UNIVERSITÀ
DI PAVIA

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Προετοιμασία σπόρων για την αντιμετώπιση της ξηρασίας κατά την βλάστηση: Μια μελέτη περίπτωσης στο βιο-ενισχυμένο για Zn ρύζι

Seed priming to mitigate drought stress during germination: A case study in zinc biofortified rice

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Academic Year 2022/2023

Η παρούσα εργασία εκπονήθηκε στο τμήμα Βιολογίας και Βιοτεχνολογίας του Πανεπιστημίου της Παβίας στα πλαίσια του προγράμματους Erasmus+ για σπουδές, με αποστολέα (sending University) το Πανεπιστήμιο Θεσσαλίας. Αναγνωρίστηκε ως πτυχιακή εργασία από την Γενική Συνέλευση του Τμήματος Βιοχημείας και Βιοτεχνολογίας.

Περίληψη

Η παγκόσμια κλιματική κρίση απαιτεί καινοτόμα εργαλεία για την αντιμετώπιση των γεωργικών προκλήσεων. Η καλλιέργεια ρυζιού, ενός ζωτικής σημασίας βασικού προϊόντος σε περιοχές που απειλούνται από κλιματικές κρίσεις, απαιτεί καινοτόμες λύσεις για θέματα όπως η ξηρασία και οι διατροφικές ελλείψεις. Η μελέτη αυτή διερευνά τις δυνατότητες του πολυ-γαμμα-γλουταμινικού οξέος (PGA) που παράγεται από βακτήρια ως μια περιβαλλοντικά βιώσιμη μέθοδος προετοιμασίας σπόρων, ιδίως για βιοενισχυμένες ποικιλίες ρυζιού πλούσιες σε ψευδάργυρο. Εν μέσω αυξανόμενων φαινομένων ξηρασίας, η γεωργική βιοτεχνολογία προσπαθεί να ενισχύσει την παραγωγή ρυζιού και τη διατροφική αξία. Η προετοιμασία των σπόρων, μια αποδεδειγμένη τεχνική, αναδεικνύεται για τη δυνητική περιβαλλοντική βιωσιμότητά της. Η έρευνα μελετά την επίδραση του PGA στη βλάστηση σε βιοενισχυμένες με ψευδάργυρο ποικιλίες ρυζιού υπό συνθήκες υδατικής καταπόνησης, αξιολογώντας πολλαπλές παραμέτρους καθώς και την γονιδιακή έκφραση που σχετίζεται με την καταπόνηση. Καθώς ο κόσμος αναζητά αποτελεσματικές, φιλικές προς το περιβάλλον και οικονομικά προσιτές λύσεις, το PGA αναδεικνύεται ως μια πολλά υποσχόμενη οδός για την αντιμετώπιση της διπλής πρόκλησης του ρυζιού, δηλαδή της παραγωγής και της κρυφής πείνας.

Abstract

The global climate crisis necessitates innovative tools to tackle agricultural challenges. Rice, a vital staple in crisis-prone regions, demands novel solutions for issues like drought and nutritional deficiencies. This study explores the potential of bacteria-derived poly-gamma-glutamic acid (PGA) as an environmentally sustainable seed-priming method, particularly for biofortified zinc-rich rice varieties. Amid increasing drought occurrences, agricultural biotechnology strives to enhance rice production and nutritional value. Seed priming, a tested and proven technique, is highlighted for its potential environmental sustainability. This research delves into PGA's impact on germination in zinc-biofortified rice varieties under water stress, assessing multiple parameters and stress-related gene expression. As the world seeks efficient, eco-friendly, and affordable solutions, PGA could emerge as a promising avenue in addressing rice's dual challenge of production and hidden hunger.

Contents

Introduction

Rice cultivation is endangered globally by drought

Rice is a monocot plant belonging to the family *Poaceae* and the genus *Oryza*, and it has played a vital role in world history as a staple food. From the approximately 22 species of rice, two are most important for human consumption: *Oryza sativa*, cultivated in Asia, America, Europe, the Middle East and Africa, and *Oryza glaberrima*, cultivated in Africa, although it starts to be fast replaced by *O. sativa* (Muthayya et al., 2012). Rice plants are characterised by long, slender leaves and a tall, upright stem that can reach up to 6 feet in height. The plant produces edible grains, which are used as a staple food by more than half of the world's population and has been cultivated for thousands of years. The roots of rice cultivation can be traced 8.000 to 9.000 years back, in South Central Asia (Callaway, 2014), where the two main varieties, indica (long-grained) and japonica (short-grained), were first domesticated. From there it spread to the rest of the world, where it now holds a central role in the diet of many cultures. It has been estimated that rice provides more than 20% of the calories consumed worldwide, a percentage that can reach up to 80% in Asian regions (Manikanta et al., 2022).

In a world with a population of 8 billion, estimated to arrive to 9.8 billion by 2050 (United Nations, 2022), most increase happening in developing nations, it is evident how important rice production is, and will be for the future. The current annual production of milled rice is estimated at around 500 million metric tons (Muthayya et al., 2014), a number that must increase significantly within a few years in order to be able to feed the growing population (Korres et al., 2017) and many challenges will need to be addressed to scale-up rice production.

The sessile nature of plants makes them susceptible to all kinds of environmental stress. Even though they have evolved several response mechanisms, these may not suffice to address the rapid climatic changes, and in the years to come, crop production will be greatly challenged by rising temperatures and changes in precipitation patterns. Climate models predict that some regions will experience increased rainfall, while others will experience decreased rainfall. These variabilities can make it challenging for farmers to plan and manage their crops effectively. In addition, climate change is expected to increase the frequency and intensity of extreme weather events, such as droughts, floods, and storms, which can cause significant crop losses (Korres et al., 2017). Since rice is a semi-aquatic plant, the most important danger for rice cultivation is drought. Drought is defined as a prolonged period of abnormally low rainfall or reduced soil moisture levels that results in a water shortage (CDC, 2023). In particular, rice plants are drought-susceptible also due to the small root system, thin cuticular wax, and swift

stomata closure (Sahebi et al., 2018). It is a water-intensive crop and requires a substantial amount of water to grow and develop, with the optimal amount required to produce 1 kg of rice being estimated at about 3000 litres (Oladosu et al., 2019). When considering rice cultivation methods, some of them require the use of modern tools, but most farmers opt for the traditional, time-tested methods of wet and dry rice growing. This is because most rice farmers operate on a small scale, with limited resources, and this subjects them to even further pressure in the near future. Wet growing is performed in flooded paddies, areas where rice seedlings are planted and covered with water until completely submerged, while the water is usually drained before harvest. This method ensures adequate moisture levels and helps in weed and pest growth control. Dry growing, also known as upland growing, is performed in regions where rainfall and other water sources are scarce. Specific cultivars are used by farmers, which may lead to lower yield, but are well adapted to their local environment's rainfall and temperature variabilities (Arraundeau, 1995).

The impact of drought on rice production are complex and depend on various factors, including the severity and duration of the drought periods, type of soil, and growth stage. Some of its effect include reduced yield, poor grain quality, and increased susceptibility to pests and disease. The yield reduction can vary depending on drought severity, but it can be as much as 25,4% or more in extreme cases (Zhang et al., 2018). Drought can become particularly damaging if it occurs during the critical growth stages of the rice plant, such as germination, flowering, and grain filling, whereas if it takes place during the reproductive stage, it can lead to complete crop failure (Korres et al., 2017). Most rice is currently cultivated in regions where temperatures are already above the optimal for growth (28/22 °C), so any further increase in average temperature could severely affect crop production (Krishnan et al., 2011).

To mitigate the impacts of drought on rice production, various measures can be taken. Improved water management practices can help reduce the impact of drought on rice production. These practices can include the use of efficient irrigation systems, such as drip irrigation, which can reduce water loss through evaporation and runoff. Water-saving techniques, such as alternate wetting and drying, can also be used to reduce water consumption without affecting yield and quality. Early sowing, or selection of early maturing cultivars to avoid high temperatures during grain filling, is another strategy (Krishnan et al., 2011). Plant breeding programs can develop rice varieties that are more tolerant to drought, either through conventional crop-breeding strategies or through genetic engineering. Besides, throughout history, farmers have always dealt with environmental changes by adopting new cultivars and adjusting their cultivation schemes. These varieties have characteristics such as deeper root

systems, which allow them to access water from deeper soil layers, and reduced water requirements, which can help them survive in low rainfall conditions (Yadav et al., 2013). However, their development is considerably limited by insufficient knowledge on the genetic mechanisms of plant drought tolerance, as well as the cumbersome regulatory framework that restricts the use of genetically engineered drought-tolerant varieties. Exogenous application of numerous agents, like growth regulators, osmoprotectants, nutrients, plant growth promoting bacteria (PGPB), are also considered even if these also require further equipment and technical knowledge (Venkateswarlu and Shanker, 2009). A feasible strategy for mitigation of drought stress in crops, that combines low cost, ease of use and efficiency, can be seed priming.

Seed priming to improve rice germination

A very old practice gives modern solutions

Seed priming is a simple, cost-effective technique that involves the treatment of seeds with various substances such as water, hormones, micronutrients, and osmoprotectants. The goal of seed priming is to improve general seed performance under various abiotic stresses, including drought (Hussain et al., 2022).

