UNIVERSITÀ
DI PAVIA

Dipartimento di Biologia e Biotecnologie
"Lazzaro Spallanzani"

Laurea Magistralis in Molecular Biology and Genetics

Seed priming with plant extracts as means to enhance drought tolerance in maize

Supervisor:
Prof. Anca Macovei

Co-supervisor:
Hisham Wazeer

Experimental thesis by
Ahmad Zeidan

Academic Year 2024/202

TITLE PAGE

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Trattamenti con estratti vegetali per migliorare la tolleranza alla siccità nei semi di mais

Summary

Climate change poses a major threat to global agriculture, particularly through drought stress, which significantly impairs plant growth and crop productivity. During the different developmental stages, germination and grain filling are the most susceptible to water stress. While the effects of drought during the reproductive phase are more investigated, the germination process is much overlooked even if highly sensitive to drought.

In this context, this work of thesis aimed at investigating the effects of drought stress, imposed during germination, on a panel of local maize (*Zea mays*) varieties, along with the development of sustainable seed priming treatments using plant-derived extracts, or biostimulants (PBs), to enhance drought tolerance.

A soil-based drought simulation system was used to mimic realistic field conditions. Three priming treatments were tested: hydropriming (HP), priming with red chicory (RC), and cauliflower (CF) extracts, alongside an untreated control (UP, unprimed). Morpho-physiological parameters such as germination percentage, shoot and root length, and the Germination Stress Tolerance Index (GSTI) were measured. The FOX-1 biochemical assay was used to quantify the levels hydrogen peroxide (H_2O_2) released from seeds, while gene expression profiling was conducted via qRT-PCR to assess the impact on antioxidant and drought-responsive genes.

The obtained data allowed to distinguish between drought tolerant and susceptive varieties, along with selecting priming protocols able to mitigate the effects of drought stress. Results showed that three landraces - Nostrano di Storo, Marano, and Mais da Polenta Chatillon - exceeded the 20% GSTI threshold under drought, indicating some degree of tolerance. Seed priming with RC significantly improved GSTI and reduced the mean germination time in several genotypes, particularly in the drought-sensitive variety B73_Blu. RC treatments also reduced the H_2O_2 levels release from seed and modulated gene expression, suggesting for a possible enhanced protection against stress.

In conclusion, seed priming with plant extracts may represent a promising, sustainable strategy to improve drought tolerance in maize. The study supports the integration of plant-based biostimulants into sustainable agriculture practices, contributing to developing more climate-resilient crops.

Riassunto

Il cambiamento climatico rappresenta una grave minaccia per l'agricoltura globale, in particolare a causa dello stress da siccità, che compromette significativamente la crescita delle piante e la produttività delle colture. Durante le diverse fasi di sviluppo, la germinazione e la fase riproduttiva sono altamente sensibili allo stress idrico. Sebbene gli effetti della siccità durante la fase riproduttiva sono ampiamente studiati, il processo di germinazione è spesso trascurato, nonostante sia altamente sensibile alla siccità. In questo contesto, il presente lavoro di tesi si propone di indagare gli effetti dello stress da siccità, imposto durante la germinazione, su delle varietà locali di mais (*Zea mays*), insieme allo sviluppo di trattamenti sostenibili di priming dei semi utilizzando estratti vegetali o biostimolanti (PBs), al fine di aumentare la tolleranza alla siccità. Per riprodurre condizioni realistiche di campo, è stato utilizzato un sistema di simulazione della siccità nel suolo. Sono stati testati tre trattamenti di priming: idropriming (HP), priming con estratti di cicoria rossa (RC) e cavolfiore (CF), insieme a un controllo non trattato (UP). Sono stati misurati parametri morfo-fisiologici come la percentuale di germinazione, la lunghezza della radice e del germoglio, e l'indice di tolleranza allo stress durante la germinazione (GSTI). Il saggio biochimico FOX-1 è stato utilizzato per quantificare i livelli di perossido di idrogeno (H_2O_2) rilasciati dai semi, mentre l'analisi dell'espressione genica è stata condotta tramite qRT-PCR per valutare l'impatto dei trattamenti su determinati geni. I dati ottenuti hanno permesso di distinguere tra varietà tolleranti e suscettibili alla siccità, oltre a selezionare protocolli di priming in grado di mitigare gli effetti dello stress idrico. I risultati hanno mostrato che tre varietà locali – Nostrano di Storo, Marano e Mais da Polenta Chatillon – hanno superato la soglia del 20% di GSTI in condizioni di siccità, indicando un certo grado di tolleranza. Il trattamento con RC ha migliorato significativamente il GSTI e ridotto il tempo medio di germinazione in diversi genotipi, in particolare nella varietà sensibile B73_Blu. Il trattamento con RC ha inoltre ridotto i livelli di H_2O_2 rilasciati dai semi e modulato l'espressione genica, suggerendo una possibile protezione contro lo stress. In conclusione, il priming dei semi con estratti vegetali può rappresentare una strategia promettente e sostenibile per migliorare la tolleranza alla siccità nel mais.

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1. Introduction

1.1 Sustainable agriculture

Sustainable agriculture is a holistic approach to farming that aims to meet the food and fiber needs of the present without compromising the ability of future generations to meet their own needs. It integrates plant and animal production practices that enhance environmental quality, make efficient use of nonrenewable resources, and sustain the economic viability of farm operations (Muhie, 2022). The need for sustainable agriculture arises from the significant environmental and social challenges posed by intensive farming. Modern agriculture often leads to ecosystem degradation, biodiversity loss, and pollution. Sustainable agriculture seeks to mitigate these impacts by promoting practices that conserve natural resources and the environment (Ayeni et al., 2024). It ensures that farming remains profitable while reducing dependency on nonrenewable resources and minimizing costs associated with environmental damage. Additionally, sustainable agriculture supports the well-being of families and communities, providing durable employment and improving quality of life (Polcyn et al., 2023). Within the climate change challenge, sustainable agriculture is needed for producing sufficient food to meet human needs without depleting resources (Paz et al., 2020).

The concept of sustainable agriculture gained prominence following the Brundtland Report in 1987 (Aslam et al., 2022). Although its definition can be somewhat vague, the United States Department of Agriculture (2012) described it as involving livestock and crop production with environmental impacts. The core idea is to balance food needs with the protection of environmental resources. Sustainable agriculture (**Fig. 1**) aims to reduce the use of inorganic fertilizers, protect water resources, conserve biodiversity, and minimize waste production (Velten et al., 2015). Eco-friendly fertilizers have been shown to improve crop yields and soil conditions (Singh, et al., 2010). Additionally, financial support for farmers to adopt new techniques and improve their quality of life is a key objective of this approach (Campbell et al., 2014).

Figure 1. Advantages of sustainable agriculture (Aslam et al., 2022).

Agriculture has played a crucial part in the growth of the human community. Domesticated crop species, such as maize, have evolved through selective breeding for desired traits and high yields (Dias et al., 2015). The urgent need to develop fresh crop enhancement strategies has prompted more research on plants. One of the major challenges for agriculture is related to the effect of climate change on crop yields (Pulighe et al., 2024). Global food security is a critical concern, with the world population projected to reach 9.5 billion by 2050 and hunger impacting over 840 million people by 2030 (Miranda et al., 2024). Achieving the ZERO HUNGER target (SDG2) necessitates increasing agricultural productivity along with reducing waste production, thus applying sustainable and resilient farming practices. To meet this ambitious goal while mitigating environmental damage, alternative approaches to intensive farming, which relies heavily on chemicals, are continuously sought. Despite the high yields, intensive farming severely affects the environment (Shankar et al., 2021). Agricultural yields should be increased and the gap between achievable and actual yields should be closed by developing innovative methods and technologies. For instance, ensuring global food security may depend on effective nitrogen management and appropriate fertilizer application. However, several global environmental and geopolitical problems, including nutrient imbalances, nutrient leaking into the environment, and growing fertilizer costs, have been brought on by the intensification of fertilization in recent decades to enhance yields (Penuelas et al., 2023).

Globally, the three most grown crops are maize (*Zea mays*), wheat (*Triticum aestivum*), and rice (*Oryza sativa*). These crops account for a significant portion of the world's total cereal production and are major sources of food and calories for many populations. Among these, maize is cultivated in diverse climates and regions, making it a versatile crop. Maize is a significant source of energy, providing essential nutrients such as starch, protein, and micronutrients, important for human health (Prasanthi et al., 2017). Additionally, it plays a relevant role in animal feed and biofuel production. Continuous advancements in maize genetics have facilitated widespread cultivation and enhanced value across the entire production chain, benefiting farmers, processors, and consumers alike (Palacios Rojas et al., 2020). Due to global population growth, maize is being intercropped with other crops to provide safe and sufficient output in limited space (Manasa et al., 2021). However, this species faces significant challenges, including drought, high temperatures, soil salinity, high concentrations of tropospheric ozone, and various pathogens (Singh et al., 2021). Sustainable agricultural practices, such as conservation tillage, efficient water management, and climate-smart techniques, are essential to mitigate these challenges and ensure the resilience of maize production (Regmi et al., 2024). These sustainable practices are crucial for maintaining maize production while addressing environmental and resource challenges.

1.2. Maize as a key crop for food, forage and biofuel industry

Maize is one of the most widely cultivated cereal crops and serves as a staple food for billions worldwide. It plays a crucial role in ensuring food security and sustainable agriculture. Originating from Mexico over 9,000 years ago, maize has become a cornerstone of modern agriculture due to its high adaptability, nutritional value, and versatility in food, feed, and industrial applications (Awika, 2011; Kennett et al., 2020). Maize cultivation (**Fig. 2**) spans both emerging economies and the developed world, including 165 countries distributed across the Americas, Asia, Europe, and Africa (Erenstein et al., 2022)**.**

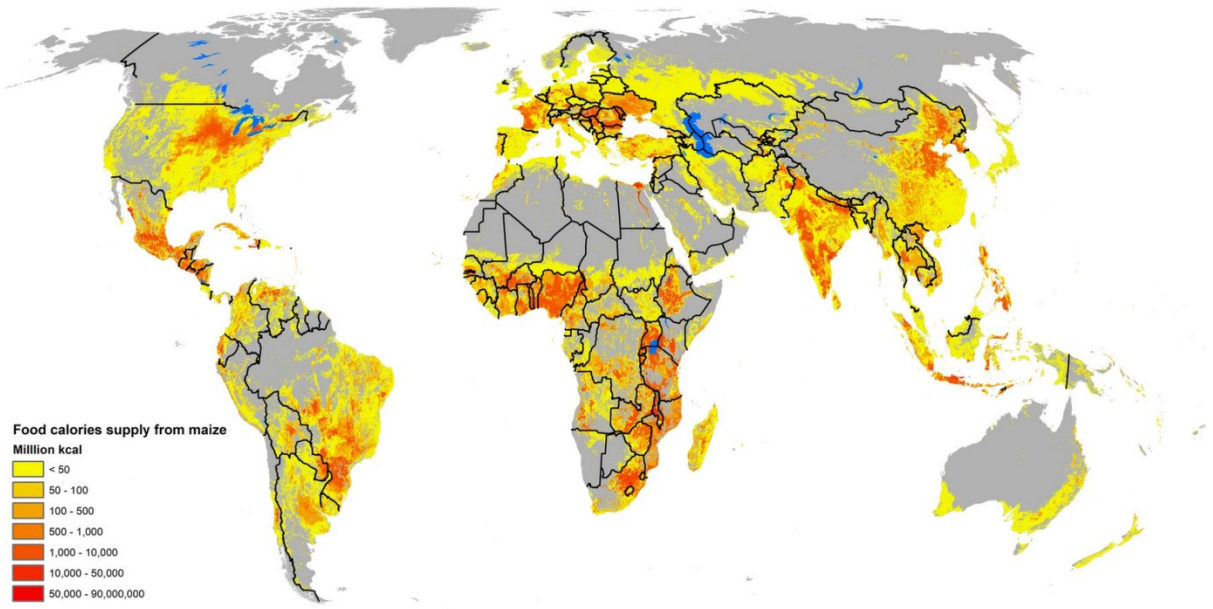

Figure 2. Geographical representation of maize cultivation and its production estimated in mega-calories (M kcal) per pixel, cca 10 × 10 km^2 (adapted from Erenstein et al., 2022).

To meet the growing demand for food in the face of the challenges raised by climate change (rising temperatures, erratic rainfall patterns, soil degradation) and population dynamics, agricultural productivity needs to increase and adapt in a sustainable manner (Benďáková, et al.,2024). Sustainable agricultural practices, e.g., precision farming, conservation tillage, and cultivation of stress-tolerant varieties, including maize, are essential to achieving food security while reducing environmental impact (Prasanna et al., 2021).

Despite significant advances in maize breeding and agronomic practices, challenges such as soil nutrient depletion, excessive chemical inputs, and land degradation, still persist. Addressing these challenges requires an integrated approach, including improved nitrogen-use efficiency, drought-resistant hybrids, and precision agriculture technologies (Riache et al., 2022).

Maize is an important food source, particularly in Sub-Saharan Africa and Latin America, where it provides a significant portion of daily caloric intake. Rich in carbohydrates, maize serves as an energy-dense staple, but its protein content is relatively low compared to legumes and animal-based proteins. However, biofortification efforts, such as the Quality Protein Maize (QPM) program, have improved maize nutritional value by increasing the content in essential amino acids like lysine and tryptophan (Gunaratna et al., 2019).

In addition to direct human consumption, maize is a major component of livestock feed. Nearly 60% of global maize production is used for animal feed, supporting the poultry, dairy, and meat industries (Grote et al., 2021). The increasing demand for maize in feed production highlights the need for sustainable practices that optimize yield without compromising soil and water resources (Khalfi et al., 2024)

Maize is also an important industrial crop, serving as a raw material for bioethanol production, starch, and other processed food products. The global push for renewable energy has increased interest in maize-based biofuels, although concerns about food production and environmental sustainability persist (Xavier et al., 2010). In 2023, approximately 40% of the maize produced in the United States was used for ethanol production (Skoufogianni et al., 2020). The transformation process involves milling the maize grains into flour, breaking down the starch into shorter carbohydrate chains and fermentable sugars, fermentation to produce ethanol, distillation to purify the ethanol, and dehydration to achieve fuel-grade ethanol (Schwietzke et al., 2009)

1.2.1 Maize production in Italy

Maize has played a central role in Italian agriculture since its introduction in the 16th century, following its arrival from the Americas (Brandolini & Brandolini, 2009). Today, Italy is among the leading maize producers in Europe, with cultivation primarily concentrated in the northern regions - Lombardy, Veneto, Friuli Venezia Giulia, and Emilia-Romagna. These regions benefit from alluvial soils, temperate climate conditions, and a well-developed irrigation infrastructure, making them ideal for intensive maize farming.

According to FAOSTAT, in 2023 Italy cultivated maize on approximately 598,400 hectares, yielding about 6.5 million tonnes of grain. Additionally, around 2.2 million tonnes of maize silage were produced for livestock feed. The average national yield for grain maize reached 10.9 tonnes per hectare, placing Italy among the top European performers.

The majority of Italy's maize production is used for animal feed, especially for high-quality dairy production. Silage maize is a crucial component of the diet for dairy cattle involved in the production of Parmigiano Reggiano and Grana Padano, two iconic Protected Designation of

Origin (PDO) cheeses. High forage quality is critical to maintaining the nutritional standards required by these traditional products (Capstaff & Miller, 2018). Apart from animal husbandry, maize is also important in Italian culinary tradition. Polenta, a staple in northern Italian cuisine, is made from local flint maize varieties, many of which have deep historical and cultural roots (Pedrotti et al., 2024).

Despite its strengths, Italian maize agriculture faces mounting challenges, including climate variability, water scarcity, and increasingly strict EU pesticide regulations. As a response, Italian farmers and researchers have been transitioning toward drought-resistant hybrids, precision irrigation, and integrated pest management (Pulighe et al., 2024). At the policy level, there is a strong push toward sustainable agriculture, including crop rotation, conservation tillage, and organic maize farming, aiming to improve soil health and enhance biodiversity (Salinger et al., 2022).

Italy also plays a pivotal role in maize genetic research. Recent studies have emphasized genome-wide association mapping, genomic selection, and phenotyping technologies to develop stress-tolerant and high-yielding varieties (Consonni et al., 2022; Pulighe et al., 2024). Italian researchers are increasingly incorporating antique and landrace varieties into modern breeding programs. These include traditional flint maize landraces like Nostrano dell'Isola, Scagliolo, and Marano, which are not only adapted to local environments but also offer valuable traits such as resilience to drought and disease, and distinct nutritional or processing qualities (Brandolini & Brandolini, 2009; Pedrotti et al., 2024).

Preserving maize biodiversity is important, not just for resilience against climate change but also for sustaining the agro-cultural heritage of Italy. There are over 100 identified local maize varieties still conserved through in situ and ex situ strategies, many of which are being re-evaluated for their potential use in modern genetic improvement programs (Pedrotti et al., 2024; Consonni et al., 2022).

