Study on the Alleviative Effects of Gypsum Application to Rhyzotoxic Stressor in Acid Soil

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（酸性土壌における石膏施用による根圏ストレスの緩和効果に関する研究）

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KOUAME KOFFI PACOME

Declaration

I at this moment declare that the work towards this thesis entitled "**Study on the Alleviative Effects of Gypsum Application to Rhyzotoxic Stressor in Acid Soil**" has been carried out by me under the supervision of Prof. Kobayashi Yuriko, Faculty of Applied Biological Sciences, at Gifu University, Japan. This work is original and has not been submitted in part or full for any other degree or diploma of any other University or Institution.

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ABBREVIATIONS

Al	Aluminum
Al^{3+}	Aluminum trivalent
$Al(SO_4)^+$	Aluminum Sulfate
$AlCl_3$	Aluminum Chloride
$Al(OH)_3)$	Aluminum hydroxide
ABA	Abscisic Acid
APX	Ascorbate peroxidase
Al exch.	Exchangeable Aluminum
ALMT1	*ALUMINUM ACTIVATED MALATE TRANSPORTER1*
AtALMT*1*	*Arabidopsis thaliana ALUMINUM ACTIVATED MALATE TRANSPORTER1*
Ca	Calcium
Ca^{2+}	Calcium divalent
CEC	Cation Exchange Capacity
$CaSO_4$	Calcium Sulfate
$CaCO_3$	Calcium Carbonate
CA	Convention Agriculture
CAT	Catalase
CuZnSOD	Copper–zinc superoxide dismutase
CECR	Cation Exchange Capacity Ratio
DREBs	Dehydration-responsive element-binding proteins
DNA	Deoxyribonucleic acid
Fe^{2+}	Iron divalent
GPX	Glutathione peroxidase
H2O2	Hydrogen Peroxide
ISFM	Integrated soil fertility management
INPM	Integrated Nutrient Management
KO	KnockOut
MATE	Multidrug and toxic compound extrusion
MYB	Myeloblastosis
N	Nitrogen
$Na_2SO_4._$	Sodium Sulfate
OAs	Organic Acids
*Os*ALS1	*Oryza sativa* ALUMINUM SENSITIVE 1
P	Phosphate
pH	Negative log H^+ ion
PM	Plasma membrane

RT-PCR	Reverse transcription polymerase chain reaction
RNA	Ribonucleic Acid
RRL	Relative Root length
ROS	Reactive oxygen species
STOP1	SENSITIVE TO PROTON RHIZOTOXICITY1
SULTR.	Sulfur transporter
SSA	Sub-Saharan Africa
SOM	Soil Organic Matter
SAP	Sustainable Agriculture Production
SGCS	Speciation Gouy-Chapman-Stern
SO_4^{2-}	Sulfate
S	Sulfur
TCA	Tricarboxylic acid
TPX	Thioredoxin peroxidase
UBQ1	Ubiquitin 1
WT	Wild Type

Units

μg	microgram
μl	microliter
μM	micromolar
°C	degree celsius
Bp	basepair
Cm	centimeter
g	Gram
Kg	kilo basepair
mg	milligram
mg/L	milligram per liter
mL	Millilitre
mM	Millimolar
Mm	Millimetre
rpm	Revolution per minute
S	Second
v/v	volume/volume(concentration)
w/v	weight/volume(Concentration)
Min	Minute
h	Hour
ng	Nanogram

Dissertation Summary (学位論文要旨)

Gypsum (calcium sulfate) is commonly used to improve subsoil acidity. Aluminum (Al) toxicity in acid soil reduces crop yields worldwide, especially in the tropical regions. In soil, sulfate decreases Al toxicity and improves plant growth and yield. Here, we aimed to investigate the effects of $CaSO_4$ on Al stress an Al-tolerant accession Col-0 as well as the Al-sensitive accessions, Wei-0 and Ts-5, of in *Arabidopsis thaliana* using hydroponics. In addition, we investigate the effects of $CaSO_4$ in acid soil (Andsoil) on Wheat, soybean, and Al-sensitive (Ts-5, Aitiba-2). Our findings indicate that $CaSO_4$ application has significantly increased root elongation and malate secretion compared to $CaCl_2$ under Al at pH= 5.0. Furthermore, in response to Al stress, $CaSO_4$ supply enhanced the expression of malate transporter gene *AtALMT1* and sulfate transporter genes *SULTR3,5* as well indicated that they may be regulated by the Al-resistant transcription factor STOP1. On the other hand, the knockout lines of *SULTR3;5* and *SULTR2;1*, which are involved in sulfate absorption and translocation from root to shoot were found to be more sensitive to Al stress without $CaSO_4$. Malate and *AtALMT1* were reduced indicating the role of these sulfur transporter genes in the regulation of Al stress. we observed an increase in sulfur content by $CaSO_4$ supply in roots and shoots, although we also noticed a decrease in Al content in the shoot. Soil experiment indicates that the fresh weight of wheat and soybeans shoot and root increases significantly with $CaSO_4$ and $CaCO_3$ treatment compared to no treatment. Interestingly, $CaSO_4$ treatments showed higher fresh weight in wheat and soybeans shoots and roots than $CaCO_3$. Al content was reduced in Wheat root and shoot as well with both treatments ($CaSO_4$ and $CaCO_3$) compared to no treatment whereas S content was increased as well. In conclusion, one of the alleviative effects of gypsum application on Al rhizotoxicity is promoted by sulfate through organic acid release mechanism

enhancing *AtALMT1* expression, alongside the Ca^{2+} reduction Al^{3+} activity on the plasma

membrane.

石膏（$CaSO_4$）は、土壌の酸性度を改善するために一般的に使用されている。酸性土壌におけるアルミニウム（Al）の毒性は、世界的に、特に熱帯地域で作物の収量を減少させる。土壌中では、硫酸塩がAl毒性を低下させ、植物の成長と収量を改善する。ここでは、シロイヌナズナのAl耐性アクセッションCol-0およびAl感受性アクセッションWei-0とTs-5のAlストレスに対する$CaSO_4$の影響を水耕栽培で調査することを目的とした。さらに、酸性土壌（Andsoil）への$CaSO_4$施用がコムギ、ダイズ、およびAl感受性シロイヌナズナ（Ts-5、Aitiba-2）に及ぼす影響についても調査した。

　水耕栽培の結果、Alストレス下（pH 5.0）において、$CaSO_4$施用は$CaCl_2$施用と比較して根の伸長とリンゴ酸分泌を有意に増加させた。さらに，Alストレスに応答して，$CaSO_4$供給はAl耐性転写因子STOP1によって制御されているリンゴ酸トランスポーター遺伝子*AtALMT1*および硫酸トランスポーター遺伝子*SULTR3;5*の発現を亢進した。一方、硫酸の吸収と根から地上部への移行に関与する*SULTR3;5*と*SULTR2;1*のノックアウト系統は、$CaSO_4$供給がない場合はAlストレスに対してより感受性が高いことがわかった。また、両系統は、リンゴ酸分泌とおよび*AtALMT1*発現量は減少しており、これらの硫酸トランスポーター遺伝子がAlストレスの制御において役割を果たしていることが示された。根および地上部において$CaSO_4$供給による硫黄含量の増加が観察されたが、地上部におけるAl含量の減少も認められた。

　酸性土壌を用いた栽培実験によると、コムギとダイズの地上部と根の新鮮重は、$CaSO_4$と$CaCO_3$添加によって、無処理に比べて有意に増加した。興味深いことに、$CaSO_4$添加区では$CaCO_3$添加区よりもコムギとダイズの地上部と根の新鮮重量が高いことを示した。また，コムギの根および地上部の Al 含有量は、無処理に比べて $CaSO_4$ および $CaCO_3$ の両添加区で減少したが，S 含有量は増加した。結論として、石膏施用によるAlの根毒性緩和効果の一つは、硫酸塩による*AtALMT1*発現向上を通じた有機酸放出メカニズムの亢進によって、細胞膜上のCa^{2+}活動度が促進、Al^{3+}活動度が低下されることである。

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General introduction

Over the last two decades, the world population has continued to grow at an unrelenting rate and is projected to peak at 10 billion by 2050 [1]. On the other hand, abiotic and biotic stresses, and climate change with the arrival of new diseases on the agricultural scene, severely threaten the world's food security [2]. In tropical regions especially in low-economic development countries, the situation is alarming because of Soil acidity, low fertilizer input and mismanagement [3] nutrient deficiencies [4], drought and water stress [5]. In fact, at low pH (<5), Aluminum ($AlCl_3$ and $Al(OH)_3$) is dissolved in soil solution into a trivalent form (Al^{3+}) which inhibits plant root growth. This inhibition results in phosphate (P) and Calcium (Ca) deficiency, nutrient imbalances, water stress, drastic reduction of crop yields [6], and fertilizer use inefficiency. Along the walking way of Agriculture science against abiotic stress, farmers, plant biologists, and agronomists have tried several solutions to cope with soil acidity. In plant molecular physiology, experiments are conducted in two major ways. First, abiotic stress was applied to plants to elucidate the strategies evolved to mitigate the stress at physiological and molecular levels [7]. Two major mechanisms have emerged in the case of Al stress. Internal Al-detoxification and external exclusion from the symplasm [8]. Internal tolerance consists of internal Al-sequestration into the vacuole decreasing the Al concentration at the plasma membrane surface (PM) [9]. For instance, *Os*ALS1 a homolog of ALUMINUM SENSITIVE 1 (ALS1) from rice has been characterized as a vacuolar Al-transporter [10]. The external Al-exclusion mechanism is mainly caused by organic acid secretion from plant roots. Therefore, several gene families encoding malate (Al-Activated Malate Transporters; ALMT1) and citrate (MULTIDRUG AND TOXIC COMPOUND EXTRUSION; MATE) have been identified. These are AtALMT*1* in Arabidopsis (*Arabidopsis thaliana*) [11,12] *TaALMt1* in wheat (*Triticum aestivum*) [13] , *SbMATE* in Sorghum (*Sorghum bicolor*) [14], and in barley (*Hordeum vulgare*) [15]. Second, the use of mineral or organic compounds such as boron

