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PhD Thesis

Enhancement of cold tolerance and growth of tomato plants using endophytic bacteria isolated from cold-adapted plants

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ABSTRACT

Cold stress is a key abiotic factor that adversely impacts crop production. Climate change is responsible for warm winter periods that allow crop production under partial protection, such as commercial greenhouses and tunnels used for tomato production in Sicily. However, temperature conditions are not constant under commercial greenhouse conditions, and cold stress damage can occur. Microbial communities associated with plants in cold environments can enhance plant growth at low temperatures and boost their tolerance to cold stress. Thus, the use of cold-tolerant endophytic bacteria could be a promising approach to protect crop plants from cold stress. This work aims to investigate the effect of bacterial endophytes isolated from wild cold-adapted alpine plants on the tolerance of tomato seedlings to cold stress. A total of 41 cold tolerant endophytic bacterial isolates collected from the roots of three cold-adapted *Rosaceae* plants were tested for their growth-promoting ability by root inoculation on tomato plants. Seedlings were grown at 25°C under controlled conditions, and the most promising isolates were selected according to their ability to increase dry and fresh plant biomass. Moreover, the ten best-performing plant growth-promoting isolates were tested for their ability to promote tomato growth at 10°C. In particular, two bacterial isolates, *Chryseobacterium* sp. GRCS301 (*Chryseobacterium* sp.) and *Pseudomonas* sp. GRCS202 (*Pseudomonas* sp.), were able to improve tomato growth under cold stress conditions, reducing the content of H_2O_2 and modulating the expression of cold stress-related genes. RNA-Seq was then performed at two different time points, one and 14 days after stress (DAS), and gene ontology analysis was carried out to detect differences in gene modulation related to bacterium inoculation under cold conditions. Genes modulated by cold stress in mock-inoculated and bacterium-inoculated plants showed the regulation of response to acid chemicals at both time points. Moreover, *Chryseobacterium* sp. inoculation showed the upregulation of gene pathways associated with osmotic and oxidative stress

protection at 1 DAS, while *Pseudomonas* sp. inoculation caused up-regulation of gene pathways associated with osmotic stress and calcium ion transporting, indicating that treatments with cold tolerant bacteria can boosts early and late cold-stress response. Cold tolerant bacteria were also tested under commercial greenhouse conditions (ITAKA Crop Solutions, Ragusa, Italy; partner of the PON project) greenhouse conditions and one bacterial isolate (*Pseudomonas* sp. GRCS202) confirmed the plant growth promotion activity (enhanced root dry weight compared to mock-inoculated plants) during winter tomato production. Cold tolerant bacteria isolated from Alpine environments represent a valuable resource to enhance plant growth and resilience to cold stress in crop plants.

CHAPTER 1. INTRODUCTION

1.1 Food and fertilizers supply in a growing-population world

Agriculture in the 21st century faces multiple challenges: it has to produce more food to feed a growing population with a smaller rural labor force, more feedstocks for a potentially huge bioenergy market, contribute to overall development in the many agriculture-dependent developing countries, adopt more efficient and sustainable production methods and adapt to climate change (FAO, HLEF2050_Global_Agriculture, 2009). Among these challenges, a growing population might be considered the root of the others. More population means more food, but also more energy, and consumption that exacerbate the ongoing climate crisis to which agriculture has to deal with. The global human population reached 8.0 billion in mid-November 2022 from an estimated 2.5 billion people in 1950, adding 1 billion people since 2010 and 2 billion since 1998. The world's population is expected to increase by nearly 2 billion persons in the next 30 years, from the current 8 billion to 9.7 billion in 2050 and could peak at nearly 10.4 billion in the mid-2080 (Nations, n.d.) (Figure 1.1).

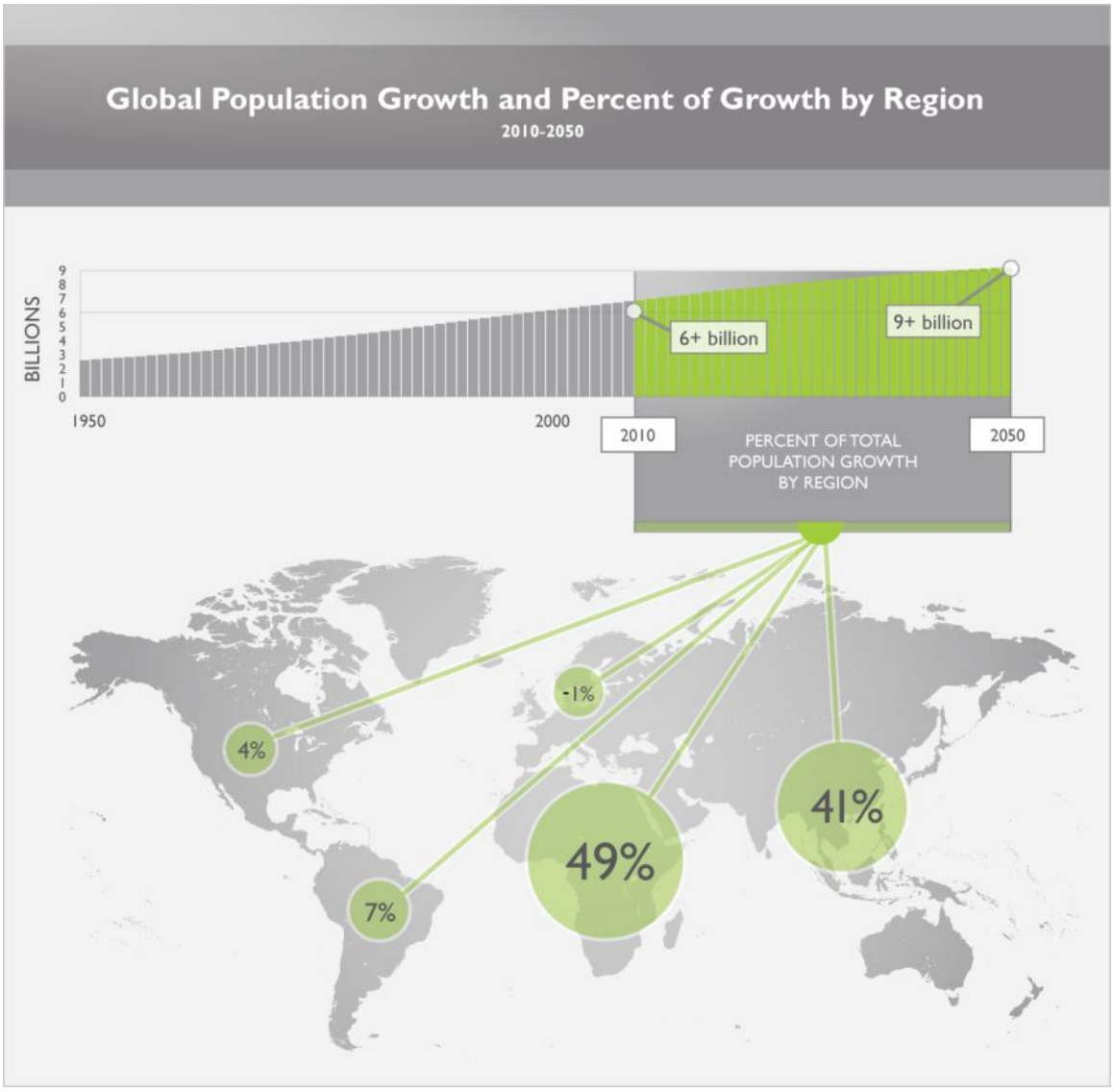


Figure 1.1. Global population growth by regions 2010 – 2050. (Feeding the world in 2050 and beyond – Part 1: Productivity challenges, 2018)

In particular, population increase results to be asymmetric through the world (Figure 1.2). The population in the developing countries will be roughly 8 billion (Feeding the world in 2050 and beyond – Part 1: Productivity challenges, 2018). The population in the developed countries would be 1.2 billion (Feeding the world in 2050 and beyond – Part 1: Productivity challenges, 2018). It is apparent that all of the population growth is taking place in the developing countries. In particular,

Asia will contribute a staggering 41% and Africa 47% towards this growth in 2050 (Feeding the world in 2050 and beyond – Part 1: Productivity challenges, 2018). Due to the growing population, the total global food demand is expected to increase by 35% to 56% between 2010 and 2050, while the population at risk of hunger is expected to change by −91% to +8% over the same period *(*van Dijk *et al.*, 2021). If climate change is taken into account, the ranges change slightly (+30% to +62% for total food demand and −91% to +30% for population at risk of hunger) (van Dijk *et al.*, 2021). More specifically, van Dijk *et al.* carried out a deep analysis of 57 global food security projections and quantitative scenario studies to address the questions of how to eradicate global hunger and feed the future world population. They studied how the distribution of food security outcomes is influenced by varying assumptions on key drivers and differences in methods. To understand these factors, projections were mapped to the Shared Socio-economic Pathways (SSPs) and Representative Concentration Pathways (RCPs), a combination of socio-economic and climate change scenarios, frequently used in global assessments. The study analyzed per capita food demand and population at risk of hunger to better delineate future food security. They found that nearly all SSP scenarios project an increase in per capita and total food consumption compared to 2010 levels, with variations depending on the considered scenario. For example, in future worlds that are characterized by fragmentation (SSP3) and inequality (SSP4), per capita consumption will increase by 4% to 7%, while in scenarios that assume sustainability (SSP1), business-as-usual development (SSP2) and rapid growth (SSP5), the increase is 12% to 16%. Taking population growth into account, the total food consumption increase is the lowest in SSP1 (+41%) and the highest in SSP2 (+51%) (Figure 2). Figure 2 also shows the climate change impact on food demand: the ranges of food demand projections change slightly when considering extreme climate change (RCP8.5) versus no climate change (NOCC), but no significant differences were found. If the uncertainty related to climate change is also taken into account, the ranges of per capita (−1% to +20%) and total food demand (+30% to +62%)

projections change slightly. Similar considerations were made when it comes to determining population at risk of hunger. The projected change in population at risk of hunger lies between −91% and +8% for NOCC projections and between −91% and +30% for climate change projections, although with no evidence that climate change projections are statistically different from NOCC projections.

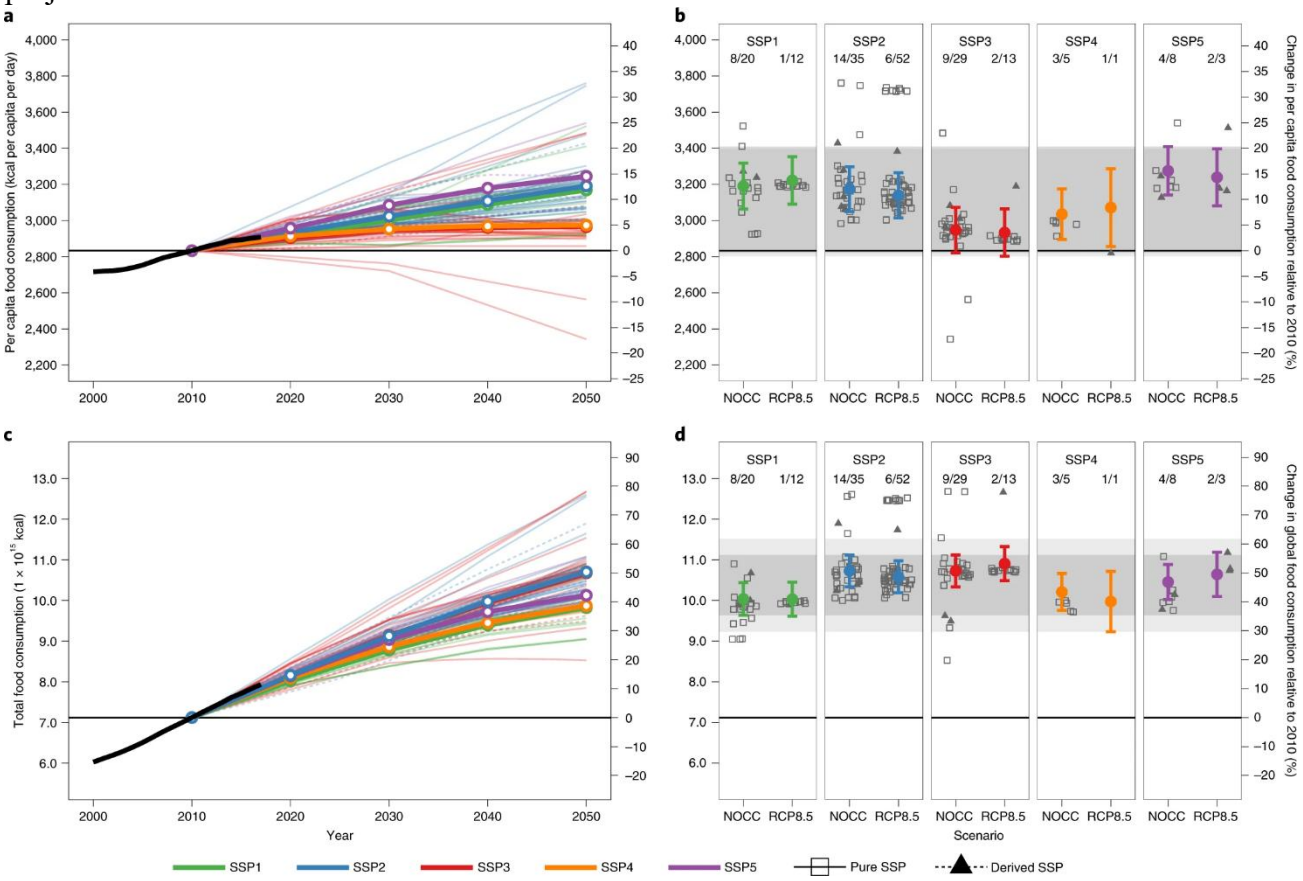


Figure 1.2. Per capita and total food consumption baseline projections for 2010–2050. a,b, Per capita food consumption baseline projections. c,d, Total food consumption baseline projections. All figures show the level of the selected food security indicator (left y axis) and the percentage increase for the period 2010–2050 (right y axis). Panels a and c show the baseline projections for the SSPs under NOCC (thin colored lines), the average for each SSP (thick colored lines with circles) and the three-year average historical trend (thick black line). Panels b and d present point estimates and 95% confidence intervals taken from the meta-regression as well as all observations in 2050, comparing NOCC projections with projections based on the most extreme climate scenario (RCP8.5). The numbers at the top refer to the number of studies/number of projections in the figure. The dark and light grey shaded areas demarcate the plausible range of baseline projections using the 95% confidence interval across all NOCC SSP and all RCP SSP projections, respectively (Extended Data Fig. 4). Pure SSPs are projections that take their assumptions from the SSPs, where relevant combined

with RCP-based climate impact scenarios. Derived SSPs are projections that belong to the same SSP and RCP scenario families but use somewhat different assumptions (van Dijk *et al.*, 2021).

If food demand seems to be more impacted by the number of people rather than climate change, climate change is impacting and will impact the agricultural sector, and crop productivity will be affected dramatically in the next few decades due to variations in integral abiotic factors such as temperature, solar radiation, precipitation, and CO_2 (Abbass *et al.*, 2022).

Agriculture should face also the soil problem, since almost 15 billion tons of fertile soil is lost every year due to anthropogenic activities and climate change (Arora, 2019). Modern agriculture is dependent on the chemical pesticides and fertilizers (Prashar and Shah, 2016) that remarkably raised food production in the past decades. However, chemical fertilizers and pesticides do positively affect the soil properties in terms of nutrient content, predominant soil species, structural and functional diversity of microbial populations, and activities of soil-enzymes (Prashar and Shah, 2016). Moreover, inadequate global management of fertilization produces areas with serious nutrient deficits in croplands linked with insufficient access to fertilizers that clearly limit food production, and areas that are overfertilized with the consequent problems of environmental pollution affecting human health (Penuelas *et al.*, 2023). Thus, proper management of fertilizers has a direct impact on crop efficiency and productivity (Randive *et al.*, 2021). However, current winds of global climate change have been posing unprecedented challenge before the agriculture sector and thereby on the fertilizer industry (Randive *et al.*, 2021). Chemical fertilizers are sources of the main macronutrients for plants, such as nitrogen, potassium, phosphorus, micronutrients, and additives (Pahalvi *et al*., 2021). Nitrogen (N), phosphorous (P), and potassium (K) are also macronutrients which form structural constituents of building block materials (N P), or regulator or activator of enzymes (K) (Tewari *et al.*, 2021). These nutrients can be found as straight fertilizers, which means that they have a declarable content of only

one of the three primary nutrients (e.g. N: urea, ammonium sulphate, ammonium nitrate; P: superphosphates; K: potassium chloride) (FAO, 2024). Other fertilizers are compound fertilizers, which means that they have a declarable content of more than one of the three primary plant nutrients (e.g., NP: diammonium phosphate, NK: potassium nitrate; all three nutrients: NPK fertilizers) (FAO, 2024). In 2022, agricultural use of inorganic fertilizers globally fell by 7% compared to 2021, to 185 Mt of nutrients (Figure 1.3). Each of the three main plant nutrients showed declines from the previous years: the strongest one was for potassium (−12 percent, to 35 Mt), followed by phosphorus (−8 percent, to 42 Mt), and nitrogen use (−4 percent, to 108 Mt).

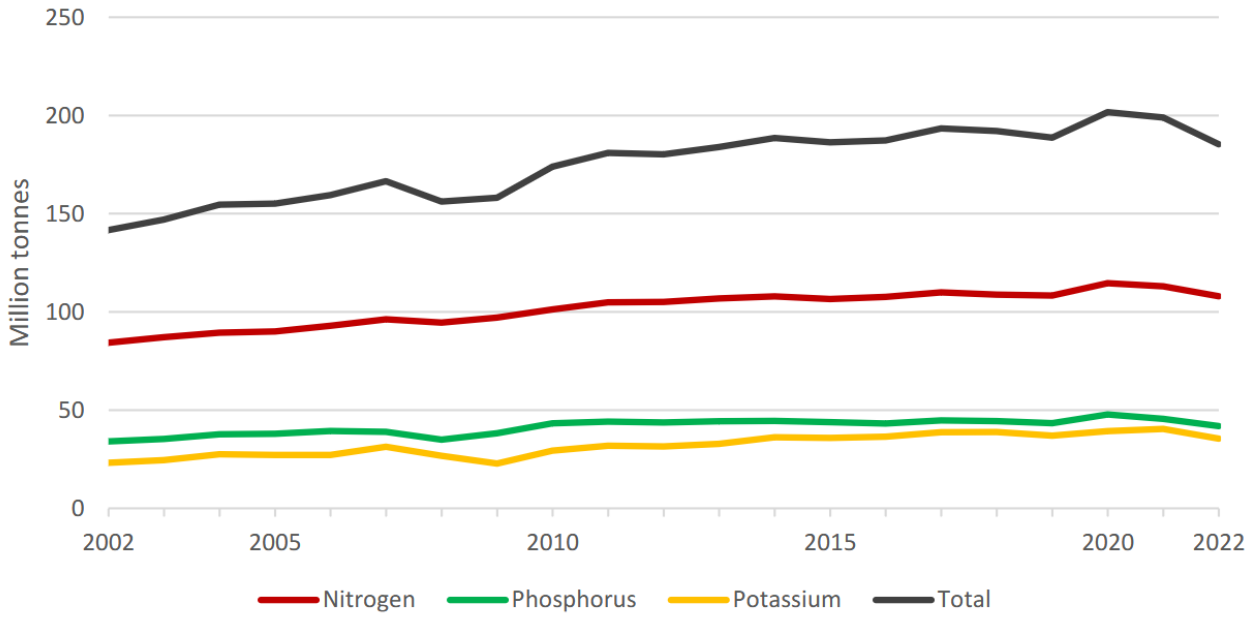


Figure 1.3. World agricultural use of inorganic fertilizers by nutrient (as N, P2O5 and K2O) (FAO, 2024).

Between 2002 and 2022, the world's agricultural use of fertilizers per area of cropland increased from 90 kilograms per hectare of cropland (kg/ha) to 113 kg/ha (Figure 1.4), indicating an overall intensification of agricultural production (FAO, 2024). At the same time, inorganic fertilizer use per value of agricultural production indicated possible improvements in fertilizer use efficiency,

decreasing from 53 kg per thousand international dollars (kg/1000 I$) to 44 kg/1000 I$, while fertilizer use per capita remained roughly constant at around 23 kg per capita (FAO, 2024).

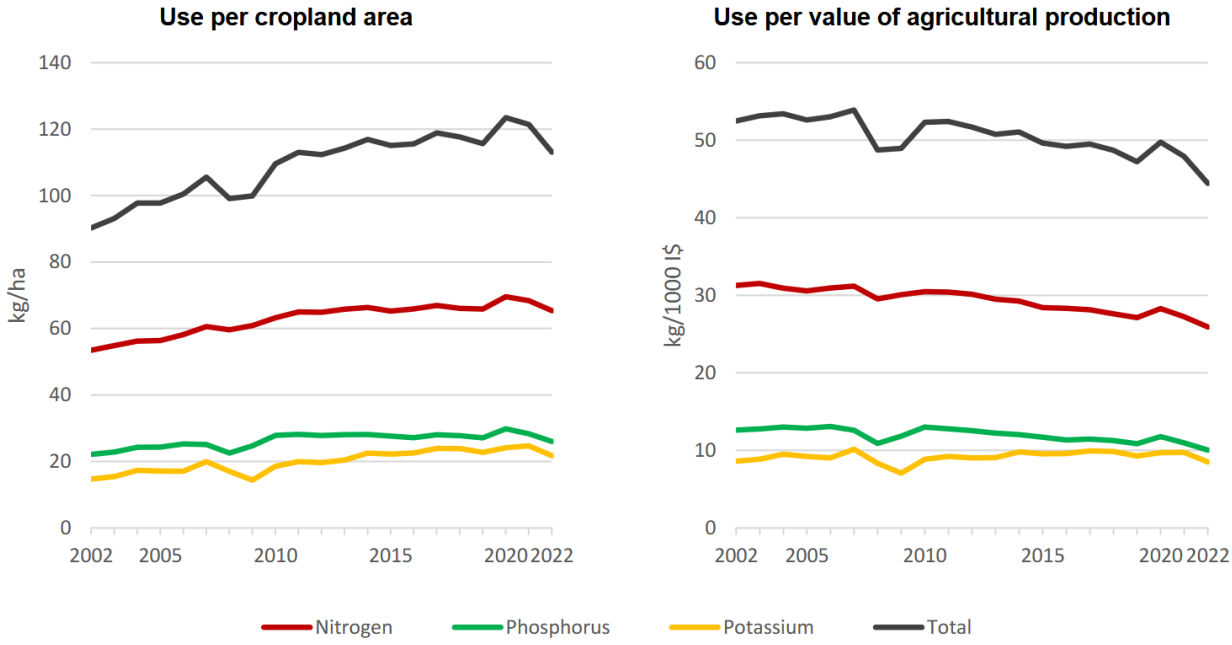


Figure 1.4. Inorganic fertilizers agricultural use indicators by nutrient (FAO, 2024).

If it true that the use of fertilizers per cropland area highlights differences in agricultural systems across the regions (FAO, 2024), it is also true that nitrogen, phosphorus, and potassium production is held by few country worldwide (Figure 1.5).

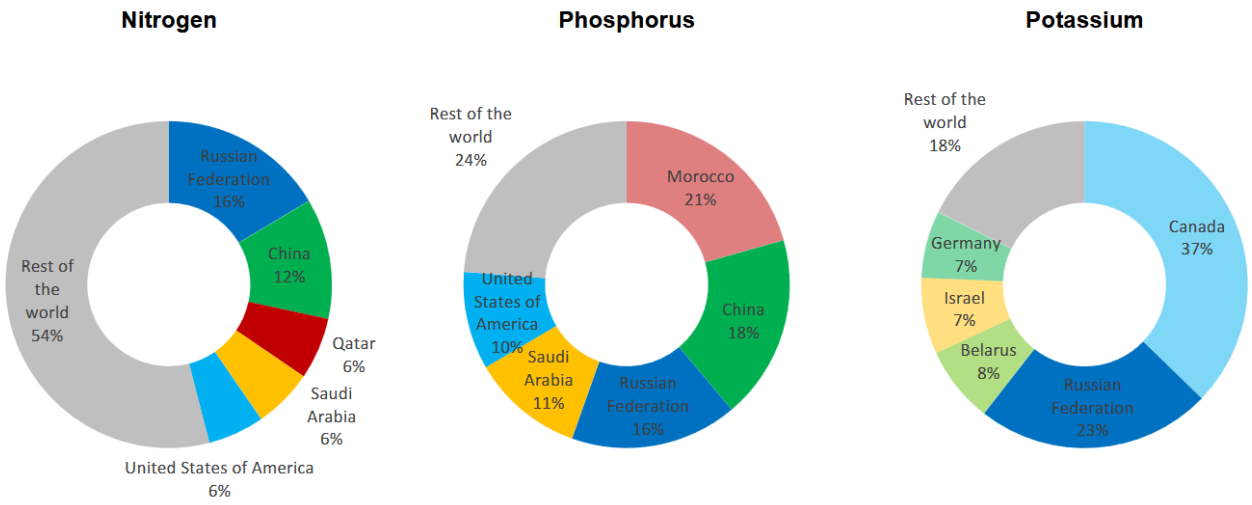


Figure 1.5. Fertilizers exports by nutrient, top countries (2022) (FAO, 2024).

In spite fertilizer affordability was much improved in the first half of 2024 compared to the previous 18 months, fertilizers remain exposed to global risks spanning geopolitics, conflict, economics and climate (IFA, 2024). Macroeconomic drivers remain highly influential as inflation and high interest rates have reduced borrowing power for producers and consumers alike (IF,2024). Moreover, continuous use of chemical fertilizers is responsible for the decline of soil organic matter content coupled with a decrease in the quality of agricultural soil (Pahalvi *et al*., 2021). The overuse of chemical fertilizers hardens the soil, reduces soil fertility, pollutes air, water, and soil, and lessens important nutrients of soil and minerals, thereby bringing hazards to environment. Sole utilization of chemical fertilizers led to weak microbial activity in the cropping system (Pahalvi *et al.*, 2021). It is crucial to investigate new and local solutions which are less dependent on the geopolitics of large-scale producers and, at the same time, ensure respect for soil and biodiversity. It is in this panorama, that strategies such as the Green Deal raise up. Among the goals, emphasis is also placed on have healthy soils in the EU by 2050, reducing the use of pesticides and herbicides in favor of more sustainable ways of treating crops (Agriculture and the Green Deal - European Commission, n.d.).

1.2 Climate change and abiotic stresses

Climate change refers to long-term shifts in temperatures and weather patterns (Nations, n.d.). The average temperature of the Earth's surface is now about 1.2°C warmer than it was in the late 1800s (before the industrial revolution) and warmer than at any time in the last 100,000 years (Nations, n.d.). The last decade (2011-2020) was the warmest on record, and each of the last four decades has been warmer than any previous decade since 1850 (Nations, n.d.). The consequences of climate change now include, among others, intense droughts, water scarcity, severe fires, rising sea levels, flooding, melting polar ice, catastrophic storms, and declining biodiversity (Nations, n.d.). Alterations in critical abiotic parameters such as temperature, precipitation, solar radiation, and CO_2 emissions are poised to substantially affect crop yields over the coming decades (Terán *et al.*, 2024). According to the FAO report "The impact of disasters on agriculture and food security 2023" , on a global scale, extreme temperatures and droughts are the hazards that exert the most significant impact per event, followed by floods, storms and wildfires (Figure 1.6) (The impact of disasters on agriculture and food security 2023, 2023).

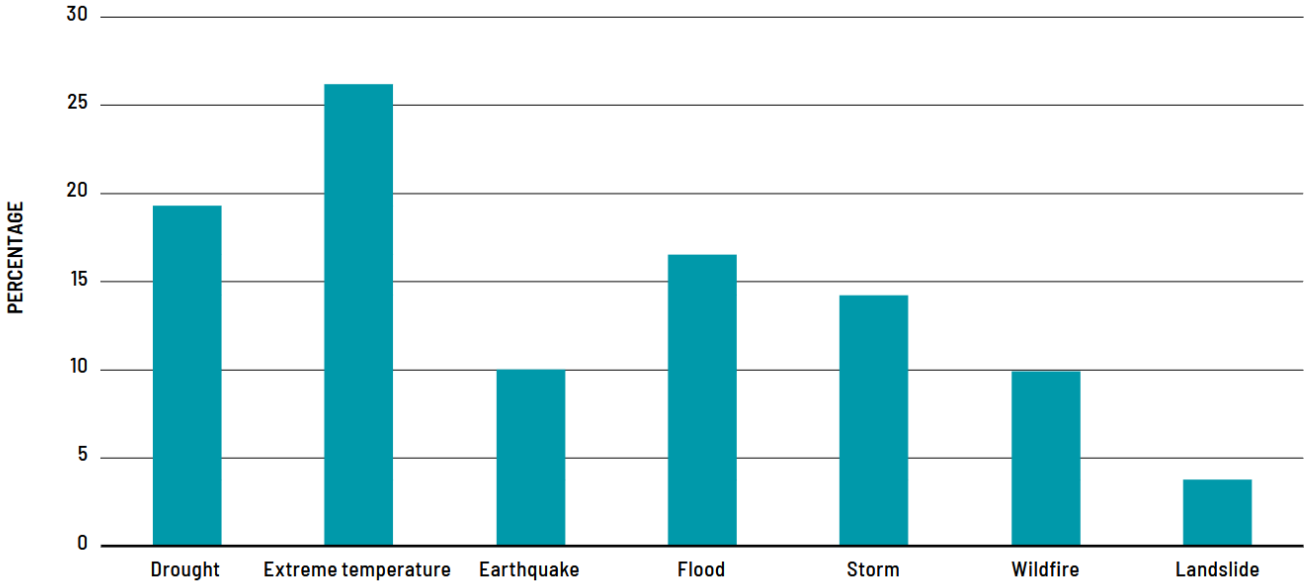


Figure 1.6. Production loss per event by hazard type in crops and livestock (1991-2021) (The impact of disasters on agriculture and food security 2023, 2023).