1.3. Breeding advances: from traditional selection to genomics

Maize has undergone extensive genetic improvement, from traditional selective breeding to modern molecular techniques. Studies regarding hybrid vigor in the early 20th century have

revolutionized maize production, leading to the development of high-yielding hybrid varieties (Duvick, 2021). Advances in molecular breeding and genomics have further enhanced maize resilience to biotic and abiotic stresses (Zhang et al., 2022). The evolution of maize breeding over time is summarized in **Fig. 3.**

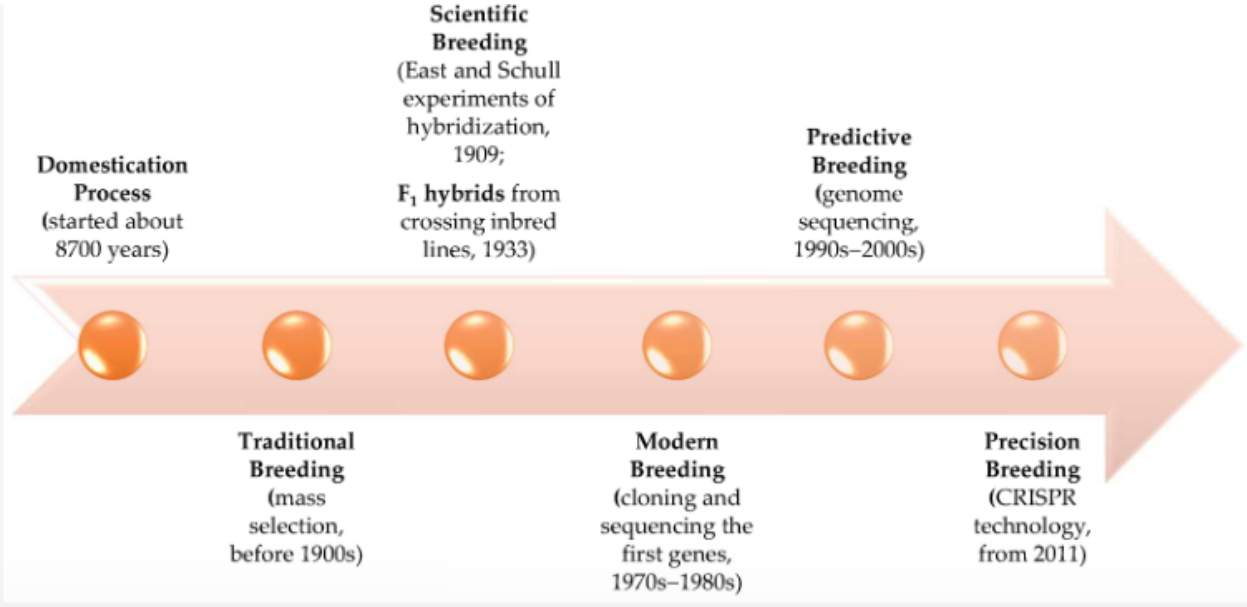

Figure 3. Timeline of maize domestication and breeding evolution (adapted from Muntean et al.,2022).

Traditional selective breeding involved choosing plants with desirable traits and crossbreeding them over multiple generations. This method relied heavily on phenotypic selection, where visible characteristics were used to guide breeding decisions. The discovery of hybrid vigor marked a significant milestone, as it demonstrated that crossing two inbred lines have produced offspring with superior qualities, such as higher yield and greater resilience (Duvick, 2021).

One of the central aspects of these advanced breeding programs is the integration of Genome-Wide Association Studies (GWAS) with high-throughput phenotyping. For instance, Maldonado et al. (2021) and Zhou et al. (2023) emphasize how GWAS has identified candidate genes involved in root architecture, chlorophyll content, pollen viability, and early seedling vigor—all critical for performance under heat and drought stress. In a notable example, the *ZmNAC111* (NAM, ATAF1/2, and CUC2) transcription factor has been linked to enhanced drought tolerance

by modulating root development and water retention capacity (Mao et al., 2015). Another gene, *ZmVPP1* (vacuolar H+-translocating pyrophosphatase), was associated with osmotic adjustment under water-limited conditions and has become a target in pre-breeding pipelines for climate resilience (Wang et al., 2016). Advances in molecular breeding and genomics have further enhanced maize resilience to biotic (pests and diseases) and abiotic (drought, heat) stresses (Zhang et al., 2022). The sequencing of the maize genome in 2009 was a pivotal moment, providing a comprehensive map of maize's genetic makeup (Schnable et al., 2009). This achievement paved the way for precision breeding and genetic engineering, allowing scientists to identify and manipulate specific genes associated with desirable traits (Jiao et al., 2017).

New breeding techniques, such as CRISPR-Cas9 genome editing, have enabled targeted modifications to improve yield, disease resistance, and drought tolerance (Erdoğan et al., 2022). CRISPR-Cas9 allows precise edits to the DNA sequence, making it possible to introduce or remove specific genes with high accuracy. This technology has accelerated the development of improved maize varieties, addressing the challenges posed by climate change and increasing global food demand (Chilcoat et al., 2017). For instance, the targeted editing of the *ARGOS8* gene promoter, which negatively regulates ethylene response, led to maize lines that produced an average of 5 more bushels per acre under drought stress without yield penalty under normal conditions (Shi et al., 2017).

Climate change poses significant risks to maize production, particularly in tropical and subtropical regions. Breeding efforts have focused on developing drought-tolerant maize (DTM) varieties, which have shown promising results in water-scarce environments. These stress-tolerant varieties, developed through both conventional breeding and genetic modification, have improved yields under challenging conditions (Sheoranet al., 2022). A prominent example of a commercialized genetically modified DTM is DroughtGard® (event MON 87460), which expresses the *CspB* (cold shock protein B) gene from *Bacillus subtilis*. Studies across Kenya, Uganda, and South Africa have demonstrated that MON 87460-traited hybrids significantly out yielded non-modified counterparts under high drought stress, by as much as 36-62%, without any significant yield penalty under optimal moisture conditions (Obunyali et al., 2024). This transgenic variety represents a critical advancement in climate-resilient agriculture, supporting maize yield stability in drought-prone regions.

The future of maize breeding lies in the integration of advanced genomic tools and data analytics. High-throughput phenotyping, which involves the rapid measurement of plant traits using sensors and imaging technologies, is expected to enhance the efficiency of breeding programs (Gill et al., 2022). Additionally, the use of machine learning algorithms to analyze genetic and phenotypic data will enable breeders to make more informed decisions, accelerating the development of superior maize varieties (Crossa et al., 2024).

The breeding efforts targeting drought tolerance converge with seed quality enhancement, as stress-resilient seeds have better germination performance under suboptimal field conditions. Advanced phenotyping, transcriptomics, and marker-assisted selection are now being deployed to develop seed lots that not only resist stress post-germination but also maintain viability during storage, critical in a changing climate and in marginal agricultural areas.

The integration of seed quality traits into breeding pipelines ensures better crop stand, lower reseeding costs, and improved yield stability, which are all vital for food security in drought-prone regions (Araus et al., 2018). These innovations align with global goals to develop climate-resilient crops and guarantee food availability for a growing population under increasingly erratic environmental conditions.

1.4. Seed germination: relevance for crop production

Seed germination is a pivotal event in the plant life cycle, significantly influencing crop productivity and the entire food chain (Nonogaki, 2017). Understanding this process is important for both agricultural and commercial industries (Corbineau, 2022). As in the case of all agronomic crops, also for maize, germination can be directly linked to early crop establishment as well as final yield (Murungu et al., 2004).

Maize seeds exhibit distinct morphological features (**Fig. 4)**, comprising two primary compartments: the embryo and the endosperm, both products of double fertilization (Silvana et al., 2007). The embryo includes a well-differentiated embryonic axis surrounded by the scutellum, a single big cotyledon (Fontanet et al., 2008). The endosperm, constituting the bulk of the seed, contains reserve proteins, complex carbohydrates, and oils (Sabelli & Larkins, 2009). The seed coat, or pericarp, originates from maternal tissue (Olsen, 2004). The developmental

stages of maize seeds involve histo-differentiation, morphogenesis, maturation, dehydration, and quiescence (Olsen, 2004). Mutations affecting endosperm development can lead to significant delays in seed development processes (Sabelli & Larkins, 2009). Additionally, the elongation of primary roots and mesocotyls in maize seedlings follows antagonistic growth patterns below the soil surface and is influenced by genetic and environmental cues (Sáenz Rodríguez & Cassab, 2021).

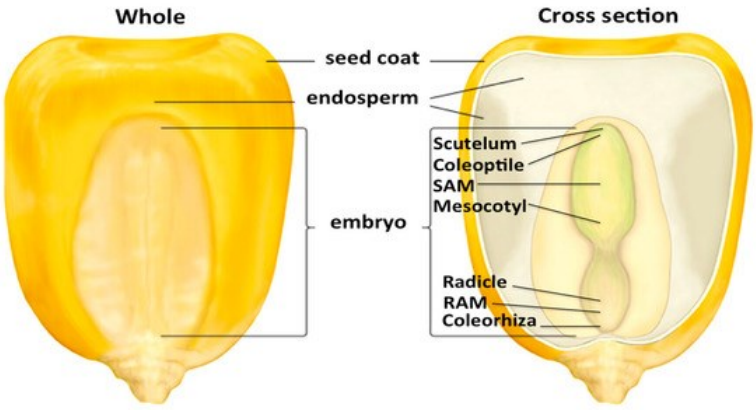

Figure 4. Maize seed (kernel) structure presented as whole seed and a cross section. The main parts of the seed are embryo, endosperm and seed coat (Sáenz Rodríguez & Cassab, 2021).

Seed germination is a complex process of physiological and biochemical changes that lead to the emergence of the radicle through the seed coat (Ma et al., 2019). Germination initiates with water absorption, which leads to the activation of cellular and metabolic processes (Gonçalves et al., 2024). This process can be divided into three stages (**Fig. 5**) **(**Szczerba et al., 2021)**:**

- **Phase I: Imbibition**. Characterized by rapid water uptake, gas release, and temperature changes, imbibition is primarily a physical process occurring in both viable and nonviable seeds (Macovei et al., 2016). This phase involves water absorption by the cell, leading to the activation of cellular metabolism. The rate of imbibition is temperature-dependent, where higher temperatures accelerate water absorption (Szczerba et al., 2021).
- **Phase II: Lag Phase**. This phase witnesses the activation of ATP synthesis through glycolysis, the Krebs cycle, and the respiratory chain, alongside the translation of stored mRNA (Finch-Savage & Leubner-Metzger, 2006). At the metabolic level, enzyme

activation is linked to the hydrolysis and mobilization of reserves for embryonic growth (Gonçalves et al., 2024).

- **Phase III: Embryo expansion**. This final phase, occurring after 48-72 hours in maize, is marked by the emergence of the radical, developing from the embryonic root, through crack in the seed coat (Gong et al., 2024).

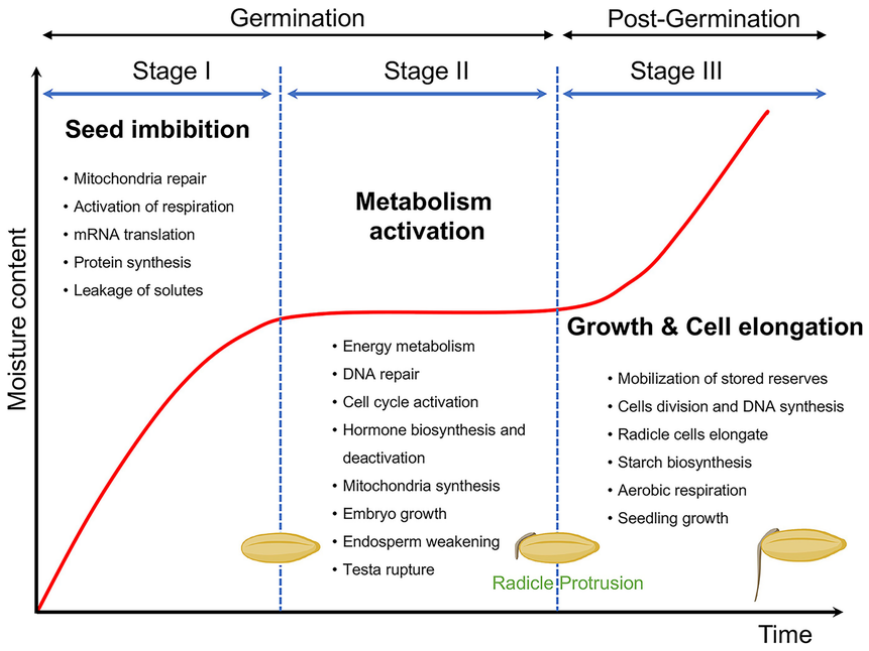

Figure 5. Seed hydration curve and germinating phases (Nogueira et al., 2023).

Considering maize seed morphology and germination physiology, enzymes such as amylases, proteases, and lipases play an important role in mobilizing stored reserves, enabling cell expansion and differentiation (Nonogaki, 2014).

Several factors influence maize seed germination, including environmental conditions and seed quality. Water availability is essential for metabolic activation, while temperature plays a significant role in determining germination speed, with an optimal range of 20-30°C (Basra et al., 2005). Oxygen is necessary for aerobic respiration, process that provides the energy required for cell division and elongation, whereas hypoxic conditions can delay germination. Unlike some plant species, maize seeds are non-photoblastic, meaning they do not require light for

germination. Other factors, like viability, dormancy or seed coat integrity, also impact germination success. Seed viability declines with aging and improper storage, while dormancy, regulated by the balance between gibberellins (GA) and abscisic acid (ABA), can prevent immediate germination even under favorable conditions (Reed et al., 2022). Damaged seed coats may lead to increased pathogen susceptibility and impaired water uptake, reducing germination efficiency.

1.4.1. The hormonal regulation of germination

Upon imbibition, the seed starts germinating, process during which the storage reserves are broken down to produce energy and nutrients needed for the growth of the embryo. GA and ABA are the main hormones that regulate this process having opposite functions. While GA promotes germination, ABA inhibits it by favoring seed dormancy. GA promotes germination by stimulating the production of α-amylase to hydrolyze the endosperm starch into glucose. The produced glucose is then used to provide energy for the elongation of the germ cells. The energy derived from glucose, through processes like oxidative phosphorylation involving dehydrogenases, fuels the growth of the cotyledons and radicles. During that process, ABA acts as an antagonist to GA (Ma et al., 2017).

ABA and ethylene are important players in the control of maize seed germination, as they are antagonistic to each other. Although ethylene is known as a growth inhibitor, it appears to be a growth stimulator during germination. In line with this, ethylene released in reaction to reactive oxygen species (ROS) governs the embryo hypocotyl-radicle axis expansion, permitting the germination of maize seeds (El-Maarouf et al., 2014).

Although GA does not directly contribute to the initial mobilization of the stored proteins and lipids, the number of genes responsible for its synthesis increases during germination while those of ABA synthesis decrease. This happens as follows: first, GA binds to its receptor, and the new complex GA-GID1 (GIBBERELLIN INSENSITIVE DWARF1) causes the degradation of the DELLA (name derived from the five conserved amino acids - aspartic acid, glutamic acid, leucine, leucine, and alanine - present in their N-terminal domain) proteins which prohibit GA signaling. Afterward, the SLY1 (F-box family protein SLEEPY1) protein recognizes the GA-GID1-DELLA complex and utilizes the 26S proteasome to deactivate the DELLA degradation

repressors. All this allows the transcription of the GA-responsive genes. However, mutations of the *SLY1* gene result in reduced germination and increased ABA sensitivity. Consequently, this shows that GA, whose production is enhanced by a properly functioning *SLY1* gene, is necessary for the complete germination of maize (Shai et al., 2010).

1.4.2 Nutrient mobilization and seedling growth

During maize seed germination, the quantity of essential nutrients such as isoflavones, saponins, and vitamin C increases, while antinutrients like lipoxygenase and phytic acid decrease (Liu et al.,2023). Lipoxygenase (LOX), which constitutes about 2% of maize seed protein, plays a key role in lipid remobilization by catalyzing the oxidation of polyunsaturated fatty acids in linoleic and linolenic acids (Singh et al., 2022). While LOX activity supports membrane remodeling and energy production, its involvement in the formation of volatile compounds such as 2-pentyl furan can negatively affect the flavor of maize, necessitating careful control during the early stages of germination. Simultaneously, carbohydrates such as oligosaccharides are metabolized, regulated by enzymes like galactoside synthase and raffinose synthase, leading to a decrease in their overall content.

Phosphorus remobilization is also important, as seeds contain phytate reserves that account for up to 90% of their phosphorus (Nadeem et al., 2022). During germination, phytase and phosphatase enzymes hydrolyze phytic acid, releasing phosphorus for ATP synthesis, essential for many metabolic processes. Furthermore, studies indicate that protein remobilization during germination significantly reduces trypsin inhibition by up to 32%, as endogenous hydrolases and C1 protease are synthesized and secreted to facilitate the breakdown of stored proteins (Müntz et al., 2001).

Beyond nutrient mobilization, seedling growth involves a sequence of tightly regulated developmental phases. During germination, the radicle emerges and establishes the root system, followed by the elongation of the coleoptile and the shoot apical meristem. Gene networks involving growth-regulating factors (GRFs), hormonal signaling pathways, and environmental cues coordinate these early events to ensure successful autotrophic transition. Functional gene modules regulate root and shoot differentiation, allowing the seedling to shift from stored nutrient reliance to photosynthetic independence (Zhao et al., 2011).

The life cycle of maize progresses through seed formation, germination, vegetative growth, and reproductive development. The cycle begins with double fertilization, forming a diploid embryo and triploid endosperm. The seed, once dormant, initiates germination under favorable conditions, and the diploid sporophyte develops into a mature plant. Meiosis in the tassels and ears produces haploid gametes, and pollination completes the cycle by generating new seeds (Candela & Hake, 2008).

Maize development is also divided into well-characterized vegetative (V) and reproductive (R) stages, as shown **(Fig. 6)**. The vegetative stages range from VE (emergence) through V1–V10 (leaf collar development), culminating at VT (tasseling). These stages capture the plant's structural development and photosynthetic capacity. The reproductive stages, from R1 (silking) to R6 (physiological maturity), describe kernel formation and maturation, with critical stages such as R2 (blister), R3 (milk), and R5 (dent) marking specific grain development milestones. These stages are essential for monitoring plant health and optimizing field management practices (Zhao et al., 2011).