(B) (Trifoliate orange; [16]; Pea: [17]; Wheat; [18]; Citrus grandis: [19], Magnesium (Mg) [20], and Silicon (Si) (In sorghum: [21]; In maize: [22]. These findings brought a clear understanding of the different mechanisms evolved by plants to cope the Al damage effects. Some of these mechanisms are Mechanism I, electrostatic displacement of Al3+ from the plasma membrane surface; mechanism II, restoration of Ca^{2+} on the plasma membrane; and third ionic interaction between Al^{3+} and Ca^{2+} at the cell surface [23]. Furthermore, he reported that mechanism III includes mechanisms I, II, and many physiological responses and unknown mechanisms. For example, Ca^{2+} decreased Al accumulation, regulated tricarboxylic acid (TCA) cycle-related protein abundances, and affected organic acid concentrations and related enzyme activities under Al stress [24].

Agronomists emphasized that mineral and organic fertilizers can compensate for the poor nutrients of soil. Lime ($CaCO_3$) and Gypsum ($CaSO_4$) amendments are used to correct acid and sodic soil [5]. Lime sometimes called the foundation of crop production or "workhorse" is famously known to increase soil pH and has been widely used by developed countries [25] but in developing like Sub-Saharan Africa the situation is in stark because of poverty. However, lime application on the soil surface is inefficient in improving subsoil acidity (75% worldwide) because it moves slowly and cannot act up to 10cm of the soil layer [26]. Therefore, incorporating lime in conventional agriculture is known to be effective but this practice is expensive and can disturb soil physical properties, soil structure and porosity [27]. Gypsum or Calcium sulfate ($CaSO_4$) is widely used in sodic soil [28] and can improve soil's physical and chemical properties [29]. Calcium sulfate ($CaSO_4$) application in tropical savannas of Brazil had an efficiency on acid soil [30]. In saline soils, Gypsum improved sulfur (S), Nitrate (N), Phosphate (P), and Calcium (Ca) uptake [31]. In addition, they reported that the combined amendment of gypsum and organic fertilizer increased

microbial activity and plant biomass. Cereals (maize [*Zea mays L.*], wheat [*Triticum aestivum L.*], white oat [*Avena sativa L.*], barley [*Hordeum vulgare L.*], and rice [*Oryza sativa L.*]) have increased their grain yield at about 77–97% by gypsum application to soils, with Al saturation exceeding 5% in the 0.20- to 0.40-m layer [32]. Using sodium sulfate (Na_2SO_4) in Wheat showed a possibility of Al mitigation by the complexation of Calcium (Ca^{2+}) and sulfate (SO_4^{2-}) to form a less toxic compound an aluminum sulfate $(ALSO_4)^+$ [33]. In complement, Calcium was shown to alleviate Al toxicity at moderately acidic conditions via Mechanism I [34]. Therefore, we suggest that the alleviation effect of gypsum on sodic or acidic soil may not be only because of the improvement of soil's physical and chemical properties but also the gypsum (Ca^{2+} and SO_4^{2-}) effect on plant growth and yield. More importantly, especially the sulfur-containing compound may be involved in many physiological and molecular mechanisms pathway of the plants. Sulfur(S) belongs to the group of secondary macronutrients (Ca, Mg, S) and has been proven to play an important role in regulating plant responses to various biotic and abiotic stress [35]. In general, sulfate concentration in soil is low and causes growth retardation, decreases cysteine, Glutathione (GSH), and Glucosinolate (GSL), increases O-acetyl-serine (OAS), and reduces crop yields and quality [36].

In Arabidopsis, *SULTR3:5* was colocalized with *SULTR2:1* low-affinity sulfate transporter in the xylem parenchyma and pericycle cell root [37]. They showed that the expression of *SULTR3:5* increased the sulfate uptake activity of the couple *SULTR3:5-SULTR2:1* from root to shoot [38]. This suggests that the co-expression of these two genes (*SULTR3:5, SULTR2:1*) could play an important role in the mechanism of Al alleviation.

Gypsum has been widely used in soil by scientists and farmers, to improve the sulfur utilization efficiency in plants, more effort is required to know the regulatory mechanisms of plant's response

toward Calcium Sulfate ($CaSO_4$) application in hydroponic and soil culture and the different stresses. This study aims to elucidate the molecular physiology mechanism of gypsum application in Hydroponic culture and the effect of gypsum in soil culture.

Ability of Nutrient Management and Molecular Physiology Advancements to Overcome Abiotic Stress: A Study on Sub-Saharan African Crops

1.1. Introduction

The global population is estimated to reach approximately 10 billion by 2050 [1]. However, many challenges, such as food insecurity, abiotic stress, and increased pest outbreaks, attributed to climate change demand global attention, particularly in tropical regions [39]. Approximately 50% of the global population lives in tropical regions, and more than two-thirds live in extreme poverty [40]. However, in recent years, this situation has been exacerbated by the continuous decline in crop yield owing to increased abiotic stress [41]. The Food and Agriculture Organization (FAO) has emphasized the need for a 50% increase in food production to meet the rising demand by adopting sustainable farming practices [42]. The efficient management of nutrients and irrigation using seeds of high-yielding crop varieties will be necessary to meet the increasing food demand in tropical regions and promote green agriculture. The use of high-yield crop varieties, inorganic fertilizers, irrigation systems, and synthetic pesticides has substantially enhanced crop productivity in various Asian and developed countries. These agricultural practices have played a vital role in meeting the increasing food demand to ensure sufficient food supply (43,44). Many studies have focused on the detrimental effects of these practices on surface and groundwater pollution in Asia [45-47] and Europe [48]. Among the developing countries, Sub-Saharan Africa (SSA), which accounts for 13% of the total arable land worldwide [49], is characterized by various factors, such as limited fertilizer availability, high soil acidity [3,50,51], drought, water stress [5], and nutrient deficiency [52]. The International Fertilizer Association has strongly emphasized the critical role of fertilizer inputs in promoting food production and ensuring food security in Africa [53]. However, the adverse effects of pesticide misuse, excessive reliance on synthetic inputs, abiotic stress, and climate change in the tropical regions of both developed and developing countries have raised concerns [54]. Therefore, proactive and emergency strategies that prioritize green, efficient, and sustainable agricultural practices are necessary to ensure food security for future generations.

Over the past decade, studies have focused on the use of organic compounds to promote sustainable agriculture while mitigating the ecological consequences of increasing global food demand [55,56], especially in tropical regions [57]. Consequently, alternative strategies based on research findings in the fields of plant nutrition, climate change, and molecular physiology have been developed for different geographical areas, especially tropical regions. The synergistic application of inorganic and organic compounds can increase the crop yield [58]. Several studies have elucidated the molecular mechanisms underlying various stresses, such as drought [59], high temperature [60], and soil acidity [61]. These findings offer valuable insights on plant nutrient management, fertilizer use, and molecular breeding to enhance the agricultural yield in tropical regions. This review provides comprehensive information on the various abiotic stresses affecting tropical crops, with a particular focus on soil acidity, Al and Fe toxicity, drought and heat stress, and climate change. Furthermore, this review highlights the recent advancements in plant nutrient management and the molecular breeding strategies used to enhance crop yields, fortify sustainable agricultural practices, and ensure food security.

1.2. Effects of Abiotic Stress on Nutrient Imbalance and Crop Yield

Abiotic stress, namely soil acidity, Al and Fe toxicity, drought and heat stress, and climate change, pose serious environmental challenges that affect and reduce the production of crops worldwide [2]. Crop yields are expected to reduce owing to climate change and the side effects of the increased world population that force the extension of urban areas, thereby limiting agriculture to areas less appropriate for crop cultivation [62]. Among various abiotic stresses, soil acidity, drought, elevated temperatures, and salinity are recognized as the predominant limiting factors [63]. These stresses, in combination with climate change and the emergence of new pests and diseases, have a significant effect on global agricultural production, particularly in tropical regions [64]. Abiotic stresses frequently induce morpho-anatomical and physiological growth constraints,

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further exacerbating challenges in crop production [65]. Soil acidity, heat stress, drought, and climate change are the most critical limiting factors for maize (*Zea mays*), millet (*Panicum milliaceum*), and sorghum (*Sorghum bicolor*) production, but not cassava (*Manihot esculenta*), which is mainly limited by floods in SSA [66]. In this article, we aimed to outline the adverse effects of these factors, specifically focusing on their impact on tropical crops.