Although current climatic trends will continue in the next decades, their exact magnitude cannot be predicted, because of natural variability (Intergovernmental Panel On Climate Change (Ipcc), 2023). Climate change will not affect all the parts of the globe evenly: rather, distinct regional patterns of temperature and precipitation change can be identified, and these changes are projected to amplify as the level of global warming increases (Intergovernmental Panel On Climate Change (Ipcc), 2023). For example, the Arctic warms more than other regions, land areas warm more than the ocean surface, and the Northern Hemisphere more than the Southern Hemisphere (Intergovernmental Panel On Climate Change (Ipcc), 2023). Precipitation increases over high latitudes, tropics and large parts of the monsoon regions, but decreases over the subtropic (Figure 1.7) (Intergovernmental Panel On Climate Change (Ipcc), 2023). Moreover, some regions that are already dry and warm, such as southern Africa and the Mediterranean, are expected to become progressively drier and drastically

warmer at higher levels of global warming (Intergovernmental Panel On Climate Change (Ipcc), 2023).

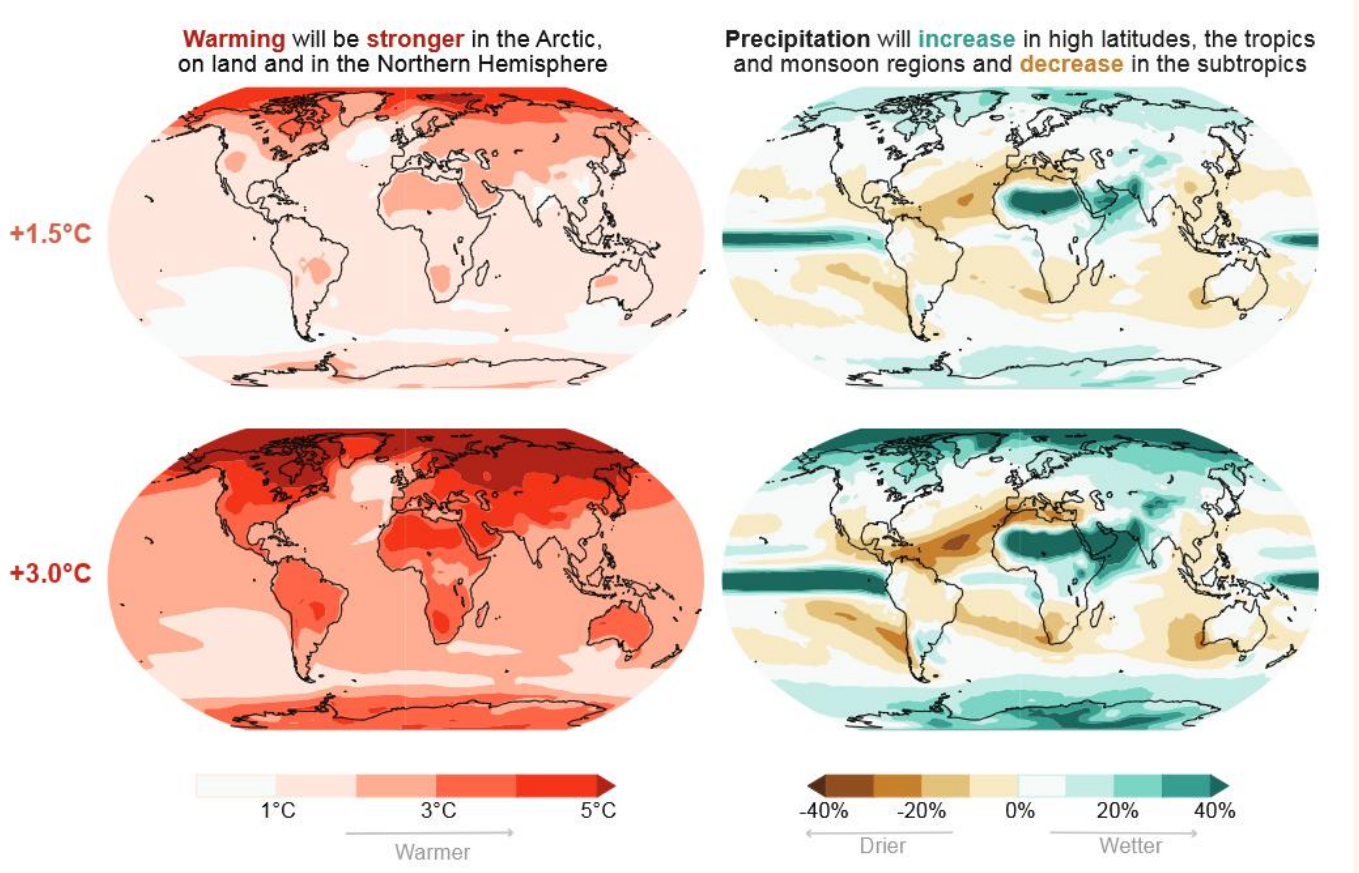


Figure 1.7. Temperature and precipitation pattern. Regional changes in temperature (left) and precipitation (right) are proportional to the level of global warming, irrespective of the scenario through which the level of global warming is reached. Surface warming and precipitation change are shown relative to the 1850–1900 climate, and for time periods over which the globally averaged surface warming is 1.5°C (top) and 3°C (bottom), respectively (Intergovernmental Panel On Climate Change (Ipcc), 2023).

Over the last 30 years, an estimated USD 3.8 trillion worth of crops and livestock production has been lost as a result of disaster events, corresponding to an average loss of USD 123 billion per year or 5% of annual global agricultural gross domestic product GDP (The impact of disasters on

agriculture and food security 2023, 2023). Research demonstrated that average losses over 30 years have increased across all the main agricultural product groups, with an average of 69 million tons of cereals, 40 million tons of fruits and vegetables and 16 million tons of meat, dairy products and eggs lost annually due to extreme events related to climate change (The impact of disasters on agriculture and food security 2023, 2023).

Climate change influences various physiological processes in plants: alterations in critical abiotic parameters such as temperature, precipitation, solar radiation, and CO_2 emissions are poised to substantially affect crop yields over the coming decades (Figure 1.8) (Terán *et al.*, 2024). For example, heat stress adversely affects normal plant growth and development depending on the sensitivity of each crop species (Kaushal *et al*., 2016). Climate change also intensifies the impact and frequency of cold stress on plants as it is responsible for an increase in cold-air outbreaks and cold duration (Gusain *et al*., 2023). Specifically, mild winter temperatures due to climate change are triggering advances in plant spring phenology with possible exposure of vulnerable plant tissues to subsequent cold and freezing temperatures (Lamichhane, 2021), possibly increasing the cold stress injury. Low temperatures damage is particularly evident in cold-sensitive species, such as tomato (Ding *et al.,* 2019).

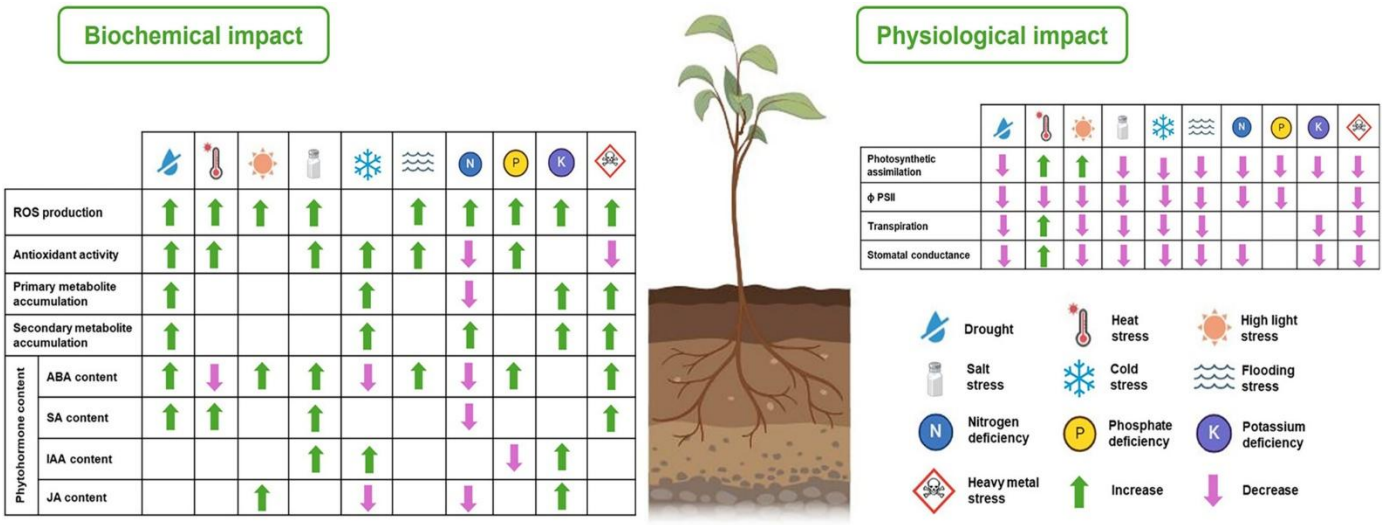


Figure 1.8. Effects of climate change-induced stress conditions on plant physiology and biochemistry (Terán *et al*., 2024).

However, climate change is not only responsible for temperature stress, but it is also the driver of salinity stress. Rising temperatures due to climate change have resulted in increased water evaporation and alterations in precipitation patterns (Figure 1.9) (Eswar *et al.*, 2021). This leads to reduced soil drainage, which, together with rising sea levels, might outcome in flooding and thus in salt stress, particularly in coastal areas (Figure 1.9) (Eswar *et al.*, 2021). Saline soils have a high concentration of soluble salts (Munns, 2005), and salinity exerts its detrimental effect on plants by two mechanisms: osmotic stress and ion toxicity (Ullah *et al.,* 2021). Moreover, increased temperatures and concentrations of atmospheric CO_2 cause them, will affect soil microbial physiology, thus altering nutrient cycles and the availability of nutrients for crop growth (Pilbeam, 2015). Different studies have demonstrated that environmental abiotic stressors, such as drought, heat, cold, nutrient deficiency, and salinity, cause oxidative stress (Tewari *et al.*, 2007; Zhou *et al.*, 2019; Wei *et al.*, 2022), and osmotic stress (Heidarvand and Maali Amiri, 2010; Yadav, 2010; Sanders and Arndt, 2012; Qu *et al.,* 2013; Wang *et al*., 2013; Belgarou*i et al.*, 2018; Mahmoud *et al.*, 2020), which can decrease the plant productivity (Upadhyaya *et al.,* 2013).

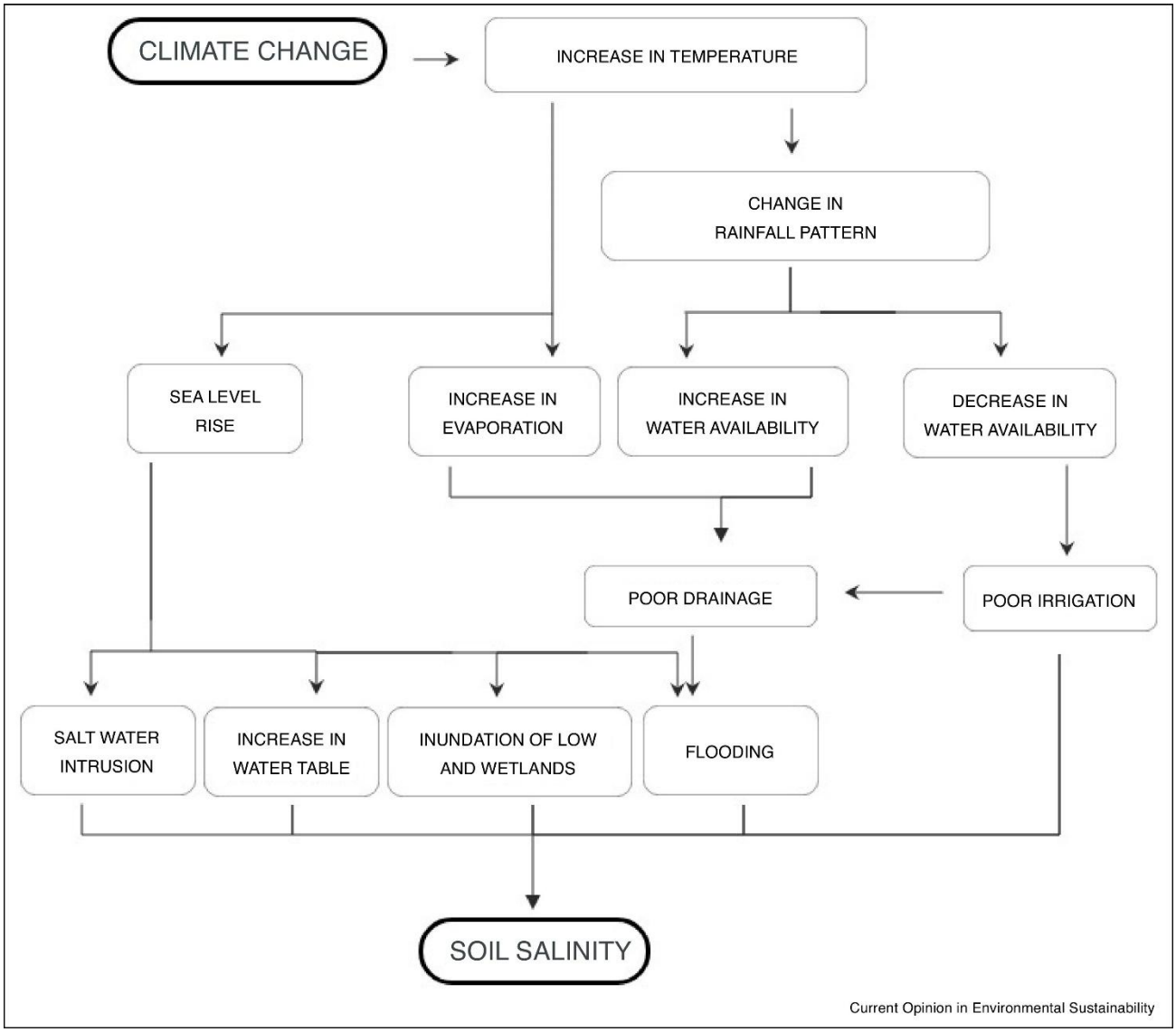


Figure 1.9. Drivers of soil salinity under climate change (Eswar et al., 2021).

Thus, abiotic consequences of climate change represent a big challenge for modern agriculture: there is a pressing need to discover sustainable solutions that both enhance agricultural production and effectively mitigate the potential damage caused by climate change.

1.3 Cold stress impacts on crop plants

Cold stress is one of the major abiotic stresses that negatively affect plant growth (Askari-Khorasgani *et al.,* 2019). It can significantly affect plant physiological activity, biochemical metabolism, growth, development, and crop productivity (Manasa S et al., 2022). Cold stress is classified as chilling stress (0 – 15 °C) and freezing stress (<0 °C) (Yang et al., 2020). Thus, cold stress refers to a wide range of temperatures ranging from 15°C to below zero. Low temperatures are indeed one of the most important factors limiting the productivity and distribution of plants *(Theocharis et al.*, 2012b). However, several plant species can increase their degree of freezing tolerance in response to low, non-freezing temperatures, and this phenomenon is known as cold acclimation *(Theocharis et al.,* 2012b). Thus, low temperatures and the ability of plants to adapt to them are key factors limiting the distribution of plant species worldwide. In general, plants from temperate climatic regions are considered chilling tolerant with a variable degree and can increase their freezing tolerance by exposure to chilling temperatures (Sanghera *et al.,* 2011). Many important crops, such as rice, maize, soybean, cotton, and tomato, are chilling sensitive (Chinnusamy *et al.*, 2007), as they are originated in the tropical and subtropical areas (Jin *et al.*, 2017). Nonetheless, they are cultivated largely outside their native geographical range, oftentimes close to their climatic limit where chilling tolerance becomes crucial (Barrero-Gil *et al.*, 2016). As a result, cold stress becomes hard to manage and has more severe effects on plants that are not naturally adapted to cold environments, such as tomato.

1.4 Plant response to cold stress

According to their ability to cope with cold stress, plants can be classified into three categories of varying tolerance and sensitivity to sub-zero (<0 °C) and chilling temperatures (0–15 °C), with plants grouped as either freezing tolerant, chilling tolerant, or chilling sensitive (Guy, 1990). For example, tomato plants are considered to be chilling sensitive, whereas potato plants are chilling tolerant (Table 1.1), among other examples of cold adaptation of different species that originated from dissimilar environments. Chilling tolerance refers to plants that can survive at low temperatures and high sub-zero temperatures that would kill chilling-sensitive plants. Moreover, chilling-sensitive crops (e.g., rice, maize, and tomato) can scarcely acclimate to cold temperatures compared to chilling-tolerant crops (e.g., wheat and barley) (Soualiou *et al.,* 2022).

Table 1.1. Examples of plants classified according to susceptibility to freezing or chilling and low-temperature capacity (defined as the lowest survivable temperatures recorded based on treatments or cultivars following cold acclimation, if capable), defined as the lowest survivable temperature, modified from (Juurakko *et al.,* 2021).

Species	**Classification**	**Low temperature capacity**	**References**
Triticum aestivum (wheat)	Freezing tolerant	-20°C	(Thomashow, 1998)
Solanum tuberosum (potato)	Chilling tolerant	-3°C	(Chen *et al*., 1976)
Solanum lycopersicum (tomato)	Chilling sensitive	10°C	(Saltveit and Morris, 1990)
Oryza sativa (rice)	Chilling sensitive	17°C	(Shakiba *et al.*, 2017)
Arabidopsis thaliana (thale cress)	Freezing tolerant	-6 to -11°C (depending on accession)	(Kaplan *et al.*, 2004) (Hannah *et al*., 2006)
Zea mays (maize)	Chilling sensitive	10°C	(Rodríguez *et al*., 2014)
Nicotiana tabacum (tobacco)	Chilling sensitive	2°C	(Zhao *et al*., 2009)
Rhododendron catawbiense (purple ivy)	Freezing tolerant	-54°C	(Wei *et al*., 2005)

Cold acclimation is a multifaceted process that encompasses the perception and transduction of environmental cold stress signals, the expression of cold-related genes, and specific metabolic and physiological changes (Qian *et al.*, 2024a). The acclimation capability can be species-dependent, and

the genomic pool seems to be one of the main drivers of cold adaptation (Soualiou *et al.*, 2022). In temperate latitudes, cold acclimation is established in the autumn, when the temperatures are low but positive and the photoperiod decreases, and it reaches a maximum in winter (Ruelland *et al.*, 2009). It has been demonstrated that cucumber (a tropical vegetable) and tomato (a tropical vegetable) can activate mechanisms related to cold acclimation (Barrero-Gil *et al.*, 2016; Qi *et al*., 2022). In general, plant cells improve cold tolerance by accumulating osmotic regulators such as proline, soluble sugar (Ss), and soluble protein (Sp), as well as increasing the activities of antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), and peroxidase (POD) (Qian *et al.*, 2024a).

Plant cells can sense cold stress through low temperature-induced changes in membrane fluidity, protein and nucleic acid conformation, and/or metabolite concentration (a specific metabolite or redox status) (Chinnusamy *et al*., 2007). More specifically, low temperatures induce rigidification of membranes, leading to a disturbance of all membrane processes (e.g. opening of ion channels, membrane-associated electron transfer reactions, etc.) (Ruelland *et al*., 2009, p. 2). Several studies have shown that the membrane protein COLD1 is involved in the very early stages of low-temperature sensing (Ma *et al*., 2015; Zhang *et al*., 2020a; Zhou *et al*., 2023). COLD1 interacts with the G-protein α subunit to activate the Ca^{2+} channels (Ma *et al.,* 2015) (Figure 1.10). Studies have reported that calcium channels can be found int the permeable transporter ANNEXIN1 *(Yadav et al., 2018; Liu et al.,* 2021; Faizan *et al.,* 2024; Peng *et al.*, 2024), and cyclic nucleotide-gated channels (CNGCs) (Peng *et al.*, 2024; Chang *et al*., 2025) (Figure 1.10). More specifically, the open stomata 1 (OST1) kinase interacted with and phosphorylated ANNEXIN1, which consequently enhanced its calcium transport activity (Liu *et al.*, 2021), whereas the leucine-rich repeat receptor-like kinase plant peptide containing sulfated tyrosine1 receptor (PSY1R) is activated by cold, phosphorylating and enhancing the activity

of cyclic nucleotide-gated channels (Peng e*t al.,* 2024). Thus, cold stress is perceived at the plasmatic membrane level by two calcium channels, which, being phosphorylated, open and allow calcium to enter and trigger cytosolic calcium concentration. Transient changes in intracellular calcium concentration have been well recognized to act as cell signals coupling various environmental stimuli to appropriate physiological responses with accuracy and specificity in plants (Zeng *et al.*, 2015). Calmodulin (CaM) and calmodulin-like proteins (CMLs) are major calcium sensors, playing critical roles in calcium signal transduction. Calmodulin/calmodulin-like proteins interact and regulate a broad spectrum of targets (Zeng *et al*., 2015). Calcium/calmodulin activated the kinase activity of the calcium/calmodulin-regulated receptor-like kinase (CRLK), which is localized in the plasma membrane (Yang *et al.*, 2010a), as well as the calmodulin-binding transcription activator (CAMTA) (Doherty *et al.*, 2009) (Figure 1.11). Yang *et al.* reported that calcium/calmodulin binds to receptor-like kinase 1 and upregulates its activity as a positive regulator of plant response to chilling and freezing temperatures (Yang *et al*., 2010b) (Figure 1.11). They demonstrated that MEKK1, a member of the mitogen-activated protein kinase (MAPK) family, interacts with receptor-like kinase 1both in vitro and in plant, triggering the mitogen-activated protein kinase cascade, which consists of three consecutively acting protein kinases, a mitogen-activated protein kinase kinase kinase kinase (MAPKKK), a mitogen-activated protein kinase kinase kinase (MAPKK) and a mitogen-activated protein kinase kinase (MAPK) (Yang *et al.*, 2010b). Specifically, the MEKK1-MKK2-MPK4 cascade is activated during cold acclimation (Furuya *et al.,* 2014), and positively regulates freezing tolerance through MPK3/6-mediated phosphorylation of the inducer of CBF expression 1 (ICE1), which promotes the inducer of CBF expression 1 degradation (Zhao *et al.*, 2017), a MYC-like bHLH transcriptional activator (Chinnusamy *et al.,* 2003) (Figure 1.11). receptor-like kinase activates also the MKK4/5-MPK3/6 cascade which negatively regulates freezing tolerance by through phosphorylation of inducer of CBF expression 1, which is consequently degraded (Zhao *et al.*, 2017).

The inducer of CBF expression 1 protein is constitutively present in the nucleus, but the *c-repeat binding factor (CBF)* genes – whose expression is enhanced by inducer of CBF expression 1 – are only upregulated under cold stress (Zhao *et al.*, 2017). Thus far, how the inducer of CBF expression 1 (ICE) is activated to induce the expression of *c-repeat binding factor(CBF)* genes under cold stress is unknown. It is likely that posttranslational modification of inducer of CBF expression 1 (ICE) is involved in this process (Zhao *et al.*, 2017). Besides Calmodulin / calmodulin-like proteins, increased calcium levels trigger also calcineurin B-like proteins (CBLs) and their interacting kinases (CIPKs) (Meena *et al.*, 2018) to increase the expression levels of dehydration responsive element binding (DREB) transcription factors and *cold responsive* genes (*COR*) in order to enhance cold tolerance (Huang *et al*., 2011).

In addition, calcium also play a crucial role in regulating the cold response pathway in chloroplasts (Luo *et al.,* 2021). For instance, calcium signaling initiated by *COLD1* enhances the expression of *VTE4* (the gene responsible for α-tocopherol, or vitamin E) and *VKORC1* (the key gene in the vitamin K1 pathway) (Luo *et al.*, 2021). This promotes the biosynthesis of vitamins E and K in chloroplasts, leading to the production of 3′-phosphoadenosine 5′-phosphate (PAP). 3′-phosphoadenosine 5′-phosphate is then transported into the nucleus, where it regulates the production of miRNAs, ultimately boosting the expression of cold-responsive genes and enhancing cold tolerance (Luo *et al*., 2021). Additionally, Lie *et al*. discovered that chilling-tolerance in Geng/japonica rice 3 (COG3), a putative calmodulin-binding protein, interacts with the proteolytic enzyme FtsH2 to remove the cold-damaged photosystem II protein D1 (Liu *et al.,* 2024a). This interaction helps balance the turnover of the D1 protein in chloroplasts, thereby maintaining photosynthetic efficiency and regulating cold tolerance (Liu *et al.*, 2024a).

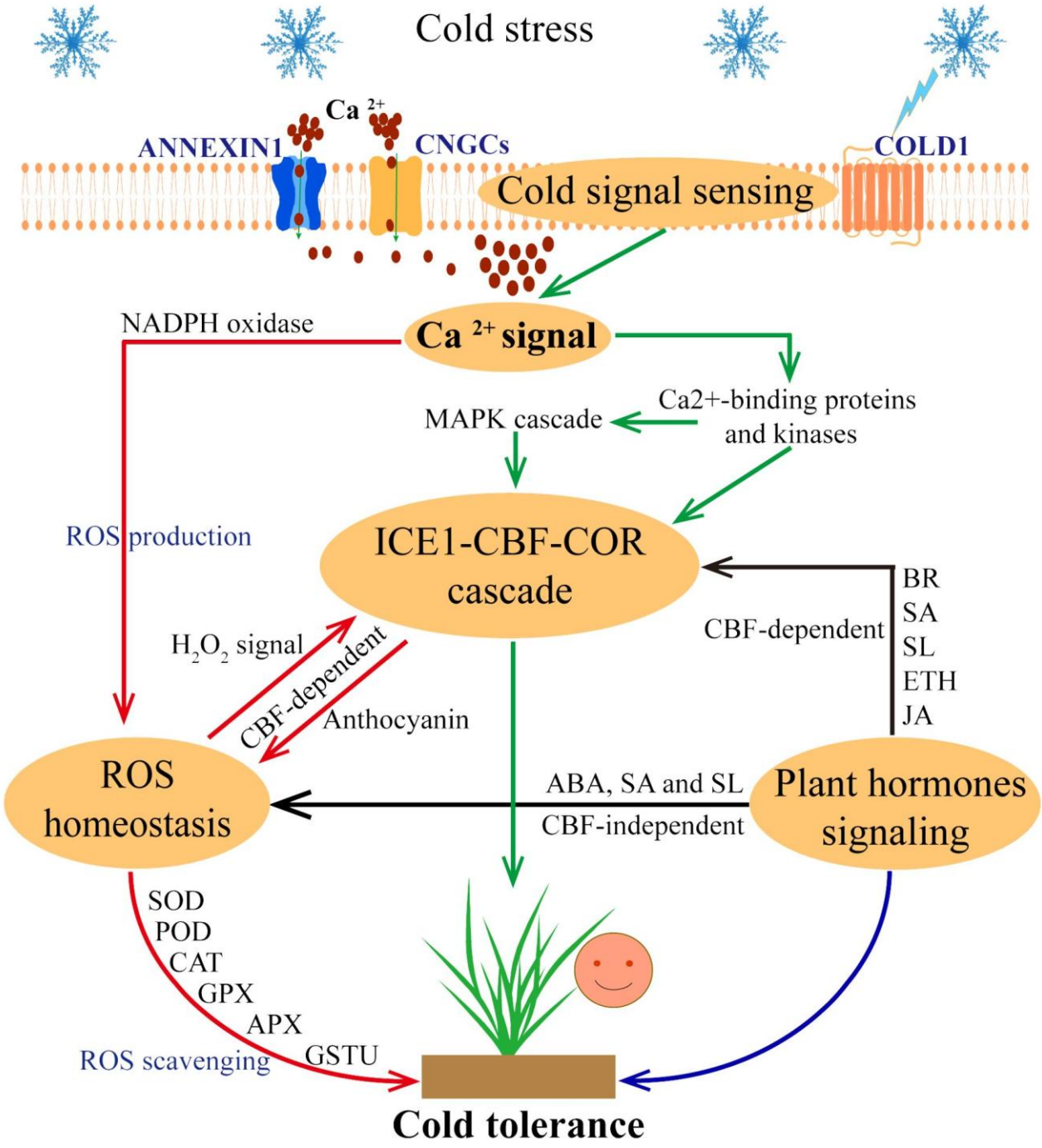


Figure 1.10. Overview of cold stress-induced mechanisms in plants (Qian *et al*., 2024a).