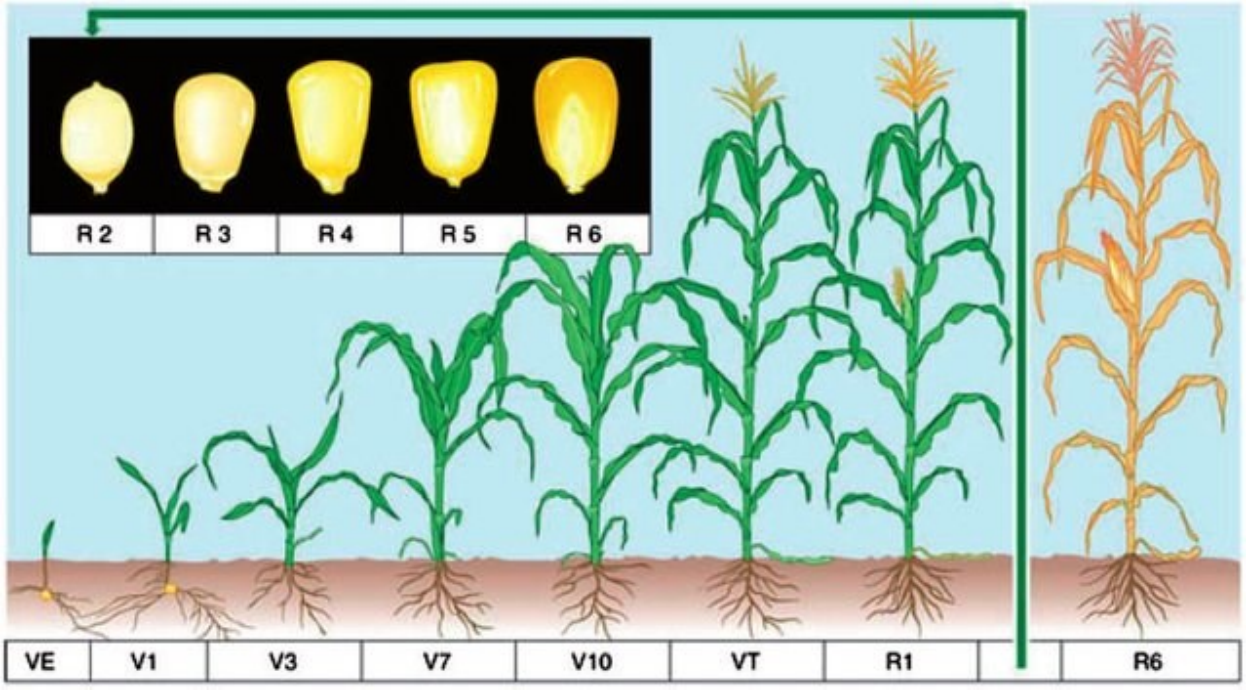

Figure 6. Different growth stages of a maize plant, including vegetative (V) and reproductive (R) stages (from Zhao et al., 2011).

Understanding the physiological and biochemical events of seed germination provides a foundational basis for improving seed performance. One such enhancement technique is seed priming, which aims to manipulate the germination process to improve uniformity, stress tolerance, and seedling vigor.

1.5. Seed priming

Seed priming is defined as a pre-sowing technique that improves germination and enhances crop resilience. By pre-treating seeds with water, nutrients, or other agents, seed priming accelerates germination and improves seedling vigor, enabling crops to better withstand abiotic stresses such as drought, salinity, and temperature extremes (Kumar et al., 2024). For instance, hydropriming and osmo-priming have been shown to significantly improve germination rates and seedling growth in plants under sub-optimal conditions (Pagano et al., 2023). This technique not only enhances the initial growth stages but also contributes to the overall health and productivity of the crop throughout its lifecycle.

Seeds are treated before sowing, allowing for a controlled hydration during the imbibition stage followed by seed drying, or the dry backstage. A comparison of the seed hydration phases and curves of primed and unprimed seeds reveals that primed seeds absorb more water and germinate faster than unprimed seeds (**Fig. 7**). This is due to the movement of water being facilitated by water channel proteins known as aquaporins, such as plasma membrane (PIP) and tonoplast (TIP) aquaporins (Kshetrimayum et al., 2017). Aquaporins are activated when seeds are treated with priming procedures based on the use of aqueous solutions. Phase I involves rapid physical water uptake, regardless of seed dormancy or viability. Phase II exhibits minimal water uptake while the seed transitions from dormancy to germination. In Phase III, significant water uptake occurs culminating in radicle protrusion and subsequent growth. Priming treatments have to be stopped before the end of phase II and start of phase III when the radicle emerges. This is because, while phases I and II are reversible, phase III is not. Once the radicle has emerged, seeds cannot be dried back as they lose their desiccation tolerance ability (Pagano et al., 2022). Upon re-imbibition, primed seeds rapidly transition to the growth phase, leading to enhanced germination potential, characterized by increased germination percentage, rate, and uniformity (Thakur et al., 2022).

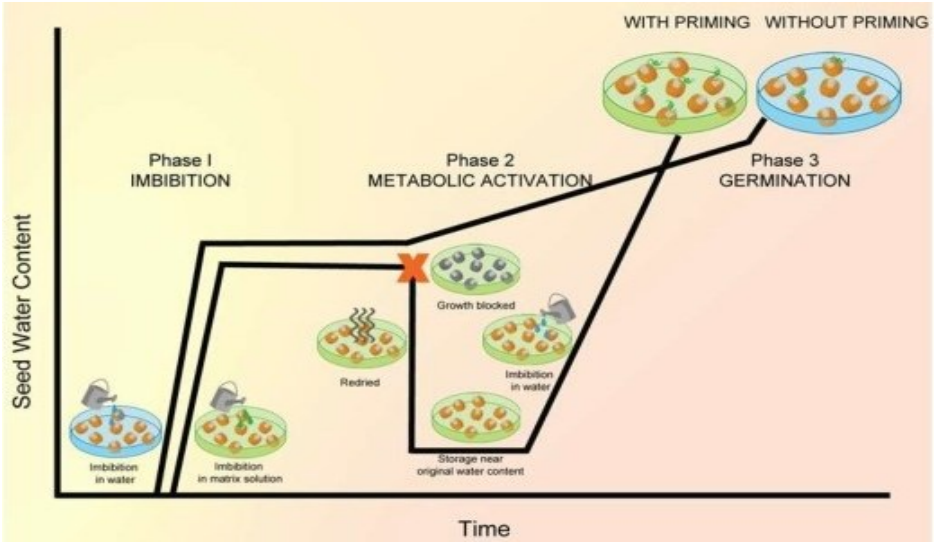

Figure 7. Model of priming treatments and germination stages in non-primed and primed maize seeds (adapted from Hacisalihogluet al., 2018).

In addition to improving resilience, seed priming can enhance nutrient uptake efficiency, thereby reducing the input of chemical fertilizers. Studies have demonstrated that primed seeds exhibit improved phosphorus use efficiency, leading to higher yields even with reduced fertilizer application (Ceritoğlu et al., 2024). This is particularly beneficial in regions with poor soil fertility or where the cost of fertilizers is prohibitive. By optimizing nutrient uptake, seed priming helps in maintaining soil health and reducing the environmental impact of excessive fertilizer use.

Moreover, seed priming offers a sustainable solution to modern agricultural challenges. Techniques like on-farm priming are cost-effective and accessible to farmers with limited resources, making advanced agricultural practices more widely adoptable (Sime & Aune, 2020). By reducing reliance on chemical inputs and enhancing resistance to soil-borne diseases, seed priming promotes sustainable farming practices. This dual benefit of increased resilience and reduced fertilizer dependency makes seed priming a valuable strategy for achieving food security and sustainability in agriculture.

1.5.1. Seed priming techniques

Seed priming can be conducted using various methods, categorized into biological, physical, and chemical approaches (**Fig. 8**).

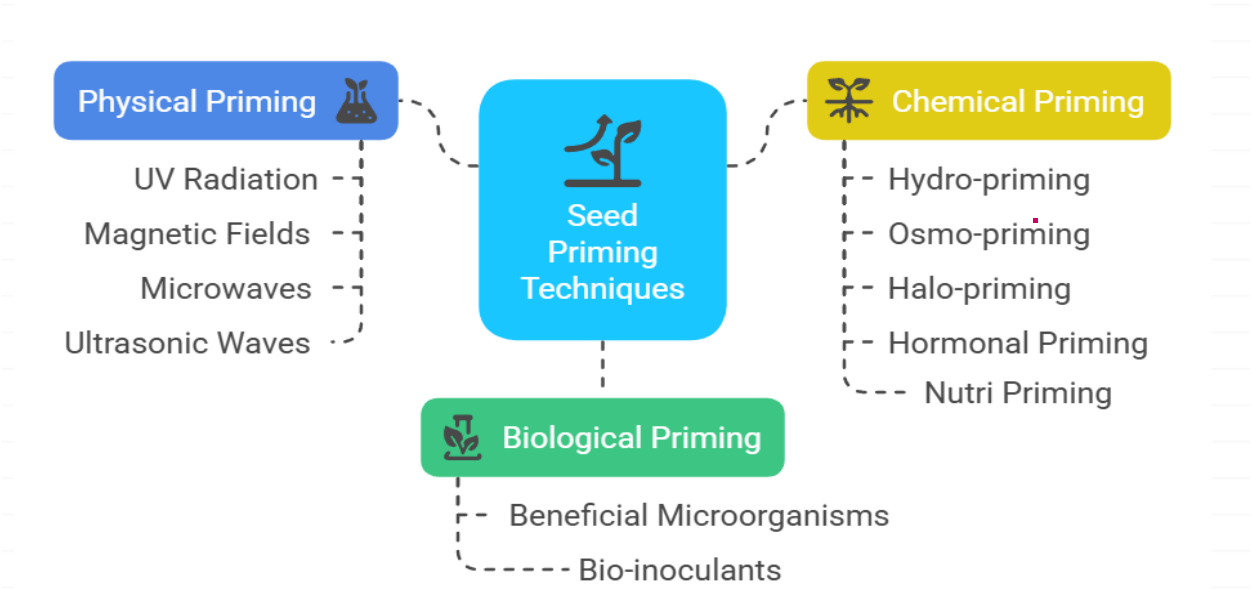

Figure 8. Classification of seed priming methodologies. The diagram was generated using the Napkin free online software (https://www.napkin.ai/).

In recent years, seed bio-priming has been recognized as a low-cost, eco-friendly technology that promotes growth, induces stress tolerance, and achieves desired crop yields. Bio-priming involves the application of beneficial microorganisms to seeds to stimulate plant growth while maintaining environmental balance. This technique has shown promising results on numerous plants, including wheat, maize, pepper, tomato, eggplant, and pulse crops (Vurukonda et al., 2016; Singh & Jha, 2017). Additionally, the use of bio-inoculants as priming agents accumulates stimuli in the rhizosphere, facilitates biochemical nutrient cycling, promotes enzymatic activity, and improves translocation, thereby enhancing crop performance under changing climatic conditions (Sarkar et al., 2021). Various bio-inoculants, such as beneficial microbes (e.g., bacteria, fungi), or biological products (e.g., humic acid, fulvic acid, chitosan), are used as bio-stimulating agents to promote plant growth and improve stress response (Ugena et al., 2018; Malik et al., 2021).

Physical seed priming techniques using non-ionizing agents such as UV radiation, magnetic fields, microwaves, and ultrasonic waves, can also offer eco-friendly and cost-effective alternatives to traditional chemical methods (Bera et al., 2022). These techniques enhance seed germination, seedling vigor, and crop resilience by stimulating various physiological and biochemical processes. For instance, UV radiation can increase antioxidant activity and stress tolerance, magnetic fields can improve nutrient uptake and enzymatic activity (Ouhibi et al., 2014), microwaves can enhance water absorption and metabolic processes (Maswada et al., 2021), and ultrasonic waves can facilitate seed hydration and oxygen uptake (Bera et al., 2022). These methods represent promising advancements in seed technology, contributing to sustainable agricultural practices and improved crop yield.

Chemical seed priming involves various techniques that enhance seed germination and seedling vigor by modulating physiological and biochemical processes. Hydro-priming, a simple and low-cost method, involves soaking seeds in water to initiate pre-germination metabolic activities (Pill & Necker, 2001). Osmo-priming uses osmotic solutions like polyethylene glycol (PEG) to control water uptake and improve germination under stress conditions (Leiet al., 2021). Halo-priming, which involves soaking seeds in salt solutions such as sodium chloride (NaCl), enhances salt tolerance and antioxidant enzyme activity (Serafy et al., 2021). Chemical priming with solutions like potassium nitrate (KNO_3) and hydrogen peroxide (H_2O_2) improves germination under adverse conditions. Hormonal priming is based on the use of main germination hormones, GA and ABA, to enhance seedling vigor by affecting cell division, elongation, differentiation, and dormancy (Rhaman et al.,2020). Nutri-priming with plant extracts, such as seaweed or moringa extracts, can also enhance seed germination and stress tolerance by providing essential nutrients and bioactive compounds (Ugena et al., 2018). These techniques contribute to sustainable agricultural practices and better crop yields

1.6. Plant extracts: valorizing agri-food waste

The valorization of agri-food waste through the extraction of plant biomolecules is a promising approach within the framework of the circular economy. This strategy not only addresses waste management issues but also provides valuable compounds for various industries, including nutraceuticals, pharmaceuticals, and biological agrochemicals.

The circular economy aims to develop a closed-loop system where waste is minimized, and resources are continuously reused and recycled (Pal et al., 2024). In the context of agri-food systems, this involves transforming waste products from food processing into valuable resources. By adopting circular economic principles, agri-food waste can be repurposed to extract important biomolecules, thus reducing environmental impact and promoting sustainability. This approach not only mitigates waste but also generates high-value products, contributing to economic growth and environmental sustainability.

For instance, the circular economy model emphasizes the reuse, recycling, and recovery of materials, which can significantly reduce the environmental footprint of agri-food systems (Pomoni et al., 2024). The implementation of circular economy practices in the agri-food sector can lead to the development of innovative solutions for waste management, such as converting food waste into bioenergy or compost, which can then be used to enhance soil fertility and crop productivity (Hamam et al., 2021). Additionally, the circular economy encourages the use of sustainable agricultural practices that minimize resource use and waste generation, thereby supporting the long-term viability of food production systems (Rabbi & Amin, 2024).

1.6.1. Extraction of biomolecules

Agri-food waste, such as fruit and vegetable peels, seeds, stalks, pods, and pulp, is a rich and underutilized source of bioactive compounds, including anthocyanins, betalains, carotenoids, polyphenols, flavonoids, and dietary fibers. These compounds exhibit a wide range of biological activities, such as antioxidants, anti-inflammatory, antimicrobial, and anticancer properties, making them highly valuable for food, pharmaceutical, and cosmetic industries. To recover these compounds efficiently while preserving their functional integrity, advanced extraction techniques have been developed **(Fig. 9)** (Choo & Saik, 2021).

Among these, supercritical fluid extraction utilizes supercritical CO_2 to selectively extract non-polar compounds with high efficiency and minimal solvent residues (Qu et al., 2020). Ultrasound-assisted extraction improves cell wall disruption and accelerates mass transfer, enhancing both extraction yield and energy efficiency (Murphy et al., 2020). Meanwhile, enzyme-assisted extraction employs hydrolytic enzymes to break down complex plant cell walls, allowing greater recovery of intracellular bioactive molecules (Gligor et al., 2019). These

techniques offer eco-friendly advantages by reducing solvent use, processing time, and environmental impact, aligning with the principles of green chemistry (Koel & Kaljurand, 2006).

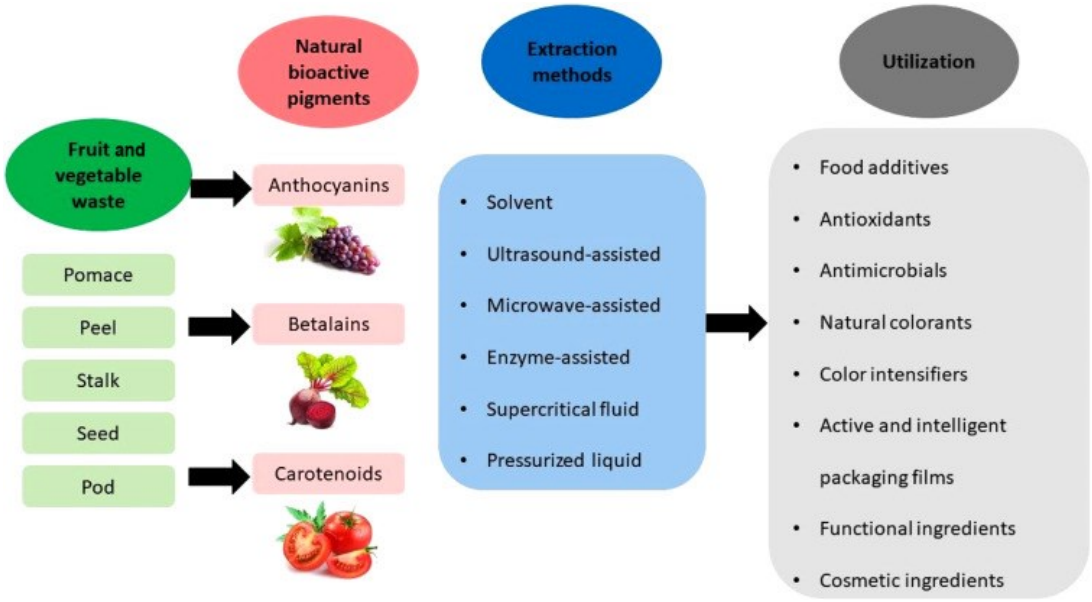

Figure 9. A schematic representation of fruit waste valorization (Choo & Saik, 2021).

By integrating such extraction technologies into circular bioeconomy models, researchers and industry stakeholders can transform agricultural waste into high-value, sustainable products, contributing to both environmental protection and economic development. Continued research and optimization of these processes are essential to enhance scalability, reduce costs, and promote widespread adoption of waste valorization technologies (Nirmal et al., 2003).