The simplicity of this technique has allowed it to be practised since ancient times. The first recorded use of seed priming can be found in the text of the Greek philosopher Theophrastos, who, after observing a faster germination of water-soaked cucumber seeds, wrote down his findings in the seventh book of his *Enquiry into Plants* (Theophrastos). Pliny the Elder, the famous Roman naturalist, mentioned the same method of cucumber seed soaking in water and honey, in his opera *Naturalis Historia* (Pliny the Elder). The pathway for rigorous research on seed priming in our modern world was paved by visionaries like the French agronomist Olivier de Serres around the beginning of the 18th century, Charles Darwin and his germination experiments on various seeds with salt water (Darwin, 1856), Ells' experiments on tomato seed treatment with a nutrient solution (Ells, 1963), Ingenhousz's studies into the impact of light, and May's observations on the effects of seed drying (May et al., 1962), among many others (Singh et al, 2020, Lutts et al, 2016). Studies that aim to standardise the measurement of germination parameters have been made and these provided general guidelines to help uniformising the results between laboratories (Ranal and de Santana, 2006). Currently, the fields of seed biology and biotechnology is recognised for the impact that it has on the future, and seed priming methods are among the major tools to address upcoming challenges related to climate uncertainty and agricultural production demands.

How does priming work?

Seed priming is a technique used for enhancing seed vigour through the application of various seed treatments before sowing. This activates the metabolic processes that occur during the early developmental stages of young seedlings, at a pre-germination level. The primed seeds can acquire many benefits, compared to their non-primed counterparts. These may include uniform and rapid seedling emergence, a wider range of germination temperatures, better plant growth, node formation, and productivity, and increased resistance to weeds and pathogens, among others (Paul et al., 2022).

Priming protocols have been established for a wide variety of seeds. These include rice, wheat, legumes, okra, sunflower, melon, as well as many vegetables like carrot, leek, onion, celery, lettuce, endive, pepper and tomato (Paparella et al., 2015, Marthandan et al., 2020). More and more seeds are quickly being contributed for the expansion of this list. With the current market size of conventional and genetically modified seeds at over 60 billion euros, and a forecast of more than 90 billion before 2028 (MordorIntelligence, 2023), the economic value of priming is in continue expansion.

The molecular and cellular mechanisms behind seed priming have been the subject of an increasing number of studies. In general, seed germination advances through three phases, as described by Bewley (**Fig. 1**):

- Phase I - the imbibition phase - where the dry tissues of the seed quickly absorb water.
- Phase II - the activation phase - where molecular mechanisms are activated and water absorption is slowed down.
- Phase III - the germination phase - where cell elongation and radicle protrusion are the main physiological events (Bewley, 1997).

Priming involves the preparation of seeds by getting them through the first two phases of imbibition and metabolism activation, and then stopping before the radicle protrusion starts, followed by dehydrating the seeds to store them for future use (**Fig. 1**). In particular, the molecular activities concern DNA damage repair, antioxidant defence mechanisms initiation, energy production, and changes in phytohormone biosynthesis, described with what has been termed “pre-germinative metabolism” (Macovei et al., 2017). First, the early processes of respiration and ATP synthesis start to provide energy for the translation mechanism, using the reserved resources of the seed. The proteins that are synthesised are responsible for DNA, lipid and protein damage repair and antioxidant protection. The damage is due partly to the high

levels of reactive oxygen species (ROS) that are produced from the mitochondrial electron transport chain within the first moments of water imbibition. It is important to underline that ROS act both as signalling molecules, necessary to initiate the germination programs, and a source of cellular damage that can lead seed tissue deterioration during storage (Dey and Bhattacharjee, 2023).

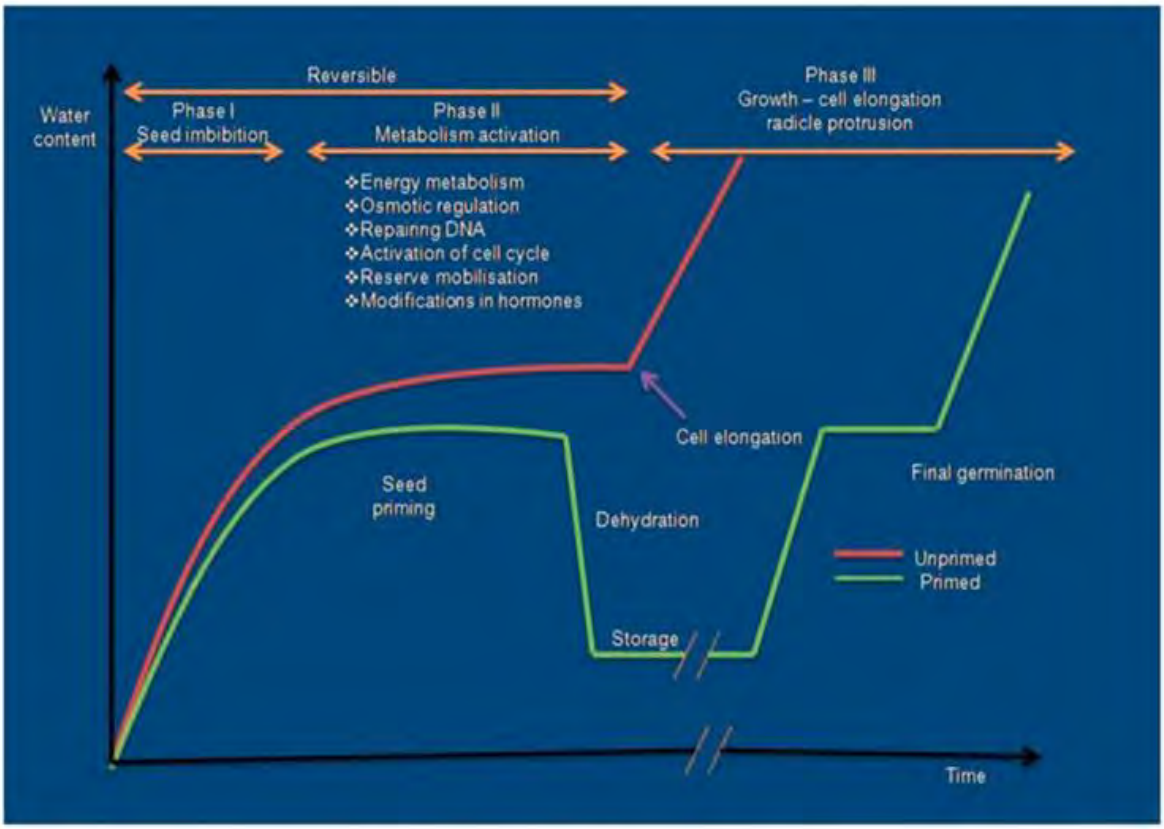

Figure 1. Seed priming mechanism explained based on main seed germination phases (Marthandan et al., 2020).

Priming methods

The simplest, and therefore the oldest, practised priming method is hydropriming. In this method, the seeds are placed in an aqueous environment, at certain temperatures (usually around 5-20ºC) for a time period that depends on the seed type, and that ensures adequate seed hydration, but not germination. It is important that the hydration happens in a controlled manner, and the critical point between priming and germination has to be defined for each species, genotype, and seed lot (Khalid et al., 2019). A common variant of hydropriming is drum-priming, where a drum is used to keep the seeds in, while water in the form of vapour comes in and hydrates the seeds (Waqas et al., 2019).

Another common priming method is osmopriming. With this method, controlled seed water uptake takes place, owing to the osmotic solution which generates a low water potential and allows for activation of the metabolic processes in Phase II. The most widely used osmotic agents are salts (halopriming) like KH_2PO_4, KNO_3, $CaCl_2$, $MgSO_4$, NaCl and KCl, mannitol, glycerol, and polyethylene glycol (PEG). As PEG has a large molecular weight, it is ideal for this type of practice as it cannot enter inside the seed, contrary to the others (Sher et al., 2019).

These methods can also be practised in a solid matrix arrangement, where less water is used because of the solid matrix substances (sawdust, charcoal, vermiculite) that generate an environment of limited water supply and adequate aeration, contrary to complete seed water submergence, thus achieving controlled imbibition and resource saving (Hilhorst et al., 1997).

Chemopriming concerns the use of a variety of chemical substances that include naturally derived molecules, like chitosan, choline, and amines, nutrients like vitamins, potassium, zinc, magnesium, and boron (Sako et al., 2020). The use of phytohormones has also been studied, as hormopriming, with abscisic acid, gibberellic acid and spermidine being the most effective in preparing the seeds for drought stress (Sytar et al., 2019). Redox priming is also used, with hydrogen peroxide as the most common agent (Dey and Bhattacharjee, 2023).

The application of growth promoting microorganisms, also known as biopriming, has been studied extensively, with the best-known examples being the use of nitrogen fixating bacteria and endomycorrhizal fungi that can help the developing plants acquire nutrients and improve stress response (Chackraborti et al., 2022).

The most recent addition to the different seed priming strategies is nanopriming. In this case, active nanoparticles or nanocarrier systems with sustained release of active molecules are either taken up by the seeds or they generate a coat around them. This helps with water uptake during imbibition, it can grant protection against pathogens and aid in overall plant development, depending on the load of active molecule (Espirito et al., 2021).

Finally, all the above methods can be combined and applied in parallel, to give optimal results for each type of seed.

Priming to mitigate drought stress

There are several mechanisms through which seed priming can mitigate the effects of drought stress on crop cultivation. As already mentioned, the processes that follow water imbibition during priming include DNA and lipid damage repair, defence against oxidation, energy production and protein synthesis (Marthandan et al., 2020). Moreover, activation of specific genes and epigenetic modifications, that aid in drought tolerance, occur (Bruce et al.,

2007). This is especially the case when a priming protocol that simulates stress condition is used, aiming to trigger a phenomenon termed "priming memory" (Aswathi et al., 2022) (**Fig. 2**).