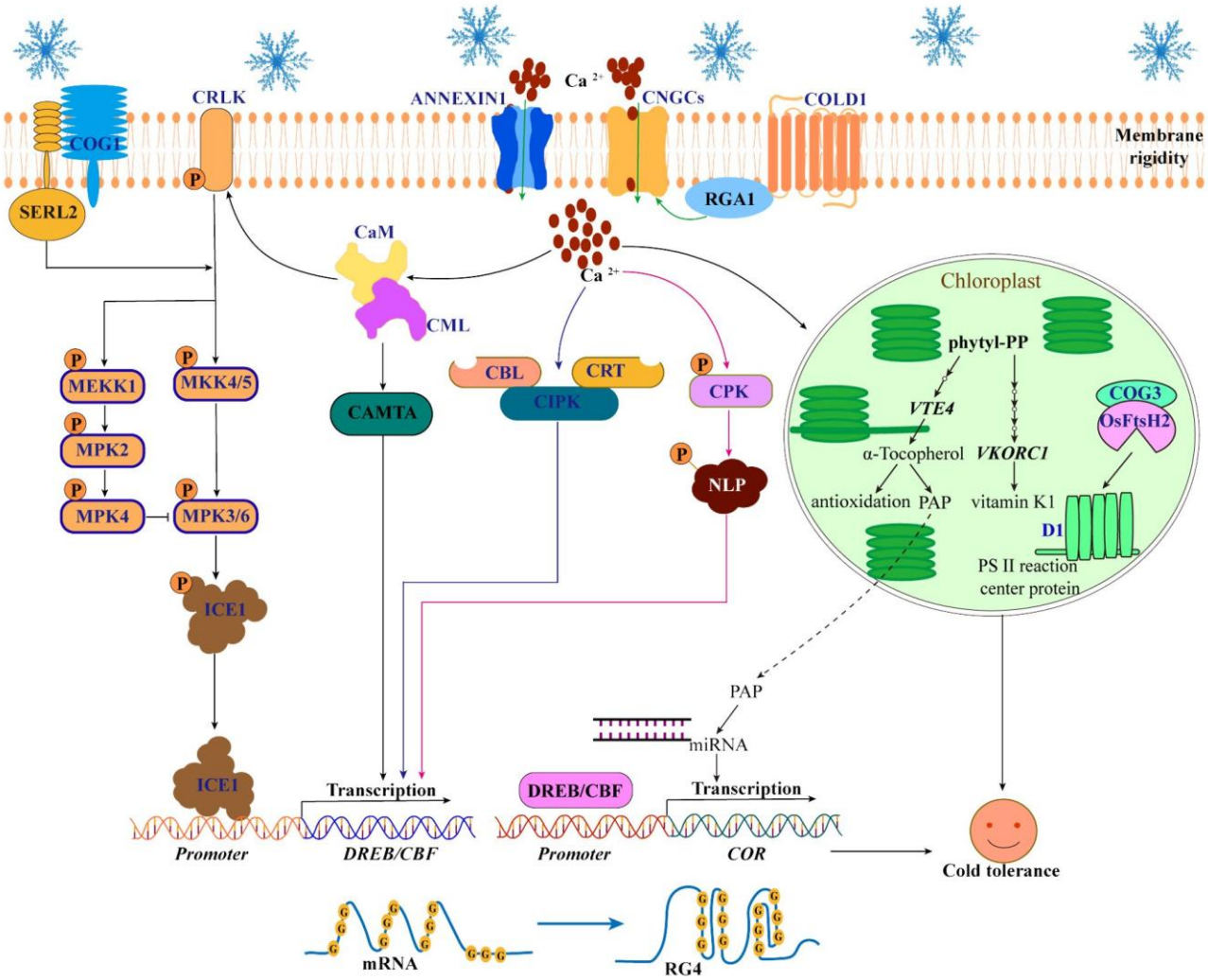


Figure 1.11. Perception and transduction of cold signals in plants (Qian *et al.*, 2024a)

The inducer of CBF expression 1- c-repeat binding factor - cold responsive (ICE1-CBF-COR) transcriptional cascade, a key component of the downstream COLD1-calcium-binding protein-mitogen activated protein kinase (MAPK) signaling cascade, is widely recognized as the prototypical and evolutionarily conserved response pathway to cold stress in plants (Qian *et al.,* 2024a). In this complex network, various transcription factors (TFs) contribute to the transcriptional level of the inducer of CBF expression 1- c-repeat binding factor - cold responsive (ICE1-CBF-COR) cascade, and modifications at the protein level regulate its stability (Qian *et al.*, 2024a). For instance, clock-

related myeloblastosis proteins (MYBs) bind to the promoters of both The inducer of CBF expression 1 (ICE1) and c-repeat binding factors (CBFs), increasing their transcription levels (An *et al*., 2018). Other transcription factors (TFs), such as B-box (BBX), NAC, WRKY, and basic leucine zipper (bZIP), also regulate CBF transcription (Hao *et al*., 2011; An *et al*., 2018; Zhang *et al.*, 2022a; Kamble, 2024) (Figure 1.12). Post-translational modifications, such as phosphorylation, myristoylation, and ubiquitination, can alter the structure, stability, activity, and function of proteins, which is essential for protein diversity (Qian *et al*., 2024a). For example, the MPK cascade phosphorylates ICE1 to regulate its stability, which is crucial for the cold stress response in Arabidopsis and rice (Zhao *et al.*, 2017). OST1 also phosphorylates ICE1 to increase its stability, while BRASSINOSTEROID-INSENSITIVE2 (BIN2) phosphorylates ICE1 to promote its degradation (Ye *et al.,* 2019) (Figure 1.12). Light signaling pathways also regulate CBF expression and cold stress response: PHYTOCHROME-INTERACTING FACTORS (PIFs) act as negative regulators targeting CBF, with PIF3 binding to CBF promoters to downregulate their expression (Jiang *et al.*, 2020) (Figure 1.12). The EIN3-BINDING F-BOX 1 and 2 (EBF1 and 2) interact with PIF3 to promote its degradation (Hao *et al.,* 2021). Specifically, PHYTOCHROME B (phyB) regulates plant growth through perceiving light and temperature signals: under warm conditions CBF pathway is actively repressed by PHYB, PIF4, and PIF7 (Lee and Thomashow, 2012) (Figure 1.12). Cold stress induces the expression of CBF genes, and the accumulated CBF proteins interact with PIF3 (Jiang *et al*., 2020). This interaction prevents PIF3 and phyB proteins from undergoing the co-degradation that occurs under light at warm temperatures (Jiang *et al.*, 2020). Cold-stabilized phyB promotes the degradation of PIF1, PIF4, and PIF5, and consequently mediates the expression of a set of stress-related and growth-related COR genes, thus increasing freezing tolerance (Jiang *et al*., 2020) (Figure 1.12). Previous studies established that CBF1, CBF2, and CBF3 are subject to circadian regulation and that their cold induction is gated by the circadian clock (Dong *et al.,* 2011) (Figure 1.12). The clock-related

myeloblastosis (MYB) proteins REVEILLE4 and REVEILLE8 (RVE4 and RVE8) were quickly transferred from the cytoplasm to the nucleus in response to cold stress and functioned as direct transcriptional activators of *DREB1* expression (Kidokoro *et al.,* 2021). The other clock-related MYB proteins, CIRCADIAN CLOCK-ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), suppressed *DREB1* expression under unstressed conditions and were rapidly degraded under cold stress, which suggests that these factors indirectly regulate cold-inducible *DREB1* expression (Kidokoro *et al.,* 2021) (Figure 1.12). Four NIGHT LIGHT–INDUCIBLE AND CLOCK-REGULATED (LNK) proteins acted as coactivators of RVEs in cold stress responses in addition to regulating circadian-related genes at normal temperatures (Kidokoro *et al.*, 2023). LNK1 and LNK2 function under normal and high-temperature conditions (Kidokoro *et al.,* 2023). In contrast, LNK3 and LNK4 specifically function under cold conditions: they are specifically phosphorylated under cold conditions, suggesting that phosphorylation is involved in their activation (Kidokoro *et al.*, 2023) (Figure 1.12). PSEUDORESPONSE REGULATOR (PRRs) is a core component of the circadian oscillator which also play a crucial role in freezing tolerance (Kim *et al.*, 2024). HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE 15 (HOS15), controls the protein accumulation pattern of PRR7 through direct protein–protein interactions at low temperatures (Kim *et al.*, 2024) (Figure 1.12). At low temperatures, HOS15 promotes the degradation of PRR7 protein through a CULLIN4-based E3 ubiquitin ligase complex (proteasome icon) during the night, resulting in the transcriptional de-repression of the cold stress-responsive genes *C-REPEAT/DRE BINDING FACTOR 1* (*CBF1*) and *COLD-REGULATED 15A* (*COR15A*) (Kim *et al.*, 2024). These studies have shown that interacting proteins are very important for the conformational changes and stability of CBF (Qian *et al.,* 2024a). However, the current understanding of CBF-interacting proteins is still insufficient (Qian *et al.,* 2024a). Generally, protein structure and stability are closely related to PTMs (Qian *et al*., 2024a). Therefore, it is necessary to further study whether PTMs directly act on CBFs (Qian *et al.,* 2024a).

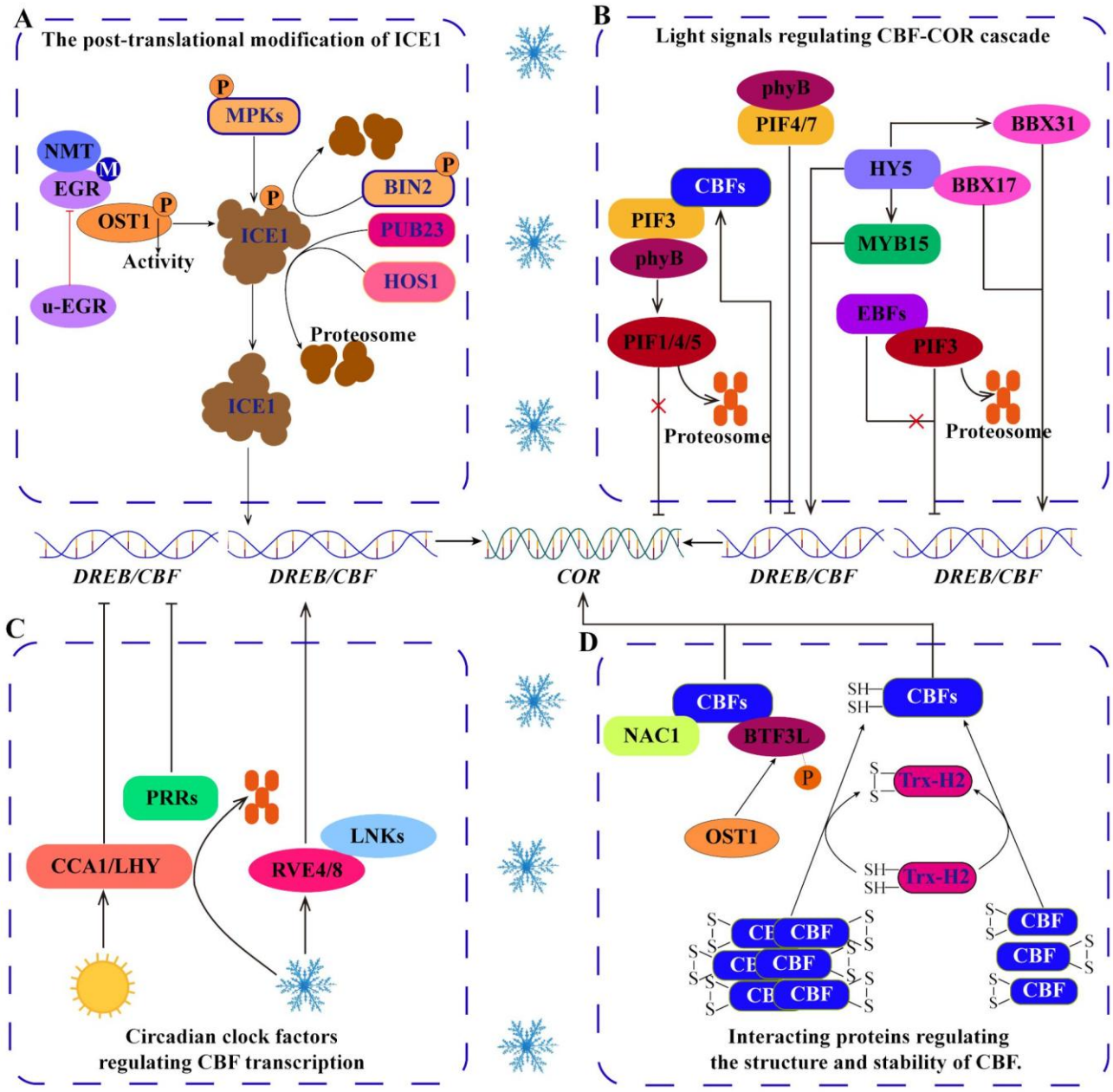


Figure 1.12.- Regulation of the ICE1/CBF/COR genes pathway (Qian *et al*., 2024a).

Chilling is also associated with the accumulation of reactive oxygen species (ROS) (Ruelland *et al.*, 2009). The activities of scavenging enzymes will be decreased by low temperatures, and the scavenging systems will then not be able to counterbalance the ROS formation that is always associated with mitochondrial and chloroplast electron transfer reactions (Ruelland *et al*., 2009). Moreover, the chloroplast electron transfer chain will be over-reduced during chilling, which will lead

to increased ROS formation (Ruelland *et al.*, 2009). The accumulation of ROS will have deleterious effects, especially on membranes (Ruelland *et al.,* 2009). This will result in ion leakage. In addition, the low temperatures will favor the formation of secondary structures in RNA, thus affecting gene and protein expression (Fig. 1.13) (Ruelland *et al*., 2009).

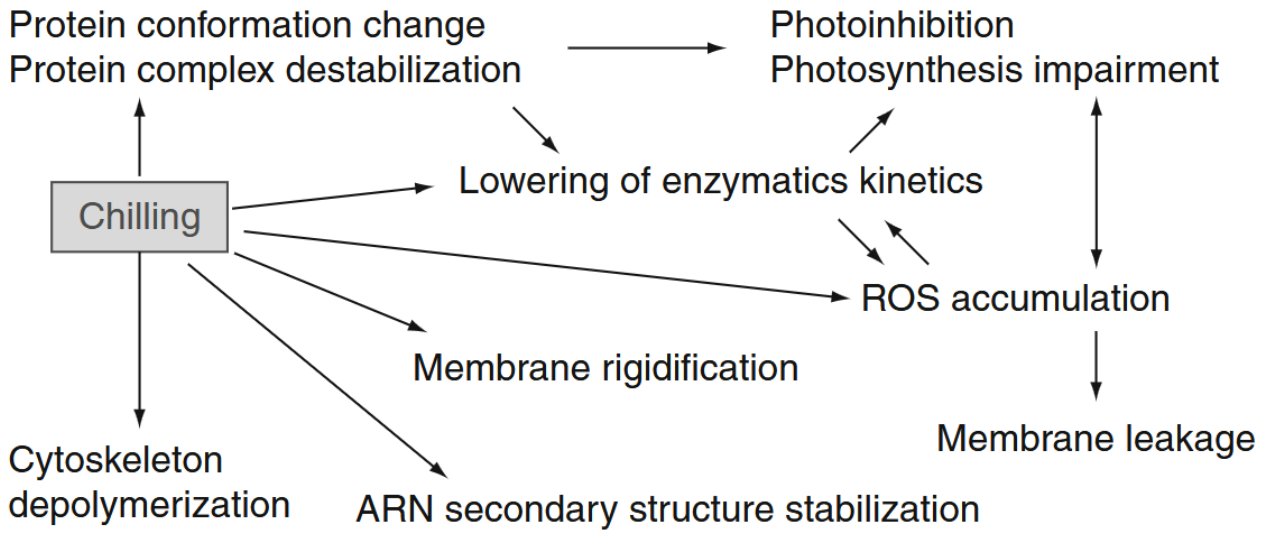


Figure 1.13. Chilling effects on plant cells (Ruelland et al., 2009).

Kawarazaki et al. demonstrated that abiotic stress-induced ROS are produced by NADPH oxidases in *Arabidopsis* (Kawarazaki *et al*., 2013). Increased levels of ROS cause the activation of ROS scavenger systems, alterations in protein and sugar synthesis, proline accumulation, and biochemical changes that affect photosynthesis (Theocharis *et al.*, 2012b). In particular, to scavenge ROS, including superoxide anion (O_2^-) and H_2O_2, to alleviate the oxidative damage in plant cells under cold stress plants exhibit enhanced activities of antioxidant enzymes, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), glutathione peroxidase (GPX), and ascorbic acid peroxidase (APX) (Wei *et al.*, 2022). Moreover, H_2O_2 acts as a signaling molecule and positively

regulates the expression of *CBF1* (Qian *et al*., 2024a). Furthermore, *CBF* is involved in the biosynthetic pathway of anthocyanins and promotes ROS clearance (Qian *et al.*, 2024a).

1.5 Plant-associated microorganisms: a promising strategy for plant growth promotion and cold stress mitigation

Some plants can adapt to low temperatures through mechanisms based on protein synthesis, membrane composition changes, and activation of ROS scavenging systems (Ruelland *et al.*, 2009). However, it is claimed that plant-associated microbial communities can play a pivotal contribution in plant cold stress tolerance. For example, in harsh environmental conditions, alpine plant microbiomes help plants adapt to low temperatures (Hou *et al*., 2024). Plants are associated with a wide range of microorganisms that can promote plant growth and survival under stress conditions by producing phytohormones and/or enhancing the uptake of nutrients (Marian e*t al*., 2022). The beneficial effects of plant-associated microbial communities on plants include promoting plant growth and development, helping plants resist biotic and abiotic stresses, and improving plant growth performance (Hou *et al.*, 2024). Plant growth-promoting bacteria (PGPB) have several properties, such as iron chelating ability, phosphate solubilization, 1-aminocyclopropane-1-carboxylate (ACC) deaminase enzyme production, and phytohormone production under stress (Rai *et al.*, 2020). Beneficial microorganisms in association with tomato plants have been demonstrated to increase tolerance to cold stress, such as *Paraburkholderia phytofirmans* PsJN (*Paraburkholderia* sp.) (Perazzolli *et al.,* 2022), *Flavobacterium* sp. OB146 and *Pseudomonas* sp. OB155, OS261, and OS211 (Subramanian *et al.,* 2015, 2016), *Trichoderma asperellum* HK703 (Cornejo-Ríos *et al*., 2021), *T. harzianum* AK20G (Ghorbanpour *et al.,* 2018), *Streptomyces* sp. TOR3209 (Ma *et al.*, 2022), *Mrakia* sp. Mr1, *Naganishia* sp. Ng1, *Pseudomonas* sp. Ps1, *Rhodotorula* sp. Rh2 and Rh3 (Tapia-Vázquez

et al., 2020), and bacterial consortia, such as *Bacillus* sp. and *Serratia* sp. Isolates (Wang *et al*., 2016) or *Curtobacterium* sp., *Frondihabitans* sp., and *Pseudomonas* sp. Isolates (Vega-Celedón *et al.*, 2021). Particularly, culturable cold tolerant bacteria of *C. quitensis* were able to endophytically colonize tomato seedlings and promote shoot growth at low temperatures (Marian et al., 2022), suggesting the potential of cold tolerant bacteria in alleviating cold stress symptoms on tomato seedlings.

In harsh environments, plants respond to stress by recruiting beneficial microbes to help resist stress. The beneficial effects of alpine and subalpine plant-associated microbial communities on plants include promoting plant growth and development, helping plants resist biotic and abiotic stresses, and improving plant growth environments (Hou *et al.,* 2024). There are several examples of bacterial strains isolated from extreme environments that demonstrated a positive effect on plants, such as desertic strains of *Bacillus* and *Brevibacillus* strains on *Zea mays* L. growth (ALKahtani *et al.*, 2020), desertic *Pantoea* spp. on winter wheat, , volcano mud *Ochrobactrum* sp. on maize (Mishra *et al*., 2023), geothermal strains J19, TP22, A20, and A3 on tomato , alpine isolates *Duganella*, *Erwinia*, *Pseudomonas* and *Rhizobium* on strawberry (Marian *et al.,* 2023).

1.5.1 Mechanisms of plant growth promotion

Direct mechanisms are defined as employing those bacterial traits that result in the direct promotion of plant growth (Olanrewaju *et al*., 2017). They include the production of auxin, ACC deaminase, cytokinin, gibberellin, nitrogen fixation, phosphorus solubilization, and mobilization of iron by bacterial siderophores (Olanrewaju *et al.,* 2017). Indirect mechanisms refer to bacterial traits that inhibit the functioning of plant pathogenic microorganisms (Olanrewaju *et al.,* 2017). Indirect mechanisms include antibiotics, cell wall degrading enzymes, competition, hydrogen cyanide,

induced systemic resistance, quorum quenching, and siderophores (Olanrewaju *et al*., 2017). Although many natural and synthetic compounds show auxin-like activity in bioassays, indole-3-acetic acid (IAA) is the best known and physiologically the most active natural auxin in the plant and is recognized as the key auxin in most plants (Olanrewaju *et al.*, 2017) (Figure 1.14). IAA is an important phytohormone with the capacity to control plant development in both beneficial and deleterious ways (Duca *et al*., 2014). Bacterial IAA stimulates root hair formation while increasing the number and length of lateral and primary roots when it is within an ideal concentration range (Duca *et al*., 2014). Plants harbor millions of IAA-producing bacteria from genera such as *Pseudomonas*, *Rhizobium*, *Azospirillum, Enterobacter, Azotobacter, Klebsiella, Alcaligenes, Pantoea,* and *Streptomyces* (Apine and Jadhav, 2011). ACC is a precursor molecule of ethylene whose concentration is elevated in the plant subjected to biotic and abiotic stress (Tiwari *et al.*, 2018). Several soil microorganisms are reported to produce ACC deaminase, which degrades ACC, thereby reducing stress ethylene in host plants (Tiwari *et al.*, 2018) (Figure 1.14). PGPB have also been proven to produce siderophores (Loaces *et al.*, 2011), siderophores chelate iron and supply it to the bacterial cell by outer membrane receptors (Ali and Vidhale, 2013), contributing to the plant nutrition (Scavino and Pedraza, 2013). Moreover, common mechanisms through which PGPB can promote plant growth are nitrogen fixation and phosphorus solubilization. In cultivated soils, nitrogen availability is a key factor often limiting crop productivity (Muratore *et al.*, 2021), and crop productivity relies heavily on nitrogen fertilization (Xu *et al*., 2012). A nitrogen-fixing bacterium can exist freely or in symbiosis and either case entraps atmospheric nitrogen and converts the unreactive N_2 molecule to ammonia (NH_3), a form that is readily utilized by plants (Bhattacharjee *et al.*, 2008). The biological process responsible for reduction of molecular nitrogen into ammonia is referred to as nitrogen fixation (Bhat *et al*., n.d.).

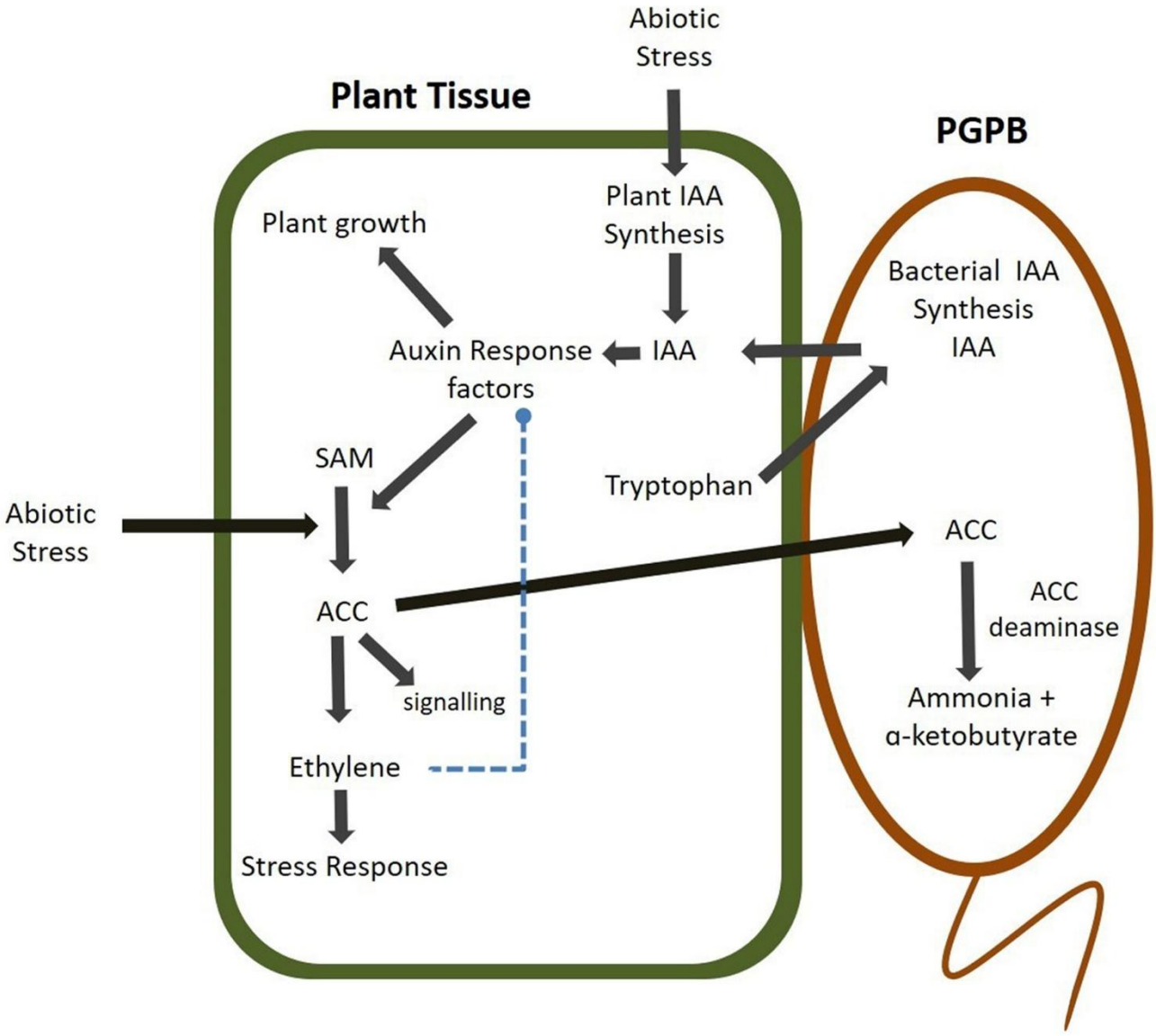


Figure 1.14. IAA and ACC production in PGPB and their interactions with plant cell (Naing *et al.*, 2021).

Phosphorus is the second most critical macronutrient after nitrogen required for the metabolism, growth, and development of plants (Rawat *et al.*, 2021). Its availability is one of the major growth-limiting factors in many ecosystems around the world (Raghothama, 2005). Phosphate uptake is strongly influenced by the nutrient availability and inorganic phosphate (P_i) status of plants (Raghothama, 2005). Mineralization, solubilization, and immobilization are the predominant ways of dissemination of phosphorus in soil by phosphate-solubilizing microorganisms, which are influenced

by available inorganic minerals in the soil (Rawat *et al*., 2021). Major strategies adopted by phosphate-solubilizing microorganisms for dissolution of phosphates include (a) exudation of organic acids, protons, and siderophores; (b) excretion of extracellular enzymes; and (c) degradation of the substrate via mineralization (Rawat *et al.*, 2021) (Figure 1.15).

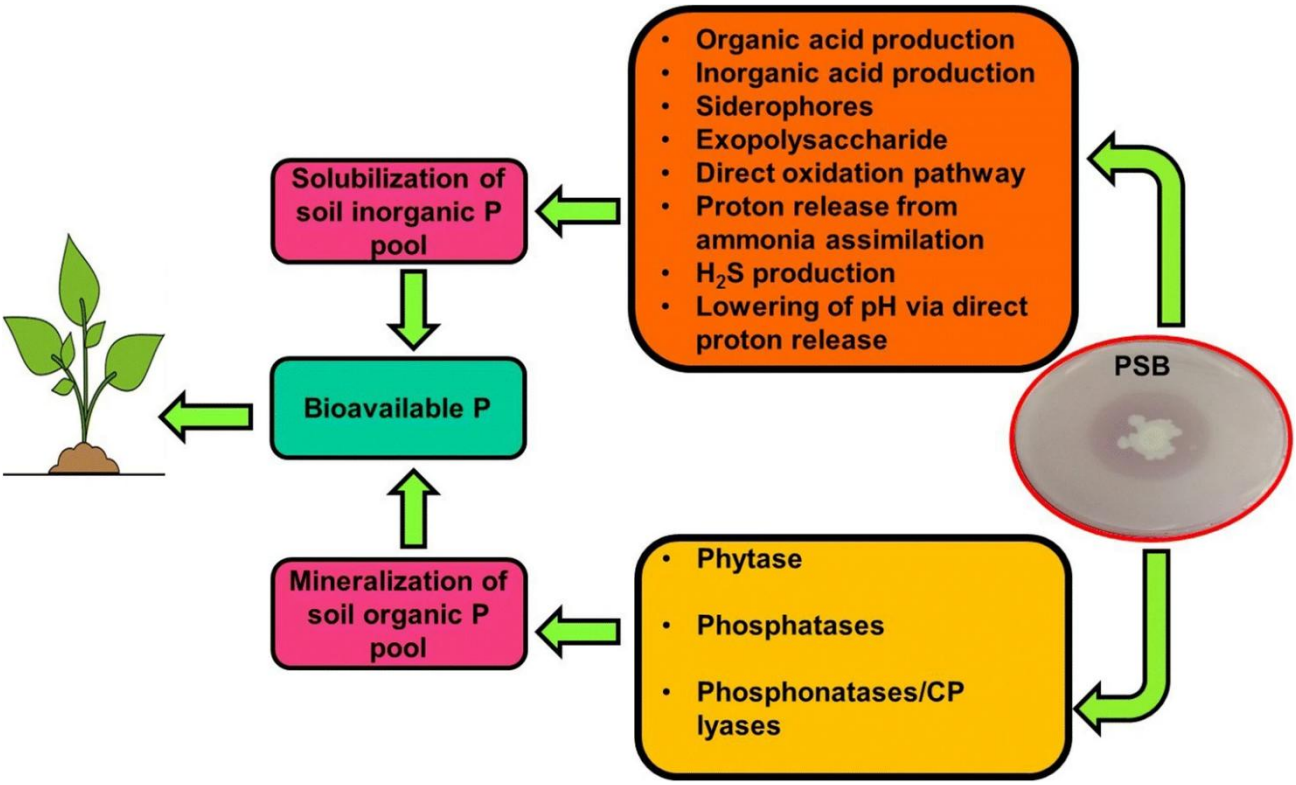


Figure 1.15. Strategies of phosphate-solubilizing microorganism to solubilize soil phosphorus (Rawat *et al.*, 2021).

1.5.2 Mechanisms of cold stress mitigation

Crop yields are affected by various types of abiotic stresses that reduce agricultural productivity, causing challenging food-security issues (Muralibabu *et al.,* 2024). The agricultural importance of cold-tolerant microbes stems from the fact that the world over temperate agroecosystems is

characterized by low temperatures and short growing seasons that subject both plant and microbial life to cold-temperature induced stress (Mishra *et al.,* 2012).

