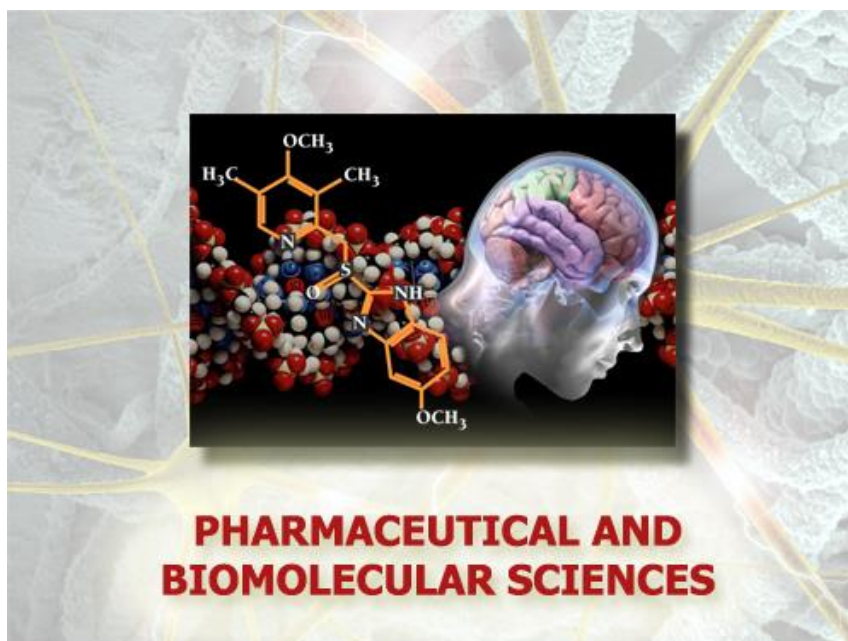# Università degli Studi di Torino

Scuola di Dottorato in
Scienze della Natura e Tecnologie Innovative

Dottorato in
Scienze Farmaceutiche e Biomolecolari
(XXXII ciclo)

Identification of new products with biostimulant action and evaluation of their effects on plant growth and development by using genomic and metabolomic approaches

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Università degli Studi di Torino

Dottorato in
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CICLO: XXXII

TITOLO DELLA TESI: Identification of new products with biostimulant action and evaluation of their effects on plant growth and development by using genomic and metabolomic approaches

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Ai miei nonni, parte di ogni traguardo.

SUMMARY

3

1. <u>GENERAL INTRODUCTION</u>

In the last decades, increasing the food production paying attention to the environment became one of the most challenging goals for agriculture (Tester, Mark and Langridge 2010). World population is exponentially growing, from 600 million in 1700 to 6.3 billion in 2003 and now it is estimated that it will reach about 12.8 billion by 2050 (Cohen 2003). The population enlargement, in addition to the increase of the food demand, is leading to direct and indirect losses of arable lands. Direct aspects concern the urban expansion due to an increased demand for habitations, infrastructures, roads, airports, etc. (Doos 2002). The indirect effects are related to the increasingly intensive use of the soil that compromises its fertility. Moreover, mechanization, use of pesticides, herbicides and forced irrigation strongly compromise its composition, structure, microbiological balance (Gaudino et al. 2014) and have a great environmental impact (Foley et al. 2011). In addition to lands used for food crop cultivation, also those used for feed and biofuel production must be taken into consideration (www.fao.org). On the other hand, the climate change is leading to the increase of biotic and abiotic stresses to which plants are exposed (Anwar et al. 2013). Finally, the cropland expansion often replaces natural ecosystems, like forests, savanna, grasslands or tropical lands, producing awful impacts on habitats and biodiversity (Foley et al. 2011).

For all these reasons, scientists from all around the world are looking for new solutions useful to increase the final production yield of crops more or less widespread, interfering as little as possible with the environment. Breeding and genetic engineering are largely studied techniques to improve plant traits, but also expensive and time-consuming (Nogué et al. 2016). Therefore, helping plants quickly is an everyday-challenge for farmers. Among the new generation products available on the market, biostimulants could be useful for this purpose.

1.1   Biostimulants: definition and main categories

The "biostimulant" concept is relatively new. It has been defined for the first time in 1997 in a web journal (http://grounds-mag.com/), while in the 2007 in a scientific paper (Kauffman, Kneivel, and Watschke 2007). Since then, many definitions have been reported, with some modifications (Yakhin et al. 2017).
The European Biostimulants Industry Council (EBIC), founded in 2011 to support the use of biostimulants in helping agriculture to produce "more with less", defined them as "*substance(s) and/or micro-organisms whose function when applied to plants or the rhizosphere is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality*" (Yakhin et al. 2017). This functional definition highlights the difference with fertilizers and pests. Indeed, these new generation products do not directly supply nutrients to the plant and do not act against pathogens, but their function is focused on plant physiology improvement and abiotic stress tolerance increase (Ricci et al. 2019).

Different methods of application, based on characteristics and purposes of the biostimulant, can be used (Drobek, Frąc, and Cybulska 2019). Three are the main known methods: seed treatment, fertigation or foliar spray. The first one is mainly used as priming induction, by soaking the seeds in a biostimulant solution before sowing. This method allows to protect seeds by abiotic stress, increase the germination rate and enhance the later phases of growth (Yildirim et al. 2002). Fertigation consists in adding the biostimulant directly to the irrigation process. These products

normally affect root structure and nutrient uptake (García-Gaytán et al. 2018). Finally, through foliar spray the biostimulant is direcly applied to leaves, flowers or fruits. In this way the product is rapidly absorbed by the plant and is useful to increase stress tolerance, reproductive efficiency and post-harvest fruit storage time (Saa et al. 2015). However, biostimulants based on mycorrhizal fungi represent an exception. Indeed, these types of products can be either supplied directly in the soil before transplanting or applied by fertigation (if soluble).

With regard to biostimulant composition, even if these products are usually complex matrices composed by waste or non-waste materials mixed together, some categories have been identified. Since the past, these have been largely used in agriculture and, in recent years, their biostimulant effects have been observed (**Figure 1**). All these compounds help plants, directly or indirectly, in facing abiotic stress and improve nutrient uptake and natural physiological aspects.
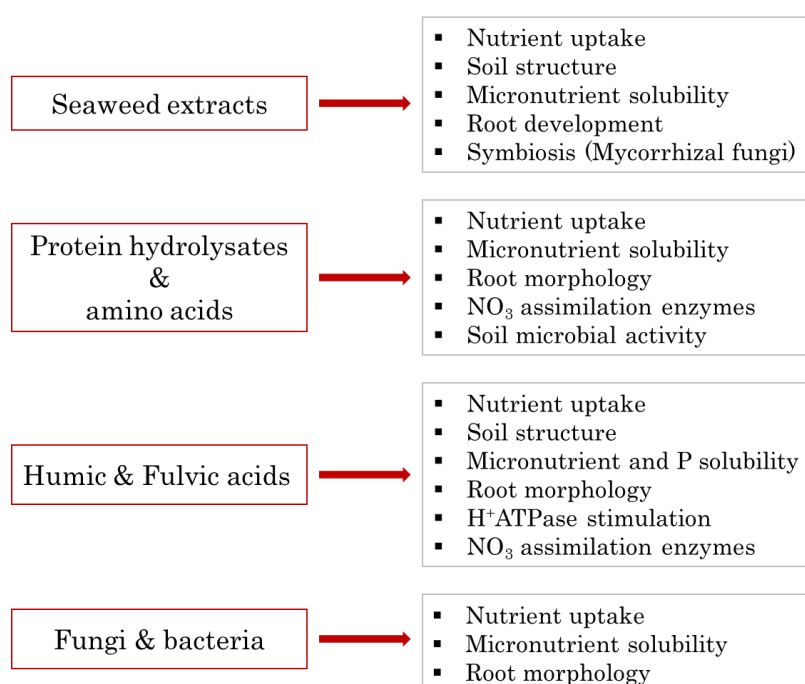
Fig.1 Summary of main categories and biostimulant effects on plants

1.1.1   Seaweed extracts (SE)

The use of algal extracts in agriculture has very ancient origins, but only recently their biostimulant potential has been defined. Seaweeds include red, brown and green macro algae that represent 10% of marine productivity (Van Oosten et al. 2017) (**Figure 2**). Seaweed extracts mainly contain molecules like hormones, alginate, fucoidans, betaines, proteins and carbohydrates, whose effects on plant are strongly recognized (H. S. S. Sharma et al. 2014). Seaweed extracts increase nutrient uptake by acting on soil structure, micronutrient solubility, and root development (Halpern et al. 2015). Moreover, these extracts promote the symbiosis with mycorrhizal fungi, by increasing their infection rate (Khan et al. 2009).
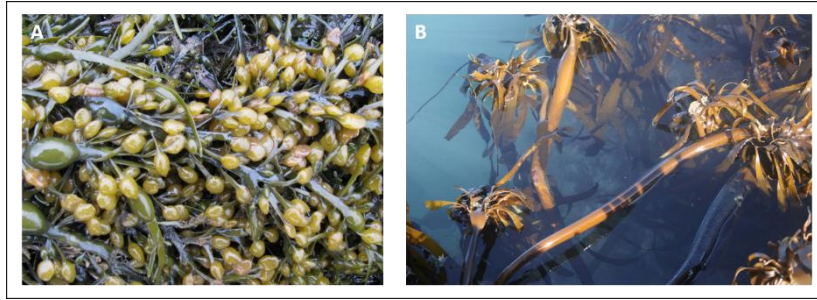
Fig.2 (A) *Ascophyllum nodosum* (www.sciencephoto.com) and (b) *Ecklonia maxima* (http://southafrseaweeds.uct.ac.za), two of the main species used for seaweed extract production

1.1.2 Protein hydrolysates (PHs) and amino acids (AAs)

Protein hydrolysates can be obtained from plant or animal waste products. The latter have been known for longest time, while in the last period the attention is growing for those of plant origin, also for an ethical aspect and because considered more environmental-friendly (L. Xu and Geelen 2018). Protein hydrolysates are classified based on source and method of hydrolysis. Chemical hydrolysis is normally used for animal-based PHs, while the enzymatic hydrolysis is used plant-based PHs (Colla et al. 2015). Peptides and amino acids can be absorbed by roots and leaves and their effects are mainly related to the increase of nutrient solubility and nitrate enzymes (**Figure 3**). Protein hydrolysates and AAs are also involved in root morphology and soil microbial activity improvement, helping plants to counteract abiotic stress and adverse conditions (Halpern et al. 2015).
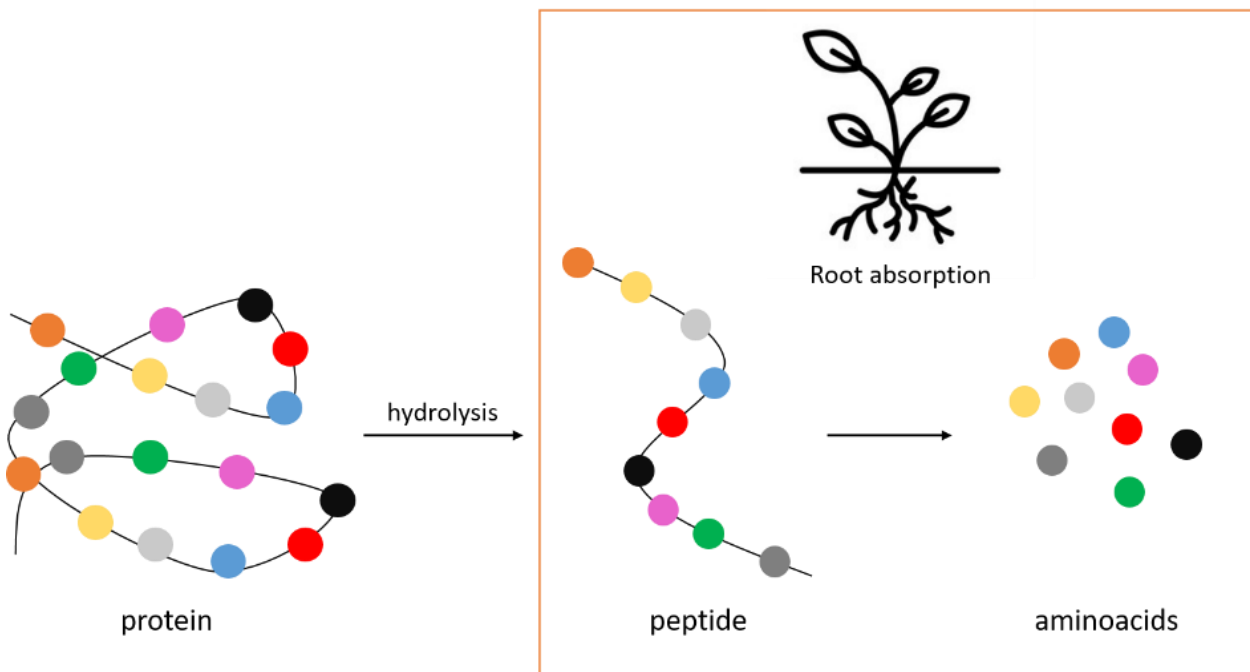
Fig.3 Protein hydrolysis leads to the production of peptides and single amino acids, more easily absorbed by the plant

1.1.3 Humic and fulvic acids

Humic and fulvic acids (**Figure 4**) derive from plant, animal and microorganism decomposition, thanks to biotic and abiotic processes (Nardi et al. 2016). These substances constitute about 60% of the organic matter in the soil and the main difference between the two classes concerns the molecular weight, lower in fulvic than humic acids (Muscolo et al. 2007; Van Oosten et al. 2017). Their effects are linked to the increase of micronutrient and P solubility, NO_3 assimilation enzymes, and H^+-ATPase activity and to changes in soil structure, root morphology and soil microbial activity (Halpern et al. 2015).
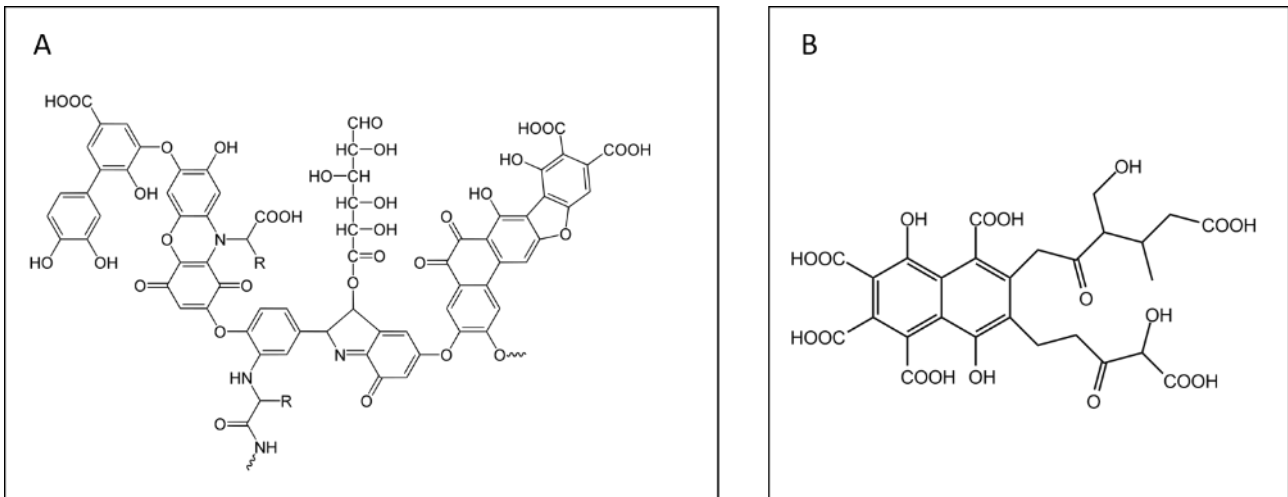
Fig. 4 Humic (**A**) and Fulvic (**B**) acids

1.1.4 Fungi and bacteria

Mycorrhizal fungi and Plant Growth Promoting Rhizobacteria (PGPR) (**Figure 5**) are known since a long time for their positive effects on plant growth. Fungi build a mutualism interaction, while bacteria extend their niches to the internal part of the cells (du Jardin 2015). These symbioses are positive for the microorganisms and allow the plant to increase nutrient availability and improve root morphology (Halpern et al. 2015). Fungi and bacteria are both useful to increase nutrient availability and use efficiency by transforming insoluble forms into others more available to the plant (du Jardin 2015).
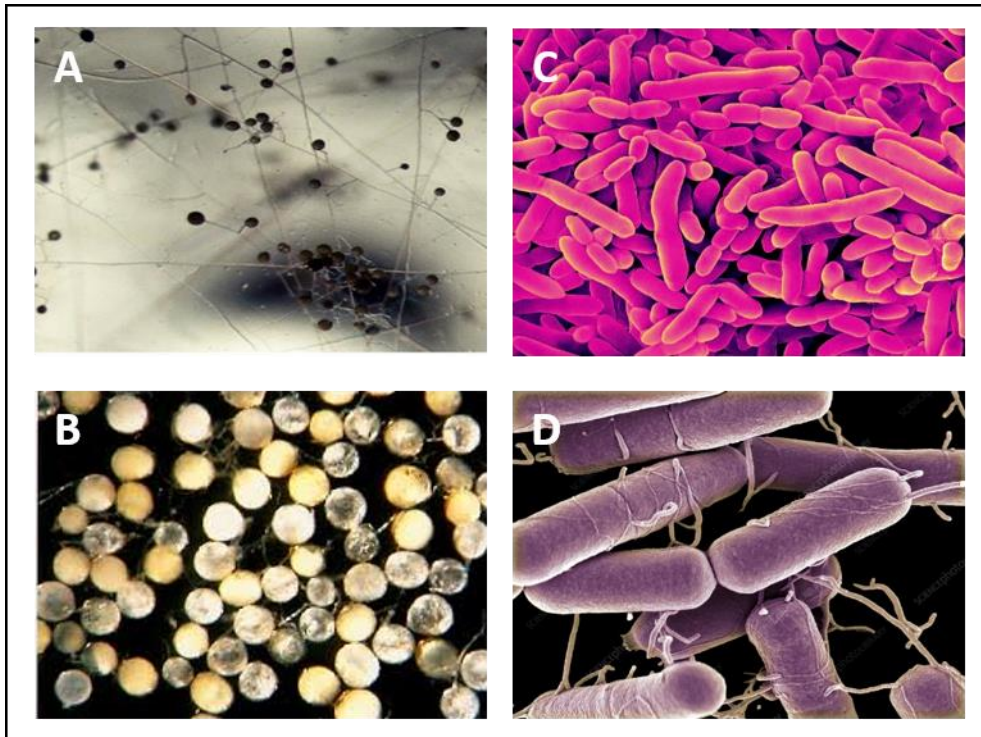
Fig. 5 Mycorrhizal fungi (**A**) *Rhizophagus irregularis* (Tamayo et al. 2014)(**B**) *Funneliformis caledonium* (http://fungi.invam.wvu.edu) and Plant Growth Promoting Rhizobacteria (**C**) *Pseudomonas putida* (www.sciencephoto.com) (**D**) *Bacillus subtilis* (www.sciencephoto.com)

1.2 Circular economy: by-products new life

The "circular economy" concept exists since 1970s, but recently is acquiring much more importance in comparison to the linear economy (**Figure 6**) (Geissdoerfer et al. 2017). Walter R. Stahel described the linear economy like a river in which different steps, starting from the use of natural resources, lead to waste production. Differently, circular economy is like a lake. In this case products used are put back into the market thanks to recycling and re-using processes that reduce waste production and energy consumption (Stahel 2016).
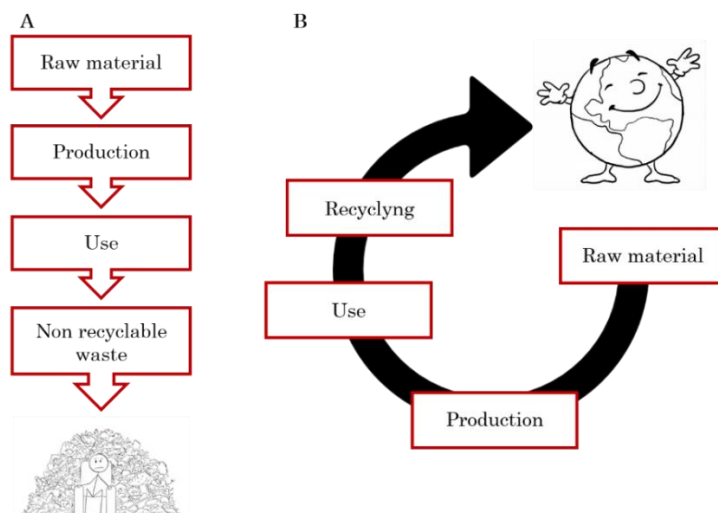
Fig.6 Schematic representation of linear (A) and circular (B) economy concept

Biostimulants perfectly fit with this concept. Indeed, as mentioned before, different substances known to be involved in plant growth and development have been identified, but these products can be obtained from a multitude of products, including those of agricultural and industrial by-products

Europe is generating more than 1.3 billion tonnes of waste annually and about 80% of these is represented by agricultural waste (Toop et al. 2017). Finding the way to re-use them, as biostimulants, once again useful in agriculture, is a perfect example of circular economy. There are several reports in literature about the use of by-products for the development of new biostimulants. Vermicompost (from organic matter degradation) (Aremu, Masondo, and Van Staden 2014), municipal organic waste and sewage sludge (Sorrenti, Toselli, and Marangoni 2012), protein hydrolysates from animal and plant by-products (Baglieri et al. 2014), sugarcane vinasse (from sugar-ethanol industry) (Christofoletti et al. 2013), vine shoots (Sánchez-Gómez et al. 2017), aqueous extracts of plant wastes, like fennel, lemon and barley (Abou Chehade et al. 2018), are all examples of reusable by-products.

Important aspects have to be evaluated to transform a by-product in a new resource. These include economical aspects like low cost of collection, storage and transport of the material, as well as the sufficient availability to satisfy the market demand (Xu and Geelen 2018). On the other hand, ensuring the safety of the by-products used, guaranteeing the absence of pesticides or pathogens, is mandatory for human, animal and environment health (La Torre, Battaglia, and Caradonia 2016).

1.3 European regulation: what news?

The biostimulant regulation, in and outside Europe, has always been quite complex and confused. In Europe, national laws that regulate these compounds are highly variable from state to state. This aspect represents a limit to free internal market movements, and creates many difficulties for producers, suppliers and controlling authorities (Caradonia et al. 2019). The difficulty in finding a unified regulation derived from the heterogeneity that distinguishes biostimulants. Indeed, it is known how they can be obtained from many waste or non-waste resources, with different characteristics and mode of action (Parađiković et al. 2019). In each European country, biostimulants were defined and classified in a different way.

In Italy, the biostimulants are classified according to the Fertilizer Legislation, D.Lgs. 75/2010 and subsequent amendments, attachment nº 6, as "Products with Specific Action on plants" (4.1). Ten different categories are admitted.

The European Biostimulants Industry Council (EBIC) worked for a long time, together with the European Commission, in order to find a solution and promote the biostimulant market, whose evaluation shows a Compound Annual Growth Rate (CAGR) of 13.58% in the forecast period 2017-2022 (http://www.marketsandmarkets.com).

In June 2019 the Regulation (EU) 2019/1009, which recognizes biostimulants at European level, was finally promulgated. It amends the Regulation (EC) N° 1069/2009 that lays down health rules as regards animal by-products and derived products not intended for human consumption and the (EC) N° 1107/ 2009 that concerns the placing of plant protection products on the market (https://eur-

lex.europa.eu). In this new regulation, for the first time, biostimulants are recognized as a distinct category of agricultural inputs and regulated by precise rules in the European Union.

In the Annex I, Part 1, the Product Function Categories (PFCs) are described. At point 6, biostimulants are reported and divided in microbial and non-microbial. In Annex I, Part 2, requirements related to PFCs are reported. At PFC 6, plant biostimulants are described as follows (https://eur-lex.europa.eu):

"1. A plant biostimulant shall be an EU fertilising product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere:

(a) nutrient use efficiency,
(b) tolerance to abiotic stress,
(c) quality traits, or
(d) availability of confined nutrients in the soil or rhizosphere."

"2. Contaminants in a plant biostimulant must not exceed the following limit values:

(a) Cadmium (Cd): 1.5 mg/kg dry matter,
(b) Hexavalent chromium (Cr VI): 2 mg/kg dry matter,
(c) Lead (Pb): 120 mg/kg dry matter,
(d) Mercury (Hg): 1 mg/kg dry matter,
(e) Nickel (Ni): 50 mg/kg dry matter
(f) Inorganic arsenic (As): 40 mg/kg dry matter.

3. The copper (Cu) content in a plant biostimulant must not exceed 600 mg/kg dry matter, and the zinc (Zn) content in a plant biostimulant must not exceed 1500 mg/kg dry matter."

"4. The plant biostimulant shall have the effects that are claimed on the label for the plants specified thereon"

"PFC 6 (A) MICROBIAL PLANT BIOSTIMULANTS
1. A microbial plant biostimulant shall consist of a microorganism or a consortium of microorganisms referred to in CMC 7 in Part 2 of Annex II.

2. Pathogens in a microbial plant biostimulant must not exceed the limits…(not reported table)

3. When the microbial plant biostimulant is in liquid form, the plant biostimulant shall have a pH optimal for contained microorganisms and for plants."

"PFC 6 (B) NON-MICROBIAL PLANT BIOSTIMULANTS

1. A non-microbial plant biostimulant shall be a plant biostimulant other than a microbial plant biostimulant

2. Pathogens in a non-microbial plant biostimulant must not exceed the limits… (not reported table).

In the Annex II the Component Material Categories (CMC) are reported. At point 7 the kind of microorganisms usable for biostimulant formulation are listed. These include *Azotobacter* spp., mycorrhizal fungi, *Rhizobium* spp., and *Azospirillum* spp.

The list is still very short, but work is underway to expand it (https://eur-lex.europa.eu).

Finally, in the Annex III, Part 2, product-specific labelling requirements are reported. For plant biostimulants, the following information shall be provided (https://eur-lex.europa.eu):

(a) physical form;

(b) production and expiry date;

(c) application method(s);

(d) effect claimed for each target plant; and

(e) any relevant instructions related to the efficacy of the product, including soil management practices, chemical fertilisation, incompatibility with plant protection products, recommended spraying nozzles size, sprayer pressure"

"PFC 6(A): MICROBIAL PLANT BIOSTIMULANTS

All intentionally added microorganisms shall be indicated. Where the microorganism has several strains, the intentionally added strains shall be indicated. Their concentration shall be expressed as the number of active units per volume or weight, or in any other manner that is relevant to the microorganism, e.g. colony forming units per gram (cfu/g).

The label shall contain the following phrase: "Microorganisms may have the potential to provoke sensitising reactions.

The Regulation should enter into force within the 2024, obligating to follow precise rules for biostimulant development and production.

AIM AND STRUCTURE OF THE PROJECT

The aim of this work was to test and demonstrate the efficacy of two biostimulants on different crops grown under standard and abiotic stress conditions. A multidisciplinary approach, including biometric measurements, transcriptomic and biochemical analyses, was used.

The thesis is organized in two main chapters:

The work reported in the first one is focused on KIEM®, a biostimulant developed to improve the germination process, especially under heat stress conditions, tested on soybean and cucumber. Germination is the first phase of plant development and is negatively affected by high soil temperature often found in different cultivated areas, as consequence of the climate change. KIEM® is able to enhance seed traits, decrease the H_2O_2 levels acting as a stress mitigator and increase the DNA repair system. Moreover, it shows positive effects on later phases of growth, thus increasing the final yield. Therefore, this seed priming biostimulant improves tolerance to heat and crop productivity by triggering different responses, mainly related to the modulation of the antioxidant system and to the germination process.
The use of a biostimulant as a seed coating agent presents several advantages, compared to the application in post-emergence, such as the reduction of the number of treatments and the subsequent decrease of the final management costs.

The work reported in the second chapter is focused on VIVEMA® TWIN, an original mix of hydrolysable and condensed tannins tested on tomato, developed to improve the root growth, especially under high salinity, one of the most widespread abiotic stress all around the world. Based on biometric and transcriptomic results, we demonstrated that this biostimulant is able to increase the plant defence system and nutrient uptake processes through the modulation of different genes. The synergic effect of the biostimulant was also compared to the effect of gallic acid, the main compound present in the matrix and known to be involved in plant growth and root development.

The study confirmed the efficacy of these two new biostimulants, able to improve plant development, strength and productivity. Interestingly, stronger effects were observed under abiotic stress conditions, a common feature of products having biostimulant activity.
Finally, in both studies, a chemical characterization of these complex matrices was also carried out, in order to identify bioactive compounds directly involved in the final effect of the products. These analyses are of paramount importance to understand the mechanism of action of biostimulants.