1.2.1. Al and Fe Toxicity

Soil acidity, characterized by a pH level of ≤5.5, is a significant constraint to crop production worldwide [8]. This condition is particularly prevalent in tropical and subtropical regions [67,68]. The primary challenge associated with acidic soils is the toxicity of aluminum (Al^{3+}), phosphate ($PO4^{2-}$), and iron (Fe^{2+}), which can have detrimental effects on the plant [69][70]. This phenomenon adversely affects crops, such as sesame (*Sesamum indicum*), and impedes nutrient mineralization [68]. It also affects other vegetable crops, such as *Brassica juncea*, *Phaseolus vulgaris*, *Pisum sativum*, and *Vigna mungo* [71]. This phenomenon affects approximately 600 Mha of land in SSA [3]. In South America, particularly Brazil, soil acidity affects approximately 205 Mha of land [25].

In the tropics, Al toxicity affects 25–80% of crop production [52]. The detrimental effects of Al^{3+} are manifested in developing root tips, as they disrupt crucial processes related to cell division, elongation, and genotoxicity. This disturbance ultimately leads to the inhibition of root growth, hindering the ability of crops to extend their roots for nutrient uptake [8]. In SSA, Al significantly decreases the yields of several crucial crops (Table 1.1). For example, in Ethiopia, Al reduces the grain yield of wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare*), and beans (*Phaseolus vulgaris* L.) [72]. Across SSA, elevated soil acidity can trigger various indirect consequences. These include the suboptimal nodulation of legumes, the proliferation of acid-

tolerant weeds, stunted root growth, and a reduction in the yields of various crops, such as millet, sorghum, tomato (*Solanum lycopersicum*), sweet potato (*Ipomoea batatas*), and tea [73]. Soil acidity also reduces enzymatic activity, interrupts microbially mediated nutrient cycling, and hampers microbial activity [74,75]. These constraints vary depending on the degree of acidity [76]. For instance, wheat [76], maize, and canola [77] exhibit yield-specific responses to soil pH. Moreover, Al reduces nitrogen (N) uptake and decreases N use efficiency (NUE) and water use efficiency (WUE) in crops such as maize [78], reducing its yield and contributing to high drought stress and nutrient unavailability due to root growth inhibition [71]. In high-income economies, the widespread application of lime to enhance the soil pH has led to remarkable increases in crop yields over the past century [79,80]. In contrast, low-income economies, particularly those in tropical and subtropical regions, face significant challenges. Extreme poverty often prevents farmers from producing lime to ameliorate soil acidity and boost crop yields . Furthermore, these regions are characterized by iron toxicity, which causes severe damage to rice (*Oryza sativa*).

Table 1.1 Major crops sensitivity level to abiotic stresses in Sub-Saharan Africa.

Abiotic Stresses	Major Crops	References
	Sensitive	
	Barley	[57]
	Maize	[81]
Soil acidity and Al Toxicity	Wheat	[76]
	Soybean, Peanut	[52]
	Less sensitive	
	Pineapple, Sweet potato,	[52]
	Cassava, Yam	[73]
Fe toxicity	Rice (lowland)	[82]
	Sensitive	
	Soybean, Peanut	[83]
Heat and drought	Wheat	[84]
	Barley	[85]
	Rice	[82]
	Less sensitive	
	Yam, Cassava	[86]
	Sweet potato	[87]
	Sorghum	[88]
	Finger Millet	[89][90]
	Sensitive	
Climate change	Maize, Rice	[91]
	Wheat	[92]
	Barley	[86]
	Less sensitive	
	Cassava, Millet, Sweet potato	[86]
	Sorghum	[92]

Iron toxicity is another major factor that significantly limits crop yields, particularly rice production in West Africa (Table 1.1). In comparison to Asia, rice production in SSA faces significant challenges arising from additional factors, such as nutrient deficiencies, low base cation exchange, a low nutrient-holding capacity, and high levels of phosphorus fixation [93]. Iron toxicity is characterized by physiological indicators, such as leaf chlorosis and necrosis, leading to yield reductions ranging from 10% to 100% [88,94]. Excessive iron uptake and its subsequent accumulation in leaves occur when soil iron concentrations exceed the critical threshold of 500 mg Fe kg^{-1} [95]. This phenomenon is linked to the development of symptoms of iron toxicity, commencing as brown spots at the leaf tip and advancing to purple, reddish-brown, or yellow discoloration. Ultimately, the affected leaves desiccate, giving the plant a scorched appearance. Concurrently, the root architecture becomes dark brown and weakened [96]. The risk of Fe toxicity is notably high in regions characterized by high rainfall, such as sub-humid and humid zones [97], owing to the poor management of water, crops, and mineral fertilizers [96]. In semi-arid zones, the situation deviates because of the co-occurrence of drought and heat stress, which overlaps with the prevalence of Fe toxicity, ultimately resulting in reduced rice yields [98].

1.2.2. Drought and Heat Stress

Drought and heat stress are two major abiotic stresses that can occur simultaneously and severely affect crop growth and productivity, especially in arid and semi-arid zones [99,100]. Although extensive research has been conducted on drought and heat stress individually [101,102], their combined effects are gaining increasing scientific relevance because of climate change-induced water scarcity.

Several staple African crops, such as cassava (*Manihot esculenta*), potatoes (*Solanum* spp.), sweet potatoes (*Ipomoea batatas*), yams (*Dioscorea* spp.), and plantains (*Musa* spp.), have adverse

effects on yield due to rising temperatures and the prolonged effects of climate change-induced drought (Table 1.2.1). Drought affects nearly 80% of agricultural land, imposing limitations on global yield and crop production in both temperate and tropical regions [103,104]. The impact of drought on cereal production has been particularly severe [93]. Recent studies have shown that the combined effects of drought and heat are more severe on maize, barley, and sorghum yields than either stress alone [100]. Moreover, drought and heat stress are affected by climate change. Climate change is projected to have a significant impact on crop yields in the tropics, particularly in West Africa, where the projected temperature rise of 2.1 °C could severely reduce the maize yield in dry lowlands and lowlands (Table 1). Drought has been established in numerous tropical areas as a factor that leads to decreased crop yields, affecting crops, such as wheat, cowpeas (*Vigna unguiculata*) [93], millet [64], and cassava [105]. For instance, up to 84.27% of cassava mortality has been attributed to drought stress [106]. Furthermore, climate change, primarily through drought stress, is recognized for its adverse impact on maize, groundnut [87], and bean yields, as well as on their nutritional quality [107,108]. In SSA, the limited response of major staple crops, such as maize, soybean (*Glycine max*), sorghum, rice, and cassava, to chemical fertilizers, possibly due to soil acidity, drought, and heat stress, presents a substantial challenge (Table 1.1). Therefore, optimizing crop nutrient management through fertilizer application (both inorganic and organic) while mitigating stress factors such as Al^{3+} and Fe^{2+} toxicity, drought, and heat stress [109] is crucial.

1.3. Nutrient Management under Abiotic Stress: Combined Use of Inorganic and Organic Fertilizers
1.3.1. Effects of Organic and Inorganic Fertilizers on Nutrient Availability

The application of organic fertilizers, whether used in conjunction with chemical fertilizers or as a discrete method, has demonstrated efficacy in mitigating soil acidification and enhancing soil fertility [110]. For example, the incorporation of pig manure and straw as amendments in maize and wheat has been found to enhance the immobilization of abiotic NH_4^+-N and NO_3^--N by increasing the soil carbon content. Notably, manure application independently ameliorated soil acidity, whereas straw amendment did not yield a comparable effect [111]. In tobacco (*Nicotiana tabacum*) cultivation, cow manure, whether discrete or combined with synthetic fertilizer in acidic soil, significantly reduced the soil exchangeable acid content, with a substantial 51.28% reduction in exchangeable Al^{3+} when organic matter was applied, thereby mitigating soil acidification [112]. This practice further led to a 37.19% and 42% increase in exchangeable base cations for cow manure and the combined organic–inorganic fertilizer, respectively, compared to the discrete use of chemical fertilizer. The use of mixed poultry manure (50%) + NPK (50%) or 100% poultry manure significantly elevated the soil pH, cation exchange capacity (CEC), and NPK uptake compared with 100% synthetic NPK [113] (Table 1.2). Conversely, the incorporation of crop residues has demonstrated a high potential to alter soil CEC, organic carbon levels, P, K, and pH [114]. Most studies conducted in Asia have revealed the importance of combining organic and inorganic fertilizers to mitigate environmental stresses, such as water pollution, soil acidity, and plant nutrient deficiency. However, reports indicate that the levels of chemical fertilizers and organic inputs for nitrogen supply are significantly lower in Africa than in Europe and North America [115][. Taken together, it is important to highlight trends in nutrient management to mitigate environmental stress in Africa.

Table 1.2 Evaluation of different types of fertilizer application depending on crops in SSA.