1.6.2. Reusing biomolecules from agri-food waste

The extracted biomolecules from agrifood waste can be reused bymultiple applications, including nutraceuticals, pharmaceuticals and biological agro-chemicals. Moreover, direct uses can include the production of animal feeds, as well as biomass for the bioenergy sector and the production of biofuels (Fernandes et al., 2024) (**Fig .10**).

Nutraceuticals. Bioactive compounds like polyphenols and flavonoids possess antioxidant, anti-inflammatory, and antimicrobial properties, making them valuable for functional foods and dietary supplements. These compounds can help in preventing chronic diseases and improving overall health. For example, polyphenols have been shown to reduce the risk of cardiovascular

diseases and certain types of cancer, while flavonoids can enhance immune function and protect against oxidative stress (Iqbal et al., 2023).The incorporation of these bioactive compounds into nutraceutical products can provide consumers with health benefits beyond basic nutrition, supporting the growing demand for functional foods and dietary supplements (Hamam et al., 2021).

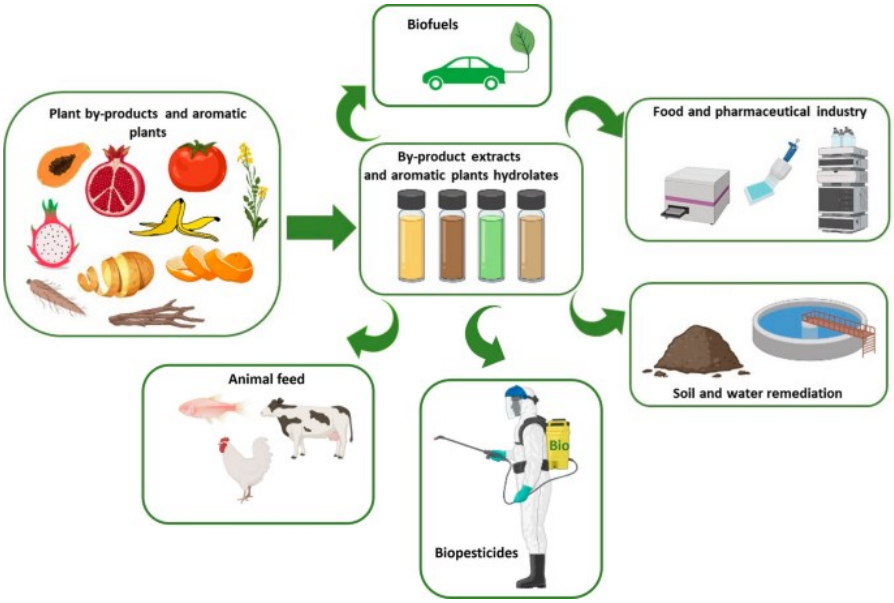

Figure 10. Valorization of plant by-products for food and pharmaceutical industries and environmental remediation applications (adapted from Fernandes et al., 2024).

Pharmaceuticals. Plant-derived biomolecules can be used in drug development for their therapeutic properties, such as anti-cancer, anti-diabetic, and cardiovascular benefits. This repurpose can lead to the development of new medications and treatments. For instance, certain plant-derived compounds have been found to exhibit potent anti-cancer activity by inducing apoptosis in cancer cells and inhibiting tumor growth (Rajabi et al., 2021). For example, paclitaxel (taxol), extracted from the bark of the Pacific yew tree (*Taxus brevifolia*), is a potent anti-cancer agent widely used in the treatment of breast, ovarian, and lung cancers. It works by stabilizing microtubules and preventing cancer cell division (Wani et al., 1971). Additionally, resveratrol, a polyphenolic compound from grape skins (*Vitis vinifera*), exhibits cardiovascular protective effects through its antioxidant and anti-inflammatory actions, helping reduce the risk of atherosclerosis (Baur & Sinclair, 2006). Furthermore, bioactive compounds from agri-food waste can be used to develop drugs that manage diabetes by regulating blood sugar levels and

improving insulin sensitivity (Tran et al., 2020) like berberine found in *Berberis* species, which has demonstrated strong anti-diabetic effects by activating AMPK pathways, thus improving insulin sensitivity and lowering blood glucose levels (Yin et al., 2008). The pharmaceutical industry can leverage these natural compounds to develop effective and safe medications, reducing the reliance on synthetic drugs and their associated side effects (Nasim et al., 2022).

Biological agrochemicals. Extracted biomolecules can be formulated into biopesticides and biofertilizers, promoting sustainable agriculture by reducing reliance on synthetic chemicals. These agrochemicals can enhance crop growth and protect against pests and diseases. For example, biopesticides derived from plant extracts can effectively control insect pests and fungal pathogens without harming beneficial organisms or the environment (Khursheed et al., 2022) like neem (*Azadirachta indica*) extracts, particularly azadirachtin, have been extensively studied for their biopesticidal activity against a wide range of insect pests, including aphids, whiteflies, and caterpillars (Adusei & Azupio, 2022). Similarly, garlic (*Allium sativum*) extracts have demonstrated antifungal properties; studies have shown that fermented garlic formulations effectively manage *Fusarium* wilt in sweet potato (Masangcay et al., 2023). Biofertilizers, on the other hand, can improve soil health and nutrient availability, leading to increased crop yields and reduced dependence on chemical fertilizers (Sreethu et al., 2023) like seaweed extracts from *Ascophyllum nodosum* have been found to significantly enhance plant growth, improve chlorophyll content, and increase yield in crops like tomato and soybean by improving nutrient uptake and hormonal stimulation (Kumari et al., 2023). By integrating these natural products into agricultural practices, farmers can achieve higher productivity while minimizing the environmental impact of farming activities.

2. Aim of the work

Climate change poses significant challenges to agriculture and plant productivity, necessitating the development of sustainable agricultural practices. One such approach is the circular economy, which emphasizes the reuse and recycling of resources to minimize waste and environmental impact. Maize cultivation, which requires substantial water input, is particularly vulnerable to drought stress.

Therefore, the aims of this work of thesis were the following:

- to assess the levels of drought tolerance/sensitivity in 26 maize landraces and one breeding reference line;
- to evaluate the impact of seed priming treatments with sustainable plant-based extracts on germination and seedling development under drought stress;
- to investigate the effects of seed priming and drought treatments at a molecular level.

To do this, a soil-based system was used to monitor germination for 14 days. Morpho-physiological parameters such as germination rate, seedling length, and stress indices, were calculated and analyzed. Additionally, the level of reactive oxygen species (ROS) released from the seeds were monitored using the FOX-1 assay to assess the oxidative stress status of primed and unprimed seeds. At a molecular level, the expression of genes involved in antioxidant defense and drought stress response was quantified though quantitative real-time PCR (qRT-PCR).

The work of this thesis is part of the project NODES Spoke 6 - FORMIDABILE (RM2 - Evaluation of forage and grain quality) funded by the PNRR European Union – NextGenerationEU program.

3. Material and Methods

3.1 Seed materials

Maize seeds from 26 Italian varieties, hereby presented in **Table 1** and **Fig. 11,** were kindly supplied by Prof. Adriano Marocco (Università Cattolica del Sacro Cuore, Piacenza).

Table 1. The Italian maize varieties.

VARIETY
1) Marano dell'Oltrepo'
2) Biancoperla
3) Marano
4) Quarantino Cremonese Sciaretta
5) Spinato di Mortara
6) RostratoValchiavenna
7) Scagliolodella Valle del Ticino
8) Dente di Cavallo Bianco
9) Nostrano_di_Storo
10) Rustico 1- Cagnacci
11) Rustico 4- Lusini
12) Mais ecotipoOrechiella
13) Mais OttofiledellaGarfagnana
14) Rosso del'Amiata
15) Nostrano -Vigo di Ton
16) Pesan -Vigo di Ton
17) Locale –Zambana
18) Marano –Gardolo
19) Giallo Comune
20) Cinquantino Bianco
21) Turco
22) Nano Precoce
23) Scagliolo Locale – Zambana
24) Mais da Polenta Chatillon
25) Mais da Polenta Entrebin
26) B73_Blu

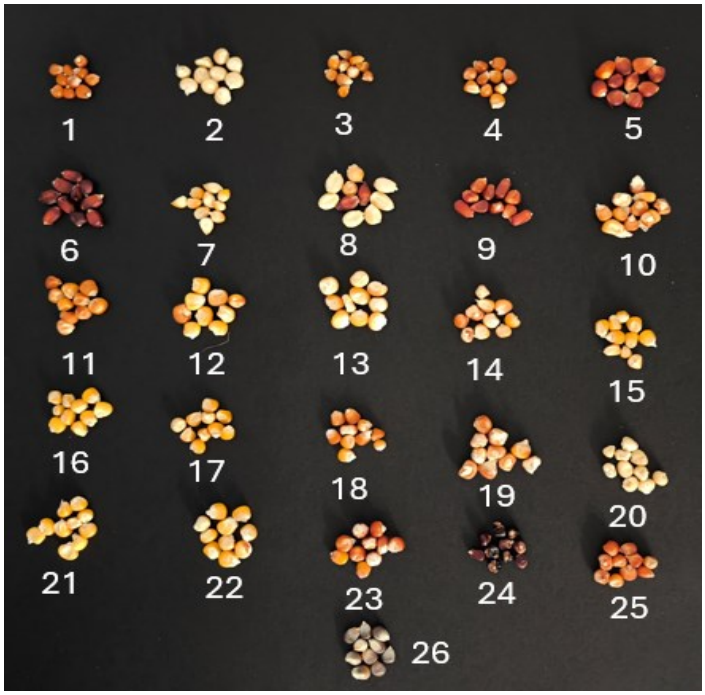

Figure 11. Seeds of the 26 Italian maize varieties used in this study.

3.2 Soil drought stress system

The soil drought stress system (Dueñas et al., 2024) used in this study was designed to simulate different moisture levels (represented as volumetric moisture content, VMC), corresponding to saturated soil (>50% VMC) for optimal growth conditions, and drought stress (30%). Maize seeds were sown in 8 cm by 11 cm trays containing 40 g of oven-dried sieved clay loam soil (VIGORPLANT ITALIA S.r.l, Piacenza, Italy). Water to be added was determined according to the soil volumetric moisture content and bulk density. Water addition was carried out daily to restore the weights to their original levels.

3.2 Seed priming treatments

The experimental system (**Fig. 12**) used in this study consisted of two seed priming treatments, namely, hydropriming (HP), and priming with plant waste extracts, in addition to the untreated control (UP, unprimed). Primed/unprimed seeds were germinated in the absence/presence of drought stress.

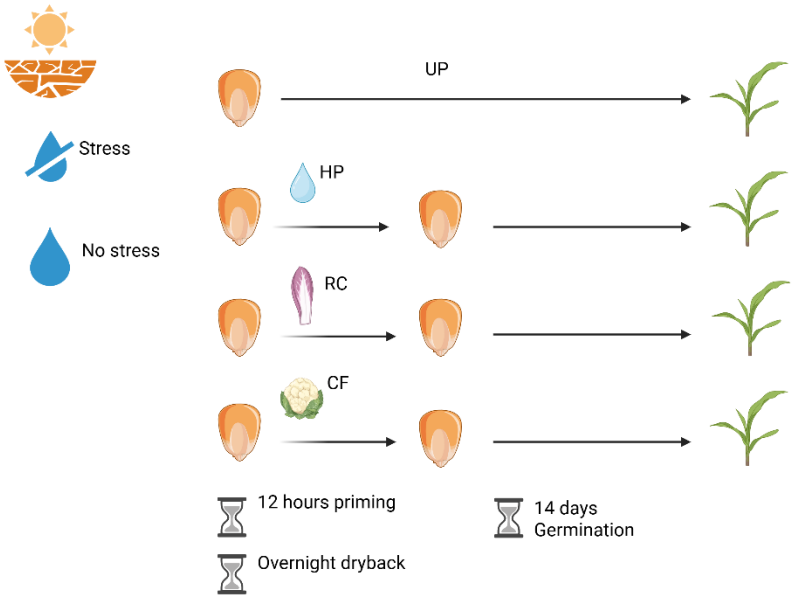

Figure 12. Schematic representation of the experimental system proposed for this work (https://www.biorender.com/).

For the priming treatments, maize seeds were imbibed in water or plant extracts for 12 h and then left to dry overnight at 20°C. For each replicate, 20 seeds were placed in soil germination trays and three replicates were used for each condition. The trays were kept in a growth chamber at 25°C under light conditions with a photon flux density of 35 µmol $m^{-2}s^{-1}$, photoperiod of 16/8 h, and monitored for 14 days.

The plant-waste extracts tested in this work, obtained from red chicory (RC) and cauliflower (CF), were kindly provided by Dr. Enrico Doria (BioRESTART, https://dbb.dip.unipv.it/en/research/innovation-and-public-engagement/spin/biorestart).

3.3 Germination parameters

Different germination parameters have been calculated as previously described (Ranal &Santana, 2006) and the respective equations are illustrated in **Table 2.** The calculated parameters are:

- G% (germination percentage), the percentage of germinated seeds.
- MGT (mean germination time), the standard time of germination of all seeds
- SL (root length), the measurement of the length of the shoot.
- RL (root length), the measurement of the length of the root.
- GSTI (germination stress tolerance index), the difference between yield under normal and stress conditions.

Table 2. Germination parameters with their corresponding significance and equations. ni, number of germinants at a specific time point; ti, a specific timepoint (in days).

Parameter	Significance	Equation
G%	Germinability	$\frac{\text{number of germinants}}{\text{total number of seeds}} x\ 100$
SL	Shoot length	(measured by ImageJ software)
RL	Root length	(measured by ImageJ software)
MGT	Mean germination time	$\frac{\sum \text{ni}}{\sum(\text{niti})}$
GSTI	Germination stress tolerance index	$\frac{\text{average number of germinants in normal condition}}{\text{average number of germinants in stress condition}} x100$

SL and RL were measured after 14 days using the ImageJ (https://imagej.nih.gov/ij/) free online software. To this purpose, six seedlings/treatment were photographed and used to determine the length.

3.4 Ferrous ion oxidation xylenol orange (FOX-1) assay

The release of hydrogen peroxide (H_2O_2) from seeds was quantified in control and treated seeds with red chicory by using the FOX-1 assay. This assay is carried out based on the use of the xylenol orange reagent (Carlo Erba), which reacts with Fe^{3+} (derived from the oxidation of Fe^{2+} induced by peroxyl radicals and H_2O_2) to give a blue-violet complex with an absorption maximum at 560 nm (Bridi et al., 2015).

The working solution (FOX-1 solution) was prepared as described by Griffo et al. (2023). A solution containing ammonium ferrous (II) sulphate $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ 25 mM (Merk) in H_2SO_4 0.25 M (Honeywell) was added to a Milli-Q water solution containing Xylenol Orange 125 μM (Carlo Erba) and D-sorbitol 100 mM (Duchefa Biochemie) in a ratio of 1:100. The solutions were mixed gently until the color became uniform.

Seed samples were incubated in 10 ml of Milli-Q water and 200 μL were transferred to 1 ml of FOX-1 solution. Three replicates of 7 seeds each were used per sample. Absorbance was determined at 560 nm by using a BiochromWPA Biowave (Biochrom Ltd.) spectrophotometer. A calibration curve was performed by using FOX-1 solution with different concentrations (0, 1.25, 2.50, 5 μM) of H_2O_2 for normalization.

3.5 DNA extraction, cDNA synthesis and qRT-PCR

Total RNA was isolated from *Z. mays* untreated seeds by using TRIzol™ (Thermo Fisher Scientific), as indicated by the provider. Subsequently, a DNase (Thermo Fisher Scientific) treatment was performed. RNA was quantified by using NanoDrop (Biowave DNA, WPA, Thermo Fisher Scientific). Subsequently, cDNAs were obtained by using the Revert AidFirst 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 MasterMix (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 95°C for 10 min, and 45 cycles of 95°C for 15 s and 60°C for 60 s. All reactions were performed in triplicates.

Oligonucleotide sequences **(Table. 3)** were designed by using Primer Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and verified with Oligo Analyzer.2 (https://eu.idtdna.com/ pages/tools/oligoanalyzer). Raw fluorescence data provided by Software 1.7 (BIO-RAD) were used to determine the threshold cycle number (C_t) values for each transcript. Relative quantification of transcript accumulation was performed as described by Thomsen et al. (2010) by using the X_0 method in which a conversion of the exponentially related C_t values is introduced to arrive at linearly related values, representing the amount of starting material in a qPCR. The *r 18s* gene was used as reference for the calculation of relative expression values.

Table 3. Oligonucleotide sequences used for the qRT-PCR reactions.

Gene	Gene ID	Forward Primer	Reverse Primer
r 18s	LOC118472325	CGTCGCTCCTACCGATTGA	CACCTACGGAAACCTTGTTACG
MSD3.4	Zm00001d009990	ATCCTGTATTCCTGCTTGCG	AAATGGTTCTTTTGGGCATCAC
CAT1	Zm00001d014848	CACCCAGAGAGCCTACACAT	AGCAAGCATTTCACACCACA
APX1.1	Zm00001d028709	CTGGGGTTTGCTGATGCTTA	ACGAGAAAGACAATACGACAATCT
GST1	Zm00001d012675	AACCACCGACCAGAAAGTTG	ACAGGCAGAGAGTGACAGAC
TRPP1	Zm00001d032298	CCACGGATGTTCCTTCTCCT	GCTATGGGCTCTGGCTC
DREB2A	Zm00001d008665	GCGTCAGAGGTGGGAGAG	GGTGGCGGTGGTATCCTA
PMP3g	Zm00001d024778	CATCATCTACGCCATCTACGC	GCAAAACAGGAACACGACTGA
LEA1	Zm00001d027740	CCACACTCACCATCAAGTCTC	CTCCTGGCTCATTCTCGCT

3.6 Statistical analyses

Statistical analyses were performed using the Microsoft Excel package. For gene expression data, one-way ANOVA followed by Tukey's *post hoc* test was used to assess significant differences between groups. For other comparisons, the Student's *t*-test was applied. In all cases, statistical significance was set at a p-value ≤ 0.05, indicated by an asterisk (*).