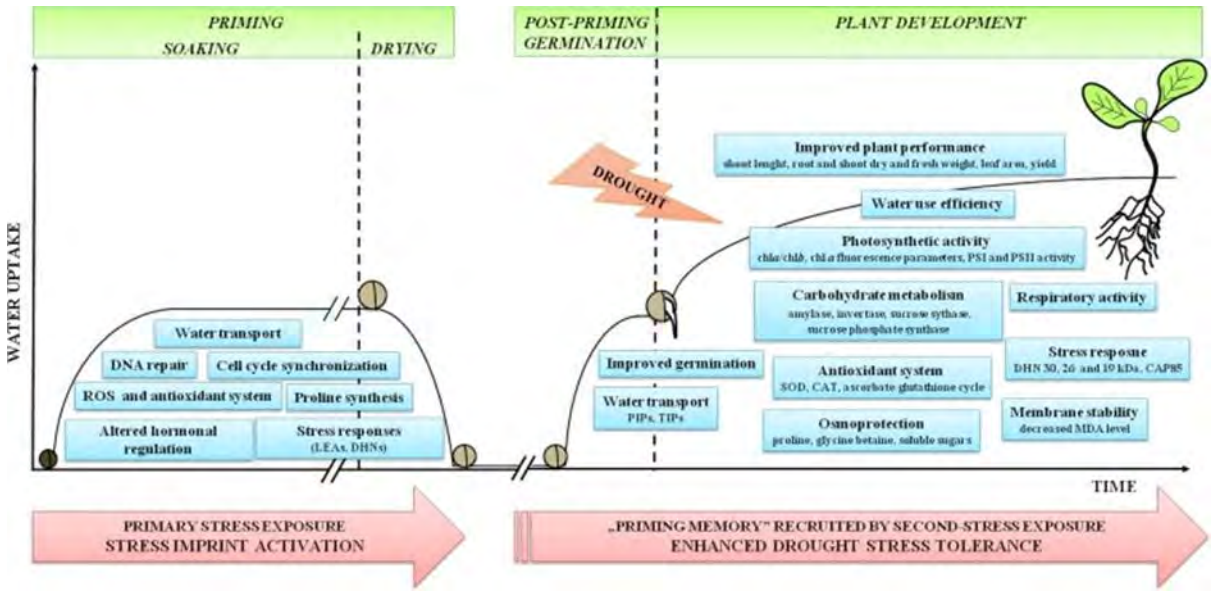

Figure 2. Schematic representation of the seed "priming memory" phenomenon, shown to be able to improve plant drought tolerance (Wojtyla et al., 2016).

For example, osmo-primed seeds have been shown to acquire both germination vigour and salinity tolerance, through the accumulation of osmoprotectants like proline (Kubala et al., 2015). Histone modifications and DNA methylation that take place during priming could aid in drought tolerance, in a similar way (Liu et al., 2022). Moreover, they have been shown to persist even after recovery from drought in *O. sativa* and *Arabidopsis thaliana*, which further facilitates recovery from future stress (Liu et al., 2022).

The accumulation of various molecules and proteins is also a very important aspect of the priming period. Osmotic potential regulators like soluble sugars and amino acids (mostly proline and glycine betaine), are present in high concentrations in primed seeds and can protect the developing seedlings from dehydration during drought stress (Khan et al., 2019). Antioxidants like ascorbate, glutathione, and carotenoids, scavenge ROS and protect the plant cells against oxidative damage (Zandalinas et al., 2017). Transcription factors (Bhargava & Sawant, 2012), signalling proteins and mRNAs (Dace et al., 1998) are also in high abundance after priming. Different genes participate in the molecular responses of drought stress recovery. DNA glycosylases like *FPG* (formamidopyrimidine glycosylase) and *OGG1* (8-oxo-guanine glycosylase 1), phosphodiesterases like the ones encoded by the *TDP1α* (tyrosyl-DNA phosphodiesterase) and *TDP1β* genes and DNA ligases are responsible for DNA repair, while

catalases (*CAT*), superoxide dismutases (*SOD*) and ascorbate peroxidases (*APX*) maintain the oxidative balance (Pagano et al., 2023).

Some other physiological adaptations that are enhanced by seed priming include a faster recovery of the photosynthetic machinery (Ammar et al., 2020) and root development, improved water efficiency and transport through tissues (Iovieno et al., 2016).

Priming with poly-gamma-glutamic acid (γ-PGA)

Poly-gamma-glutamic acid (γ-PGA) is a naturally occurring macromolecule composed of repeating units of the amino acid glutamic acid, both in the L and D configurations. It can be produced by several microorganisms in the environment, but *Bacillus* species are currently being used for commercial production (Sung et al., 2005). It is biocompatible and biodegradable, properties that are going to bring it to the forefront of new natural polymer production (Park et al., 2021). With the advent of new fermentation techniques coming from synthetic biology and metabolic engineering, γ-PGA is projected to play an important role in the new bioeconomy (Hu et al., 2022).

So far, it has been used in agriculture to address drought stress, because of its water absorbing capabilities, contribution to root growth and apparent enrichment of the plant growth promoting bacteria numbers (Ma et al., 2022), when applied as a soil conditioner. In view of this, γ-PGA could be considered as a good candidate for the development of new seed priming protocols that will mitigate drought stress.

Rice biofortification for increased zinc

The first outcome of rice production, after the steps of harvesting and threshing, is rice grain. This is usually processed through further steps of milling to remove the outer husk layer, producing the brown rice, with can be subsequently polished to remove the germ and bran layers and obtain the white rice grains. White rice is composed of the endosperm and the embryo, is rich in starch but it has very low amounts of lipids and proteins. These macromolecules are mainly found in the bran (15.0 - 19.7% and 11.3 - 14.9% of dry weight, respectively) (Bodie et al., 2019). Therefore, brown rice polishing can greatly reduce its nutritional profile.

"Hidden hunger" (HH) is a term that refers to a form of micronutrient deficiency caused by the consumption of foods with an insufficient amount of vitamins and minerals. It occurs when the quality of food that people consume does not meet the nutrient requirements, so they are

not getting the essential micronutrients needed for proper growth and development. It has been estimated that HH has affected one in three people globally, most of them being in the developing world (Dhaliwal et al., 2022). While the traditional method of fortification of staple foods, like rice, with vitamins and minerals can be an efficient way to deal with the problem (World Health Organization, 2018), biofortification is considered a more sustainable and long-lasting approach.

Biofortification is a biotechnological technique aimed at increasing the nutritional value of food crops by means of conventional breeding methods or genetic engineering (Bhardwaj et al., 2022). In rice, biofortification studies have mainly focused on the nutrients that are lacking, and that would have the highest impact on the health of consumers should their concentrations increase. These are micronutrients like zinc (Zn), iron (Fe) (Wairich et al., 2022), and provitamin A (Beyer et al., 2002).

Conventional breeding is utilized for introducing genes from closely related or wild ancestors of rice to its popular domesticated counterparts to enhance its Fe and Zn content. The success of this approach relies on rice's genetic diversity. A vast amount of accession from different cultivars can be screened for potential parentals (Guimaraes, 2009). The access to these information and collections supported considerable breakthroughs in developing the released high Zn varieties like IRRI 195 or NSIC Rc460 in the Philippines and Impa IR Nutrizinc rice in Indonesia. These varieties provide 40% EAR for women and children (Bouis et al., 2011).

In parallel, genetic engineering advances attempted to achieve the goals set by biofortification programs of 10–15 μg/g for Fe and 28 μg/g for Zn (53). Increased understanding of Fe and Zn homeostasis genes in rice has led to numerous efforts in biofortification using different transgenic approach to reach the nutritional target level in the polished grain (Gross et al., 2003; Jeong and Guerinot, 2009). Currently, the highest attained levels of Fe and Zn were obtained in the indica IR64 mega-variety with 15 mg/kg Fe and 45.7 mg/kg Zn in polished grains no significant yield penalty (Trijatmiko et al., 2016). Attaining both Fe and Zn biofortification through genetic engineering approaches is feasible because genes encoding proteins involved in metal ion transport to the embryo are common for both Fe and Zn.

Biofortified rice, obtained through conventional breeding or genetic engineering, represents a sustainable solution to HH. However, this endeavour must be accompanied by efforts to render rice cultivation resistant to environmental stresses and, most importantly, drought.

Aim of the Work

The repercussions of the current climate crisis experienced across the globe, call for new tools to help face these challenges. Novel solutions to the agricultural problems, such as drought and increasing production, are imperative. Rice, being a staple food in many areas that are most sensitive to this crisis, is an important target for the development and application of novel solutions to enhance its production. Moreover, rice is also the contributor to another problem that demands attention, namely issues related to the hidden hunger phenomenon, as it is poor in vitamins and minerals, thus causing health concerns like micronutrient deficiency. Zinc and iron are two of the minerals that breeders and scientists are trying to increase in rice. As drought phenomena are becoming more and more common, agricultural biotechnology is called to find a way to protect and increase rice production as well as improve its nutritional value.

The proposed solution must be also environmentally sustainable. One such method, is seed priming, a well-established technique that can improve crop productivity. Besides the existing priming treatments, the new trends demand for methods that combine the highest effectiveness with the lowest environmental footprint, while being also economically affordable. Bacteria-derived poly-gamma-glutamic acid (PGA) could represent such a product.