Chapter 1

Seed transcriptome analyses and antioxidant activity profiling reveal the role of the new biostimulant KIEM® in priming heat stress tolerance in soybean and cucumber

1. <u>**INTRODUCTION – heat stress and seed priming**</u>

Germination is the first phase of plant development. Different other factors such as the plant vigour, the harvest homogeneity and the final yield, directly or indirectly, depend from this process (Ashraf and Foolad 2005). Therefore, this critical phase needs to be protected from environmental stresses, such as drought, salinity or thermal stress. The high soil temperature is one of the main limiting effects on crop germination, able to considerably reduce the germination percentage, inhibit radicle emergence and therefore compromise the final yield (Probert 2000). So far, different approaches have been employed to enhance plant stress tolerance, however some treatments can be particularly time-consuming (e.g., conventional breeding) and others not accepted by all countries in the world (e.g., plant genetic modification). Due to the limits related to these techniques, it has become fundamental to find an alternative solution, faster, cost effective and less dependent on ethical issues to help plant fighting stress (Jisha, Vijayakumari, and Puthur 2013). Seed priming could represent an alternative tool to prepare plants to counteract abiotic stress conditions more successfully (Filippou et al. 2013). Recent studies suggest that different molecules have the potential to act as priming agents against different abiotic stresses. It has been demonstrated that the application of several compounds such as amino acids, hormones, reactive oxygen–nitrogen–sulphur species or just water (hydro-priming) can be effective in enhancing plant tolerance to different abiotic stresses (Savvides et al. 2016). Plants can be pre-treated at different developmental stages (e.g. vegetative or reproductive stage), however in the last few years the attention has been focused on seed priming. This approach consists in a pre-sowing treatment of seeds with synthetic or natural compounds, aimed to increase uniformity and vigour of seedlings and to enhance the tolerance of plants to different abiotic stresses. The priming treatment at seed stage leads to a reduction of application costs (a single treatment instead than multiple treatments) and often to a prolonged potential protection (Savvides et al. 2016). Priming treatments generally cause a faster germination and a faster field emergence, which have practical agronomic implications, notably under adverse conditions.

Among the new generation products present on the market, biostimulants could play a key role as seed-treatment agents. They are considered safe for the environment and possess a broad spectrum of possible biological activities. In the last 25 years, plant biostimulants have received considerable attention since these innovative products offer a potentially novel approach for the modulation of physiological processes in plants to stimulate growth, to enhance stress tolerance, and to increase yield (du Jardin 2015; Yakhin et al. 2017).

In the studies described below, we evaluated the potential priming effects of KIEM®, an innovative aqueous-based biostimulant containing plant-derived amino acids and molybdenum on soybean and cucumber seed germination and development under heat stress conditions. This product was especially studied for the Brazilian market, since in this country adverse conditions such as high temperatures for long periods are often present at sowing time.

In order to determine the effects and the metabolic targets of this innovative product, morphological, molecular and biochemical analyses were carried out on untreated and KIEM®-treated seeds incubated in controlled conditions (cucumber and soybean) and cultivated in open field (soybean).

The results obtained on both plant systems provided insights on the mechanism of action of KIEM®
and on its application as a seed priming biostimulant able to increase tolerance to heat stress and
potentially to other abiotic stress typical of adverse environmental conditions.

Glycine max (L.) Merr – Soybean

Soybean (*Glycine max* (L.) Merr) is one of the most important crops in the world for food, feed and industrial applications (Lima et al. 2017). This crop is widely cultivated in warm countries (i.e. Brazil), but its germination, growth, root development and emergence uniformity are negatively affected by climate adverse conditions such as extreme temperatures, drought and salinity stress. In particular, temperature is one of the most crucial climatic factors influencing seed germination (Wang et al. 2014).

For this plant, the biometric parameters were evaluated on both, seeds germinated in controlled conditions (35°C) and plants cultivated in Brazil, in a natural heat stress conditions.

2. MATERIALS AND METHODS

2.1 Plant material and biostimulant

For this study, soybean seeds, variety PR91M10, (Pioneer Hi-Bred Italia Srl) were used. KIEM®, developed by Green Has Italia S.p.A (Canale, CN, Italy) is an acqueous based biostimulant containing 2% w/w of organic nitrogen, 2% w/w of molybdenum and 21% w/w of organic carbon. The pH (1% acq. sol. w/w) and Electrical Conductivity (in acq. sol. 1g L^{-1}) are 4.00±0.5 u. pH and 200 µS cm^{-1} respectively.

2.2 Seed treatment

Soybean seeds were treated by following the protocol developed by Green Has Italia S.p.A. KIEM® solution was diluted in distilled water using 1:3 (v/v) ratio, then added drop by drop to 50 g of dried seeds (to reach a final dosage of 2 mL kg^{-1}), kept in continuous shaking to allow the homogeneous distribution of the product. Following the treatment, seeds were dried at room temperature and then placed in glass Petri dishes (20 cm Ø) containing two filter papers saturated with 15 mL of distilled water. For treatment and control, 15 seeds per three Petri dishes (45 seeds for each condition) were incubated for 24 hours at 35°C in the dark. The seeds were then collected, homogenized in liquid nitrogen with mortar and pestle, and stored at -80°C for further analyses.

The same treatment was performed for in field plant parameter evaluation using the same dosage (2 mL kg-1 seeds). Trials were carried out in four replicates in randomized complete block. Each replicate was represented by a 32 m^2 plot, with a density of 24 plants/m^2. Plants were cultivated in 2017 in São Paolo State at Detec Experimental Station (Taquarituba, 23°31'33.7"S 49°15'16.6"W).

2.3 Biometric data analysis

Morphological analysis was conducted in order to evaluate differences between KIEM®-treated and untreated seeds. The germination protocol used for these tests was the same used for biochemical and molecular analyses. Three Petri plates with 15 seeds each for KIEM®-treated and untreated seeds were used. Each seed was weighed, measured and statistically evaluated by using the Smart Grain software (Tanabata et al. 2012). Projected area, perimeter, length, width and weight were measured. All measurements were

taken immediately after treatment (T0) and after 24 h incubation at 35°C in the dark (T1). The changes were calculated on values obtained from the difference between T1 and T0. In addition, germination percentages were measured for control and KIEM®-treated seeds at 24, 48 and 72 hours incubation at 35°C in the dark. Three biological replicates, each composed by 45 seeds (15 seeds per three different Petri dishes) were used.

Plants in field were evaluated, at 7 days after sowing, by measuring root length and plant height. Furthermore, pod number/plant, thousand seeds weight (TSW) and yield were measured at harvesting time. About 1700 control and 1700 treated plants were analysed. Data were analysed by means of Student's t test ($p \leq 0.05$). Different letters indicate statistical differences between treatments.

2.4 RNA isolation

Total RNA was isolated from powdered germinated seeds by using TRIzol® reagent (Thermo Fisher Scientific, MA, USA). In order to increase RNA yield, the manufacturer's protocol was modified according to Wang and colleagues (Wang et al. 2012). To 50-100 mg of powdered germinated seeds, 0.4 mL of extraction buffer (Agilent Plant RNA Isolation Mini Kit, Agilent Technologies, CA, USA) were added. After an incubation of 15 min at room temperature, samples were centrifuged 10 min at 12000 x g (4°C), the supernatant transferred into a new microcentrifuge tube and 0.8 mL of TRIzol® reagent were added. Samples were incubated 10 min at room temperature and then 240 µL of chloroform were added. After 10 min centrifugation at 12000 x g (4°C) the upper phase (aqueous) was transferred into a new microcentrifuge tube. To this phase, 0.7 mL of isopropanol were added and solutions were mixed by inversion. Total RNA was precipitated 20 min at -20°C. Then, samples were centrifuged 20 min at 12000 x g (4°C) and the supernatant discarded. Two washing steps were made with 0.5 mL of cold 75% (v/v) ethanol. The samples were centrifuged 5 min at 12000 x g (4°C) and the supernatant was discarded. The pellet was dried at room temperature for 10 min and re-suspended in 30 µL of nuclease-free water. The isolated total RNA was purified by using the RNeasy® Mini Kit (Qiagen, Hilden, Germany), following the RNA clean-up protocol. RNA concentration was evaluated using an UV/visible spectrophotometer Ultrospec 3000 (Pharmacia Biotech, Sweden). Sample quality was checked by using the RNA 6000 Nano kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) according to manufacturer's instructions. Three biological replicates, each composed by 45 seeds (15 seeds per three different Petri dishes) were used.

2.5 RNA-seq analysis

Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) at Novogene (Hong Kong, China) and index codes were added to attribute sequences to each sample. Library were sequenced on an Illumina Hiseq platform and 150 bp paired-end reads were generated. Three biological replicates were used for RNA-seq analysis.
Raw data (raw reads) of fastq format were firstly processed through custom scripts and clean reads were obtained by removing reads containing adapter and low quality reads from raw data, running Sickle (https://github.com/najoshi/sickle), and trimmed for the presence of residual adapter sequences through Scythe (https://github.com/vsbuffalo/scythe). All the downstream analyses were based on the clean data with high quality. Reference genome and gene model annotation files

were directly downloaded from Genbank (ftp://ftp.ncbi.nlm.nih.gov/genomes/*Glycine_max*). Index of the reference genome was built using Bowtie v2.2.3 and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12. We selected TopHat as the mapping tool for that TopHat which can generate a database of splice junctions based on the gene model annotation file and thus a better mapping result than other non-splice mapping tools. HTSeq v0.6.1 was used to count the reads numbers mapped to each gene. The FPKM (fragments per kilobase of transcript per million fragments mapped) of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels (Trapnell, Williams, Pertea, Mortazavi, Kwan, van Baren, et al. 2010). A similarity matrix of the control and treatment (+KIEM®) data was built up calculating as 1-cosine-distance (http://amp.pharm.mssm.edu/clustergrammer)

Differential expression analysis of two conditions/groups (three biological replicates per condition) was performed using the DESeq R package (1.18.0). DESeq provides statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq were assigned as differentially expressed.

A Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented using the enrichment term engine (GO terms, KEGG pathways/INTERPRO domain) implemented in STRING (https://string-db.org). To characterize the function of genes differentially expressed in treated plants, the soybean genes were organized in list of up-regulated and down-regulated genes and compared to Arabidopsis proteome (TAIR version 10), using reciprocal blast best hits, to find species orthologs. The list of orthologs was submitted to STRING and enrichments were recorded when terms that are more enriched in the set of query proteins than the background, considering a FDR value less than 0.05. An interactome map was built up using DEG (up/down regulated) based on the STRING database, with known and predicted Protein-Protein Interactions and the networks were built accordingly.

2.6 Extraction and activities of antioxidant enzymes

Total proteins and antioxidant enzymes were extracted according to (Contartese et al. 2016). Half a gram of powdered germinated seeds was used and all the steps were carried out at 4°C. The extraction buffer used contained: 50 mM sodium phosphate, pH 7.5, 250 mM Sucrose, 1.0 mM EDTA, 10 mM KCl, 1 mM $MgCl_2$, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM dithiothreitol (DTT), and 1% (w/v) polyvinylpolypyrrolidone (PVPP) in a 1:10 proportion (w/v). The homogenate was mixed by pipetting and then centrifuged 20 min at 25000 x *g* (4°C). The supernatant, containing total proteins, was used for enzymatic assays. Soluble protein content was evaluated by the method of (Bradford 1976) using bovine serum albumin as a standard. Three biological replicates, each composed by 45 seeds (15 seeds per three different Petri dishes) were used.

SOD (EC 1.15.1.1) – Superoxide dismutase activity evaluation was based on the ability of this enzyme to inhibit the reduction of nitro blue tetrazolium, thanks to the superoxide anion, generated photochemically. The reaction consisted in 1 mL containing 50 mM sodium phosphate buffer, pH 7.8, 13 mM methionine, 75 µM nitro blue tetrazolium (NBT), 2 µM riboflavin, 0.1 mM EDTA, and enzyme extract. To avoid degradation, riboflavin was added last. The samples were placed 30 cm

under a light source (4000 lux) and the reaction was run for 15 min. Two blanks were prepared: one without enzyme extract, placed under the light to totally develop the reaction and, the other one, containing the enzyme extract placed in the dark to avoid the reaction. The last one was used as control. The absorbance was detected at 560 nm.

CAT (EC 1.11.1.6) - Catalase activity was detected spectrophotometrically. The absorbance at 240 nm was measured for 120 sec for evaluating the change due to the decreased absorption of H_2O_2 (ε = 39.4 mM-1 cm-1). The reaction was prepared in 1 mL final volume, containing 50 mM sodium phosphate, pH 7.0, 15 mM H_2O_2, and enzyme extract. The reaction was initiated by addition of H_2O_2.

GST (EC 2.5.1.18) - The 1-Chloro-2,4-dinitrobenzene (CDNB) was used as reaction substrate. The enzyme activity was evaluated by monitoring the absorbance variation at 340 nm for 15 min. One mL of reaction solution contained 100 mM potassium phosphate buffer (pH 7.0), 1 mM reduced glutathione (GSH), 1 mM 1-chloro-2,4-dinitro-benzene (CDNB) (10 mM CDNB dissolved in 50% acetone stock solution), and enzyme extract. The reaction was started by adding CDNB (Jain and Bhalla-Sarin 2001).

2.7 Non-protein-thiol content

Non-protein thiols were evaluated by monitoring the free glutathione formation after adding 500 μL of protein extracts to 100 μL of 25% (w/v) TCA (Thrichloroacetic acid). The mixture was then centrifuged at 12000 g for 20 min at 4°C and 300 μL of supernatant were added to 2.7 mL of 0.6 mM DTNB (5,5'-dithiobis-2-nitrobenzoic acid; Ellman's reagent, ε = 14.150 mM^{-1} cm^{-1}) dissolved in pH = 8.0 buffer containing 0.1 mM $NaPO_4$. The absorbance was read spectrophotometrically at 412 nm (Jain and Bhalla-Sarin 2001). Three biological replicates, each composed by 45 seeds (15 seeds per three different Petri dishes) were used.

2.8 Hydrogen peroxide content

The hydrogen peroxide level was detected according to (Velikova, Yordanov, and Edreva 2000). Powdered seeds (0.5 g) were homogenized with 5 mL of 0.1% (w/v) TCA. The samples were centrifuged at 12000 x *g* for 15 min and 0.5 mL of supernatant was added to 0.5 mL 10 mM potassium phosphate buffer (pH 7.0) and 1 mL 1 M KI. The absorbance was read at 390 nm and the H_2O_2 content was determined based on a standard curve. Three biological replicates, each composed by 45 seeds (15 seeds per three different Petri dishes) were used.

2.9 Statistical analysis

For biometric and antioxidant system analyses, three biological replicates were used for the statistical treatment of the data which are expressed as mean values ± standard deviation (SD). Significance of differences observed in data sets was tested by Student's *t* test ($p \leq 0.05$).

3. RESULTS AND DISCUSSION

3.1 KIEM® improves some seed traits and shows a late effect on soybean development and yield

The use of biostimulants to counteract the effect of abiotic stress is well recognized and their function in priming plant defence against adverse environmental conditions has been studying lately (Masondo et al. 2018). Priming has been revealing a good method to counteract the ever-increasing environmental stress presence, improving the yield of productions, starting from seed germination (Reddy 2015). This is a faster method in comparison to conventional breeding or plant genetic modification and could be useful for seed treatment in countries, such as Brazil, where high temperature at sowing could be a limiting factor (Savvides et al. 2016). Among the crops whose cultivation occurs under this adverse environmental condition, soybean (*Glycine max*) is affected in terms of poor germination, increased incidence of pathogen infection, and decreased economic value (Lima et al. 2017).

In order to evaluate the biostimulant treatment effect on seed morphology at the early germination phase under heat stress conditions, biometric parameters were monitored on control and KIEM®-treated seeds, at time 0 and 24 hours after incubation at 35°C in the dark. Most part of the evaluated parameters showed a significantly higher percentage increase in treated seeds compared to the control. Seed projected area had increased by 50.34%, perimeter 18.28%, length 20.39% and width 14.74% (Table 1). In contrast, the weight increase of treated seeds was only 0.63% (not statistically significant).

Tab 1. Increase of biometric parameters (%) in KIEM®-treated and control seeds. Measurements were recorded at time 0 (just before sowing) and at time 1, 24 h after incubation at 35°C in the dark. Values are expressed as a mean percentage of three biological replicates of 15 seeds each. P value indicates significant differences ($p \leq 0.05$). Different letters indicate statistical differences

PARAMETER	CONTROL	KIEM®
	mean %	mean %
Seed projected area	84.46 ± 4.91[b]	134.80 ± 8.90[a]
Seed perimeter	40.71 ± 2.72[b]	58.99 ± 2.71[a]
Seed length	55.62 ± 2.74[b]	76.01 ± 3.35[a]
Seed width	13.08 ± 1.55[b]	27.82 ± 3.17[a]
Seed weight	101.86 ± 2.33[b]	102 50 ± 3.49[b]

This aspect could be related to the absence of changes in water uptake. The result is supported also by transcriptomic data (fully reported in the paragraph 3.2), since the gene *TIP2-1* (*LOC100803901*), coding for an aquaporin, resulted to be down/regulated. Aquaporins are proteinaceous channels known to regulate transmembrane water transport, and therefore play a key role in the imbibition process during seed germination. Previous studies conducted on spinach (*Spinacia oleracea* L.) unprimed and osmoprimed-seeds subjected to chilling and drought stress showed a down-regulation of the genes coding for aquaporins (including TIP subfamily) compared to seeds germinated in optimal conditions (Chen et al. 2012). There is evidence that different seed-priming treatments cause a decrease in water absorption owing to the lower water potential of the priming solution. In this way, the treated seeds have more time to complete DNA repair processes and also have reduced cellular damage, which often occurs during rapid seed rehydration in the germination process (Savvides et al. 2016).

Besides aquaporin down-regulation, we could not exclude that the differences observed for some biometric parameters such as projected area, perimeter, length and width of the seeds treated with KIEM® could be related to a different level of gas exchange due to the coating during the germination process. Further analysis should be done to better evaluate this aspect and to confirm this hypothesis.

Together with biometric data, the germination percentage was evaluated. This parameter was collected at three different time points, 24, 48 and 72 hours after incubation at 35°C in the dark (**Table 2**). The biostimulant treatment prompted a significant increase in the germination percentage with respect to control at 72 h. After 24 h, seeds were imbibed, but no visible germination (emergence of a radicle) was detected. At 48 h, both control and KIEM®-treated seeds showed a similar germination percentage (**Table 2**).

Table 2. Germination percentage of control and KIEM®-treated soybean seeds incubated at 35°C in the dark. Values are the means of three biological replicates. Different letters indicate significant differences (Student's t test, $p \leq 0.05$). N.d. not detected

	24 h (%)	48 h (%)	72 h (%)
CONTROL	n.d.	68.8 ± 1.16[a]	82.2 ± 0.58[b]
KIEM®	n.d.	77.7 ± 0.58[a]	91.1 ± 0.58[a]

With regard to experiments in field, 7 days after sowing, KIEM® (2 mL kg^{-1} seeds) positively influenced root length and plant height. Both parameters were significantly modified by the biostimulant application compared to control (**Figure 1**). At harvest, pod number/plant, thousand seeds weight (TSW) and consequently yield, were significant higher compared to control.
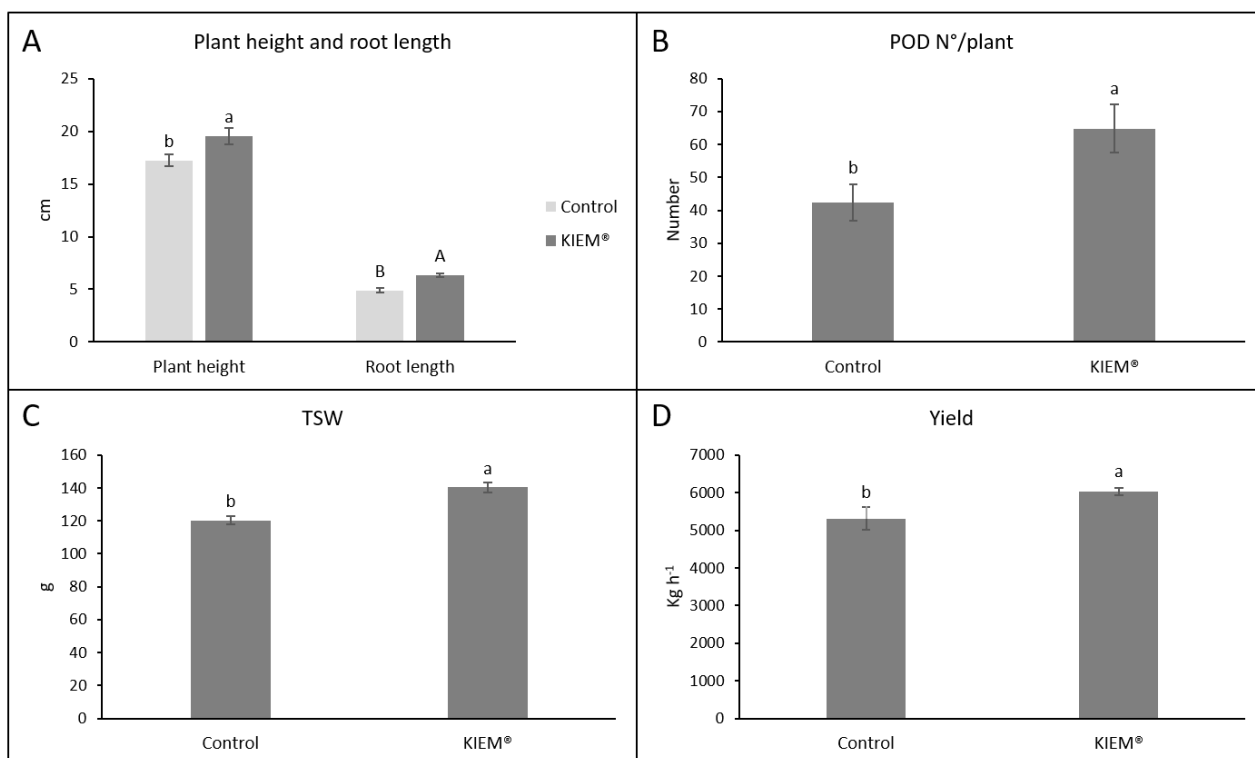
Fig. 1. Effects of the application of KIEM® on soybean grown in field. Root length (root system total length measured after plant harvesting) and plant height (**A**) were measured at 7 days after sowing, pod number/plant (**B**), thousand

seeds weight (TSW) (**C**) and yield (**D**) were evaluated at harvesting time. Data were analysed by means of Student's *t* test (*p* ≤ 0.05). Different letters indicate differences between treatments. The bars represent the means ± SD.

Since KIEM® showed the best performance in treated seeds 72 h after sowing, in 7-days-old plants and at the harvest time, it is possible to hypothesize a biostimulant late effect in promoting plant vegetative and reproductive growth under heat stress. In this regard, the priming effect is already known to be prolonged during later plant growth stages. It has been demonstrated that in salt stress, the activation of different biochemical mechanisms related to stress response, promote a 'priming memory' in seeds which can be recruited upon a later stress-exposure and provokes higher stress tolerance of germinating primed seeds (Ibrahim 2016). Wei and co-workers observed that 7-day old soybean seedlings grown from melatonin-treated seeds and subjected to drought stress showed an enhanced tolerance compared to untreated seeds (Wei et al. 2015). Moreover, Amirkhani and colleagues showed that broccoli seeds coated by plant protein lysates enhanced seedling shoot and root growth compared to uncoated seeds, whereas their germination was negatively affected by the treatment (Amirkhani et al. 2016). This negative effect could be related to a barrier for water uptake due to the coating during the whole germination process.

3.2 KIEM® treatment modulates the expression of genes involved in DNA repair and heat-stress tolerance

RNAseq analysis was performed to evaluate gene expression changes in treated seeds germinated at 35°C, in comparison to the control. The results showed 879 genes differentially expressed, 51 up regulated and 828 down regulated by the treatment (**Figure 2**).
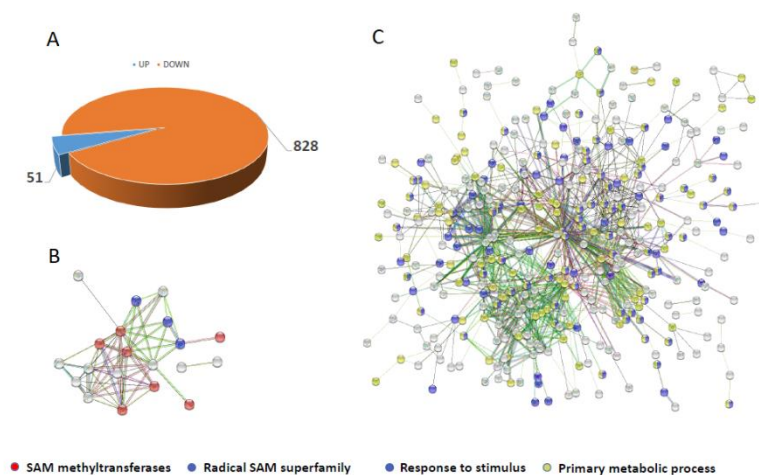
Fig. 2 (**A**) KIEM® up/down regulated genes. Interactome graph of the: (**B**) KIEM® up regulated genes, (**C**) KIEM® down regulated genes. For each interactome subset, enrichment are highlighted as red/blue dots

Considering the 51 up-regulated genes following KIEM® treatment (**Table 3**), most part of them encode for methyltransferases, genes involved in different processes, like DNA repair and methylation, pectin methylation, molecule biosynthesis, protection from enzymatic degradation and response to abiotic stress (Struck et al. 2012; S. C. Wang and Frey 2007; Ishikawa et al. 2000). DNA methylation is known to affect developmental processes and to be activated in response to abiotic stress (Rivero, Ruiz, and Romero 2004). Indeed, it is strongly connected to the perception of

external environmental stimuli, to changes in stress-responsive gene expression and to priming effect activation (Conrath 2011; Kim et al. 2015). Therefore, the identified categories of up-regulated genes could highlight the presence of a priming defence effect induced by the treatment in terms of protection of the seed DNA against heat stress. Among them, the most represented families are two: the superfamily adenosyl-L-methionine-dependent methyltransferase (IPR029063S, 8 genes, FDR = $6.65e^{-8}$) and the pFAM domain "Radical SAM superfamily" (PF04055, 3 genes, FDR=$8.76 e^{-5}$).

The first family (IPR029063) contains genes coding for 5 classes of proteins, divided on their structure (Struck et al. 2012). These are involved in the methylation of small and macromolecules, an important process in primary and secondary metabolism, cell signalling and response to stimuli (Grove et al. 2011). The main genes regulated and included in this group are *LOC100796633*, *LOC100807160* and *LOC100809923*.

The radical SAM superfamily includes more than 2800 proteins containing the amino acid sequence motif CxxxCxxC that nucleates a [4Fe–4S] cluster. These proteins participate in many biochemical transformations and reactions (Frey, Hegeman, and Ruzicka 2008). Genes included here are *LOC100813692*, *LOC100818638* and *LOC100804869*.

Other up-regulated genes after the treatment include ribosomal RNA large subunit methyltransferases RlmN/Cfr (IPR004383, 3 genes, FDR = $5.73e^{-06}$), Dual-specificity RNA methyltransferase RlmN (IPR027492, 2 genes, FDR = 0.00212), Ribosomal RNA large subunit methyltransferase E (IPR015507, 2 genes, FDR = 0.00509), Ribosomal RNA methyltransferase FtsJ domain (IPR002877, 2 genes, FDR = 0.00847), Ribosomal RNA methyltransferase FtsJ domain (IPR002877, 2 genes, FDR=0.00847), RNA (C5-cytosine) methyltransferase (IPR023267, 2 genes, FDR = 0.0263), FtsJ-like methyltransferase (PF01728, 2 genes, FDR = 0.0149), Elongator protein 3/MiaB/NifB (IPR006638, 2 genes, FDR = 0.0106) and SAM-dependent methyltransferase RsmB/NOP2-type (IPR001678, 2 genes, FDR = 0.0304). They are all genes coding for methyltransferases, attributable to the above mentioned functions.

Moreover, few genes involved in stress response are also up-regulated by KIEM® application. In particular, our analysis revealed the up-regulation of *NF-X Like 1* (*LOC102669482*) and *pentatricopeptide repeat containing protein* (*LOC100788313*). The human NF-X1 protein and homologous proteins in eukaryotes represent a class of transcription factors whose common feature is the cysteine-rich region, which possess a variable number of repeated motifs, defined as NF-X 1 zinc finger motifs. It has been demonstrated in Arabidopsis that NF-X Like 1 protein is involved in a regulatory mechanism able to improve the physiological status of plants and to support growth and survival under salt stress (Lisso, Altmann, and Müssig 2006). Pentatricopeptide Repeat Proteins (PPRs) constitute one of the largest gene families in Arabidopsis. Several genes belonging to this family are involved in tolerance to different biotic and abiotic stresses (Sharma and Pandey 2016). The mitochondrial PPR protein PGN (PENTATRICOPEPTIDE REPEAT PROTEIN FOR GERMINATION ON NaCl) was identified to positively regulate biotic and abiotic stress response. Arabidopsis plants with mutation in PGN show low resistance against necrotrophic fungi as well as toward ABA, glucose and high salinity (Laluk, Abuqamar, and Mengiste 2011).