AEZ	Crops	Fertilizer Use
Semi-arid zone	**Cereals** maize (*Zea mays*), millet (*Panicum milliaceum*), sorghum. (*Sorghum bicolor*), soybean (*Glycine max*), bean (*Phaseolus vulgaris* L.), wheat (*Triticum aestivum* L.) pigeon pea (*Cajanus cajan*) **Root tubers** cassava (Manihot esculenta) **Perennial crops** cotton (*Gossypium herbaceum*)	SI+N [116] CA+mulch+Manure [117] Manure [118] Biological N-fixation (*Acacia mangium*) [119] Urea/DAP/TSP/KCl [41] NPK [120] Sulfur(S) [67] ISFM [121]
Sub-humid zone	**Cereals** rice (*Oryza sativa*), maize (*Zea mays*), millet (*Panicum milliaceum*), sorghum (*Sorghum bicolor*), soybean (*Glycine max*), bean (*Phaseolus vulgaris* L.), cowpea (*Vigna unguiculata*) **Root tubers** cassava (*Manihot esculenta*), yam (*Dioscorea alata*), sweet potato (*Ipomoea batatas*), groundnut (*Arachis hypogaea*) **Perennial crops**	Lime and Gypsum [122] [123] NT+NPK/CT+NPK+Manure/NT+NPK+Manure [124][125][126] Biochar [127] NPK+Ca+Zn+B/N+Manure [128][129] Biological N-fixation (*Acacia mangium*, *Casuarina equisetifolia* [119] ISFM [130][113][131][131] NPK/Urea/DAP/TSP/KCl/ISFM [41] INPM [121][132]
	cotton (*Gossypium herbaceum*), cashew nut (*Anacardium occidentale*), cocoa (*Theobroma cacao*), coffee (*Coffea canephora*), sugar cane (*Saccharum officinarum*),	

| | **Cereals** | |
	rice (*Oryza sativa*), maize (*Zea mays*) wheat (*Triticum aestivum* L.) sweet potato (*Ipomoea batatas*)	
	Root tubers	
	cassava (*Manihot esculenta*), yam (*Dioscorea alata*)	
	Perennial crops	
Humid zone	rubber tree (*Hevea brasiliensis*), oil palm tree (*Elaeis guineensis*), cocoa (*Theobroma cacao*), coffee (*Coffea canephora*), plantain banana (*Musa paradisiaca*), desert banana (*Musa acuminata*), mango (*Mangifera indica*), avocado (*Persea americana*), ananas (*Ananas comosus*)	Lime and Gypsum [122][123] INPM [121][132] Crop residue [133]

1.3.2. Crop Responses to Fertilizer Management Practices in SSA

In SSA, the implementation of Integrated Soil Fertility Management (ISFM), which strategically combines organic and inorganic fertilizers, is progressively recommended for African agricultural practices across distinct agroecological zones [134,130]. ISFM is an approach aimed at enhancing crop yields and sustaining long-term soil fertility by strategically combining fertilizers, recycled organic resources, responsive crop varieties, and improving agronomic practices [132]. In semi-arid zones, where drought and heat stress are severe, plant nutrient management differs significantly from that in sub-humid and humid regions [135] (Table 1.2). ISFM enhances N and P efficiency in maize by 54 and 16%, respectively [136]. The combination of organic input and urea for maize cultivation led to a 64% increase in N uptake and an 84% increase in yield, while the synergistic effects of both (organic input and urea) nearly doubled the yield to 114% [132]. Studies conducted in Ethiopia have demonstrated that the simultaneous application of inorganic and organic fertilizers yielded significantly higher crop production in tropical agroecosystems than using either fertilizer alone. Furthermore, they concluded that the synergy between manure and NP fertilizer, coupled with practices, such as crop rotation, green

manuring, and crop residue management, resulted in substantial increases in wheat and faba bean grain yields, emphasizing the economic incentives for farmers to adopt ISFM practices [137]. For example, the yields of maize and sorghum were significantly enhanced by the co-application of NPK, manure, and micronutrients in Mali, Kenya, Nigeria, and Tanzania [137]. The efficient uptake of N and P owing to an increase in soil organic matter (SOM) has also been reported in southern Nigeria [117]. Furthermore, the utilization of local fertilizers, such as crop residue application, and the implementation of techniques such as mulching or straw application [138] have been shown to notably mitigate soil temperature and drought stress, resulting in enhanced crop yields (Table 2). Recently, African agronomists have emphasized the use of blended fertilizers, such as NPK+S or NPK+Zn, to enhance rice yield [139]. This approach is driven by the potential of certain compounds, such as sulfur (S), to significantly increase agronomic N-use efficiency [140]. This approach may be because of S, Si, Zn, and P deficiencies in most West African countries [141,142].

In sub-humid and humid zones, a substitutive approach for ISFM is strongly recommended. This approach involves the application of 50% of the recommended inorganic N or P combined with total manure [113,136] (Table 2). This practice is recommended to compensate for the loss of organic matter and soil nutrients. For example, crop N uptake can be enhanced by 26% by combining synthetic N with manure in maize cultivation [143]. Seasonal variations in crop production, climate change, and abiotic stresses have led researchers, farmers, and governments to diversify organic fertilizer sources, provide guidance, and offer fertilizer subsidies (Figure 1.1). The utilization of a rock-based fertilizer (phosphate rock) in conjunction with compost resulted in enhanced Maize and Soybean yields of 2.5 t·ha^{-1} during both the dry and rainy seasons. Similarly, Yam yields increased to 2.5 t·ha^{-1} during the rainy season and 3.0 t·ha^{-1} during the dry season

[144]. In Nigeria, rock phosphate combined with poultry manure increased the P content and yield of maize and cowpea [145]. Furthermore, N, P, and K uptake was significant in sorghum in the presence of combined rock phosphate and farmyard manure [145]. Sustainable agricultural productivity can be improved through effective disease management, optimized soil and water resources, the use of organic fertilizers, the utilization of new tools and facilities (Figure 1.1), and the adoption of improved plant varieties with good-quality seeds from traditional or biotechnological sources, including transgenic breeding. Transgenic varieties are considered a promising approach for doubling or tripling African crop yields [146]. However, their successful utilization for abiotic and biotic stress tolerance requires a clear understanding of the molecular physiological mechanisms related to stresses, such as Al and Fe toxicity, nutrient deficiency, drought, and heat stress, whether occurring individually or in combination, within specific crop species.
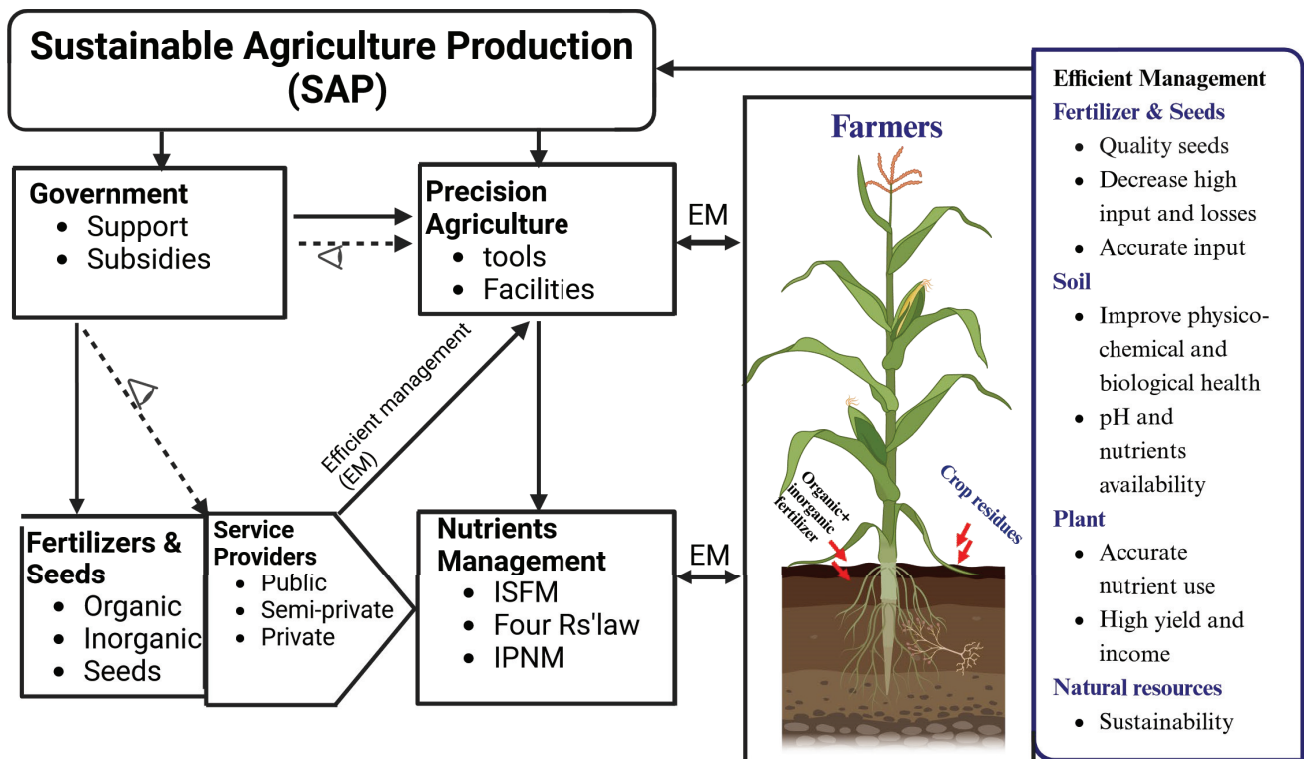
Figure 1.1 Model for sustainable agricultural production adapted for tropical regions, especially in Sub-Saharan Africa. ISFM, integrated soil fertility management; IPNM, integrated plant nutrition management; EM, efficient management. The Four Rs' Law (right time, right source, right rate, and right place) in fertilizer input indicates the practices that must be promoted (black arrow) and supervised (dotted arrow) by the government through private, public, and semi-private sectors to ensure food security. Farmers can also co-operate with these sectors for sustainable crop production.