The aim of this study was to investigate the effect that PGA could have in the germination of two rice varieties. To address hidden hunger, these varieties are biofortified with zinc, obtained through conventional breeding methods, and deemed as environmentally sustainable and promising for the future of rice nutritional enhancement. The germination behaviour was investigated under water stress and PGA treatments, along with the use of additional materialled generated by using denatured PGA (dPGA), and water as control samples. To assess the effect of each treatment, multiple germination parameters and stress-related gene expression data were evaluated, analysed and discussed.

Materials and Methods

Plant materials and treatments

Two zinc-biofortified rice varieties were used in this work of thesis. The Impa IR Nutrizinc (from here on referred to as Inpari) variety was provided by the National Rice Research Institute of Indonesia (ICAR), and the NSIC Rc460 (from here on referred to as Rc460) variety provided by the International Rice Research Institute (IRRI), Philippines. Both varieties have a high grain Zn content and were obtained using conventional breeding approached within the respective research centres.

A priming protocol was developed to treat rice seed based on the use of γ-PGA. This component was isolated from the *Bacillus subtilis* strain 5625 in the lab of Dr. Cinzia Calvio (Department of Biology and Biotechnology, University of Pavia, Italy). The denatured PGA (dPGA) was prepared after incubation of PGA at 120 ℃ for 72 hours. The seeds were primed by soaking in 6 ml of γ-PGA 1 mg/ml or dPGA 1 mg/ml, for 16 hours.

Following priming treatments, the seeds were sown in germination trays supplied with specific clay loam soil, prepared in such a way to replicate natural field capacity (FC) or mild drought (MD) conditions. The soil used for the mild drought simulation system was sieved and dried in the oven until moisture was not observed. For the preparation of the sowing process, 20 g of soil was placed in 8 cm by 10 cm aluminium trays. Then 7.96 g of water was added to the soil to simulate mild drought stress, while 11.46 g to those that simulated field capacity levels. After sowing 30 seeds in each tray, the trays were sealed with cellophane and were placed in a growth room, under the conditions of 23 ± 3°C and relative humidity of 43 ± 7%. Weight measurements were taken each day at the same time, throughout a 7-day period. Whenever water levels were lower than the limits described by Ahmad et al. (2019), water was added to replenish the shortage.

Phenotypic analyses

The number of germinated seeds was recorded at 24 hours intervals. Seeds were considered as germinated when shoots sprouted from the soil (Kader, 2005). Different parameters were collected and calculated (Kader, 2005; Ranal and de Santana, 2006; Mahmood et al., 2022).

The parameters that were calculated from these measurements were germination percentage (G%), germination index (GI), mean germination time (MGT), germination synchronicity (Z), coefficient of germination velocity (CVG), seedling vigour index (VII), root, shoot and plant length (RtLn, StLn and PlLn), germination drought tolerance index (GDTI), Shoot Length

Drought Tolerance Index (SLDTI), Root Length Drought Tolerance Index (RLDTI) and Total Plant Length Drought Tolerance Index (TPLDTI). The calculations were performed according to the equations in **Table 1**.

Table 1. Germination parameters calculated in this study.

Parameter	Equation
Germination Percentage (G%)	G% = (Number of germinated seeds/numbers of sown seeds) *100
Germination Index (GI)	GI = (7*No1) +(6*No2) +...(1*No7), where the numbers are the germinated seeds per day
Mean Germination Time (MGT)	MGT=∑ f ·x/ ∑ f, where f is the number of germinated seeds on day x
Germination Synchronicity (Z)	Z = ∑Cni, 2 / N, where ∑Cni, 2=∑(ni(ni-1)/2) while N= ∑ni(∑ni-1)/2
Coefficient of Germination Velocity (CVG)	CVG = N1 + N2 + · · · + Nx/100 × N1T1 + · · · + NxT, where N=No. of seeds germinated per day, T=No. of days from seeding corresponding to N
Seedling Vigour Index (VII)	VII = Plant Length * G%
Root. Shoot and Plant Length (RtLn, StLn and PlLn)	The lengths in mm, measured with ImageJ
Germination Drought Tolerance Index (GDTI)	(G% under stress/G% under normal condition) *100
Root, Shoot and Plant Length Drought Tolerance Indices (RLDTI, SLDTI and TPLDTI)	(Tissue length under stress / Tissue length under normal conditions) *100

Seedling measurements for root and shoot length were performed with the ImageJ (https://imagej.net/ij/index.html) software.

RNA extraction

At the end of the 7th day, seedlings were collected from each tray, washed, placed in aluminium foil and frozen in liquid nitrogen. The materials were then grinded in sterilised clay mortars and the powder were preserved in Eppendorf tubes in liquid nitrogen. From the samples that did not germinate, RNA was collected from the seed embryos.

The RNA extraction protocol was performed using TRIZOL® Reagent (Thermo Fisher Scientific, Milan, Italy) according to the instructions given by the supplier. The protocol started

with addition of 750 ml of Trizol to the grinded powder, incubated in ice for 5 min, then 150 ml of chloroform, and another incubation in ice for 3 min. The samples were then centrifuged at 4°C, 10.000 rpm for 15 min, 300 ml of the supernatant was collected and an equal amount (here 300 ml) of isopropyl alcohol was added. After an incubation in -20°C for 15 min and a centrifugation at 4°C, 10.000 rpm for 15 min, the supernatant was now discarded, and 500 ml of ice-cold 70% ethanol were added. The samples were again centrifuged at 4°C, 10.000 rpm for 15 min and this step was repeated to thoroughly wash the samples. Then another wash with 100% ethanol was performed followed by a centrifugation at 4°C, 10.000 rpm for 15 min. Subsequently, the ethanol was removed, and the samples were left open in ice, for the ethanol to evaporate completely. Finally, the samples were resuspended in 25 ml of diethylpyrocarbonate (DEPC)-treated cold water. Measurements of RNA concentration were taken after the resuspension, using a Nanopore spectrometer (Biowave DNA, WPA, ThermoFisher Scientific).

cDNA synthesis and quantitative RealTime Polymerase Chain Reaction (qRT-PCR)

For the cDNA synthesis, each sample was first treated with DNAse I (Thermo Fisher Scientific). For this, 1,5 μL per sample of DNAse I and 1,5 μL of buffer were added, and they were incubated in a thermocycler at 37°C for 30 min. The reaction was then paused shortly, to add 1,5 μL of EDTA in each sample, and was then resumed for 10 more min at 65°C. cDNAs were then obtained by using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's suggestions.

The qRT-PCR reactions were performed with the Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific) according to the supplier's indications, by using a CFX Duet, Real Time PCR System (BIO-RAD, Milano, Italy). Amplification conditions were as follows: denaturation at 95ºC for 10 min, and 45 cycles of 95ºC for 15 s and 60ºC for 60 s. Oligonucleotide sequences were designed by using Primer3Plus1 (https://primer3plus.com/) and verified with Oligo Analyzer.2 (https://eu.idtdna.com/pages/tools/oligoanalyzer).
Relative quantification was carried out by using the Os25SrRNA as reference gene (Jain et al., 2006). Raw fluorescence data provided by Software 1.7 (BIO-RAD) were used to determine the threshold cycle number (Ct) values for each transcript quantification. Relative quantification of transcript accumulation was performed as described by Thomsen et al., 2010. All reactions were performed in triplicate.

Statistical Analysis

The germination parameters calculations and the gene expression heatmaps were performed in Microsoft Excel files.

The statistical analysis was performed with the software tool developed by Assaad et al. (2015), available online at https://houssein-assaad.shinyapps.io/TwoWayANOVA/. A two-way analysis of variance (ANOVA) was performed to identify statistically significant differences between the parameter means in the different varieties and treatments, followed by Tukey's Honest Significant Difference Test (Tukey's HSD).

The Principal Component Analysis (PCA) was conducted using the software resources available at MetaboAnalyst 5.0 (www.metaboanalyst.ca) following user's guide specifications (Xia and Wishart, 2016).

Results

Phenotyping analysis of rice germination following γ-PGA priming

For the purpose of this study, four different treatments (PGA, dPGA, water soakin and no treated control), were applied on two zinc-biofortified rice varieties germinated under two different water level conditions (mild drought and field capacity). To simulate drought, a water shortage of 3,5 g was applied to the growth trays, for a water level of 7,96 g in 20 g of soil to achieve "mild drought" as described by Lad'anyi et al. (2021) and 11,96 in the rest of the trays, achieving field capacity watering levels.

Throughout the phenotyping analysis, two types of measurements were collected. The first is a set of germination parameters, calculated from the observations that were made each day, for a period of seven days. Every visible germinant was counted as a germination event. From the first set of data, the following parameters were calculated: germination percentage (G%), germination index (GI), mean germination time (MGT), germination synchronicity (Z), coefficient of germination velocity (CVG) and the germination drought tolerance index (GDTI). The second set consisted in the measurements of the seedling lengths at the end of the seventh day. These are root length (RtLn) and shoot length (StLn), along with seedling vigour index (VII), which is calculated from plant length and germination percentage.