Tab.3 Up regulated gene categories after KIEM® treatment at 35°C

Pathway ID	Pathway description	Nº of genes	FDR
IPR029063	S-adenosyl-L-methionine-dependent methyltransferase	8	$2.2e^{-07}$
PF04055	Radical SAM superfamily	3	0.000134
IPR004383	Ribosomal RNA large subunit methyltransferase RlmN/Cfr	3	$5.73e^{-06}$
IPR027492	Dual-specificity RNA methyltransferase RlmN	3	0.00212
IPR015507	Ribosomal RNA large subunit methyltransferase E	2	0.00509
IPR002877	Ribosomal RNA methyltransferase FtsJ domain	2	0.00847
IPR023267	RNA (C5-cytosine) methyltransferase	2	0.0263
PF01728	FtsJ-like methyltransferase	2	0.0149
IPR006638	Elongator protein 3/MiaB/NifB	2	0.0106
IPR001678	SAM-dependent methyltransferase RsmB/NOP2-type	2	0.0304

Considering the 828 down-regulated genes following the KIEM® treatment a large number of specific GO enrichments were observed (**Table 4**). Many genes related to response to stress (GO:0006950, 80 genes, FDR = $5.49e^{-06}$) were down-regulated by the biostimulant treatment. In particular, treated seeds showed a reduced expression of genes coding for heat shock transcription factors, such as *LOC100527682*, *LOC100786140* and *LOC100789792*. These genes are also part of two other GOs: response to chemical (GO:0070887, 90 genes, FDR = $8.83e^{-11}$) and response to stimulus (GO:0050896, 135 genes, FDR = $2.24e^{-10}$). These GOs also grouped genes coding enzymes involved in the redox regulation, such as glutathione S-transferase (*LOC100808374*), glutaredoxins (*LOC100792704*) and thioredoxins (*LOC100810192*, *LOC100800129*).

Among the genes responding to chemical, *peroxidases 3* (*LOC100547872*) and *peroxidase 5* (*LOC100811641*) are involved in removal of H_2O_2, oxidation of toxic reductants, biosynthesis and degradation of lignin, suberization, auxin catabolism and response to environmental stresses. In the same category, the biostimulant treatment down-regulated the expression of genes coding protein kinase superfamily protein (*LOC100794703*) which acts as a positive regulator of abiotic stress response and zinc finger transcription factors (*LOC100806997*). The response to stimulus category (GO:0050896) included other genes coding for transcription factors, such as NAC domain containing protein 42 (*LOC100795553*) and genes involved in the redox response, such as *peroxidase 52* (*LOC100803637*) and *Respiratory burst oxidase-B* (*LOC100799682*). Moreover, it grouped genes whose coding proteins are involved in stress promoted calcium-dependent signaling like Ralf-like 34 (*LOC100527368*) and MAP kinase kinase 2 (*LOC100789241*). A common function in protecting against oxidative or heat stress is also performed by other genes included in molecular functions (GO:0003674) category, like uncoupling protein 5 (*LOC100816412*) at the mitochondrial membrane level and stress responsive A/B Barrel Domain (*LOC100795145*). Among the genes grouped in the response to stress (GO:0006950), *ethylene-responsive transcription factors* (ERFs) (*LOC100785936*, *LOC100500502*, *LOC100793410*) can be also gathered inside the hormone-mediated signalling pathway (GO:0009755, 34 genes, FDR = 0.00117). Together with ERF transcription factors, other 31 genes belonging to this GO were significantly down-regulated in treated seeds. The biostimulant treatment seemed to influence the expression of genes coding repressors of hormone signalling, such as AUX/IAA proteins (*LOC100802759*, *LOC100791342*, *LOC100799875*) and DELLA proteins (*LOC100805968* and *LOC100791952*), together with protein kinases (*LOC100794703*) and receptor kinases (*LOC102668647*) acting into hormone-induced pathways. Along with a general down-regulation of stress-related response and the influence on hormone signalling, the biosynthesis of secondary metabolites (Kegg pathway 01110, 10 genes, FDR = 0.000697) was decreased in the presence of the biostimulant. Among the others, KIEM® treatment down-regulated genes coding for cytochromes P450s and enzymes like beta glucosidases (*LOC100820528*, *LOC100777773*) and O-methyltransferases (*LOC100787536*) which act on phenolic compounds. The GO analysis highlighted also a global down-regulation of primary metabolic process (GO:0044238, 160 genes, FDR = 0.000933), including carbohydrate metabolic process (GO:0005975, 35 genes, FDR = 0.00117). In particular, it regulated the expression of genes which encode enzymes involved in the promotion of cell wall organization or biogenesis (GO: 0071554, 21 genes, FDR = 0.00993) like pectinesterases (i.e. *LOC100792319*, *LOC100794948*), glucosyl transferases (*LOC100812586*) and xyloglucan:xyloglucosyl transferases (*LOC100778482*). Moreover, the biostimulant acted on genes involved in sucrose metabolism, such as *sucrose synthase 4* (*LOC100819730*), and trehalose accumulation, such as *haloacid dehalogenase-like hydrolase domain-containing protein* and *trehalose-6-phosphate phosphatase J* (*LOC100803119*). The glycolysis and fermentation pathways are also affected by the biostimulant treatment, since *phosphofructokinase 3* (*LOC100818755*) and *alcohol dehydrogenase 1* (*LOC100800668*) transcript level was reduced. The enriched primary metabolic process GO also grouped genes involved in protein and amino acid metabolism. In particular, our analysis showed the down-regulation of *glutamate decarboxylases* (*LOC100812201* and *LOC100781791*) and aspartate aminotransferases (*LOC100780254*), able to catalyse the biosynthesis of GABA and aromatic amino acids, respectively (Weitbrecht, Müller, and Leubner-Metzger 2011; Luo et al. 2018). Last, but not least, lipid metabolism resulted also negatively regulated by the biostimulant. For instance, the primary metabolic process GO also contained *lipid phosphate phosphatase 2* (*LOC100782531*), *Long-chain acyl-CoA synthetase 2* (*LOC100806645*) and *Lipoxygenase 4* (*LOC100811820*).

Based on these results, KIEM® treatment seems to negatively affect the primary metabolism of soybean seeds by inducing a dramatic global down-regulation of primary metabolism process (GO:0044238, 160 genes, (**Figure 2**), whose activation is directly related to the promotion of germination (Bellieny-Rabelo et al. 2016). Concerning carbohydrate metabolism, sucrose synthase 4 is one of the most important enzyme directly involved in the germination process, since it catalyses the reversible cleavage of sucrose to glucose and fructose. Its downregulation induced by KIEM® seems to confirm the reduction of the isolation of sugar-nucleotide precursors for structural and storage polysaccharide biosynthesis, which indeed appears to be negatively affected by the biostimulant treatment. Moreover, some of the carbohydrate metabolism regulated genes control trehalose accumulation, which has a role in improving the abiotic stress tolerance (Fernandez et al. 2010). Interestingly, the glycolysis pathway is also affected by the biostimulant treatment. A decrease of the transcript level of phosphofructokinase 3, encoding the enzyme which catalyses the phosphorylation of D-fructose 6-phosphate to fructose 1,6-bisphosphate by ATP, the first committing step of glycolysis. Moreover, alcohol dehydrogenase 1 downregulation suggests also a reduced alcohol fermentation in seeds treated with KIEM®. Together with the carbohydrate metabolism, protein and amino acid metabolism is also affected by the biostimulant treatment. Seed storage proteins are critical to provide amino acids for protein synthesis and energy production, particularly glutamate and aspartate, which are the most abundant amino acids in soybean seeds. In particular, our analysis shows the downregulation of glutamate decarboxylases and aspartate aminotransferases, both encoding enzymes whose activity is promoted by imbibition and whose role is essential along seed germination (Bellieny-Rabelo et al. 2016). While glutamate decarboxylase (*LOC10081220*) promotes the accumulation of GABA (Luo et al. 2018), aspartate aminotransferase (*LOC100780254*) directly promotes the biosynthesis of aromatic amino acids (De La Torre et al. 2014). Since imbibition is a critical step in seed germination, the down-regulation of the primary metabolism genes observed in KIEM®-treated soybean seeds might be attributable to the lower water absorption linked to the priming process. However, this slowing down in germination has been already demonstrated to be important for DNA repair and cellular damage prevention (Balestrazzi et al. 2011).

Also the hormone mediated signalling pathway (GO:0009755) has a key role in modulating seed germination, development and response to stress (Han and Yang 2015). In particular, during soybean germination, auxins and ABA appears to repress germination (Shuai et al. 2017), while gibberellin and ethylene promote germination (Bellieny-Rabelo et al. 2016). Our trascriptomic analysis highlight a complex influence of the biostimulant treatment on seed hormone cascade. ERF transcription factors, which act as key integrators of ethylene signal (Xu et al. 2011), result downregulated. Concerning auxin signalling, the biostimulant treatment appears to significantly reduce the expression of different AUX/IAA proteins (IAA29, IAA19, ATAUX2), which are known to act as repressor of early auxin response genes under low auxin concentration (Han and Yang 2015). On the contrary, KIEM® seems to positively affect gibberellin signalling pathway by negatively modulating the expression of genes coding for DELLA proteins (*LOC100805968*, GAI and *LOC100791952*, RGL3), which act as repressor of gibberellin signalling. Last but not least, ABA signaling is also regulated by the biostimulant treatment, in terms of decrease in the expression of gene encoding protein kinases (*LOC100794703*) and receptor kinases (RK1) acting along its signalling pathway.

Tab. 4 Selected GO categories of down-regulated genes after KIEM® treatment

GO category	Pathway description	N° of genes	FDR
GO.0042221	response to chemical	90	$8.83e^{-11}$
GO.0050896	response to stimulus	135	$2.24e^{-10}$
GO.0044699	single-organism process	186	$9.73e^{-09}$
GO.0008152	metabolic process	211	$1.21e^{-07}$
GO.0044763	single-organism cellular process	154	$2.49e^{-07}$
GO.0009987	cellular process	213	$3.13e^{-06}$
GO.0006950	response to stress	80	$5.49e^{-06}$
GO.0010033	response to organic substance	60	$1.13e^{-05}$
GO.0071704	organic substance metabolic process	175	$2.3e^{-05}$
GO.0009628	response to abiotic stimulus	52	$3.51e^{-05}$
GO.0044238	primary metabolic process	160	0.000933
GO.0005975	carbohydrate metabolic process	35	0.00117
GO.0009755	hormone-mediated signalling pathway	34	0.00117
GO.0009058	biosynthetic process	100	0.00211
GO.0006952	defense response	39	0.00228
GO.0007165	signal transduction	42	0.00993
GO.0071554	cell wall organization or biogenesis	21	0.00993

3.3 KIEM® is able to mitigate heat stress by acting on the antioxidant system

To gain more insight the seed response to heat stress and H_2O_2 production during the early phases of seed germination, the level of H_2O_2 and the activity of several ROS scavenging enzymes namely, superoxide dismutase (SOD), catalase (CAT) and glutathione S transferase (GST) were analysed following the application of KIEM®. In general, the biochemical analysis conducted on biostimulant-

treated soybean seeds at 35°C suggested a better response to stress in comparison with control (stressed seeds). In seed physiology, reactive oxygen species (ROS) are usually considered as toxic molecules, whose accumulation leads to cell injury with consequent problems in seed germination and development. However, there is increasing evidence that ROS, at low concentrations, can function as signalling molecules involved in a wide range of responses to various stimuli (Bailly 2004). The dual function of ROS in plants mainly relies to the cellular antioxidant machinery, which involves detoxifying enzymes and antioxidant compounds. Such mechanisms can scavenge potentially toxic ROS, generally produced under stressful conditions, or rather tightly control ROS concentrations in order to regulate various signalling pathways. Among ROS, hydrogen peroxide plays a key role during germination process, however too high levels of H_2O_2 can be toxic for the seeds (Wojtyla et al. 2016).

In general, exposure to high temperature causes oxidative stress, leading to a significant increase in H_2O_2 accumulation. This aspect was known and reported in literature for different crop species, like tomato, wheat or spinach (Rivero, Ruiz, and Romero 2004; Jeevan Kumar et al. 2015; Gómez et al. 2008).

The treatment with KIEM® strongly decreased the H_2O_2 amount in treated seeds germinated at 35°C (**Figure 3**). The lower amount of H_2O_2 observed in treated seeds could be related to CAT activity (**Figure 4**). This enzyme, along with peroxidase (PRX), is directly involved in the disruption of H_2O_2, and its activation indicates the effort of plants to reduce oxidative damage. A lower activity of CAT can be an indication of the biostimulant capability in mitigating stress effects on seed germination process.
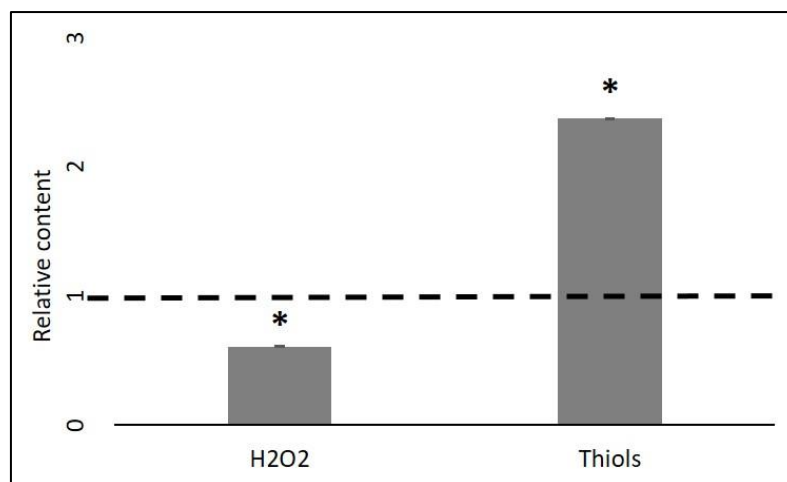
Fig. 3 Effect of KIEM® on H_2O_2 and thiol levels at 24 h after seed imbibition at 35°C. Values are expressed as a relative content obtained by comparing KIEM®-treated samples with the corresponding untreated controls (dotted line). Bars represent the means ± SD of three biological replicates. Asterisks (*) indicate significant differences between KIEM®-treated samples and the corresponding untreated controls (Student's t test, $p \leq 0.05$).

The trend of CAT activity, the main H_2O_2-scavenging enzyme, in seeds incubated at high temperature, was very similar to that observed for H_2O_2 (Dat, Inzé, and Van Breusegem 2001).

As for H_2O_2, KIEM® strongly reduced CAT activity in treated seeds germinated under heat stress (**Figure 4**). These two data are in correlation, confirming the involvement of the biostimulant in oxidative stress mitigation. Differently, SOD and GST did not shown such evident changes after the treatment compared to control.

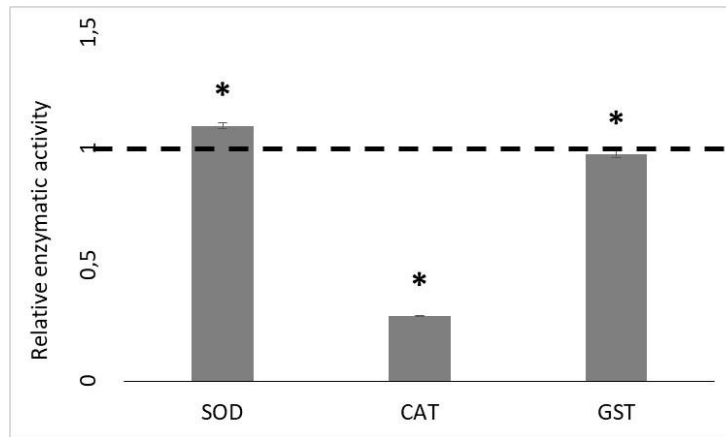**Fig. 4** Effect of KIEM® on enzymatic activities of SOD, CAT and GST at 24 after seed imbibition at 35°C. Values are expressed as a relative enzymatic activity obtained by comparing KIEM®-treated samples with the corresponding untreated controls (dotted line). Bars represent the means ± SD of three biological replicates. Asterisks (*) indicate significant differences between KIEM®-treated samples and the corresponding untreated controls (Student's *t* test, $p \leq$ 0.05).

Thiols, among which glutathione (GSH) is the most common, are non-protein compounds able to be rapidly oxidized and regenerated. Therefore, they have a role, together with scavenger enzymes, in counteracting the oxidative stress (Dickinson and Forman 2002). In addition to this, in soybean thiols are involved in sulphur metabolism. Sulphur is an essential plant nutrient, normally metabolized in sulphur-containing amino acids, like methionine and cysteine. Soybean is widely used for food and feed thanks to its protein richness (38% by weight) (Yi et al. 2010). In spite of this, it is a crop poor in sulphur containing amino acids, so the increase of this aspect is object of study to improve quality and final yield (Yi et al. 2010). Cysteine is an intermediate in the glutathione and homoglutathione synthesis pathway. Based on this, thiol increase could be related to an increase on this sulphur-containing amino acid. Based on the above mentioned observations, cysteine and, consequently, glutathione production seem to be strongly related to both oxidative stress tolerance increase and soybean nutrient content enhancement. Youssefian and co-workers showed that transgenic plants modified to produce higher values of cysteine and thiols resulted to be more tolerant to oxidative stress in comparison to the control group (Youssefian et al. 2001). At the same time, genetic engineering has often been used to increase sulfur-containing-amino acids to improve the protein content and quality and it confirms the importance of this aspect at a commercial level (Krishnan 2005).

Surprisingly, the treatment performed with KIEM® strongly affected the thiol level in stressed seeds by increasing the amount of these important soluble antioxidant compounds (**Figure 3**). Based on these results, we can hypothesized that KIEM® priming effects on antioxidant capacity appear to be more correlated with thiol levels rather than on an increased transcription and/or scavenging enzyme activity.

These data, together with those observed for H_2O_2 content and CAT activity, suggest the role of KIEM® in heat stress mitigation. Moreover, the increase of thiols in treated seeds germinated at 35°C could be a good indication of a high product quality also in adverse conditions.

4. CONCLUSIONS

The innovative biostimulant KIEM® tested in this study acts as a priming agent in soybean grown under heat stress. In particular, this biostimulant was able to enhance the germination rate of soybean seeds incubated at 35°C in controlled conditions, although the effects were evident only at later stages (72 h after sowing). Field trials confirmed the laboratory data. Soybean plants grown from KIEM®-treated seeds cultivated in high temperature conditions (Brazilian soils) showed better biometric parameters at 7 days after sowing (root length and plant height) and at harvesting time (pod number/plant, thousand seed weight and yield) compared to untreated plants.

Furthermore, sequencing analysis carried out on seeds incubated 24 h at 35°C showed the modulation of about 900 genes (51 up-regulated and 828 down-regulated). Interestingly, almost half of the up-regulated genes encoded for different methyl-transferase families, proteins involved in abiotic stress response and in various processes, including DNA repair, a mechanism crucial for a successful seed germination. On the other hand, some metabolic pathways, such as carbohydrate metabolism, response to stress and hormone signalling were negatively regulated by KIEM® treatment. This unexpected down-regulation might be related to the activity of KIEM®, a priming agent probably able to decrease the water uptake in favour of cellular damage prevention.

In addition, the antioxidant system activity (ROS scavenging enzymes) evaluated on the same seeds employed for RNA sequencing, revealed a lower amount of hydrogen peroxide in KIEM®-treated seeds, correlated to a lower activity and lower expression level of the corresponding detoxification enzymes, thus confirming the protective action of this biostimulant.

Cucumis sativus (L.)– Cucumber

Cucumber (*Cucumis sativus* L.) is an important vegetable crop, mainly produced in Asia and Europe, also used as a model organism. Along with tomato, onion and melon, cucumber is the most widely cultivated vegetable species in the world (Bisognin, 2002). Cucumber germination and development is negatively affected by adverse conditions, including high temperature (Wang et al., 2014).
In this study, we evaluated the potential priming effects of KIEM® on cucumber seed germination under heat stress conditions. In order to determine the effects and the metabolic targets of this product, biometric, gene expression (qPCR) and biochemical (ROS-scavenging system) analyses were carried out on both cucumber untreated and KIEM®-treated seeds incubated for 24 or 48 h at 28°C (optimal condition) or 35°C (heat stress condition). Finally, in order to correlate the composition of KIEM® with its possible mechanism of action, a partial chemical characterization of the amino acid fraction of this product was obtained by GC-MS analysis.

2. MATERIALS AND METHODS – Cucumber (*Cucumis sativus* L.)

2.1 Plant material and biostimulant

Cucumis sativus L. (cucumber) seeds var. Vert Petit de Paris were purchased and certified OGM free by OLTER® (Piacenza, Italy) and treated with the biostimulant Kiem®, as described at paragraph 2.1 (Soybean).

2.2 Seed treatment and germination parameters

Cucumber seeds were treated by following the protocol developed by Green Has Italia S.p.A. KIEM® solution was diluted in distilled water using 1:3 (v/v) ratio, then added drop by drop to 2.5 g of dried seeds (to reach a final dosage of 2 mL kg^{-1}), kept in continuous shaking to allow the homogeneous distribution of the product. Following the treatment, seeds were dry at room temperature and then placed in glass Petri dishes (20 cm Ø) containing two filter papers saturated with 15 mL of distilled water. For treatment and control, 15 seeds per three Petri dishes were incubated in the dark at standard (28°C) or heath stress (35°C) conditions for 24 or 48 h. At 48 h, germination percentage and fresh biomass were measured in order to evaluate differences between KIEM®-treated and untreated seeds.

2.3 Total RNA isolation and cDNA synthesis

Total RNA was extracted by using the NucleoSpin® RNA Plant Isolation Kit (Macherey-Nagel, Germany) according to manufacturer's instructions. RNA concentration was measured using an UV/visible spectrophotometer Ultrospec 3000 (Pharmacia Biotech, Sweden). Total RNA quality was checked by using the RNA 6000 Nano kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, USA) according to manufacturer's instructions. Three biological replicates, each composed by 45 seeds (15 seeds per three different Petri dishes) were used.

First strand cDNA synthesis was accomplished with 1 μg of total RNA and random primers using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA), according to the manufacturer's instructions.

2.4 Gene expression analysis by qPCR

All qPCR analyses were run on a Stratagene Mx3000P Real-Time System (Agilent Technologies, USA) using SYBR Green I with ROX as reference dye. The reactions were performed with 10 μL of mixture consisting of 5 μL of 2XMaxima™ SYBR Green qPCR Master Mix (Thermo Fisher, USA), 0.5 μL of cDNA and 100 nM primers (Integrated DNA Technologies, USA). Thermal conditions were as follows: 10 min at 95°C, 40 cycles of 15 sec at 95°C, 20 sec at 57°C, and 30 sec at 72°C. Fluorescence was read after each annealing and extension phase. All runs were followed by a melting curve analysis from 55 to 95°C. *Ubiquitin* (*UBI*) was used as a reference gene to normalize the results. Primers for *Respiratory Burst Oxydase* (*RBOH*)*, Copper-Zinc Superoxide Dismutase* (*CuZnSOD*), *Manganese Superoxide Dismutase* (*MnSOD*), *Iron* Superoxide Dismutase (*FeSOD*), *Catalase* (*CAT*), *Glutatione-S-Transferase* (*GST*), *Isocitrate lyase* (*ICL*) and *Ubiquitin* (*UBI*) used in this work are reported in **Table 1**. All amplification plots were analysed with the MX3000P™ software (Agilent Technologies, USA) to obtain Ct values. The relative expression levels of each gene were estimated using the method previously described by Pfaffl (Pfaffl 2001).

Tab. 1 List of primers used for qPCR analyses. **ICL* primers were designed with Primer3 software (Koressaar and Remm, 2007)

Accession number	Gene	Primer pairs	Reference
NM_124165.3	*RBOHD*	F 5'-TCTTCTTCTTCTTCCTCCCTCAAAGCC-3' R 5'-GAAAGTTCAGGGTCTTCAAGAGAGTTGG-3'	(Jakubowska et al. 2015)
XM_004146841.2	*FeSOD*	F 5'-ATGAAAACATACAAAAAAGG-3' R 5'-ATGGACTCCCAGAGAAAATC-3'	(Xia et al. 2011)
XM_011651083.1	*MnSOD*	F 5'-CAATGGCGGAGGTCACATTA-3' R 5'-AGAGCAAGCCACACCCATC-3'	(Gao, LI, and YU 2009)
NM_001280768.1	*CuZnSOD*	F 5'-GACTGGGCCACATTTCAACC-3' R 5'-GCCTTGCCATCTTCACCAA-3'	(Gao, LI, and YU 2009)
GU248529.1	*CAT2*	F 5'-ACAATCACCACGAGGGTTTC-3' R 5'-GACAAAGCATCCACCCATCT-3'	(Hu et al. 2016)
XM_011652599.1	*GST*	F 5'-TTTGAGGAGGTGAAGGTAA-3' R 5'-ACGCACAAGAAATGTAGAT-3'	(Xia et al. 2011)
XM_004151832.2	*ICL*	F 5'-TGGCACATCAGAAATGGTCT-3' R 5'-GGACTTGGCTACCACCACAT-3'	*
AF104391	*UBI*	F 5'-CCTTATTGACCAACCAGTAGT-3' R 5'-GGACAATGTTGATTTCCTCG-3'	(Migocka and Papierniak 2011)
AB010922	*ACT*	F 5'-TGGACTCTGGTGATGGTGTTA-3' R 5'-CAATGAGGGATGGTGGAAAA-3'	(Qi et al. 2012)

2.5 Extraction and activities of antioxidant enzymes

Antioxidant (ROS-producing and scavenging) enzymes were extracted and analysed according to Contartese et al., 2016 using 0.5 g of powdered seeds (Contartese et al., 2016), as described in paragraph 2.6.). Total soluble protein content was measured according to (Bradford 1976).
NADPH oxidase (*RBOH; EC 1.6.3.1*). The activity of RBOH was measured spectrophotometrically by reading the changes in absorbance at 530 nm (Ozawa et al. 2009). A standard assay mixture contained 40 mM NADPH, 0.02 % (w/v) Triton X-100, 100 mM nitroblue tetrazolium (NBT) and buffer (20 mM Tris-chloride, pH 7.5, 3 mM $MgCl_2$) to make a total volume of 1 mL in a quartz cuvette. An additional 30 µM DPI (diphenyl iodonium) was added to the reaction mixture. The specific activity was calculated using an absorption coefficient of 12.8 mM^{-1} cm^{-1}.
Superoxide dismutase (SOD; EC 1.15.1.1) – the method used is the same described at 2.6 (Soybean).
Catalase (CAT; EC 1.11.1.6) - the method used is the same described at 2.6 (Soybean).
Glutathione-S-transferase (GST; EC 2.5.1.18) - the method used is the same described at 2.6 (Soybean). Three biological replicates, each composed by 45 seeds (15 seeds per three different Petri dishes) were used.

2.6 Extraction and activity of isocitrate lyase

All steps were carried out at 4°C. The plant material was homogenized in two volumes of extraction buffer containing 40 mM Hepes (N-[2-hydroxyethyl] piperazine-N%-[2-ethanesulfonic acid]) buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT and Tween 20 (1% v/v) (Maffei et al. 1999). The homogenate was centrifuged 30 min at 15000 x *g* (4°C) and the resulting supernatants were brought to 30% saturation with solid $(NH_4)_2SO_4$. After stirring for 2 h the solution was centrifuged at 10000 x *g* for 20 min and solid $(NH_4)_2SO_4$ was added slowly to the supernatant to 50% saturation. After stirring for 2 hours, the enzyme-enriched pellets were collected by centrifugation (10000 x *g* for 20 min), resuspended in a small volume of 40 mM Hepes (pH 7.0) and used for enzymatic assays.
Isocitrate lyase (ICL; EC 4.1.3.1) activity was recorded following NADH oxidation at 340 nm in the presence of an excess of lactate dehydrogenase (LDH) according to the protocol of Giachetti et al., 1983 (Giachetti et al. 1987). The reaction mixture, in a final volume of 1 ml, contained: 40 mM buffer (pH 7.0), 6 mM $MgCl_2$, 45 IU LDH (Lactic dehydrogenase), 0.28 mM NADH, 2 mM isocitric acid and enzyme extract. The reaction was started with isocitric acid. Three biological replicates, each composed by 45 seeds (15 seeds per three different Petri dishes) were used.

2.8 Non-protein-thiol content

The method used is the same described at 2.7 (Soybean).

2.9 Hydrogen peroxide content

The method used is the same described at 2.8 (Soybean).

2.10 KIEM® chemical analysis

Targeted and untargeted metabolomics was performed on KIEM® in order to identity polar metabolites as previously described by Lisec *et al.* (Lisec et al. 2006). Some adaptations were made to the protocol according to Villafort Carvalho (Villafort Carvalho et al. 2015). Briefly, polar metabolites were extracted from 50 mg of the biostimulant using methanol, followed by a 2-phase separation using chloroform. Aliquots of the polar phase were dried by vacuum centrifugation and (the dried samples were) derivatized online according to the protocol of Lisec et al. (2006) using a Triplus RSH autosampler system (Thermo Fischer Scientific, USA) that was coupled to the GC/MS system (Lisec et al. 2006). The derivatized samples were analyzed by gaschromatography (GC) (Thermo Trace 1300) coupled to mass spectrometry (MS) (Thermo TSQ Duo) system.