Singh et al. discovered that ACC-deaminase producing four PGPB, including *Brevibacterium halotolerans* (Sd-6), *Bacillus subtilis* (Ldr-2), *Achromobacter xylosoxidans* (Fd-2) and *Burkholderia cepacia* (Art-7) *Ocimum sanctum*, considerably decreased ACC content over the control (Singh *et al.,* 2020). ACC-deaminase hydrolyzes the ethylene precursor 1-aminocyclopropane-1-carboxylate to ammonia and α-ketobutyrate, reducing the damaging effects of cold by lowering the production of ethylene in plants (Theocharis *et al.*, 2012a). *Pseudomonas vancouverensis* OB155 and *Pseudomonas frederiksbergensis* OS261 inoculation activated the antioxidant capacity of plants evidenced through significant increase in proline content in the leaves and induction of antioxidant enzymes superoxide dismutase, ascorbate peroxidase and glutathione synthase (Subramanian *et al.,* 2016). In addition, PGPB can decrease freezing injury, ice nucleating activity, and lipid peroxidation content in parallel with the decrease of reactive oxygen species level such as O_2^- and H_2O_2 in the inoculated seedlings compared to the control (Tiryaki *et al.*, 2019). Moreover, inoculating the maize plants with *L. fusiformis* and *L. sphaericus* alone or in combination resulted in a higher level of chlorophyll a, chlorophyll b, carotenoid, and total photosynthetic pigments as well as a higher photosynthetic rate in comparison to control under normal temperature conditions (27 °C) and cold stress conditions (4 °C). However, when the maize plants were exposed to cold stress conditions (4 °C), there was a dramatic drop in Chl a, Chl b, carotenoid, total photosynthetic pigments, and photosynthetic rate (25.0%) was detected in non-inoculated control plants. The most pronounced increases in Chl a, Chl b, carotenoid, total photosynthetic pigments, and photosynthetic rate (29.6% and 44.4%) were detected in maize plants inoculated with a combination of two bacterial strains compared to control plants and cold stressed plants, respectively (Jha and Mohamed, 2023).

In general, symbiotic microbes positively influence the physiology of cold-exposed plants under controlled conditions, with consequent effects on aboveground biomass accumulation and fitness (Figure 1.16) (Acuña-Rodríguez *et al.,* 2020).

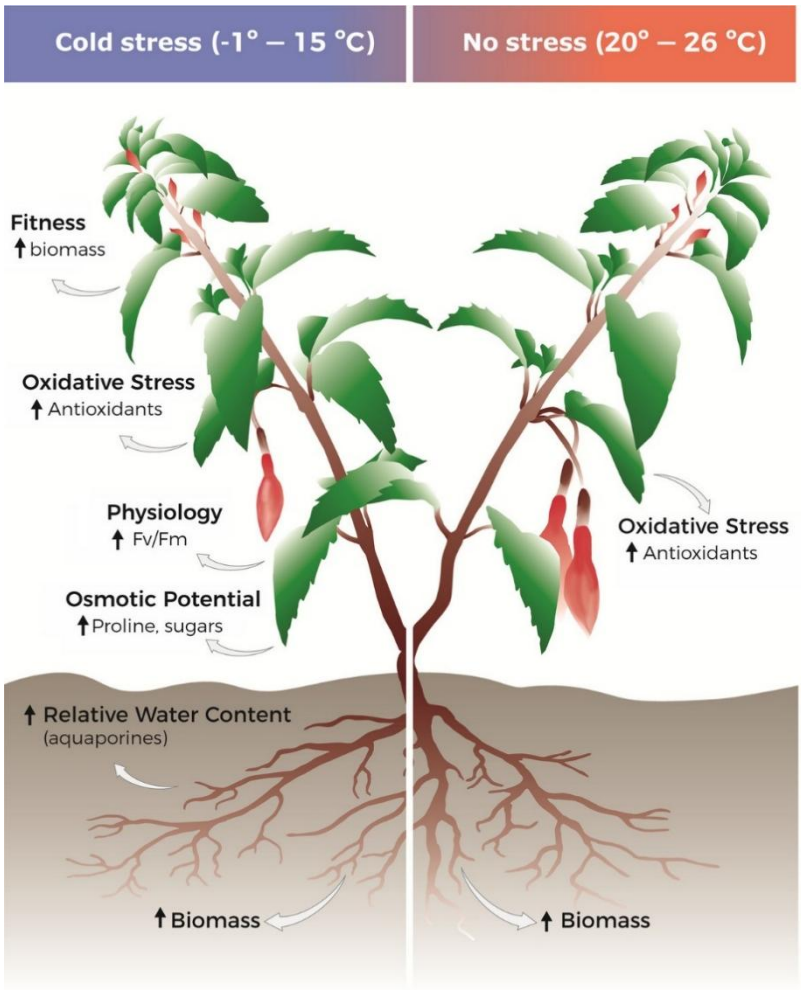


Figure 1.16. Plant growth promoting bacteria can enhance plant growth and fitness under cold stress (Acuña-Rodríguez *et al.*, 2020).

1.6 Tomato is an economically relevant crop plant susceptible to cold stress

Tomato (*Solanum lycopersicum*) is a crop of great importance, both for its economic value and its nutritional benefits. Tomato is the most produced vegetable in the world with 192 million tons in

2023, followed by onions (111 million tons, including shallots), cucumbers (98 million tons, including gherkins), cabbages (74 million tons), and eggplants (61 million tons) (FAO, 2024, n.d.). China led global production with 70 million tons, representing 35.5% of the world's total output (FAO. 2024. FAOSTAT: Production: Crops and livestock products). India followed with 20 million tons, and Turkey produced 13 million tons (FAO. 2024. FAOSTAT: Production: Crops and livestock products). Italy was the top European producer, ranking sixth globally with 6 million tons, accounting for 3% of world production. Remaining in Europe and within the top-10 global ranking, Spain secured the eighth position with nearly 4 million tons, accounting for 2% of the world's production. (FAO. 2024. FAOSTAT: Production: Crops and livestock product). The reason for its popularity lies in the versatility of the tomato within the food chain, from table tomatoes to processed products, and in the nutritional potential of the fruit. Tomato contains different antioxidant molecules such as carotenoids, particularly lycopene, ascorbic acid, vitamin E, and phenol compounds, particularly flavonoids (Frusciante *et al.*, 2007). These characteristics make it a food with excellent nutritional properties, and it is therefore well accepted that tomato fruits are an important source of bioactive compounds with known beneficial effects, including vitamins, antioxidants, and anticancer substances (Raiola *et al*., 2014).

Cultivated tomato is an annual crop (Wang *et al.*, 2023) and it is a member of the Solanaceae family, which includes more than 3000 species, and contains many taxa of importance, both agronomically (potatoes, tomatoes, peppers) and medicinally (mandrake, tobacco, deadly nightshade, henbane) (Knapp, 2002). Originating from South America, tomato plants were imported to Europe in the 16th century: it is now a diploid plant with 2n = 24 chromosomes (Gerszberg *et al*., 2015). Tomato plants are sensitive to temperatures below 12°C and above 32°C (Mesa *et al.,* 2022). Under commercial conditions, seedlings take 60-80 days from transplanting into the soil to fructify.

1.6.1 Tomato cold susceptibility and winter cultivation challenges in Sicily

Tomato is a cold-sensitive plant, but it is cultivated all around the world. Besides this, tomato plants can develop a chilling acclimation response, which includes comprehensive transcriptomic and metabolic adjustments leading to increased chilling tolerance (Barrero-Gil *et al.,* 2016). Strong resemblances between this response and cold acclimation, the process whereby plants from temperate regions raise their freezing tolerance after exposure to low, non-freezing temperatures *(Barrero-Gil et al.,* 2016). Cold acclimation is regulated by transcription factors and hormones that lead to defense mechanisms, including the accumulation of compatible solutes, the mobilization of antioxidant systems and the rearrangement of the photosynthetic machinery (Barrero-Gil *et al.,* 2016), indicating an attempted activation of acclimation processes in tomato plants.

Sicily is an Italian island located in the central part of the Mediterranean Sea. The top five products affecting the value of regional agri-food imports ingredients of the Sicilian culinary tradition: fish, pistachios, crustaceans, and mollusks. As far as on the other hand, the predominant products predominant products are table grapes and fruit juices, followed fruit juices, followed at some distance by tomatoes followed at some distance by tomatoes (excluding dry tomatoes) oranges and virgin and extra virgin oil (Pubblicato l'opuscolo "L'agricoltura in Sicilia in cifre 2024," n.d.). Particularly, in the Vittoria area (Ragusa province) in Sicily, protected horticulture occupies 4 thousand hectares, mainly cultivated with tomatoes, Thus, in the Ragusa area, the most important agricultural sector is that of greenhouses (Berti, 2020). These structures follow one another on mostly sandy soils, from Vittoria to Pozzallo, touching Ispica, the area between Donnalucata and Scicli, as well as the coast between Punta Secca and Santa Croce Camerina and that between Marina di Acate and Acate (Berti, 2020). Over the years, the areas planted with vegetables in a protected environment have increased in

Sicily; the transformed zone hosts about 4 thousand hectares (out of 9 thousand total). Second in terms of production, only to Spain, the Ragusano area in 2019 recorded a production of 188 thousand tons of fruits. In the foreground is the tomato (120 thousand tons harvested), which accounts for more than 50% of production of Sicily. This is followed by zucchini, the quantities of which have grown a lot in the last three years, reaching 38 thousand tons. At the tail end are peppers and aubergines (both 15 thousand tons harvested) (Berti, 2020). At a national level, given the excessively high running costs, almost all greenhouses are unheated. Generally, the most common are simple plastic tunnels, not too long (maximum 40-45 meters) to favor ventilation and without openings in the eaves, again in order to keep running costs down (FruitJournal.com, 2023).

Tomato annual production has been increasing in recent years and is explained by the higher yield per square meter under commercial greenhouse conditions (especialistasweb, 2024). If conditions are favorable, the greenhouse tomato can achieve two harvests per year, which provides a large production of quality fruit (FruitJournal.com, 2023). For example, in Sicily, transplanting takes place between September and October (FruitJournal.com, 2023) and transplanted crop has a cycle of 100-120 days (Pomodoro Lycopersicon esculentum Mill. - Piante da tubero e orticole - Coltivazioni erbacee, n.d.). Thus, plants need to overcome winter. In the Vittoria area, the average of the seasons affected by transplanting goes from 19°C-28°C in September - the hottest month - to 14°C-7°C in January - the hottest month (Figure 1.17). In this panorama, therefore, tomato plants are faced with sub-optimal temperatures for a large part of their cycle, even if they are in a greenhouse.

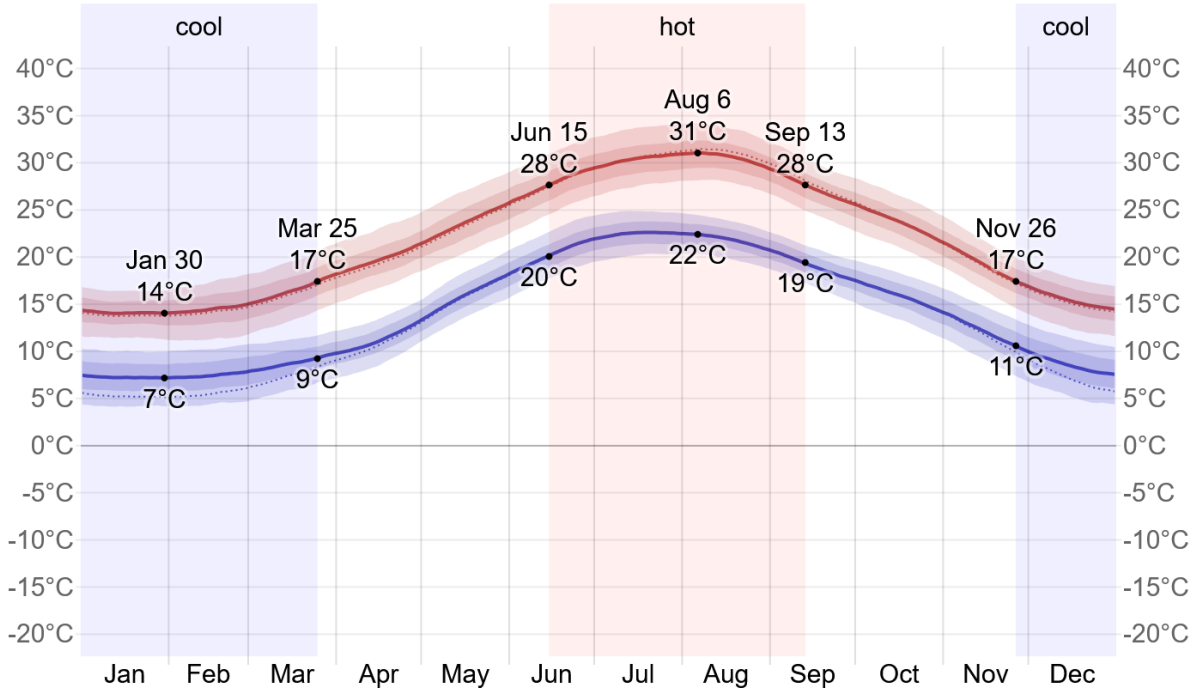


Figure 1.17. Average high and low temperatures in Vittoria. The daily average high (red line) and low (blue line) temperature, with 25th to 75th and 10th to 90th percentile bands. The thin dotted lines are the corresponding average perceived temperatures_The importance of plant growth promoting bacteria in soil and stress management (Vittoria Climate, Weather By Month, Average Temperature (Italy) - Weather Spark, n.d.).

1.6.2 Use of growth-promoting and stress-tolerant bacteria in tomato cultivation

Fertilization is essential for the optimal growth and development of crops; however, the amount of fertilizer can cause positive or negative effects depending on its rate. In addition, the cultivation system plays a significant role in determining vegetative growth and fruit quality (Terada *et al*., 2023). The yield and fruit quality of tomato are affected by the type of fertilization (Li *et al*., 2023). For example, Wu et al. found that the combined use of 25% cow manure and 75% inorganic fertilizer was the best condition for tomato production, explained by balanced soil physicochemical variables and microbial nitrification process (Wu *et al.*, 2022). Another study pointed out that the conjunctive use of compost and inorganic fertilizer made it possible to reduce inorganic fertilization by about 40%

while obtaining similar fruit quality and amounts in addition to improving soil characteristics (Hernández *et al.*, 2014). Li et al. showed that the application of digestate significantly increased the growth and fruit quality of tomato, including height, stem diameter, leaf chlorophyll content index, and photosynthetic rate of tomato plant and sugar–acid ratio, protein content, and ascorbic acid content of the fruit (Li *et al*., 2023). The nitrate contents in tomato fruit were lower in the digestate treatment and digestate combined with chemical fertilizer treatment than in the chemical fertilizer (Li *et al.*, 2023). The digestate combined with chemical fertilization resulted in the greatest increase in tomato yield, up to 26.29% and 10.78% higher than that in the chemical fertilizer treatment under field and greenhouse conditions, respectively (Li *et al.,* 2023). The effects of digestate treatments to maintain a stable tomato yield and improve fruit quality may be due to the enhanced soil enzymatic activities and chemical properties (Li *et al*., 2023).

Numerous studies highlight the efficacy of plant growth-promoting bacteria (PGPB) in tomatoes, both as agents that promote growth and as key players in mitigating abiotic stresses. Strains of *Pseudomonas, Serratia, Azotobacter, Bacillus, and Actinobacter* have been proven to increase tomato growth (Almaghrabi *et al.*, 2013; Tahiri e*t al.*, 2022; Valencia-Marin *et al.*, 2025). PGPB provided protection to tomato under cold stress (Subramanian *et al*., 2016), salt stress (Sánchez *et al*., 2023), water stress (Mannino *et al.,* 2020), and drought stress (Abdelaal *et al.*, 2021). Thus, PGPB represents a promising approach to enhance plant growth, valorize the soil, and mitigate abiotic stress, particularly cold stress under commercial greenhouse and field conditions (Figure 1,18).

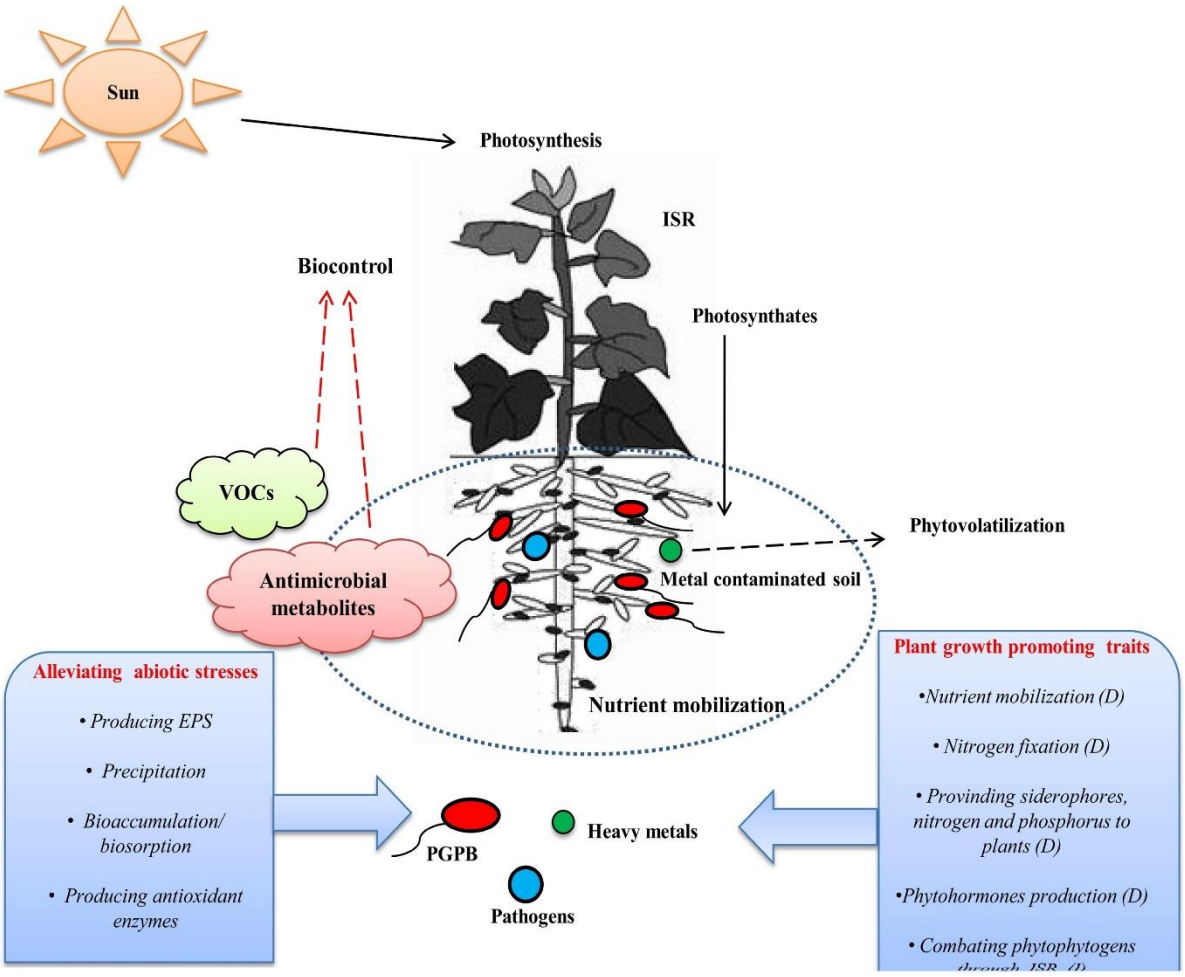


Figure 1.18. The importance of plant growth promoting bacteria in soil and stress management (Singh *et al*., 2018)

1.7 Aims of the project

The aim of this PhD project can be summarized as follows:

1. To select the best-performing plant growth-promoting bacteria for tomato plants under controlled conditions. This first part of the study aims to test bacterial isolates from a laboratory collection to identify PGPBs. The objective was achieved through growth assessment tests, in which tomato plants were inoculated with bacterial isolates and grown under controlled conditions. The biomass was measured and used as a parameter to establish the success of bacterial inoculation. The initial screening with 41 bacterial isolates was conducted under non-stressed conditions (25°C), and ten isolates that met predefined selection criteria were subsequently analyzed in a validation trial under both non-stressed (25°C) and cold-stressed (10°C) conditions to confirm their efficacy as PGPBs. After the validation trial, two isolates were selected for the subsequent analyses.
2. To investigate functional and transcriptional mechanisms activated by plant growth-promoting bacteria in tomato plants exposed to cold stress and incubated under optimal conditions. The objective was achieved through the functional characterization trial, in which biomass and H_2O_2 assessement were carried out on the two selected isolates under non-stressed and cold-stressed conditions. Moreover, transcriptomics analysis was performed on shoots of tomato plants inoculated with the two isolates under non-stressed and cold-stressed conditions.
3. To validate the plant growth-promoting activity of selected bacteria under commercial greenhouse conditions used for winter tomato production in Sicily. Specifically, growth trials were conducted in a commercial greenhouse using tomato plants of the local variety typically cultivated under greenhouse conditions. Parameters such as root biomass and fruit count were assessed at the end of the growth cycle to evaluate the bacterial inoculation.

When planning the PhD project, it was necessary to find an experimental strategy (Figure 1.19) aimed at identifying versatile bacterial isolates that would prove to be efficient PGPBs both in non-stressed conditions and under cold-stressed conditions. For this reason, the initial growth tests were carried out under non-stressed conditions, and subsequently was introduced the stress parameter. In addition, we wanted to investigate the molecular mechanisms by which tomato plants inoculated with certain isolates appear to be more resilient to cold stress, mechanisms that may form the basis for future investigations and applications.

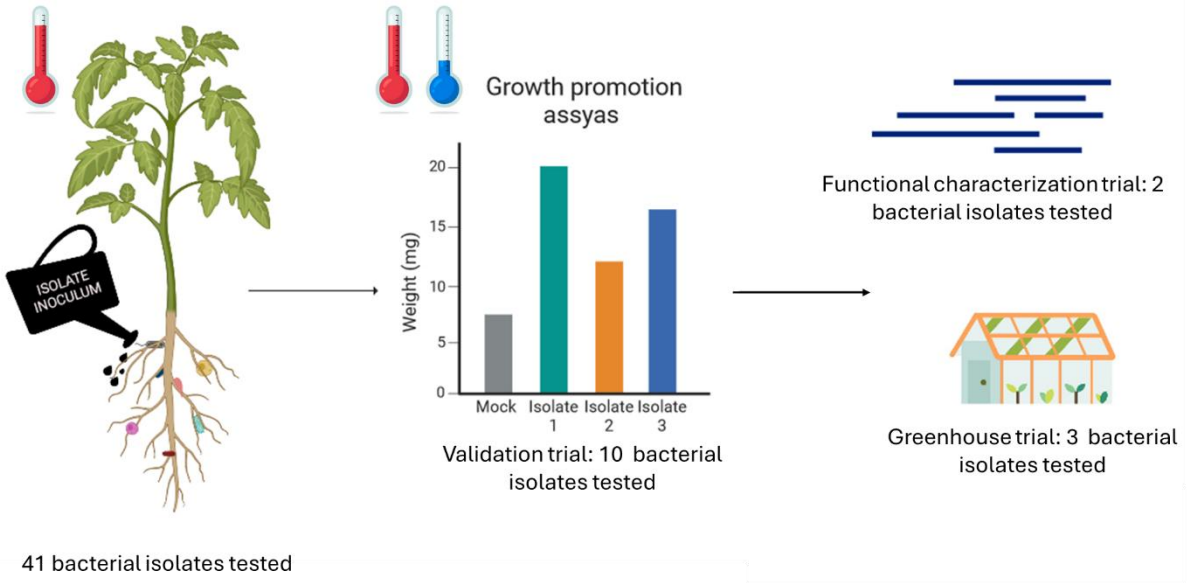


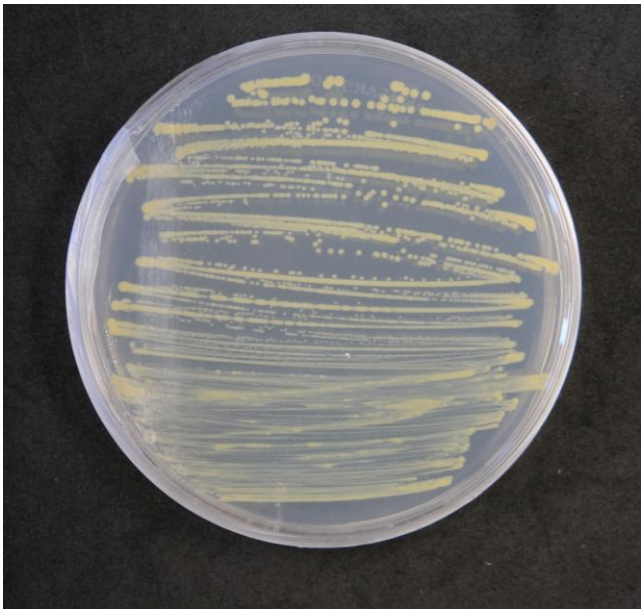
Figure 1.19. Overview of the experimental workflow. The study involved three phases: (1) initial screening of 41 bacterial isolates under non-stressed conditions, leading to the selection of ten candidates and subsequent validation under non-stressed (25°C) and cold-stressed (10°C) conditions; (2) functional and transcriptomic characterization of two selected isolates under both temperature regimes; and (3) greenhouse trials in Sicily using three selected isolates to assess root biomass and fruit yield in winter tomato production.

CHAPTER 2. MATERIALS AND METHODS

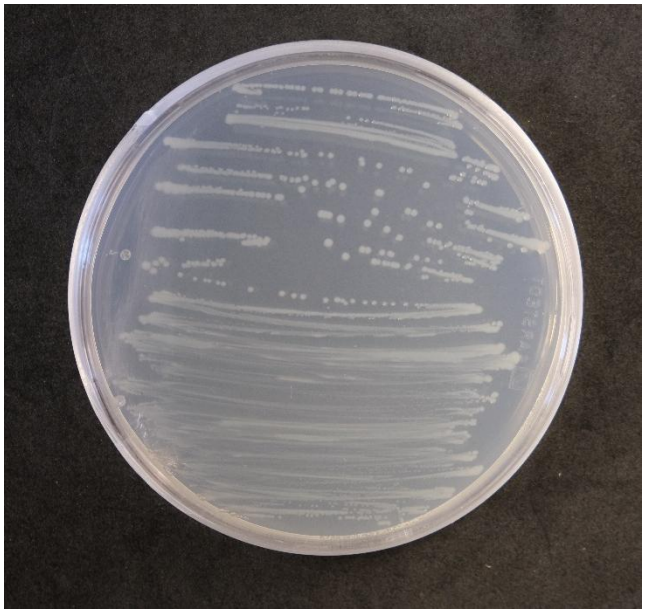
2.1 Cold-tolerant bacterial isolation and growth conditions

Cold-tolerant bacterial endophytes were previously isolated from the roots of three alpine *Rosaceae* plants (*Alchemilla sp., Dryas octopetala,* and *Geum montanum*) (Marian *et al*., 2025). Bacterial isolates were stored in 40% glycerol at -80 °C and grown in Reasoner's 2A agar (R2A, Sigma-Aldrich, Merch, Rahway, NJ, USA) at 25 °C ± 1 °C. The 16S gene sequence of each cold-tolerant bacterial isolate was analyzed by nucleotide alignment against the 16 ribosomal RNA database of the National Center for Biotechnology Information (NCBI). The first ten best hits of the alignments were used to verify the non-pathogenicity for humans and crops according to the literature search, and a total of 41 isolates were selected for the functional characterization in tomato plants (Table S1).

To prepare the bacterial suspension for plant inoculation, each isolate was grown overnight (18 h) in liquid Reasoner's 2 Broth (R2B, Sigma-Aldrich) at 25 ± 1 °C under orbital shaking at 180 rpm. Bacterial cells were collected by centrifugation (3,500 × g for 10 min) and washed three times with sterile 10 mM $MgSO_4$. The bacterial suspension was then adjusted to 1.0×10^8 colony forming units (CFU) per unit of volume (CFU mL^{-1}) based on the conversion of optical density (OD) at 600 nm (OD_{600}) optimized for each isolate using an Ultrospec 3100 spectrophotometer (GE Healthcare, Chicago, IL, USA). For the two cold-tolerant bacterial isolates used in the functional characterization experiments, the OD_{600} 0.1 corresponded to $9.8 \times \pm 0.8 \times 10^7$ CFU mL^{-1} for *Chryseobacterium* sp. GRCS301 (*Chryseobacterium* sp.) and $8.7 \pm 1.0 \times 10^7$ CFU mL^{-1} for *Pseudomonas* sp. GRCS202 (*Pseudomonas* sp.) (Figure 2.1).



Chryseobacterium sp. GRCS202



Pseudomonas sp. GRCS301

Figure 2.1. Photos of cold-bacterial isolates. *Chryseobacterium* sp. GRCS301 and *Pseudomonas* sp. GRCS202 grown on solid medium Reasoner's 2A agar. Photos were taken 48 hours after plating the bacterial isolates from the stocks stored at -80°C.

2.2 Plant material and growth conditions

Seeds of *Solanum lycopersicum* L. cultivar Moneymaker (Justseed, Wrexham, UK) were surface disinfected as previously reported (Perazzolli *et al.,* 2022). Briefly, seeds were treated with 70% ethanol for 1 min, 2% sodium hypochlorite for 5 min, and 70% ethanol for 1 min, followed by three washes with sterile distilled water (3 min each) in a 50 mL-tube with moderate shaking. Surface disinfected seeds were transferred into a 100 cm^2-square dish (100 seeds per dish; Sarstedt, Nümbrecht, Germany) containing 10 g L^{-1} water agar (0.8% Agar Technical, Oxoid, Basingstoke, Hampshire, UK) and they were incubated for two days at 25 ± 2 °C in the dark to allow seed germination. Germinated seeds with the same root length (2 mm) were selected and transplanted in pots (45 cm^3) containing a sterilized soil:sand (1:2) mixture (Semina 80 Tecno Grow, Tercomposti,

Calvisano, Italy and silica sand, Type 519, Bacchi, Boretto, Italy) and incubated in a growth chamber (Ekochl 700, Angelantoni Life Science, Milano, Italy) at 25 ± 2 °C with a 14:10 light:dark photoperiod (photon flux density of 0.033 mmol s^{-1} m^{-2}) Plants were treated with 1.5 ml of sterile 10 mM $MgSO_4$ (mock-inoculated) or inoculated with 1.5 ml of the suspension (1.0×10^8 CFU mL^{-1}) of the respective cold-tolerant bacterial isolate (bacterium-inoculated) in a randomized block design. Treatments were applied at the root collar every seven days starting from the transfer into the soil:sand mixture for a total of four applications to each plant (Figure 2.2).