1.4. Molecular and Physiological Mechanisms of Abiotic Stress

In this section, we explore the molecular and physiological mechanisms developed by plants to alleviate Al toxicity and cope with drought and heat stress. Additionally, we provide examples of major crops cultivated in tropical regions that hold potential for the future molecular breeding of crop varieties.

1.4.1. Molecular and Physiological Mechanisms Underlying Al Stress

It has been observed that the mechanisms regulating Al tolerance are different in various phytospecies under Al stress conditions [147]. In some species, various mechanisms can function simultaneously to generate Al resilience through their combined effects. Although the type of tolerance generation mechanism for Al^{3+}-induced phytotoxicity remains controversial, Al exclusion mechanisms are widely accepted to be involved in Al^{3+} detoxification [148]. However, the molecular and physiological mechanisms underlying Al phytotoxicity have been extensively studied, primarily utilizing model plants, such as *Arabidopsis*, and important crops such as wheat and rice [11]. Based on these studies, two primary categories of plant tolerance mechanisms have been proposed to mitigate the toxic effects of Al: "Exclusion" and "Internal Al tolerance" [6,149]. In the context of Al, exclusion mechanisms are characterized by their capacity to reduce the presence of rhizotoxic Al ions (Al^{3+}) within the symplasm of plant cells, whereas internal tolerance mechanisms effectively mitigate Al toxicity and damage within the cytosol. Furthermore, additional mechanisms have been identified, such as the alteration of rhizosphere pH, Al efflux across the plasma membrane [8], and the removal of Al by the sufficient application of calcium at

the plasma membrane surface, which creates a negatively charged screen between Al and the plasma membrane [150].

Numerous studies have provided evidence supporting the Al exclusion mechanism, that is, the excretion of organic acids (OAs) that effectively chelate Al^{3+} toxic ions in various plants, including staple food crops commonly grown in tropical regions. This phenomenon is mediated by specific transporters for OAs, such as aluminum-activated malate transporter1 (ALMT1), which is encoded by the *ALMT1* gene in wheat [13]. This gene has been characterized in several other plants and crops (Table 3), including *AtALMT1* in *Arabidopsis* [12], *BnALMT1* and *BnALMT2* in rapeseed (*Brassica napus*) [151], and *VrALMT1* in mung beans (*Vigna radiata*) [152]. In addition, similar patterns of Al-activated citrate transporter genes from the multidrug and toxic compounds extrusion (MATE) family, such as HvAACT1 [15] and SbMATE [14][153], have been observed, with their constitutive expression reported for the first time in barley (*Hordeum vulgare*) and sorghum (*Sorghum bicolor*). Moreover, citric acid has been shown to have a strong affinity for Al and enhance phosphorus availability from insoluble Al phosphate in snap beans (*Phaseolus vulgaris* L.) [154]. Recently, several *MATE* family genes associated with citrate secretion have been identified in various crops, including maize (*Zea mays*), rice (*Oryza sativa*), peanut (*Arachis hypogaea*), and soybean (*Glycine max*) (Table 1.3). Studies in *Arabidopsis* have provided strong evidence that the expression of *AtALMT1* and *AtMATE* is regulated by several transcription factors [7]. A notable example is the involvement of the master regulator SENSITIVE TO PROTON RHIZOTOXICITY1 (STOP1), which has been identified as a key regulator of the Al-inducible expression of both *AtALMT1* and *AtMATE* under Al stress [155-157]. In contrast, STOP1 was highly conserved among plants [158]. Recently, it was suggested that SbSTOP1 in Sorghum activates the transcription of the β-1,3glucanase, which reduces callose

deposition under Al toxicity [159]. In addition to its role in the Al stress response, STOP1 has demonstrated pleiotropic regulation under various stresses, such as salt, drought, hypoxia, low pH, and nutrient management [160,161]. For example, in maize, ZmSTOP1 plays a crucial role in Al and drought tolerance by exhibiting hypersensitivity to abscisic acid (ABA) treatment in the roots and insensitivity to stomatal hormones, consequently promoting stomatal closure [162]. Therefore, STOP1 is a useful genetic factor for alleviating Al stress and other growth-limiting factors. Therefore, further studies should analyze the STOP1-mediated environmental stress tolerance in various crops.

Table 1.3 Transporters responsible for Al-responsive organic acid secretion from roots in various plants.

Plant Species	Organic Acid Transporter	Reference
Malate secretion		
Triticum aestivum	TaALMT1	[13]
Arabidopsis thaliana	AtALMT1	[12]
Brassica napus	BnALMT1, 2	[151]
Secale cereale	ScALMT1	[163]
Medicago sativa	MsALMT1	[164]
Holcus lanatus	HlALMT1	[165]
Vigna radiata	VrALMT1	[152]
Citrate secretion		
Sorghum bicolor	SbMATE	[14]
Hordeum vulgare	HvMATE (HvAACT1)	[15]
Arabidopsis thaliana	AtMATE	[166]
Phaseolus vulgaris	MATE-a, -b	[167]
Secale cereale	ScMATE2 (ScFRDL2)	[168]
Zea mays	ZmMATE1, ZmMATE6	[169,170]
Oryza sativa	OsFRDL4, OsFRDL2 (OsMATE2)	[171,172]
Eucalyptus camaldulensis	EcMATE1	[173]
Triticum aestivum	TaMATE1B	[174]
Vigna umbellata	VuMATE1	[174]
Brassica oleracea	BoMATE	[175]
Amaranthus hypochondriacus	AhMATE1	[176]
Fagopyrum esculentum	FeMATE1	[177]
Medicago truncatula	MtMATE66	[178]
Populus trichocarpa	PtrMATE1	[179]

Brachypodium distachyon	BdMATE	[180]
Cajanus cajan	CcMATE1	[149]
Glycine soja	GsMATE	[181]
Glycine max	GmMATE75, 79, 87, GmMATE13	[182,183]
Arachis hypogaea Oxalic secretion	AhMATE (AhFRDL1)	[184]
Hevea brasiliensis Al-responsive transcriptome	HbOT1, 2	[185]
Populus tremula	MATE	[186]
Camellia sinensis	MATEs, ALMTs, CsMATE1,CsALMT1	[179,187]
Citrus sinensis	MATEs, ALMTs	[188]
Stylosanthes	MATE family	[189]
Nicotiana tabacum	NtMATE	[190]
Populus trichocarpa	PoptrALMT10, 54	[191]
Solanum lycopersicum	SlALMT3	[192]
Saccharum officinarum	ALMT2,4,5,7,9,11	[153]
Lens culinaris	ALMT-1, MATE-a,b,c	[193]
Triticum aestivum	TaMATE85,100,114	[194]
Cicer arietinum	CaMATE2,4	[195]
Chenopodium quinoa	CqALMT6	[196]

1.4.2. Drought and Heat Stress

1.4.2.1. Physiological Adaptation

Abiotic stresses, such as high temperatures and water deficits, can adversely affect plant growth and development, resulting in irreversible declines in crop yields [197,198]. According to the Intergovernmental Panel on Climate Change [199], the synergistic effects of drought and heat stress are expected to increase. Consequently, it is crucial to gain a comprehensive understanding of the mechanisms utilized by plants to respond to both stresses. Drought-induced molecular physiological dysfunctions include stomatal closure, oxidative stress, reduced photosynthesis the, disruption of cell walls, and a reduction in root length and plant growth [102,200]. Numerous plant species have developed multiple mechanisms, including the alternative oxidase (AOX) [201], to mitigate or withstand drought stress [202]. This stress triggers the activation of numerous genes and transcription factors, leading to the synthesis of a wide array of proteins and enzymes [203-

205]. Extensive research has been conducted on diverse plant species, including *Arabidopsis* [206], wheat [207], barley [208], and tobacco [97], to investigate their responses to combined drought and heat stress, as well as their individual responses to each stress condition. These studies revealed similar physiological responses, with more severe damage being observed in plants exposed to both stresses than in those subjected to a single stress. These findings highlight the existence of shared defense mechanisms among these plant species in response to drought and heat stress [206]. In this section, our primary emphasis is on the prominent crops cultivated in tropical regions, highlighting the molecular and physiological mechanisms that have evolved to mitigate the combined effects of drought and heat stress.

Plants adopt three primary strategies for coping with drought stress: escape, avoidance, and tolerance [209]. Avoidance involves stomatal closure, reduced photosynthesis, enhanced respiration, and suppressed transpiration to maintain the plant's water status and prevent water loss [97]. For example, morphophysiological mechanisms in maize and sorghum under heat or drought stress are characterized by leaf wax, a lower leaf angle, compact tassels, and a lower cob angle, all of which aim to prevent evapotranspiration [210,211]. An important physiological adaptation in plants is the increase in photosynthetic rates. The maintenance of optimal photosynthetic activity contributes to membrane stability and enhances heat tolerance [212]. Moreover, stomatal conductance is significantly reduced under both stress conditions in *Arabidopsis* and citrus plants [98,198]. Plants exhibit time-dependent responses to both drought and high temperatures. Initially, low levels of reactive oxygen species (ROS), such as H_2O_2 and O_2^-, are observed within the first 24 h, accompanied by an increase in antioxidant enzyme activity. However, at later time points (after 24 h), ROS levels increase substantially, while the antioxidant enzyme activity gradually decreases, potentially indicating the disruption of the antioxidant

pathway [213]. Stress-dependent ROS detoxification mechanisms are also observed with heatstress-inducing cytosolic ascorbate peroxidase (APX) and thioredoxin peroxidase (TPX), whereas drought stress leads to an increase in catalase (CAT) and glutathione peroxidase activities. However, a combination of these stresses uniquely induces glutathione S-transferase (GST), glutathione reductase (GR), copper–zinc superoxide dismutase (CuZnSOD), and glutathione peroxidase (GPX) enzymes [214].