The chromatographic separation was performed using a VF-5MS capillary column [Agilent, 30 m × 0.25 mm (internal diameter) × 0.25 μm (film thickness)] including a 10-m guardian column with helium as carrier gas at a constant column flow rate of 1 ml min^{-1}. The GC oven temperature was isothermal for 2 min at 70°C, followed by a 10°C min^{-1} ramp to 310°C, and then held at this temperature for 10 min. The transfer line temperature was set at 280°C. The column effluent was ionized by electron impact at 70 eV. Mass spectra were acquired using selected reaction monitoring (SRM) as scan type, with preselected SRM transitions and collision voltage. The ion source was set at a temperature of 290°C. A solvent delay of 420 s was set up. The detector voltage was set at 1500 V.

Each sample was injected in two different concentrations in order to better detect and quantify the different compounds. External calibration curves of each amino acid were used for the identification and quantification. On the other hand, other polar compounds were tentatively identified using the in-house metabolite database.

2.11 Statistical analysis

Data are expressed as mean values ± standard deviation (SD) of three different biological replicates. For biometric data significant differences were evaluated by performing one-way ANOVA, Tukey-Kramer's post-hoc test, ($p \leq 0.05$). For biochemical (H_2O_2 and non-protein thiol content, and enzymatic activities) and molecular biological data (gene expression levels) results are expressed as a relative values (values obtained with KIEM®-treated seeds compared to the respective untreated seeds) and significant differences were evaluated by performing Student's *t*-test (p<0.05).

3. RESULTS AND DISCUSSION

3.1 KIEM® increases germination percentage and fresh weight under heat stress conditions

As observed in soybean, also the cucumber germination was positively affected by KIEM® application. In order to evaluate whether the biostimulant treatment had a visible influence on cucumber seeds at the early germination phase under heat stress conditions, germination rate and fresh weight were measured on control and KIEM®-treated seeds at 48h after incubation at 28°C and 48h after incubation at 35°C. At this time, both untreated and KIEM®-treated seeds incubated at 28°C showed similar germination percentage (**Table 2**). This result can be considered a good indication of not toxic effects exerted by the pre-sowing treatment with KIEM®. With regard to fresh weight, a significant lower value was recorded for treated seeds compared to untreated ones ($p \leq$ 0.05). Different results were obtained at 35°C. At this temperature, KIEM® treatment prompted a significant increase in the germination percentage and in the fresh biomass with respect to controls (**Table 2**).

Tab. 2 Germination percentage and fresh weight at 48 h after seed imbibition. Values are expressed as a mean (±SD). Different letters indicate significant differences among samples (ANOVA, Tukey-Kramer's post-hoc test, $p \leq 0.05$)

Seed treatment	Germination (%)	Fresh weight (g)
28°C Untreated	100±0.00 [a]	2.34±0.04 [a]
28°C Treated	100±0.00 [a]	2.13±0.006 [b]
35°C Untreated	93.4±0.11 [c]	1.30 ±0.00 [d]
35°C Treated	97.7±0.04 [b]	1.47±0.02 [c]

3.2 KIEM® decreases H_2O_2 levels in treated seeds germinated under heat stress conditions

In seed physiology, reactive oxygen species (ROS) are usually considered as toxic molecules, whose accumulation leads to cell injury with consequent problems in seed germination and development (Jeevan Kumar et al. 2015).
However, there is the increasing evidence that ROS, at low concentrations, can act as signalling molecules involved in a wide range of responses to various stimuli (El-Maarouf-Bouteau and Bailly 2008; Barba-Espín, Hernández, and Diaz-Vivancos 2012; Bailly 2004). The dual function of ROS in plants mainly relies to the cellular antioxidant machinery, which involves detoxifying enzymes (Alscher and Hess 2017) and antioxidant compounds (Gershenzon 1984). Such mechanisms can scavenge potentially toxic ROS, generally produced under stressful conditions, or rather tightly control ROS concentrations in order to regulate various signalling pathways. Among ROS, hydrogen peroxide plays a key role during germination process, however high levels of H_2O_2 can be toxic for the seeds (Wojtyla et al. 2016). The ability of seeds to survive to this oxidative condition during germination phases is related, at least partly, to their ability to activate different detoxification systems, including both the neo-synthesis of soluble antioxidants and the activation of gene expression of enzymatic defense (Lehner et al. 2006). To evaluate this aspect, the levels of endogenous H_2O_2 were evaluated in untreated and KIEM®-treated cucumber seeds incubated in standard (28°C) and heat stress condition (35°C) for 24 and 48 h. The results are reported in **Figure**

1. Values are expressed as a relative content obtained by comparing KIEM®-treated samples with the corresponding untreated controls.

Interestingly, in biostimulant-treated seeds, the endogenous H_2O_2 level was drastically reduced at both incubation temperatures with respect to untreated controls (**Figure 1**). These results suggest a potential protective role of KIEM® against the effects of oxidative stress.
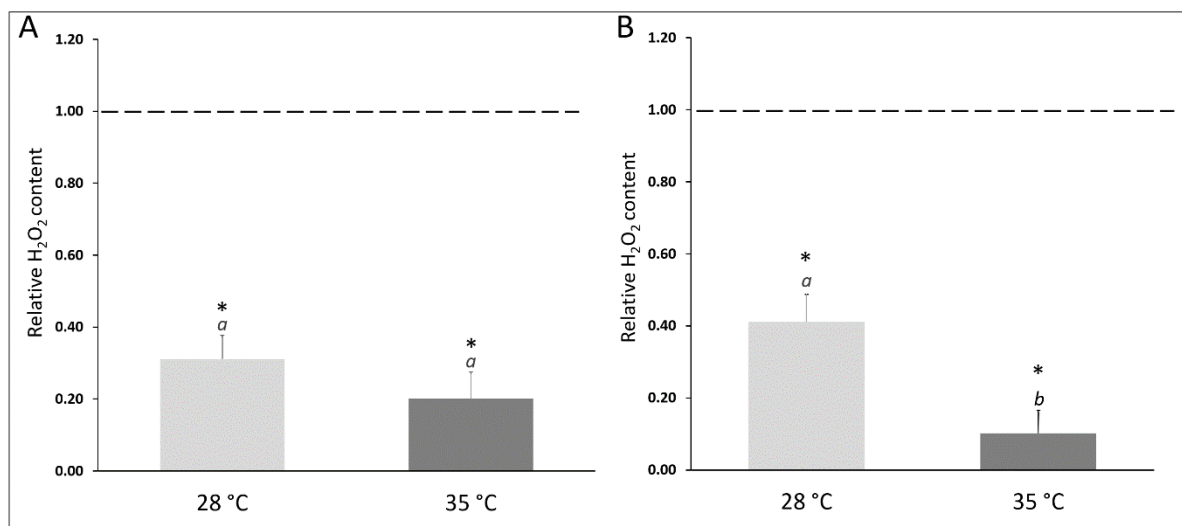

Fig. 1 Effect of KIEM® on H_2O_2 levels at 24 (**A**) and 48 h (**B**) after seed imbibition. Values are expressed as a relative content obtained by comparing KIEM®-treated samples with the corresponding untreated controls (dotted line). Bars represent the means ± SD of three biological replicates. For each bar, different letters indicate significant differences (p ≤ 0.05) between treatments at the two different temperatures (28 and 35°C), as measured by Student *t*-test. Asterisks (*) indicate significant differences between KIEM®-treated samples and the corresponding untreated controls (Student *t*-test, *p* ≤ 0.05).

In the end, the lower amount of H_2O_2 observed in KIEM®-treated seeds is linked to the expression level of genes coding for ROS-scavenging enzymes. The effect of KIEM® was particularly evident at 48 h and at 35°C (**Figure 3**).

3.3 KIEM® increases non-protein thiol production

In addition to the scavenging enzyme machinery, plants possess a number of antioxidant molecules that are able to counteract the effects of different stress, such as non-protein thiols, the most important source of sulfur in different seeds, a fundamental element involved in metabolic pathways, nutritional quality and plant productivity. Thiol metabolism was shown to play a key role in plant growth, development, and defense against a range of environmental stresses (Colville and Kranner 2010; Yi, Galant, et al. 2010).

It has been demonstrated that thiols, such as glutathione (GSH) together with its regulation in redox signalling and defense processes, are important components for the heat stress tolerance (Szalai et al. 2009). The glutathione pool was shown to be associated with the response to heat stress of maize (Kocsy, Szalai, and Galiba 2002), *Coleus blumei* and *Fagus sylvatica* L. (Peltzer, Dreyer, and Polle 2002), *Triticum aestivum* (L.) (Nieto-Sotelo and Ho 1986) and *Vigna radiata* (Nahar et al. 2015).

Directly linked to thiols are glutathione-S-transferases (GSTs), proteins playing important roles in enzymatic thiol-dependent ROS scavenging mechanisms (Zagorchev et al. 2013) since they catalyse the conversion of H_2O_2 by using glutathione or homoglutathione as substrates.

In our experiments, after 24 h incubation, non-protein thiol levels were higher in KIEM®-treated seeds incubated at 28°C (**Figure 2A**). Seeds treated at 35°C displayed an opposite effect, and clearly lead to a reduction in thiols, compared to the non-biostimulant control. Interestingly, an opposite trend was observed after 48 h, where an increase of the level of non-protein thiols was observed at 35°C in KIEM®-treated cucumber seeds (**Figure 2B**). In this case, an increase of the level of non-protein thiols was recorded at 35°C in KIEM®-treated cucumber seeds, suggesting a possible activation in the production of these molecules at later time and induced by the biostimulant application. These observations are in accordance with the results obtained in soybean, in which an accumulation of thiols was measured in treated seeds incubated at 35°C (Paragraph 3.3).

The obtained results are in correlation with the expression levels of *GST* (**Figure 3**).
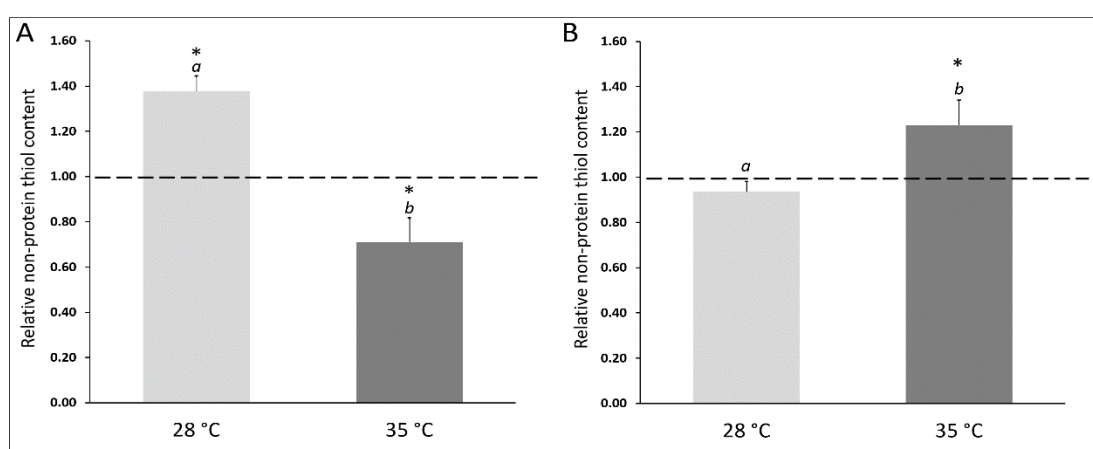
Fig. 2 Effect of KIEM® on non-protein thiol content at 24 (**A**) and 48h (**B**) after seed imbibition. Values are expressed as a relative content obtained by comparing KIEM®-treated samples with the corresponding untreated controls. Bars represent the means ± SD of three biological replicates. For each bar, different letters indicate significant differences (p ≤ 0.05) between treatments at the two different temperatures (28 and 35°C), as measured by Student *t*-test. Asterisks (*) indicate significant differences between KIEM®-treated samples and the corresponding untreated controls (Student *t*-test, $p \leq 0.05$).

3.4 KIEM® modulates the antioxidant enzyme activity and gene expression

To gain more insight into the seed response to heat stress and H_2O_2 production during the early phases of germination, the transcript levels and the activities of several ROS producing and scavenging enzymes namely, NADPH-Oxidase (RBOH), superoxide dismutase (SOD), catalase (CAT) and glutathione S transferase (GST) were evaluated.

The gene expression analysis was carried out on *RBOHD,* three *SOD* isoforms (*CuZnSOD, MnSOD, FeSOD*)*, CAT* and *GST* on untreated and KIEM®-treated cucumber seeds incubated in standard (28°C) and in heat stress conditions (35°C) for 24 and 48 h. **Figure 3** reports the data expressed as relative gene expression obtained by comparing each biostimulant-treated sample with the corresponding untreated control incubated in the same experimental conditions.
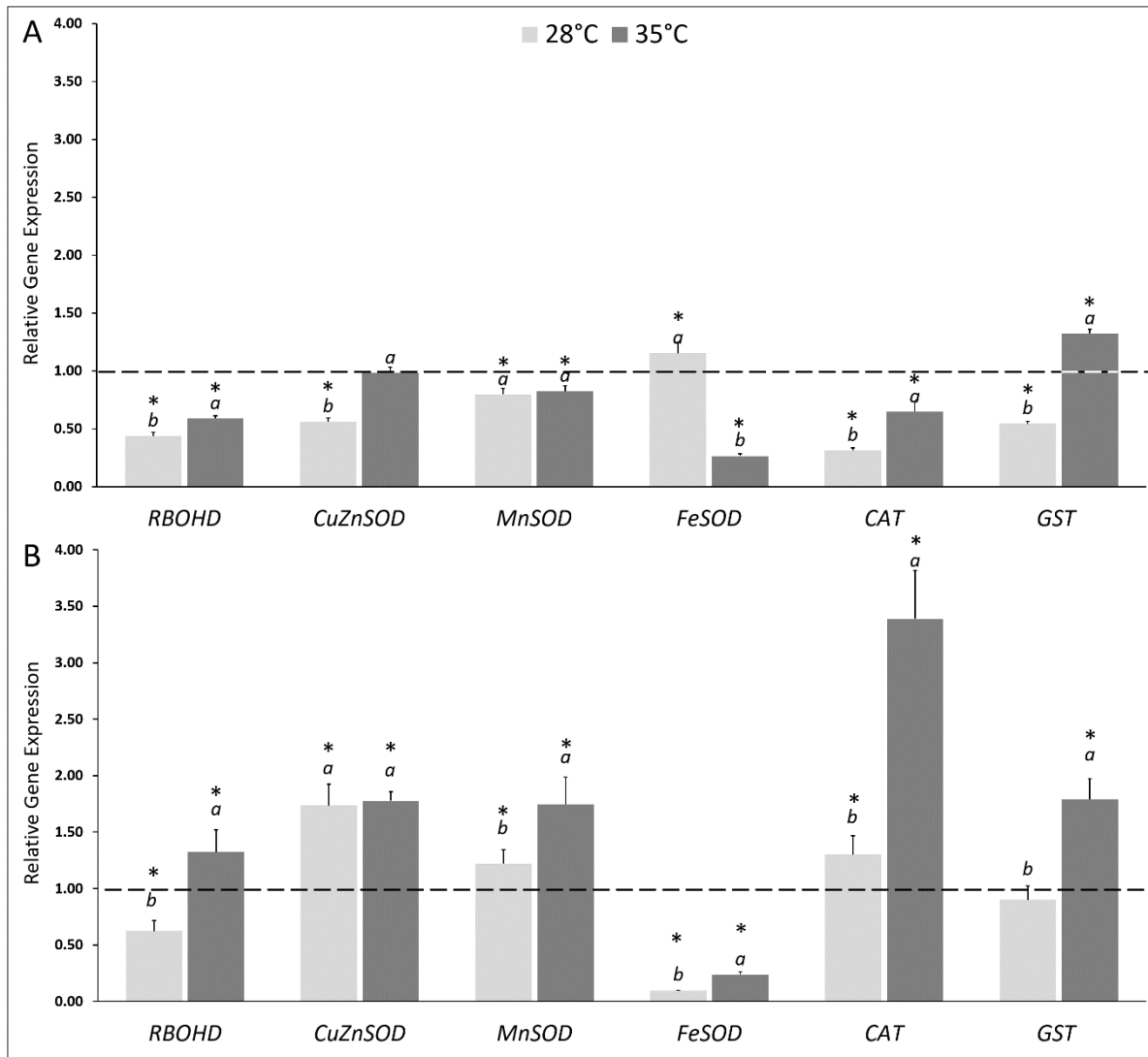
Fig.3 Effect of KIEM® on expression levels of genes coding for ROS producing (*RBOHD*) and scavenging (*CuZnSOD*, *MnSOD*, *FeSOD*, *CAT* and *GST*) enzymes after 24 (**A**) and 48 h (**B**) from seed imbibition. Values are expressed as a relative gene expression obtained by comparing KIEM®-treated samples with the corresponding untreated controls (dotted line). Bars represent the means ± SD of three biological replicates. For each bar, different letters indicate significant differences ($p \leq 0.05$) between treatments at the two different temperatures (28 and 35°C), as measured by Student *t*-test. Asterisks (*) indicate significant differences between KIEM®-treated samples and the corresponding untreated controls (Student t-test, $p \leq 0.05$).

In general, the treatment with KIEM® at 28°C did not exert a strong effect on antioxidant gene expression level, after 24h. After this incubation time, only two *SOD* isoforms (*MnSOD* and *FeSOD*) were slightly activated, while the other antioxidant genes were downregulated with respect to the control (**Figure 3A**). A different expression profile was obtained at 48 h, in which the biostimulant treatment stimulated a higher accumulation of three scavenger gene transcripts (*CuZnSOD* and *MnSOD* isoforms and *CAT*). Interestingly, the *FeSOD* isoform, upregulated at 24 h, was dramatically downregulated at 48 h (**Figure 3B**).

Also, when cucumber seeds were treated with KIEM® and incubated at 35°C for 24 h, downregulation of antioxidant genes, similar to that recorded at 28°C, was observed (**Figure 3A**). The downregulation of the genes coding for antioxidant enzymes might be correlated to the capacity

of this biostimulant to slow down the consequence of heat stress. At 24 h, all the genes coding for the antioxidant enzymes in analysis, except *GST*, were downregulated. On the other hand, at 48 h, KIEM® exerted a stronger effect at expression level (**Figure 3B**). Indeed, the treatment led to upregulation of the expression of all the antioxidant genes. This effect is probably due to the priming potential of KIEM® in preparing the seedlings to be more active to counteract the effects of heat stress.

With regard to the enzymatic assays, in general, a lower activity of the ROS producing and scavenging enzymes compared to the controls was recorded at both temperatures and incubation times (**Figure 4**). The data, expressed as relative enzymatic activity obtained by comparing each biostimulant-treated sample with the corresponding untreated sample, indicated a positive action of KIEM® in mitigating the effects of the oxidative stress. At 24 h (**Figure 4A**), the enzymatic activity profile followed the same trend as the gene expression pattern, since all enzymes showed a very low activity compared to the controls. This effect was particularly evident at 35°C, in which significant differences compared to the activity registered at 28°C were observed. After 48 h from the application of the biostimulant in heat stress conditions, a higher activity was observed for all antioxidant enzymes, compared to 24h. The enzymatic profiles follow the observations for gene expression and data are also in agreement with the lower amount of H_2O_2 measured in KIEM®-treated seeds (**Figure 1**).
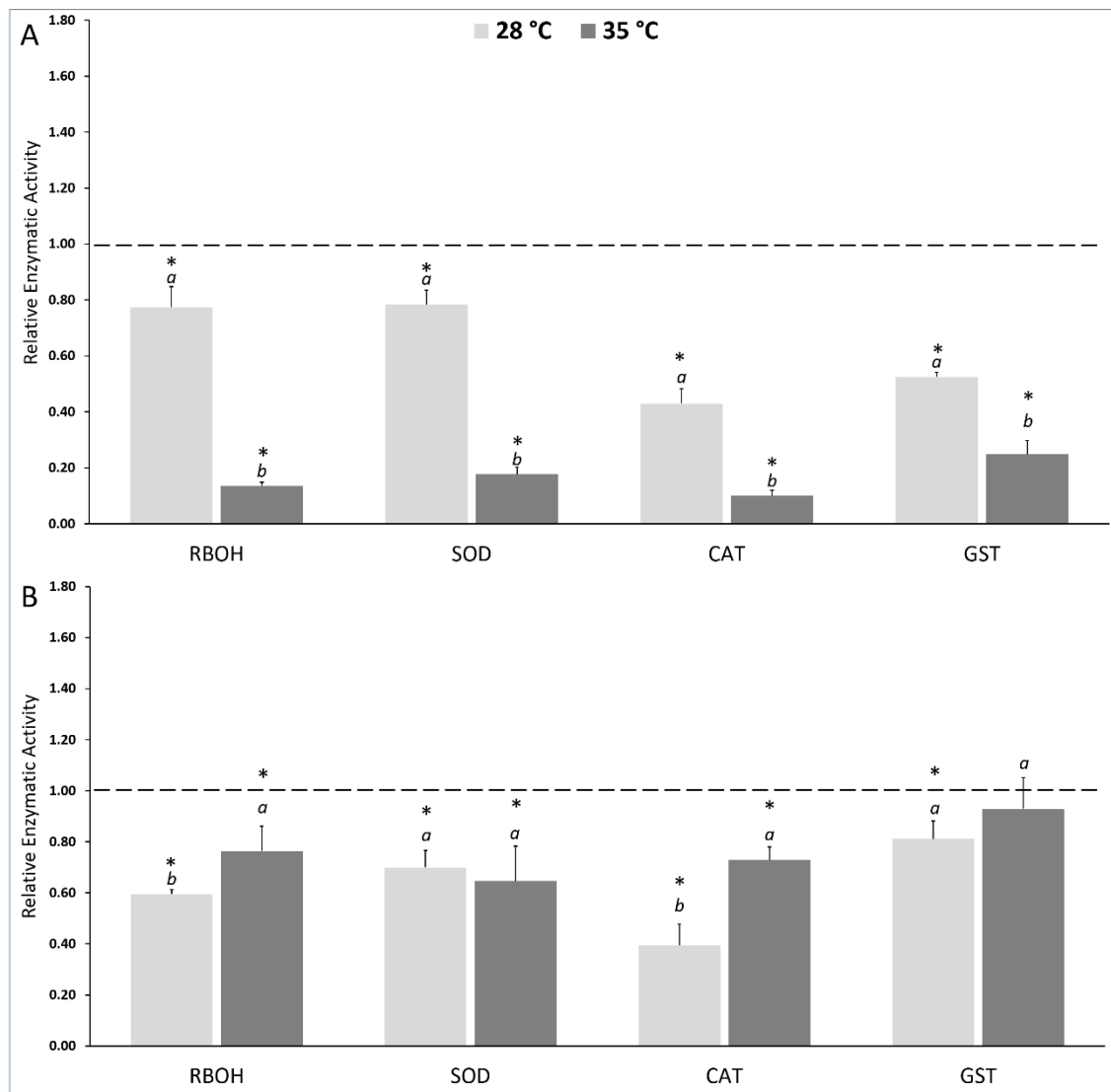
Fig. 4 Effect of KIEM® on enzymatic activities of RBOH, SOD, CAT and GST at 24 (**A**) and 48 h (**B**) after seed imbibition. Values are expressed as a relative enzymatic activity obtained by comparing KIEM®-treated samples with the corresponding untreated controls (dotted line). Bars represent the means ± SD of three biological replicates. For each bar, different letters indicate significant differences (p ≤ 0.05) between treatments at the two different temperatures (28 and 35°C), as measured by Student t-test. Asterisks (*) indicate significant differences between KIEM®-treated samples and the corresponding untreated controls (Student t-test, $p \leq 0.05$).

3.5 KIEM® increases the isocitrate lyase activity and gene expression

During cucumber seed germination, the glyoxylate cycle plays a key role in the mobilisation of triacylglycerides located in storage tissue during post-germinative growth to effect net gluconeogenesis from the acetyl-CoA derived by β-oxidation (Lamb et al. 1978; Reynolds and Smith 1995; Dunn, Ramirez-Trujillo, and Hernández-Lucas 2009). During early germination phases, enzymes of the glyoxylate cycle such as isocitrate lyase increase their activity during maximum fat metabolism in specialized microbodies (glyoxysomes) located in the storage tissue of germinating seeds (McLaughlin and Smith 1994). In cucumber, several factors affect the synthesis, activity and regulation of ICL. It has been demonstrated that the amount and developmental pattern of ICL

activity may vary depending on germination conditions such as temperature, light, hormones and secondary metabolites (Giachetti et al. 1987).

For these reasons, the upregulation of the gene coding for ICL is essential for the seed health status, and its down-regulation might be linked to particular stress conditions.

In standard conditions (28°C), KIEM® positively affected the level of *ICL* expression at 24 h. This effect was even stronger at 48 h in which a significant ($p \leq 0.05$) up-regulation compared to control was observed (**Figure 5**). The most pronounced effect was obtained with cucumber seeds treated with KIEM® at 35°C. At this temperature condition, the application of the biostimulant promoted a significant increase in *ICL* transcript levels compared to control and compared to the level of transcripts observed at 28°C, suggesting a positive effect of this biostimulant in enhancing the germination process, at least when judged from ICL expression level, at high temperature conditions (**Figure 5**).

With regard to biochemical results, in general a lower ICL enzymatic activity was observed in cucumber seeds treated with KIEM® compared to controls at both incubation times and temperatures. However, the observed values showed a similar trend of the gene expression profile (**Figure 6**).

Based on these results, the application of KIEM® promoted a strong accumulation of *ICL* transcripts, especially at 48 h under heat stress conditions, suggesting a positive action of this biostimulant in enhancing cucumber seed germination.

Fig. 5 Effect of KIEM® on *ICL* expression levels at 24 (**A**) and 48 h (**B**) after seed imbibition. Values are expressed as a relative gene expression obtained by comparing KIEM®-treated samples with the corresponding untreated controls (dotted line). Bars represent the means ± SD of three biological replicates. For each bar, different letters indicate significant differences (p ≤ 0.05) between treatments at the two different temperatures (28 and 35°C), as measured by Student *t*-test. Asterisks (*) indicate significant differences between KIEM®-treated samples and the corresponding untreated controls (Student *t*-test, $p \leq 0.05$).
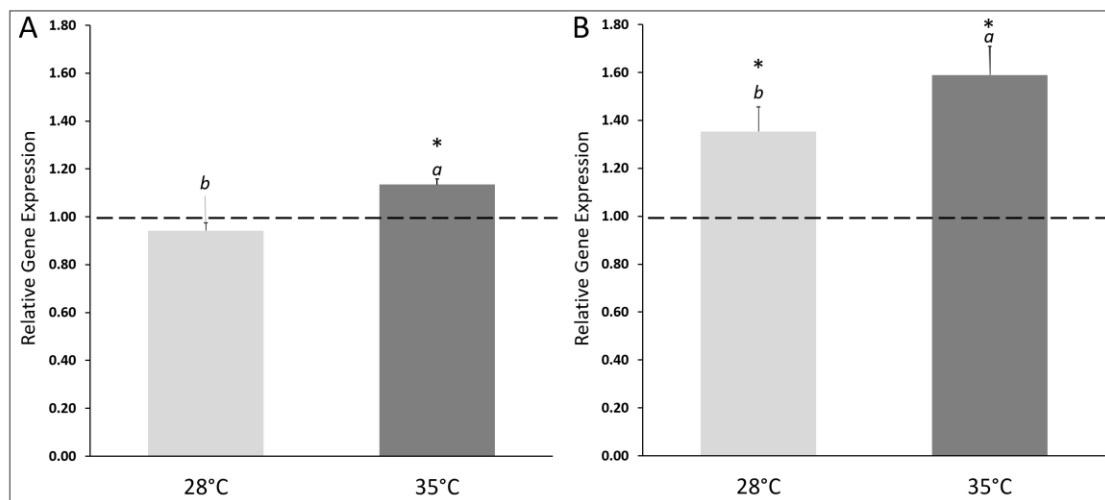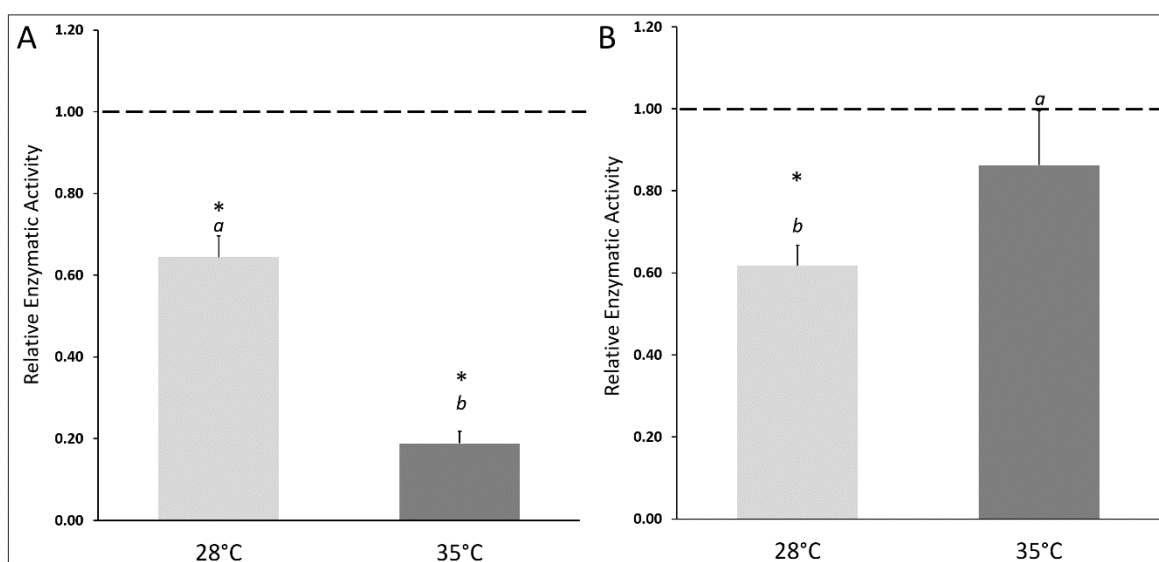
Fig. 6 Effect of KIEM® on ICL enzymatic activity at 24 (**A**) and 48 h (**B**) after seed imbibition. Values are expressed as a relative enzymatic activity obtained by comparing KIEM®-treated samples with the corresponding untreated controls. Bars represent the means ± SD of three biological replicates. For each bar, different letters indicate significant differences (p ≤ 0.05) between treatments at the two different temperatures (28 and 35°C), as measured by Student *t*-test. Asterisks (*) indicate significant differences between KIEM®-treated samples and the corresponding untreated controls (Student *t*-test, $p ≤ 0.05$).