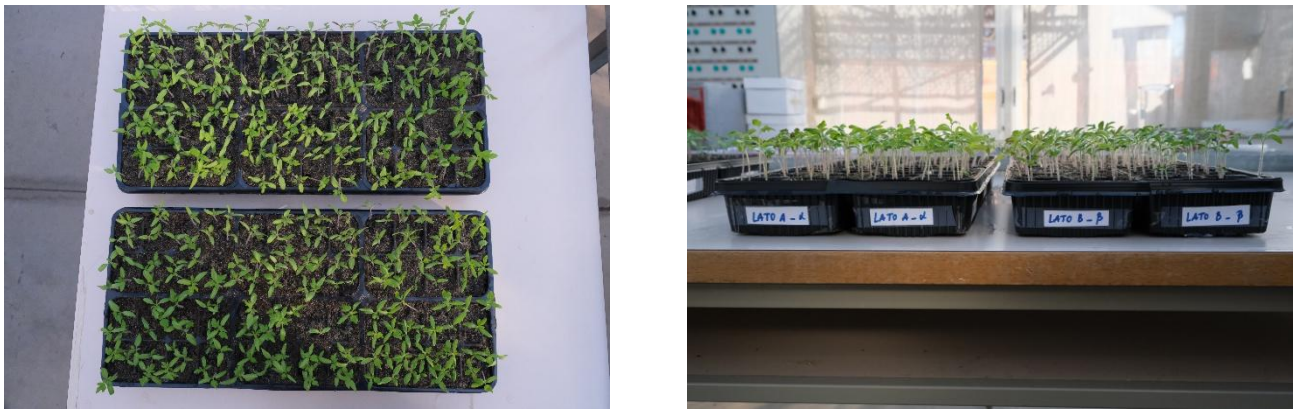


Figure 2.2. Tomato seedlings after 4 weeks in the chamber growth. This figure shows two illustrative images of four-week-old tomato seedlings, featuring an aerial view on the left and a side view on the right.

To select plant growth promoting bacteria, screening trials with the 41 cold-tolerant bacterial isolates were carried out, and plants were incubated in the growth chamber for 28 days at 25 °C ± 2 °C with a 14:10 light:dark photoperiod. The plant growth promotion activity of the best performing bacterial isolates was further analyzed under non-stressed and cold-stressed conditions in validation trials and functional characterization trials. Therefore, the selected isolates were tested to confirm their ability to act as plant growth-promoting bacteria in the validation trial, and their effects on the plant were subsequently investigated in the functional characterization trial. In particular, mock-inoculated and bacterium-inoculated plants were grown for 14 days in the growth chamber at 25 ± 2 °C with a 14:10 light:dark photoperiod. Two groups of plants were then obtained by random selection within

each inoculation condition, and they were incubated at 25 °C ± 2 °C in the growth chamber (non-stressed plants) or at 10 °C ± 2 °C in another growth chamber (cold-stressed plants) for a further incubation of 14 days with a 14:10 light:dark photoperiod (Figure 2.3). Four (screening trials and validation trials) or three (functional characterization trails) replicates were analyzed for each inoculation condition and temperature regime, and each replicate consisted of a pool of four plants (screening trials) or seven plants (validation trials and functional characterization trials).

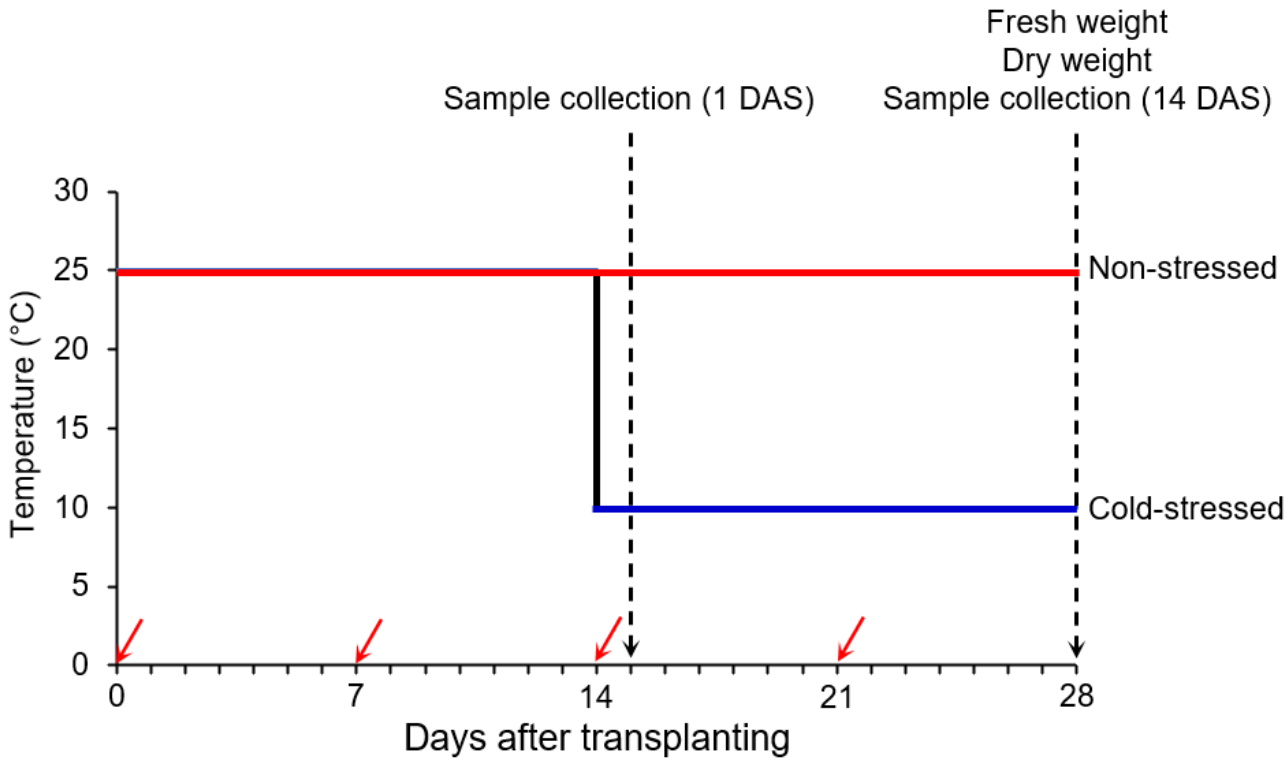


Figure 2.3. Growth conditions and sampling time points for validation trials and functional characterization trials. Germinated seeds were transplanted in pots containing sterilized soil:sand mixture and grown in a growth chamber at 25 °C ± 2 °C with a 14:10 light:dark photoperiod. Plants were treated with 1.5 ml of sterile 10 mM $MgSO_4$ (mock-inoculated) or inoculated with 1.5 ml of the suspension of the respective cold-tolerant bacterial isolate (bacterium-inoculated) at 0, 7, 14, and 21 days after transplanting (red arrows) for a total of four applications at the root collar using a randomized block design. In the screening trials, mock-inoculated and bacterium-inoculated plants were grown for 28 days at 25 °C ± 2 °C with a 14:10 light:dark photoperiod. In the validation trials and functional characterization trials, mock-inoculated and bacterium-inoculated plants were grown for 14 days in the growth chamber at 25 ± 2 °C with a 14:10 light:dark photoperiod, and two groups of plants were then incubated at 25 °C ± 2 °C in the growth chamber (non-stressed plants) or at 10 °C ± 2 °C in another growth chamber (cold-stressed plants) for further 14 days with a 14:10 light:dark

photoperiod. Four replicates (screening trials and validation trails) or three replicates (functional characterization trails) were analyzed for each inoculation condition and temperature regime, and each replicate consisted of a pool of four plants (screening trials) or seven plants (validation trials and functional characterization trials). Shoot fresh weight (screening trials, validation trials, and functional characterization trials) and dry weight (screening trials and validation trials) were assessed at the end of the experiment. In the functional characterization trials, shoots of mock-, *Chryseobacterium*-, and *Pseudomonas*-inoculated plants were collected at one day and 14 days after stress exposure (DAS; dotted arrows) from non-stressed and cold-stressed plants for H_2O_2 analysis (1 DAS) and RNA-seq analysis (1 DAS and 14 DAS).

2.3 Sample collection and assessment of fresh weight and dry weight

In the screening trials, validation trials, and functional characterization trials, tomato shoots of each replicate (pool of at least four plants each) were collected at the end of the incubation in the growth chamber. The fresh weight was immediately assessed with a precision balance (E42, Gibertini, Milan, Italy) and expressed as mg of each plant. In the screening trials and validation trials, each shoot sample was then incubated at 60 °C for 48 h, the dry weight of each replicate was assessed with the precision balance and expressed as mg of each plant.

In the functional characterization trials, tomato shoots of mock-, *Chryseobacterium*-, and *Pseudomonas*-inoculated plants were collected at one day and 14 days after stress exposure (DAS) from non-stressed and cold-stressed plants (Figure 2.3). These time points were chosen to analyze the early (Barrero-Gil et al., 2016) and late (Reimer et al., 2021) transcriptional response of tomato plants to cold stress. Moreover, 1 DAS was chosen to assess the content of hydrogen peroxide (H_2O_2) in response to cold stress (Diao et al., 2017). Each replicate (pool of seven plants each) of shoot samples was immediately frozen in liquid nitrogen and stored at -80 °C. Tomato samples were ground to a fine powder using a mixer mill disruptor (MM 400, Retsch, Haan, Germany) at 25 Hz for 60 s with 2 ml-tubes and 6 mm-beads refrigerated in liquid nitrogen, and the resulting powder was stored at −80 °C until further analysis. Three replicates pool (pool of seven plants each) were obtained for each

inoculation condition (mock-, *Chryseobacterium*-, and *Pseudomonas*-inoculated plants), temperature regime (non-stressed and cold-stressed plants), and time point (1 DAS and 14 DAS).

2.4 Bacterial re-isolation from tomato roots

In the functional characterization trials, tomato roots from each replicate (pool of six plants each) were collected at the end of the incubation in the growth chamber (14 DAS) and rhizosphere samples were obtained as previously described (Bulgarelli *et al*., 2012) with slight modifications. Briefly, fresh weight of tomato roots was immediately assessed using a precision balance (E42, Gibertini, Milan, Italy) and large soil aggregates were removed by shaking the roots. Root samples were transferred to a 15 ml-tube containing 2.5 ml sterile 10 mM $MgSO_4$ and 0.01% Tween 20 buffer and incubated for 20 min under orbital shaking at 180 rpm. Each suspension was serially diluted, and aliquots (10 µl) were plated in triplicates on R2A agar plates containing 2.5 µg mL^{-1} cycloheximide, 2 µg mL^{-1} erythromycin, 10 µg mL^{-1} gentamycin, and 5 µg mL^{-1} kanamycin to isolate *Chryseobacterium* sp. (*Chryseobacterium* medium), or R2A agar plates containing 2.5 µg mL^{-1} cycloheximide, 30 µg mL^{-1} chloramphenicol, and 5 µg mL^{-1} tobramycin sulfate to isolate *Pseudomonas* sp. (*Pseudomonas* medium). CFU values of bacterial isolates were assessed for mock-, *Chryseobacterium*-, and *Pseudomonas*-inoculated plants in each growth medium and expressed per unit of root fresh weight (CFU g^{-1}) two days after incubation at 25 ± 1 °C. Four replicates (with five plants each) with two technical replicates (plates) were analyzed for inoculation condition (mock-, *Chryseobacterium*-, and *Pseudomonas*-inoculated plants) and temperature regime (non-stressed and cold-stressed plants).

2.5 Assessment of H_2O_2 content in tomato shoots

H_2O_2 content in tomato shoots was assessed using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) as previously described (Chakraborty *et al.,* 2016). Briefly, each ground shoot sample (50 mg) was mixed with 250 µl of phosphate buffer (50 mM, pH 7.4), incubated in ice for 30 minutes under orbital shaking and centrifuged at 12,000 × g for 5 min at 4 °C. The supernatant (5 µL) was mixed with 45 µL of phosphate buffer (50 mM, pH 7.4) in a flat-bottom-black 96-well plate (Greiner 655076, Sigma-Aldrich). A calibration curve of H_2O_2 was obtained (0, 0.078, 0.156, 0.313, 0.625, 1.25, 2.5, and 5 µM) by mixing the H_2O_2 standard (10 µM) with phosphate buffer (50 mM, pH 7.4) in a final volume of 50 µL in each well. The reaction buffer (50 µL) containing the AmplexRed reagent and horseradish peroxidase (HRP) was added to each well for a total volume of 100 µL and mixed by pipetting. Samples were incubated in the dark at room temperature for 30 min and fluorescence was measured using a Synergy 2 Multi-Mode Microplate Reader (Biotek, Winooski, VT, USA) with excitation range from 525 nm to 540 nm and emission range from 520 nm to 595 nm. Three replicates (pool of seven plants each) and two technical replicates were analyzed for each inoculation condition and temperature regime.

2.6 RNA extraction and sequencing

Total RNA was extracted from 80 mg of ground shoot samples using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, Merck) with an on-column DNase treatment with RNase-Free DNase Set (Qiagen, Hilden, Germany). Total RNA was quantified using a Qubit (Thermo Fisher Scientific) and RNA quality was checked using a Tapestation 4150 (Agilent Technologies, Santa Clara, CA, USA). The effectiveness of the DNase treatment was confirmed by running a PCR with primers of a tomato

gene encoding ankyrin repeat domain containing protein 2 (*ARD2*; Supplementary Table S2) in the absence of retro-transcription, and no amplification signals were detected. Three replicates (pool seven plants each) were analyzed for each inoculation condition (mock-, *Chryseobacterium*-, and *Pseudomonas*-inoculated plants), temperature regime (non-stressed and cold-stressed plants) and time point (1 DAS and 14 DAS).

RNA samples were subjected to RNA-Seq library construction using the TruSeq Stranded Total RNA with Ribo-Zero Plant library preparation kit (Illumina, SanDiego, CA, USA) according to the manufacturer's instructions. Paired-end reads of 150 nucleotides were obtained using a Novaseq Instrument (Illumina) at Fasteris (Plan-les-Ouates, Switzerland) and sequences were deposited at the Sequence Read Archive of the NCBI (https://www.ncbi.nlm. nih.gov/sra) under the BioProject number PRJNA1079540.

2.7 Bioinformatic analysis, identification and functional annotation of differentially expressed genes

After assessing the quality of the RNA-Seq raw reads with FastQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc), adapter sequences and low-quality reads were removed using Fastq-mcf v1.05 with the settings as follows: minimum quality threshold of 30 (-q 30) and a minimum read length of 50 base pairs (-l 50) (Aronesty, 2013). Filtered read pairs were mapped to the *S. lycopersicum* reference genome version ITAG5.0 (https://phytozome-next.jgi.doe.gov/) (Zhou *et al*., 2022) using STAR version 2.7.11 a with default parameters, and read counts of each tomato gene were assessed using htseq-count version 1.99.2 with default parameters. Differential expressed genes (DEGs) were identified with the DESeq2 package version 1.47.1 in R with default parameters (Love *et al.*, 2014) imposing a false discovery rate (FDR) lower than 0.05

(adjusted *P*-value ≤ 0.05) and a Log_2-transformed fold change (LFC) higher than 1 or lower than -1 in the pairwise comparisons between the cold-stressed and non-stressed condition of mock-inoculated plants (cold-stressed mock-inoculated *vs* non-stressed mock-inoculated), *Chryseobacterium*-inoculated (cold-stressed *Chryseobacterium*-inoculated *vs* non-stressed *Chryseobacterium*-inoculated), and *Pseudomonas*-inoculated plants (cold-stressed *Pseudomonas*-inoculated *vs* non-stressed *Pseudomonas*-inoculated) for each time point (1 DAS and 14 DAS). The distribution of DEGs was summarized using the Venn diagram (http://bioinformatics.psb.ugent.be/webtools/Venn/) and DEGs were grouped into upregulated and downregulated genes for each time point.

2.8 Functional annotation of differentially expressed genes

Protein descriptions and Gene Ontology (GO) annotations of each DEG were obtained from the tomato reference genome (*S. lycopersicum* version ITAG5.0; https://phytozome-next.jgi.doe.gov/) (Zhou et al., 2022)(Zhou et al. 2022). DEGs upregulated and downregulated by cold stress in all inoculation conditions or exclusively modulated by cold stress in mock-, *Chryseobacterium*-, and *Pseudomonas*-inoculated plants were selected, and GO enrichment analysis was performed in R using the *topGO* package version 2.58.0 (Alexa and Rahnenführer, n.d.), and enriched categories belonging to biological processes were identified using the *classic* algorithm with the Fisher's classic test ($P \leq 0.05$) (Chowdhury et al., 2023).

2.9 Gene expression analysis by quantitative real-time RT-PCR

The first strand cDNA was synthesized from 1 μg of total RNA using Superscript III (Invitrogen, Thermo Fisher Scientific) and oligo-dT primer. Genes encoding the 9-cis-epoxycarotenoid dioxygenase (*NCED5*), C-repeat/DRE binding factor 1 (*CBF1*), chalcone synthase (*CHS2*), elongated hypocotyl 5 transcriptional factor (*HY5*), flavonoid-3'-hydroxylase (*F3'5'H*), glutathione-S-transferase enzyme (*GST*), and sucrose synthase (*SUS*) were used as markers of tomato response to cold stress (Barrero-Gil et al., 2016; Reimer et al., 2021; Ma et al., 2022) for RNA-Seq validation (Supplementary Table 2). Quantitative real-time PCR (qPCR) reactions were carried out with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Thermo Fisher Scientific) and specific primers (Supplementary Table S2) using the Light Cycler 480 (Roche Diagnostics), as previously described (Avesani et al., 2023). In particular, the PCR conditions were as follows: 50 °C for 2 min and 95 °C for 2 min as initial steps, followed by 50 cycles at 95 °C for 15 s and at 60 °C for 1 min. Each sample was examined in three technical replicates and dissociation curves were analyzed to verify the specificity of each amplification reaction. The Light Cycler 480 SV 1.5.0 software (Roche) was used to extract Ct values based on the second derivative calculation and the reaction efficiency (Eff) was calculated with the LinRegPCR 11.1 software for each gene (Ruijter et al., 2009). The expression level of each gene was calculated according to the Hellemans equation (Hellemans et al., 2007), using housekeeping genes encoding *ARD2* (Pombo et al., 2017) and glyceraldehyde-3-phosphate dehydrogenase (*GADPH*) (Ghorbanpour et al., 2018) (Supplementary Table S2). Relative quantities (RQ) were calculated according to the formula: $RQ = Eff^{(Ct-Ct')}$, where Ct is the threshold cycle and Ct' is the average Ct values of mock-inoculated plants for each temperature regime and time point. Normalized relative quantities (NRQ) were then calculated by dividing the RQ by the normalization factor, based on the RQ values of the two housekeeping genes (Hellemans et al., 2007).

Three replicates (pool of seven plants each) were analyzed for each inoculation condition, temperature regime, and time point.

2.10 Inoculum preparation for the trial under commercial greenhouse conditions

The greenhouse trial has been performed in an experimental greenhouse at ITAKA Crop Solutions (https://itakasolution.com/it/la-nostra-storia/; Comiso, Ragusa, Italy) that was partner of the PON project. This company specializes in the development and testing of natural solutions for agriculture. Three cold-tolerant bacteria that showed a significant increase in dry weight compared to the mock-inoculated plants in the validation trail were chosen for the experiment under commercial greenhouse conditions, such as *Chryseobacterium* sp. GRCS301 (*Chryseobacterium* sp.), *Paenibacillus* sp. GRFS203 (*Paenibacillus* sp.), and *Pseudomonas* sp. GRCS202 (*Pseudomonas* sp.). To prepare the bacterial suspension for plant inoculation, each isolate was grown for 72 hours on R2A medium (Sigma-Aldrich) at 25 ± 1°C. Bacterial cells were collected by scratching the agar with 5 ml 10 mM $MgSO_4$. The bacterial suspension was adjusted to 1.0×10^8 CFU mL^{-1} based on the conversion of optical density (OD) at 600 nm (OD600) using an Ultrospec 3100 spectrophotometer (GE Healthcare). For the three cold-tolerant bacterial isolates the OD_{600} 0.1 was specifically measured to correspond to $9.8 \times \pm 0.8 \times 10^7$ CFU mL^{-1} for *Chryseobacterium* sp. GRCS301 (*Chryseobacterium* sp.), $2.33 \times \pm 1 \times 10^6$ CFU mL^{-1} for *Paenibacillus* sp. GRFS203 (*Paenibacillus* sp.), and $8.7 \pm 1.0 \times 10^7$ CFU mL^{-1} for *Pseudomonas* sp. GRCS202 (*Pseudomonas* sp.). Moreover, a positive control provided by ITAKA Crop Solutions was included in the trail. The selected product is usually used as a bio-stimulant for tomato seedlings, and was prepared and supplied to the plant according to manufacturing instructions.

2.11 Plant Material and growth conditions under commercial greenhouse conditions

Seeds of *S.lycopersicum* cv. Rivaldo (CORA Seeds, Cesena, IT) were planted into a 64 cm^2 alveolus (84 seeds per tray) containing non-sterilized peat. Three-weeks old seedlings were transferred to pots (7000 cm^3) filled with non-sterilized sandy local soil and grown under commercial greenhouse conditions (Figure 2.4). Plants were treated with 50 ml of 10 mM $MgSO_4$ (mock-inoculated) or inoculated with 50 ml of the suspension (1.0×10^8 CFU mL^{-1}) of the respective cold-tolerant bacterial isolate (bacterium-inoculated) in a randomized block design. Treatments were applied at the root collar level after the transfer into pots and during the fruiting phase (15 weeks after the transplant) for a total of two treatments (Figure 2.5).

Figure 2.4. Experimental set-up under commercial greenhouse conditions. the seedlings were grown in alveoli and then transferred into pots.

Three replicates were analyzed for each inoculum condition, and each replicate consisted of a pool of five plants in a randomized block design. The greenhouse experiment was carried out during the second season of tomato production in Sicily, which roughly goes from September to February.

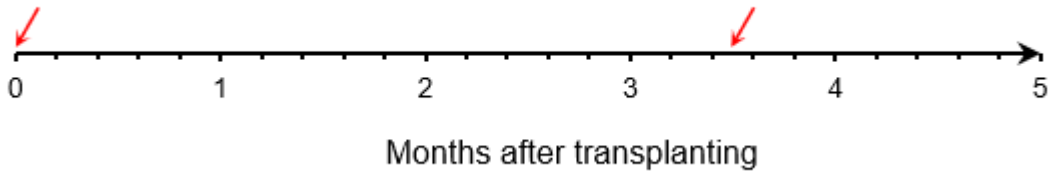


Figure 2.5. Bacterial treatments under greenhouse commercial conditions. A simplified temporal framework of the experiment is presented, depicted as a numbered horizontal timeline where each number represents a month. Red arrows above the timeline indicate the points at which the treatments were administered.

The commercial greenhouse has no controllable parameters, and temperatures are not available for each day of the winter season during which the tomato plants were grown. However, temperatures are available for the first 43 days (mid-September to end of October 2023) after transplanting into the greenhouse (Figure 2.6). The average minimum and maximum temperatures for October were recorded inside the greenhouse - 13.9 and 35.5°C respectively. Based on the fact that the greenhouse is not thermally insulated and on the local trends shown in Figure 1.17, which show a range of minimum temperatures for October compatible with that recorded in the greenhouse, we can only assume that cold stress occurred in November, December, January, and February.

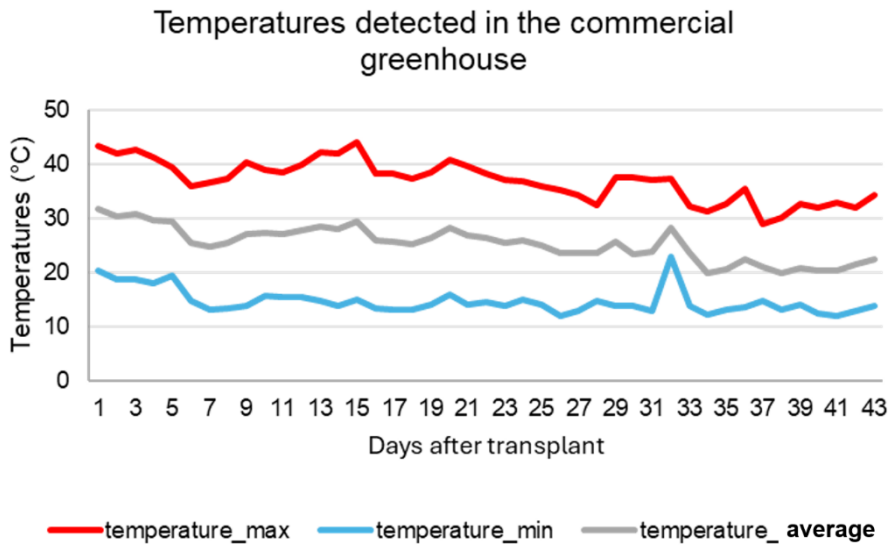


Figure 2.6. Maximum, minimum and mean of the temperatures registered in the first 43 days after transplant.

2.12 Sample collection and assessment of growth parameters under commercial greenhouse conditions

Tomato shoot length, shoot thickness, fruit number, and root dry weight were assessed for each plant during the vegetative phase, the fruiting phase, and at the end of the cycle, respectively. In particular, tomato shoot length and thickness of each plant were measured every 14 days starting from the transfer into pots for a total of 4 measurements during the first 6 weeks after the transplant (Figure 2.7). The fruit number for each plant was assessed starting from the development of the first fruit stage until the end of the production season. The time points were chosen according to the rate of growth and development of the fruiting stages, which were mainly dependent on the temperatures in December 2023 – January 2024 (Figure 2.7). Tomato roots of each plant were collected at the end of the reproductive period. Tomato roots were dried open air in the greenhouse for four days. The dry weight was assessed by using a standard electric balance and expressed as g of each plant (Figure 2.7).

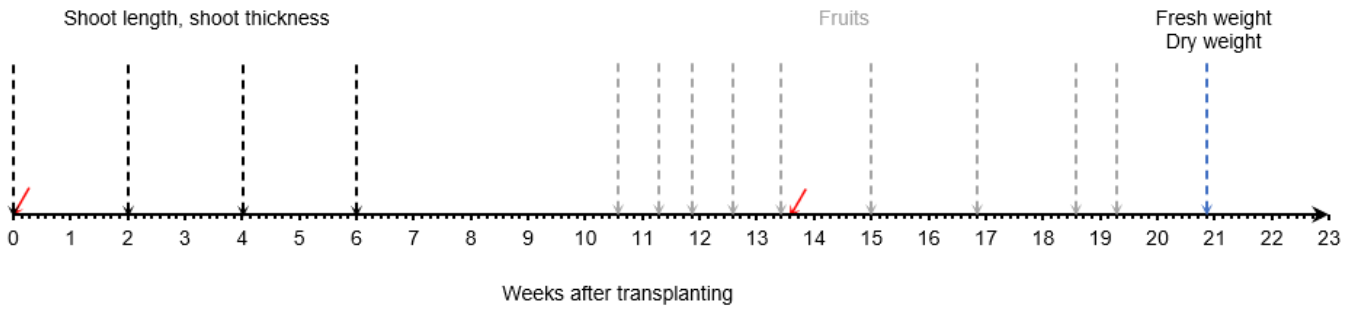


Figure 2.7. Experimental conditions under commercial greenhouse. Number of treatments (red arrows) and number of physiological samplings (dashed lines) are reported during the winter cycle under commercial greenhouse conditions.

2.13 Statistical analysis

Fresh weight, dry weight, fruit number, CFU counts, and H_2O_2 content were analyzed with R version 4.3.0 (https://www.r-project.org/1). Normal distribution (Shapiro test, $P > 0.05$) and variance homogeneity (Levene tests, $P > 0.05$) of the data were checked. When both assumptions were respected, the parametric t-test was used to detect significant differences ($P \leq 0.05$) between bacterium-inoculated and mock-inoculated plants for each temperature regime and time point, or between cold-stressed and non-stressed samples for each inoculation condition. When parametric assumptions were not respected, the non-parametric Mann-Whitney test was used to detect significant differences ($P \leq 0.05$) between bacterium-inoculated and mock-inoculated plants for each temperature regime and time point, or between cold-stressed and non-stressed samples for each inoculation condition.

CHAPTER 3. RESULTS

3.1 Cold-tolerant bacterial isolates promoted tomato growth and reduced H_2O_2 content under cold stress

The plant growth-promotion activity of 41 cold-tolerant bacterial isolates was assessed in the screening trials, and the shoot fresh weight was higher in plants inoculated with *Chryseobacterium* sp. GRCS301, *Flavobacterium* sp. ARAS206, *Microbacterium* sp. DREN103, *Paenibacillus* sp. GRFS203, *Plantibacter* sp. ARGS301, *Pseudomonas* sp. ARAN201, ARAS204, ARBN104, ARBS103, ARDN101, ARDS202, ARFN101, GRCS202, and *Stenotrophomonas* sp. ARGN203 compared to mock-inoculated plants after 28 days of incubation at 25 ± 2 °C (Figure 3.1A). Likewise, shoot dry weight was higher in plants inoculated with *Chryseobacterium* sp. GRCS301, *Flavobacterium* sp. ARAS206, *Paenibacillus* sp. GRFS203, *Plantibacter* sp. ARGS301, *Pseudomonas* sp. ARBN104, ARDS202, ARFN101, GRCS202, and *Stenotrophomonas* ARGN203 compared to mock-inoculated plants in the screening trials (Figure 3.1B). Ten cold-tolerant bacterial isolates were selected according to plant growth-promotion activity and taxonomic annotation (genera representative of the collection), and they were further characterized in the validation trials under non-stress and cold-stress conditions.

