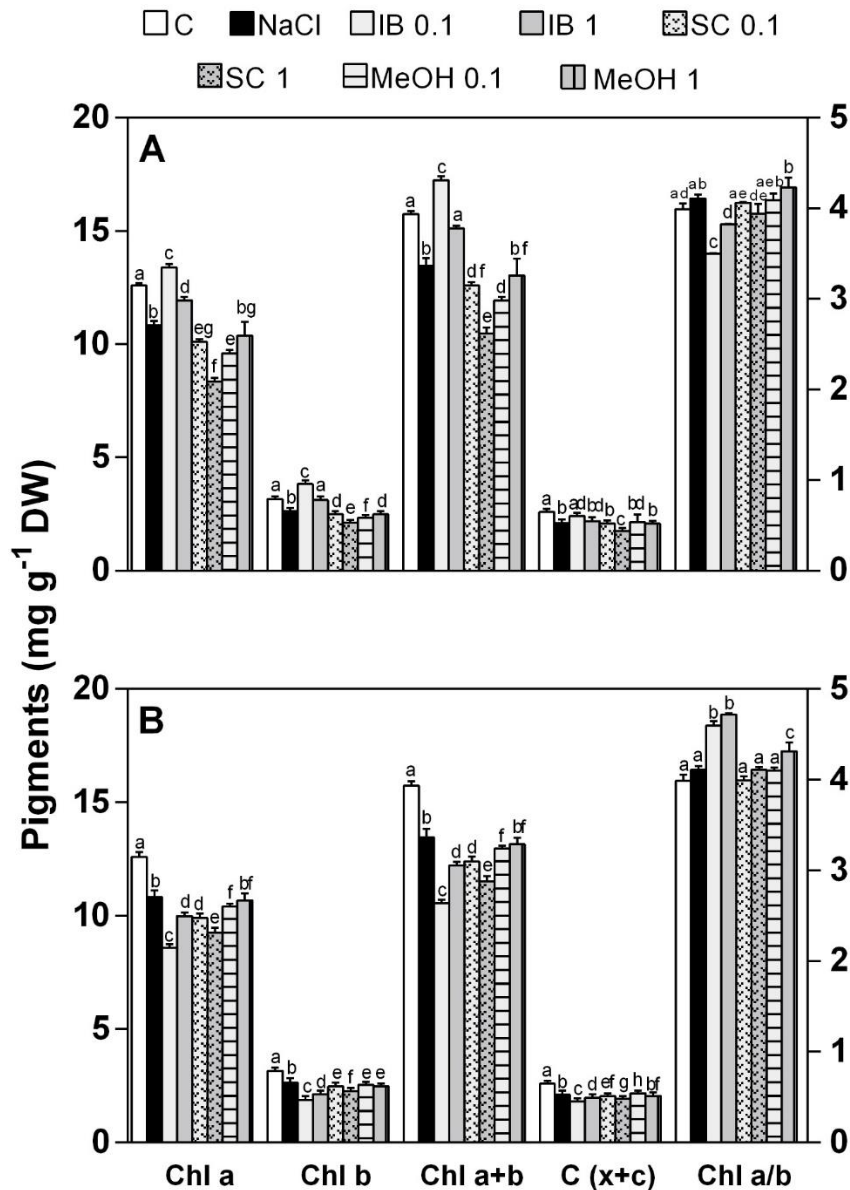

Fig. 6. Concentration of photosynthetic pigments and Chl*a*/Chl*b* ratios after 12 days of exposure to 100 mM NaCl in the presence of 0.1 or 1 ml L^{-1} IB, SC, or methanol (MeOH) added two days before (A) or two days after (B) the start of salt treatment. Data are means ± S.E. (n=3). Different letters indicate significant differences ($P<0.05$).

Upon treatment with IB (both doses), recovery of proline to control levels was observed, without differences between pre- and post-treatment; all other treatments resulted in no change relative to salt alone or even further accumulation (with methanol) of this salt stress-related compound (Fig. 7A, B).

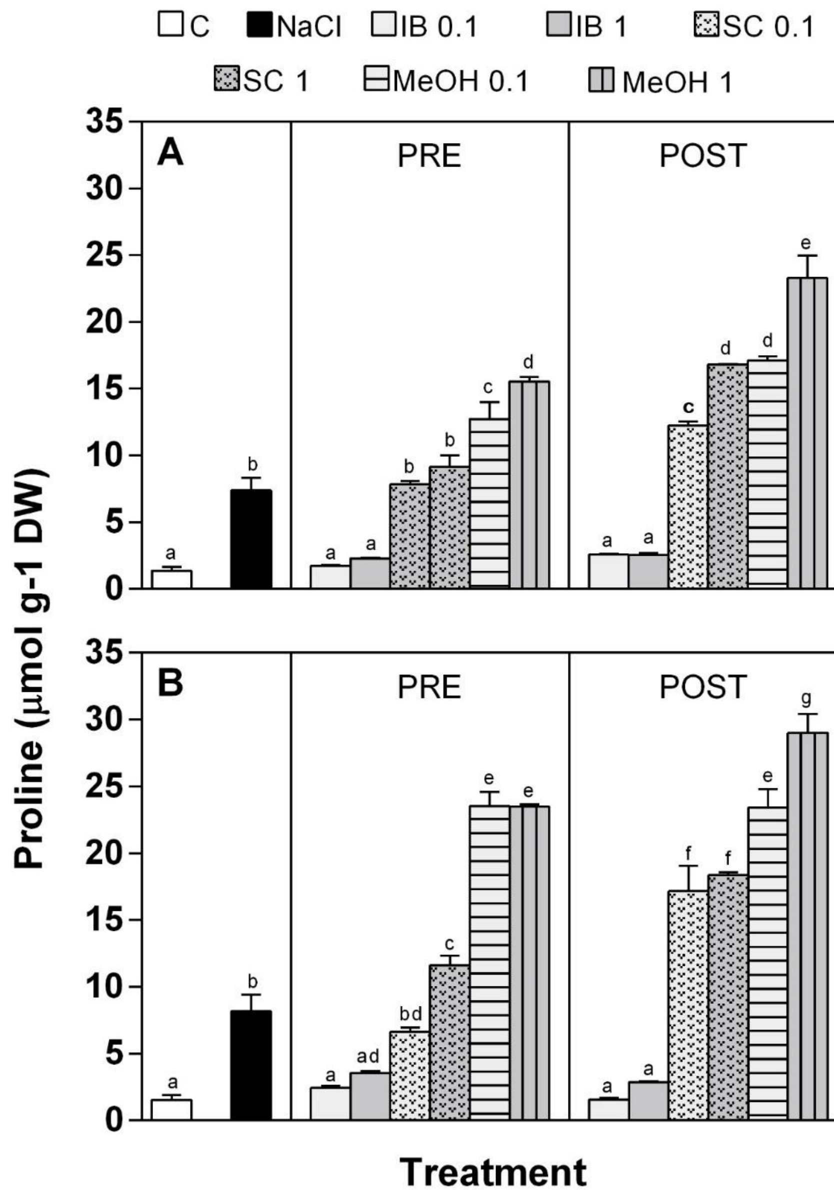

Fig. 7. Proline concentration in shoots (A) and roots (B) after 12 days of exposure to 100 mM NaCl in the presence of 0.1 or 1 ml L^{-1} IB, SC or methanol (MeOH) added two days before (PRE) or two days after (POST) the start of salt treatment. Data are means ± S.E. (n=3). Different letters indicate significant differences ($P<0.05$).

After pre-treatment with either dose of IB, MDA accumulation under saline conditions, indicative of oxidative stress, returned to control values in shoots, although the effect was not significantly different from that of PRE-MeOH 0.1 (Fig. 8A). In roots, all pre-treatments reduced MDA accumulation as compared with NaCl alone, with PRE-IB 0.1 producing the strongest ameliorative effect (Fig. 8B). POST-IB 0.1 also strongly contrasted the salt-induced MDA accumulation in both organs; in shoots, this beneficial effect was significantly stronger than with pre-treatment, while in roots pre-treatment was better than post-treatment. Post-treatment with the lower dose of SC and methanol exerted a similar stress-mitigating effect, but only in roots, so the effect was due to the solvent (Fig. 8A, B).