To guide tissue selection for expression analysis, a data mining step was conducted using the Maize eFP Browser, part of the Bio-Analytic Resource for Plant Biology (BAR), University of

Toronto (https://bar.utoronto.ca). This database provided spatial gene expression patterns across different *Zea mays* seed tissues, including embryo, endosperm, and pericarp.

4. Results

4.1 Identification of drought sensitive and tolerant maize varieties

The 26 local varieties of maize used in the current study were provided by Università Cattolica del Sacro Cuore (Piacenza) and represent an integral part of the materials used for the NODES project. Looking at the distribution of these landraces **(Table 4)**, it is possible to see that they are found mostly in several regions of North Italy, including Lombardy, Veneto, Tuscany, Trentino-Alto Adige, and Aosta Valley. Additionally, the B73_Blu variety was also selected as it represents a well-known breeding line (initially developed in USA) which is used as an international reference line for maize studies**.**

Table 4. Agronomic (kernel type and seed color) and geographical (region of origin of the obtained material) characteristics of the maize varieties evaluated.

Variety	Kernel Type	Seed color	Region
Marano dell'Oltrepo'	Flint	Yellow	Lombardy
Biancoperla	Flint	White	Veneto
Marano	Semi-flint	Orange to red	Veneto
Quarantino Cremonese Sciaretta	Flint	Yellow	Lombardy
Spinato di Mortara	Flint	Yellow	Lombardy
Rostrato Valchiavenna	Flint	Red to dark orange	Lombardy
Scagliolo della Valle del Ticino	Flint	Yellow	Lombardy
Dente di Cavallo Bianco	Dent	White	Northern Italy
Nostrano_di_Storo	Flint	Orange	Trentino-Alto Adige
Rustico 1- Cagnacci	Flint	Yellow	Tuscany
Rustico 4- Lusini	Flint	Yellow	Tuscany
Mais ecotipo Orechiella	Flint	Yellow	Tuscany
Mais Ottofile della Garfagnana	Flint	Yellow	Tuscany
Rosso del'Amiata	Flint	Red	Tuscany
Nostrano -Vigo di Ton	Flint	Yellow	Trentino-Alto Adige
Pesan -Vigo di Ton	Flint	Yellow	Trentino-Alto Adige
Locale -Zambana	Flint	Yellow	Trentino-Alto Adige
Marano -Gardolo	Flint	Orange	Trentino-Alto Adige
Giallo Comune	Flint	Yellow	Northern Italy
Cinquantino Bianco	Flint	White	Northern Italy
Turco	Flint	Yellow	Northern Italy
Nano Precoce	Flint	Yellow	Northern Italy
Scagliolo Locale -Zambana	Flint	Yellow	Trentino-Alto Adige
Mais da Polenta Chatillon	Flint	Yellow	Aosta Valley
Mais da Polenta Entrebin	Flint	Yellow	Aosta Valley
B73_Blu	Dent	Gray-blue	USA (adapted in Italy)

Concerning the kernel type, most varieties are defied as flint (the hard endosperm comprises most of the grain and forms a cap over the germ) or semi-flint, while only two (Dente di Cavallo Bianco, B73_Blu) are dent (the hard endosperm is present only as an open cylinder which partly surrounds the germ towards the base of the grain). Regarding the color of the seeds, the majority are yellow/orange, while few red (Rosso del'Amiata, Rostrato Valchiavenna, Marano), white (Dente di Cavallo Bianco, Biancoperla), or gray blue (B73_Blu) accessions are also present **(Table 4)**. This diversity in kernel type, color, and origin reflects a broad genetic base and supports a robust evaluation of drought response.

To evaluate the levels of drought tolerance or susceptibility at germination, a soil-based system was employed, following the protocol of Dueñas et al. (2024), which replicates realistic field-like conditions using VMC as an indicator of soil hydration levels. This system is especially valuable because it addresses a major gap in current literature, where most drought-related studies use artificial systems such as filter paper or PEG (polyethylene glycol) setups, which fail to capture the complexity of soil-root interactions. In this study, saturated conditions were defined as greater than 50% VMC to promote maximum germination potential, while drought stress was imposed by using 30% VMC. Water levels were adjusted daily to ensure precise control of moisture content throughout the germination period. As shown in **Fig. 13**, trays under optimal moisture (top row) supported robust germination and growth, while those under drought stress (bottom row) exhibited visibly fewer seedlings and a stunted development.

Figure 13. Representative image showing maize growth different soil moisture conditions. **(a)** Optimal moisture (>50% VMC). **(b)** Drought stress (30% VMC).

Germination Stress Tolerance Index (GSTI), calculated by reporting the germination rate under drought stress to that of control conditions, was selected as the key parameter to evaluate the levels of drought tolerance **(Fig. 14).** A threshold of 20% was imposed to define genotypes as drought-tolerant, based on their ability to maintain germination performance under reduced water availability. Out of the 26 maize varieties tested, only three surpassed this threshold. Nostrano di Storo exhibited the highest drought tolerance level with a GSTI of 28%, followed by Marano (26.67%) and Mais da Polenta Chatillon (20%). These varieties demonstrated a relative capacity to germinate under stress and could represent promising candidates for breeding programs focused on enhancing drought resilience.

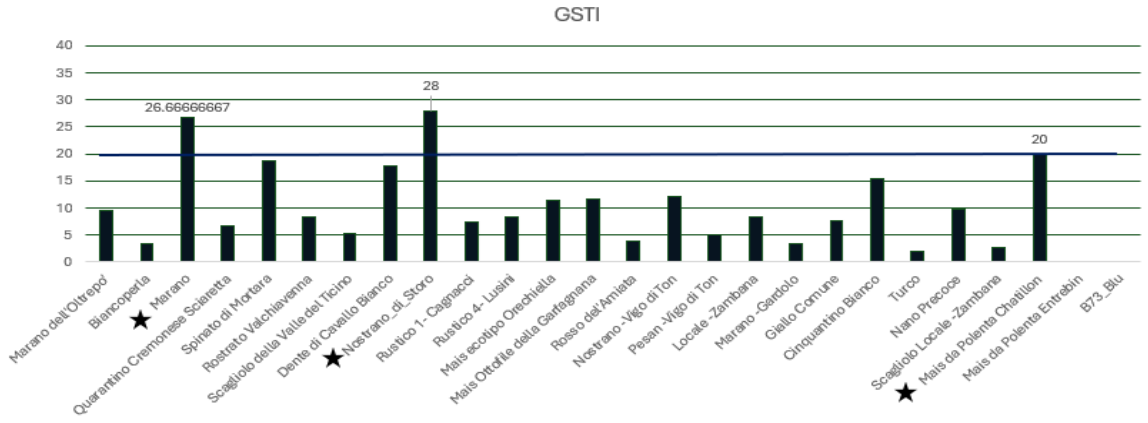

Figure 14. Graphical representation of GSTI (Germination Stress Tolerance Index) values (%) in the 26 maize varieties analyzed in the current study. The 20% threshold is indicated with a bolded line. Varieties surpassing or arriving at the threshold are also evidenced with a star.

Other genotypes showed moderate levels of tolerance, with GSTI values ranging between 10–19%. These included Spinato di Mortara (18.64%), Dente di Cavallo Bianco (17.86%), Cinquantino Bianco (15.38%), Nostrano - Vigo di Ton (12.07%), Mais Ottofile della Garfagnana (11.76%), and Mais ecotipo Orechiella (11.54%). These varieties were still able to germinate the under water deficit, albeit with reduced efficiency. On the other hand, a considerable number of varieties showed low tolerance, with GSTI values below 10%, such as Nano Precoce (9.80%), Marano dell'Oltrepò (9.52%), Rustico 1 - Cagnacci (7.41%), Giallo Comune (7.54%), Locale - Zambana (8.33%), Rustico 4 - Lusini (8.33%), Scagliolo della Valle del Ticino (5.26%), Pesan -

Vigo di Ton (5.00%), and Quarantino Cremonese Sciaretta (6.66%). These varieties showed a very limited germination capacity under reduced soil moisture. The most sensitive genotypes were Biancoperla (3.39%), Rosso dell'Amiata (3.92%), Scagliolo Locale - Zambana (2.78%), Turco (1.96%), Mais da Polenta Entrebin (0.00%), and B73_Blu (0.00%), displaying minimal or no germination under drought stress, thus indicating high vulnerability to water limitation at the germination stage. Interestingly, the common inbred line B73_Blu was among the most sensitive to drought at germination, in contrast to local Italian landraces which appear to be more resilient.

The wide variability observed in GSTI values emphasizes the presence of valuable genetic diversity among Italian maize landraces. Only 11.5% of the tested genotypes exceeded the 20% tolerance threshold, suggesting that true drought tolerance at the germination stage is relatively rare, and reinforcing the importance of early screening for stress-adaptive traits.

4.2. Seed priming with plant extracts improves germination in a treatment- and genotype-dependent manner

Following the characterization of drought sensitive and tolerant materials, the next step consistent in the development of methodologies/protocols to improve the response to drought during seed germination. To this purpose, seed priming treatments were applied keeping in consideration both the basic HP protocols as well as the addition of plant extracts to the aqueous solution. The plant extracts used in this study were obtained from agricultural waste coming from red chicory or cauliflower materials removed in the supermarket before packaging. The extraction protocol for the preparation of these extracts is based on a highly-sustainable method called enzymatic assisted selection, combined with the use of microorganisms (Doria et al., 2022). The extracts are prepared in water, which also prompted the addition of HP treatments as controls, alongside the unprimed (UP) seeds. The obtained extracts are rich in bioactive molecules, like polyphenols and flavonoids, but their exact composition cannot be yet divulged due to Intellectual Property (IP) issues. Preliminary analyses (data not shown), carried out in a previous study, allowed to select one dilution for each extract to be used for the priming treatments.

The effects of HP, RC, and CF priming on germination and seedling growth were evaluated across 26 maize varieties under control **(Fig. 15a)** and drought stress conditions **(Fig. 15b)** and compared to the unprimed seeds (UP).

a

	G%				SL				RL			
	UP	HP	RC	CF	UP	HP	RC	CF	UP	HP	RC	CF
Marano dell'Oltrepo'	70 ± 25.98	85 ± 10	86.67 ± 12.58	93.33 ± 2.89	15.58 ± 1.22	19.25 ± 3.43	18.73 ± 0.43 *	15.46 ± 1.99	12.90 ± 1.22	16.09 ± 3.43	19.50 ± 0.45 *	9.61 ± 1.99
Biancoperla	98.33 ± 2.89	95 ± 8.66	86.67 ± 2.89	83.33 ± 7.64	26.48 ± 1.50	28.77 ± 0.96 *	30.15 ± 0.89 *	29.50 ± 2.71 *	27.56 ± 1.50	31.26 ± 0.96 *	29.86 ± 0.89	33.98 ± 2.71 *
Marano	100 ± 0	98.33 ± 2.89	90 ± 8.66	100 ± 0	28.48 ± 2.08	27.99 ± 3.10	28.33 ± 1.31	27.37 ± 2.03	25.28 ± 2.08	31.58 ± 3.10 *	29.78 ± 1.31	31.33 ± 2.03 *
Quarantino Cremonese Sciaretta	100 ± 0	96.67 ± 5.77	93.33 ± 7.64	91.67 ± 2.89 *	30.76 ± 5.66	32.23 ± 2.50	31.68 ± 4.44	31.70 ± 2.04 *	26.10 ± 3.45	22.43 ± 5.09 *	32.48 ± 2.64 *	26.15 ± 3.54
Spinato di Mortara	98.33 ± 2.89	78.33 ± 10.41 *	76.67 ± 11.55 *	75 ± 5.00 *	25.29 ± 2.24	33.51 ± 8.14 *	33.67 ± 6.34 *	33.34 ± 6.58 *	25.73 ± 4.44	26.01 ± 2.92	31.55 ± 3.60 *	24.32 ± 5.35 *
Rostrato Valchiavenna	100 ± 0	90 ± 8.66	80 ± 5.00 *	91.67 ± 10.41	27.62 ± 1.85	32.37 ± 5.12 *	32.41 ± 1.33 *	34.52 ± 4.86 *	24.11 ± 3.65	34.45 ± 5.12 *	30.59 ± 1.33 *	35.09 ± 4.86 *
Scagliolo della Valle del Ticino	95 ± 5.00	98.33 ± 2.89 *	88.33 ± 7.64 *	98.33 ± 2.89 *	28.37 ± 4.61	27.49 ± 4.99	29.23 ± 0.54 *	29.27 ± 4.32 *	30.57 ± 2.84	27.00 ± 1.87	30.02 ± 4.11 *	30.75 ± 2.51 *
Dente di Cavallo Bianco	93.33 ± 7.64 *	86.67 ± 7.64 *	65 ± 15.00 *	68.33 ± 10.41 *	27.03 ± 2.55	26.80 ± 7.41 *	26.17 ± 3.07 *	26.85 ± 2.21 *	31.87 ± 1.00 *	33.10 ± 5.06 *	35.87 ± 2.64 *	35.48 ± 1.63 *
Nostrano_di_Storo	83.33 ± 11.55 *	76.67 ± 10.41 *	63.33 ± 5.77 *	90.00 ± 8.66 *	29.10 ± 2.99 *	31.71 ± 1.04 *	27.72 ± 0.93 *	27.27 ± 1.95 *	25.94 ± 3.00 *	32.91 ± 3.65 *	25.94 ± 2.63 *	32.48 ± 5.45 *
Rustico 1- Cagnacci	90.00 ± 5.00 *	86.67 ± 2.89 *	91.67 ± 2.89 *	90.00 ± 5.00 *	27.59 ± 4.84 *	28.82 ± 2.90 *	28.02 ± 3.37 *	25.19 ± 1.46 *	20.77 ± 2.64 *	34.66 ± 13.67 *	36.57 ± 5.49 *	32.33 ± 5.50 *
Rustico 4- Lusini	100.00 ± 0.00 *	88.33 ± 12.58 *	86.67 ± 11.55 *	78.33 ± 17.56 *	29.26 ± 2.03 *	22.41 ± 2.23 *	26.16 ± 1.48 *	25.21 ± 0.96 *	30.43 ± 4.07 *	25.93 ± 5.11 *	24.07 ± 7.33 *	20.69 ± 4.93 *
Mais ecotipo Orechiella	86.67 ± 12.58 *	86.67 ± 10.41 *	71.67 ± 10.41 *	76.67 ± 7.64 *	30.16 ± 2.16 *	22.88 ± 2.45 *	35.89 ± 6.16 *	33.13 ± 4.62 *	26.10 ± 3.01 *	26.97 ± 4.13 *	26.94 ± 2.87 *	30.31 ± 4.57 *
Mais Ottofile della Garfagnana	85.00 ± 5.00 *	78.33 ± 20.21 *	71.67 ± 16.07 *	71.67 ± 7.64 *	30.24 ± 3.22 *	22.78 ± 2.42 *	25.81 ± 3.15 *	25.50 ± 3.65 *	34.05 ± 3.60 *	26.90 ± 4.34 *	28.69 ± 2.91 *	29.10 ± 7.88 *
Rosso del'Amiata	85.00 ± 13.23 *	95.00 ± 0.00 *	81.67 ± 18.93 *	83.33 ± 10.41 *	19.18 ± 1.69 *	21.17 ± 1.96 *	21.48 ± 3.77 *	26.61 ± 4.65 *	25.77 ± 4.85 *	21.84 ± 2.60 *	23.98 ± 10.90 *	23.47 ± 2.02 *
Nostrano -Vigo di Ton	96.67 ± 2.89 *	86.67 ± 2.89 *	80.00 ± 13.23 *	95.00 ± 5.00 *	15.99 ± 4.06 *	21.58 ± 1.22 *	21.14 ± 4.38 *	20.82 ± 3.31 *	16.03 ± 3.72 *	22.39 ± 7.23 *	22.40 ± 5.87 *	21.72 ± 5.36 *
Pesan -Vigo di Ton	66.67 ± 24.66 *	76.67 ± 2.89 *	71.67 ± 27.54 *	75.00 ± 8.66 *	19.09 ± 1.84 *	13.27 ± 0.74 *	18.72 ± 4.79 *	18.48 ± 2.31 *	25.38 ± 3.94 *	18.72 ± 4.44 *	23.63 ± 6.09 *	26.01 ± 2.73 *
Locale -Zambana	100 ± 0	98.33 ± 2.89 *	96.67 ± 2.89 *	90 ± 8.66 *	19.23 ± 1.89 *	20.98 ± 1.30 *	24.47 ± 8.06 *	20.87 ± 4.02 *	22.65 ± 2.38 *	20.21 ± 3.51 *	10.96 ± 3.12 *	24.14 ± 3.01 *
Marano -Gardolo	96.67 ± 5.77 *	91.67 ± 2.89 *	85 ± 5.00 *	88.33 ± 7.64 *	19.15 ± 1.71 *	21.12 ± 1.38 *	20.17 ± 1.10 *	20.21 ± 0.98 *	25.15 ± 2.67 *	18.55 ± 4.83 *	25.48 ± 7.12 *	18.56 ± 4.69 *
Giallo Comune	88.33 ± 12.58 *	78.33 ± 2.89 *	80 ± 8.66 *	71.67 ± 5.77 *	21.90 ± 1.99 *	20.99 ± 0.88 *	21.34 ± 1.90 *	21.01 ± 1.64 *	17.59 ± 5.70 *	25.88 ± 1.87 *	24.41 ± 4.54 *	29.03 ± 4.50 *
Cinquantino Bianco	86.67 ± 7.64 *	91.67 ± 2.89 *	85.00 ± 5.00 *	90.00 ± 5.00 *	32.25 ± 3.28 *	35.28 ± 3.54 *	31.31 ± 1.85 *	35.84 ± 3.92 *	27.11 ± 4.07 *	27.39 ± 5.13 *	32.75 ± 5.05 *	34.11 ± 4.11 *
Turco	85.00 ± 13.23 *	66.67 ± 2.89 *	73.33 ± 10.41 *	73.33 ± 28.87 *	20.65 ± 1.71 *	19.69 ± 4.99 *	17.51 ± 1.95 *	20.97 ± 2.69 *	18.84 ± 6.31 *	16.31 ± 3.81 *	28.04 ± 3.80 *	30.02 ± 5.41 *
Nano Precoce	85.00 ± 5.00 *	90.00 ± 8.66 *	78.33 ± 2.89 *	66.67 ± 17.56 *	20.08 ± 3.40 *	19.04 ± 1.83 *	20.88 ± 0.89 *	20.98 ± 2.68 *	25.51 ± 3.58 *	25.33 ± 6.01 *	26.64 ± 2.06 *	21.02 ± 6.24 *
Scagliolo Locale -Zambana	60.00 ± 13.23 *	66.67 ± 17.56 *	50.00 ± 18.03 *	70.00 ± 5.00 *	16.48 ± 0.71 *	24.34 ± 2.14 *	17.46 ± 3.87 *	22.18 ± 2.29 *	14.56 ± 3.90 *	15.80 ± 3.22 *	16.46 ± 4.31 *	21.07 ± 1.31 *
Mais da Polenta Chatillon	83.33 ± 2.89 *	81.67 ± 23.09 *	61.67 ± 10.41 *	76.67 ± 11.55 *	26.73 ± 3.58 *	31.31 ± 2.54 *	31.20 ± 4.25 *	33.37 ± 3.96 *	27.54 ± 6.61 *	23.72 ± 6.19 *	27.32 ± 3.44 *	25.66 ± 6.56 *
Mais da Polenta Entrebin	90.00 ± 8.66 *	86.67 ± 7.64 *	63.33 ± 33.25 *	78.33 ± 16.07 *	19.81 ± 1.58 *	21.56 ± 1.57 *	21.16 ± 2.39 *	20.27 ± 0.45 *	15.40 ± 0.86 *	19.61 ± 2.78 *	19.89 ± 3.80 *	15.50 ± 3.44 *
B73_Blu	91.67 ± 5.77 *	81.67 ± 23.63 *	58.33 ± 5.77 *	68.33 ± 18.93 *	18.06 ± 0.41 *	17.84 ± 0.19 *	19.52 ± 2.16 *	20.03 ± 1.06 *	32.31 ± 2.41 *	24.73 ± 5.51 *	24.61 ± 5.31 *	22.02 ± 6.25 *