1.4.2.2. Molecular Mechanism

Transcriptomic analyses of several plants under drought and heat stress have revealed many transcripts [206] involved in mitigating these stresses. For example, the transcriptome of sorghum under combined drought and heat stress revealed 5779 transcripts (3003 upregulated and 2776 downregulated). Gene ontology analysis revealed enrichment in categories related to lipid localization, the regulation of photosynthesis, fluid transport, and protein folding. Importantly, these enriched categories overlapped with the responses observed under drought or heat stress [215]. Moreover, a unique set of genes was identified as a specific response of sorghum to combined stress. Similar trends were observed for *Arabidopsis* [97], tobacco [98], and wheat [216]. Furthermore, *OsMYB55* is tolerant to high temperatures and drought stress in maize [217]. An analysis of OsMYB55 transgenic maize revealed the significant upregulation of genes associated with abiotic stresses, such as heat, dehydration, and oxidative stress [217]. This suggests that plants perceive combined stress as a unique transcriptional response during adaptation. Interestingly, drought and heat abiotic stresses induce several transcription factors, such as the ethylene-responsive transcriptional co-activator, dehydration-responsive element-binding proteins (DREBs), and WRKY, to improve plant endurance [97,99], calcium transporter ATPase 9, and proteins involved in disease resistance [218]. Some transcription factors that are well-

known master regulators of stress-responsive genes under abiotic stresses (drought and heat) have been extensively studied because of their vital roles in crop yield improvement [219]. For example, a DREB2 transcription factor from sorghum, the *SbDREB2* gene, showed higher resistance to water deficit than the wild type in transgenic rice [220], and potato *StDREB* also showed the same resistance in transgenic cotton [221]. In *Arabidopsis* and wheat, AtDREB1A and TaDREB1A exhibit high tolerance to abiotic stress [222]. Furthermore, in barley, HD-zip genes (*HDZI-3* and *HDZI-4)* from wheat can be used in combination with DREB/CBF transcription factors to enhance abiotic stress (drought) tolerance and improve crop yield [223]. Increased levels of phytohormone ABA, which plays a key role in regulating several plant responses during abiotic stress, in dry soil helps in the maintenance of root growth, hydraulic conductivity, and water uptake [197]. ABA is also transported via the xylem to the shoot, inducing stomatal closure to reduce the water use efficiency [197]. As the transcriptome can vary depending on the type of plant, time, and severity of stress [214], the functions of proteins encoded by these genes and their associated metabolic pathways need to be further explored. This knowledge is crucial for a comprehensive understanding of the mechanisms involved in mitigating the combined effects of drought and heat stress. This understanding can be useful in arid and semi-arid regions such as Africa, tropical parts of India, and Latin America where crops such as *Sorghum bicolor* hold significant importance as grain crops [215]. These studies indicate substantial advancements in the development of crop varieties well suited for agriculture in arid and semi-arid regions.

1.5. Conclusion

In this review, the multifaceted exploration of factors affecting global crop production revealed the critical challenges posed by environmental stresses, such as soil acidity, Al and Fe toxicity, drought, and heat stress. The far-reaching consequences of these factors on nutrient balance and crop yield, particularly in tropical regions, highlight the urgent need for new strategies to address

these issues. The integration of organic and inorganic fertilizers with region-specific nutrient management practices has emerged as a key solution to enhance soil health and mitigate environmental stress. In SSA, ISFM is used as a strategic approach combining organic and inorganic fertilizers to improve nutrient efficiency. The further exploration of Al tolerance mechanisms revealed the complex strategies used by plants. The identification of key players, such as the ALMT1 and MATE family members and STOP1 transcription factor, highlighted the potential of genetic factors to overcome Al stress and other growth-limiting processes. This review lays the foundation for the further investigation of STOP1-mediated stress tolerance and facilitates the development of crop varieties resilient to different environmental conditions. The interplay between drought and heat stress poses a substantial threat to global agriculture, particularly tropical crops. The adaptive complexity of plant responses, including escape, avoidance, and tolerance strategies, emphasizes the dynamic nature of the plant defense system. The shared defense mechanisms of various plant species present new avenues for targeted research and stress tolerance interventions.

Transcriptomic analyses of various plants under combined drought and heat stress provide valuable insights into the specific gene expressions and pathways involved in stress mitigation, suggesting new targets for crop improvement. Continued research and innovative approaches are crucial to navigate the complex landscape of climate change and its impact on agriculture. This article reveals the ongoing efforts to develop sustainable strategies for food security to overcome the escalating abiotic stresses. The findings presented here may impact the agricultural practices used in various regions and aid in adapting crops to challenging environments and fostering sustainable agricultural practices.

Chapter 2

Alleviating effect of calcium sulfate on aluminum stress through enhanced malate release with increased gene expression of malate transporter and sulfate transporter

2.1. Introduction

Global agricultural production is significantly reduced by acid soil, characterized by a pH below 5, affecting approximately one-third of the world's land and nearly 50% of the arable land designated for crop cultivation [224]. This issue is particularly prominent in tropical and subtropical regions, where numerous developing nations situated within this acidic soil area experience detrimental effects on food production. Inappropriate farming practices and fertilizer use contribute to soil pH reduction, ion concentration intensification, and diminished plant growth. In acidic soil, silicon leaching results in aluminum (Al) forms such as boehmite and gibbsite, releasing phytotoxic Al^{3+}, which inhibits root growth and impacts meristem cell division, reducing plant growth. Soil acidity also influences nutrient solubility, leading to phosphorus (P) and calcium (Ca) deficiencies, coupled with water stress. Various tolerance mechanisms for abiotic stressors, including Al toxicity have been identified over the last few decades [147,225].

Plants have developed bi-directional mechanisms for Al tolerance, encompassing both Al-detoxification (internal tolerance) and exclusion (external tolerance), as adaptive strategies to counter the challenges posed by Al toxicity in acid soils [8,182]. An essential aspect of this adaptation involves the regulation of organic acid release, facilitated by the transporters such as the aluminum-activated malate transporter (ALMT) and multi-drug and toxic compound extrusion (MATE) protein families, respectively [11,14]. The pronounced affinity of citrate for Al has been demonstrated to enhance phosphate availability in various plant species, including snap beans [154], sorghum [14], maize [170], soybean [183], and rice [172]. Furthermore, the elevated expression levels of *AtALMT1* and *TaALMT1* in Arabidopsis and wheat, respectively, provide additional insights into the pivotal role of organic acid release in alleviating Al toxicity [13,12].

This intricate network of adaptive responses elucidates the strategies employed by plants to overcome challenges imposed by Al toxicity in acidic environments.

Additionally, calcium (Ca) reduces Al toxicity [226]. The Ca role in alleviating Al toxicity has been demonstrated through general mechanisms: the electrostatic displacement of Al^{3+} from the plasma membrane surface, restoration of Ca^{2+} on the plasma membrane, and ionic interaction between Al^{3+} and Ca^{2+} at the PM surface, alongside other yet-to-be-elucidated physiological mechanisms [23,227]. Simultaneously, sulfur (S) is integral in modulating plant responses to diverse stresses [35]. The upregulation of sulfate transporter (SULTR) genes, such as *SULTR1;1* and *SULTR1;2*, under S deficient conditions emphasizes the importance of maintaining plant S homeostasis [228]. *SULTR3;5*, which co-localized with *SULTR2;1* shows enhancing sulfate uptake activity [37,229]. S-application enhances citrate levels, improves phosphorus acquisition, and induces citrate transporter genes in soybeans [230]. Furthermore, S-containing compounds like hydrogen sulfide (H_2S) alleviate Al toxicity [231-233]. Recent findings suggest that S enhances Ca and P uptake, water content, and Al-induced citrate secretion, mitigating Al toxicity in *Citrus grandis* [188]. In addition, the less toxicity level of aluminum sulfate ($AlSO_4$) in wheat [33].

In agricultural fields, gypsum ($CaSO_4$) is widely applied in acid soils as fertilizers, and many studies about its ameliorative effects on soil physical-chemical properties have been reported [234-236]. Thus, understanding the mechanism of the sulfur contained in the gypsum application under Al stress could be very important for use with proper management including breeding in the tropical region. In this study, the mechanism by which $CaSO_4$ alleviates Al stress was investigated by focusing on sulfur and the responses of genes responsible for organic acid release, which are Al tolerance mechanisms, and sulfate absorption.