Salt-induced accumulation of H_2O_2 in shoots and roots was slightly, but significantly, mitigated by all pre-treatments and to the same extent. In post-treatment, however, 0.1 mL L^{-1} IB caused the strongest reduction in shoot H_2O_2 production, while other treatments had no or a negative effect (Fig. 8C, D). In roots, POST-IB 0.1 was again the most effective treatment, while all other post-treatments (except 1.0 mL L^{-1} MeOH) led to only a slight decline in H_2O_2 levels as compared with salt alone (Fig. 8D).

Salt-induced GSH content in shoots was slightly, but significantly, decreased by PRE-IB 1.0, while neither SC nor methanol had any effect. When given after the start and for the entire duration of the salt treatment, none of the extracts contrasted the rise in shoot GSH concentration (Fig. 8E). In roots, the response was quite different. In fact, a significant increase in GSH relative to NaCl was observed, both after pre- and post-treating plants with SC and methanol, while IB had no effect (Fig. 8F).

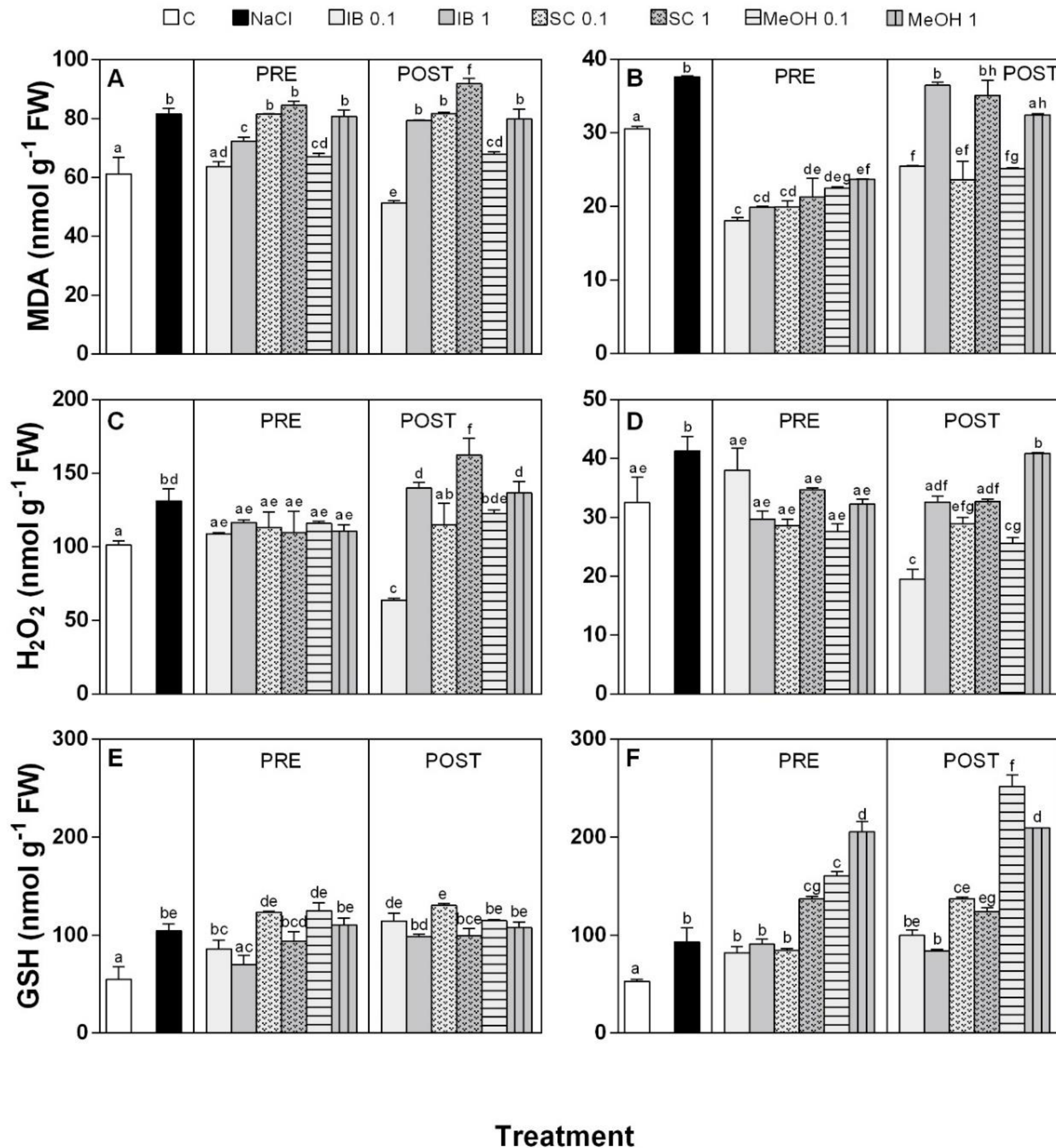

Fig. 8. Malondialdehyde (A, B), H_2O_2 (C, D) and GSH (E, F) concentrations in shoots (A, C, E) and roots (B, D, F) after 12 days of exposure to 100 mM NaCl in the presence of 0.1 or 1 ml L^{-1} IB, SC or methanol (MeOH) added two days before (PRE) or two days after (POST) the start of salt treatment. Data are means ± S.E. (n=3). Different letters indicate significant differences ($P<0.05$).

Effects of treatment with 200 mM NaCl and IB

The efficacy of the extract treatment that gave the best results with 100 mM NaCl (i.e., 1.0 mL L^{-1} IB) was tested with the higher dose of salt (200 mM NaCl) albeit for a shorter duration (8 days). As shown in Table 4, both pre- and post-treatment with IB resulted in a growth stimulation of shoots and roots relative to the salt treatment both in terms of fresh and dry biomass. Pre-treatment with IB improved shoot growth to a greater extent than the post-treatment: *ca*. 53% (FW) and 78% (DW); in

roots, FW and DW increments (relative to salt) were of the order of 50%. Although methanol also stimulated growth relative to salt, the increase was always lower than that of IB, so that net increases were around 30-40%. These positive effects on growth were not, however, accompanied by significant changes in the other parameters. Thus, the decline in photosynthetic pigments and the accumulation of proline, MDA, H_2O_2, and GSH induced by 200 mM NaCl were not mitigated, either by IB or methanol (data not shown).

	Shoot				**Root**			
	Fresh weight variation (%)							
	1 ml l^{-1} IB		**1 ml l^{-1} MeOH**		**1 ml l^{-1} IB**		**1 ml l^{-1} MeOH**	
VS	**pre**	**post**	**pre**	**post**	**pre**	**post**	**pre**	**post**
NaCl	**+ 53.1**	**+ 31.2**	**+ 25.2**	**+ 4.4**	**+ 50.9**	**+ 29.3**	**+ 10.4**	**- 5.7**
	Dry weight variation (%)							
VS	**pre**	**post**	**pre**	**post**	**pre**	**post**	**pre**	**post**
NaCl	**+ 77.8**	**+ 43.4**	**+ 44.4**	**+ 10.1**	**+ 47.5**	**+ 22.0**	**+ 16.9**	**- 5.1**

Table 4. Percent variation in shoot and root FW and DW relative to NaCl after 8 days of exposure to 200 mM NaCl and pre- or post-treatment with 1 mL L^{-1} IB or methanol.