Inpari

The results indicate that none of the treatments exerted any significant effect on the germination percentage of Inpari seeds grown under mild drought conditions (**Figure 3**). However, under field capacity conditions, the treatment with PGA and dPGA resulted in increased germinability of Inpari seeds (**Figure 4**). After PGA treatment, the first increase is noticed as soon as the fourth day, and even more pronounced on the seventh day. Seeds treated with dPGA also seem to have an increase in G%, albeit less than the PGA treatment. For PGA the effect was slightly more than 45% on the last day, and for dPGA 31%. However, based on the GDTI data (**Figure 5**), dPGA that has the biggest effect on addressing drought tolerance in Inpari, with the increase starting on the fourth day and reaching 25% by the seventh. PGA reached its maximum on day 6 (**Figure 5**). A similar situation can be observed for the rest of the parameters. In **Figure 6**, the dPGA treatment resulted into increased growth of the roots and shoots, with dPGA showing better results than PGA, with a more than 2,5 times difference. This trend is much less noticed in the field capacity germinated seedlings. The parameters of mean germination time and synchronicity showed no statistical significance, but both the

germination index and seedling vigour index pinpoint that dPGA increases the seedling vigour under mild drought (**Table 2**).

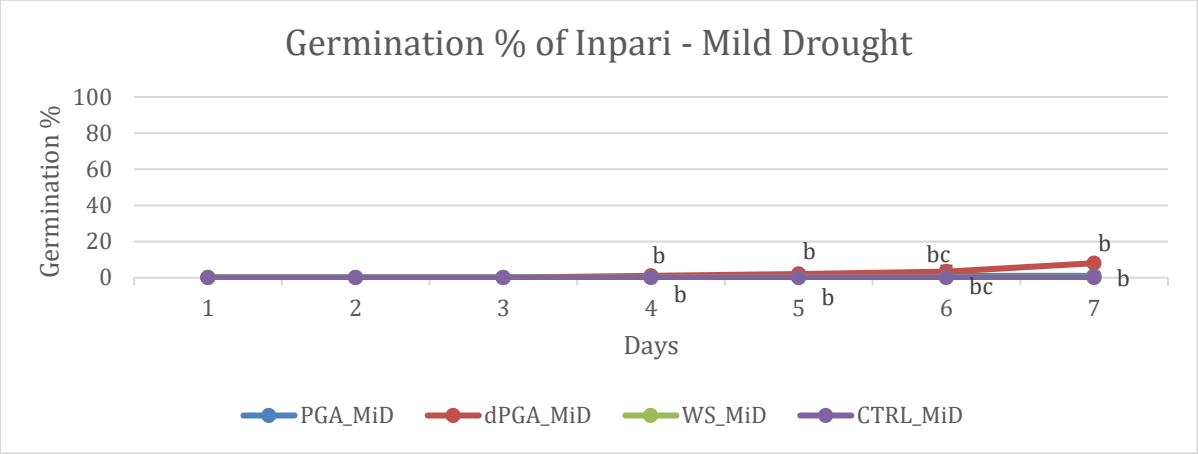

Figure 3. Germination percentage (%) of Inpari rice seed grown under mild drought stress (MiD) in the presence of PGA or dPGA priming, waster soaking (WS) and control (CTRL) conditions. Statistical significance, calculated with Tukey test, is indicated by different letters between the different treatments.

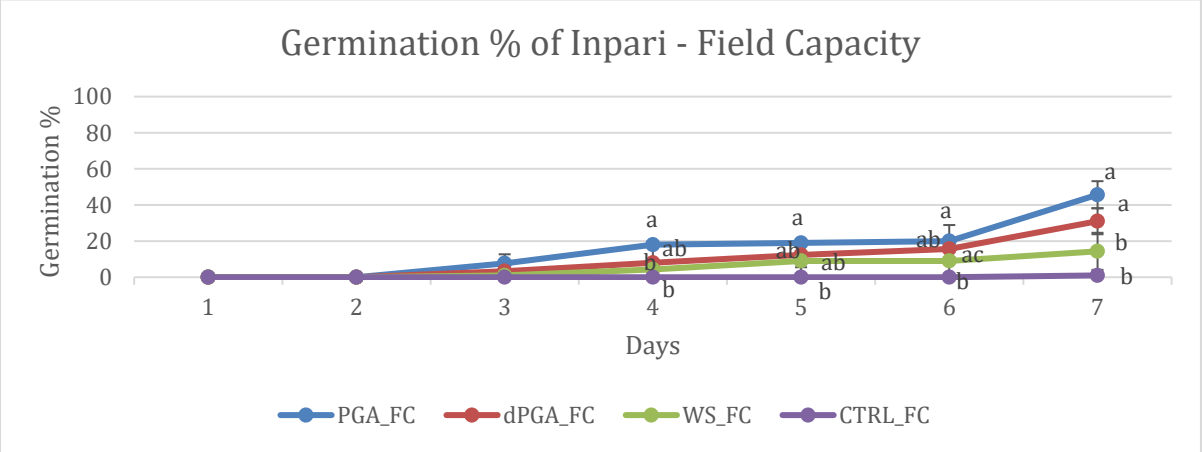

Figure 4. Germination percentage (%) of Inpari rice seed grown under field capacity (FC) in the presence of PGA or dPGA priming, waster soaking (WS) and control (CTRL) conditions. Statistical significance, calculated with Tukey test, is indicated by different letters between the different treatments.

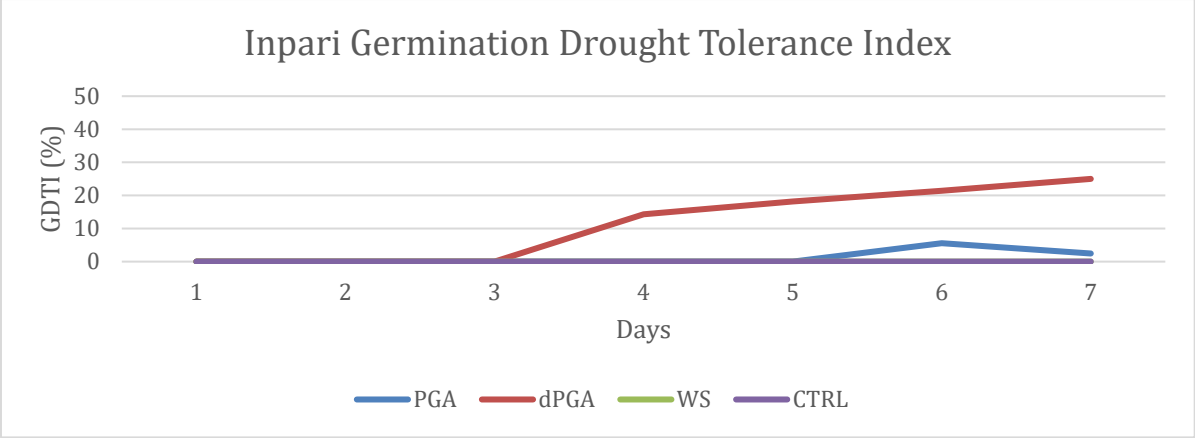

Figure 5. Germination drought tolerance index of Inpari rice seed in the presence of PGA or dPGA priming, waster soaking (WS) and control (CTRL) conditions.

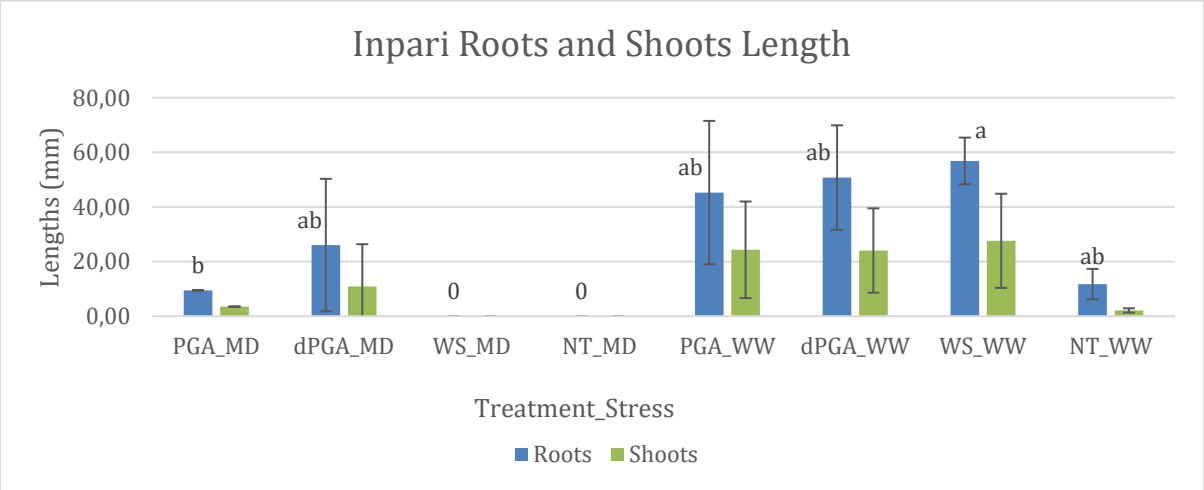

Figure 6. The root and shoot lengths of Inpari seedlings after seven days of treatment with PGA, dPGA, water soaking (WS) and control (CTRL) under mild drought (MD) or field capacity (FC) water levels. Statistical significance, calculated with Tukey test, is indicated by different letters between the different treatments.

Table 2. Germination parameters MGT, Z, VII and GI for the different priming treatments and stress conditions in Inpari rice. Statistical significance, calculated with Tukey test, is indicated by different letters between the different treatments.