3.6 KIEM® amino acid fraction characterization

In this work, the main polar metabolites present in KIEM® were analysed through targeted and untargeted analysis using GC-MS. GC-MS analysis revealed the presence of five essential (**#5**, leucine; **#7**, isoleucine; **#9**, threonine**; #10**, methionine; and **#12**, phenylalanine) and three non-essential amino acids (**#2**, alanine; **#8**, serine; **#14**, glutamic acid) in detectable amount (**Table 2**). Moreover, other polar metabolites were tentatively identified comparing their retention time (RT), molecular weight (MW) and mass fragmentation (m/z) to literature data. Among them five organic and inorganic acids (**#1**, lactic acid; **#3**, sulfuric acid; **#6**, phosphoric acid and **#15**, citric acid), two sugars (**#16,** fructose and **#18,** galactose), myoinositol (**#19**), oxoproline (**#11**) and glycerol (**#4**) were identified. However, due to the lower and not significant amount with respect to amino acid compounds, the quantification was performed only for the amino acid fraction. GC-MS data are reported in **Table 2**. The most abundant amino acid found in KIEM® is glutamic acid (**#14**) followed by methionine (**#10**). The sums of these two compounds counted for more of 55% of the total amino acid content.

Tab.3 GC-MS analysis of the polar metabolite content in the biostimulant KIEM®. Quantification of amino acids was performed using an external calibration curve of pure standards. Values are expressed as µg ± SD of three different replicates. Data with different letters indicate significantly different values at p ≤ 0.05 as measured by ANOVA test followed by Tukey-Kramer's post hoc test. The symbol "*"categorizes the compounds that were tentatively identified simply on the base on their mass fragmentation and by comparison with literature data.

#	RT	Compound(s)	µg per mL of KIEM®
1	8.21	Lactic acid	*
2	8.83	Alanine	269.23 ± 19.67[e]
3	9.79	Sulfuric acid	*
4	11.14	Glycerol	*
5	11.19	Leucine	902.32 ± 15.49[c]
6	11.19	Phosphoric acid	*
7	11.51	Isoleucine	948.45 ± 62.75[c]
8	12.32	Serine	131.23 ± 12.61[e]
9	12.65	Threonine	431.12 ± 32.44[d]
10	13.81	Methionine	1371.01 ± 60.28[b]
11	14.59	Oxoproline	*
12	15.77	Phenylalanine	743.12 ± 18.83[d]
13	15.84	Arabinose	*
14	17.27	Glutamic acid	2930.22 ± 197.91[a]
15	17.67	Citric acid	*
16	18.28	Fructose methyloxime	*
17	18.42	Glucose	*
18	18.61	Galactose methyloxime	*
19	20.29	Myo-inositol	*

In the recent years, several scientific studies reported the beneficial effects of the application of plant-derived protein hydrolysates as biostimulant in order to increase the growth, yield and fruit quality of agricultural crops (Shafeek, Helmy, and Omar 2015). Since the beneficial properties of biostimulants were largely linked to their content of amino acids and others polar metabolites (Nardi et al. 2016), investigation about the chemical profile is actually essential to elucidate the possible mechanism of action of these products. The chemical profile of these formulations depend clearly on the raw material used for their manufacture processes, and the use of different raw materials determines changes both in metabolite profile and in plant physiological activity.

Glutamic acid, that it is highly abundant in KIEM®, plays also an important role during the seed germination processes, being the precursor of glutamine, arginine and proline (Buchanan, Gruissem, and Jones 2000). These metabolites are in turn the substrates for two aminotransferases (AspAT and AlaAT) activated during seed imbition (Rocha et al. 2010). In addition, glutamic acid may be also degraded into asparagine during the germination of seed, providing the energy required for its germination (Sivaramakrishnan and Sarma 1956). On the other hand, methionine is involved in the synthesis of several enzymes correlated to DNA-maintenance and to metabolism during the highly active state in seed germination of different plants (Szczotka, Pawłowski, and Krawiarz 2003). Finally, methionine is also linked to the synthesis of polyamines and ethylene, which are involved in

countering the action of ABA during the germination phase (Szczotka, Pawłowski, and Krawiarz 2003).

Interestingly, both glutamate and methionine are important precursors for the cellular antioxidant glutathione, which is a tripeptide composed of glutamate, glycine and cysteine. Cysteine is produced from methionine in the cellular metabolism (Wirtz and Droux 2005). Possibly, the application of glutathione precursors in KIEM® provides a pool of precursors for enhanced cellular antioxidant levels, and consequently to a better tolerance against oxidative stress and temperature stress.

However, despite the high content of methionine and glutamic acid in the amino acid fraction of this biostimulant, we cannot exclude that the displayed effects both on the balance of oxidative status, and on the expression of genes coding for antioxidant enzymes and isocitrate lyase might also depend on other compounds (i.e. lignin derivatives) present in the formulation of KIEM®. Probably, the effects discussed in this paper are the consequences of a synergic action of several metabolites.

4. CONCLUSIONS

Plant-based biostimulants are an excellent choice if a more sustainable and ethical approaches have to be used in agriculture. In this work, we showed that KIEM®, an innovative biostimulant, is able to increase the germination rate and restore the oxidative balance in cucumber seeds under heat stress conditions. The balancing effect is displayed not only through the reduction of endogenous H_2O_2 but also through the activation of antioxidant defenses. Indeed, the pre-sowing treatment with KIEM® is able to restore the capacity of synthesizing the soluble antioxidants and modulate the expression of genes coding for antioxidant enzymes. Moreover, our study provided also the experimental evidence that this biostimulant is able to regulate positively the *ICL* expression, a gene coding for a key enzyme involved in the germination process. Finally, it is interesting to note that the most significant protective effects occur always after 48 hours from the application of the biostimulant in heat stress condition. Probably, the effects of the biostimulant are the consequence of a synergic action of the different and several metabolites in the formula.

5. FINAL CONSIDERATIONS

The results obtained on both soybean and cucumber suggest a potential application of KIEM® as a seed priming biostimulant. This new product may improve tolerance to heat, and likely to other abiotic stress, by triggering positive responses (i.e. decrease in H_2O_2 content, increase in thiol accumulation, different modulation of the enzymatic antioxidant machinery, etc.) in more than one crop as observed in this work. The use of KIEM® as a pre-sowing agent could be of paramount importance for reducing the number of treatments and thus the final management costs, especially in those countries characterized by adverse environmental conditions.

Chapter 2

VIVEMA® TWIN, a new biostimulant based on hydrolysable and condensed tannins, is able to enhance root development and mitigate salt stress

1. INTRODUCTION – salt stress and root development

The plant root system is involved in nutrient and water uptake, interactions with soil microorganisms and in anchoring plant to the ground. Moreover, roots are the main organ directly in contact with the surrounding environment, able to respond to different stresses (Koevoets et al. 2016). Root function is essential to maintain plant productivity, even under stressful conditions. Among these, high salinity in soil represents one of the most important limiting factors for crop production. It affects plant growth and development and causes water leakage and nutritional disorders (Läuchli and Grattan 2007). About the 20% of arable lands is affected by salinity and this represents a real problem considering the increasing population (Yamaguchi and Blumwald 2005). Plant breeding and genetic engineering are very helpful techniques, but also expensive and time-consuming (Nogué et al. 2016). For this reason, finding faster solutions, useful when needed is an everyday-challenge to help plants. Among the new generation products available on the market, biostimulants could be useful for this purpose.

In this study VIVEMA® TWIN, a new biostimulant based on an original mix of hydrolysable and condensed tannins working in synergy, was tested on tomato plants under optimal and salt stress conditions and root growth parameters were evaluated. Hydrolysable tannins, water soluble phenolic compounds of variable size able to precipitate proteins, are known since ancient times and used for leather treatment, wine and beer clarification, textile dyes, coagulants in rubber production or as a supplement in forage (Khanbabaee and van Ree 2001; Sieniawska and Baj 2017). Differently, their use in agriculture is little known. Few studies are reported in literature and most of them concerns only the use of chestnut tannin (Bargiacchi et al. 2012). Tannins are one of the most abundant secondary metabolites made by plants. Thanks to the protein-binding capacity, they are involved in protecting plants by herbivores and insects (Barbehenn and Peter Constabel 2011). Probably, the role in salt stress protection could be related to their strong antioxidant activity. Flavonoids, of which tannins are a subfamily, are known to have antitumor, anti-allergic and anti-inflammatory effects, all features connected to their antioxidant property (Vázquez et al. 2008). Moreover, gallic acid, the main building-block of hydrolysable tannins, present in high quantity in VIVEMA® TWIN, is also involved in root development thanks to its auxin-like activity (Negi et al. 2005). The root growth increase, together with an intense antioxidant activity, are factors linked to short and long-term plant adaptation to salt stress, fundamental aspects to improve plant strength (Maggio et al. 2001).

Tomato (*Solanum lycopersicum*) was selected as model system because of its global diffusion and economic relevance (Kimura and Sinha 2008). The importance of this crop as a food source, the large amount of data available in literature and the easy greenhouse cultivation, make tomato plants a good model for plant physiology.

2. <u>MATERIALS AND METHODS</u>

2.1 Plant material, biostimulant and treatment

In this study VIVEMA® TWIN , a biostimulant developed by Green Has Italia, was used. This product is made by two different matrices, mixed together in a specific ratio. One raw materials is characterized by a prevalence of hydrolysable tannins, while the other one by a prevalence of condensed tannins. Tomato Heinz 1706 (*Solanum lycopersicum* L.) seeds were obtain from the Center for Genetic Resources the Netherlands (CGN, Wageningen University and Research). They were sown in plate on wet filter paper and then incubated in growth chamber (25°C, 16/8 h light/dark) for 7 days. Seedlings were then transferred in greenhouse in plastic pots containing 100% of sand and watered three times a week with nutritional solution 1 g L^{-1} (Hyponex, Japan). For each growth condition (unstressed/untreated, unstressed/treated, stressed/untreated, stressed/treated), twenty plants were used, randomly distributed, considering each plant as a biological replicate (fully randomized experimental design). All plants were used for data collection (biometry, biochemistry and transcriptomics). Plants were treated by fertigation from the first leaf appearance (BBCH 11) once a week, for four weeks. VIVEMA® TWIN 1 mL L^{-1} and the single raw materials (1 and 2) were tested under optimal and salt stress conditions. The salt stress was induced starting after the first treatment (priming treatment) by watering plants three times a week with a 100 mM NaCl solution. Roots were collected at two time points, 24 hours after the second treatment (ten plants for each condition) and 24 hours after the fourth one (ten plants for each condition).

2.2 Biometric data collection

Root length and fresh weight were measured at both time points. Before plant collection, NDVI index was measured (Pigment Analyzer PA110 - Control in applied Physiology, Germany). NDVI index is based on light absorption and reflectance. In optimal conditions, chlorophyll absorbs red light and, conversely, cellular structure reflects light in the near infrared band. In case of stress, red band reflectance increases and near infrared band reflectance decreases. NDVI measurements can range from -1 to 1, with higher values indicating better plant health (www.specmeters.com).

2.3 Gallic acid – synergy evaluation

In order to compare the synergic effect due to the use of VIVEMA® TWIN with the effect of a pure compound known to be involved in root and plant development, gallic acid was tested on plants. The protocol was the same reported in the paragraph 2.1. The dose was defined based on a LC/MS quantification using an external standard curve. For each mL of VIVEMA® TWIN, 13 mg of gallic acid were quantified. In order to use a dose comparable to the mixture (1 mL L^{-1}) for plant treatment, 13 mg L^{-1} of gallic acid were dissolved in water and applied.

2.4 "Short-term" test for imaging analysis

The same protocol used for the above mentioned trials (paragraph 2.1) was applied in a "short-term" test, useful to evaluate the root growth in the first phase of development. Plants were treated with 1 ml L^{-1} VIVEMA® TWIN or 13 mg L^{-1} gallic acid for four times. In this case, the treatment was done every 4 days, starting 2 days after transplant in greenhouse. Control and treated groups were

grown under optimal and salt stress conditions (mild 100 and strong 200 mM NaCl). Salt was added to the treatment solution and to all the irrigations. Roots were collected 24 h after the second and the fourth treatment and length and fresh weight were measured. Moreover the picture of each root were processed on Root System Analyzer®, an automated approach employed to measure root architectural parameters from two-dimensional root images (Schnepf et al. 2015). The original photo is converted into a skeleton in which each radical order is indicated with a different colour. Total number of roots, measured according to the procedure described above, was taken into account. For each conditions, at both time points, 10 plants were analysed.

2.5 Total RNA isolation

Total RNA was extracted from powdered roots of four (first sampling) and six (second sampling) week-old plants, respectively, using TRIzol™ reagent (Thermo Fisher Scientific, USA). One mL of reagent was added to 100 mg of homogenized sample and incubated 5 min to allow the complete dissociation of nucleoprotein complex. Then, 0.2 mL of chloroform were added and after 3 min of incubation and centrifugation (15 min at 12000 x g) the mixture showed 3 phases. The upper aqueous phase, containing RNA, was transferred to a new tube. To allow the RNA precipitation, 0.5 mL of isopropanol were added and samples were incubated 20 min at -20°C. After centrifugation (10 min at 12000 x g) RNA formed a pellet (normally invisible). Supernatant was discarded and the pellet washed twice with 500 µL of ethanol 75% (v/v). Finally, RNA pellets were suspended in 40 µL of nuclease-free water. RNA quality was observed in 1% (w/v) agarose gel. RNA purity was measured using a NanoPhotometer® (IMPLEN, USA). RNA integrity was checked by using the Nano 6000 Assay Kit and the Agilent 2100 Bioanalyzer 2100 (Agilent Technologies, USA). Finally, RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Fluorometer (Life Technologies, USA). Three biological replicates (formed by at least three plant root systems) were prepared starting from the ten plants collected for each condition, at both time points.

2.6 RNAseq analysis

RNAseq analysis was performed by Novogene (Hong Kong, China) starting from roots treated with VIVEMA® TWIN and control groups, grown under optimal and salt stress conditions (100 mM NaCl), collected at both time points.

Library preparation for Transcriptome sequencing

A total amount of 3 µg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer(5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase（RNase H-）. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 150~200 bp in length, the library

fragments were purified with AMPure XP system (Beckman Coulter, USA). Then 3 μL USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. Then PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system (Agilent Technologies, USA).

Clustering and sequencing (Novogene Experimental Department)

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using HiSeq PE Cluster Kit cBot-HS (Illumina, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq platform and 125 bp/150 bp paired-end reads were generated.

Quality control

Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low quality reads from raw data. At the same time, Q20, Q30 and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

Reads mapping to the reference genome

Reference genome and gene model annotation files were downloaded from genome website directly. Index of the reference genome was built using Bowtie v2.2.3 and paired-end clean reads were aligned to the reference genome using TopHat v2.0.12. TopHat was selected as the mapping tool for that TopHat can generate a database of splice junctions based on the gene model annotation file and thus a better mapping result than other non-splice mapping tools.

Quantification of gene expression level

HTSeq v0.6.1 was used to count the reads numbers mapped to each gene. And then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels (Trapnell, Williams, Pertea, Mortazavi, Kwan, Van Baren, et al. 2010).

Differential expression analysis

Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq R package (1.18.0). DESeq provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq were assigned as differentially expressed.

GO enrichment analysis of differentially expressed genes

Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the GOseq R package, in which gene length bias was corrected. GO terms with corrected P value less than 0.05 were considered significantly enriched by differential expressed genes.

2.7 cDNA synthesis and Quantitative Real-Time PCR (qPCR)

RNAseq data were validated through qPCR analysis on genes selected among the most significantly modulated by the application of VIVEMA® TWIN. The same genes were also analysed in gallic acid-treated roots to evaluate the gene expression differences between the biostimulant and a pure compound with the same target (see paragraph 2.3). Genes and related primers, designed with *Primer3* software (Kõressaar et al. 2018), are reported in **Table 1**.

Tab.1 Primers of target genes designed with *Primer3* software

GENE	ID	Primer F	Primer R
WRKY transcription factor 61	Solyc12g056750	5'-AGTGTTCAATTCGAGGATGCA-3'	5'-AGCTTTCATGATTGTGTTTGCCT-3'
Trehalose 6-phosphate phosphatase	Solyc04g082550	5'-TGGAACAAAGGTCATGCATTGG-3'	5'-TGGTGTCTTTTGGAGCAGCA-3'
Na+/H+ exchanger 8	Solyc05g024410	5'-TGATTTGCAAGGATGTGCTCC-3'	5'-CTAGCGGCTTTCCCAATCGA-3'
C2H2 zinc finger protein	Solyc10g084910	5'-TTAGAAAACAAGGGCATGGAGC-3'	5'-GAAGTTCAAATCGTGGATCTGTG-3'
Glutathione S-transferase	Solyc02g081340	5'-AAGCCACCTCCCTAGCCTTA-3'	5'-TGGGCCGGGTAACATCTCTA-3'
Major facilitator superfamily protein	Solyc12g006050	5'-AACAACGAAGGGAAAACGCG-3'	5'-TCGAGTTGTCCAACGTAGGC-3'
ERD (early-responsive to dehydration stress)	Solyc06g084330	5'-TGAGCACCAAAGAGGCTTCC-3'	5'-ACTGTATCCTTCACCACCGC-3'
Glycosyltransferase	Solyc10g009580	5'-ATGTGTCGGTTGGTGGATTT-3'	5'-GTCTGCAAAGAGAGGCCAAG-3'
Xyloglucan endotransglucosylase-hydrolase 5	Solyc08g005610	5'-TCCCCGGTACACTCTTCAAC-3'	5'-ATGAATGACCCGAGCAAATC-3'
phosphate starvation inducible gene TPSI1	Solyc03g098010	5'-GATCATTGCCTTGAGCACAT-3'	5'-TTCAACCCTTTCACGTCTCC-3'

Half a μg of cDNA was synthesized by using the Maxima H Minus First Strand cDNA Synthesis kit (Thermo Fisher Scientific, USA), following the manufacturer's instructions.

For each qPCR reaction primers 0.3 μM, 4.1 μL of nuclease-free H_2O and 5 μL of SYBR-Green I (Maxima SYBR Green/ROX qPCR Master Mix 2X, Thermo Scientific, Thermo Fisher Scientific, USA) were used. Three biological replicates were analysed for each treatment and three technical replicates for each biological replicate. The qPCR reactions were performed using QuantStudio 1 Real-Time PCR System (Thermo Fisher Scientific, USA). All the conditions used in the amplification are reported in **Table 2**. Four different reference (housekeeping) genes (*Tubulin*, *Ubiquitin*, *Elongation Factor 1* and a catalytic subunit of *Protein Phosphatase 2A*) were used to calibrate and normalize qPCR results. The reference primers were designed based on the work of Løvdal and Lillo (Løvdal and Lillo 2009).

The most stable gene was *Elongation Factor 1* (*EF1*).

Relative expression levels of genes were calculated by using the Pfaffl method (Pfaffl 2001).

The variations in gene expression were based on relative quantification of the genes of interest in relation to the housekeeping genes used as a reference.

Tab.2 qPCR instrument conditions

Step 1 (1 cycle)	Initial denaturation	10 min, 95°C
Step 2 (45 cycles)	Denaturation	15 sec, 95°C
	Annealing	20 sec, 57°C
	Extension	30 sec, 72°C
Step 3 (1 cycle)	Melting curve	1 min, 95°C
		30 sec, 55°C
		30 sec, 95°C

2.8 VIVEMA® TWIN chemical characterization

VIVEMA® TWIN, raw material 1 and raw material 2 were analysed by using an Orbitrap Fourier Transformed Mass Spectrometer (FTMS; Thermo) hybrid system. Biostimulant and single components were extracted in methanol 50% v/v and 1% (v/v) of formic acid, 100 µL mL^{-1} for VIVEMA® TWIN and 50 µL mL^{-1} for the two raw materials. Samples were vortexed and sonicated 10 min. After 15 min of centrifugation at maximum speed in a microfuge, the supernatant was transferred in a new tube. The extracts were then incubated over-night at -20°C and again sonicated and centrifuged. The obtained supernatant were transferred in vials for the analysis. The final extract was not filtered to avoid loss of compounds of interest. A LUNA 3 µ C18 (2) 150 × 2.00 mm column (Phenomenex, USA) was used as described before (Outchkourov et al. 2014). A linear gradient from 5 to 35% B in 45 min at a flow rate of 0.19 mL min^{-1} was used. The FTMS was set at a mass resolution of 60,000 HWHM and a mass range of m/z 140-2000, using electrospray ionization in negative mode. Identification and quantification was based on retention times and accurate masses compared to the pure standards (5-20 µg mL^{-1}) of ellagic acid, gallic acid and tannic acid. Data analysis was performed in an untargeted manner, essentially as previously described (De Vos et al. 2007; Outchkourov et al. 2014).

2.9 Statistical analysis

Data, if not differently specified, were analysed through one-way analysis of the variance (ANOVA) using "Systat Version 10" (Systat Software, USA) followed by Tukey-Kramer's post-hoc test ($p \leq 0.05$). Data are expressed as mean values ± standard deviation (SD) of three different biological replicates.

3. RESULTS AND DISCUSSION

3.1 VIVEMA® TWIN increases tomato root growth and NDVI index under optimal and salt stress conditions

Biometric measurements such as root length and fresh weight were carried out in order to evaluate the effect of VIVEMA® TWIN, raw material 1 and raw material 2 on root development, under optimal and salt stress conditions. Hydrolysable and condensed tannins, main components of the biostimulant in analysis, are used in a number of industrial applications. It is reported how these compounds can be used as phenolic resin adhesive (Spina et al. 2013), wood adhesive (Ping et al. 2011), corrosion protector of iron and steel (Rahim and Kassim 2010) or in animal nutrition. This last application allows to reduce the amount of protein digested in the rumen and enhance the amount of proteins available for digestion in the small intestine (Mueller-Harvey 2006). Based on tannin characteristics, such as antioxidant activity, the possibility of using them also in agricultural field has being investigated, however at the moment only few information are available.

Fig. 1A and **1B**, show root length and root fresh weight, respectively. At the first time point, salt stress did not affect root growth, in comparison to unstressed plants. Differently, at the second time point (**Fig. 1C** and **D**), the trend was negative in stressed plants compared to the unstressed ones, even if not significantly. It is important to remember that salt stress induction started just before the first time point, so probably at the first sampling plants were not under stress yet. Indeed, normally salt stress negatively affects root and plant development. Machado and colleagues (Machado and Serralheiro 2017) reported how yield of many crops is decreased by the presence of salt in the soil. Final yield is just the last point of a chain in which each link is negatively affected. Root growth, shoot development, growth rate, water uptake, photosynthesis, biomass, flower and fruit production are all factors decreased by salt stress (Machado and Serralheiro 2017; Galvan-Ampudia and Testerink 2011; Parihar et al. 2015).
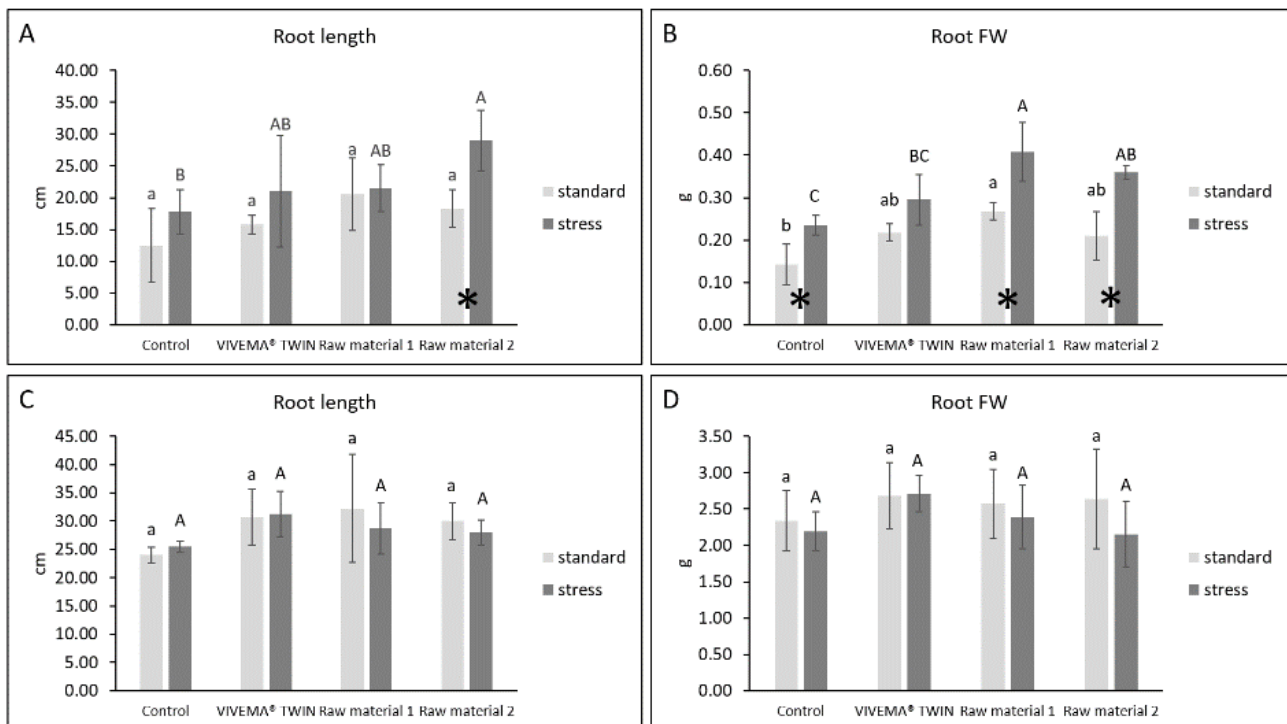
Fig. 1 Root length and fresh weight (FW) measured 24 h after the second treatment (**A** and **B**) and 24 h after the fourth treatment (**C** and **D**) in optimal and salt stress conditions. In all conditions and time points, treated plants showed a positive increase in the analysed parameters in comparison to the relative control. After two treatments, root length was higher and statistically significant in all the treated plants under salt stress (VIVEMA® TWIN=21cm ± 8.83, Raw material 1=21.5cm ± 3.7, Raw material 2=29cm ± 4.74), compared to the control (17.75cm ± 3.49) (**A**). The same significant increase was observed for fresh weight at both optimal (control=0.14g ± 0.05, VIVEMA® TWIN=0.22g ± 0.02, Raw material 1=0.27g ± 0.02, Raw material 2=0.21g ±0.06) and salt stress conditions (control=0.24g ±0.02, VIVEMA® TWIN=0.30g ± 0.06, Raw material 1=0.41g ± 0.07, Raw material 2=0.36g ± 0.02) (**B**). Bars represent the means ± SD. Statistical differences are indicated by different letters (ANOVA, Tukey-Kramer's post-hoc test, $p \leq 0.05$) and asterisks (Student's t-test, $p \leq 0.05$).