Discussion

Present results indicate that the maize genotype used in this study was moderately sensitive to salt stress. Indeed, reductions in shoot and root growth (except root DW) of 20-33% were observed with 100 mM NaCl. This is in accord with a previous study, aimed at investigating the genetic variability of salt tolerance in maize, in which a reduction in growth from 20 to 80% was already evident at 100 mM NaCl in all tested accessions (Hoque et al., 2015). In the present study, the response to 100 mM salt was characterized by changes in several biochemical parameters, including photosynthetic pigments, proline, oxidative markers such as MDA levels and H_2O_2. Salt stress also caused a nutrient imbalance resulting in a lower K^+/Na^+ ratio. Moreover, all growth, and biochemical changes observed under saline treatments were in general more evident in shoots than in roots, even though the amount of Na^+ accumulated in the two plant portions was similar in absolute terms. The analyses performed in this work also indicate that biochemical salt-induced responses were concentration- and time-dependent. Thus, photosynthetic pigments, proline, MDA, and H_2O_2 levels were, generally speaking, higher with 200 mM NaCl than with the lower doses of salt. In particular, pigments generally declined and proline accumulated in a clear dose-dependent manner. Intensity of the responses also increased with time, except for the oxidative markers (i.e., MDA, H_2O_2, GSH), which instead show a short-term response (day 5) even at the lowest dose of salt. This is in agreement with Ashraf et al. (2018),

who observed a more pronounced accumulation of MDA and H_2O_2 levels at early growth stages compared to later ones in salt-treated maize.

IB and SC were tested for their possible role as biostimulants on maize plants subjected to salt stress. The responses appear to be differentially modulated by the two extracts, and chemical composition may account for these differences. Indeed, only IB, even at the lower concentration tested, was able to recover the performance of stressed plants in terms of growth, photosynthetic pigments content, and levels of salt stress markers, such as proline, lipid peroxidation products, and H_2O_2. Thus, IB can be regarded as a potential biostimulant able to mitigate salt stress. Present results also indicate that the biostimulant activity of IB, in salt-stressed maize plants, strictly depended on timing of treatment. Thus, whereas shoot growth recovery and reduction of proline accumulation occurred when plants were either pre-treated or post-treated with the extract, POST-IB 0.1 was most effective in mitigating oxidative stress. Conversely, the positive effect on photosynthetic pigments, i.e., recovery of Chl*a* and *b* levels, only occurred when IB was applied to plants before salt stress; when applied two days after salt treatment, the stress-alleviating effect was no longer observed. Moreover, the decrease in Chl*a*/*b* ratio induced by PRE-IB relative to control and salt-treated plants may be of particular relevance, since Chl*b* is favoured over Chl*a* in exerting a protective function of the photosystems, due to the lower photo-oxidation rate of the former compared to the latter (de Souza et al., in press). A similar alleviating effect on chlorophyll content in NaCl-stressed bean plants was reported after foliar application Howladar et al. (2014) or seed pre-soaking with *Moringa oleifera* leaf extract (Rady et al., 2013). Ertani et al. (2016) also reported that Chl*a* and Chl*b* were differentially affected by treatment with various plant extracts (blueberry, hawthorn, red grape skin). In *Salicornia*, adaptation to stress was revealed by the maintenance of a high ratio (0.12 to 0.14) of photo-protective pigments (i.e., carotenoids) against light-harvesting chlorophylls (de Souza et al., in press). In our study, neither salt alone nor salt combined with HEs had any effect on this ratio.

Proline accumulation under various types of abiotic stress (Scoccianti et al., 2016), including salinity, is a common response and overproduction of this compatible solute in transgenic plants confers a higher salt stress tolerance (Kishor et al., 1995). In addition to their osmoprotective role, organic osmolytes, such as proline, contribute to contrasting oxidative stress (Szabados and Savouré, 2010). Interestingly, IB, at both doses and times of application, reverted the salt-induced proline increase in shoots and roots to control levels, a strong indication in favor of its stress-mitigating effect**.**

Sodium and chloride are responsible for both osmotic stress and ion-specific toxicity that significantly reduce crop growth and yield. The best characterized mechanisms of tolerance involve limiting Na^+ uptake, excluding Na^+ from leaves, and efficient vacuolar compartmentation of Na^+ (Munns and Tester, 2008). Soil salinity also causes ion imbalance by affecting, for example, the uptake of

potassium (K^+), which is an essential macronutrient in plants. Maintaining high K^+/Na^+ ratios is regarded as a major strategy for coping with salinity stress in salt-sensitive (glycophyte) species (e.g., cereals, such as barley) and this is often achieved by K^+ retention rather than Na^+ exclusion (Shabala and Pottosin 2014). The positive effect of moringa leaf extract on shoot K^+ under high salinity was previously reported in wheat (Yasmeen et al., 2013). As regards ion homeostasis under salt stress, here we show that none of the treatments improved the K^+/Na^+ ratio in shoots, whereas in roots, post-treatment with the higher dose of IB was able to slightly ameliorate this parameter; the ratio increased due to enhanced K^+ levels and no change in Na^+ levels.

Salt stress is known to result in extensive lipid peroxidation, a parameter that has often been used as indicator of oxidative damage in membranes (Miller et al., 2010). Depending on timing and concentration, both HEs as well as methanol were able to reduce MDA production in salt-stressed roots. In shoots, however, post-treatment with the lower dose of IB was most effective in mitigating salt-induced lipid peroxidation as well as in reducing both shoot and root H_2O_2 concentration. In spite of the dose- and time-dependent ameliorative effect of IB on oxidative stress, growth recovery under saline conditions was observed when the extract was applied at either dose and both in pre- and post-treatment. This suggests that the positive effect of IB on growth was not simply the consequence of reduced oxidative stress, as also confirmed by the results of the experiment conducted using 200 mM NaCl, in which growth recovery was observed, without a substantial change in biochemical parameters.

It is worth noting that partial recovery from salt-induced oxidative stress was also observed with methanol alone, at the lower dose and in both organs. Some authors have investigated the biological functions of solvents used in plant experiments (Savvides et al., 2016), and a clear role of ethanol and methanol in influencing several plant responses has been reported. In *Arabidopsis thaliana* and rice plants, the application of exogenous ethanol enhanced salinity stress tolerance by regulating ROS-related genes and enhancing ROS detoxification (Nguyen et al., 2017); an increase in tolerance to chilling stress has also been reported in rice plants (Kato-Noguchi, 2008). As regards methanol, its positive effects on growth and water use efficiency has been documented in various plant species (Behrouzyar and Yarnia, 2016), even though they strictly depend on exposure time, tissue morphology and, especially, application method. In tomato, Arabidopsis, and tobacco plants, foliar spraying with methanol enhanced plant growth under normal conditions, while root applications caused severe damage (Rowe et al., 1994; Ramirez et al., 2006). Nevertheless, it has to be underlined that the methanol concentrations (0.01-0.1%, v/v) applied by us to maize roots were much lower than those used in the above-cited studies, and this can explain the, sometimes, ameliorative action or ineffectiveness of this solvent.