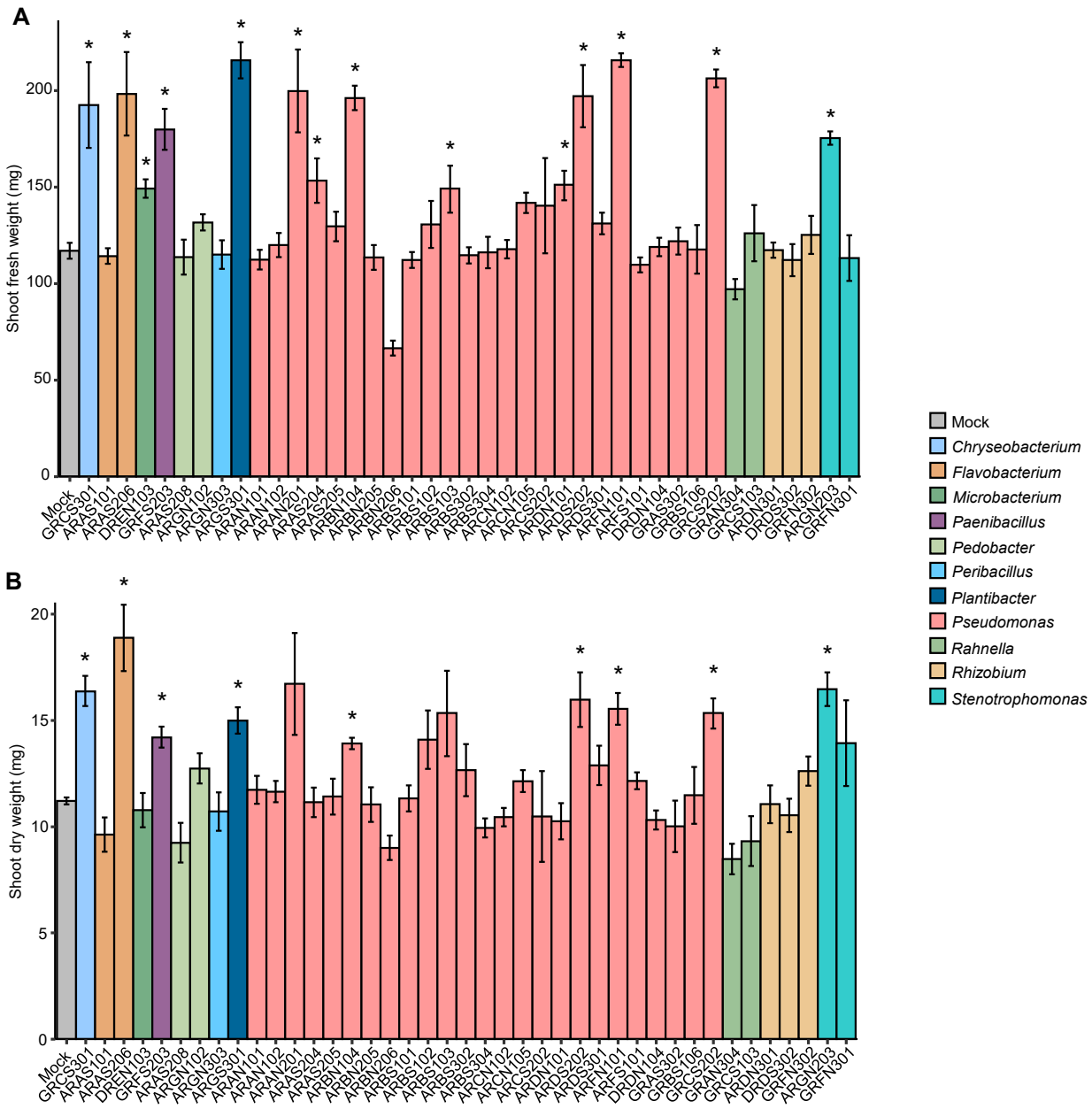


Figure 3.1. Screening trials of plant growth-promoting bacteria. Tomato plants were treated with MgSO4 (mock-inoculated) or inoculated with a bacterial suspension (bacterium-inoculated) of cold-tolerant bacterial isolates specified by the codes and taxonomic annotations (colored legend). Shoot fresh weight (A) and shoot dry weight (B) were assessed after 28 days of incubation in the growth chamber at 25 ± 2 °C and expressed as mg of each plant. Mean and standard error values of four replicates (pool of four plants) are reported for each inoculation condition. Asterisks indicate significant increases of shoot fresh weight or shoot dry weight in bacterium-inoculated compared to mock-inoculated plants, according to the Mann-Whitney test ($P \leq 0.05$).

In the validation trials, mock-inoculated and bacterium-inoculated plants were grown for 14 days in the growth chamber at 25 ± 2 °C, and further incubated at 25 ± 2 °C (non-stressed plants) or at 10 ± 2 °C (cold-stressed plants) for 14 days. The shoot fresh weight was improved by *Chryseobacterium* sp. GRCS301 and *Pseudomonas* sp. GRCS202 inoculation under non-stress conditions, and by *Chryseobacterium* sp. GRCS301, *Pseudomonas* sp. ARBN104, ARDN101, and GRCS202 inoculation under cold-stress conditions in the validation trials (Figure 3.2A). Moreover, shoot dry weight was higher in plants inoculated with three bacterial isolates (*Chryseobacterium* sp. GRCS301, *Paenibacillus* sp. GRFS203, and *Pseudomonas* sp. GRCS202) and six bacterial isolates (*Chryseobacterium* sp. GRCS301, *Paenibacillus* sp. GRFS203, *Pseudomonas* sp. ARBN104, ARDN101, ARFN101, and GRCS202) compared to mock-inoculated plants under non-stress and cold-stress conditions, respectively (Figure 3.2B).

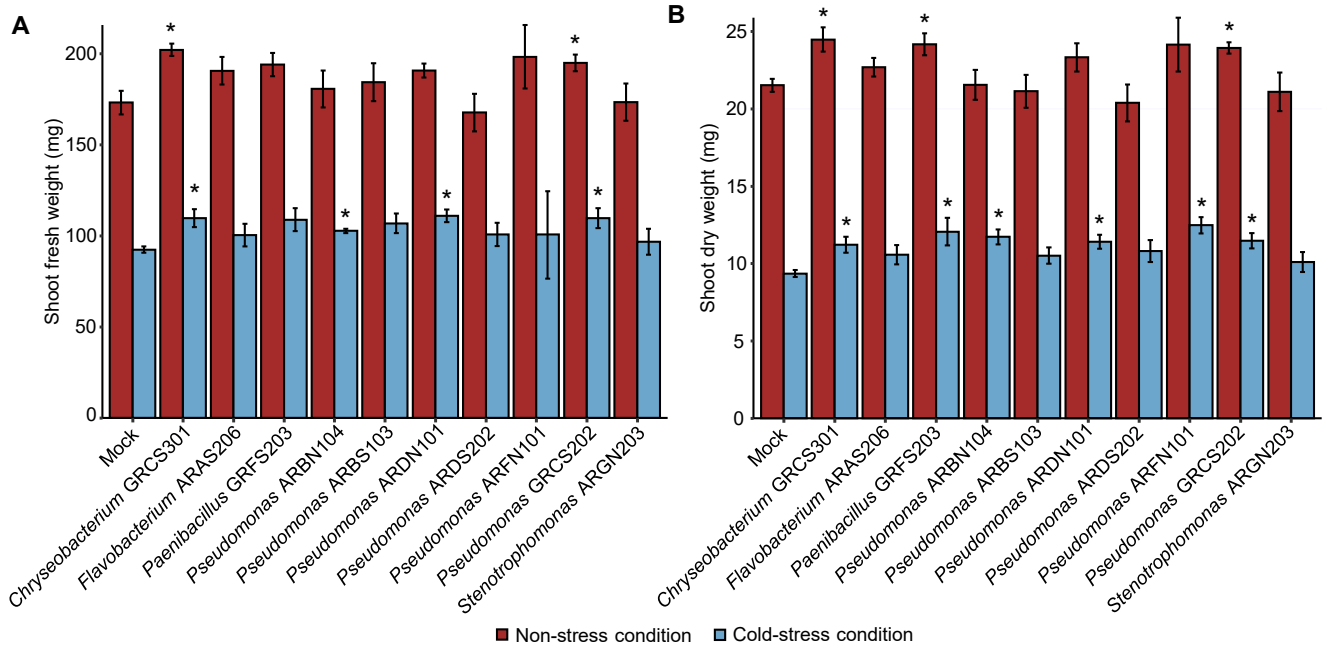


Figure 3.2. Validation trials of plant growth-promoting bacteria under non-stress and cold-stress

conditions. Tomato plants were treated with MgSO4 (mock-inoculated) or inoculated with a bacterial suspension (bacterium-inoculated) of cold-tolerant bacterial isolates specified by the codes and taxonomic annotations. Plants were incubated in the growth chamber for 14 days at 25 ± 2 °C, two groups of plants were then obtained, and they were incubated at 25 ± 2 °C (non-stressed plants; red bars) or at 10 ± 2 °C (cold-stressed plants; blue bars) for 14 days. Shoot fresh weight (A) and shoot dry weight (B) were assessed at the end of the incubation in the growth chamber and expressed as mg of each plant. Mean and standard error values of four replicates (pool of seven plants) are reported for each inoculation condition. Asterisks indicate significant increases of shoot fresh weight or shoot dry weight in bacterium-inoculated compared to mock-inoculated plants, according to the Mann-Whitney test ($P \leq 0.05$).

Chryseobacterium sp. GRCS301 and *Pseudomonas* sp. GRCS202 were selected for the functional characterization trials since they belonged to different genera and provided a consistent plant growth promotion in terms of fresh weight and dry weight under non-stressed and cold-stressed conditions. In the functional characterization trials, *Chryseobacterium* sp. GRCS301 and *Pseudomonas* sp. GRCS202 confirmed the plant growth promotion activity under non-stressed and cold-stressed conditions in the functional characterization trials (Figure 3.3A).

The content of H_2O_2 was lower in *Chryseobacterium* sp. GRCS301-inculcated compared to mock-inoculated plants under cold-stressed conditions and non-stressed conditions (Figure 3.3B). Moreover, the content of H_2O_2 tended to increase in *Pseudomonas* sp. GRCS202-inoculated compared to mock-inoculated plants under non-stressed conditions, but not under cold-stressed conditions.

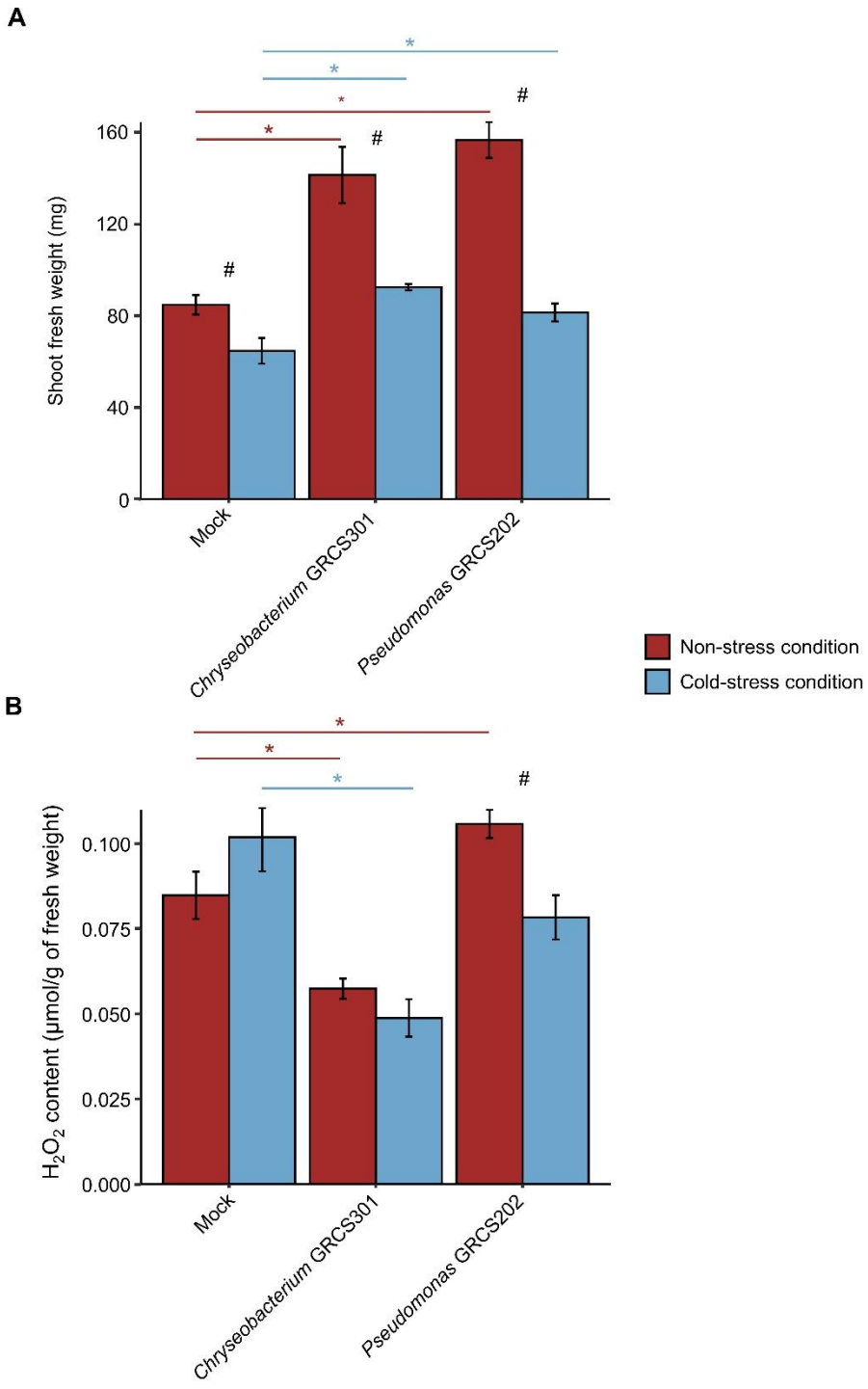


Figure 3.3. Functional effects of cold-tolerant bacterial isolates on tomato plants under non-stressed and cold-stressed conditions. Tomato plants were treated with sterile 10 mM $MgSO_4$ (mock-inoculated) and inoculated with *Chryseobacterium* sp. GRCS301 or *Pseudomonas* sp. GRCS202. Plants were incubated in the growth chamber for 14 days at 25 °C ± 2 °C, two groups of plants were

then obtained, and they were incubated at 25 °C ± 2 °C (non-stressed plants) or at 10 °C ± 2 °C (cold-stressed plants) for further 14 days. Shoot fresh weight (A) and H_2O_2 content (B) were assessed at 14 days and on day after stress exposure (DAS), respectively. Mean and standard error values of three replicates (pool of seven plants each) are reported for each inoculation condition, temperature regime, and time point. Asterisks indicate significant differences between bacterium-inoculated and mock-inoculated samples for each temperature regime and time point, according to the Mann-Whitney test ($P \leq 0.05$). Hashtags indicate significant differences between cold-stressed and non-stressed samples for each inoculation condition and time point, according to the Mann-Whitney test ($P \leq 0.05$).

On the growth media enriched with antibiotics for the semi-selective growth of *Chryseobacterium* sp. or *Pseudomonas* sp., rhizosphere from mock- and bacterium-inoculated plants were plated after 4 weeks of growth in controlled conditions. The number of colonies – log10 (CFU/g fresh weight) – from the rhizosphere of bacterial-inoculated plants was significantly higher than the number of colonies from the rhizosphere of the mock-inoculated plants under both non-stressed and cold-stressed conditions (Figure 3.4). Results show that, under both non-stressed and cold-stressed conditions, there was a significantly higher number of colonies on the media enriched for *Chryseobacterium* in samples from *Chryseobacterium*-inoculated plants, and on the media enriched for *Pseudomonas* in samples from *Pseudomonas*-inoculated plants, compared to the corresponding media plated with samples from mock-inoculated plants. Under non-stressed condition, rhizosphere sample from *Chryseobacterium*- and mock-inoculated plants plated on the medium for *Chryseobacterium* showed 7.74 ± 0.89 log10 (CFU/g of fresh weight) and 5.50 ± 0.36 log10 (CFU/g of fresh weight), respectively (Figure 3.4A). Similarly, rhizosphere sample from *Pseudomonas*- and mock-inoculated plants plated on the medium for *Pseudomonas* showed 9.02 ± 0.61 log10 (CFU/g of fresh weight) and 6.86 ± 0.46 log10 (CFU/g of fresh weight), respectively (Figure 3.4A). At cold-stressed condition, rhizosphere sample from *Chryseobacterium*- and mock-inoculated plants plated on the medium for *Chryseobacterium* showed 7.09 ± 0.62 log10 (CFU/g of fresh weight) and 1.82 ± 0.52 log10 (CFU/g of fresh weight), respectively (Figure 3.4B). Similarly, rhizosphere samples from

Pseudomonas- and mock-inoculated plants plated on the medium for *Pseudomonas* showed 8.54 ± 0.48 log10 (CFU/g of fresh weight) and 2.12 ± 0.62 log10 (CFU/g of fresh weight), respectively (Figure 3.4B). Bacterial growth on selective media for *Chryseobacterium* and *Pseudomonas* from rhizosphere samples of *Chryseobacterium* -inoculated and *Pseudomonas* -inoculated plants showed no differences in CFU counts under both non-stressed and cold-stressed conditions, suggesting the bacterial fitness at both temperatures. Conversely, bacterial growth on selective media for *Chryseobacterium* and *Pseudomonas* from mock-inoculated rhizosphere samples exhibited higher growth under non-stressed conditions, suggesting, as expected, greater bacterial proliferation of bacteria other than *Chryseobacterium* and *Pseudomonas* under non-stressed conditions compared to stressed conditions.

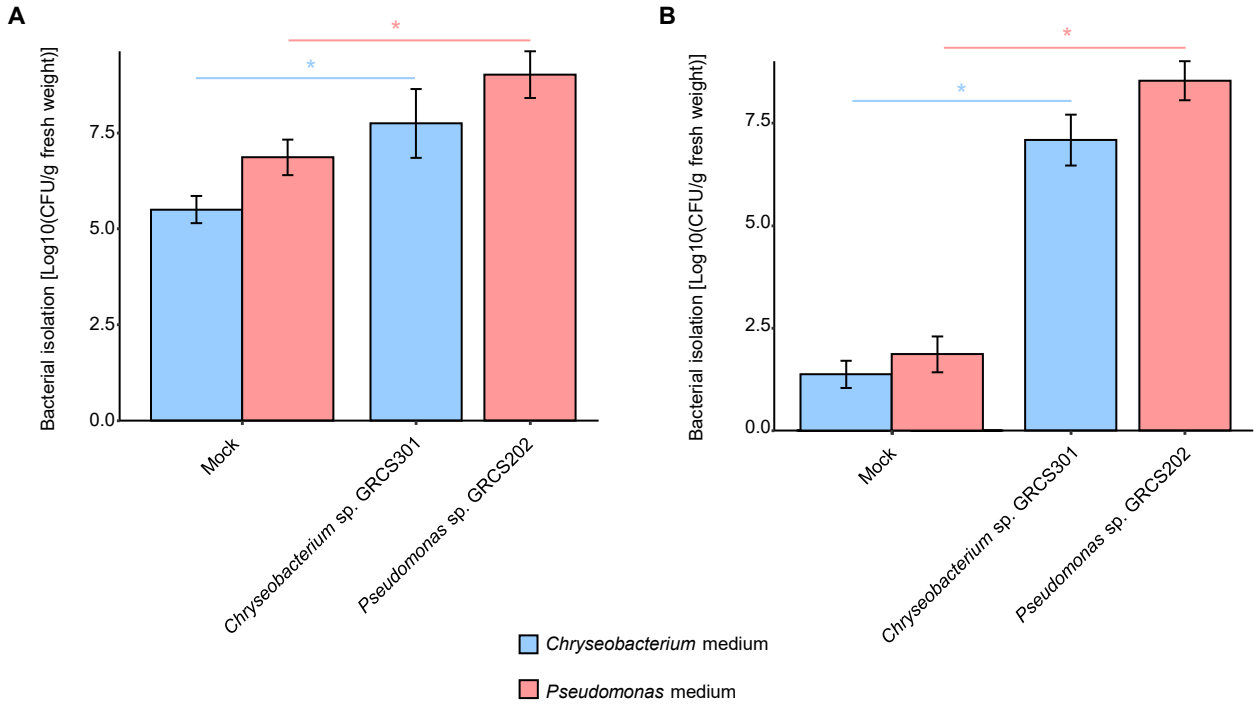


Figure 3.4. Bacterial re-isolation from tomato rhizosphere under non-stress and cold-stress conditions. Tomato plants were treated with MgSO4 (mock-inoculated) or inoculated with *Chryseobacterium* sp. GRCS301 or *Pseudomonas* sp. GRCS202, and rhizosphere samples were

collected at the end of incubation at 25 ± 2 °C (non-stressed plants; A) or at 10 ± 2 °C (cold-stressed plants; B). Colony forming units (CFU) of rhizosphere bacteria were assessed on semi-selective R2A media to allow *Chryseobacterium* spp. (*Chryseobacterium* medium) and *Pseudomonas* spp. (*Pseudomonas* medium) growth. Mean Log10-transformed CFU and standard error values of four replicates (pool of five plants) with two technical replicates are reported for each inoculation condition, temperature regime, and growth medium. Asterisks indicate significant differences between bacterium-inoculated and mock-inoculated samples for each temperature regime and growth medium, according to the t-test ($P \leq 0.05$).

3.2 Cold-tolerant bacterial isolates affected transcriptional responses of tomato shoots under cold-stress

To characterize the tomato response to bacterial inoculation under cold-stress, a RNA-Seq analysis was carried out, and sequences were deposited at NCBI (BioProject number PRJNA1079540). From 45.70% to 87.87% of filtered paired-end reads aligned to the tomato genome (*S. lycopersicum* reference genome ITAG5.0) and 21,698 genes resulted as upregulated or downregulated (normalized counts according DEseq2 >1 in at least one sample; Supplementary Table S3). The low percentage detected may indicate issues related to sample quality (e.g., RNA degradation), library preparation, or sequencing depth. Alternatively, it may reflect biological factors such as the presence of non-coding RNAs, microbial transcripts, or cultivar-specific sequences not represented in the reference genome. The accuracy of RNA-Seq results was validated by qPCR using seven genes (Supplementary Table S2), and a good correlation ($R^2 = 0.8401$) between LFC values assessed by RNA-Seq and qPCR was obtained (Figure 3.5).

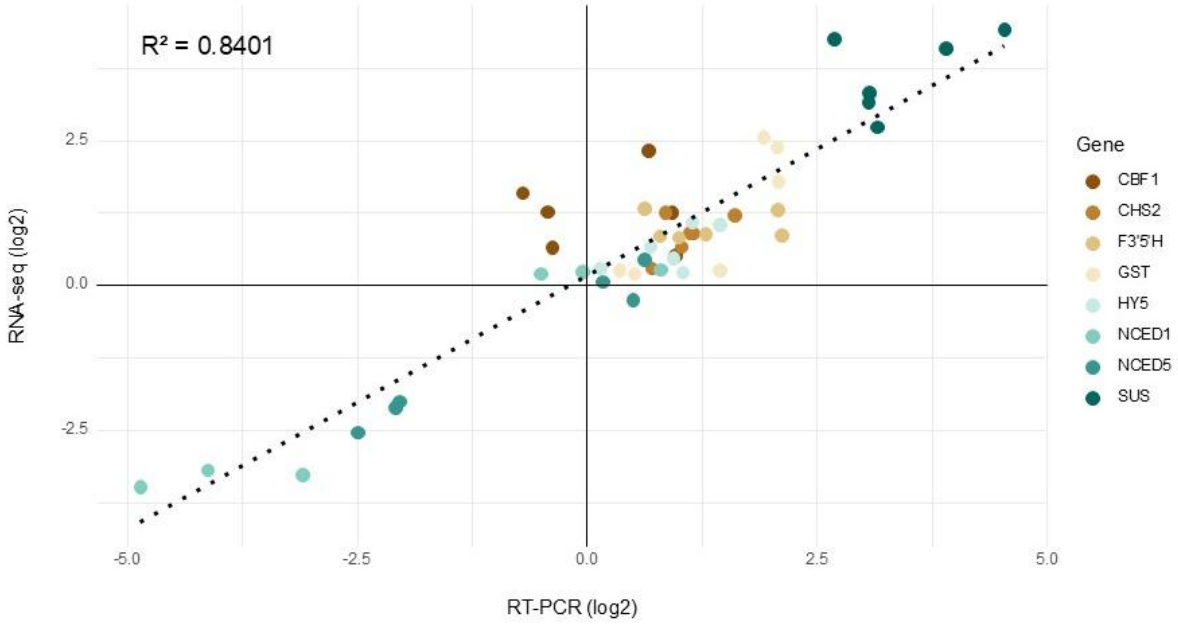


Figure 3.5. RNA-Seq data validation by qPCR. Scatter plot of Log2-transformed fold change (LFC) values assessed by RNA-Seq and qPCR analyses is reported together with the regression line equation and the R2 coefficient based on expression levels of genes encoding the 9-cis-epoxycarotenoid dioxygenase (*NCED5*), C-repeat/DRE binding factor 1 (*CBF1*), chalcone synthase (*CHS2*), elongated hypocotyl 5 transcriptional factor (*HY5*), flavonoid-3'-hydroxylase (*F3'5'H*), glutathione-S-transferase enzyme (*GST*), and sucrose synthase (*SUS*) in shoot samples of tomato plants treated with sterile 10 mM $MgSO_4$ (mock-inoculated), inoculated with *Chryseobacterium* sp. GRCS301 or *Pseudomonas* sp. GRCS202, and incubated at 25 °C ± 2 °C (non-stressed; NS) or at 10 °C ± 2 °C (cold-stressed; S) for 14 days and collected at one day (1 DAS) and 14 days (14 DAS) after stress exposure (DAS).

A total of 4,909 and 4,806 DEGs were found at 1 DAS and 14 DAS, respectively, by differential expression analysis in the pairwise comparisons between the cold-stressed and non-stressed condition of mock-inoculated, *Chryseobacterium*-inoculated, and *Pseudomonas*-inoculated plants (FDR lower than 0.05 and a LFC lower than -1 or higher than 1; Supplementary Table S4). In particular, 2,131 and 2,130 DEGs were upregulated at 1 DAS and 14 DAS, respectively, whereas and 2,778 and 2,676 DEGs were downregulated at the two time points. For each time point, upregulated and downregulated

DEGs were grouped in those modulated by cold stress in all inoculation conditions or exclusively modulated in mock-, *Chryseobacterium*-, and *Pseudomonas*-inoculated plants, to highlight possible common and specific responses to cold stress (Figure 3.6). A large fraction of DEGs was modulated in all inoculation conditions (groups 4, 8, 12, and 16), as possible consistent response of tomato shoots to cold stress. Moreover, *Chryseobacterium* inoculation (groups 1, 5, 9, and 13) and *Pseudomonas* inoculation (groups 2, 6, 10, and 14) highlight specific transcriptional response to cold stress compared to mock inoculated plants (groups 3, 7, 11, and 15) at both time points.

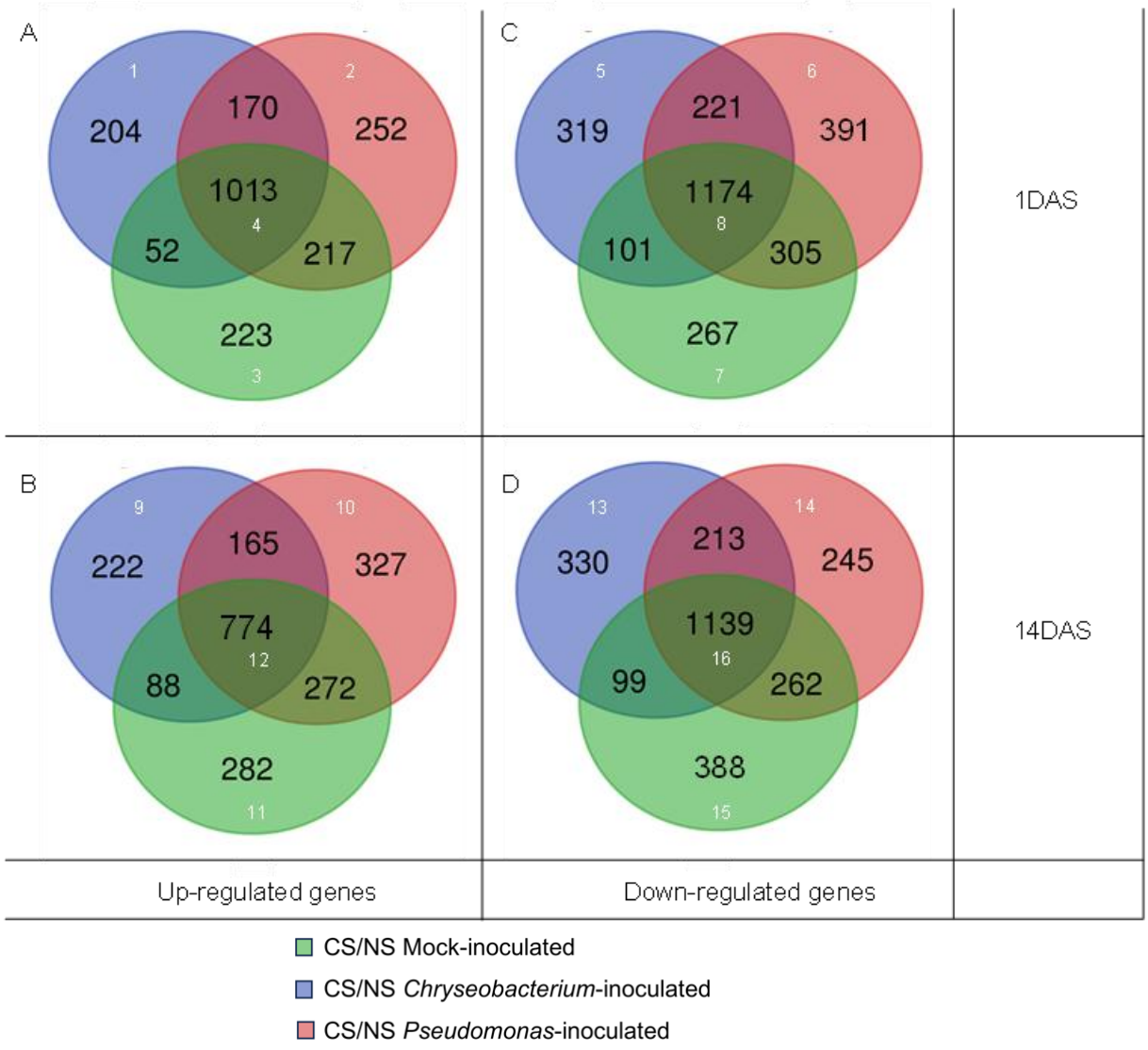


Figure 3.6. Differential expression analysis results. Tomato plants were treated with sterile 10 mM $MgSO_4$ (mock-inoculated) and inoculated with *Chryseobacterium* sp. GRCS301 (*Chryseobacterium*-inoculated) or *Pseudomonas* sp. GRCS202 (*Pseudomonas*-inoculated), and shoots samples were collected at one day (1 DAS) and 14 days (14 DAS) after stress exposure (DAS) from plants incubated at 25 °C ± 2 °C (non-stressed; NS) or at 10 °C ± 2 °C (cold-stressed; CS). Differentially expressed genes (DEGs) were identified in pairwise comparisons between CS and NS samples for each inoculation condition and time point (false discovery rate lower than 0.05 and Log_2-transformed fold change higher than 1 or lower than -1). Venn diagrams summarize the number (black number) of upregulated (A, B) and downregulated (C, D) DEGs at 1 DAS and 14 DAS in the pairwise comparisons specified by the color legend. Sixteen groups (G; white numbers) of genes modulated in CS samples compared to NS samples in all inoculation conditions or exclusively in mock-, *Chryseobacterium*-, and *Pseudomonas*-inoculated plants are selected for further analyses.