Variable		MGT	Z	VII	GI
FC	CTRL	4.67 ± 2.33	0.667 ± 0.333	27.6 ± 13.8c	1.67 ± 0.882c
	HP	3.57 ± 1.82	0.163 ± 0.0876	1160 ± 582bc	24.7 ± 15.6bc
	PGA	5.54 ± 0.116	0.334 ± 0.0478	3190 ± 484a	77 ± 9.87a
	dPGA	5.82 ± 0.266	0.297 ± 0.0195	2320 ± 359ab	44 ± 13.7ab
MD	CTRL	0 ± 0	0 ± 0	0 ± 0c	0 ± 0c
	HP	0 ± 0	0 ± 0	0 ± 0c	0 ± 0c
	PGA	2 ± 2	0 ± 0	12.9 ± 12.9c	1 ± 1c
	dPGA	6.17 ± 0.601	0.5 ± 0.289	324 ± 174c	8 ± 3.21bc
P-value	Stress	0.006	0.05	<0.001	<0.001
	Treatment	0.022	0.213	<0.001	0.002
	SXT1	0.261	0.091	0.001	0.003

Rc460

The data on Rc460 shows no significant effect of dPGA or PGA on seed germination under mild drought or field capacity conditions. In **Figure 7** and **Figure 8**, the germination percentages remained zero throughout the whole period of the experiment, evidencing a high susceptibility to drought for this variety. Under mild drought, it was PGA that reached the highest G% of 3%, and its counterpart under field capacity was again PGA, at 5%. The GDTI, on the sixth day, shows an increase equal to double the amount of the control, as can be noticed in **Figure 9**. However, this can be attributed to the way that GDTI is calculated, because this increase is caused by a difference of only one more seed on the sixth day of growth under mild drought, compared to the same day under field capacity. From **Figure 10** and **Table 3**, it can be observed that almost no seeds germinated under mild drought, except for some that had been treated with PGA. Under field capacity, the seedlings grown from PGA treated seeds had a small increase in root and shoot length, albeit smaller than that of the seeds treated with water soaking. PGA also showed an increase in seed vigour and germination index. However, these effects are not statistically different compared to controls, according to the two-way ANOVA with a level of significance at $p < 0,05$.

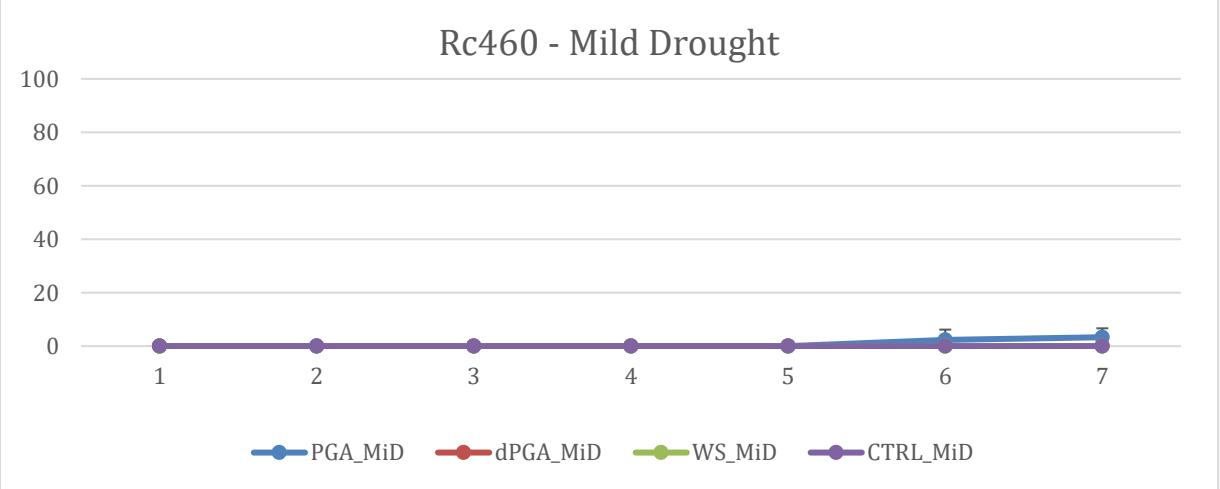

Figure 7. Germination percentage (%) of Rc460 rice seed grown under mild drought stress (MiD) in the presence of PGA or dPGA priming, waster soaking (WS) and control (CTRL) conditions. Statistical significance, calculated with Tukey test, is indicated by different letters between the different treatments.

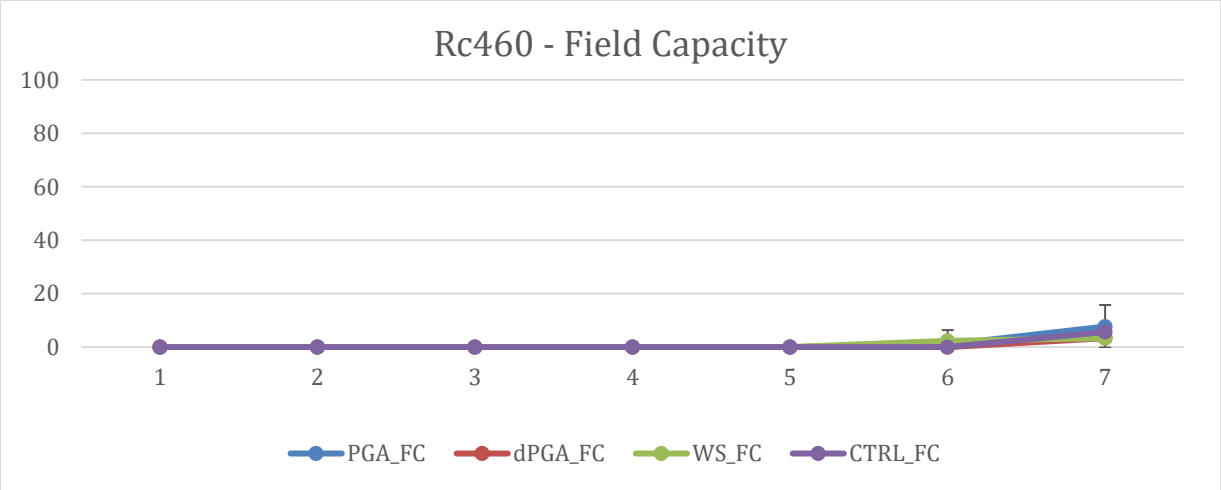

Figure 8. Germination percentage (%) of Rc460 rice seed grown under field capacity (FC) in the presence of PGA or dPGA priming, waster soaking (WS) and control (CTRL) conditions. Statistical significance, calculated with Tukey test, is indicated by different letters between the different treatments.

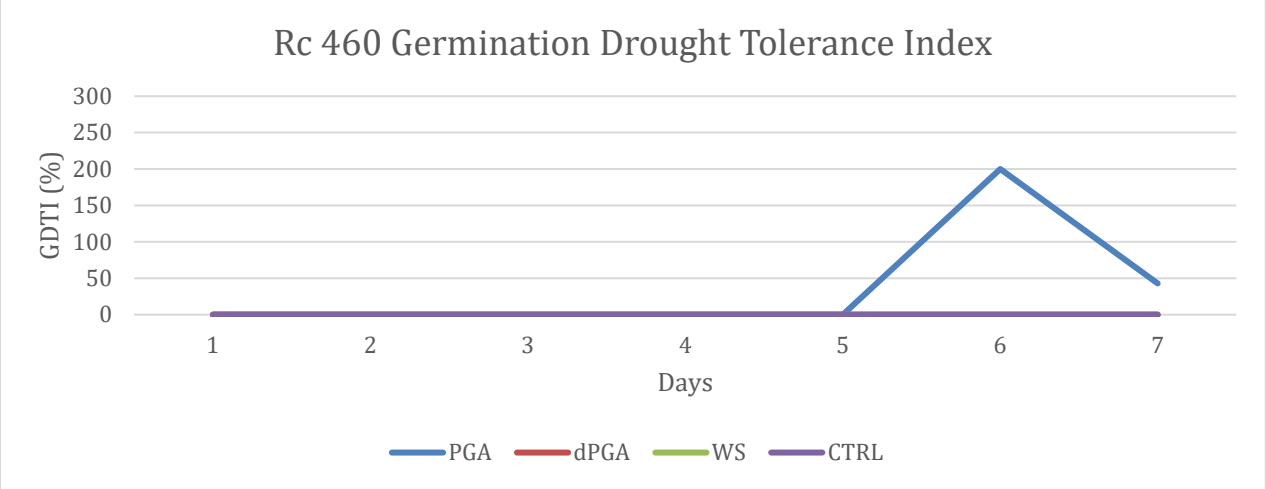

Figure 9. Germination drought tolerance index of Rc460 rice seed in the presence of PGA or dPGA priming, waster soaking (WS) and control (CTRL) conditions.