In plants, glutathione (G) is an essential component of the cellular antioxidant defense system. It is the substrate of glutathione peroxidase and glutathione-S-transferases, enzymes involved in the removal of ROS, and the ascorbate-G cycle is regarded as the principal means of superoxide and H_2O_2 removal. Reduced/oxidized forms of G (GSH/GSSG) influence the redox status of plant cells. Although increases in GSH levels have been measured in response to chilling, heat shock, and other forms of abiotic stress, the numerous publications on this topic (Tausz et al., 2004 and references therein) indicate that the GSH/GSSG ratio may change one way or another, suggesting that the role of G is particularly complex. In our study, maize plants exposed to 100 mM NaCl exhibited significantly higher GSH levels as compared with non-saline controls, with only a slight reduction in shoot FW/DW and no inhibition of root biomass, suggesting that GSH may have contributed to salt acclimation. The strongest increase in GSH levels, however, occurred with 200 mM NaCl, which significantly depressed plant biomass. Thus, under more severe conditions, although the plant activated this antioxidant response, it was not enough to ensure normal growth. The salt-induced increase in GSH concentrations could be interpreted as an "overcompensation" to keep G in its reduced, active form (Tausz et al., 2004) or an interference with cellular metabolism limiting the conversion of GSH to GSSG. Indeed, De Azevedo Neto et al. (2006) reported that G reductase activity in leaves of salt-stressed maize plants (both salt-tolerant and salt-sensitive) was greater than in control plants. Ruiz and Blumwald (2002) showed that synthesis of cysteine and GSH increased significantly when *Brassica napus* was exposed to salt stress. Similarly, the contents of GSH, MDA, $O_2^{\bullet-}$, and H_2O_2 were significantly increased in bean plants stressed with 200 mM NaCl (Latif and Mohamad, 2016). Overall, these results suggest that the processes leading to the accumulation of GSH are salt stress-elicited. The pattern of changes in GSH levels in response to spelt HEs and methanol showed that, in shoots, the higher dose of IB reverted salt-induced GSH accumulation when plants were exposed to 100 mM NaCl. On the other hand, roots responded in a relevant manner only to SC and, especially, methanol, which, instead, caused a further increase in GSH levels. Thus, although GSH is regarded as having a positive (antioxidant) role under stress conditions, our results indicate that GSH increased with increasing stress (100 *vs* 200 mM NaCl), so that the effect of IB can be considered as stress-mitigating, while SC and methanol had the opposite effect.

Extract composition plays an outstanding role in determining the efficacy of biostimulants (Bulgari et al., 2015). The phytochemical investigation of the two spelt HEs revealed that their chemical composition was rather different, both in qualitative and quantitative terms. In particular, SC contained, besides phenolic and hydroxycinnamic acids, also organic and fatty acids, which were absent in IB, while the latter had a much higher level of ferulic, *p*-coumaric, and caffeic acids compared to the former. Indeed, most hydroxycinnamic acids in spelt and einkorn (*Triticum*

monococcum L.) grains are localized in cell walls where they are bound to structural components, such as cellulose, lignin, and arabinoxylans (Hidalgo and Brandolini, 2014) and this might explain their higher levels in IB compared to SC. The greater amount of hydroxycinnamic acids, especially ferulic and *p*-coumaric acid, in IB could account for its higher efficacy, compared to SC, in counteracting the negative impact of salt stress. Besides the numerous functions of ferulic acid in plant growth and development (e.g., cell wall lignification), a major physiological role is likely to be its potent antioxidant capacity (Graf, 1992). Thus, the higher antioxidant potential of IB, revealed by attenuated lipid peroxidation and H_2O_2 accumulation, may be due to its higher ferulic acid content and may have contributed to alleviate the toxicity generated by salt stress. Moreover, the higher fatty acid content of SC may render this extract more hydrophobic, thus less prone to interact with the root surface and be absorbed. Differences in the biostimulatory effects in maize plants of two phenol-containing extracts, deriving from lignin-rich biorefinery wastes, have been recently reported; both extracts were able to act as biostimulants, but at different concentration ranges and through diverse mechanisms, for example, via modification of the plant's hormonal balance (Savy et al., 2017). Other authors also investigated the potential biostimulant effect of polyphenol-enriched fractions derived from plant by-products. Maize plants supplied with two different doses of extracts obtained from dry apple and blueberry residues displayed a significant increase in root and leaf biomass and a higher content in macronutrients and proteins; extracts also exerted a positive impact on secondary metabolism associated with the synthesis of phenolic compounds (Ertani et al., 2011b). Phenolic acids also have allelopathic properties. Allelopathic water extracts (AWE) containing, among other compounds, phenolic acids, can improve tolerance to abiotic stresses when applied to crops, such as wheat (Farooq et al., 2018).

Finally, a growth-stimulating effect of IB was also observed in maize plants grown under non-saline conditions, confirming its potential as biostimulant. This effect could be accounted for by the phytohormone (gibberellin, auxin)-like activity reported for phenols (Ertani et al., 2016; Savy et al., 2017) and their ability to influence endogenous phytohormone levels (Einhellig, 2004).

In conclusion, we show here that polyphenol-containing methanolic extracts prepared from spelt husks have a growth-stimulating and stress-mitigating effect on maize plants by acting on different targets, including accumulation of compatible solutes, photosynthetic pigments, oxidative stress, and ion balance. The plethora of mechanisms activated by the extract can be related to its chemical complexity, as occurs with all biostimulants, with IB generally exerting a more positive action than SC (Fig. 9). Work is in progress to investigate the effect of spelt HEs on plant polyphenol metabolism and regulation of genes involved in polyphenol biosynthesis. Experiments are also underway to test extracts prepared by using more environmentally compatible extraction procedures.

		PRE						POST					
		IB 0.1	IB 1.0	SC 0.1	SC 1.0	M 0.1	M 1.0	IB 0.1	IB 1.0	SC 0.1	SC 1.0	M 0.1	M 1.0
FW	S												
	R												
DW	S												
	R												
Chl*a*	S												
Chl*b*	S												
Chl*a*+*b*	S												
Cc+x	S												
proline	S												
	R												
MDA	S												
	R												
H_2O_2	S												
	R												
GSH	S												
	R												

Fig. 9 Graphical representation of the effects on growth and metabolic parameters of pre- or post-treatments with IB, SC or methanol in shoots (S) and roots (R) of maize plants grown under saline (100 mM NaCl) conditions. Darker and lighter colour shadings represent relatively higher and lower values, respectively.

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4. Effect of spelt husk extracts on other physiological parameters and as priming agents

Unpublished data

4.1 MATERIALS AND METHODS

4.1.1 Determination of total phenols content (tps)

The total phenols content of IB (insoluble-bound phenolic acid fraction) and SC (soluble-conjugated phenolic acid fraction) extracts was determined using the Folin-Ciocalteu method as previously reported by Singleton et al. (1999). The principle of this assay is the reduction of the Folin-Ciocalteu reagent (FCR) in the presence of phenolates, resulting in the production of molybdenum-tungsten blue. For the analysis, extract solutions were serially diluited (1:2) in Milli-Q water and 125 µL of each dilution added to 0.5 mL of Milli-Q water and 125 µL of FCR. After 6-min reaction, 1.25 ml of 7% aqueous sodium carbonate solution and 1 mL of distilled water were added into the mixture in sequence. After 90 min of incubation at room temperature in the dark, the developed color was measured at 760 nm using a UVIKON 930 spectrophotometer (Kontron Instruments). A calibration curve was prepared using gallic acid (from 2 to 10 µg mL^{-1}) as a standard and the total phenolic content was expressed as mg of gallic acid equivalents (GAE)/mL extract.

4.1.2 DPPH assay

The 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\cdot}$) assay was performed as reported by Brand-Williams et al. (1995), with slight modifications. The assay is based on the reduction of the purple $DPPH^{\cdot}$ to 1,1-dyphenyl-2-picrylhydrazine in the presence of an antioxidant molecule, giving rise to a yellow solution. The reduction of $DPPH^{\cdot}$ is followed by monitoring the decrease in its absorbance at 515 nm. Briefly, 100 µl of IB or SC extracts were mixed with 900 µl of freshly made DPPH solution (100 µM in methanol) and incubated for 10 minutes in the dark at room temperature. The absorbance of the mixture was measured at 515 nm using a UVIKON 930 spectrophotometer (Kontron Instruments). Initial absorbance readings for DPPH, used as control, were 1.08±0.05. A calibration curve was prepared with Trolox (from 2 to 20 µm) used as a standard. The antioxidant activity was expressed as µmol of Trolox equivalents (TE)/mL extract.