Our results suggested that the use of VIVEMA® TWIN could increase the evaluated parameters, helping root and plant growth and mitigating the salt stress. Tomato roots collected 24 h after the second and the fourth treatment with VIVEMA® TWIN and single raw materials showed an enhanced root development in comparison to the control group, under optimal and salt stress conditions. At the first time point, root length was increased in salt stressed plants and the best performance was related to the application of raw material 2 (**Fig. 1A**). The root fresh weight was instead increased in both unstressed and salt stressed plant, with a significantly better performance for all treatments (**Fig. 1B**). Statistical differences between unstressed and stressed plants were observed in root length of plants treated with raw material 2 (**Fig. 1A**) and root fresh weight in plants treated with both raw materials and in control group (**Fig. 1B**). At the second time point, results were not statistically significant, but it was evident a positive trend in both growth conditions. These results are probably related to the high variability typical of crop plants. Moreover, root length (**Fig. 1C**) and fresh weight (**Fig. 1D**) resulted to be increased by all treatments. As mentioned before, very little is known about the use and the effects of tannins in agriculture. Bargiacchi and Miele (Bargiacchi et al. 2012) reported that the exogenous application of hydrolysable tannins has a role in boosting early plant growth and root development, but the mechanism by which this happens is still unclear.

The knowledge about the possible mode of action of these molecules is mainly related to the antioxidant activity of polyphenol compounds, including tannins. The better development of treated plants, grown under salt stress conditions, could be an indirect effect due to a more functional

antioxidant defence system (Quideau et al. 2011). This hypothesis is supported also by NDVI results. The Normalized Difference Vegetation Index is a unit designed to measure both red and near infrared reflectance, two parameters useful to determine plant health. NDVI measurements can range from -1 to 1, with higher values indicating better plant health (Rahman, Islam, and Rahman 2004). As shown in **Fig. 2A** NDVI of stressed control plants resulted to be statistically decreased in comparison to the unstressed plants. At the same time, the treatment with VIVEMA® TWIN and single raw materials on stressed plants was able to re-establish values similar to those obtained in optimal conditions. At the second time point (**Fig. 2B**) all the treatments statistically increased the NDVI index in comparison to the control, in both growth conditions. NDVI is not directly linked to a better antioxidant activity, but it is conceivable that a better plant health status, observed in treated plants, could also be related to this factor.
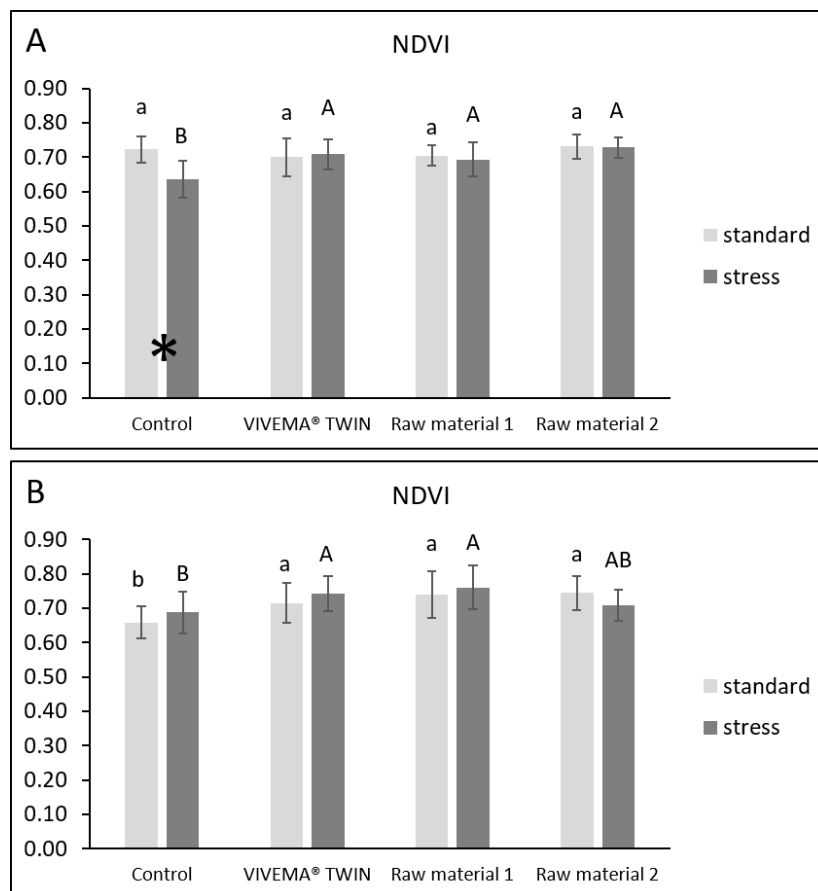

Fig. 2 NDVI data collected 24 h after the second treatment (**A**) and 24 h after the fourth treatment (**B**) in optimal and salt stress conditions. At the first time point, NDVI index was significantly increased by all treatments, under salt stress conditions (VIVEMA® TWIN=0.71 ± 0.04, Raw material 1=0.69 ± 0.05, Raw material 2=0.73 ± 0.03), compared to the control (0.64 ± 0.05) (**A**). The same significant increase was observed after four treatments, but under both optimal (control=0.66 ± 0.05, VIVEMA® TWIN=0.71 ±0.06, Raw material 1=0.74 ± 0.07, Raw material 2=0.74 ± 0.05) and salt stress conditions (control=0.67 ± 0.06, VIVEMA® TWIN= 0.74 ± 0.05, Raw material 1= 0.76 ± 0.06, Raw material 2=0.71 ± 0.04) (B). NDVI index can range from -1 to 1 and, based on light absorbance and reflectance, is a plant health indicator. Bars represent the means ± SD. Statistical differences are indicated by different letters (ANOVA, Tukey-Kramer's post-hoc test, $p \leq 0.05$) and asterisks (Student's *t*-test, $p \leq 0.05$).

3.2 Comparison between VIVEMA® TWIN and gallic acid effect on root growth and development

Gallic acid was tested on unstressed and salt stressed plants as pure compound present in high amount in the mixture and having a role in root and plant development. The protocol used was the same mentioned in paragraph 2.1. The final purpose was to compare a possible synergic effect of VIVEMA® TWIN versus a single molecule contained in the biostimulant, having the same target. **Figure 3** reports the results of this experiment. Both VIVEMA® TWIN and gallic acid exerted a positive effect on root fresh weight and length. In particular, root fresh weight resulted to be significantly increased in unstressed and salt stressed plants (**Fig. 3D**). The highest increase was observed in plants treated with the biostimulant, followed by those treated with gallic acid. The same trend was observed in root length at both time points, even if without statistical significance (**Fig. 3A** and **3C**).
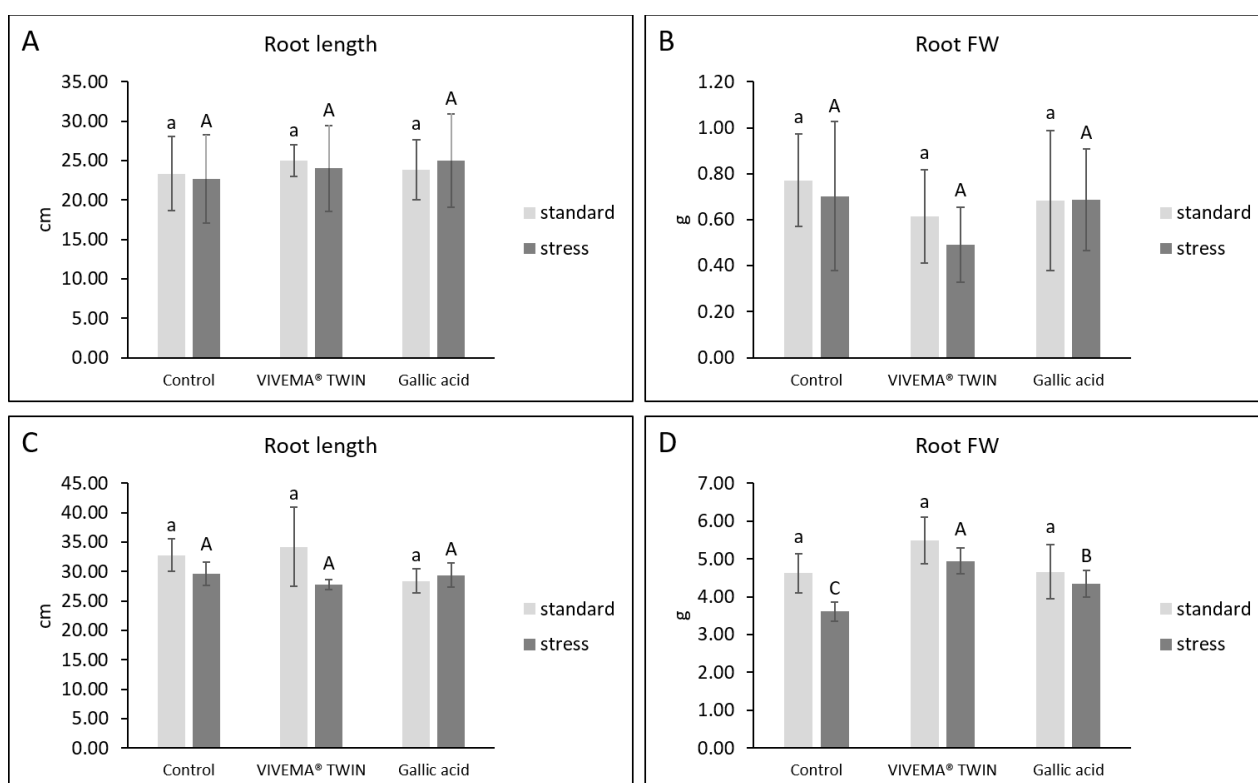

Fig. 3 Root length and fresh weight (FW) measured 24 h after the second treatment (**A** and **B**) and 24 h after the fourth treatment (**C** and **D**) in optimal and salt stress conditions. Both gallic acid and VIVEMA® TWIN significantly increased root fresh weight at the second time point, under salt stress conditions, in comparison to the control (3.62g ± 0,25) (D). VIVEMA® TWIN (4.95g ± 0.34) showed a better performance than the pure molecule (4.34g ± 0.35). Bars represent the means ± SD. Statistical differences are indicated by different letters (ANOVA, Tukey-Kramer's post-hoc test, $p \leq 0.05$).

Gallic acid is a hydrolysable tannin, building-block of multiple more complex tannins, known to be involved in early root and plant development (Bargiacchi et al. 2012). This molecule is mainly known for its antioxidant effect (Yen, Duh, and Tsai 2002) and probably these properties could explain the positive results obtained under salt stress conditions. The increase of defence against oxidative stress could also exert an indirect effect on root development. Moreover, Negi and his colleagues (Negi et al. 2005) demonstrated that gallic acid could have an auxin-like function, by directly increasing root length and development. Therefore, the mechanism of action by which gallic acid positively affects root growth is still not clear, but, based on our results, its use in synergy with other molecules seems to be more effective than the sole compound. Indeed, it is well known that the

effect of multiple molecules with a given function is higher than the sum of the effects of the individual molecules. Among the other molecules identified in VIVEMA® TWIN (paragraph 3.5), ellagic acid and hydrolysable tannin building blocks are also known to exert an antioxidant activity (Landete 2011), as well as the flavanone eriodictyol, also involved in root development (De Simón et al. 2014). These, together with the other identified and unidentified molecules, acting in synergy, make VIVEMA® TWIN more effective than single pure compounds.

3.3 VIVEMA® TWIN improves root parameters more than gallic acid in "short-term" test under mild and strong salt stress conditions

A "short-term" test was conducted on plants treated with VIVEMA® TWIN and gallic acid, grown under standard and mild/strong salt stress conditions. This test was aimed at the collection of material for imaging analysis, useful to better understand the root development in the early growth phases. To measure length, fresh weight and total root number using Root System Analyzer imaging software, roots were collected 24 h after the second and the fourth treatments.
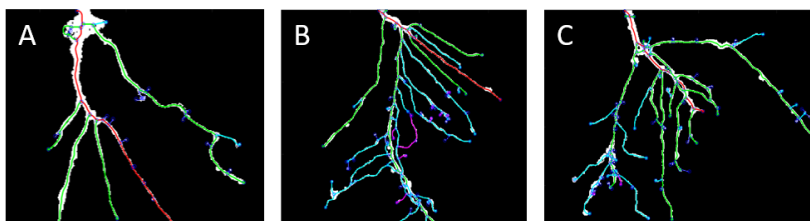

Fig. 4 Root System Analyzer® output. Example of three processed images of control (A) and roots treated with VIVEMA® TWIN (**B**) and gallic acid (**C**) at the second sampling point

Figure 4 shows the Root System Analyzer® output. The software allows to convert a picture in a black&white structure made of pixels, in which roots of different orders are coloured in different colours. In this way, it is possible to visualize the root structure and compare the total root number. **Panel A**, **B** and **C** show an example of control, VIVEMA® TWIN-treated roots and gallic acid-treated root, respectively.

At the first sampling point, 10 days after the transplant in greenhouse, a similar trend was observed for all the parameters evaluated. As shown in **Figure 5**, gallic acid seemed to work better compared to VIVEMA® TWIN in optimal conditions. Both root length and fresh weight showed a significant increase (**Fig. 5C** and **5D**). Total root number did not show statistical differences, however a positive trend was observed (**Fig. 5B**). On the other hand, under mild (100 mM NaCl) and strong (200 mM NaCl) salt stress conditions, the biostimulant worked much better than gallic acid alone, which instead gave results similar to those obtained in the control. In particular, in plants stressed with 200 mM NaCl the use of VIVEMA® TWIN led to values comparable (or better) to those obtained under optimal conditions (**Fig. 5C** and **5D**).
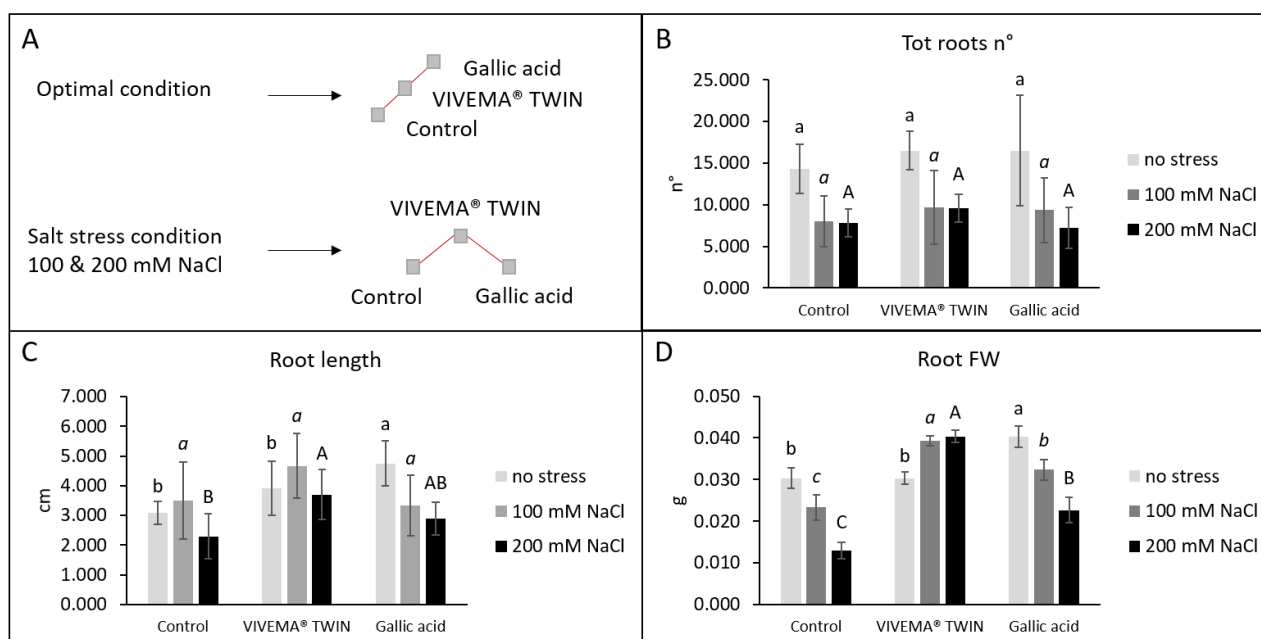
Fig. 5 Total root number (**B**), root length (**C**) and root fresh weight (FW) (**D**) measured 24 h after the second treatment. The descriptive scheme (**A**) shows the trend observed for all the parameters. Under optimal conditions ("no stress") gallic acid displayed better results in comparison to control and VIVEMA® TWIN. Total root number showed a positive trend (control=14.3±2.9, VIVEMA® TWIN=16.5±2.3, Gallic acid=16.5±6.6), while root length (control=3.1cm±0.38 VIVEMA® TWIN=3.9cm±0.92 Gallic acid=4.8cm±0.76) and fresh weight (control=0.30g±0.003 VIVEMA® TWIN=0.030g±0.002 Gallic acid=0.040g±0.003) were significantly increased. On the other hand, under mild and strong salt stress, better results were obtained in plants treated with the biostimulant. Again, total root number showed a positive trend at both 100 mM NaCl (control=8±3 VIVEMA® TWIN=9.7±4.4 Gallic acid=9.3±3.9) and 200 mM NaCl (control=7.8±1.6, VIVEMA® TWIN=9.6±1.7, Gallic acid=7.2±2.5). The most evident effect was related to root length at 100 mM NaCl (control=3.5cm±1.3, VIVEMA® TWIN=4.7cm±1.1, Gallic acid=3.3cm±1) and 200 mM NaCl (control=2.3cm±0.8, VIVEMA® TWIN=3.7cm±0.8, Gallic acid=2.9cm±0.5) and fresh weight at 100 mM NaCl (control=0.02g±0.003, VIVEMA® TWIN=0.04g±0.001, Gallic acid=0.03g±0.003) and 200 mM NaCl (control=0.13g±0.002, VIVEMA® TWIN=0.40g±0.002, Gallic acid=0.23g±0.003), in which the treatment restored values similar (or better) to those obtained under optimal conditions. Bars represent the means ± SD. Statistical differences are indicated by different letters (ANOVA, Tukey-Kramer's post-hoc test, $p \leq 0.05$).

At the second sampling, nineteen days after the transplant in greenhouse, most part of the plants grown under strong salt stress (200 mM NaCl) was dead, however data on unstressed and mild-stressed plants were collected. Again, the total root number showed a positive trend for both the growing conditions. At this time point was not observed the same trend obtained at the first one, but VIVEMA® TWIN and gallic acid showed more similar results (**Fig. 6**)
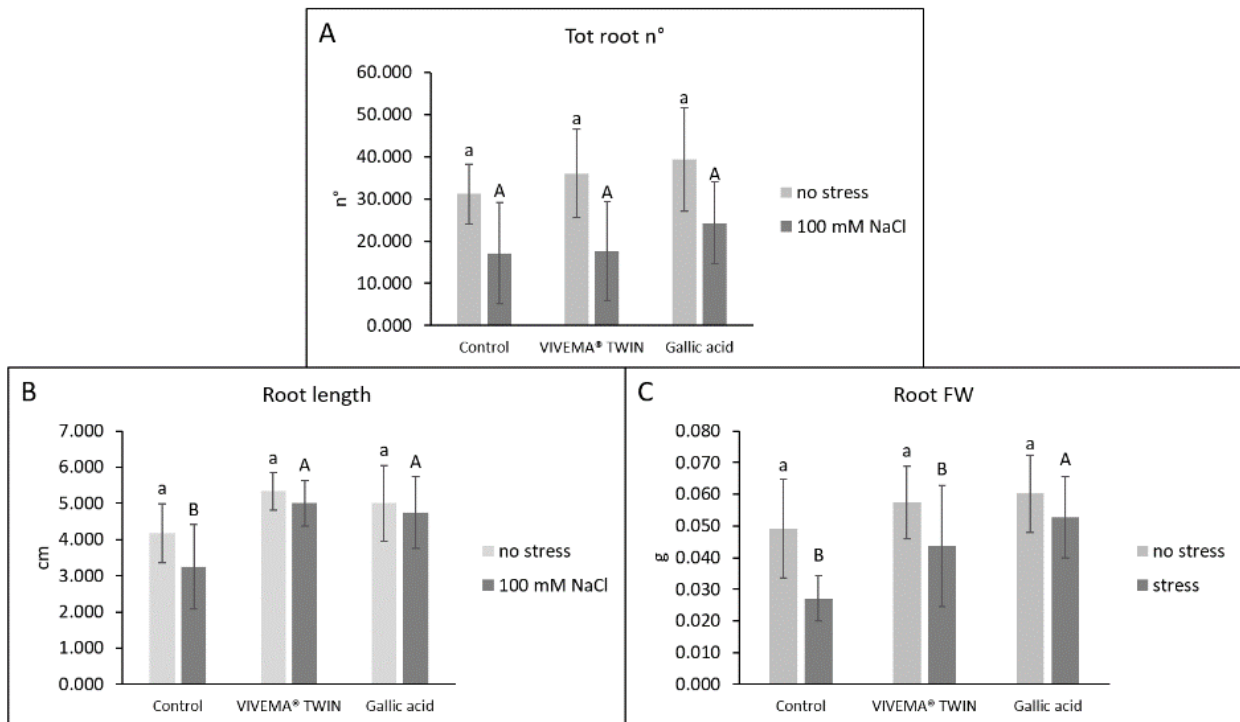
Fig. 6 Total root number (**A**), root length (**B**) and root fresh weight (FW) (**C**) measured 24 h after the fourth treatment. The total root number showed a positive trend at both optimal (control=31±7, VIVEMA® TWIN=36±10.5, Gallic acid=39±12.3) and mild salt stress conditions (control=17.2±11.9, VIVEMA® TWIN=17.7±11.7, Gallic acid=24.3±9.7). Root length showed a positive trend under optimal conditions (control=4.2cm±0.8, VIVEMA® TWIN=5.3cm±0.5, Gallic acid=5cm±1), while a statistical significant increase under salt stress (control=3.3cm±1.2, VIVEMA® TWIN=5cm±0.6, Gallic acid=4.8cm±1). The same trend was observed for fresh weight, under optimal (control=0.05g±0.02, VIVEMA® TWIN=0.06g±0.01, Gallic acid=0.06g±0.01) and salt stress conditions (control=0.03g±0.007, VIVEMA® TWIN=0.04g±0.02, Gallic acid=0.05g±0.01). Bars represent the means ± SD. Statistical differences are indicated by different letters (ANOVA, Tukey's post-hoc test, $p \leq 0.05$).

As mentioned in the paragraph 3.2, the antioxidant and early growth boosting activity of gallic acid is already known, also in presence of salt stress (Yen, Duh, and Tsai 2002; Negi et al. 2005). Singh and co-workers showed that the application of exogenous gallic acid on rice seeds led to an increase of root length of about the double compared to the control (Singh, Gupta, and Pandey 2017). In our results, we observed that gallic acid seemed to be more effective under optimal conditions in comparison to the biostimulant application. As reported above, the situation was completely different in presence of salt stress. Even if the knowledge about the use of tannins in agriculture is very limited, some works in literature reported the effect of these molecules on root development, in particular under stress conditions. Hydrolysable tannins extracted from chestnut wood, for example, are known to be involved in early plant growth, root development and phosphate uptake (see paragraph 3.4) (Bargiacchi et al. 2012). Moreover, both hydrolysable and condensed tannins have a strong antioxidant activity that could be indirectly related to their effect on plant development, mainly evident under abiotic stress (Romani et al. 2006).

3.4 VIVEMA® TWIN modulates the expression of genes involved in salt stress response, root growth and phosphate availability

RNAseq analysis was carried out on unstressed and salt stressed roots of plants treated with VIVEMA® TWIN and control group in order to evaluate changes in gene expression levels. The material analysed was collected 24 hours after the second treatment and 24 h after the fourth treatment. **Figure 7** shows Volcano plots, in which up and down regulated genes in treated groups compared to the control for each conditions are reported. X-axis shows the fold change in gene expression and y-axis shows the statistical significance of the differences. Dots represent different genes. Blue dots indicate genes without significant different expression. Red dots indicate genes significantly up regulated. Green dots indicate genes significantly down regulated. It is visible how only in treated stressed plants, harvested at the second time point (**Fig. 7D**), a high number of genes (456) were differently regulated, while in the other conditions the differences were negligible (**Fig. 7A** and **C**). The absence of significant gene regulation, at the first time point in both growth conditions and at the second one in unstressed plants, confirms that plants were not negatively affected by the application of VIVEMA® TWIN. Moreover, the absence of significant regulation at the first time point in salt stressed plant could be due to a low salt concentration at the sampling time, as reported in paragraph 3.1.
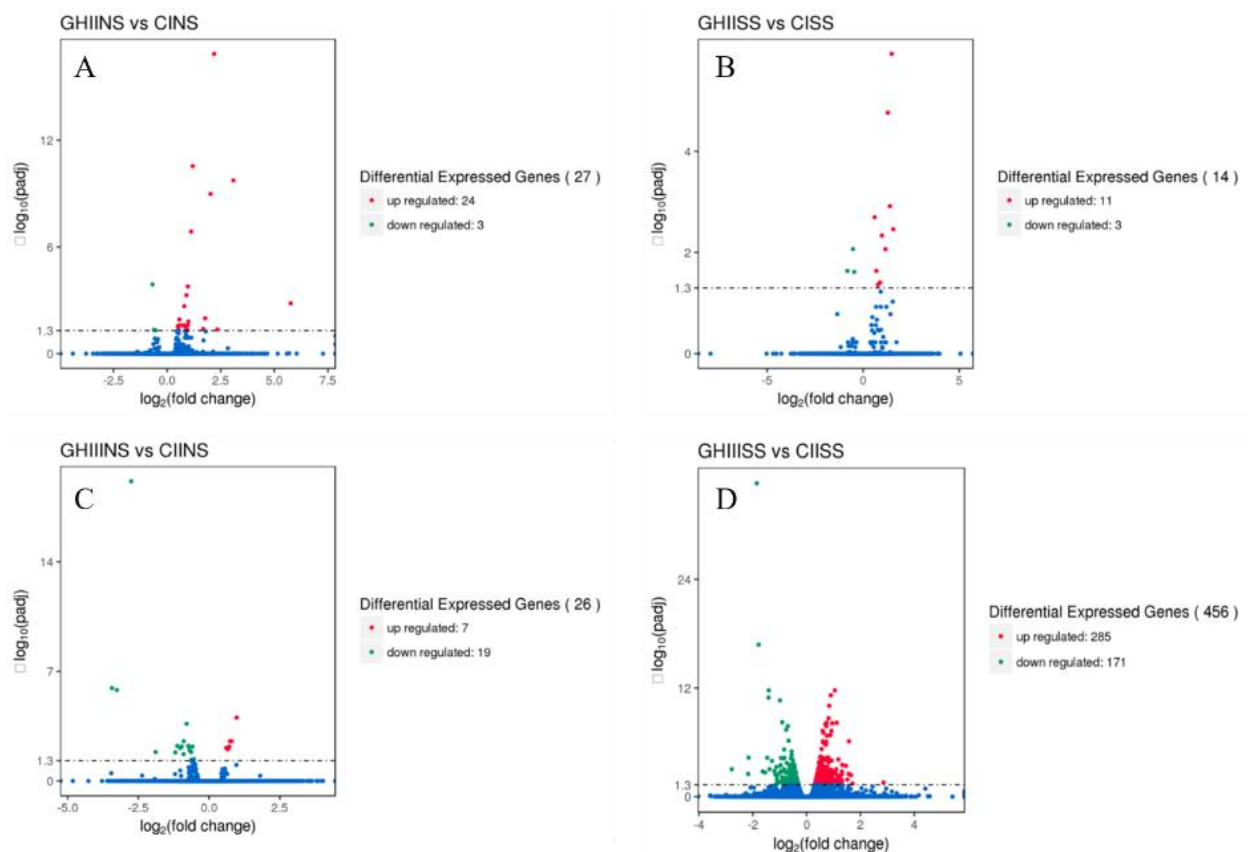
Fig. 7 Volcano plots showing the differences in gene regulation observed in control and VIVEMA® TWIN treated roots, grown under optimal and salt stress conditions, collected 24 after the second treatment (**A** and **B**) and 24 h after the fourth treatment (**C** and **D**). In these Volcano plots x-axis shows the fold change in gene expression and y-axis shows the statistical significance of the differences. Dots represent different genes. Blue dots indicate genes without significant different expression. Red dots indicate genes significantly up regulated. Green dots indicate genes significantly down regulated

RNAseq of treated salt stressed roots, collected at the second time point, showed the up-regulation of 285 genes and the down-regulation of 171. Considering the up-regulated genes, most part of them resulted involved in abiotic stress response (63%), root growth (18.5%) and another 18.5% to different metabolic functions (**Fig 8A**).