2.2. Materials and methods

2.2.1. Plant materials

Arabidopsis (*Arabidopsis thaliana*) of the Al-tolerant accession Col-0 and Al-sensitive accession, Wei-0 and Ts-5, as described by [157], were used. *SULTR2;1*-knockout (KO) (SM_3_39440) and *SULTR3;5*-KO (SM_3_24048) (Figure. S1) provided by the Arabidopsis Biological Resource Center were used. Each T-DNA insertion in the KO line and its own gene expression level was confirmed with gene-specific primers (Figure 2.1, Table 2.1). Seeds were harvested via controlled self-pollination using the single-seed descent method.
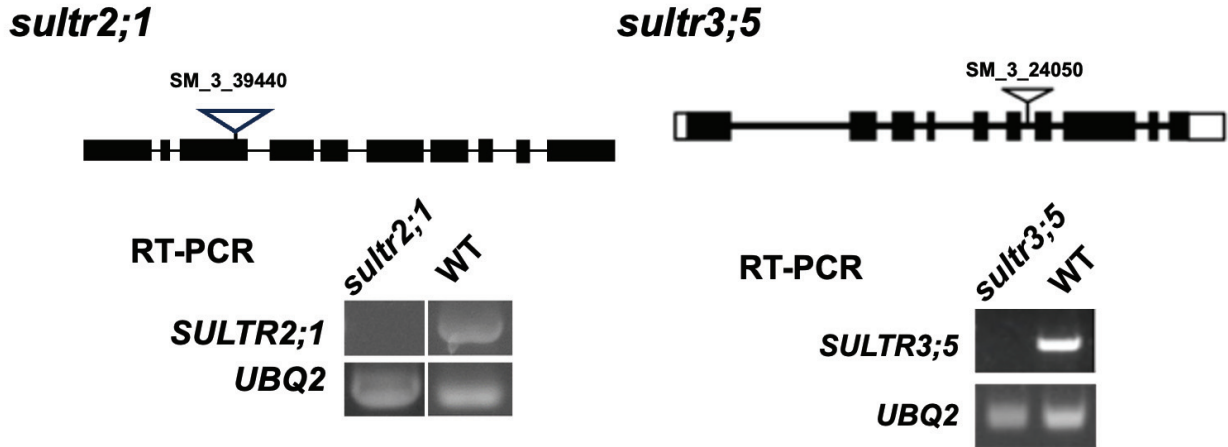
Figure 2.1 Isolation of *SULTR2;1* and *SULTR3;5* disruption line.The genomic region of *SULTR2;1* and *SULTR3;5*, T-DNA insertion sites (white triangle) in *sultr2;1* (SM_3_39440) and *sultr3;5* (SM_3_24050), and the gel image of RT-PCR analysis showing *SULTR2;1, SULTR3;5* and *UBQ2* (ubiquitin2) transcripts

Table 2.1 Primer sequences used for the isolation of *SULTR2;1* and *SULTR3;5* disruption lines

T-DNA insertion line	Purpose	Primer Name	Oligonucleotides (5' to 3')
sultr2;1 (SM_3_39440)	Confirmation of T-DNA Insertion	2;1cds-F	CACCATGGCCAACTCAGGTTCATCTGCA
		2;1cds-R	AACTTTTAATCCAAAGCAAGCATCAAGAGCTTCG
		Spm32	TACGAATAAGAGCGTCCATTTTAGAGAGA
	Confirmation of homozygous insertion	2;1cds-F	CACCATGGCCAACTCAGGTTCATCTGCA
		2;1cds-R	AACTTTTAATCCAAAGCAAGCATCAAGAGCTTCG
	Confirmation of the transcript disruption	SULTR2;1-FA	TCTTCATAGTTAAACTTCCACACAACGTC
		SULTR2;1-RA	ACATGCAATAACCCGTAACACAACTGGTC
		UBQ2-144F	CCAAGATCCAGGACAAAGAAGGA
		UBQ2-372R	TGGAGACGAGCATAACACTTGC
sultr3;5 (SM_3_24050)	Confirmation of T-DNA Insertion	SULTR3;5-KOF	GGCATTGCTACTGTGAGCAATGCAGTCAT
		SULTR3;5-KOR	GCCCATTACTACATTCGACATCGGTGTCTT
		Spm32	TACGAATAAGAGCGTCCATTTTAGAGAGA
	Confirmation of homozygous insertion	SULTR3;5-F	GGAGAATACTATAACGAGCTCTACCTCCTCA
		SULTR3;5-R	TCACACTTCCGGCTTGGTGGTGGTA
	Confirmation of the transcript disruption	SULTR3;5-F	GGAGAATACTATAACGAGCTCTACCTCCTCA
		SULTR3;5-R	TCACACTTCCGGCTTGGTGGTGGTA
		UBQ2-144F	CCAAGATCCAGGACAAAGAAGGA
		UBQ2-372R	TGGAGACGAGCATAACACTTGC

2.2.2. Hydroponic culture and measurement of root length

Arabidopsis seedlings were grown hydroponically in 2% strength-modified MGRL medium without Pi as described by [11]. $MgSO_4 \cdot 7H_2O$ (0.03 mM) was replaced by $MgCl_2$ (0.03 mM). $CaSO_4 \cdot 2H_2O$ (200, 600, 800, 1000 μM) was added to the solution in the presence and absence of $AlCl_3$ (5 μM). The pH of the medium was adjusted to 5.0. The seedlings were grown for 7 days, and the medium was replaced every 2 days to maintain the pH at 5.0 under a 12 h illumination at 22 ± 2 °C. The top five seedlings with the longest roots were measured using Liar software. The relative root length (RRL) was calculated according to the following formula: RRL (%) = Root length ($+AlCl_3$) / Root length ($-AlCl_3$) × 100.

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2.2.3. Malate release and measurement

Twenty-five Arabidopsis seedlings per treatment condition were grown in a liquid medium in plastic pots under aseptic conditions, according to [12]. The growth conditions were the same as those used for hydroponic culture. The basal nutrient solution was composed of MGRL with 1% (w/v) Suc. at pH 5.6. Four-day-old seedlings were treated with a 2% MGRL solution containing 1% (w/v) Suc. set as follows: $\pm$ 5 μM $AlCl_3$; 600 μM $CaSO_4 \pm$ 10 μM $AlCl_3$, 600 μM $CaCl_2 \pm$ 10 μM $AlCl_3$. After treatment, the solution was collected as described by [11]. Malate in the collected sample was quantified using the enzyme reaction of dehydrogenase coupled with NADH/NAD+ cycling methods developed by [237]. Absorbance at 570 nm was measured using absorbance microplate reader Sunrise (TECAN, Japan). All experiments were performed in three biological replicates.

2.2.4. Morin Staining

Morin (100 μM) was used to stain the Al contained in root tips according to the method of [238]. Arabidopsis seedlings were grown in 2% MGRL plus phosphate for five days. After 5 growing days, the roots of the seedlings were carefully washed in distilled water and incubated in a new 2% MGRL nutrient solution containing the following treatments: 5 μM $AlCl_3 \pm$ 600 μM $CaSO_4$ at pH 5.0 for 24 h. The root was stained with 100 μM morin for 15 min, rinsed with distilled water, and fluorescence was observed with All-in-One Fluorescence microscope BZ-X800 (Keyence, Osaka, Japan).

2.2.5. RNA extraction and quantitative RT-qPCR

Approximately 120 seedlings were precultured in 2% MGRL nutrient solution for 10 d. After 10 growing days, the seedlings were washed in distilled water and incubated into a 2% MGRL 10 µM $AlCl_3$ stress medium with or without 600 µM $CaSO_4$, where $MgSO_4$ was replaced by $MgCl_2$. After the 24 h stressing period, the seedlings were gently wiped with tissue, harvested with scissors, and immediately frozen in liquid nitrogen. Total RNA was isolated from the roots using Sepasol-RNA I Super G (Nacalai Tesque, Kyoto, Japan) with a High-Salt Solution for Precipitation (plant) (Takara Bio, Japan) following the methods indicated by the manufacturer's instructions. Reverse transcription was conducted using the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). qPCR was performed using SYBR Premix Ex Taq II (Takara Bio, Japan) and the Thermal Cycler Dice Real Time System II (Takara Bio, Japan) following the manufacturer's instructions using gene-specific primer pairs (Table 2.2). The amplification program consists of 94 °C for 3 min, followed by 20 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a final extension period of 72 °C for 3 min.

Table 2.2 Primer sequence used for RT-qPCR.

Gene name	FW primer seqence	RV primer sequence
SULTR2;1	TCAAGCAAAGTTCGTCGACAGA	CCCGTAACACAACTGGTCCTT
SULTR3;5	ATCTTACCACCACCAAGCCG	TCGGTTACAACTCTTTCATAGCCA
AtALMT1	TCTTCATGTTTTTCATGGTTTGAGTT	CACAGTTTTACATGACGTTGATAATGAT
UBQ1	TCGTAAGTACAATCAGGATAAGATG	CACTGAAACAAGAAAAACAAACCCT

2.2.6. Analysis of Mineral Content

Arabidopsis was grown on agar plates containing 1/2 MS medium [239] with 1% (w/v) Suc.. Plants were cultivated for three weeks to get >10 mg of weight for mineral analysis under a 12-h-light / 12-h-dark photoperiod (45 μmol m^{-2}s^{-1}) at 22°C $\pm$ 2°C. The seedlings were gently washed and transferred to a 2% MGRL solution including 10 μM AlCl$_3$ with 600 μM CaSO$_4$ for 3 to 7 days. After treatment, elemental analysis of harvested shoots and roots was performed as previously described. After 24 hours, fresh shoots and roots were collected, weighed, dried in an oven at 60°C for 3 days, and then mineral content was measured as described by [240].