b

	G%				RL				SL			
	UP	HP	RC	CF	UP	HP	RC	CF	UP	HP	RC	CF
Marano dell'Oltrepo'	6.7 ± 2.89	1.7 ± 2.89	1.7 ± 2.89	6.7 ± 7.64	7.3 ± 1.55	11.5 ± 0	3.0 ± 0	3.8 ± 3.85	3.7 ± 3.47	7.6 ± 0	2.9 ± 0	6.4 ± 1.55
Biancoperla	3.33 ± 2.89	3.33 ± 2.89	8.33 ± 5.77	13.33 ± 2.89	7.75 ± 8.03	6.69 ± 2.81	8.58 ± 7.50	10.94 ± 0	7.59 ± 0.87	7.60 ± 0	12.21 ± 1.97	14.04 ± 4.26
Marano	26.67 ± 5.77	30 ± 0	25 ± 10	23.33 ± 2.89	16.81 ± 2.05	16.68 ± 0	11.32 ± 0 *	13.33 ± 0 *	16.76 ± 2.05	20.61 ± 6.76	20.17 ± 2.44 *	18.34 ± 6.08
Quarantino Cremonese Sciaretta	6.67 ± 7.64	6.67 ± 5.77	20 ± 5	1.67 ± 2.89	7.04 ± 0	6.50 ± 0	11.24 ± 0 *	7.24 ± 0	11.06 ± 4.65	6.71 ± 3.93	25.02 ± 2.20 *	13.17 ± 0
Spinato di Mortara	18.33 ± 10.41	15 ± 5	21.67 ± 16.07	25 ± 5	16.89 ± 8.03	14.26 ± 5	12.57 ± 2.89	10.90 ± 5	22.67 ± 8.03	16.35 ± 6.20	21.43 ± 1.99	22.09 ± 8.77
Rostrato Valchiavenna	8.33 ± 10.41	15 ± 5	18.33 ± 2.89	10 ± 0	11.35 ± 10.41	12.35 ± 5	13.01 ± 2.89	12.32 ± 0	13.59 ± 10.13	18.66 ± 7.48	18.50 ± 7.08	9.48 ± 7.72
Scagliolo della Valle del Ticino	5 ± 5	13.33 ± 7.64	20 ± 15	6.67 ± 2.89	7.43 ± 5	9.06 ± 7.64	15.81 ± 15	9.13 ± 2.89	11.32 ± 8.34	18.89 ± 7.01	25.76 ± 2.64 *	16.06 ± 4.69
Dente di Cavallo Bianco	16.67 ± 2.89	25 ± 5	21.67 ± 2.89	26.67 ± 5.77	8.02 ± 2.89	13.84 ± 5 *	12.76 ± 2.89 *	11.83 ± 5.77 *	17.49 ± 5.90	17.93 ± 6.15	22.62 ± 3.98	20.05 ± 2.25
Nostrano_di_Storo	23.33 ± 7.64	20 ± 8.66	25 ± 5	21.67 ± 2.89	17.00 ± 7.64	12.97 ± 8.66 *	8.91 ± 5 *	8.65 ± 2.89 *	24.66 ± 4.85	19.72 ± 6.36	16.71 ± 8.36	15.40 * ± 7.11
Rustico 1- Cagnacci	6.67 ± 2.89	10 ± 8.66	15 ± 5	10 ± 0	6.17 ± 2.89	11.32 ± 8.66 *	7.87 ± 5	11.41 ± 0 *	7.62 ± 6.33	10.95 ± 4.54	12.41 ± 5.68	17.64 ± 8.52
Rustico 4- Lusini	8.33 ± 7.64	18.33 ± 5.77	21.67 ± 7.64	5 ± 5	11.52 ± 7.64	6.01 ± 5.77	10.06 ± 7.64	7.17 ± 5	11.74 ± 10.57	19.64 ± 3.48 *	13.63 ± 5.34 *	10.87 ± 5.79 *
Mais ecotipo Orechiella	10.00 ± 2.37	5.00 ± 4.40	3.33 ± 1.14	11.67 ± 1.81	7.18 ± 5	10.91 ± 5	8.33 ± 2.89	5.40 ± 5.77	9.81 ± 7.83	17.27 ± 4.89	19.00 ± 4.72 *	6.46 ± 3.21
Mais Ottofile della Garfagnana	10.00 ± 3.83	23.33 ± 1.05 *	13.33 ± 1.08	13.33 ± 2.65	9.65 ± 5.00	6.58 ± 2.89	5.94 ± 7.64	9.28 ± 2.89	14.61 ± 11.56	14.61 ± 2.44	9.93 ± 6.32	11.31 ± 5.85
Rosso del'Amiata	3.33 ± 6.08	11.67 ± 1.49	15.00 ± 1.77 *	6.67 ± 2.80	7.09 ± 2.89	13.54 ± 5.77	5.78 ± 0.00	5.21 ± 7.64	6.23 ± 3.57	11.21 ± 6.88	8.85 ± 1.94	2.98 ± 1.43
Nostrano -Vigo di Ton	11.67 ± 2.07	5.00 ± 5.92	18.33 ± 2.51 *	8.33 ± 4.33	4.25 ± 2.89	6.54 ± 5.00	8.81 ± 2.89 *	6.39 ± 7.64	10.79 ± 6.00	7.30 ± 5.83	14.19 ± 3.51	7.71 ± 4.97
Pesan -Vigo di Ton	3.33 ± 2.89	13.33 ± 10.41	10.00 ± 5.00	8.33 ± 7.64	8.42 ± 2.53	9.08 ± 2.51	4.62 ± 3.43	4.19 ± 2.76	7.45 ± 4.77	9.48 ± 4.41	2.74 ± 0.42	4.02 ± 2.76
Locale -Zambana	8.33 ± 2.89	6.67 ± 5.77	11.67 ± 5.77	0.00 ± 0.00 *	5.06 ± 2.02	9.45 ± 3.27	5.00 ± 1.35	0.00 ± 0.00	9.15 ± 2.02	11.20 ± 3.50	6.84 ± 0.67 *	0.00 ± 0.00
Marano -Gardolo	3.33 ± 2.89	13.33 ± 5.77	8.33 ± 10.41	8.33 ± 2.89	7.16 ± 4.24	13.03 ± 5.44	6.90 ± 3.84	9.89 ± 1.33	10.62 ± 4.06	13.25 ± 3.99	6.85 ± 1.53	10.07 ± 2.57
Giallo Comune	6.67 ± 7.64	3.33 ± 5.77	6.67 ± 7.64	3.33 ± 2.89	5.73 ± 2.60	6.10 ± 0	7.23 ± 2.03	3.54 ± 0.70	8.04 ± 4.04	6.56 ± 0	8.42 ± 4.62	3.61 ± 1.53
Cinquantino Bianco	13.33 ± 2.89	10.00 ± 5.00	13.33 ± 7.64	13.33 ± 5.77	9.70 ± 2.89	8.68 ± 5.00	5.98 ± 7.64	11.34 ± 5.77	10.03 ± 4.09	15.93 ± 4.89	13.67 ± 2.64	14.16 ± 6.13
Turco	1.67 ± 2.89	1.67 ± 2.89	6.67 ± 2.89	1.67 ± 2.89	13.42 ± 0	8.10 ± 0	7.38 ± 4.45	4.07 ± 0	11.33 ± 0	6.39 ± 0	8.30 ± 4.45	1.38 ± 0
Nano Precoce	8.33 ± 7.64	3.33 ± 2.89	6.67 ± 7.64	1.67 ± 2.89	6.69 ± 0.46	5.14 ± 2.56	4.28 ± 0.99	5.97 ± 0	8.19 ± 2.69	10.73 ± 4.30	6.68 ± 4.33	2.50 ± 0
Scagliolo Locale -Zambana	1.67 ± 2.89	3.33 ± 2.89	3.33 ± 2.89	8.33 ± 2.89	4.97 ± 2.89	0.00 ± 2.89	4.22 ± 2.22	5.83 ± 5.15	15.37 ± 2.89	0.00 ± 2.89	7.01 ± 5.65	8.59 ± 6.78
Mais da Polenta Chatillon	16.67 ± 2.77	20.00 ± 4.10	21.67 ± 3.32	20.00 ± 4.98	12.05 ± 12.58	8.23 ± 8.66	8.03 ± 2.89 *	9.32 ± 0.00	15.51 ± 8.48	13.15 ± 7.19	14.24 ± 2.91	15.55 ± 4.15
Mais da Polenta Entrebin	0.00 ± 0.00	1.67 ± 2.89	6.67 ± 0.95	3.33 ± 0.63	0.00 ± 0.00	3.72 ± 2.89	4.38 ± 2.89	4.53 ± 2.89	0.00 ± 0.00	6.21 ± 1.57	5.50 ± 4.22	4.21 ± 1.57
B73_Blu	0.00 ± 0.00	3.33 ± 1.95	3.33 ± 1.17	5.00 ± 5.13	0.00 ± 0.00	4.63 ± 2.89	4.68 ± 2.89	6.12 ± 0.00	0.00 ± 0.00	3.95 ± 0.27	3.94 ± 1.25	6.40 ± 6.69

Figure 15. Evaluation of the germination behavior of the 26 maize varieties subjected to priming and grown under **(a)** no stress conditions or **(b)** drought stress. Data are represented as a two-color scale heatmap (where red represents low values and green high values) using mean ± SD values. Asterisks indicate statistically significant differences (*, $p \leq 0.05$) as determined through Student's *t*-test**.** G%, germination percentage; SL/RL, shoot/root length; HP, hydropriming; RC/CF, priming with red chicory or cauliflower extracts; UP, unprimed seeds.

The data obtained evidenced that, under control conditions, G% varied significantly among treatments. UP seeds of Marano dell'Oltrepo showed moderate germination (70.00 ± 25.96%), which increased with HP (85.00 ± 10.00%), RC (86.67 ± 12.58%), and CF (83.33 ± 2.89%). In contrast, high-germinating varieties like Marano (100.00 ± 0.00% UP) exhibited no further improvement (**Fig. 15a**). Shoot length (SL) and root length (RL) were significantly enhanced by HP and RC treatments. For example, Marano dell'Oltrepo seedlings obtained from HP seeds had longer shoots (19.26 ± 3.42 cm) compared to UP (15.58 ± 1.22 cm), while RC treatments further maximized root growth (19.50 ± 0.45 cm vs. 12.90 ± 1.22 cm UP). However, these effects were not observed also for the CF treatments, where the Turco variety was even showing reduced RL (17.51 ± 1.95 cm) compared to UP control (20.65 ± 1.71 cm). Notably, priming treatments resulted in reduced germination also in other cases, such as in Spinato di Mortara (UP: 98.33 ± 2.89% vs. HP: 78.33 ± 10.41%). Statistical analysis ($p \leq 0.05$p) confirmed significant differences between UP and treated seeds in responsive varieties (like Biancoperla, Spinato di Mortara, Rostrato Valchiavenna, Rustico 1- Cagnacci for RL). These findings highlight that while HP and RC generally improve germination and early growth, optimal priming strategies must be genotype-specific to maximize benefits.

When looking at the stress conditions, the priming treatments demonstrated to be more effective in improving germination and seedling development. The germination of UP seeds was significantly affected by drought, as seen for instance in Marano dell'Oltrepo, where substantial root length reduction was observed (from 12.90 ± 1.22 cm in optimal conditions to 3.7 ± 3.47 cm under stress). Priming treatments effectively mitigated the impact of stress, with HP maintaining root length at 7.5 ± 0.0 cm and RC at 19.50 ± 0.45 cm for the same variety. Germination percentage (G%) was significantly improved by priming treatments in several maize varieties. For instance, Biancoperla showed marked enhancement after RC and CF treatments (UP: 3.33 ± 2.89% vs. RC: 8.33 ± 5.77%, CF: 13.33 ± 2.89%), while Rustico 4 - Lusini and Rostrato Valchiavenna also exhibited increased G% following RC priming (RC: 21.67 ± 7.64% and 18.33 ± 2.89%, respectively). Notably, CF treatment strongly boosted G% in Spinato di Mortara (CF: 25 ± 5%) compared to UP (18.33 ± 10.41%). Statistical analysis ($p \leq 0.05$) confirmed that these increases were significant in selected genotypes (e.g., Biancoperla, Rustico 4 - Lusini, Scagliolo della Valle del Ticino), indicating that stress priming can effectively enhance germination.

The stress-protective effects were particularly pronounced for RC treatments in varieties like Scagliolo della Valle del Ticino, where the stress-induced decline of seedling growth were almost completely prevented (25.76 ± 2.84 cm under stress versus 29.23 ± 0.54 cm control). HP showed consistent stress protection across multiple varieties, while the effectiveness of CF treatments was more limited and variable, occasionally exacerbating stress sensitivity as observed in Spinato di Mortara (6.09 ± 8.77 cm under stress versus 33.34 ± 6.58 cm control). These findings underscore the important role of priming in drought mitigation, with HP and RC offering more positive results than CF.

Therefore, the results presented in **Fig. 15** underline a genotype- and treatment-specific response to priming, along with the potential of some treatments to mitigate the negative effects of drought imposed at germination. These differential patterns can be better visualized when calculating the GSTI values, therefore reporting the tolerance index (**Fig. 16)**.

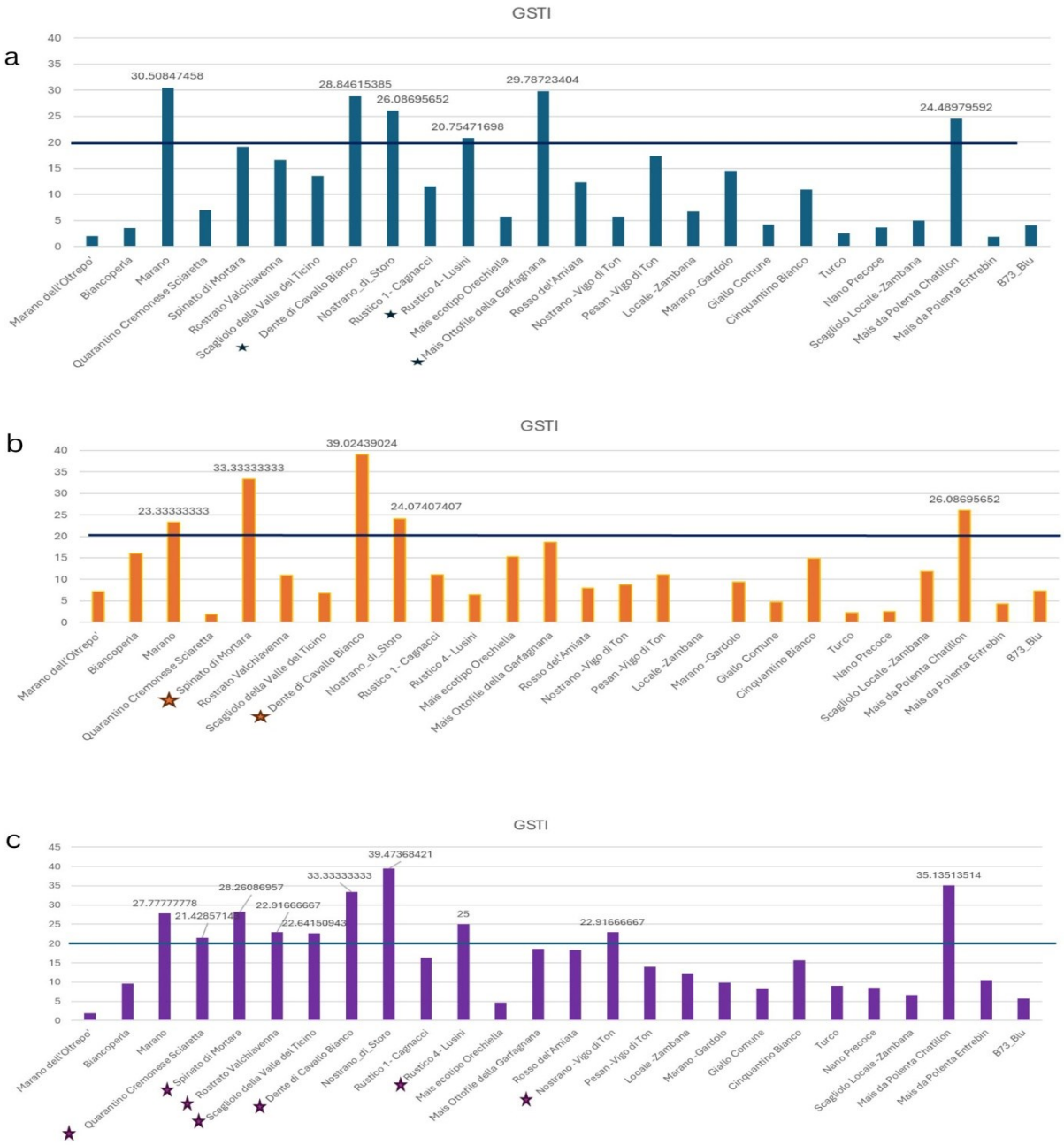

Figure 16. Germination Stress Tolerance Index (GSTI) calculated for the 26 maize varieties following priming treatments. **(a)** Hydropriming. **(b)** Priming with cauliflower extract. **(c)** Priming with red chicory extract. The 20% threshold is indicated with a bolded line. Varieties surpassing or arriving at the threshold are also evidenced with a star.