4.1.3 ABTS assay

The 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity of IB and SC extracts was measured as previously reported by Ferri et al. (2013), with slight modifications. The assay is based on the generation of a blue/green $ABTS^{\cdot+}$ radical cation that can be reduced by antioxidants. The ABTS working solution was prepared by dissolving ABTS salt in water to a final concentration of 7 mM and then treating with 140 mM potassium persulfate. The

solution was allowed to stand for 12-16 hours in the dark, at room temperature, to produce the $ABTS^{\cdot+}$ radical. Immediately before the analysis, the $ABTS^{\cdot+}$ radical solution was diluted 1:90 in ethanol to reach the absorbance value of 0.70±0.02 at 734 nm (control). The assay was performed as follows: 990 µl of diluted $ABTS^{\cdot+}$ radical solution was mixed with 10 µl of IB (diluted 1:10, 1:20 and 1:30) or SC (diluted 1:2, 1:5 and 1:10) extracts. A calibration curve was prepared with Trolox (from 2 to 20 µm) used as a standard. After 5 min incubation, the absorbance was read at 734 nm, using a UVIKON 930 spectrophotometer (Kontron Instruments), and the antioxidant activity expressed as µmol of Trolox equivalents (TE)/mL extract.

4.1.4 ORAC assay

The Oxigen Radical Absorbance Capacity (ORAC) assay was performed using a Fluostar Optima plate reader fluorimeter (BMG Labtech, Offenburgh, Germany), equipped with a temperature-controlled incubation chamber and automatic injection pump, as previously reported (Ninfali et al., 2009). The assay is based on the free radical damage to a fluorescent probe such as fluorescein with a consequent reduction in fluorescent intensity. Addition of an antioxidant absorbed the generated ROS, allowing the fluorescent signal to persist. The ROS generator is AAPH (2,2′-azobis (a-amidinopropane)) which produces a peroxyl free radical upon termal decomposition at 37 °C. The reaction mixture consisted of: 200 µL of fluorescein 0.06 µM in 75 mM sodium phosphate buffer, pH 7.0, and 20 µL of IB or SC extracts tested at different dilutions. Parallel reaction mixtures were set with 20 µL of Trolox (from 50 to 500 µM) to obtain the standard curve. For the blanck 20 µL of phosphate buffer were added to the fluorescein solution. The reaction was initiated by adding 80 µL of AAPH 0.33 M and fluorescence was read at 485 nm (excitation) and 520 nm (emission) until complete extinction. ORAC values were expressed as µmol of Trolox equivalents (TE)/mL extract.

4.1.5 Plant treatment

Plant growth and treatments were performed as described in Ceccarini et al. (2019). Afther germination, plants were grown for 10 days in the pots and exposed or not to 100 mM NaCl for 12 additional days. Based on previous results indicating that pre-treatment was more effective than post-treatment and that both extracts were already active at the lower dose, plants were pre-treated with 0.1 ml L^{-1} IB or SC extracts for two days before the beginning of salt treatment. At the end of the treatment the following parameters were determined.

4.1.6 Leaf chlorophyll content index (CCI)

Chlorophyll content index was measured between 9.00 and 11.00 a.m. with a portable chlorophyll meter (CCM-200, Opti-Science) which calculates the CCI by measuring leaf absorbance at two different wavelengths. One at 653 nm (red light), that falls within the chlorophyll absorbance range, and one at 931 nm to correct the red light absorption from other cell components. CCI values were taken from the second fully expandend functional leaf on each plant. A total of 4 plants were randomly measured from each treated group, and 4 measurements were taken in different areas of each leaf, around the midpoint of the leaf blade. These values were averaged for the mean CCI reading of each leaf.

4.1.7 Stomatal conductance (G_S)

Stomatal conductance was measured between 9.00 and 11.00 a.m. with a porometer (SC-1 porometer Decagon Devices, MRETR group) which measures the rate of passage, in terms of conductance, of carbon dioxide (CO_2) or water vapor through leaf stomata. Measurements were taken as described for CCI determination.

4.1.8 Leaf water potential (Ψ_{leaf})

The leaf water potential was measured in a plant water status console (model 3000 F01H12G2P40, Soilmoisture Equipment Corp.) by using the pressure chamber technique as described by Scholander et al. (1965). A fully expanded leaf was collected with only one incision with a sharp razer blade and immediately sealed in a polyethylene bag to prevent water loss due to transpiration. The leaf was then placed in the pressure chamber with the cut side visible outside the chamber. Pressurizing the leaf until the xylem sap just appears at the cut surface gives a measure of the hydrostatic presure. Indeed, the pressure value recorded equals the negative water potential of the leaf. The second fully expanded leaf of 4 different plants for each treatment was collected. At the end of measurements the cut leaves were stored at -80 °C and used for the determination of leaf osmotic potential and abscisic acid (ABA) content.

4.1.9 Leaf osmotic potential (Ψ_π)

For the osmotic potential determination leaves were thawed for 15 min at room temperature. The leaf sap was extracted by squeezing it with forceps and the resulting liquid collected with filter paper. The collected sap was then analysed using a psychrometer (C-52 sample chambers, Wescor Inc.) connected to a data logger (Wescor's Dew Point Microvoltmeter, model HR-33T).

4.1.10 ABA content

For the determination of ABA, leaf samples were crushed using mortar and pestle in liquid nitrogen. Thirty mg of the powder were dissolved in 1.0 mL distilled water, homogenised by using an end-over-end rotatory shaker for 24 h at 4 °C and then centrifuged at 10.000 x*g* for 5 min at 4 °C. The clear supernatant was collected and stored at 4 °C until analysis. Leaf ABA concentration was determined through an enzyme-linked immunosorbent assay (ELISA) using a monoclonal antibody for ABA (AFRC MAC252) as described by Asch (2000).

4.1.11 Seed priming

Maize seeds were surface-sterilized with 3% calcium hypoclorite for 10 min and then washed five times with sterile distilled water. Seeds were then soaked in distilled water or in a solution containing 0.1 mL L^{-1} IB, SC or MeOH and incubated for 7 h at 25 °C in the dark. Afther removal of the priming solution, seeds were washed with sterile distilled water, spread on filter paper and air-dried overnight at room temperature.

4.1.12 Germination test and pot study

The germination test was performed following the guidelines provided by the International Seed Testing Association (ISTA, 2020). Briefly, primed seeds were transferred on a sheet of filter paper previously soaked with distilled water or 100 mM NaCl, covered with another wet sheet, and rolled together into a loose cylinder. This was wrapped in plastic bags to maintain high humidity and put in a germination chamber at 25 °C in the dark. Four replicates with 50 seeds per replicate were used for each treatment. The evaluation of germination was performed, according to ISTA, after 4 (first count) and 7 (final count) days from the beginning of the test. Seeds were considered normally germinated when the radicle had emerged at least 3 mm from the seed coat.

For pot studies, primed seeds were germinated in Petri dishes, tranferred to plastic pots, grown until maturity (10 days old) and exposed to salinity stress following the protocol described in Ceccarini et al. (2019). Afther 12 days of exposure to 100 mM NaCl plants were harvested and CCI, G_S, Ψ_{leaf} and Ψ_{π} were measured as described above.

4.2 RESULTS AND DISCUSSION

4.2.1 Evaluation of the antioxidant properties of the extracts and their effects on physiological parameters

The antioxidant properties of polyphenols have been widely studied and it has become clear that their biological activities go beyond their antioxidant capacity (Scalbert et al., 2005; Virgili and Marino, 2008). Salt stress causes a redox imbalance and, in this context, phenol-enriched biostimulants may also have protective effects as ROS scavengers. In the light of these evidences, we sought to characterize the antioxidant properties of IB and SC extracts not only in terms of phenol composition, as reported in Ceccarini et al. (2019), but also of antioxidant capacity. Numerous methods that allow to measure total antioxidant capacity *in vitro* exist in the literature. These can be classified into two groups, depending on the reaction mechanisms: assays based on hydrogen atom transfer (HAT) and assays based on electron transfer (ET). Most of HAT-based methods apply a competitive scheme, in which antioxidant and substrate compete for thermally generated peroxyl radicals deriving from the decomposition of azo-compounds (Zulueta et al., 2009). An example of these assays is the Oxygen Radical Absorbance Capacity (ORAC) assay. Conversely, ET-based assays measure the antioxidant's capacity in reducing an oxidant, which changes colour upon reduction, and the degree of colour change is correlated with the antioxidant concentration. ET-based assays include, among others, the Trolox Equivalence Antioxidant Capacity (TEAC, also called ABTS; Miller et al., 1993), and the DPPH assay (Brand-Williams et al., 1995). Each of these methods has its advantages, limitations, and large variability, depending on the matrix. Thus, no single method is sufficient for a valid evaluation of the antioxidant capacity of a plant extract, given the wide range of natural antioxidants with different modes of action (Young, 2001). An interlaboratory comparison of six different methods demonstrated that DPPH and ABTS assays are the easier and more reproducible tests to measure antioxidant potential (Buenger et al., 2006), however, the ORAC assay is considered more reliable than others, since it involves the action of the peroxyl radical, which is a rather common radical in biological systems.