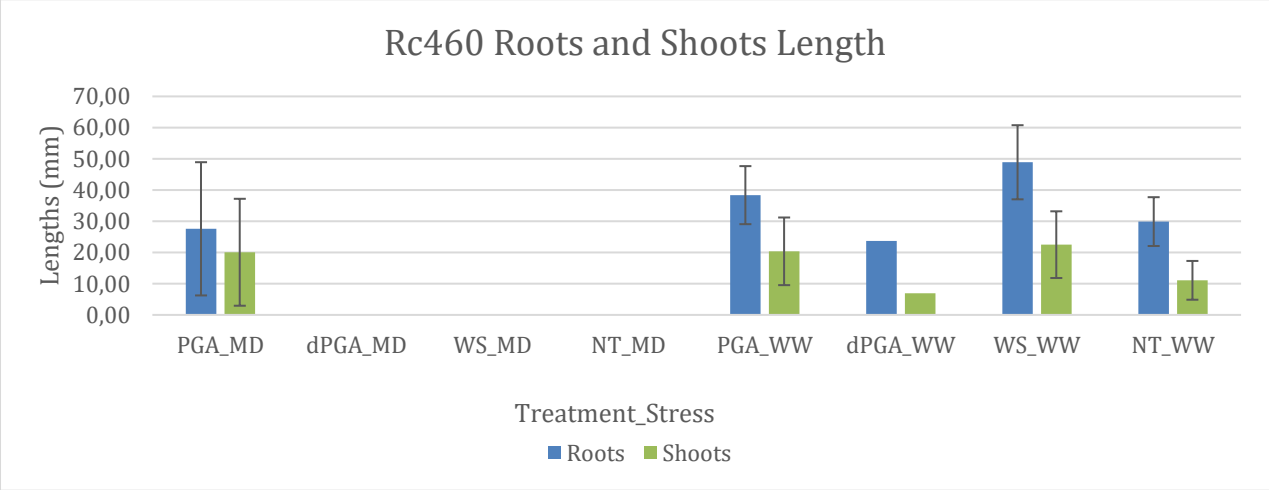

Figure 10. The root and shoot lengths of Rc460 seedlings after seven days of treatment with PGA, dPGA, water soaking (WS) and control (CTRL) under mild drought (MD) or field capacity (FC) water levels. Statistical significance, calculated with Tukey test, is indicated by different letters between the different treatments.

Variable		MGT	Z	VII	GI
FC	CTRL	4.67 ± 2.33	0.333 ± 0.333	138 ± 93.2	1.67 ± 0.333
	HP	4.33 ± 2.19	0 ± 0	259 ± 183	1.33 ± 0.882
	PGA	4.6 ± 2.3	0.2 ± 0.2	392 ± 310	3 ± 2
	dPGA	2.33 ± 2.33	0 ± 0	30.6 ± 30.6	1.33 ± 0.333
MD	CTRL	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	HP	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	PGA	4.33 ± 2.19	0.333 ± 0.333	165 ± 151	2.33 ± 1.86
	dPGA	0 ± 0	0 ± 0	0 ± 0	0 ± 0
P-value	Stress	0.036	0.701	0.123	0.105
	Treatment	0.342	0.395	0.316	0.187
	SXT1	0.598	0.622	0.853	0.968

Table 3. Germination parameters MGT, Z, VII and GI for the different priming treatments and stress conditions in Rc460 rice. Statistical significance, calculated with Tukey test, is indicated by different letters between the different treatments.

Changes in gene expression profiles in treated and untreated rice varieties

After seven days of growth in the growth chamber, the samples were collected to perform a gene expression analysis study using qRT-PCR. The tested genes were *OsNRMP1*, *OsCu/ZnSOD* and *OsC3H50*. These genes were selected based on specific processes in which they are involved, namely metal transport (*OsNRMP1*), ROS homeostasis (*OsCu/ZnSOD*), and drought resistance (*OsC3H50*). The results of the qRT-PCR are represented with heatmaps in **Figure 11** and **Figure 12**.

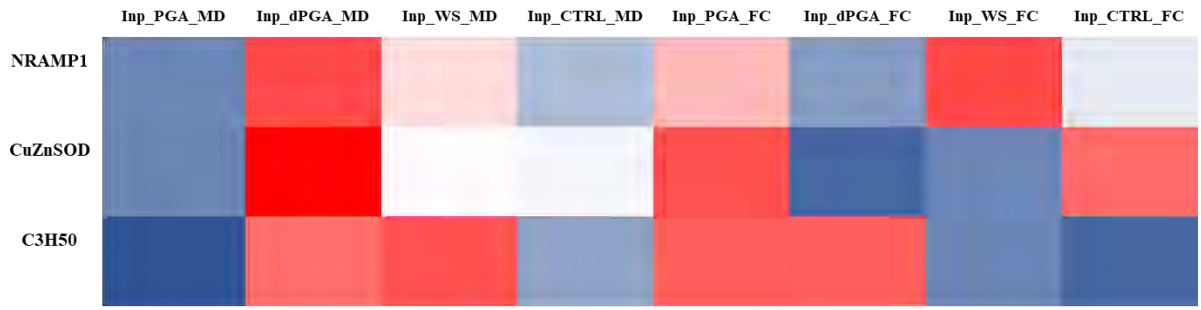

Figure 7. Gene expression heatmap relative to the Inpari rice variety.

In Inpari, the samples that appear to have the highest expression of the genes of interest are the dPGA under drought and PGA under field capacity conditions. In these samples, all three genes are expressed more than in the others. There are other instances, where only two or even one of the genes is overexpressed. These are the water-soaked seeds in mild drought and the dPGA, water-soaked and untreated seeds in field capacity.

Specifically, the *OsNRAMP1* gene is highly expressed under drought conditions in response to the dPGA treatment, and under field capacity in response to water-soaking, while its expression was limited in PGA-treated seeds under drought and dPGA under field capacity. The *OsCu/ZnSOD* gene was highly expressed in dPGA-treated seeds growing under mild drought, PGA-treated and untreated seeds in field capacity, and was weakly expressed in PGA-treated seeds in mild drought and dPGA-treated and untreated seeds in field capacity. The *OsC3H50* gene showed higher expression levels in dPGA-treated and water-soaked seeds growing under mild drought, and PGA and dPGA-treated seeds under field capacity. It showed lower expression levels in PGA-treated and untreated seeds in mild drought and water-soaked and untreated seeds in field capacity.

Figure 8. Gene expression heatmap relative to the Rc460 rice variety.

In Rc460, the samples with the highest expression are PGA treated seeds that grew under mild drought, and the untreated seeds that grew under field capacity. In the rest, the expression remained in relatively low levels. There is also a noticeable dip in expression for the OsCuZnSOD gene in the seeds that grew untreated under mild drought.

Specifically, the *OsNRAMP1* gene is highly expressed under drought conditions in response to the PGA treatment, and under field capacity in the dPGA-treated and untreated seeds, while its expression was limited in the rest of the samples. The *OsCu/ZnSOD* gene was highly expressed in PGA and dPGA-treated seeds growing under mild drought, dPGA-treated and untreated seeds in field capacity, and was weakly expressed in the rest of the samples, especially in the untreated seeds in mild drought. The *OsC3H50* gene showed higher expression levels in PGA-treated seeds growing under mild drought, and in the untreated seeds that grew under field capacity. It showed lower expression levels in all the other samples.

Integrative data analysis

To better elucidate the association of the phenotyping and the gene expression data, an integrative data analysis approach has been performed. This resulted in the generation of clustering graphs with both hierarchical and partitional methods.

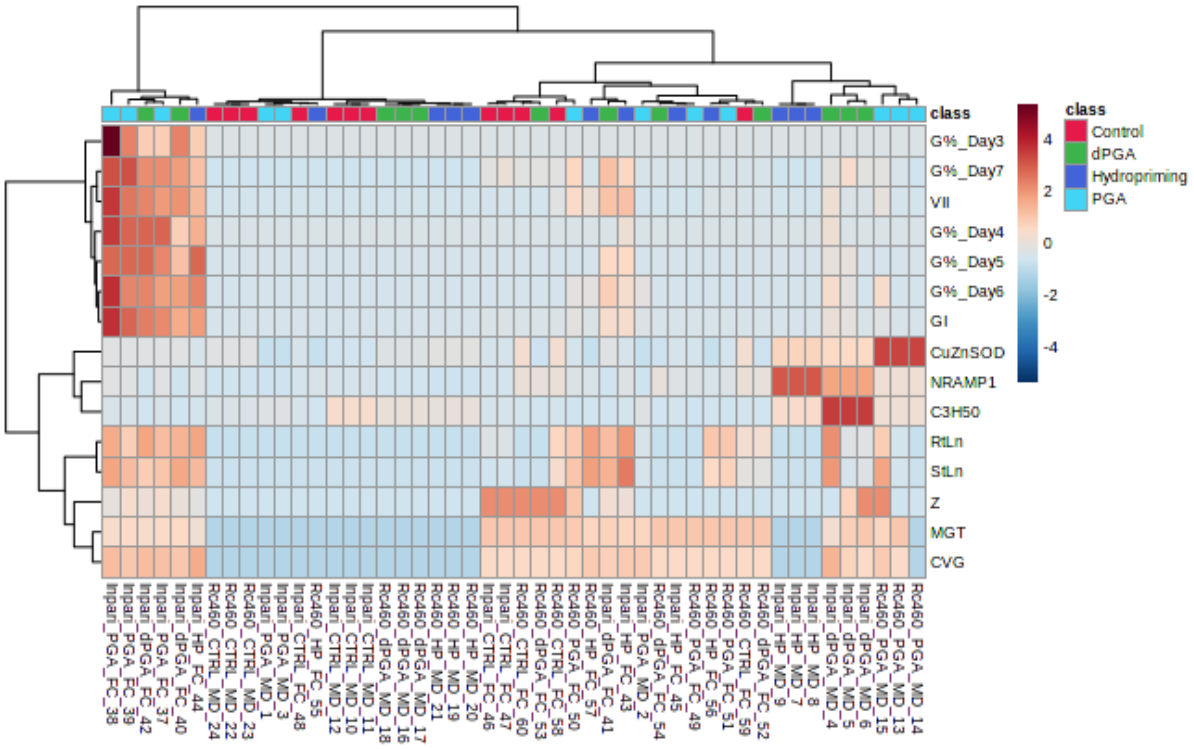

Figure 9. Hierarchical clustering performed using the phenotyping and gene expression data for all rice varieties and treatments.