The GSTI analysis revealed that HP improved stress tolerance in three varieties (**Fig. 16a**), with Marano showing the strongest response (30.51%), followed by Mais Ottofile della Garfagnana (29.79%) and Dente di Cavallo Bianco (28.85%). Priming with CF subsequently improved the

GSTI values in fewer varieties (**Fig. 16b**), namely Dente di Cavallo Bianco (39.02%) and Spinato di Mortara (33.33%). Differently, priming with RC proved most effective as it improved the GSTI values in seven varieties (**Fig. 16c**): Dente di Cavallo Bianco (33.33%), Spinato di Mortara (28.26%), Rustico 4-Lusini (25.00%), Quarantino Cremonese Sciaretta (21.43%), Rostrato Valchiavenna (22.92%), Scagliolo della Valle del Ticino (22.64%), and Nostrano -Vigo di Ton (22.92%). Still, seven varieties remained below the 20% tolerance threshold for all treatments, and these included Turco (HP:2.50%, CF:2.27%, RC:9.09%), Nano Precoce (HP:3.70%, CF:2.50%, RC:8.51%), and Locale-Zambana (HP:5.00%, CF:0%, RC:6.67%).

Overall, these values support for a clear benefit of seed priming with plant extracts in enhancing drought tolerance during the germination phase. Notably, RC priming proven to be the most efficient, resulting in the highest number of responsive varieties. Based on this distinction taking into consideration the GSTI values, a Venn diagram (**Fig. 17**) was generated to highlight similarities or differences in the responsiveness to priming per variety.

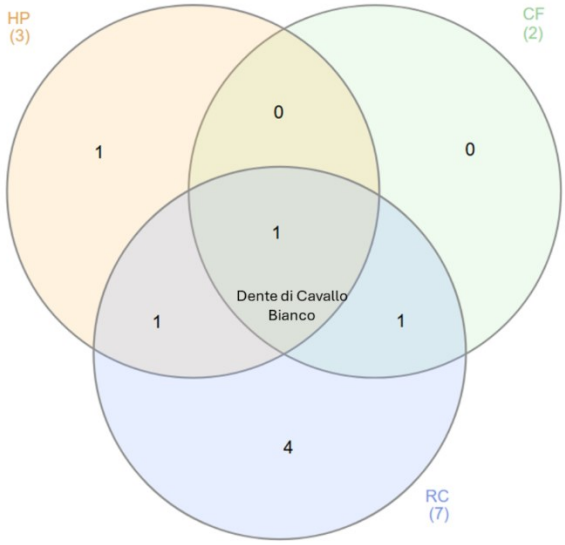

Figure 17. Venn diagram representing the grouping of varieties based on different seed priming treatments. HP, hydropriming; RC/CF, priming with red chicory or cauliflower extracts.

These results showed that RC elicited the highest number of unique responses (4 varieties), suggesting its ability to activate stress resilience in a broad range of genotypes. In contrast, HP triggered only one unique response, indicating more limited effects. CF did not produce any

unique response, instead showing an overlap with RC- and HP-responsive varieties. The only genotype exhibiting an universal responsiveness to all priming treatments was Dente di Cavallo Bianco, suggesting the existence of mechanisms worthy of further investigation.

While GSTI analysis identified genotype-specific patterns in stress tolerance, investigating another parameter, namely the Mean Germination Time (MGT, **Fig. 18**), could provide further insights into the temporal dynamics of germination under optimal or stress conditions.

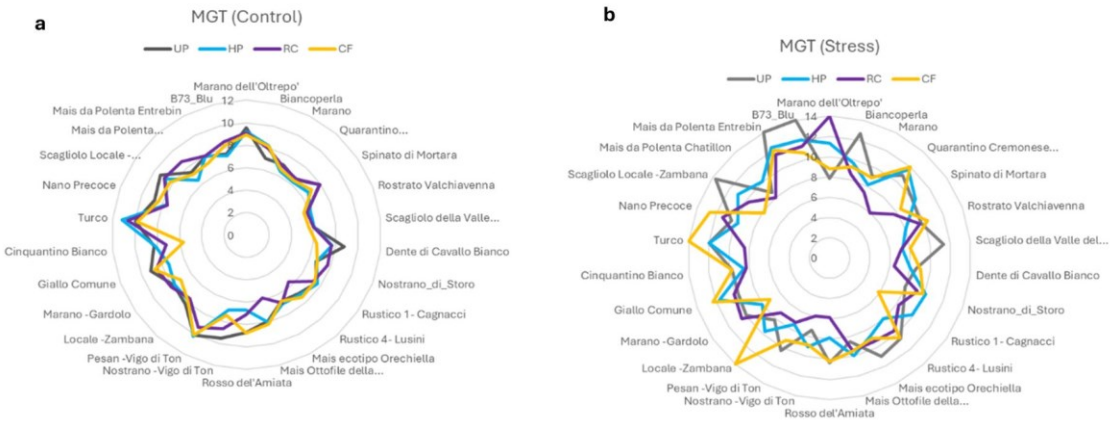

Figure 18. Plotted representation of calculated MGT (Mean Germination Time) values in the 26 maize varieties following priming and stress treatments. **(a)** Optimal (control) conditions. **(b)** Drought stress. HP, hydropriming; RC/CF, priming with red chicory or cauliflower extracts; UP, unprimed seeds.

Under no stress conditions, MGT values were relatively constant across treatments, with most genotypes showing moderate velocity. On average, RC and CF treatments tended to slightly reduce MGT compared to UP and HP, suggesting a mild enhancement in germination speed (**Fig. 18a**). For instance, Scagliolo della Valle del Ticino and Rostrato Valchiavenna showed the lowest MGT values following RC and CF priming, indicating faster germination. In contrast, genotypes like Turco and Pesan - Vigo di Ton had higher MGTs, particularly under HP, suggesting slower germination. These data indicates that even under optimal conditions, priming with RC and CF can offer a slight advantage in promoting quicker seedling emergence. Importantly, under stress conditions, the differences between treatments became more pronounced. Priming with RC emerged once more as the most effective treatment, as it reduced the MGT value in several genotypes, including Quarantino Cremonese Sciaretta (5.98 days) and Rosso del'Amiata (5.89

days), indicating a strong stress-mitigating effect (**Fig. 18b**). In contrast, UP and HP treatments often resulted in increased MGTs, reflecting delayed germination under stress. Notably, Biancoperla and Mais da Polenta Entrebin showed significantly higher MGTs in untreated seeds (12.67 and 14 days, respectively), while RC considerably reduced it. Priming with CF showed mixed results, being effective in some genotypes like Rustico 1 - Cagnacci (5.83 days), but less so in others like Locale - Zambana (14 days).

Overall, all the collected data regarding the morpho-physiological response to priming and stress, highlight the superior performance of RC extract in promoting rapid and uniform germination under drought stress imposed during germination.

4.3 Measurement of ROS molecules released from seeds

Following the characterization of varieties tolerant or sensitive to drought and responsive or non-responsive to priming, the next analyses focused on investigating why these responses are more present in some genotypes than others. Since priming with the red chicory extract emerged as the most effective treatment in enhancing germination performance under stress, we further investigated whether this effect could be linked to its antioxidant activity. To test this, the levels of H_2O_2, a key ROS molecule with dual roles in seed germination (acting both as a stimulus or stressor depending on its concentration), released from seeds were measured using the FOX-1 assay.

Measurements were taken at four time points (15, 30, 45, and 60 minutes) during early hydration from dry seeds, comparing unprimed (UP) seeds to seeds primed with RC extract (RC). The analysis was conducted across four maize varieties (**Fig. 19**): Marano and Mais di Polenta Chatillon, classified as drought-tolerant, and Dente di Cavallo Bianco and B73_Blu classified as drought-sensitive**.**

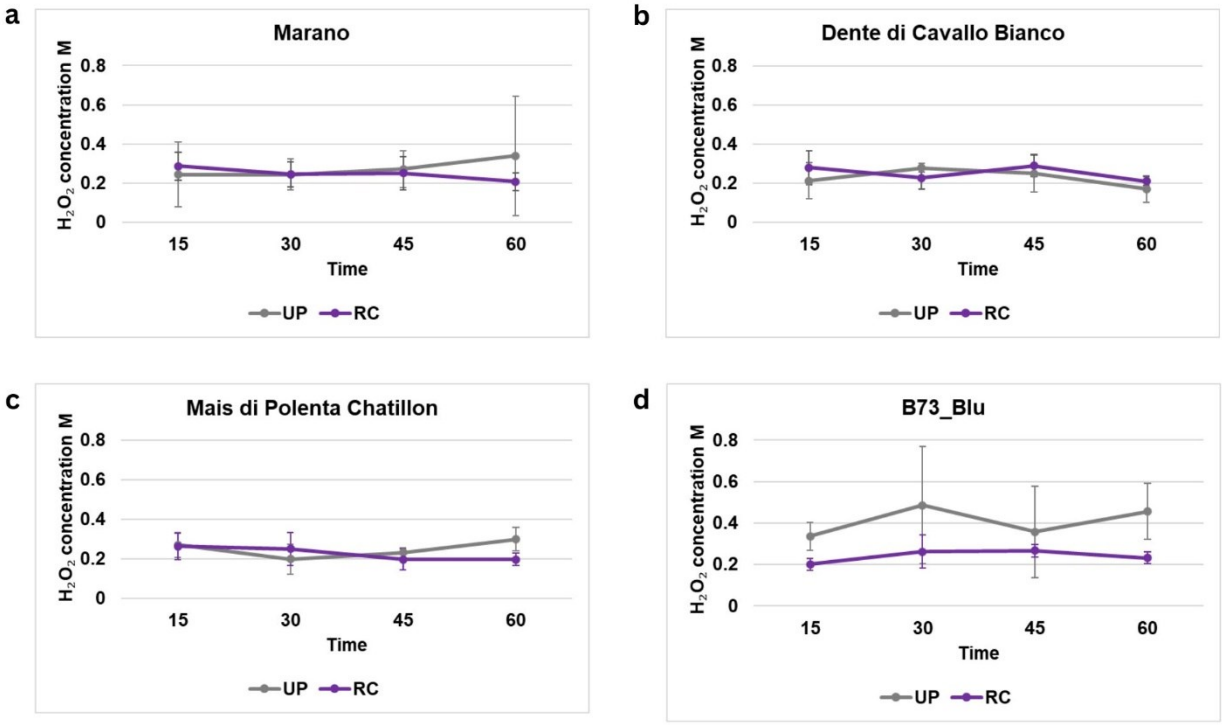

Figure 19. FOX-1 assay results showing the variation of hydrogen peroxide concentration [H_2O_2] in *Zea mays* varieties. **(a)** Marano. **(b)** Dente di Cavallo Bianco. **(c)** Mais di Polenta Chatillon. **(d)** B73_Blu. Data are represented as means ± SD of three replicates. Asterisks indicate statistically significant differences (*, $p \leq 0.05$) as determined through Student's *t*-test, using UP as control. UP, unprimed; RC, priming with red chicory extract.

In the UP seeds, the B73_Blu hybrid, identified as drought-sensitive, exhibited the highest ROS release levels, peaking at 60 min with 0.456 ± 0.136 M (**Fig. 19d**). Earlier points also showed elevated ROS release: 0.336 ± 0.067 M at 15 min and 0.486 ± 0.282 M at 30 min. This pattern indicates a strong oxidative burst, possibly correlated with susceptibility to stress. In contrast, Dente di Cavallo Bianco, also drought-sensitive but an Italian traditional landrace, showed the lowest H_2O_2 release across all time points, with levels declining from 0.210 ± 0.092 M at 15 min to 0.168 ± 0.067 M at 60 min (**Fig. 19b**). The drought-tolerant varieties, Marano and Mais di Polenta di Châtillon, exhibited intermediate H_2O_2 release levels. Marano showed a progressive increase, peaking at 60 min (0.339 ± 0.306 M), while Mais di Polenta reached 0.299 ± 0.059 M at 60 min (**Fig. 19a,c**).

Following priming with RC, a reduction in the levels of ROS release was observed in all genotypes, although this reduction did not appear to be statistically significant. In B73_Blu, H_2O_2 levels at 60 min dropped to 0.230 ± 0.028 M (**Fig. 19d**), nearly half of what observed in the UP seeds. At 15 min, B73_Blu primed seeds showed an even more pronounced effect, with levels reduced to just 0.200 ± 0.079 M compared to 0.336 ± 0.067 M in untreated seeds (**Fig. 19d**). Marano also responded positively to RC priming, showing a decrease from 0.339 ± 0.306 M (UP) to 0.210 ± 0.046 M (RC) at 60 min (**Fig. 19a).** Similarly, Dente di Cavallo Bianco and Mais di Polenta both exhibited lower H_2O_2 release after RC treatment. For example, Dente di Cavallo Bianco showed a reduction from 0.168 ± 0.067 M to 0.210 ± 0.023 M at 60 min, while Mais di Polenta dropped from 0.299 ± 0.059 M to 0.200 ± 0.031 M (**Fig. 19b,c**).

The antioxidant effect of RC was mostly observed in B73_Blu and Marano varieties after priming; it is thus possible to hypothesized that priming with RC may lower the H_2O_2 release due to the presence of antioxidant biomolecules in this extract. These results may indicate that RC priming effectively mitigates the release of ROS from dry seeds, supporting its potential as a pre-sowing treatment to reduce the oxidative stress status and possibly enhance seed vigor.

4.4. Expression of ROS-scavenging and drought-related genes in maize embryos

Building upon the observed differences in ROS release between primed and unprimed maize seeds, next, the expression of key antioxidant and drought-responsive genes were investigated in the four selected maize varieties: Marano, Dente di Cavallo Bianco, Mais di Polenta di Châtillon, and B73_Blu.

The list of genes used for this analysis are presented in **Table 5**. The *MSD 3.4* gene, encoding for the manganese superoxide dismutase 3.4 enzyme, is involved in the detoxification of superoxide radicals, protecting cells from oxidative damage. The *CAT1* (catalase 1) gene product is known to break down H_2O_2 into water and oxygen, thus maintaining redox balance. The APX1.1 (ascorbate peroxidase 1.1) is responsible for a reaction that reduces H_2O_2 using ascorbate. The *GST1* (Glutathione S-Transferase 1) gene encodes an enzyme that conjugates glutathione to toxic compounds, aiding in detoxification and stress signaling. The *TRPP1* (Tonoplast Intrinsic Protein 1) gene is involved in water transport across the tonoplast, maintaining cellular water balance. The *DREB2A* (Dehydration-Responsive Element-Binding Protein 2A) gene encodes a

transcription factor (TF) that activates stress-responsive genes under dehydration and salinity conditions. The *PMP3g* (Plasma Membrane Protein 3g) gene is involved in the drought stress tolerance mechanisms by maintaining membrane potential and ion homeostasis. The LEA1 (Late Embryogenesis Abundant Protein 1) gene function related to the stabilization of proteins and membranes during desiccation, aiding in seed maturation and stress protection.

Table 5. Functional Classification of Selected Genes for qPCR Analysis

Gene Symbol	Full Name	Functional Category	Biological Role
MSD 3.4	Manganese Superoxide Dismutase 3.4	Antioxidant	Detoxifies superoxide radicals in mitochondria, protecting cells from oxidative damage.
CAT1	Catalase 1	Antioxidant	Breaks down hydrogen peroxide into water and oxygen in peroxisomes, maintaining redox balance.
APX1.1	Ascorbate Peroxidase 1.1	Antioxidant	Reduces hydrogen peroxide using ascorbate in the ascorbate-glutathione cycle.
GST1	Glutathione S-Transferase 1	Antioxidant	Conjugates glutathione to toxic compounds, aiding in detoxification and stress signaling.
TRPP1	Tonoplast Intrinsic Protein 1	Drought Tolerance	Facilitates water transport across the tonoplast, maintaining cellular water balance.
DREB2A	Dehydration-Responsive Element-Binding Protein 2A	Drought Tolerance	Activate stress-responsive genes under dehydration and salinity conditions.
PMP3g	Plasma Membrane Protein 3g	Drought Tolerance	Maintains membrane potential and ion homeostasis during osmotic stress.
LEA1	Late Embryogenesis Abundant Protein 1	Drought Tolerance	Stabilizes proteins and membranes during desiccation, aiding in seed maturation and stress protection.