Based on these considerations, the antioxidant activity of IB and SC extracts was measured using ORAC (Fig. 8A), DPPH (Fig. 8B) and ABTS (Fig. 8C) assays.

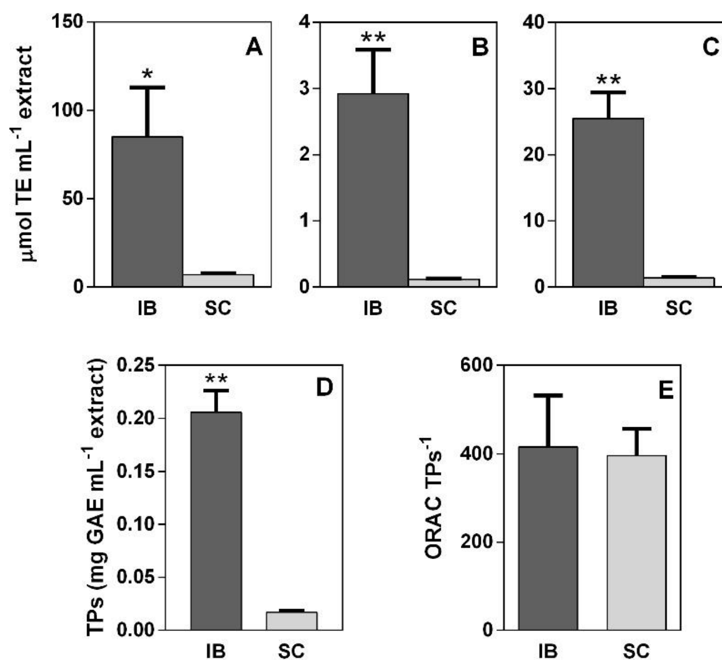

Fig. 8 *Oxygen radical absorbance capacity (A); DPPH (B) and ABTS (C) radical scavenging activity; total phenolic content (D); ORAC/phenolic ratio (E) of SC and IB extracts. Data are means ± S.E. Asterisks indicate significant differences (P < 0.05).*

The antioxidant potential of both extracts was quantitatively different depending on the assay used in the order: ORAC > ABTS > DPPH, with IB showing, in all cases, a significantly higher antioxidant capacity than SC. In agreement with these results, total phenolic concentration was 10-fold higher in IB compared to SC (Fig. 8D), confirming our previous results deriving from HPLC determinations (Ceccarini et al., 2019). Based on these considerations, one would expect that the effects of IB at 0.1 ml $L^{-1}$and those of SC at a 10-fold higher dose would be similar, assuming that their actions were mainly attributable to their antioxidant capacity. However, results previously obtained (Ceccarini et al., 2019) clearly indicated that IB and SC differentially affected several parameters (such as fresh and dry weight, concentration of photosynthetic pigments, and proline, among others), with IB having a higher ameliorative action compared to SC; in a few cases, however, SC had a similar or stronger effect. This suggests that plant protection from salinity stress cannot be merely ascribed to the antioxidant activity of the extracts, but probably involves a biostimulant activity as well.

To further support this hypothesis, a new pot trial was conducted by selecting the lower dose and the pre-treatment protocol as the best conditions to obtain the highest protection (Ceccarini et al., 2019). These experiments were performed during my stay at the Department of Plant and Environmental Sciences, University of Copenhagen, where suitable equipment was available to

analyse several plant physiological parameters. A marked decrease in chlorophyll content index (CCI), determined *in vivo* using a portable chlorophyll meter, was observed under salt stress (Fig. 9A) but it was partially prevented when the plants were pre-treated with IB or SC.

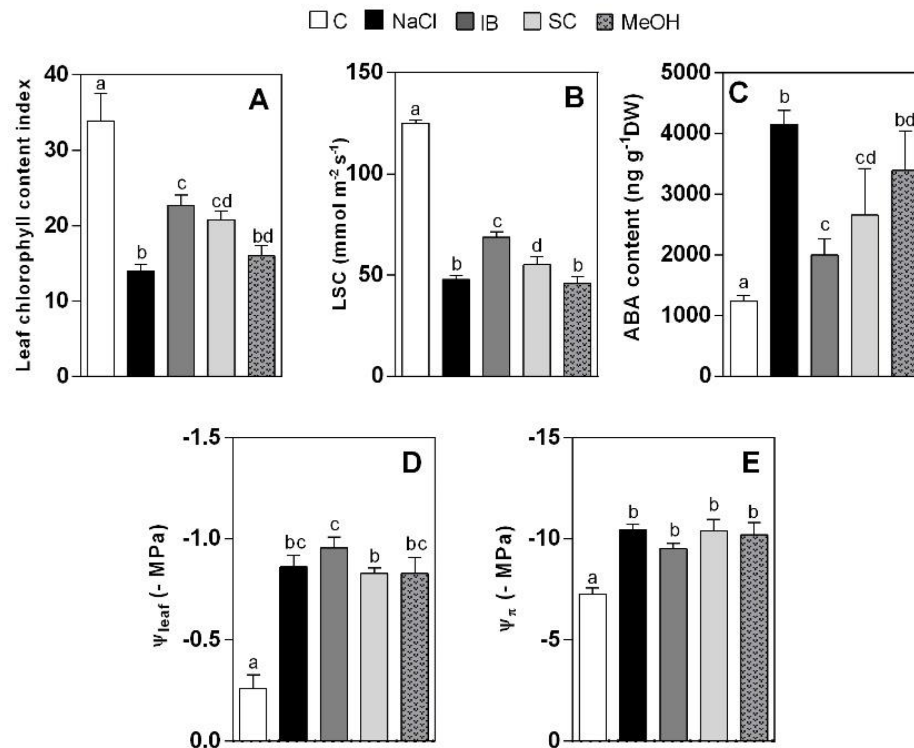

Fig.9 *Chlorophyll content index (A), leaf stomatal conductance (B), ABA content (C), water (D) and osmotic (E) potentials of maize leaves after 12 days of exposure to 100mM NaCl in the presence of 0.1 ml L^{-1} IB, SC, or methanol (MeOH) added two days before (pre-treatment) the start of salt treatment. Data are means ± S.E. (n=4). Different letters indicate significant differences ($P < 0.05$).*

These observations are in agreement with those reported by Ceccarini et al. (2019) where the total leaf chlorophyll was determined. Although faster and non-destructive, the evaluation of the CCI gives less accurate results than the spectrophotometric determination of chlorophyll content, but it was useful as a preliminary screening to highlight differences among treatments.

Our results show that salt stress caused a marked reduction in both water and osmotic potentials (Ψ_{leaf} and Ψ_{π}) in leaf tissues (Fig. 9D), thus confirming previous reports (Alarcón et al., 2006; Álvarez et al., 2012). Indeed, when plants grow under high salinity conditions they are actually exposed to an osmotic stress. High salt concentrations decrease the osmotic potential of soil, which, in turn, decreases the availability of water, thus impairing water and nutrient uptake into plant roots. This

event results in a decreased leaf water potential, which is usually accompanied by a significant reduction in the leaf osmotic potential through passive dehydration and/or active accumulation of organic and/or inorganic osmolytes (Munns, 2002; Tester and Davenport, 2003).These effects were not reverted by treatment with either IB or SC extracts (Fig. 9D and 9E).