A Up-regulated   **B** Down-regulated

18.5%
18.5%
63%

100%

■ Abiotic stress response   ■ Root growth   ■ Other functions   ■ Nutrients

Fig. 8 Genes up regulated (**A**) in roots treated with VIVEMA® TWIN and grown under salt stress conditions, are involved in abiotic stress response (63%), root growth (18.5%) and other metabolic functi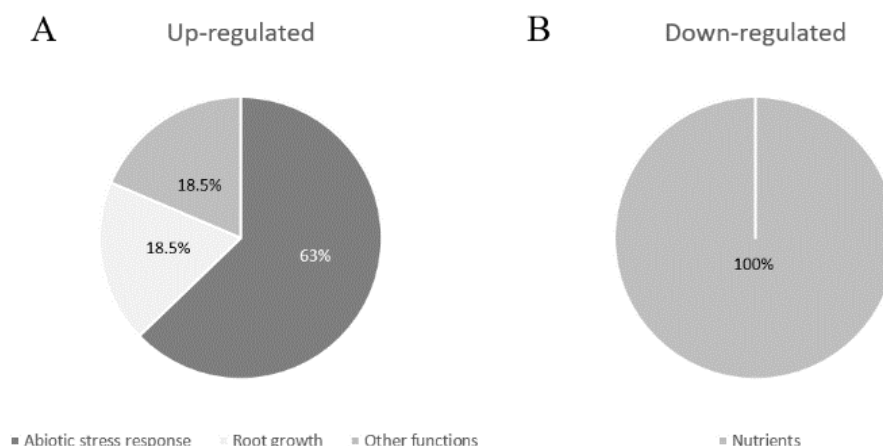ons (18.5%). Differently, the totality of the most significant down-regulated genes (**B**) are involved in nutrient availability, in particular phosphate. These genes are normally up regulated in presence of phosphate starvation, while the transcripts rapidly decrease when this element is again available to the plant

In **Table 1** the most significantly up-regulated genes (FC > 1.7) are reported. Among those involved in stress response, *WRKY transcription factor* showed the highest up-regulation (FC = 2.95), together with *ABA 8'-hydroxylase* (FC = 2.25), *protein phosphatase 2C* (FC = 1.89) and *HVA22-like protein* (FC = 1.81), involved in abscisic acid (ABA) signal transduction and catabolism. Abscisic acid, also called "stress hormone", is fundamental in plant development and plays an important role in the integration of stress signals and in the control of the response (Tuteja 2007). The overexpression of these genes is related to the increase of ABA activity, leading to an increase of stress tolerance (Ryu and Cho 2015). In particular, WRKY proteins may act as activator or repressor of ABA signalling based on plant status and play a crucial role in plant adaptation to salt (Chen et al. 2012; Golldack, Lüking, and Yang 2011). ABA 8'-hydrolase, a cytochrome P450, is involved in ABA catabolism, and helps to maintain hormone balance (Kushiro et al. 2004). Protein phosphatase 2C has also a role in ABA signal transduction (Meyer, Leube, and Grill 1994) while HVA22-like protein is an ABA/stress induced protein. The overexpression of this gene inhibits the formation of gibberellins (GA)-induced large vacuoles, correlated to GA-induced programmed cell death (Guo and Ho 2008). Other up-regulated genes identified are already known to be involved in salt and drought stress in tomato plants. *Na$^+$/H$^+$ exchanger 8* (FC = 2.19) is a gene coding for a plasma membrane antiporter. This protein is essential to maintain Na$^+$/K$^+$ homeostasis, that in particular helps plant during salt stress, when Cl$^-$ interferes with K$^+$ efflux (Olías et al. 2009). Overexpression of this gene usually leads to salt stress tolerance increase in tomato plants (Apse et al. 1999). The overexpression of genes coding for stress-activated transcription factors, like *C2H2 zinc finger protein* (FC = 2.08) or *Drought responsive Zinc finger protein* (FC = 1.93), may also control the regulation of downstream genes, resulting in a stress resistance enhancement (Rai, Singh, and Shah 2013). Finally, the up-regulation of *Glutathione S-transferase* (FC = 1.92) is an evidence of the antioxidant effect of VIVEMA® TWIN. It is known that the protein coded by this gene is involved in abiotic stress responses, even if few information are available about the gene function in tomato (Xu et al. 2015). Glutathione-S-transferases catalyse the

conjugation between reduced glutathione (GSH) and electrophilic substrates. In particular, they have been largely studied for their capacity to bind toxic exogenous compounds, protecting plants from multiple types of stress (Csiszár et al. 2014).

Among the up-regulated genes involved in root growth development *R2R3MYB transcription factor 41* (FC = 2.34), *Major facilitator superfamily protein* (FC = 1.81) and *xyloglucan endotransglucosylase-hydrolase 5* (FC = 1.76) were found. Different studies show that these genes are involved in primary and lateral root formation, acting on cellulose deposition (*xyloglucan endotransglucosylase-hydrolase 5*) (Liu et al. 2007)*,* auxin transport (*Major facilitator superfamily protein*) (Remy et al. 2013) and different other aspects such as root hair development, root elongation and root architecture (*R2R3MYB transcription factor 41*) (Ambawat et al. 2013).

Tab. 1 The most significantly up regulated genes (FC>1.7) in VIVEMA® TWIN treated roots, grown under salt stress conditions. Most part of these genes is involved in abiotic stress response and root growth

Gene ID

Abiotic stress response	gene description	FC
Solyc12g056750.2	WRKY transcription factor 61	2.95
Solyc04g082550.2	Trehalose 6-phosphate phosphatase	2.76
Solyc02g077980.2	Late embryogenesis abundant protein (LEA) family protein	2.50
Solyc00g272810.1	Tyramine N-feruloyltransferase 4/11, putative	2.26
Solyc04g078900.3	ABA 8'-hydroxylase	2.25
Solyc05g024410.3	Na+/H+ exchanger 8	2.19
Solyc05g050220.3	TAF-3	2.16
Solyc04g009910.3	phosphoenolpyruvate carboxylase kinase 1	2.09
Solyc10g084910.2	C2H2 zinc finger protein	2.08
Solyc04g071770.3	Ethylene-responsive transcription factor	2.07
Solyc12g010820.2	Late embryogenesis abundant protein-like	1.97
Solyc04g007470.3	Drought responsive Zinc finger protein	1.93
Solyc06g066540.1	Ethylene-responsive transcription factor TINY	1.93
Solyc02g081340.3	Glutathione S-transferase	1.92
Solyc07g040680.3	SolycHsfA9	1.92
Solyc06g076400.3	Protein phosphatase 2C	1.89
Solyc12g044390.2	ethylene-responsive transcription factor	1.87
Solyc12g006050.2	Major facilitator superfamily protein	1.81
Solyc11g010930.2	HVA22-like protein	1.81
Solyc02g089190.2	R2R3MYB transcription factor 29	1.79
Solyc08g075320.3	Cytochrome P450 family ABA 8'-hydroxylase	1.78
Solyc06g084330.3	ERD (early-responsive to dehydration stress) family protein	1.77
Solyc10g081840.2	Nuclear transcription factor Y subunit	1.77
Solyc01g087590.3	Polyamine oxidase	1.76
Solyc12g006640.2	Lactoylglutathione lyase / glyoxalase I family protein	1.75
Root growth		
Solyc10g009580.3	Glycosyltransferase	2.48
Solyc12g042600.2	Glycosyltransferase	2.34
Solyc07g054840.3	R2R3MYB transcription factor 41	2.34
Solyc10g084910.2	C2H2 zinc finger protein	2.08
Solyc06g072870.2	Glycosyltransferase	2.01
Solyc04g076710.3	COBRA-like protein 11 precursor	1.90
Solyc12g006050.2	Major facilitator superfamily protein	1.81
Solyc08g005610.3	xyloglucan endotransglucosylase-hydrolase 5	1.76

The most significantly down-regulated genes (1/FC > 1.7, FC < 0.6) are involved in nutrient availability (**Fig. 8B**) and are reported in **Table 2**. *Phosphate starvation inducible gene TPSI1* (FC = 0.145), *phosphate transporter* (FC = 0.377), *SPX domain-containing protein* (FC = 0.384), *bHLH transcription factor 037* (FC = 0.465), *IDS4-like* (FC = 0.504), *myb-like protein* (FC = 0.514) and *purple acid phosphatase* (FC = 0.534) are all genes involved in phosphate deficiency. They are normally up-regulated in presence of phosphate starvation, while their transcript decreases rapidly when Pi-(inorganic phosphate) starved tomato plants are resupplied with this nutrient. Phosphorus is an essential macronutrient for plant growth and development and Pi is the main source of phosphorus uptaken by plants (Muneer and Jeong 2015). Therefore, plants treated with VIVEMA® TWIN and grown under salt stress condition seemed to have a better capacity to uptake phosphorous in comparison to the control group.

Tab. 2 The most significantly down-regulated genes (1/FC>1.7, FC<1.6). These genes are involved in nutrient availability, in particular phosphate

Gene ID

Nutrients uptake	gene description	FC
Solyc03g098010.3	phosphate starvation inducible gene TPSI1	0.15
Solyc03g005530.1	Phosphate transporter	0.38
Solyc01g090890.3	SPX domain-containing protein	0.38
Solyc05g009640.3	bHLH transcription factor 037	0.47
Solyc08g060920.3	IDS4-like	0.50
Solyc02g091890.2	myb-like protein X	0.51
Solyc09g091910.2	Purple acid phosphatase	0.53
Solyc08g007800.3	SPX domain-containing family protein	0.54

3.5 RNAseq data validation and gene expression evaluation in gallic acid-treated roots

RNAseq data were validated through quantitative Real Time PCR analysis on genes selected among the most significantly regulated by the application of the biostimulant.
qPCR analysis were performed on samples derived from VIVEMA® TWIN-treated roots of plants grown under salt stress conditions and collected at the second time point (see paragraph 2.1), the conditions in which RNAseq gave the most interesting results. The analysis was also carried out on roots developed under the same conditions, but treated with gallic acid (see paragraph 2.3). Besides verifying the RNAseq results, the goal was also to observe the differences between the synergic effect of VIVEMA® TWIN and the effect due to the application of a pure molecule present in the biostimulant and involved in root growth and plant development (see paragraph 2.3)

qPCR analyses confirmed RNAseq data, while gallic-acid treated roots showed a quite different results. VIVEMA® TWIN is a complex mixture of compounds, of which gallic acid is a molecule present in high quantity, but just one of the components. In **Table 3** are reported the FC values obtained in the RNAseq and qPCR analyses on VIVEMA® TWIN and gallic acid-treated roots.

Tab. 3 FC obtained in the RNAseq and qPCR analyses on VIVEMA® TWIN and gallic acid-treated roots. Statistical differences between treated and controls of each group in qPCR analysis are indicated by asterisks (Student's *t*-test, *p* ≤ 0.05)

	VIVEMA® TWIN		GALLIC ACID
	RNAseq	qPCR	qPCR
	FC	FC	FC
WRKY transcription factor 61	2.95	5.06*	4.23*
Trehalose 6-phosphate phosphatase	2.76	14*	0.81
Na⁺/H⁺ exchanger 8	2.19	1.8	1.19
C2H2 zinc finger protein	2.08	1.83	1.01
Glutathione S-transferase	1.92	27.03*	2.13*
Major facilitator superfamily protein	1.81	1.01	0.6
ERD (early-responsive to dehydration stress)	1.77	10.46*	1.99
Glycosyltransferase	2.48	21.59*	3.15*
Xyloglucan endotransglucosylase-hydrolase 5	1.76	1.55*	1.02
phosphate starvation inducible gene TPSI1	0.15	0.81	1.5

As shown in **Table 3** the up/down regulation of selected genes observed in RNAseq results was confirmed by qPCR. Differently, the expression of most part of the genes analysed in gallic acid-treated roots seems not to be significantly modulated by the treatment. Only *WRKY transcription factor 61*, *Glutathione S-transferase* and *Glycosyltransferase* showed a significant up-regulation, as in VIVEMA® TWIN-treated roots ($p \leq 0.05$). Based on these results, the biostimulant seems to be more effective on the expression of genes involved in stress response, root growth and nutrient uptake, in comparison with the pure molecule treatment. It is possible to hypothesize that gallic acid has a role in the biostimulant activity, but is the synergy with other components that makes the difference on its efficacy. The study of synergies is a current topic in the biostimulant field and since there are still not many reports on it (Rouphael and Colla 2018), it is important to investigate this aspect.

3.6 VIVEMA® TWIN chemical characterization

VIVEMA® TWIN and single raw materials were analysed by Orbitrap LC/MS in order to identify the most important active compounds responsible for the biostimulant activity. One of the raw materials forming VIVEMA® TWIN is mainly composed by hydrolysable tannins, while the other one has a prevalence of condensed tannins. An original mix, stable also at low temperatures, is obtained by merging these two matrices in a precise ratio. The mix of different types of tannins allows them to work in synergy, leading to effects other than those due to the individual components. A preliminary qualitative analysis allowed the detection of different compounds and later to the quantification of gallic acid. This molecule represented the highest peak in the VIVEMA® TWIN chromatogram and, for this reason, was also tested on plant as a pure compound (paragraphs 3.2 and 3.3). The quantity of gallic acid identified in the biostimulant and based on an external standard curve, was 13 mg mL^{-1}. Moreover, in both the final biostimulant and single raw materials, different molecules of interest were qualitatively identified (**Table 4**). The most part of the identified compounds are tannin building blocks, such as gallic and ellagic acid, digalloylglucose, 1,3,6-trigalloyl glucose, 1,2,3,6-tetra-O-galloyl-beta-D-glucose (Khanbabaee and van Ree 2001). Valoneic acid is

also a hydrolysable tannin (Vieira et al. 2011). Other molecules of interest are eriodictyol, involved in root length development and plant growth enhancement and phloionic acid, a common compound deriving from wood hydrolysis (De Simón et al. 2014). Most part of the compounds found in VIVEMA® TWIN derived from raw material 1, which represents the main fraction of the final biostimulant formulation. Nevertheless, raw material 2, containing a prevalence of condensed tannins, contributes to modify the composition of VIVEMA® TWIN, but its chemical nature makes the characterization more difficult. Indeed, some peaks identified in both biostimulant and raw material 2 are absent in raw material 1, but no information useful for their identification have been found in literature so far.

Tab. 4 Main compounds identified in VIVEMA® TWIN, Raw material 1 and Raw material 2

m/z [M-H]-	VIVEMA® TWIN	Raw material 1	Raw material 2
193	Mannuronic acid	Mannuronic acid	
195			Gluconic acid
169	Gallic acid	Gallic acid	Gallic acid
483	Digalloylglucose	Digalloylglucose	
593			Procyanidin
635	1,3,6-Trigalloyl glucose	1,3,6-Trigalloyl glucose	
787	1,2,3,6-Tetra-O-galloyl-beta-D-glucose	1,2,3,6-Tetra-O-galloyl-beta-D-glucose	
301	Ellagic acid	Ellagic acid	Ellagic acid
463			Quercetin glucoside
469	Valoneic acid dilactone	Valoneic acid dilactone	
287	Eriodictyol	Eriodictyol	
187			Azelaic acid
317			2',3,3',4',5,7-Hexahydroxyflavone
551	5,6,7,4'-Tetrahydroxy-3,3',5'- trimethoxy flavone 3-glucuronide	5,6,7,4'-Tetrahydroxy-3,3',5'- trimethoxy flavone 3-glucuronide	5,6,7,4'-Tetrahydroxy-3,3',5'- trimethoxy flavone 3-glucuronide
725	Gluco kamaloside	Gluco kamaloside	
345	Phloionic acid	Phloionic acid	Phloionic acid
329			Vanillic acid glucoside
517	Perulactone	Perulactone	
311			3-O-α-D-Galactopyranosyl-L-arabinose

4. **CONCLUSIONS**

VIVEMA® TWIN is a new biostimulant able to increase salt stress tolerance and root development in treated plants grown under salt stress conditions. The synergy obtained by mixing two different raw materials seems to improve the biostimulant performance, as also demonstrated by the results compared with gallic acid treatment. The antioxidant and early root development activity of hydrolysable and condensed tannins makes VIVEMA® TWIN a positive solution for helping plants under abiotic stress conditions during the vegetative growth. Future field trials will allow to understand the direct effect on the final yield, for tomato and other crops of commercial interest.

FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

The final goal of this PhD project was to develop and study new biostimulant products, starting from raw materials to the market introduction.

The increasing use of these new generation products makes the study of their main characteristics and modality of action very important. Moreover, the new legislation (Regulation (EU) 2019/1009), requires the drafting of registration dossiers, designed to certify the chemical characteristics and recognized effects of these innovative products.

In this work, the effects of two biostimulants acting at different plant developmental stages and on different organs (seeds and roots), developed by the Green Has Italia S.p.A, KIEM® and VIVEMA® TWIN, were analysed, by collecting biometric, transcriptomic and biochemical data on treated and control plants, grown under standard and abiotic stress conditions (heat and salt stress).

A chemical characterization of the products was also performed in order to identify the main active compounds involved in the biostimulant effect. Both biostimulants studied in this thesis are today present on the market, demonstrating that the objectives of this project were achieved.

Further studies will be focused on testing these products on different crops (corn, wheat, horticultural crops, fruit trees, etc.) also in open field conditions. The data collected will be useful to verify the effects on plant development and productivity in presence of multi stress conditions, typical of outdoor cultivation.

The ever increasing demand for new biostimulants products, able to help plant development in an increasingly difficult situation due to the reduction of arable land and the presence of multiple types of stress, makes this field extremely important for agricultural sciences.

In this perspective, the collaboration between companies and universities becomes of paramount importance in order to carry out proper research able to meet the commercial needs.

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General introduction

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Chapter 1

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https://github.com/vsbuffalo/scythe

ftp://ftp.ncbi.nlm.nih.gov/genomes/*Glycine_max*

http://amp.pharm.mssm.edu/clustergrammer

https://string-db.org

Chapter 2

www.specmeters.com

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Phytochemical profile and antioxidative properties of *Plinia trunciflora* fruits: A new source of nutraceuticals

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ABSTRACT

This study evaluated the polyphenol profile and the antioxidative properties of *Plinia trunciflora* (O. Berg) Kausel fruits. Folin-Ciocalteau and pH-jumping methods indicated that these berries are a major source of antioxidant polyphenols (1201.05 mg GAE/100 g FW), particularly anthocyanins. HPLC-DAD-ESI-MS/MS analysis identified cyanidine glycosides as the main components. Flavon-3-ols and hydrolysable-tannins were also found. CAA assay showed that extracts of *P. trunciflora* fruits prevent lipid peroxidation in HepG2 cells with higher efficacy than other colourful fruits (CAA_{50} 935.25 mg FW/mL cell medium). Moreover, our results suggested that the observed antioxidant protection involve both redox active properties of *P. trunciflora* components, as measured by ABTS, DPPH and FRAP assays, and upregulation of the genes coding for the antioxidant enzymes MnSOD and GPx, as evaluated by qRT-PCR. Collectively, our data provided evidence on the potential of *P. trunciflora* fruit as a very rich source of natural antioxidant molecules.

1. Introduction

The intake of foods rich in phenolic compounds produces several health benefits mainly linked to the prevention of the oxidative damage to cells (Bjørklund & Chirumbolo, 2017). Dietary polyphenols may contribute also in the prevention of several pathologies, such as cardiovascular (Manach, Mazur, & Scalbert, 2005), neurodegenerative (Albarracin et al., 2012), and inflammatory diseases (Gentile, Perrone, Attanzio, Tesoriere, & Livrea, 2015).

The increasing consumption of tropical fruits in Europe, due to their nutritional and nutraceutical value, has recently sparked a growing interest in the characterization of their phytochemical profile (Gentile et al., 2019). Consequently, in the Italian market the presence of tropical fruits is constantly increasing. These fruits are not only imported from traditional producing countries but are often of local origin. In fact, experimental studies have shown that the Italian territory, in particular Sicily and Calabria, is suitable for cultivation of tropical species (Migliore, Farina, Tinervia, Matranga, & Schifani, 2017). However, while the cultivation of species such as mango, lychee, and avocado achieved significant commercial success, the cultivation of other exotic fruit species is still in the experimental phase.

The *Myrtaceae* family includes about 5900 species, with a widespread distribution in tropical and subtropical regions. *Myrtae* is a tribe belonging to the *Myrtaceae* family and comprises 53 genera, including *Plinia* and *Myrciaria*. The evergreen trees of the species belonging to the *Plinia* and *Myrciaria* genera are all native to Central and South America (Borges, Conceição, & Silveira, 2014). They produce small dark-coloured berries directly on the trunk that are grapelike in size, flavour, and appearance. The fruits are known also with the name of jaboticabas or Brazilian berries. (Wu, Dastmalchi, Long, & Kennelly, 2012). These fruits are consumed also in form of juices, jams, wines and liqueurs, and represent a great potential for food industry (Borges et al., 2014).

Fruits and leaves of species belonging to the *Plinia* and *Myrciaria* genera are largely used in folk medicine for the treatment of several diseases, including angina, erysipelas, dysenteries, and asthma, and they show also activity against acute and chronic mouth inflammations (de Araújo, Neri-Numa, de Paulo Farias, da Cunha, & Pastore, 2019). On the other hand, several terpenoid compounds were identified in essential oils of the leaves of species belonging to the *Plinia* and *Myrciaria* genera (Tietbohl et al., 2012) and some studies showed that Brazilian berries are also rich in phenolic constituents, in particular anthocyanins, flavonols, and ellagitannins (Wu et al., 2012).

Furthermore, several works have shown functional properties of fruit extracts of these species. In particular, antioxidant, anti-inflammatory, antimicrobial, and antiproliferative activity has been reported for *Plinia cauliflora*, *Myrciaria dubia* and *Myrciaria vexator* (Akter, Oh, Eun, & Ahmed, 2011; Kaneshima, Myoda, Toeda, Fujimori, & Nishizawa, 2017; Leite-Legatti et al., 2012). Nevertheless, not all the species belonging to the *Myrciaria* and *Plinia* genera have been equally studied. In particular, few works evaluated the phytochemical profile and the potential functional properties of *P. trunciflora* (Calloni, Santos, Martínez, & Salvador, 2016; Sacchet et al., 2015). Previously, this jabuticaba was classified in the genus *Myrciaria*, but in 1985 it was reclassified in the genus *Plinia* due to its inflorescences (Danner et al., 2007). However, the term *Myrciaria* is still used in scientific fields and for this reason *P. trunciflora* is also known with the name of *Myrciaria trunciflora*.

The aim of this study is the evaluation of phytochemical profile and the antioxidant properties of *P. trunciflora* fruits grown in Sicily. Chemical analyses included the identification of anthocyanins, flavonols, and hydrolysable tannins by both HPLC-DAD-MS/MS and spectrophotometric assays. Antioxidant properties of fruit extracts were assessed in a cellular model of lipid peroxidation and the involved mechanism evaluated. In particular, we assessed the radical scavenging and reducing abilities of the extracts in *in solution* assays and their ability to modify the expression of the genes coding for the antioxidant enzymes by qRT-PCR.

2. Materials and methods

2.1. Standards and chemicals

[2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid)]-diammonium salt (ABTS), 2,2′-azobis(2-methylpropionamidine)-dihydrochloride (ABAP), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), Folin-Ciocalteu's reagent, Hanks' balanced salt solution (HBSS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), cyanidine-3-glucoside, gallic acid (GA), potassium chloride, sodium acetate, potassium persulfate, 2,4,6-tripyridyl-S-triazine (TPTZ), iron (III) chloride hexahydrate ($FeCl_3$ $6H_2O$), were purchased from Sigma-Aldrich. Ethanol (LC–MS grade) was purchased from Biosolve B.V. (Valkenswaard, The Netherlands). RPMI, fetal bovine serum (FBS), phosphate buffered saline (PBS), L-glutamine solution (200 mM), trypsin-EDTA solution (170,000 U/l trypsin and 0.2 g/l EDTA), and penicillin/streptomycin solution (10,000 U/mL penicillin and 10 mg/mL streptomycin) were purchased from Lonza (Verviers, Belgium). All other materials and solvents were of analytical grade unless otherwise indicated.

2.2. Plant material and fruit extract preparation

The freshly harvested fruits of *P. trunciflora* (O. Berg) Kausel were obtained from Vivai Torre (Milazzo, Sicily, Italy; 38°19′ N, 15°24′ E; 20 m a.s.l.) and taxonomically identified. Fruits were frozen at −80 °C until extract preparation. The frozen fruits were thawed, finely chopped, and then homogenized. Fruit extracts were prepared as previously described with minor changes (Gentile et al., 2019). Briefly, ten grams of the whole homogenate were weighted and then extracted with EtOH using a 1:10 (w/v) ratio. Samples were mixed by vortex for 5 min and sonicated at room temperature for 15 min. After centrifugation (10 min at 8000g, 4 °C) the supernatants were filtered and stored at −20 °C. The extraction procedure was repeated to obtain three different technical replicates.

2.3. Total phenolic content

The total phenolic content (TPC) was determined by reduction of phosphotungstic-phosphomolybdic acid (Folin-Ciocalteu's reagent) to blue pigments in alkaline solution (Singleton & Rossi, 1965).

Quantification was performed using an external calibration curve with GA and results were expressed as mmol GA equivalents (GAE) per 100 g of fresh weight (FW). All measurements were repeated four times.

2.4. Total anthocyanin content

The total anthocyanin content (TAC) of *P. trunciflora* fruit extracts were determined using the pH-differential method (Elisia, Hu, Popovich, & Kitts, 2007) with some modifications (Lee, Rennaker, & Wrolstad, 2008). Twenty μl of extract were added separately to either 980 μl of 0.025 M potassium chloride (pH 1.0) or 980 μl 0.4 M sodium acetate (pH 4.5) buffers. The absorbance was measured at 535 nm and 700 nm for both solutions, using EtOH:H_2O (v/v) as a blank. TAC was calculated using the equation

$$TAC = \frac{(\Delta_{Abs} \times MW \times DF \times 1000)}{\varepsilon} \times l$$

where Δ_{Abs} is the difference between the Abs recorded at 515 nm and 700 nm at both pH 1.00 and pH 4.5; MW and ε are respectively the molecular weight (449.2 g mol^{-1}) and molar extinction coefficient (26.900 mM^{-1} cm^{-1}) of the cyanidine-3-glucoside, which was used as standard; DF is the sample dilution factor; l is the path length (1 cm). The total anthocyanins were expressed as mg of cyanidin-3-glucoside equivalents in 100 g of FW.

2.5. Identification and quantification of phenolic compounds by HPLC-ESI-DAD-MS/MS

HPLC analysis was performed as previously reported (Vigliante, Mannino, & Maffei, 2019b). The HPLC system consisted of an Agilent Technologies 1100 coupled to a DAD and a 6330 Series Ion Trap LC–MS System (Agilent Technologies, USA) equipped with an electrospray ionization (ESI) source. The chromatographic separation was carried out at constant flow rate (0.2 mL min^{-1}) using a reverse phase C18 Luna column (3.00 μm, 150 × 3.0 mm i.d., Phenomenex, USA), thermo-maintained at 25 °C by an Agilent 1100 HPLC G1316A Column Compartment. The UV–VIS spectra were recorded between 220 and 650 nm and the chromatographic profiles were registered at 280, 360, and 520 nm. Tandem mass spectrometry analyses were performed operating in negative mode for all compounds, except for anthocyanins, which were analyzed in positive mode. Compound identification was carried out by comparing the obtained retention time and UV–VIS/MS spectra with those of reference compounds.

2.5.1. Analysis of Flavon-3-ols and hydrolysable-tannins

The binary solvent system for analysis of Flavon-3-ols and hydrolysable tannin was MilliQ H_2O acidified with 0.1% (v/v) formic acid (Solvent A) (Millipore, Billerica, MA, USA) and ACN acidified with 0.1% (v/v) formic acid (Solvent B) as previously reported (Mannino, Gentile, & Maffei, 2019). The samples were separated by the following gradient: 97% A and 3% B as initial conditions, 70% A and 30% B for 35 min, and then 2% A and 98% B for 5 min. The concentration of A was maintained at 2% for 5 min and eventually raised to the initial condition before the next injection. Sample injection volume was 5 μl.

2.5.2. Analysis of anthocyanins

The binary solvent system for the analysis of anthocyanins was MilliQ H_2O acidified with 0.1% (v/v) formic acid (Solvent A) and MeOH 50% (v/v) acidified with 10% (v/v) formic acid (Sigma-Aldrich, USA) (Solvent B) as previously described (Mannino et al., 2019). The elution method involved a multistep linear solvent gradient varying from the initial concentration of 85% A and 15% B to 55% A and 45% B in 15 min. Then, the gradient reached the 30% A and 70% B concentration in 20 min. The concentration of solvent A was decreased to 2% and maintained for 5 min before the next injection. Sample injection volume was 15 μl.

2.6. Cell culture

Cancer cell line HepG2 (hepatocarcinoma cells, American Type Culture Collection ATCC, Rockville, MD, USA) were cultured in RPMI supplemented with 5% (v/v) FBS, 2 mM L-glutamine, 50 IU/mL penicillin, and 50 μg/ mL streptomycin and maintained in a humidified atmosphere with 5% CO_2 at 37 °C (Gentile et al., 2019). Cells were routinely cultured in 75 cm² culture flasks and were trypsinized using trypsin-EDTA.