In **Figure 13**, the hierarchical clustering analysis has divided the data into groups that correspond to samples with similar germination, seedling growth and gene expression patterns. Starting from the left side of the figure, the presence of Inpari seedlings treated mainly with PGA, dPGA and water soaking, grown under field capacity can be noticed with red colour. There is a significant gap that corresponds to the gene expression data for these samples, which appears to be quite low. Following these samples, is an area of relatively low clustering. It is comprised by Inpari seeds untreated of treated with PGA, and Rc460 samples that were treated dPGA, water soaking (WS) or untreated, all grown under mild drought conditions. The next group consists of the rest of samples grown under field capacity, and the last one is composed of the samples that showed the highest gene expression, compared to the rest. These are PGA-treated Rc460 and dPGA-treated and WS-treated Inpari, all grown under mild drought.

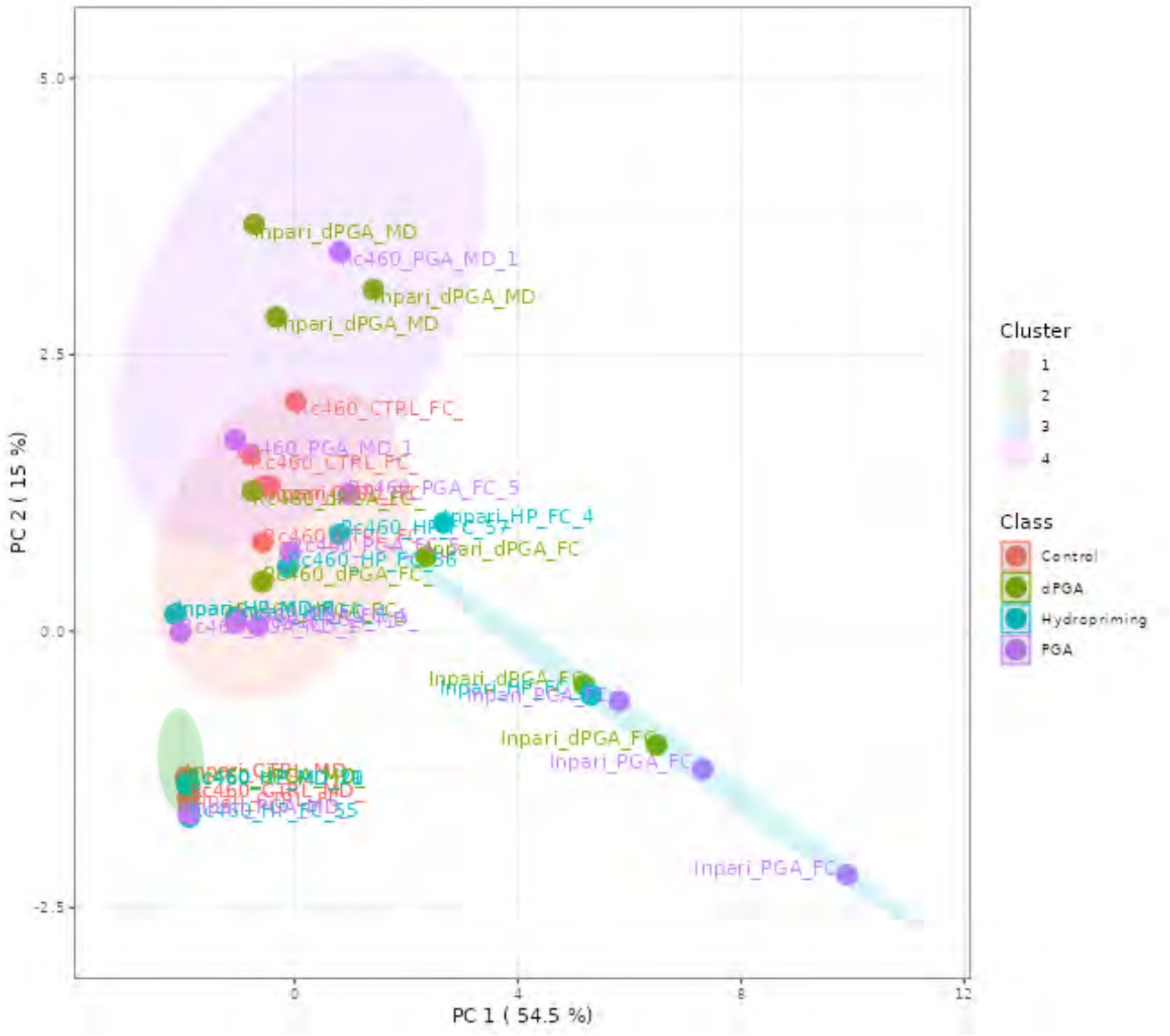

Figure 10. Principal Component Analysis (PCA) clustering obtained using the phenotyping and gene expression data for all rice seed varieties and treatments.

Figure 14 draws the PCA clustering graph representation which better evidenced the effects of the imposed treatments on the two rice varieties. The four clusters that can be identified have emerged from: (1) a variety of samples with treatments that showed at least some effect on germination or gene expression, (2) samples that had close to zero effect, all grown under mild drought, (3) Inpari seeds treated with PGA or dPGA grown under field capacity, and (4) the dPGA-primed Inpari seeds with the PGA-primed Rc460 grown under mild drought. The latter appears to be the only categories of samples that show the beneficial effect of priming on mitigating the consequences of drought.

Discussion and Conclusion

The need for environmentally friendly agricultural approaches that will help farmers grow crops in a sustainable way, is more urgent than ever. Practices that focus solely on the increase of production without taking into consideration other problems, will have to be overcome. In the case of rice, sensitivity to drought and lack of nutrients (hidden hunger) are emerging as big crises in developing countries. The solutions proposed to solve these challenges have to be sustainable both for environment and economy. Seed priming can be a good promise to this end, as already established in the scientific literature (Subhabrata et al., 2021).

In this work of thesis, the experiments carried out looked into the role of γ-PGA as a priming agent to alleviate the stress caused by drought during seed germination, using two different varieties of zinc-biofortified rice. The results of this study indicate that priming treatments hav improved germination only under field capacity conditions, whereas the imposed drought stress levels appeared to be too detrimental for rice germination. Moreover, these effects were mainly encountered in the Inpari variety, while the Rc460 appears to be even more sensitive to drought stress as germination was low even under field capacity conditions.

The measured germination parameters of germination %, germination index, germination synchronicity, mean germination time, germination velocity, drought tolerance index and the lengths of the germinants showed limited increase when the seeds germinated under mild drought conditions. For Inpari, the seeds that grew under field capacity appeared responsive to the treatments, but the most effective one, PGA, still did not improve G% by more than 45%, with the rest of the parameters following a similar trend. During mild drought, it was dPGA that appeared to have an effect on germination parameters. These samples were also the most active regarding gene expression.

All three genes were differentially expressed during germination, especially *OsCu/ZnSOD* that has an important role in ROS homeostasis (Pan et al., 2001). PGA-treated, field capacity grown Inpari seeds also showed relatively high gene expression compared to the rest Inpari samples. For Rc460, the samples that had the highest expression of the three genes were those obtained from PGA-treated mild drought-grown seeds and untreated field capacity grown seeds.

There are some key similarities and differences between the behaviour of Inpari and Rc460 that can be noted. Both varieties seem susceptible to drought, as only a small number of seeds germinated in mild drought conditions and in both cases, it was the water-soaked seeds that showed the most plant growth (root and shoot length). Considering the different treatments, dPGA had more effect on Inpari, while PGA was more effective for Rc460. The difference

between these molecules is that PGA represents the chain of glutamic acid residues, while dPGA consists of single molecules of glutamic acid. These have different properties, where PGA acts as a polymer that can increase the nutrient bioavailability and uptake for plants by acting as a fertilizer, source of N and acidic agent that reduces soil pH (Zhang et al., 2017). On the other hand, dPGA may act more as a nutrient itself or being perceived as a possible signalling molecule. This was previously evidenced in studies where GABA, a known enhancer of plant stress tolerance (Kumari and Vaneet, 2020), was used.

The different genetic backgrounds of these varieties could explain the variability in the responsiveness to the priming treatments. Other differences are evidenced is the gene expression patterns. In Inpari, there are generally more samples where the gene expression is induced compared to Rc460, that may be attributed to the differences in sensitivity to drought evidenced between the two varieties. As Rc460 appears to me more sensitive, the genes seem to be more downregulated in response to stress and the priming treatments could not overcome this obstacle.

To conclude, this study evidenced positive effects of dPGA priming on Inpari rice seed germination while PGA priming was more effective on the Rc460 variety. This was evidenced both on the phenotype and transcriptional level, mostly under field capacity. Future studies should include additional controls where seed germination happens under optimal conditions, under flooded soil, since rice is defined as a semiaquatic plant. Moreover, different levels of drought can be proposed where the amount of water in the soil is better compatible with seed germination. Optimization of the priming protocols, in terms of concentration and treatment time, should be also considered. Further examination of these effects should be carried out throughout the life cycle of the plant under field conditions to be able to also quantify rice productivity.

PGA-seed priming for biofortified seed varieties could fill the necessity for low-cost, environmentally friendly materials that will aid the agricultural industry in achieving future goals of providing high-nutrient foods all around the globe.

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