Alteration in the balance between water and osmotic potentials in leaves generally leads to a change in turgor pressure, which regulates stomatal opening. Although extract application did not affect the water status of maize plants, a slight enhancement in leaf stomatal conductance (LSC) was observed in salt-stressed plants treated with both IB and SC (Fig. 9B). Stomatal conductance indicates not only the rate of water loss by transpiration, but also the CO_2 diffusion into the leaf for photosynthesis. Because of this role, the value of stomatal conductance provides preliminary information on the photosynthetic performance of the plant. Thus, the improvement of stomatal conductance is in accord with increased CCI values and total chlorophyll content (Fig. 9A and 9B; Ceccarini et al., 2019). Stomatal opening and closure is controlled by the hormone abscisic acid (ABA). It is well known that under stressful conditions such as high salinity, ABA content increases leading to stomatal closure in order to prevent water loss (Sah et al., 2016; Niu et al., 2018). In line with this evidence, in maize plants exposed to salt stress, ABA levels were significantly increased compared to controls (Fig. 9C). Interestingly, a significantly lower ABA concentration was found in the leaves of the plants treated with the extracts when compared to salt-stressed plants with a more pronounced effect of IB compared to SC. Solvent treatment (MeOH) was ineffective in lowering ABA levels. A decreased level of free ABA after application of biostimulants was reported in plants grown under different stressful conditions, including salinity stress (Przybysz et al., 2010; Ladan Moghaddam and Soleimani, 2012; Bulgari et al., 2019), suggesting that low ABA levels are correlated with a positive effect on plant growth and physiology under stress.

Overall, these results confirm that IB and SC act as biostimulants able to ameliorate several biochemical and physiological parameters on maize plants exposed to salinity stress.

4.2.2 Seed priming experiments

In addition to seedling growth, seed germination is also a critical stage sensitive to abiotic stress and essential for the establishment of crops (Petrović et al., 2016). In order to enhance seed germination under extreme temperatures, drought and salinity stress conditions, seed priming could be considered as a useful technique for obtaining stress-tolerant plants (Yadav et al., 2011; Jisha et al., 2013; Paparella et al., 2015). Furthermore, seed priming could enable glycophyte species to cope with saline conditions (Kazemi and Eskandari, 2012; Gholami et al., 2015). Since the use of biostimulants to counteract abiotic stress is well recognised, their use as seed priming agents could

be an innovative solution to protect seeds and stimulate their germination in the presence of stress. Based on these evidences, the potential efficacy of IB and SC extracts as priming agents was evaluated on maize seeds. The priming technique was performed using the lower dose of extracts (0.1 mL L^{-1}), which positively affected most of the parameters previously evaluated in maize plants exposed to salt stress (Ceccarini et al., 2019). After the priming procedure, both germination test and pot studies were performed. The germination test was carried out according to the ISTA guidelines and counting of germinated seeds was recorded at 4 and 7 days (Fig. 10).

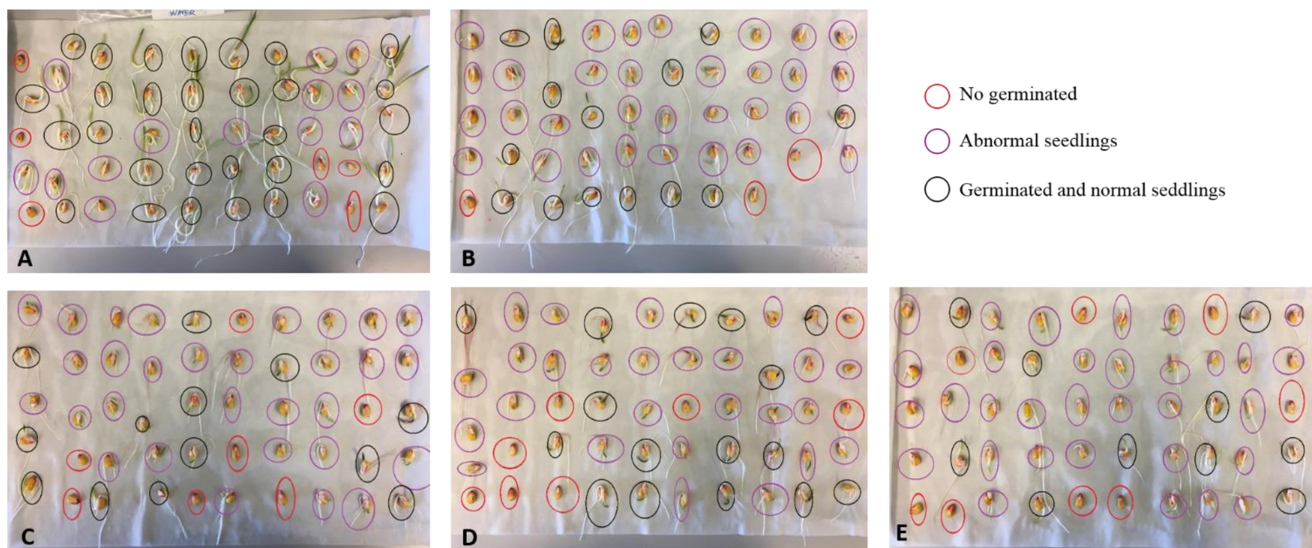

Fig. 10 *Representative images of maize seeds germinated in the absence (A) or in the presence (B-E) of NaCl (100 mM). Water (A, B), IB (C), SC (D) or MeOH (E) were used as priming agent, respectively.*

As expected, salinity treatment significantly decreased germination of maize seeds. In fact, germination percentage under saline conditions was 24.7% and 32.7% at 4 and 7 days, respectively, compared to a 60% and 71.5% in control condition that is in absence of salt stress (Fig. 11A and 11B).

The priming treatment with IB, SC or MeOH had no effect on the germination percentage of maize seeds under non-saline conditions nor was it able to counteract the inhibition of seed germination in the presence of NaCl. The effect of the extracts on seed priming was the same for both time points (4 and 7 days from the beginning of treatment).

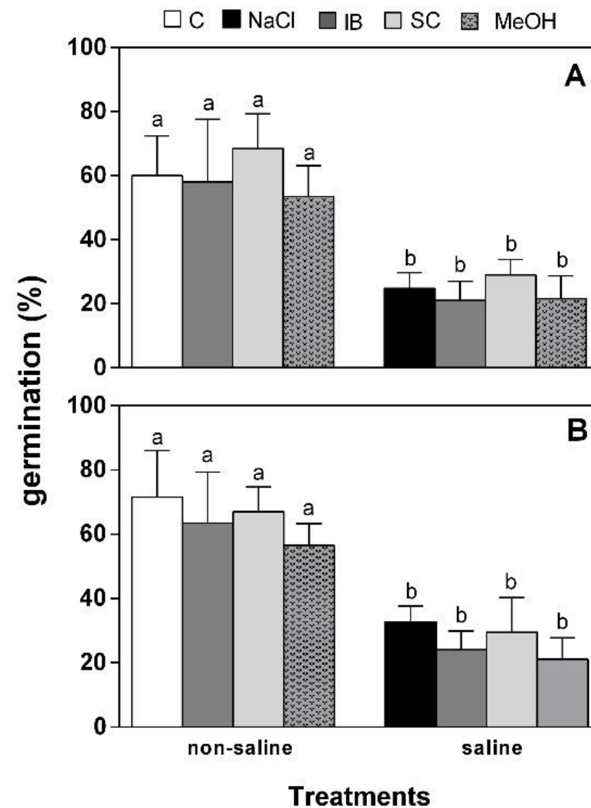

Fig. 11 *Effect of maize seed priming on germination percentage evaluate after 4 (A) and 7 (B) days from the beginning of the test in non-saline (0 mM NaCl) and saline (100 mM NaCl) conditions. Priming was performed with 250 ml of water (control) or IB, SC and MeOH at the concentration of 0.1 ml* L^{-1}*. The statistical analysis was performed on raw data. Different letters indicate significant differences (*$P < 0.05$*).*

These results are in agreement with those reported by Masondo et al. (2018), who evaluated how seed priming with different biostimulants modulated germination and growth of *Ceratotheca triloba* seedlings exposed to several stresses. In particular, with regard to salt stress, these authors observed a decrease in germination by increasing salt concentrations, which was not reverted by the application of biostimulants

Based on evidences by Chunthaburee et al. (2014), who observed that seed priming with phytohormones was able to confer salt tolerance to rice seedlings when exposed to stress conditions 17 days after treatment, we also evaluated whether IB and SC, used as priming agents, were able to confer a delayed tolerance to salt stress. Consequently, salt treatment was applied for 12 days to 10-day-old maize seedlings, derived from primed seeds. At seedling maturity (23-day-old plants), CCI, LSC, Ψ_{leaf} and Ψ_{π} were evaluated (Fig. 12).