2.7. Cellular antioxidant activity (CAA) assay

Fruit extracts were submitted to CAA assay, which was performed as previously described (Wolfe & Liu, 2007) with minor changes (Gentile et al., 2019). Briefly, the cells were seeded in 96-well plates in complete culture medium at 6.0×10^4 cells/well. 24 h after seeding, the medium was removed and the cells were treated for 2 h with 25 μM DCFH-DA plus the ethanolic extracts at different concentrations. Ethanol concentration never exceeded 0.25% (v/v) and culture medium with 0.25% EtOH (v/v) was used as control. After the incubation time, the cells were washed with PBS, then 600 μM ABAP in HBSS was added, and the microplate was placed into a plate-reader at 37 °C. Emission at 538 nm was measured with excitation at 485 nm every 5 min for 1 h. Each plate included triplicate control and blank wells. Control wells were pre-incubated with DCFH-DA and then incubated with ABAP in HBSS; blank wells contained cells treated with DCFH-DA and HBSS without oxidant. The area under the curve of fluorescence versus time was integrated to calculate the CAA value at each concentration of fruit extracts as follows:

$$CAA = 100 - \left[\frac{\int SA}{\int CA} \right] * 100$$

where: $\int SA$ is the integrated area of the sample curve and $\int CA$ is the integrated area of the control curve. The concentration necessary for 50% of DCF formation inhibition (CAA_{50}) for each fruit extracts was calculated from concentration-response (CAA) curves using linear regression analysis. Each result was obtained as the mean value of three separate experiments performed in quadruplicate.

2.8. Gene expression of antioxidant enzymes

HepG2 cells were seeded at a density of 5×10^5 cells/well in 24-multiwell plates. The media were discarded after 24 h and the cells were treated for 2 h with the fruit extracts in fresh FBS-free DMEM. Then, cells were exposed to 200 μM H_2O_2 for 24 h. After the incubation time, the cells were collected and total cellular RNA was isolated with a commercial kit (RNA-XPress™ Reagent, HiMedia), according to the manufacturer's instructions. One microgram of the total RNA was reverse-transcribed using oligo (dT) and OneScript® Reverse Transcriptase (HiMedia, China), according to the manufacturer's instructions. The resulting cDNA was used as a template for quantitative real-time PCR, using the BrightGreen 2X qPCR MasterMix-Low ROX (Abm, Canada) and a Stratagene® Mx3000 Real-Time PCR system, according to the manufacturer's instructions. Primers for human *CuZnSOD*, *MnSOD*, *GPx* and *CAT* genes and for the reference gene *β-Actin* are listed in Table 1. Real Time PCR was performed according to Sowndhararajan, Hong, Jhoo, Kim, and Chin (2015) (Sowndhararajan et al., 2015), and the relative expression levels of each gene were estimated using the method of Pfaffl (Pfaffl, 2001).

2.9. Radical scavenging and reducing activities in solution

2.9.1. ABTS assay

ABTS radical cation decolorization assay was performed as previously described (Miller & Rice-Evans, 1996). The assay is based on

Table 1
PCR primer sequences used in quantitative real-time PCR analysis.

Genes		Primer sequences	Accession
CuZnSOD	F	5′-ACGGTGGGCCAAAGGATGAA-3′	AC026776.4
	R	5′-TCATGGACCACCAGTGTGCG-3′	
MnSOD	F	5′-AGAAGCACAGCCTCCCCGAC-3′	NM_000636.4
	R	5′-GGCCAACGCCTCCTGGTACT-3′	
GPx	F	5′-TCGGTGTATGCCTTCTCGGC-3′	NM_000581.4
	R	5′-CCGCTGCAGCTCGTTCATCT-3′	
CAT	F	5′-CCAACAGCTTTGGTGCTCCG-3′	NM_001752.4
	R	5′-GGCCGGCAATGTTCTCACAC-3′	
β-Actin	F	5′-CGGGAAATCGTGCGTGACAT-3′	NM_001101.5
	R	5′-GGACTCCATGCCCAGGAAGG-3′	

monitoring the colorization decay of ABTS·⁺ at 515 nm. The ABTS radical was produced by reacting ABTS with potassium persulfate. Samples were analyzed at five different dilutions, within the linearity range of the assay. Radical scavenging activity was expressed as mmol Trolox Equivalent (TE) per 100 FW. All measurements were repeated three times.

2.9.2. DPPH assay

The assay is based on monitoring of colorization decay of DPPH at 735 nm (Ozgen, Reese, Tulio, Scheerens, & Miller, 2006). The radical scavenging activity of each sample was expressed as TE per 100 g of FW. Samples were tested at five different dilution, and all the measurement were repeated three times.

2.9.3. Ferric reducing antioxidant power (FRAP) assay

The reducing activity of fruit extracts was assessed by FRAP assay measuring the reduction of the Fe^{3+}–TPTZ complex to the ferrous form (Benzie & Strain, 1996). Briefly, the FRAP reactive, prepared by mixing 0.3 M acetate buffer (pH 3.6), 10 mM TPTZ, and 20 mM $FeCl_3$ in 8:1:1 (v/v/v) ratio, was incubated at 37 °C for 30 minutes with an opportune sample dilution and the absorbance was measured at 595 nm. All measurements were repeated three times and expressed as mmol TE per 100 g of FW.

2.10. Statistical analysis

All results were expressed as mean ± standard deviation (SD). ANOVA followed by Tukey's post-hoc test was applied to qRT-PCR data. A value of $p \leq 0.05$ was pre-determined as the criterion of significance. All the statistical analyses were carried out using SPSS Statistics 24 (SPSS, Chicago, IL, USA).

3. Results and discussion

3.1. Phytochemical characterization of P. trunciflora fruits

This study investigated the chemical profile of *P. trunciflora* fruits by spectrophotometric assays and HPLC-DAD-ESI-MS/MS analysis. The total amounts of polyphenols and anthocyanins were estimated by Folin-Ciocalteu and pH differential methods respectively (Table 2). Interestingly, the TPC value measured in *P. trunciflora* fruits exceeds those reported for all the fruits included in the Phenol-Explorer Database (Díaz-García, Obón, Castellar, Collado, & Alacid, 2013).

Anthocyanins are the main molecules responsible for the color of several fruits. Our results indicate that TAC in *P. trunciflora* is higher than in other berries, including blackberries, cranberries, raspberries, and strawberries (Szajdek & Borowska, 2008), and comparable with those evaluated for other species belonging to the *Myrciaria* genera (Santos, Veggi, & Meireles, 2010). Moreover, our results are similar to those obtained by Sacchet and colleagues, who evaluated TPC and TAC in extracts of whole fruits of *P. trunciflora* prepared in acidified water

Table 2
Total polyphenol content (TPC), total anthocyanin content (TAC), radical scavenging activity (ABTS and DPPH), ferric reducing antioxidant power (FRAP) and cellular antioxidant activity (CAA) of the *Plinia trunciflora* fruit extracts. Values are expressed as mean ± SD of three experiments carried out in triplicate.

TPC	1201.05 ± 89.49	mg GAE per 100 g of FW
TAC	195.78 ± 35.19	mg CCE per 100 g of FW
ABTS	5.19 ± 0.11	mmol TE per 100 g of FW
DPPH	8.49 ± 0.39	mmol TE per 100 g of FW
FRAP	15.34 ± 0.32	mmol TE per 100 g of FW
CAA	935.25 ± 4.81	mg of FW per mL medium

(Sacchet et al., 2015).

In order to separate and determine individual polyphenols, HPLC-DAD-ESI-MS/MS was employed. Although in colorful fruits anthocyanins generally account for the most of the TPC, in species of the *Myrciaria* genera others phenols may be present too (Reynertson, Yang, Jiang, Basile, & Kennelly, 2008). On the other hand, the really high TPC value in comparison with TAC, suggests the presence of other polyphenolic compounds, in addition to anthocyanins, in our extracts. HPLC-DAD-MS/MS analysis identified sixteen different compounds (Fig. 1). Among them, four were anthocyanins [Empetrin (**1**), Myrtillin (**2**), Ideain (**3**), and Chrysontemin (**4**)], seven were flavon-3-ols [Hyperoside (**5**), Astragalin (**6**), Isoquercetin (**11**), Quercetrin (**12**), Myricitrin (**13**), Quercetin (**14**), Vincetoxicoside B (**15**), and Rutin (**16**)], and four were hydrolysable-tannins [Casuariin (**7**), Tellimagrandin I (**8**), Tellimagrandin II (**9**), and Casuarinin (**10**)]. The qualitative and quantitative determination of each identified compound was reported in Table 3. The weight of the identified polyphenol compounds represent 1.10% of the total FW, with anthocyanins accounting for more than 70% of this amount. The two glycosylated forms of cyanidine, **3** (0.42% of FW) and **4** (0.29% of FW), contribute for about 90% of the total content of anthocyanins. Sacchet and collegues evaluated the anthocyanin profile of *P. trunciflora* fruits (Sacchet et al., 2015) too. However, in contraposition to our results, they also found low amounts of cyanine and malvidin.

About 25% of the identified polyphenol weight is represented by the flavon-3-ols. The most concentrated flavon-3-ols in *P. trunciflora* fruits are **6**, **5**, and **13**. Although the most abundant flavon-3-ols in plant matrices, is normally the **14**, together with its glycosylate-forms, in our *P. trunciflora* fruit extracts, the **14** was found in very small amounts.

While condensed tannins, also named proanthocyanidins (PACs) are the most distributed polyphenols in food, hydrolysable tannins are rarely found in edible plant matrices (Okuda & Ito, 2011). Only few red fruits, such as pomegranate and some nuts, contain a discrete amount of these compounds (Vigliante, Mannino, & Maffei, 2019a). Our analysis revealed that *P. trunciflora* fruits contain ellagitannins (**7**, **8**, **9**, and **10**), but the total amount of these molecules does not exceed the 0.6% of the identified polyphenols weight. Among hydrolysable tannins, **7** and **10** were the most abundant. Finally, also the presence of PACs was investigated by both BL-DMAC assay and HPLC-DAD-MS/MS but no traces were detected.

3.2. Cellular antioxidant activity (CAA) of P. trunciflora fruit extracts

In order to evaluate the antioxidant properties of *P. trunciflora* fruit extracts, we used a cell-based lipid peroxidation model. CAA assay, in contraposition to other lipid peroxidation models, beside evaluating the possible ability of redox-active compounds to interact with biological membranes, also measures the stability of antioxidant compounds at cellular metabolic conditions (Wolfe & Liu, 2007). The CAA_{50} of *P. trunciflora* fruit extracts was 935.25 ± 4.80 μg FW mL^{-1} of cell medium. In comparison with the values determined by Wolfe and Liu for several common fruits (Wolfe et al., 2008), the antioxidant capacity measured in our extracts was much higher. In particular, comparing our

results with the CAA_{50} value of colorful fruits, including several berries, cherries, and pomegranate, *P. trunciflora* fruits extracts had an antioxidant activity 3–30 times higher.

In order to limit the consequences of overproduction of reactive species, living organisms have developed effective endogenous defence systems against oxidative stress conditions (Halliwell, 1991). These are sophisticated mechanisms that maintain redox homeostasis through both directly scavenging reactive species (soluble antioxidant defences) and preventing their production (antioxidant enzymes) (Matés, Pérez-Gómez, & De Castro, 1999). The antioxidant benefits of the intake of food with high concentration of polyphenols have been attributed not only to their redox-active properties but also to their ability to regulate the activity or expression of antioxidant enzymes (Yeh, Ching, & Yen, 2009). In order to clarify the mechanism underlying the antioxidant action observed in HepG2 cells, we evaluated both the radical scavenging activity of *P. trunciflora* extracts via *in solution* assays and its effects on the expression of antioxidant enzymes.

3.3. Radical scavenging and reducing activities of P. trunciflora fruit extracts

Foods contain several molecules with redox active properties, including classical antioxidant vitamins and several antioxidant phytochemicals such as phenolic compounds. These molecules contribute synergistically to the overall antioxidant properties of each single food. Therefore, the concept of the total antioxidant capacity (TAC) of foods takes into account the additive, synergistic, or antagonistic redox interactions between the different molecules in the food. To measure the TAC, a variety of assays, most of which were *in solution* assays, has been devised (Pellegrini, Vitaglione, Granato, & Fogliano, 2018).

In order to evaluate the overall intrinsic reducing capability of *P. trunciflora* fruit extracts, we employed three *in solution* antioxidant assays. As it was previously reported that a single assay is not sufficient to predict the antioxidant potential of plant extracts and that the results from different assays can help to elucidate the mechanism involved in the observed activities (Ozgen et al., 2006), redox active properties of *P. trunciflora* fruit extracts were investigated through ABTS, DPPH, and FRAP assays.

Despite the criticisms, due to obvious limitations of *in vitro* chemical TAC methods, those assays are very popular for technological and nutritional purposes (Pellegrini et al., 2018), and provide valuable information about the intrinsic reducing properties of the complex mixture of redox active molecules contained in foods. In this view, our results showed that *P. trunciflora* fruit extracts possess high reducing properties (Table 1). Concerning the radical scavenging activity, the value obtained via DPPH was higher than via ABTS. The observed differences could be explained by the variability in pH or in the hydrophilicity of the reaction mixtures, and by the relative difference in the radical-scavenging ability of antioxidant compounds present in the extracts (Gentile et al., 2016). Moreover, the ABTS value was comparable to those reported for pomegranate and was one order of magnitude higher than the values recorded for common fruits, including apple, banana, blueberry, grapefruit, and orange (Fu et al., 2011). Regarding metal reducing ability, a very high FRAP value was recorded. According to our HPLC-DAD-MS/MS results, this high activity of *P. trunciflora* fruit extracts can be explained by the presence of several flavonoids having a meta or ortho hydroxyl substitution on B-ring (**1**, **2**, **3**, **4**, **5**, **11**, **12**, **13**, **14**, **15**, **16**) that is able to chelate metal-ions, thus easing their reduction (Ozgen et al., 2006).

3.4. Gene expression of antioxidant enzymes in HepG2 cells after treatment with P. trunciflora fruit extracts

The antioxidative properties of *P. trunciflora* fruit extracts in HepG2 cells, beside involving the redox active properties of its components, could also depend on the effects on the activity or the expression of
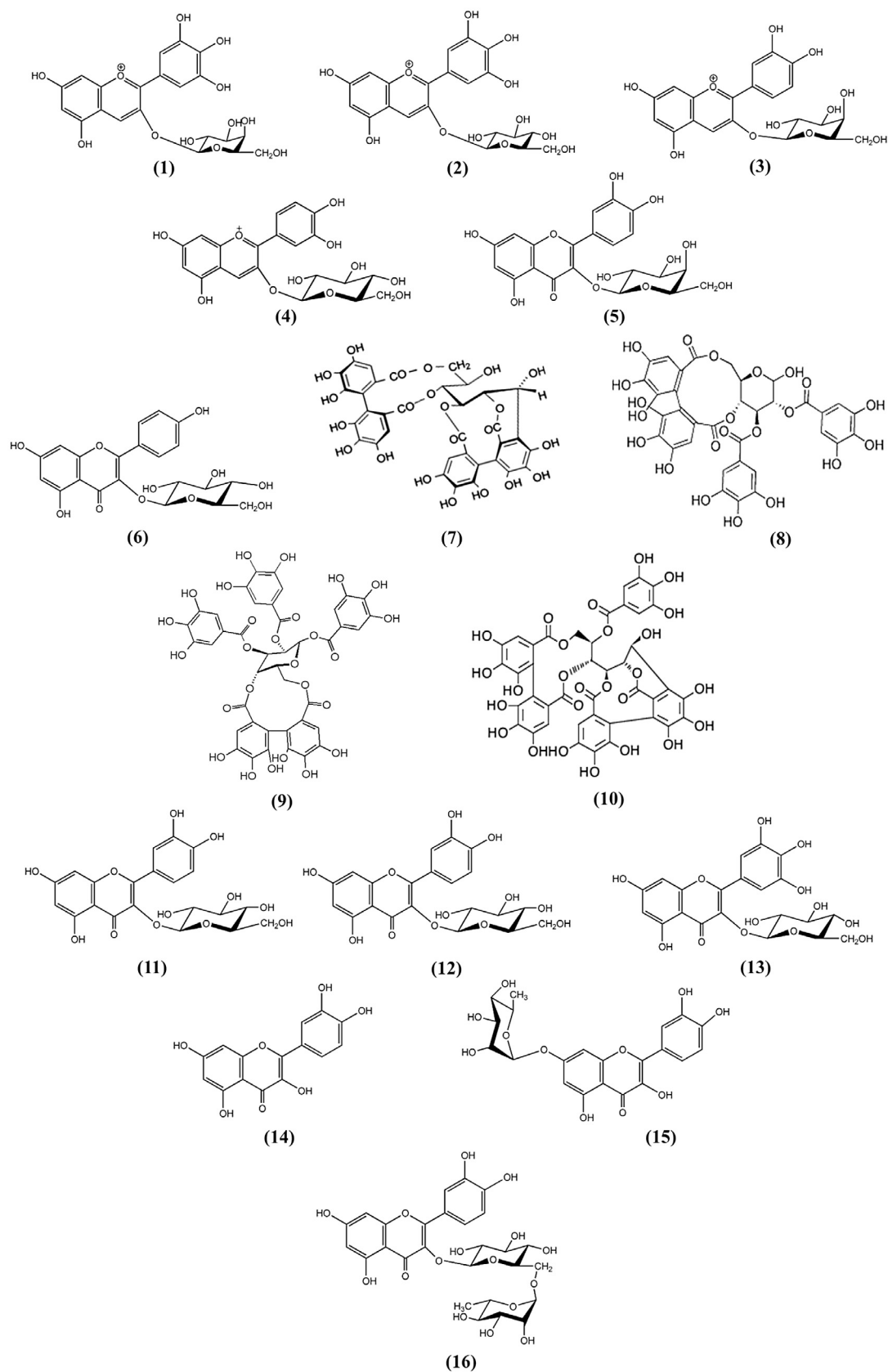
Fig. 1. Structure formulae of the phenolic compounds characterized and quantified in *Plinia trunciflora* fruit extracts.

Table 3
Qualitative and quantitative chemical analysis of the phytochemical profile of *Plinia trunciflora* fruit extracts. Quantification is expressed as mean ± SD of three experiments carried out in triplicate and the values are expressed as mg of each compound per 100 g of fresh weight (FW). Supplementary Table 1 reports statistical analysis carried out by SPSS v.24 statistical software.

#	RT [min]	Compound(s)		λ	MW	m/z	mg per 100 g of FW
1	12.1	Delphinidin-3-O-galactoside	Empetrin	525	465	303	61.57 (1.86)
2	14.3	Delphinidin-3-O-glucoside	Myrtillin	525	465	303	35.00 (0.58)
3	15.1	Cyanidin-3-O-galactoside	Ideain	525	449	289	418.35 (12.99)
4	16.7	Cyanidin-3-O-glucoside	Chrysontemin	525	449	289	287.11 (7.09)
5	18.6	Quercetin-3-O-galactoside	Hyperoside	360	463	301	52.65 (0.94)
6	19.9	Kampherol-3-O-glucoside	Astragalin	360	447	285	174.17 (12.27)
7	20.8		Casuariin	360	783	481; 319; 319; 301	1.25 (0.05)
8	25.9		Tellimagrandin I	360	785	633; 578; 483; 301	3.32 (0.16)
9	26.6		Telligramadin II	360	937	766; 617; 452	0.50 (0.01)
10	27.6		Casuarinin	360	935	782; 632; 451	1.51 (0.03)
11	27.7	Quercetin-3-O-glucoside	Isoquercetin	360	463	301	5.43 (0.20)
12	29.4	Quercetin-3-O-rhamnoside	Quercetrin	360	447	300	2.18 (0.04)
13	30.1	Myricetin-3-O-rhamnoside	Myricitrin	360	463	316	36.53 (0.86)
14	30.4		Quercetin	360	301		10.65 (1.01)
15	31.3	Quercetin-7-O-rhamnoside	Vincetoxicoside B	360	447	301	12.10 (0.30)
16	32.9	Quercetin-3-O-rutinoside	Rutin	360	609	463; 301	1.65 (0.04)

antioxidant enzymes. Sacchet and colleagues (2015), measuring the enzymatic activity of *SOD, CAT,* and *GPx* on mice brain homogenate after treatment with a hydroalcoholic extract from *P. trunciflora* fruits, excluded a direct action of its phytochemicals on the enzymatic activity modulation (Sacchet et al., 2015). Accordingly, in this work we focused on the effect of *P. trunciflora* fruit extracts on the gene expression of antioxidant enzymes in HepG2 cells submitted to pro-oxidant action of H_2O_2. HepG2 cells, retaining several functional properties of normal hepatocytes, including the basal and induced expression of antioxidant enzymes (Lee et al., 2015), are widely used to study the cellular response to oxidative stress. In our experiments the working concentrations of *P. trunciflora* fruit extracts were chosen taking into account the CAA_{50} value previously measured in the same cellular model. According to literature data, we stimulated HepG2 cells with 200 μM H_2O_2 for 24 h. Moreover, in pilot studies we excluded the cytotoxic action of this treatment by MTT assay (data not shown). The gene expression of *MnSOD, CuZnSOD, CAT* and *GPx* was monitored via qRT-PCR in control cells and after treatments.

In our experimental conditions, control cells expressed all target genes. When cells were exposed to 200 μM H_2O_2 for 24 h, accordingly to Sowndhararajan (2015), we also found a strong down-regulation of *MnSOD* (53% inhibition), *CuZnSOD* (48% inhibition) and *CAT* (44% inhibition), and a lower inhibition of *GPx* (32% inhibition) in comparison with control cells (Fig. 2, panel A). Moreover, cell exposure to the *P. trunciflora* extracts alone did not change the basal expression of target genes (Fig. 2, panel B). On the other hand, when HepG2 cells were pre-treated for 2 h with *P. trunciflora* fruit extracts and then incubated with 200 μM H_2O_2 for 24 h, we observed a reactivation of *MnSOD* and *GPx* genes (Fig. 3). The effects were dose-dependent and when the cells were exposed to the highest concentration of the extracts the gene expression levels of *GPx* and *MnSOD* increased 4.96 and 1.75 times respectively in comparison to H_2O_2 exposed cells. While SOD prevents the oxidative damage related to superoxide radical anion producing H_2O_2, GPx neutralizes H_2O_2 to water and organic peroxides (ROOH) to their related-alcohols respectively. Thus, the effects of *P. trunciflora* fruit extracts on gene expression of SOD and GPx in HepG2

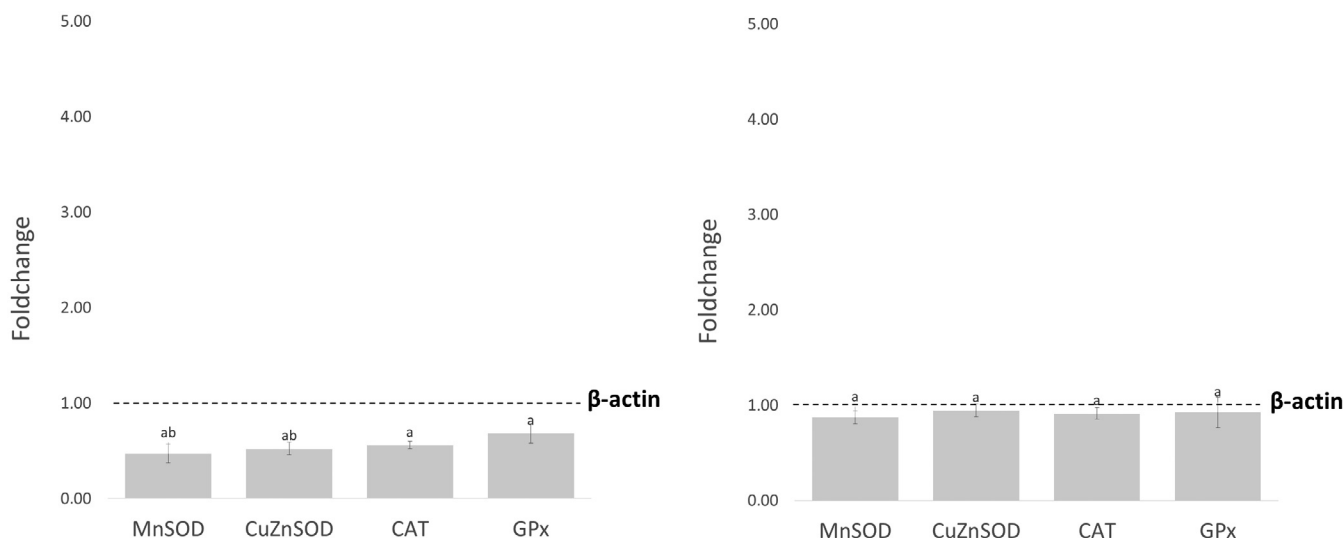**Fig. 2.** Effect of H_2O_2 or *Plinia trunciflora extracts* on the gene expression of SOD, CAT and GPx on HepG2 cells. After the seeding, cells were incubated in the absence of additional treatments (untreated cells), exposed for 24 h to 200 μM H_2O_2 (Panel A) or treated with 1 mg of FW per mL of cell medium *Plinia trunciflora* fruit extracts (Panel B). After the treatments, the cells were collected and total cellular RNA was isolated and retro-transcribed in cDNA. The cDNA was used as template for quantitative real-time PCR using β-actin as reference gene. Bars represent the mean ± SD of three qRT-PCR analyses carried out in triplicate. Values are expressed as fold change with respect to untreated cells, calculated as described in the paragraph 2.8 of section "Materials and Methods". Bars with different lowercase letters indicate significantly different values at p ≤ 0.05 as measured by Tukey's (see Supplementary Table 2 for further information).
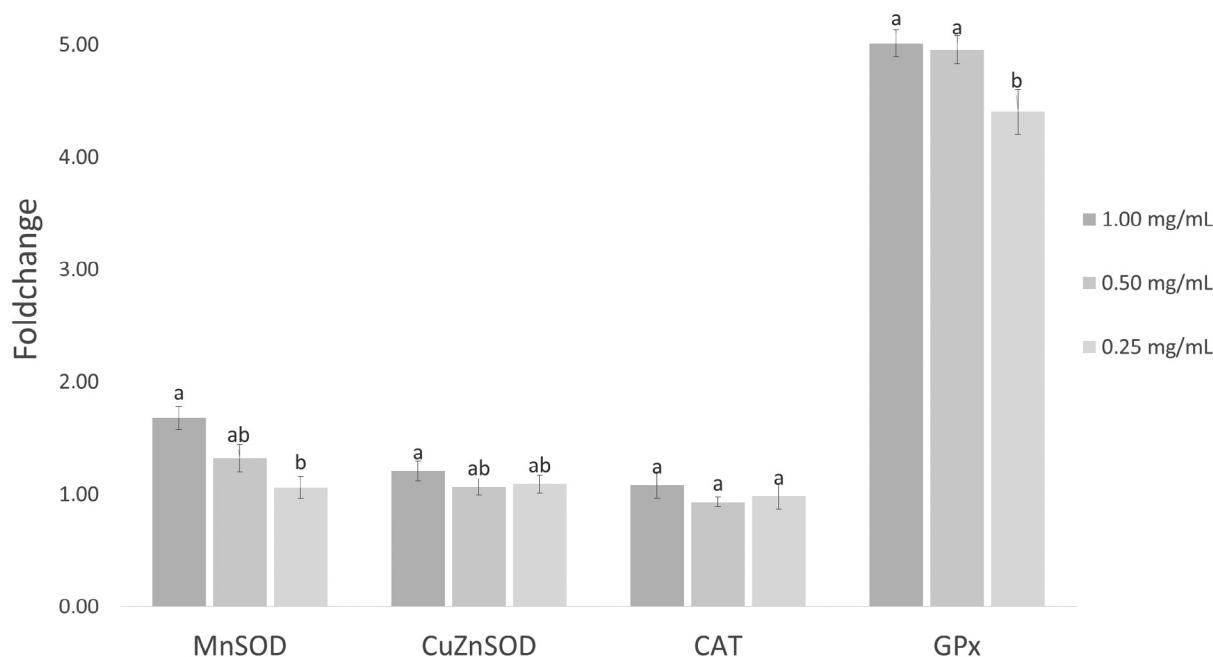
Fig. 3. Effect of *Plinia trunciflora fruit extracts on gene expression of SOD, CAT and GPx on HepG2 cells.* After the seeding, cells were treated for 2 h with indicated concentrations of *Plinia trunciflora* fruit extracts and then were exposed to 200 μM H_2O_2 for 24 h. Cells untreated but exposed to H_2O_2 were used as positive control. After the treatments, the cells were collected and total cellular RNA was isolated and retro-transcribed in cDNA. The cDNA was used as template for quantitative real-time PCR using β-actin as reference gene. Bars represent the mean ± SD of three qRT-PCR analyses carried out in triplicate. Values are expressed as fold change with respect to positive control, calculated as described in the paragraph 2.8 of section "Materials and Methods". Bars with different lowercase letters indicate significantly different values at $p \leq 0.05$ as measured by Tukey's (see Supplementary Table 2 for further information).

cells, when exposed to H_2O_2, suggest that specific phytochemicals in the extracts activate an adaptive response helping those cells in defending themselves from oxidative stress.

4. Conclusion

Our results provided information on the functional properties of *P. trunciflora* fruits. Specifically, we have shown that these fruits are a very rich source of antioxidant polyphenols, including some with limited occurrence in nature. For this reason these berries could become an interesting raw material for the nutraceutical industry in addition to gaining acceptance as health-promoting food. In particular, our data indicating antioxidant activity at very low concentrations in a biological environment suggested that even a moderate daily intake of this fruit may provide physiologically significant antioxidant protection. Finally, our results suggest a real possibility to obtain high quality tropical fruits also in the Mediterranean climate.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2019.125515.

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