In particular, DEGs upregulated by cold stress in all inoculation conditions at 1 DAS (group 4) revealed the enrichment manly of amino acid catabolic process, embryo development, phenylalanine metabolic process, polysaccharide catabolic process, response to acid chemical and sucrose metabolic process ($P \leq 0.05$, DEG/expected genes ration ≥ 4 with DEGs per category ≥ 2; Figure 3.7A). DEGs upregulated by cold stress exclusively in mock-inoculated plants at 1 DAS (group 3) revealed the enrichment of carbohydrate biosynthetic process, glucan biosynthetic process, multicellular organism development, phosphatidylinositol metabolic process, and response to wounding (Figure 3.7B). Functional categories of cell wall polysaccharide metabolic process, glucose metabolic process, siderophore metabolic process, tricarboxylic acid cycle were enriched in DEGs exclusively upregulated in *Chryseobacterium*-inoculated plants (group 1; Figure 3.7C), while functional categories of calcium transport, glucan metabolic process, and galactose metabolic process were enriched mainly in DEGs exclusively upregulated in *Pseudomonas*-inoculated plants at 1 DAS (group 2; Figure 3.7D). DEGs downregulated by cold stress at 1 DAS showed the enrichment of photosynthesis/light harvesting, DNA replication initiation, NA-templated DNA replication, l-phenylalanine metabolic process, and cytoplasmic microtubule organization in all inoculation conditions (group 8; Figure 3.7E), mismatch repair, reproductive process, and chemical homeostasis

in mock-inoculated plants (group 7; Figure 3.7F), ammonium transmembrane transport, oxidative phosphorylation, and sulfate reduction in *Chryseobacterium*-inoculated plants (group 5; Figure 3.7G), and regulation of cell cycle, and carbohydrate catabolic process in *Pseudomonas*-inoculated plants (Figure 3.7H).

At 14 DAS, DEGs upregulated by cold stress in all inoculation conditions (group 12) revealed the enrichment mainly of cell wall polysaccharide and macromolecule metabolic processes, glucan and xyloglucan metabolic processes, polysaccharide metabolic process, response to acid chemical, and steroid biosynthetic process ($P \leq 0.05$, DEG/expected genes ration ≥ 4, and more than 2 genes per category; Figure 3.8A). Moreover, DEGs upregulated by cold stress exclusively in mock-inoculated plants at 14 DAS (group 11) revealed the enrichment of carbohydrate catabolic process, dicarboxylic acid metabolic process, fatty acid biosynthetic process, and secondary metabolite biosynthetic process (Figure 3.8B). Functional categories of DNA metabolic process, DNA replication, and phenylpropanoid metabolic process were enriched in DEGs exclusively upregulated in *Chryseobacterium*-inoculated plants (group 9; Figure 3.8C), while protein translation and regulation of biological process were enriched mainly in DEGs exclusively upregulated in *Pseudomonas*-inoculated plants at 14 DAS (group 10; Figure 3.8D). DEGs downregulated by cold stress at 14 DAS showed the enrichment of sulfate reduction, photosynthesis, response to wounding, branched-chain amino acid biosynthetic processes ,and light reaction in all inoculation conditions (Figure 3.8E), photosynthetic electron transport chain, photosynthesis, and secondary metabolic process in mock-inoculated plants (Figure 3.8F), biological processes involved in interspecies interactions, response to biotic stimulus, and cell recognition in *Chryseobacterium*-inoculated plants (Figure 3.8G), and G-protein-coupled receptor signaling pathway, response to endogenous stimulus, and response to organic substance in *Pseudomonas*-inoculated plants (Figure 3.8H).

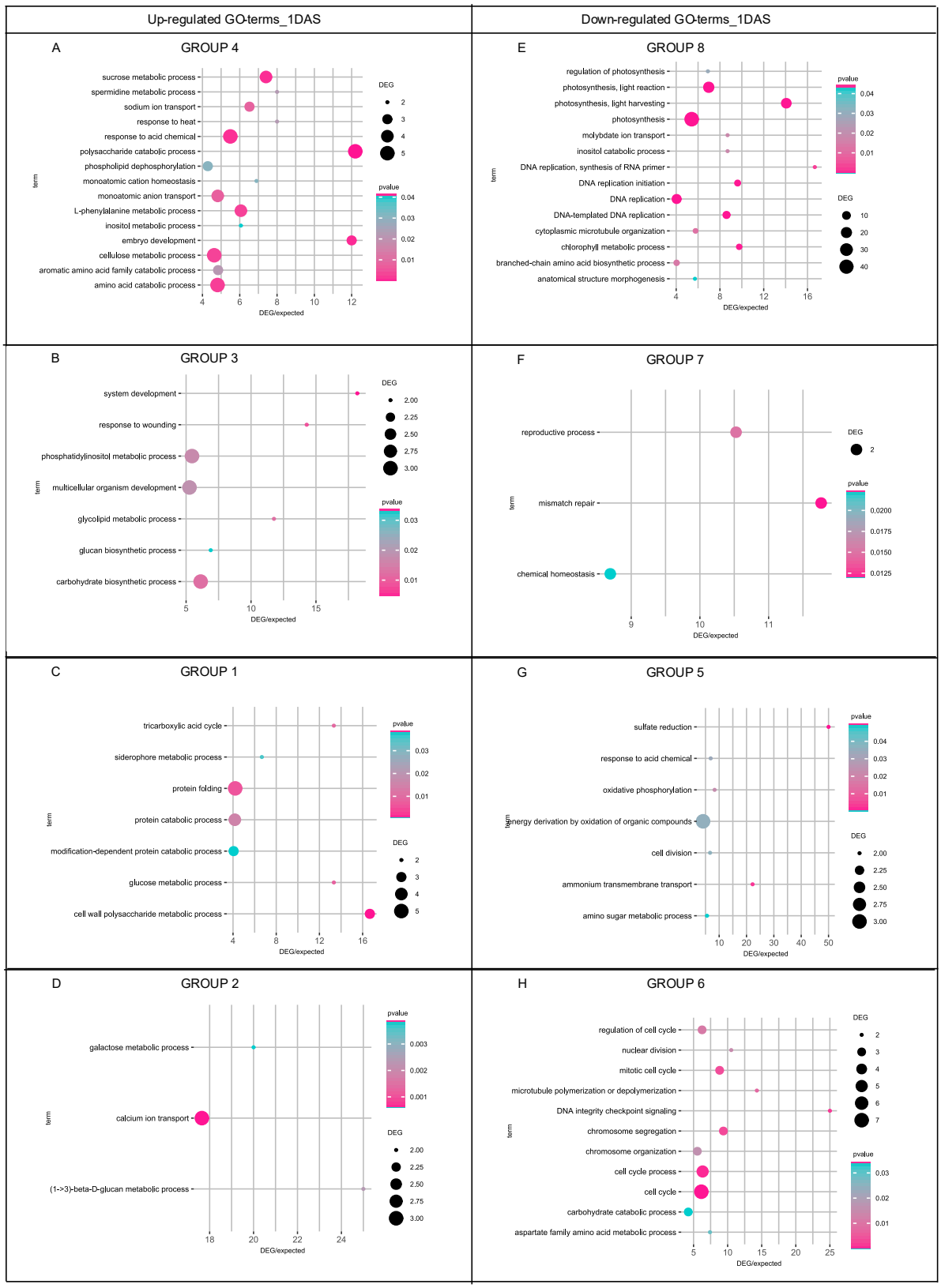

Up-regulated GO-terms_1DAS
Down-regulated GO-terms_1DAS
A
GROUP 4
sucrose metabolic process
spermidine metabolic process
sodium ion transport
response to heat
response to acid chemical
polysaccharide catabolic process
phospholipid dephosphorylation
monoatomic cation homeostasis
monoatomic anion transport
L-phenylalanine metabolic process
inositol metabolic process
embryo development
cellulose metabolic process
aromatic amino acid family catabolic process
amino acid catabolic process
term
DEG/expected
DEG
pvalue
E
GROUP 8
regulation of photosynthesis
photosynthesis, light reaction
photosynthesis, light harvesting
photosynthesis
molybdate ion transport
inositol catabolic process
DNA replication, synthesis of RNA primer
DNA replication initiation
DNA replication
DNA-templated DNA replication
cytoplasmic microtubule organization
chlorophyll metabolic process
branched-chain amino acid biosynthetic process
anatomical structure morphogenesis
B
GROUP 3
system development
response to wounding
phosphatidylinositol metabolic process
multicellular organism development
glycolipid metabolic process
glucan biosynthetic process
carbohydrate biosynthetic process
F
GROUP 7
reproductive process
mismatch repair
chemical homeostasis
C
GROUP 1
tricarboxylic acid cycle
siderophore metabolic process
protein folding
protein catabolic process
modification-dependent protein catabolic process
glucose metabolic process
cell wall polysaccharide metabolic process
G
GROUP 5
sulfate reduction
response to acid chemical
oxidative phosphorylation
energy derivation by oxidation of organic compounds
cell division
ammonium transmembrane transport
amino sugar metabolic process
D
GROUP 2
galactose metabolic process
calcium ion transport
(1->3)-beta-D-glucan metabolic process
H
GROUP 6
regulation of cell cycle
nuclear division
mitotic cell cycle
microtubule polymerization or depolymerization
DNA integrity checkpoint signaling
chromosome segregation
chromosome organization
cell cycle process
cell cycle
carbohydrate catabolic process
aspartate family amino acid metabolic process

Figure 3.7. GO terms enrichment analysis at 1DAS. Leaves of mock-inoculated and bacteria-inoculated (*Chryseobacterium* sp. GRCS301 and *Pseudomonas* GRCS202) tomato plants were collected at 1 days after stress (1DAS), two weeks after the onset of cold stress (10°C). Gene Ontology (GO) term enrichment analyses were conducted on the DEGs resulting from pairwise comparisons between stressed/non-stressed mock-inoculated plants, stressed/non-stressed *Chryseobacterium*-inoculated plants, and stressed/non-stressed *Pseudomonas*-inoculated plants for each group identified in the Venn diagram (G 9-16). The analyses were performed using the topGO package in R version 2.58.0, employing the Fisher's classic algorithm and Fisher's exact test ($P \leq 0.05$). The predominant functional categories (DEG/expected genes ration ≥ 4, and more than 2 genes per category) of upregulated DEGs common to all groups (A), enriched only in mock-inoculated plants (B), only in *Chryseobacterium*-inoculated plants (C), and only in *Pseudomonas*-inoculated plants (D) are reported. Similarly, the predominant downregulated categories were common to all groups (E), enriched only in mock-inoculated plants (F), only in *Chryseobacterium*-inoculated plants (G), and only in *Pseudomonas*-inoculated plants are reported (H).


Up-regulated GO-terms_14DAS
Down-regulated GO-terms_14DAS
A
GROUP 12
xyloglucan metabolic process
steroid biosynthetic process
sodium ion transport
response to temperature stimulus
response to acid chemical
response to abiotic stimulus
protein refolding
primary alcohol metabolic process
positive regulation of RNA biosynthetic process
polysaccharide metabolic process
organic acid catabolic process
monoatomic ion homeostasis
monoatomic anion transport
intracellular monoatomic ion homeostasis
intracellular chemical homeostasis
glucan metabolic process
fructose 6-phosphate metabolic process
flavonoid metabolic process
chemical homeostasis
cell wall polysaccharide metabolic process
cell wall macromolecule metabolic process
carbon fixation
biological process involved in interaction with host
aromatic amino acid family catabolic process
2'-deoxyribonucleotide metabolic process
term
DEG/expected
DEG
pvalue
E
GROUP 16
sulfur compound transport
sulfate reduction
response to wounding
regulation of multicellular organismal process
photosynthesis, light reaction
photosynthesis, light harvesting
monocarboxylic acid transport
branched-chain amino acid metabolic process
branched-chain amino acid biosynthetic process
ammonium transmembrane transport
amine biosynthetic process
B
GROUP 11
secondary metabolite biosynthetic process
potassium ion transmembrane transport
malate metabolic process
fatty acid biosynthetic process
dicarboxylic acid metabolic process
carbohydrate catabolic process
F
GROUP 15
terpenoid metabolic process
shoot system development
secondary metabolic process
response to biotic stimulus
positive regulation of RNA metabolic process
photosynthetic electron transport chain
photosynthesis, light reaction
photosynthesis, light harvesting
photosynthesis
phenylpropanoid metabolic process
oxidative phosphorylation
generation of precursor metabolites and energy
electron transport chain
developmental process
amino acid transmembrane transport
C
GROUP 9
steroid metabolic process
regulation of cell cycle
phenylpropanoid metabolic process
DNA replication initiation
DNA replication
DNA repair
DNA recombination
DNA metabolic process
DNA-templated DNA replication
chemical homeostasis
G
GROUP 13
response to biotic stimulus
cell recognition
biological process involved in interspecies interaction
D
GROUP 10
tRNA aminoacylation for protein translation
riboflavin metabolic process
positive regulation of nitrogen compound metabolic process
positive regulation of biological process
photosynthesis
mitotic cell cycle process
DNA replication
DNA conformation change
H
GROUP 14
response to organic substance
response to endogenous stimulus
glycerol ether metabolic process
G protein-coupled receptor signaling pathway

Figure 3.8. GO terms enrichment analysis at 14DAS. Leaves of mock-inoculated and bacteria-inoculated (*Chryseobacterium* sp. GRCS301 and *Pseudomonas* GRCS202) tomato plants were collected at 1 days after stress (1DAS), two weeks after the onset of cold stress (10°C). Gene Ontology (GO) term enrichment analyses were conducted on the DEGs resulting from pairwise comparisons between stressed/non-stressed mock-inoculated plants, stressed/non-stressed *Chryseobacterium*-inoculated plants, and stressed/non-stressed *Pseudomonas*-inoculated plants for each group identified in the Venn diagram (G 9-16). The analyses were performed using the topGO package in R version 2.58.0, employing the Fisher's classic algorithm and Fisher's exact test ($P \leq 0.05$). The predominant functional categories (DEG/expected genes ration ≥ 4, and more than 2 genes per category) of upregulated DEGs common to all groups (A), enriched only in mock-inoculated plants (B), only in *Chryseobacterium*-inoculated plants (C), and only in *Pseudomonas*-inoculated plants (D) are reported. Similarly, the predominant downregulated categories were common to all groups (E), enriched only in mock-inoculated plants (F), only in *Chryseobacterium*-inoculated plants (G), and only in *Pseudomonas*-inoculated plants are reported (H).

3.3 Tomato physiological parameters are positively affected by cold-bacterial isolates under commercial greenhouse conditions

To evaluate the effectiveness of bacterial isolates in commercial greenhouse conditions (plastic tunnel with no controlled parameters of temperature and humidity), a growth trial was conducted in autumn-winter 2023 at ITAKA in Ragusa, Sicily, Italy, a leading Italian company specializing in green agricultural solutions on seeds of *S.lycopersicum* cv. Rivaldo (CORA Seeds, Cesena, IT). The experiment focused on three bacterial isolates that demonstrated the highest potential as plant growth-promoting bacteria (PGPB) in preliminary tests (Figure 3.2). In this context, it should be noted that two different tomato cultivars were used, one for the laboratory part (cv. Moneymaker) and one for the greenhouse part (cv. Rivaldo). This was mainly due to the company's commercial objectives: in fact, the Rivaldo cultivar is the one most commonly used in greenhouse cultivation. The isolates tested were *Chryseobacterium* sp. GRCS301 (*Chryseobacterium*-inoculated), *Paenibacillus* sp. GRFS203 (*Paenibacillus*-inoculated), *Pseudomonas* sp. GRCS202 (*Pseudomonas*-inoculated), and a consortium of these three bacteria (consortium-inoculated). Additionally, a positive control using a bio-stimulant previously validated by the company as a biofertilizer for tomatoes (ITAKA control-

inoculated) was included – no information about the product is allowed to be published. To minimize the impact of variability in tomato plants, which leads to uneven apical growth among seedlings, stem growth was expressed as a percentage relative to each biological replicate (Figure 3.9). In other words, tomatoes germinated from seeds show high genetic variability, which can affect the downstream analysis of results. For this reason, it was decided to measure the height of the tomato plants at the time of inoculation (t0) up to 6 weeks later (t6) and calculate the percentage growth using the formula: [t6 (cm) - t0 (cm)]/t0 (cm)*100. Both *Pseudomonas* sp. GRC202 and *Chryseobacterium* sp. GRCS301 demonstrated their ability to enhance tomato growth at the end of the first six weeks after seedling transplantation, with *Pseudomonas* sp. GRCS202 showing significant enhance in stem growth. Conversely, none of the bacteria-inoculated plants showed an increase in stem thickness compared to the mock-inoculated plants (Figure 3.10), nor in the number of fruits per stage (Figure 3.11) or in the number of total fruits per biological replicate (Figure 3.12).

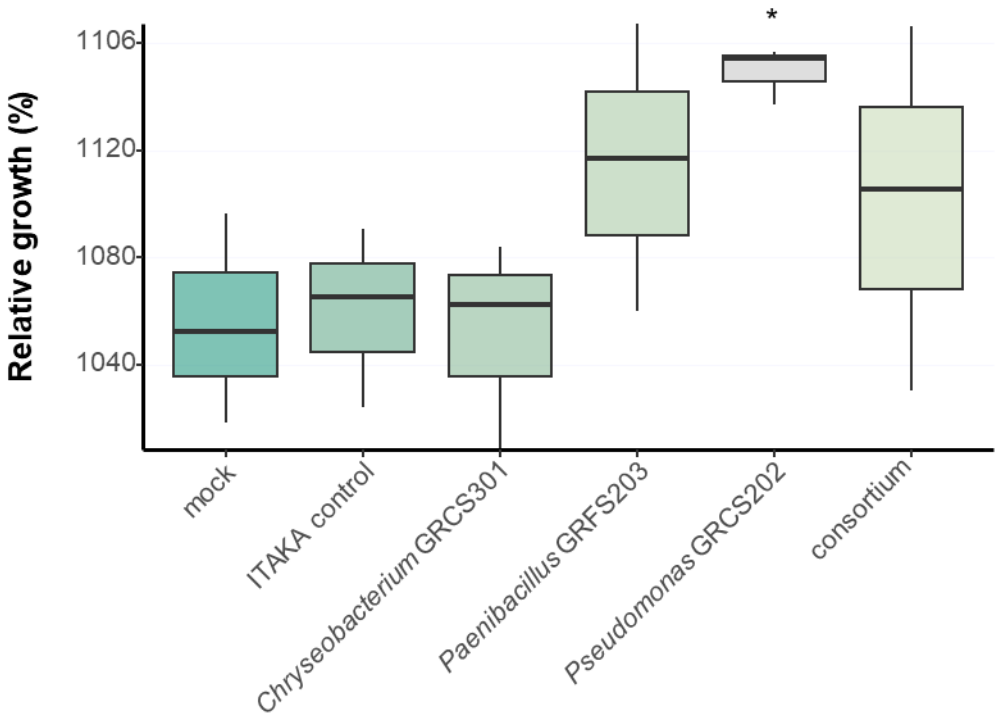


Figure 3.9. Greenhouse trail results: stem growth. Tomato plants were treated with non-sterile 10 mM $MgSO_4$ (mock-inoculated) or ITAKA control (ITAKA control-inoculated), and inoculated *Paenibacillus* sp. GRFS203 (*Paenibacillus*-inoculated), *Pseudomonas* sp. GRCS202 (*Pseudomonas*-inoculated), and a consortium of these three bacteria (consortium-inoculated). Measurements were take every two weeks, for a total of six weeks after plant soil transplantation, thus the results refer to the increase in growth in the first six weeks. Relative growth was calculated as a percentage of height increase at t6 compared to the initial height at t0. Mean and standard error values of 3 biological replicates are reported for each treatment. Asterisks indicate significant changes compared to mock-inoculated samples, according to the t-test ($P \leq 0.05$).

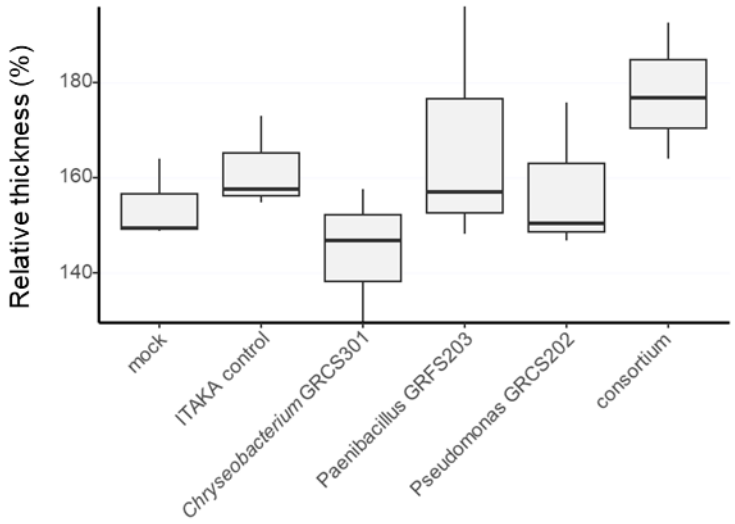


Figure 3.10. Greenhouse trail results: stem thickness. Tomato plants were treated with non-sterile 10 mM $MgSO_4$ (mock-inoculated) or ITAKA control (ITAKA control-inoculated), and inoculated *Paenibacillus* sp. GRFS203 (*Paenibacillus*-inoculated), *Pseudomonas* sp. GRCS202 (*Pseudomonas*-inoculated), and a consortium of these three bacteria (consortium-inoculated). Measurements were taken every two weeks, for a total of six weeks after plant soil transplantation, thus the results refer to the increase in growth in the first six weeks. Relative thickness was calculated as a percentage of thickness. increase at t6 compared to the initial height at t0No significance according to the parametric t-test.

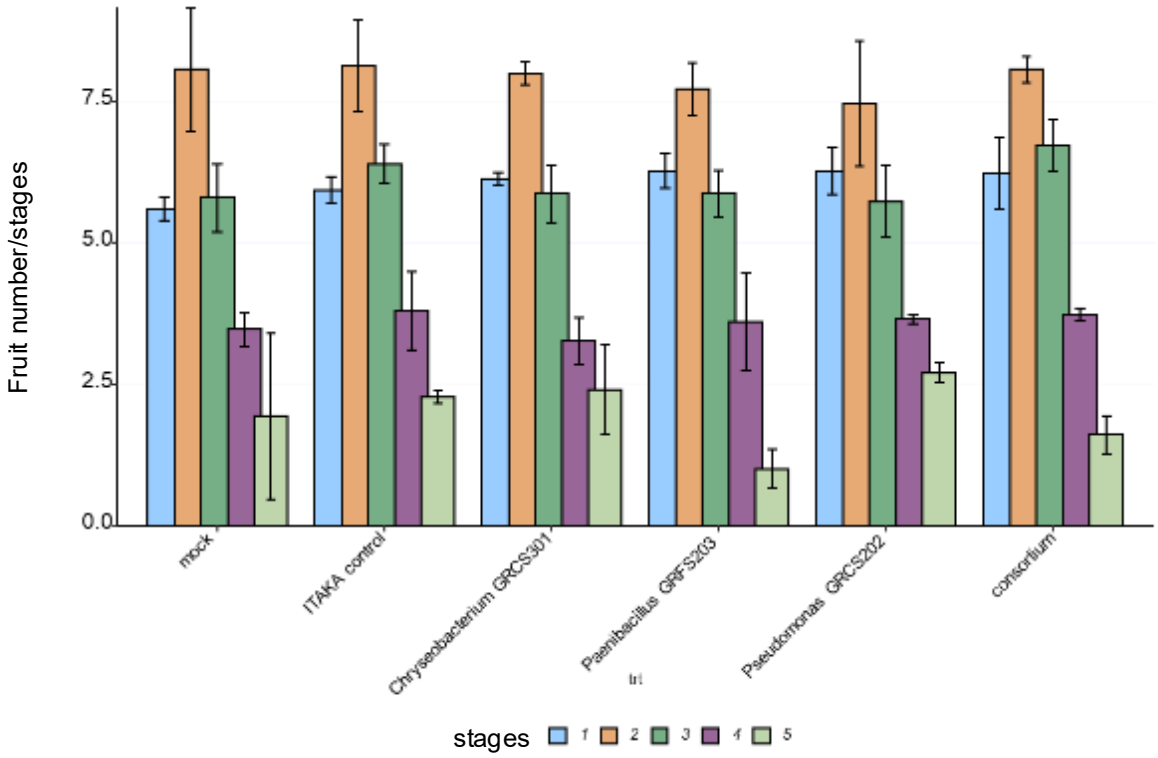


Figure 3.11. Greenhouse trial results: number of fruit per tomato stage. Tomato plants were treated with non-sterile 10 mM $MgSO_4$ (mock-inoculated) or ITAKA control (ITAKA control-inoculated), and inoculated *Paenibacillus* sp. GRFS203 (*Paenibacillus*-inoculated), *Pseudomonas* sp. GRCS202 (*Pseudomonas*-inoculated), and a consortium of these three bacteria (consortium-inoculated). The measurements were taken during the fruiting stage of the tomato, for a total of nine measurements, divided into a total of five stages. No significance according to the parametric t-test.

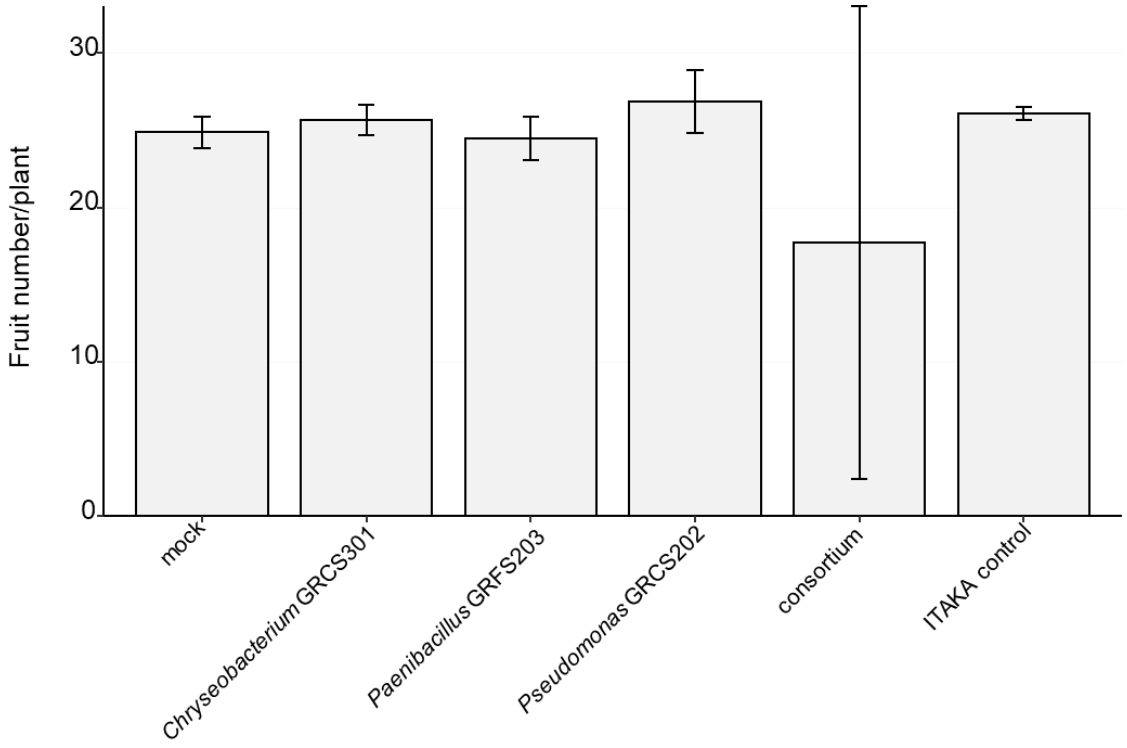


Figure 3.12. Greenhouse trail results: number of fruit per tomato plant. Tomato plants were treated with non-sterile 10 mM $MgSO_4$ (mock-inoculated) or ITAKA control (ITAKA control-inoculated), and inoculated *Paenibacillus* sp. GRFS203 (*Paenibacillus*-inoculated), *Pseudomonas* sp. GRCS202 (*Pseudomonas*-inoculated), and a consortium of these three bacteria (consortium-inoculated). The measurements were taken during the fruiting stage of the tomato, for a total of nine measurements. No significance according to the parametric t-test.

In order to define whether bacterial inoculations had an impact on the final biomass, a destructive survey was carried out at the end of the tomato winter cycle (February 2024) with the aim

of assessing the difference in weight between the mock-inoculated and the bacterial-inoculated plants. *Chryseobacterium*-inoculated plants and *Pseudomonas*-inoculated plants possess a more developed root system (root dry weight) than mock-inoculated plants, confirming the ability of *Chryseobacterium* sp. GRCS301 and *Pseudomonas* sp. GRCS202 as growth promoters under commercial greenhouse conditions (Figure 3.13).