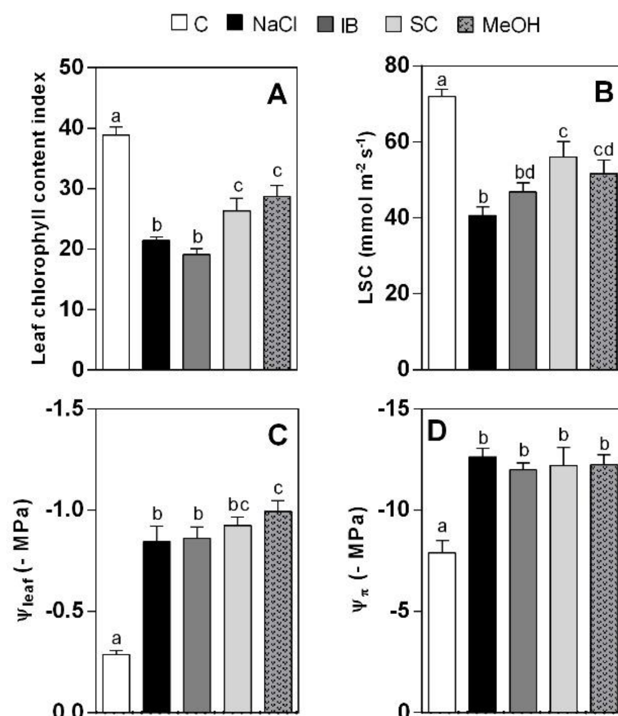

Fig. 12 *Chlorophyll content index (A), stomatal conductance (B), water (C) and osmotic (D) potentials in leaves of maize plants deriving from germinated primed seeds exposed to 100 mM NaCl for 12 days. Priming was performed with 250 ml of water (control) or IB, SC and MeOH at the concentration of 0.1 ml L^{-1}. Data are means ± S.E. (n=4). Different letters indicate significant differences ($P < 0.05$).*

Both CCI (Fig. 12A) and stomatal conductance (Fig. 12B) were affected by salinity, with a reduction of about 40% compared to controls. Priming with SC, but not IB, was associated with a slight, although significant, increase of CCI and of stomatal conductance in salt-stressed plants compared to plants derived from hydro-primed seeds (control) and subjected to salinity stress. Both leaf water potential and osmotic potential decreased due to the stress imposed by 100 mM NaCl (Fig. 12C and 12D), and no ameliorative effects were observed in the case of priming treatments.

Priming with MeOH alone exerted similar effects as SC on CCI, LSC, and leaf water potential with no significant differences, suggesting that the solvent, rather than other components of the extract, was responsible for the observed effects. As described in Ceccarini et al. (2019), it has been reported that priming with alcoholic solutions (e.g., ethanol) resulted in earlier and more uniform germination although no improvement was recorded for final germination percentage, shoot length, and seedling dry weight (Farooq et al., 2006). Considering this, our observation that MeOH priming could have beneficial effects on plant tolerance to salinity is worthy of further investigations

5. CONCLUSIONS AND FUTURE PROSPECTS

Since crops are frequently exposed to adverse environmental conditions, which limit their development and yields, it has become essential to identify and develop strategies to protect them, especially in a global change scenario. It is now clear that approaches directed to improve agricultural technologies (e.g. irrigation, re-cultivation, fertilization, etc.) are insufficient, thus plant protection should be based on the stimulation of their growth and innate protective capacities. Biostimulants offer a potentially novel approach for modulating biochemical and physiological processes in plants, with the final goal to stimulate growth, mitigate stress and, ultimately, increase yield. The application of biostimulants in crucial phases of plant development, such as before seed germination or during seedling establishment, appears fundamental to provide protection from stress. Furthermore, the possibility to extract biostimulants from crop residues and agro-industrial by-products makes this approach low-cost and environment friendly.

Spelt, one of the oldest cereals cultivated in Italy, is considered the ancestor of wheat. In the last centuries, its cultivation has been greatly reduced because of changes in dietary habits that set aside foods considered as "poor", the discovery of new higher-yielding wheat varieties and the high cost of ancient grains. Only recently, there has been a return of this grain in Italian cropping systems due to the greater attention for the environment and an increasing interest in preserving local agro-biodiversity and in the nutritional properties of spelt. Because of its recently developed uses in the food industry, spelt husk has become easily available. This agro-industrial residue represents an interesting source of biostimulants because it contains bioactive molecules, including phenolics, which modulate physiological processes in plants. Ertani et al. (2016) already investigated the application in agriculture of biostimulants enriched in phenols, obtained from food by-products, and reported their efficacy in stimulating plant growth in the absence of stress.

In this thesis, I evaluated the biostimulant activity of two methanolic extracts, termed SC and IB, obtained from spelt husks and containing soluble conjugated and insoluble bound polyphenols, respectively. Polyphenols act as potent antioxidants, but they also have a role in plant growth as physiological regulators or chemical messengers, in plant protection from UV-B radiation, and in allelopathic processes. When applied to maize seedlings growing in semi-hydroponic conditions, IB and SC extracts were able to positively affect many biochemical and physiological parameters, which are usually heavily altered by salinity stress, thus conferring a greater salt tolerance and stimulating growth. Both doses of extracts tested were effective in ameliorating the stress-related parameters and, for some of these, the lower dose tested exhibited a more pronounced effect. Between the two extracts, IB generally showed a more positive protective effect than SC, which could be ascribed to their different chemical composition. Application of the extract prior to or after treatment with salt also led to relevant differences in growth and biochemical parameters.

Interestingly, when used as priming agents, the same extracts had no effects on maize seed germination and plant growth both under physiological and salt stress conditions. These results suggest that the effectiveness of these biostimulants is strictly dependent on the developmental stage of plants at the time of their application.

Future prospects

Another method for biostimulants application is the foliar spray technique. Its positive effects on growth and chemical composition of different plants has been already reported by several authors (Ghoname et al., 2009; Abdelgawad et al., 2018; Luziatelli et al., 2019), both in the absence or in the presence of abiotic stresses. Thus, it would be interesting to evaluate the activity of IB and SC extracts when administered as foliar spray. Moreover, the detrimental or beneficial role of alcoholic solvents, such as methanol and ethanol, in the growth of different plants has been reported in the literature (Rowe at al., 1994; Ramírez et al., 2006; Behrouzyar and Yarnia, 2016; Nguyen et al., 2017). For this reason, the possibility of using an inert solvent in the extraction procedure should be taken into consideration.

Finally, the molecular mechanisms through which IB and SC act as biostimulants remains an open question. This aspect is difficult to study due to the complex composition of these extracts. Nonetheless, in the future it would be worth to investigate whether they can affect gene or protein relative abundance by using “omics” approaches focusing on antioxidant and hormonal signaling pathways, which are known to mediate the early molecular response to salt stress (Bartoli et al., 2013).

6. REFERENCES

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