To guide tissue selection for gene expression analysis, a transcriptomics data mining approach was carried out on the Maize eFP Browser (https://bar.utoronto.ca). The decision to focus on embryo tissue was supported by the observation that our genes of interest showed higher expression levels in the embryo compared to other seed compartments (**Fig. 20**). While tissues like the scutellar aleurone layer and pericarp showed relatively high average expression levels, the embryo exhibited consistent expression across multiple genes, including *APX1.1* (0.49), *MSD2.3* (0.65), and *GST1* (0.49). This uniformity suggests a coordinated regulatory environment, making the embryo a biologically relevant tissue for validating gene expression patterns.

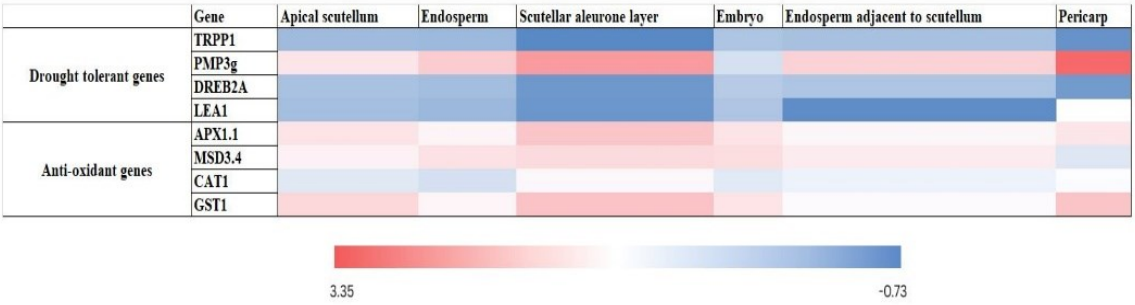

Figure 20. Gene isoform expression across maize seed tissues. The gene expression data were obtained from the Maize eFP Browser, part of the Bio-Analytic Resource for Plant Biology (BAR) at the University of Toronto (https://bar.utoronto.ca).

Therefore, based on these preliminary data, the embryo was chosen for qRT-PCR experiments**.** The obtained data are presented as relative expression values, normalized to the *18s rRNA* reference gene and visualized through a heatmap (**Fig. 21**), The data was grouped into two functional categories related to antioxidant-related genes (*MSD2.3, CAT1, APX1.1, GST1*) and drought-tolerance-related genes (*TRPP1, DREB2A, PMP3g, LEA1*).

Among antioxidant genes (**Fig, 21a**), *APX1.1* showed higher expression levels in B73_Blu and Dente di Cavallo Bianco, compared to Mais di Polenta di Châtillon and Marano, with some differences confirmed by Tukey's test ($p \leq 0.05$). Although the expression in Dente di Cavallo Bianco was lower than in B73_Blu, it was still markedly elevated relative to the drought-tolerant landraces, suggesting that both sensitive varieties may rely on early activation of antioxidant mechanisms. This trend was mirrored in the *MSD3.4* expression, which was highest in B73_Blu, followed by Polenta di Châtillon, Marano and Dente di Cavallo Bianco. These findings may suggest that the *APX1.1* and *MSD3.4* genes could serve as potential indicators of stress sensitivity in the investigated maize materials.

For the drought-related genes (**Fig. 21b**), *PMP3g* expression was elevated in both B73_Blu and Dente di Cavallo Bianco, with significantly lower values in Polenta di Châtillon and Marano. Given the membrane-protective role of PMP3-type family mambers, this higher expression suggests an anticipatory stress response in both sensitive genotypes. The shared pattern between B73_Blu and Dente di Cavallo Bianco further reinforces their classification as more responsive to drought stimuli at the transcript level. However, the overall expression profile of Dente di

Cavallo Bianco characterized by moderately elevated *APX1.1*, *PMP3g*, and notably high *DREB2A* expression, may indicate that it is less drought-sensitive than B73_Blu, possibly due to a more effective or balanced stress response system.

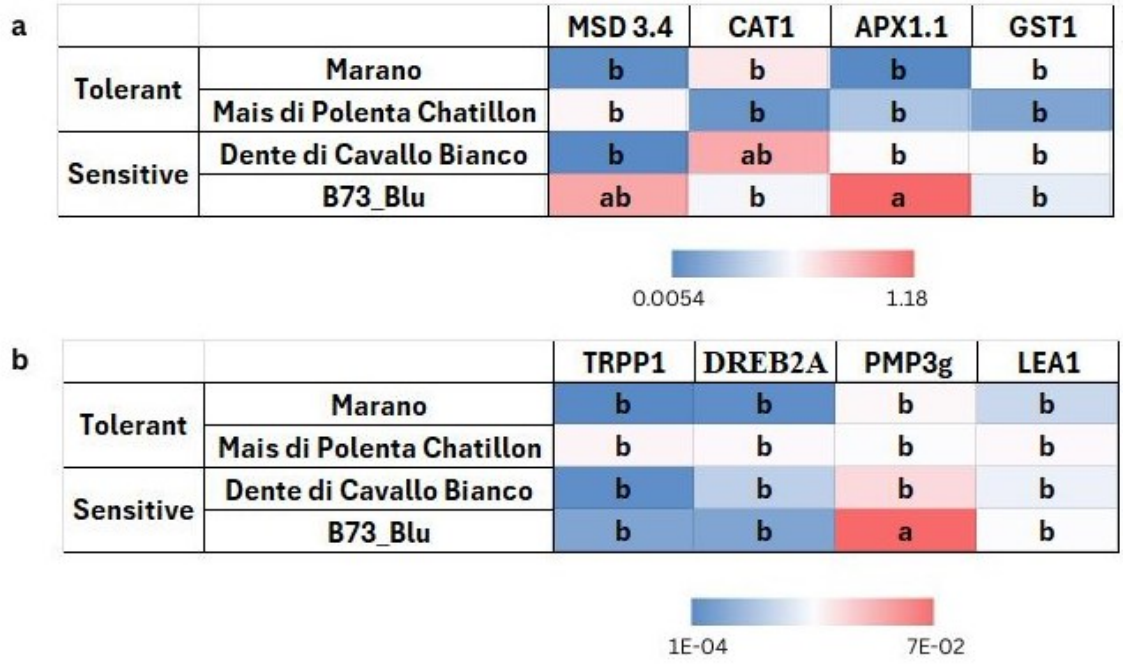

Figure 21. Heatmap showing the variation in gene expression in four *Zea mays* varieties (Marano, Dente di Cavallo Bianco,Mais di Polenta Chatillon &B73_Blu). The values are normalized on the reference gene *18s rRNA*. **(a)** Relative expression of antioxidant genes *MSD3.4* (Mn superoxide dismutase 3.4), *CAT1* (catalase 1), *APX1.1* (ascorbate peroxidase 1.1), and *GST* (glutathione S-transferase). **(b)** relative expression of drought tolerance-related genes *TRPP1* (trehalose-6-phosphate phosphatase), *DREB2B* (dehydration-responsive element-binding 2B), *PMP3g* (plasma membrane proteolipid 3g), *LEA1* (late embryogenesis abundant 1). Statistical significance ($p \leq 0.05$) is indicated by the occurrence of different letters, as determined through one-way ANOVA and TUKEY test.

In contrast, the drought-tolerant landraces Marano and Mais di Polenta di Châtillon showed consistently lower expression across nearly all genes, including *TRPP1*, *DREB2A*, and *LEA1*. For example, *DREB2A* gene amplicon was nearly absent in Polenta di Châtillon and remained low in Marano, suggesting that these genotypes may not rely on the prevemptive transcriptional activation of this TF under non-stressed conditions. Similarly, *LEA1* expression was highest in Dente di Cavallo Bianco further supporting its capacity for stress preparedness.

Overall, the data suggest that elevated expression of *APX1.1* and *PMP3g* in dry seeds may serve as early indicators of drought sensitivity or stress responsiveness, as seen in both B73_Blu and Dente di Cavallo Bianco. However, the relatively moderate expression in Dente di Cavallo Bianco, particularly across antioxidant and drought-responsive genes, points to a less extreme sensitivity compared to B73_Blu. These findings highlight the complex transcriptional landscape of drought responses and underscore the importance of evaluating both gene expression magnitude and genotype context.

5. Discussion

Climate change is increasingly disrupting global agricultural systems through altered precipitation patterns and rising temperatures, which intensify abiotic stresses such as drought. These environmental challenges are particularly detrimental to crops like maize, since its productivity is highly related to water availability (Pulighe et al., 2024). Drought stress during early developmental stages, especially germination, can severely impair seedling establishment and ultimately reduce yield (Regmi et al., 2024). While conventional breeding and genetic engineering have contributed to improving drought tolerance, these approaches are often time-consuming, resource-intensive, or constrained by regulatory frameworks. In this context, seed priming has emerged as a promising, low-cost strategy to enhance plant resilience by preconditioning seeds to better withstand environmental stress (Aswathi et al., 2022).

In this work of thesis, the drought tolerance profiles of 26 maize genotypes were evaluated. These included a diverse panel of Italian landraces and the widely used inbred line B73_Blu, which were screened using the Germination Stress Tolerance Index (GSTI) under soil-based drought conditions. The obtained results revealed a high variability in drought response, with only 11.5% of genotypes, namely Nostrano di Storo, Marano, and Mais da Polenta Chatillon, surpassing the 20% GSTI threshold. This finding aligns with previous studies showing that early-stage drought resilience is often governed by conserved physiological traits and is not uniformly distributed across germplasm (Liu et al., 2022). For instance, Nostrano di Storo originates from Trentino-Alto Adige, a region characterized by variable rainfall patterns that may have selected for drought-adaptive alleles. Similar findings were reported by Marone et al. (2021), who observed that landraces from marginal environments often show enhanced abiotic stress tolerance. This suggests that environmental pressures in such regions may drive the selection of genotypes with superior resilience traits, which can be harnessed in breeding programs.

Notably, the superior performance of traditional landraces compared to B73_Blu (GSTI: 0%) underscores the value of genetic diversity in breeding programs aimed at enhancing stress resilience (Dube et al., 2023). Moderate tolerance observed in genotypes such as Spinato di Mortara (18.64%) and Dente di Cavallo Bianco (17.86%) further suggests the presence of partial adaptive mechanisms that could be harnessed through targeted selection (Ribaut et al., 2009).

To explore whether seed priming could enhance drought tolerance, three treatments were tested: hydropriming (HP), and priming with cauliflower (CF) or red chicory (RC) extracts. Notably, the extracts used in this study were obtained from agricultural by-products, demonstrating a practical application of circular bioeconomy principles. Valorizing agri-food waste through the extraction of bioactive compounds not only reduces environmental impact but also generates high-value inputs for sustainable agriculture (Pal et al., 2024; Pomoni et al., 2024). This approach supports a closed-loop system where waste is repurposed into functional products, contributing to both ecological sustainability and economic resilience.

Among the extracts used in this work of thesis, RC consistently outperformed the CF, significantly improving GSTI in seven genotypes and reducing mean germination time (MGT) under stress. For instance, Dente di Cavallo Bianco reached a GSTI of 33.33% with RC, while Quarantino Cremonese Sciaretta exhibited a shortened MGT of 5.98 days compared to 8.7 days in unprimed seeds. This is aligned with research where plant derived extracts help to mitigate abiotic stresses (Di Sario et al., 2025). The superior performance of RC is likely due to its rich content of polyphenols and other bioactive compounds, such as anthocyanins and chlorogenic acids, which are known to modulate ROS levels and stabilize cellular structures under stress conditions (Papetti et al., 2017). This mechanism could be compared to the action of melatonin, a well-studied plant biostimulant. Melatonin has been shown to enhance drought tolerance in maize by stimulating antioxidant enzyme activity (e.g., SOD, CAT, APX), maintaining stomatal conductance, and regulating ABA metabolism to prevent excessive ROS accumulation and chlorophyll degradation (Li et al., 2021). Similarly, in the current work of thesis, priming with RC extracts significantly reduced the release of H_2O_2 from seeds in several genotypes, and specifically in B73_Blu.

HP showed more limited but still consistent benefits, improving GSTI in three varieties (Dente di Cavallo Bianco, Rustico 4- Lusini & Mais Ottofile della Garfagnana), suggesting a role in water-mediated metabolic activation. While CF priming proved highly effective in Dente di Cavallo Bianco (GSTI: 39.02%), it failed to enhance germination performance in most genotypes, suggesting a narrow spectrum of compatibility. This variability may be attributed to differences in genotype sensitivity or uptake of signaling molecules. According to Doria et al. (2022), cauliflower extracts contain a complex mixture of bioactive compounds, including

glycoalkaloids, glucosinolates, and phenolic acids, which are known for their antifungal, antibacterial, and allelochemical properties. While these metabolites can confer protective effects, their concentration-dependent activity may also induce stress responses in seeds, particularly if present at phytotoxic levels. This dual nature could explain the inhibitory effects observed in some genotypes, where the extract may have acted more as a stressor than a stimulant. These findings highlight the importance of optimizing extract composition and dosage to ensure compatibility with specific genotypes and avoid unintended negative effects.

To investigate the basis of the observed responses, the amount of ROS (specifically H_2O_2) released from seeds was measured using the FOX-1 assay. Priming with RC appeared to slightly reduce the release of these molecules in the four investigated accessions, though the results were not statistically significant. The most pronounced effect was observed in the drought-sensitive B73_Blu variety. This possible antioxidant effect was also evident in moderately tolerant and tolerant landraces such as Marano and Mais da Polenta Chatillon, which maintained lower ROS release levels under stress. Interestingly, Dente di Cavallo Bianco exhibited low basal ROS levels yet showed strong priming responsiveness, suggesting that RC may confer additional benefits beyond ROS scavenging, such as osmotic adjustment or enhanced membrane integrity (Gill & Tuteja, 2010)

By analyzing the expression trends in a subset of genes selected for their roles in the antioxidant and drought response, it can be suggested that *APX1.1* and *PMP3g* may serve as early indicators of drought sensitivity in maize. Notably, both B73_Blu and Dente di Cavallo Bianco, classified as drought-sensitive, exhibited elevated expression of *PMP3g*, a gene linked to membrane stabilization during osmotic stress (Lie et al., 2023). The *APX1.1* gene, associated with cellular protective responses, also showed marked upregulation in these varieties, particularly in B73_Blu. Although Dente di Cavallo Bianco demonstrated slightly lower expression than B73_Blu, its gene activation profile suggests it may be less sensitive which proved earlier. By contrast, the drought-tolerant landraces Marano and Mais di Polenta di Châtillon maintained lower and more consistent expression across all drought-related genes, including *TRPP1, LEA1*, and *DREB2A*. This more balanced expression may reflect an inherent stress resilience that reduces reliance on early transcriptional activation mechanisms.

The genotype-specific nature of priming responses was particularly evident in all the types of analyses carried out in this work of thesis. Dente di Cavallo Bianco responded positively to all three treatments, suggesting the presence of unique stress-adaptive pathways, potentially linked to its high basal expression of antioxidant genes such as *APX1.1* and *CAT1,* both critical for maintaining ROS homeostasis. While *APX1.1* encodes the ascorbate peroxidase enzyme involved in H_2O_2 detoxification in the cytosol, *CAT1* encodes for the catalase enzyme which breaks down H_2O_2 into water and oxygen in peroxisomes (Mittler et al., 2004). Conversely, genotypes like Turco and Locale-Zambana showed minimal improvement (<10% GSTI), indicating a lack of priming-sensitive triggers.

Taking together, these results indicate that seed priming, particularly with red chicory extract, is an effective strategy to enhance drought tolerance in maize at the germination stage. The observed improvements in germination performance, ROS homeostasis, and gene expression are strongly genotype-dependent, highlighting the importance of optimizing priming treatments to specific genetic backgrounds. Moreover, the superior performance of traditional landraces under both physiological and molecular assessments underscores their potential as valuable genetic resources for breeding programs targeting climate resilience.

Further studies, including a more detailed molecular characterization of these responses along with testing the efficiency of the priming methods under field conditions, will be necessary to validate these results.

6. Conclusion

The present work of thesis provides compelling evidence that seed priming, particularly with plant-derived biostimulants such as red chicory and cauliflower extracts, can significantly enhance drought tolerance in maize during the germination stage. By evaluating 26 genetically diverse maize varieties under both optimal and drought-stressed conditions, it was demonstrated that priming treatments not only improve germination rates and seedling vigor but also mitigate the adverse effects of water deficit in a genotype-dependent manner.

Among the treatments tested, red chicory extract (RC) emerged as the most effective, enhancing the Germination Stress Tolerance Index (GSTI) in the highest number of varieties and eliciting unique responses in several genotypes. This suggests that RC may play a role in modulating different pathways related to stress perception and response, possibly through the regulation of ROS homeostasis and activation of antioxidant defense mechanisms. Hydropriming (HP) showed consistent but more limited benefits, while cauliflower (CF) extract demonstrated variable effects, highlighting the importance of optimizing priming agents to specific genotypes.

The universal responsiveness of Dente di Cavallo Bianco to all priming treatments suggests the presence of unique stress-adaptive traits, making it a valuable candidate for further physiological and molecular studies.

Importantly, this work supports the use of sustainable, low-cost priming agents derived from agricultural waste, offering an environmentally friendly alternative to synthetic chemicals. Such practices not only reduce ecological impact but also contribute to circular economic principles in agriculture.

To build on these findings, future research should delve deeper into the biochemical and molecular mechanisms underlying priming-induced stress tolerance, with particular attention to ROS dynamics and the expression of antioxidant-related genes. Ultimately, combining seed priming with other sustainable agricultural practices can contribute to the development of a more resilient and productive cropping system, one that is better equipped to face the challenges of climate change and water scarcity, while supporting food security and environmental sustainability.

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