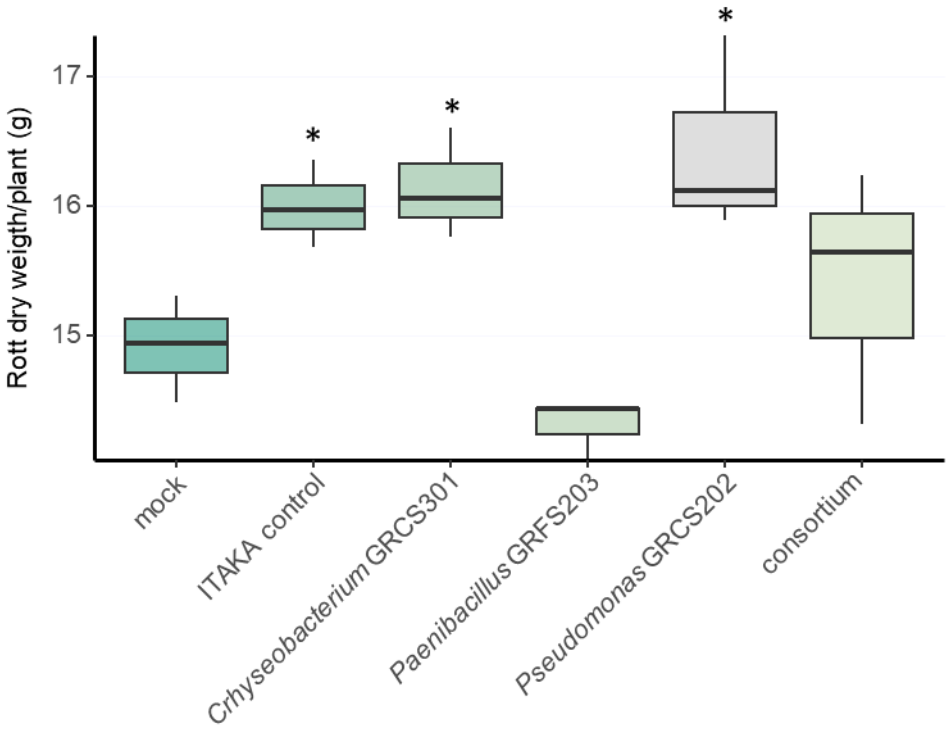


Figure 3.13. Greenhouse trail results: root dry weight**.** Tomato plants were treated with non-sterile 10 mM $MgSO_4$ (mock-inoculated) or ITAKA control (ITAKA control-inoculated), and inoculated with *Chryseobacterium* sp. GRCS301(*Chryseobacterium*-inoculated), (*Paenibacillus* sp. GRFS203 (*Paenibacillus*-inoculated), *Pseudomonas* sp. GRCS202 (*Pseudomonas*-inoculated), and a consortium of these three bacteria (consortium-inoculated). Measurements were taken at the end of the winter cycle. Mean and standard error values of 3 biological replicates are reported for each treatment. Asterisks indicate significant changes compared to mock-inoculated samples, according to the t-test ($P \leq 0.05$).

CHAPTER 4. DISCUSSION

Tomato plants are sensitive to temperatures below 12°C and above 32°C (Mesa *et al*., 2022), and they are also valuable plants both nutritionally and economically. Due to the need to lower the use of chemical fertilizers, the need to find greener solutions becomes increasingly urgent, and PGPB represents a promising solution. They indeed can both promote plant growth (Rai *et al*., 2020), but also enhance plant resilience against biotic and abiotic stresses (Acuña-Rodríguez et al., 2020).

Among the bacterial isolates tested, resulting from natural col-adapted plant sampling (Marian et al., 2023),– *Chryseobacterium* sp. GRCS301 and *Pseudomonas* sp. GRCS202 were able to enhance plant growth under non-stressed and stressed conditions. Moreover, *Chryseobacterium*-inoculated plants showed lower H_2O_2 production at 1 DAS. These findings are in line with previous research work highlighting how plant growth promoting bacteria can enhance plant growth and improve plant fitness under cold stress (Acuña-Rodríguez et al., 2020). Indeed, transcriptomic data revealed some quite interesting information about up-regulated genes in bacterial-inoculated plants, both at 1 and 14 DAS. In all inoculation conditions at 1 DAS, the Gene Ontology (GO) terms analysis revealed up-regulations of genes associated with the biosynthetic pathways of flavonoids (L-phenylalanine metabolic process), sucrose (sucrose metabolic process), and dehydrin (response to acid chemicals) among others. In particular, *Solyc09G00274* (sucrose-phosphate synthase 3-related), and *Solyc09G002889* (sucrose synthase 2) are involved in the sucrose pathway, which plays a pivotal role in maintaining the homeostasis under abiotic stresses (Wang e*t al.*, 2018; Ahmad *et al.*, 2020; Saddhe *et al*., 2021; Li *et al.*, 2024). Similarly, *Solyc10G002904* (phenylalanine ammonia-lyase) encodes the first rate-limiting enzyme in the biosynthetic pathway of flavonoids, another class of molecules involved in the homeostasis maintenance under stressed conditions (Liu *et al.,* 2023; Li *et al.*, 2025). Interestingly, a pool of genes (*Solyc01G004178*, *Solyc02G000724*, *Solyc02G002102*,

Solyc02G002103, *Solyc04G002967*) related to the dehydrin pathway was found, although no information is available on the exact protein they encode, according to the Phytozome database (https://phytozome-next.jgi.doe.gov/). Dehyndrin are involved in the cold stress tolerance, and their activation seems to be the consequence of an alternative pathway, different from the *Sl-CBF1* regulation (Hara *et al*., 2003; Weiss and Egea-Cortines, 2009). As expected, genes related to the photosynthesis processes were found to be down regulated in the early stages of cold stress (Paredes and Quiles, 2015; Zhang *et al.*, 2022b). Indeed, at 1 DAS, *Solyc01G003718* (light-harvesting complex II chlorophyll a/b binding protein 6 LHCB6), *Solyc03G000076* (light-harvesting complex II chlorophyll a/b binding protein 1 LHCB1), *Solyc03G000077* (light-harvesting complex II chlorophyll a/b binding protein 1 LHCB1) resulted down-regulated in all the inoculum conditions. Focusing on the up-regulated genes at 14 DAS, an increase in genes associated with cell wall polysaccharide metabolism was observed. For instance, *Solyc03G001837*, *Solyc03G001840*, and *Solyc03G001841* all encode for xyloglucan endotransglucosylase. Xyloglucan endotransglucosylase/hydrolase (XTH) family cleaves and reconnects xyloglucan molecules (Ishida and Yokoyama, 2022). Since xyloglucan molecules have been hypothesized to tether cellulose microfibrils, forming the main load-bearing network in the primary cell wall, xyloglucan molecules have been thought to play a central role in cell wall loosening for plant cell expansion (Ishida and Yokoyama, 2022). However, *Solyc05G000551,* which encodes for chalcone synthase, was found as well. Chalcone synthase is the second rate-limiting enzyme and the first reported enzyme involved in the biosynthetic pathway of flavonoids (Yin *et al.,* 2019). Based on these findings, it can be suggested that under all treatment conditions, at 1 DAS, the plant limits photosynthesis and activates osmoprotectans mechanisms, such as the synthesis of flavonoids and sugars. At 14 DAS, however, it can be hypothesized that the plant has reactivated mechanisms related to cell growth while still maintaining protective mechanisms against cold stress, which is still ongoing

With regard to GO terms and related genes modulated exclusively in mock-inoculated plants, it is noted that the genes upregulated at 1 DAS are associated with the biosynthetic pathways of trehalose (*Solyc03G000232*, encoding trehalose-6-phosphate synthase), callose synthase (*Solyc11G000094*, encoding callose synthase), and phosphatidylinositol metabolism (*Solyc12G001877*, encoding phosphatidylinositol glycan), notably related with abiotic stress management (Nedukha, 2015; Beihammer *et al.,* 2020; Mollavali and Börnke, 2022; Liu *et al.*, 2024b). In the GO terms and related genes concerning exclusively mock-inoculated plants, it is interesting to note the involvement in carbohydrate catabolism and fatty acid biosynthesis terms. Pyruvate kinase (*Solyc08G001997*) is an important enzyme of glycolytic pathway that also functions in providing carbon skeleton for fatty acid biosynthesis (Ambasht and Kayastha, 2002); whereas glycoside hydrolase (*Solyc11G000354*) has been reported to affect cell wall polysaccharides, mobilization of storage reserves, and can be the basis of various secondary metabolites (Minic, 2008).

Interestingly, in the gene ontology terms analysis and corresponding up-regulated genes at 1 DAS exclusively of *Chryseobacterium* -inoculated plants, the relevant GO terms are associated with the metabolic processes of cell wall polysaccharides, protein folding, glucose metabolism, and the tricarboxylic acid cycle. Specifically, *Solyc01G000013* and *Solyc03G001837* encode for two xyloglucan endotransglucosylase, which have been proposed to be involved in the process of cell expansion (Campbell and Braam, 1999; Ishida and Yokoyama, 2022). *Solyc05G000603* (heat shock protein 90kDa beta) and *Solyc01G003830* (molecular chaperone DnaK) may be responsible for protein folding, assembly, translocation and degradation in many normal cellular processes, stabilize proteins and membranes and may assist protein folding under stress conditions (Wang *et al.,* 2004). Instead, *Solyc02G002146* (succinate dehydrogenase flavoprotein subunit), *Solyc06G002032* (mitochondrial pyruvate carrier 1), *Solyc07G002282* (2-polyprenyl-6-hydroxyphenol methylase / 2-

octaprenyl-6-hydroxyphenol methylase), *Solyc11G000194* (cytochrome c oxidase assembly protein subunit 15), *Solyc12G002366* (are involved in the respiratory chain (Leegood and Walker, 2003; Fernie *et al*., 2004; Liu and Lu, 2016; Zhu *et al*., 2016; Mansilla *et al.*, 2018; Zhang and Fernie, 2023). However, in the exclusively up-regulated genes of *Chryseobacterium*-inoculated plants at 1DAS, other genes deserve to be mentioned to better understand the whole picture. For example, *Solyc02G001890* (3-hydroxy-3-methylglutaryl-coenzyme a reductase 1) is the first rate-limiting enzyme regulating the synthesis of terpenoids upstream of the mevalonate (MVA) pathway *(Kevei et al.*, 2007; Zhang *et al.*, 2020b). Zhang *et al.* claim that under oxidative stress, transgenic *A. thaliana* plants over-expressing of *Malus domestica 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (MdHMGR)* had a higher germination rate, a longer main root length, higher chlorophyll and proline content, and higher activities of antioxidant enzymes *(Zhang et al.,* 2020b). On the other hand, malondialdehyde (MDA) content, relative conductivity, and ROS production rate were significantly lower than in wild type plants (Zhang *et al.*, 2020b). These results indicated that over-expression of *MdHMGR5 gene* enhanced plant tolerance to oxidative stress by ROS scavenging in transgenic plants (Zhang *et al.*, 2020b). Indeed, the glutamate 5-kinase (*Solyc06G000525*) has been founded as well in *Cryseobacterium*-inoculated plants at 1DAS, and the enzyme is reported to control the first step of proline biosynthesis (Pérez-Arellano *et al.,* 2006). Proline can act as an osmo-protectant under abiotic stress, scavenging reactive oxygen species (Ghosh *et al.*, 2022). Moreover, *Solyc02G002279* (alpha-dioxygenase α-DOX enzyme), *Solyc05G000551* (chalcone isomerase), and *Solyc10G002676* (S-adenosylmethionine synthetase) were found up-regulated. These enzymes are involved in the biosynthetic pathways of oxylipins, flavonoids, and methionine, respectively *(Muir et al.*, 2001; Ravanel *et al*., 2004; Tirajoh *et al*., 2005). If flavonoids are well known to be involved in abiotic stress management, oxylipins are less: a newly identified group of oxylipins, derived from alpha-dioxygenase (α-DOX) enzymes catalyze activity of oxygenation of fatty acids plays a role in

protecting tissues from oxidative damage and cell death (Muir *et al.,* 2001; Tirajoh *et al.,* 2005). S-adenosylmethionine synthetase is at the base of phytosiderophores, ethylene and nicotianamine, and the modulation of these molecules levels, which are important for iron uptake, can therefore be attributed on the activity of this enzyme. Thus, it is possible to speculate that, as the enzyme is up-regulated, there is a better iron nutrition in the plant mediated by *Chryseobacterium* (Hesse and Hoefgen, 2003; Zuchi *et al.*, 2009; Coppa *et al.*, 2018). Conversely, at 14DAS exclusive *Chryseobacterium*-inoculated plants enriched GO terms refer mainly to DNA replication, which suggest an intense cellular replication, and thus plant growth.

Interestingly, at 1 DAS exclusive *Pseudomonas*-inoculated plants enriched GO terms revealed an enrichment in calcium ion transport and galactose metabolic processes, as suggested by *Solyc07G002308* (calcium:hydrogen antiporter) and *Solyc02G000743* (galactinol synthase 1-related) genes. Calcium is involved in the very first step of cold sensing, whereas galactinol synthase is a member of the glycosyltransferase eight family that catalyzes the first step in the biosynthesis pathway of the raffinose family of oligosaccharides, known to be osmotic protectors (Zhou *et al*., 2014; Qian *et al*., 2024b). However, among the up-regulated genes exclusive *Pseudomonas*-inoculated plants, *Solyc10G001669* (mitochondrial pyruvate carrier 1) and *Solyc09G000287* (aspartate kinase) might help the plant in coping with the cold stress (Shen *et al*., 2017; Han *et al.*, 2021). More specifically, L-aspartate (Asp) serves as a central building block, in addition to being a constituent of proteins, for many metabolic processes in most organisms, such as biosynthesis of other amino acids, nucleotides, nicotinamide adenine dinucleotide (NAD), the tricarboxylic acid (TCA) cycle and glycolysis pathway intermediates, and hormones, which are vital for growth and defense (Han *et al.,* 2021). At 1DAS exclusive *Pseudomonas*-inoculated plants enriched GO terms, besides GO term enriched in protein translation and DNA metabolic processes, genes related to photosynthesis such as *Solyc09G002347*

(photosystem ii d1 precursor processing protein psb27-h2) can be found, suggesting an active plant growth.

Up to now, it can be established that in bacterial-inoculated plants, particularly in *Chryseobacterium*-inoculated plants, seedlings can cope with cold stress better compared to mock-inoculated plants and can keep growth-related genes active at 1 DAS. The plants inoculated with bacteria seem to show an array of up-regulated genes to mitigate oxidative and osmotic stress, *Chryseobacterium*-inoculated plants. This is therefore in line with what has been observed in growth trials and ROS content: plants inoculated with *Chryseobacterium* sp. show greater growth under cold stress compared to both plants inoculated with *Pseudomonas* sp. and mock, and a greater reduction in ROS.

Solyc06G002269 turned out to be highly up-regulated in Chryseobacterium-inoculated plants under at 14 DAS compared to mock- or Pseudomonas-inoculated plants. Solyc06G002269 encodes for a sterol reductase, which is the last step of the sterol biosynthetic process (Horling *et al*., 2012). Sterol at low temperatures inserts into phospholipids and prevents them from interfering with each other to avoid aggregation (Zhang *et al.*, 2019). Sterol is well-known for maintaining membrane fluidity, thus by ensuring the physiological processes that take place through them. The 7-dehydrocholesterol reductase seems also to be involved brassinosteroids biosynthesis, hence promoting plant growth (Zhang *et al.*, 2021). At 14 DAS, Solyc06G002269 is up-regulated by cold stress in all inoculation conditions, suggesting tomato adaptation to the cold condition. Solyc06G002032 (mitochondrial pyruvate carrier 1), Solyc02G002146 (succinate dehydrogenase flavoprotein subunit), Solyc07G002282 (2-polyprenyl-6-hydroxyphenol methylase / 2-octaprenyl-6-hydroxyphenol methylase) are involved in the tricarboxylic acid cycle (Shen *et al*., 2017; Zhang and Fernie, 2023). Tricarboxylic acid cycle is responsible for the oxidation of respiratory substrate to drive

ATP synthesis (Zhang and Fernie, 2023), pertaining to the central metabolic pathway, which use carbon for energy generation in the form of Adenosine Triphosphate (ATP). This molecule serves as the central energy source for cellular metabolic processes through the release and addition of phosphate groups. As a component of the central metabolic pathway, it is linked to glycolysis and photosynthesis, thereby constituting a fundamental pathway for the study of plant energy stability. It is sensitive to the generation of ROS, both in the mitochondrial intermembrane space and in the thylakoids due to photophosphorylation, both directly associated with ATP generation. These three TCA-related genes mentioned before are upregulated in *Chryseobacterium*-inoculated plant at 1 DAS. Interestingly, Solyc06G002032 is overexpressed in mock-inoculated plants on 14 DAS. Solyc03G001161 (Galactinol--sucrose galactosyltransferase / Raffinose synthase) and Solyc11G002117 (flavonoid 3',5'-hydroxylase) can be found up-regulated in bacterial-inoculated (both *Chryseobacterium* and *Pseudomonas*) plants at 1 DAS. Raffinose family oligosaccharides (RFOs) accumulate under stress conditions in many plants and have been suggested to act as stress protectants (Gu *et al*., 2018); whereas flavonoid 3′,5′-hydroxylase (F3′,5′H) is a key enzyme in the flavonoid biosynthetic pathway (Zhang *et al.,* 2021). Both genes led to the synthesis of osmoprotectants. Overall, we could argue that bacterial inoculation can lead to a better fitness compared to mock-inoculated plants at 1 DAS, and plants seem to be more ready to face cold stress.

Moreover, we can summarize DEGs possibly related to oxidative stress response were modulated by cold stress in mock-, *Chryseobacterium*-, or *Pseudomonas*-inoculated plants, such as three catalase, nine ferredoxin, ten glutaredoxin, 14 glutathione S-transferase, 21 oxidoreductase, 40 oxygenase, 20 peroxidase, 19 reductase, two superoxide dismutase, and thioredoxin genes (Figure 4.1A). Transcriptional reprogramming of mock-, *Chryseobacterium*-, or *Pseudomonas*-inoculated plants to cold stress included the modulation of genes possibly related to stress response (14 cold-related

proteins, 35 heat shock-related proteins, five heat stress transcription factors, 29 late embryogenesis abundant proteins, and 30 stress-related proteins; (Figure 4.1B) and hormonal signaling (17 abscisic acid-, 81 auxin-, 13 cytokinin-, 43 ethylene-, 20 gibberellin-, four jasmonic acid-, and three salicylic acid-related genes; Figure 4.1C).

Greenhouse trials conducted under commercial conditions during the winter cycle of tomatoes have yielded encouraging results. These trials aimed to evaluate the efficacy of bacterial strains *Chryseobacterium* and *Pseudomonas* as plant growth promoters. The findings indicate that, even in the challenging context of winter cultivation, plants inoculated with these bacteria exhibited significantly enhanced fitness compared to control plants. Notably, the *Pseudomonas*-inoculated plants demonstrated a marked increase in stem height during the first six weeks post-transplantation. Additionally, at the end of the reproductive cycle, the dry weight of the roots in inoculated plants was substantially higher than that of the control plants. This increase in root biomass suggests that the bacteria may facilitate more efficient nutrient uptake and storage, contributing to overall plant health. However, no differences were spotted in terms of fruit production during the season. These findings underscore the potential of bacterial inoculation as a targeted strategy for improving plant growth under commercial greenhouse conditions, although further research is needed to elucidate the mechanisms by which these bacteria interact with the local microbiota.

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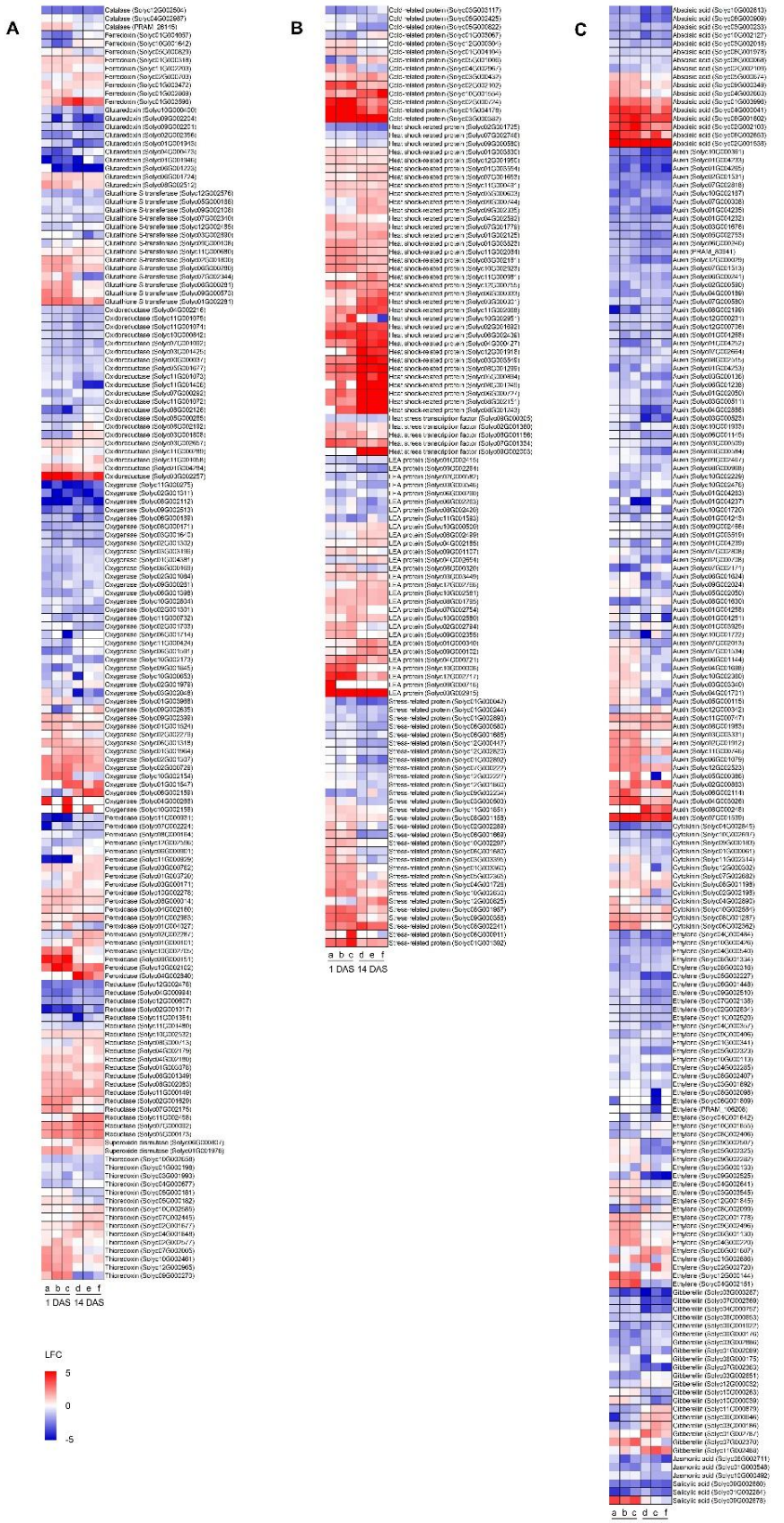

Figure 4.1. Heat map of the genes clustered by function and treatments The figure presents three

distinct heatmaps, all adhering to the following data representation structure: The X-axis displays the two time points, 1 and 14 Days After Stress (DAS). Within each of these DAS groups, three sub-groups are represented: the first, labeled "a" or "d", corresponds to mock-inoculated plants; the second, labeled "b" or "e", corresponds to Chryseobacterium-inoculated plants; and the third, labeled "c" or "f", corresponds to Pseudomonas-inoculated plants. Each cell within the heatmaps displays the Log2(Fold Change) value, as represented in the accompanying legend. These values compare gene expression under control conditions versus cold stress conditions, with the red scale indicating up-regulation and the blue scale indicating down-regulation. The three heatmaps are categorized according to cellular functions: (A) genes related to ROS scavenging, (B) genes related to stress response, and (C) genes related to the biosynthesis or catabolism of phytohormones.

CHAPTER 5. CONCLUSION

Functional and transcriptomic analysis allowed the identification of two bacteria - *Chryseobacterium* sp. and *Pseudomonas* sp. - able to promote tomato growth under normal and stress conditions. In particular, *Chryseobacterium*-inoculated plants showed a decrease in H_2O_2 production one day after cold stress, modulating the expression of genes related to ROS scavenging enzymes, as well as modulating the gene expression of the respiratory chain, a known site of ROS formation under stress conditions. Furthermore, it seems to activate a series of biosynthetic pathways aimed at the production of osmoprotectans, while continuing to maintain active pathways that indicate ongoing cellular growth. *Pseudomonas*-inoculated plants seemed to promote mechanism related to calcium ion transport as early cold stress response, as well as osmoprotectans molecules. More specifically, *Chryseobacterium*- and *Pseudomonas*-inoculated plants exhibit distinct early and late responses to cold stress, yet they share some common adaptive strategies. In the early phase (1 DAS), both treatments activate genes involved in stress mitigation, such as those related to osmotic protection and energy metabolism. However, *Chryseobacterium*-inoculated plants show a broader and more proactive response, with up-regulation of genes linked to cell wall remodeling, protein folding, antioxidant defense, and metabolic activity, suggesting enhanced growth and stress tolerance. In contrast, *Pseudomonas*-inoculated plants primarily activate calcium signaling and galactose metabolism, indicating a focus on cold sensing and osmoprotection. At the late stage (14 DAS), both treatments promote growth-related processes, but with different emphases: *Chryseobacterium* enhances DNA replication, implying active cell division, while *Pseudomonas* up-regulates genes related to protein translation and photosynthesis, reflecting recovery and biomass accumulation.

Overall, *Chryseobacterium* appears to confer a more robust and sustained cold stress resilience compared to *Pseudomonas.*

While *Chryseobacterium* showed great fitness under cold stress conditions, *Pseudomonas* demonstrated the ability to compete in commercial greenhouse conditions, increasing stem growth in the first six weeks post-transplant and, like *Chryseobacterium*, enhancing the root mass of treated plants. Individually, both *Chryseobacterium* and *Pseudomonas* demonstrate plant growth-promoting effects under commercial conditions. However, this beneficial impact is not observed when they are applied in consortium with *Paenibacillus*. On the contrary, the combined treatment appears to reduce root biomass compared to the mock control. This outcome may be attributed to the fact that *Paenibacillus* alone does not enhance root development, potentially interfering with or negating the positive effects of the other two strains. A more comprehensive explanation likely lies in the complex interaction — or lack thereof — that these bacterial isolates establish with the native microbiota, which plays a central role in determining the success of artificial inoculation. Additionally, the discrepancy in *Pseudomonas* performance between laboratory and greenhouse trials may be partly explained by the use of different tomato cultivars. It is also important to consider that temperature fluctuations in the greenhouse environment (e.g., between day and night), which could not be replicated in the controlled laboratory setting, may have influenced the final outcomes.

In recent years, the use of plant growth-promoting bacteria has captured the attention of both the agricultural and scientific communities, offering a promising green and adaptable approach to crop management. However, for bioinoculants to deliver meaningful results, they must be tailored not only to specific plant species but ideally to specific environmental challenges. Effective bio-stimulants should be developed with local soil conditions, target crops, and particular stressors—such as cold, heat, or drought—in mind. Although abiotic stresses often trigger similar physiological

responses in plants, making it tempting to seek broadly effective bacterial strains, the reality is that true efficacy across diverse crops and soils remains elusive. Compounding this issue is the commercial hesitation to invest in and patent bacterial consortia containing more than two or three strains, largely due to cost concerns. This limits the development of complex yet effective microbial solutions and pushes the industry toward overly generalized products. While scientific research increasingly emphasizes the value of localized, context-specific strategies, commercial demands still lean toward universal solutions – often at the expense of real effectiveness.

To ensure the reproducibility and robustness of the experimental outcomes, it is first essential to implement a systematic monitoring of greenhouse environmental conditions, with particular attention to temperature fluctuations, which may significantly influence plant physiology and the efficacy of bacterial inoculants. Building upon the findings of the present study, it would be of considerable scientific interest to undertake a more comprehensive and targeted investigation into fruit quality parameters, with the aim of identifying potential alterations in organoleptic and nutritional traits. Such analyses should include, but not be limited to, detailed profiling of flavor-related metabolites and quantification of carotenoid content, both of which are critical indicators of fruit quality and consumer appeal. In light of the results obtained, it would also be advisable to design a more calibrated and temporally homogeneous inoculation strategy. Furthermore, it would be beneficial to monitor the persistence of bacterial inoculants throughout the growing season and to conduct metagenomic analyses to assess whether these inoculants induce significant shifts in the native microbial community. This approach would enable a deeper understanding of the functional potential of the selected PGPB strains within a dynamic microbial ecosystem and provide valuable insights for optimizing their application to the plant.

In addition, a joint metabolomic investigation of both the plant and the inoculated bacterial strains, complemented by transcriptomic analyses of the bacteria, would allow for the identification of key metabolic pathways that could be either autonomously exploited or selectively triggered to enhance the beneficial effects of PGPB. Such integrative omics approaches would offer a more mechanistic understanding of the molecular dialogue between plant and microbe, potentially revealing novel targets for biostimulant development and precision agriculture strategies.

Finally, more detailed studies should be conducted to elucidate the spatial localization and colonization patterns of the bacterial strains within the rhizosphere. This would contribute to a more refined comprehension of their ecological behavior, persistence, and interaction with the root system, ultimately informing the development of more effective and sustainable microbial inoculation protocols.

Bridging the gap between scientific research and agricultural practice requires not only updated legislation, but also a paradigm shift in how biostimulants are conceived and applied. Studies like this thesis play a crucial role in deepening our understanding of how bacteria interact with plants and respond to environmental stressors. This knowledge is essential for optimizing the formulation of PGPB-based products and for designing targeted solutions that modulate specific biosynthetic pathways. By aligning scientific insights with practical applications, we can move toward more effective, sustainable, and context-sensitive use of microbial biostimulants in agriculture.

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- **Milanese I.**, Marian M., Bombarely A., Perazzolli M. (2024) Enhancement of cold tolerance of tomato plants using endophytic bacteria isolated from cold-adapted plants. Short oral communication and Poster: International Symposium Microbe-Assisted Crop Production (miCROPe), 15-18 July 2024, Vienna, Austria
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- **Milanese I.**, Marian M., Alussi M., Perazzolli M. (2023). Bacteria associated with wild cold-adapted alpine plants can enhance cold tolerance in tomato seedlings. Poster presentation and Abstract: 16th Symposium on Bacterial and Genetics and Ecology (BAGECO). 26-30 June, Copenhagen, Denmark.
- **Milanese I.**, Marian M., Perazzolli M. (2022). Enhancement of cold stress tolerance of tomato using endophytic bacteria from wild Rosaceae plants. Poster presentation and Abstract "Plant ProBioTech" conference. 28-30 June, Reims, France.

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Other conference proceedings as coauthor

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