2.2.7. Simulation of ionic activity

Ions speciation in hydroponics was performed out as described by [11]. Test solutions containing different concentrations of CaCl$_2$ and CaSO$_4$ and 5 μM AlCl$_3$ in 2% MGRL [241] nutrient salts without P at pH 5.0 were used for ions speciation. The following the free ion concentrations in the bulk were calculated using Geochem-EZ [242], these values were inputted into the SGCS [243] to calculate the activity of the ions at the surface of the plasma membrane ({Ions}PM) and in the bulk ({Ions}BULK).

2.3. Results

2.3.1. Promotion of root growth with CaSO$_4$ under Al stress

To evaluate the effects of CaSO$_4$ on Al stress, An Al-tolerant Col-0 and Al-sensitive Ts-5 and Wei-0 were grown under different concentrations of CaSO$_4$ (Figure 1(a-d)). Root length was suppressed with Al exposure compared to the control without Al, and Al sensitivity was greater in sensitive accessions than in tolerant accession (Figure 2.2). Inhibition of root length by Al was gradually ameliorated by the addition of CaSO$_4$.

CaSO$_4$ responsiveness of root growth in sensitive accessions was high, the addition of more than 600 μM of CaCO$_4$ to Ts-5 resulted in more than two-fold as long root length as that of the no-added condition (Figure 2.2 (c)). The root length did not increase above 600 μM CaCO$_4$. In contrast, root length under control conditions without Al increased slightly or did not change with the addition of CaSO$_4$ (Figure 2.2(b-d)). Meanwhile, to examine the effect of sulfate, CaSO$_4$ treatment was compared to CaCl$_2$ and found that both treatments significantly enhanced root growth (Figure S2). However, CaSO$_4$ supply suppressed the inhibition of root growth by Al than CaCl$_2$ supply (Figure 2.2 (e)), suggesting that the sulfate effect of CaSO$_4$, in addition to Ca, promoted root growth.
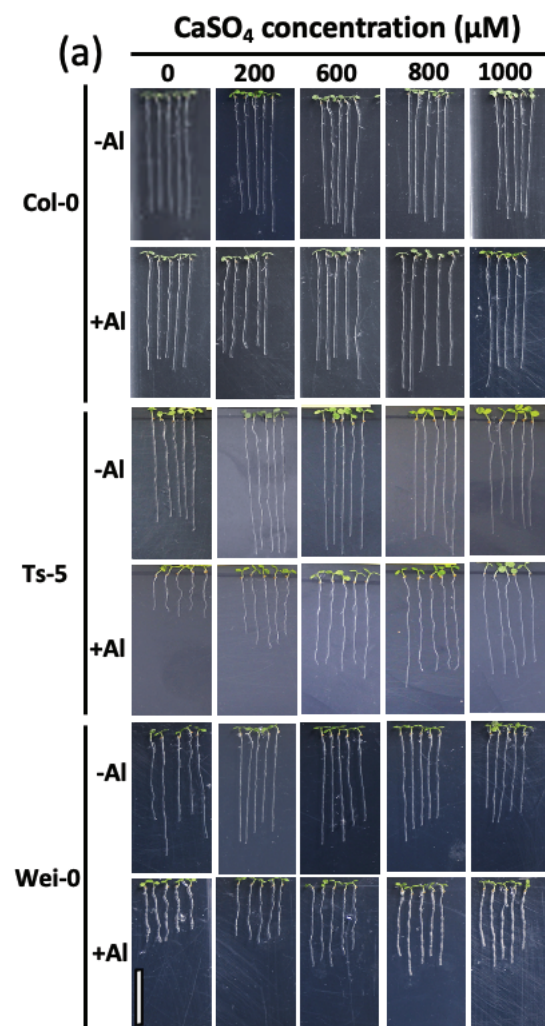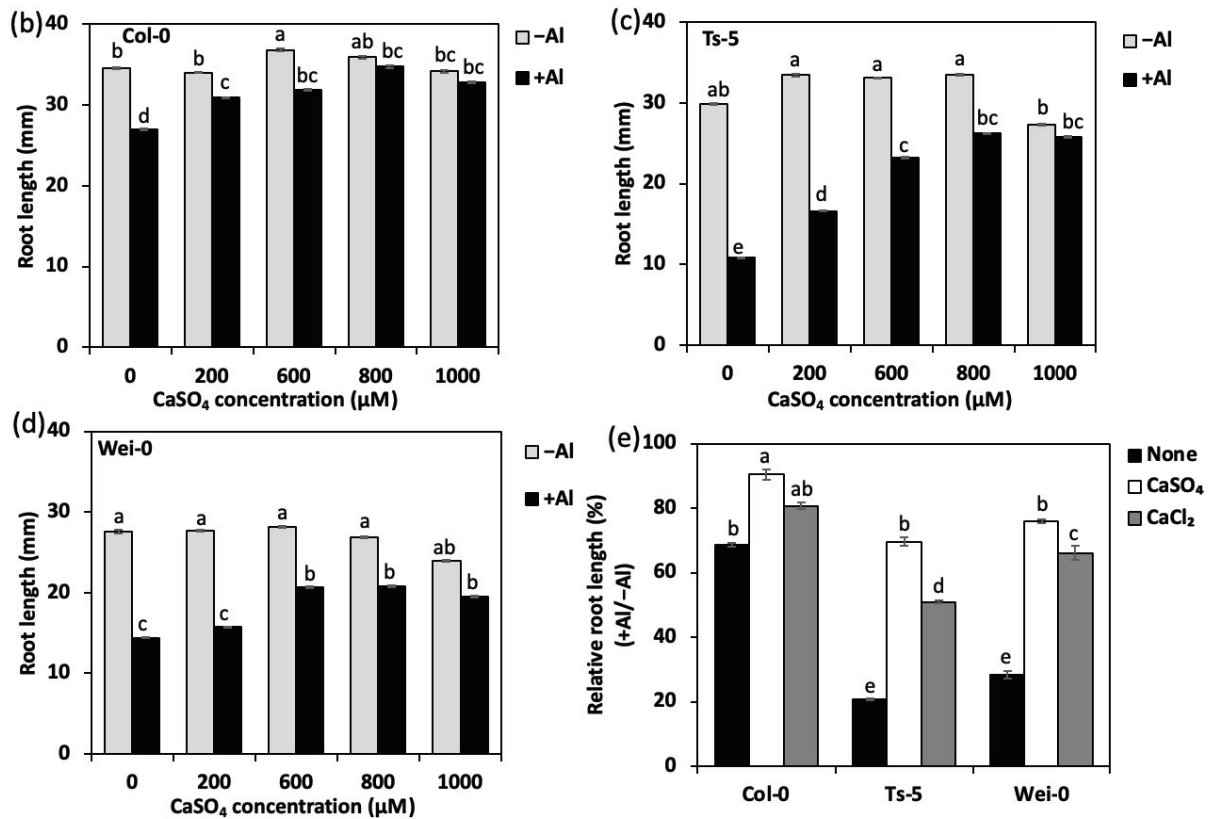
Figure 2.2 Root growth of an Arabidopsis accessions in response to different concentration of $CaSO_4$ treatments with or without Al (a-d). An Al-tolerant accession Col-0 (b), Al-sensitive accessions Ts-5 (c) and Wei-0(d) were grown for 7 days in the presence (open bar) or absence of 5 μM Al (solid bar) at pH 5.0 with the various concentration of $CaSO_4$. (e) Relative root length (RRL (%) = Root length $(+AlCl_3)$ / Root length $(-AlCl_3) \times 100$) in response to different treatments of 600 μM $CaSO_4$ or $CaCl_2$. Mean values ± SE are shown ($n = 5$). Bar = 1 cm (a). Different letter indicates a significant difference ($P < 0.05$, Tukey's test).

2.3.2. Effect of $CaSO_4$ on malate release and *AtALMT1* expression

Aluminum-induced malate release was lower in the sensitive accessions (Ts-5 and Wei-0) than in the tolerant accessions (Col-0) (Figure 2.3 (a)). However, malate release in sensitive accessions was significantly greater increased by approximately 2- to 3-fold by $CaSO_4$ treatment under Al stress (Figure 2.3 (a)). The addition of $CaSO_4$ reduced Al at the root tip that is malate-release region (Figure 2.3(b)). However, no increase in malate release under Al + $CaCl_2$ treatment was observed (Figure 2.3(a)). Therefore, we

measured the expression of *AtALMT1*, which is responsible for malate release in Arabidopsis, following the addition of $CaSO_4$. The expression level was significantly increased by $CaSO_4$ treatment with Al compared to the control without $CaSO_4$ (Figure 2.3 (c)). This suggests that the increase in the expression level of *AtALMT1* with the addition of $CaSO_4$ is one of the causes of increased malate release. Incidentally, under without Al condition, Ts-5 secreted much higher malate than Col-0, while expression of *AtALMT1* was comparable (Figure 2.3 (a) (c)). The malate secretion of Ts-5 without stress may be higher than that of Col, which may be mediated by transporters other than Al-responsive *AtALMT1*.
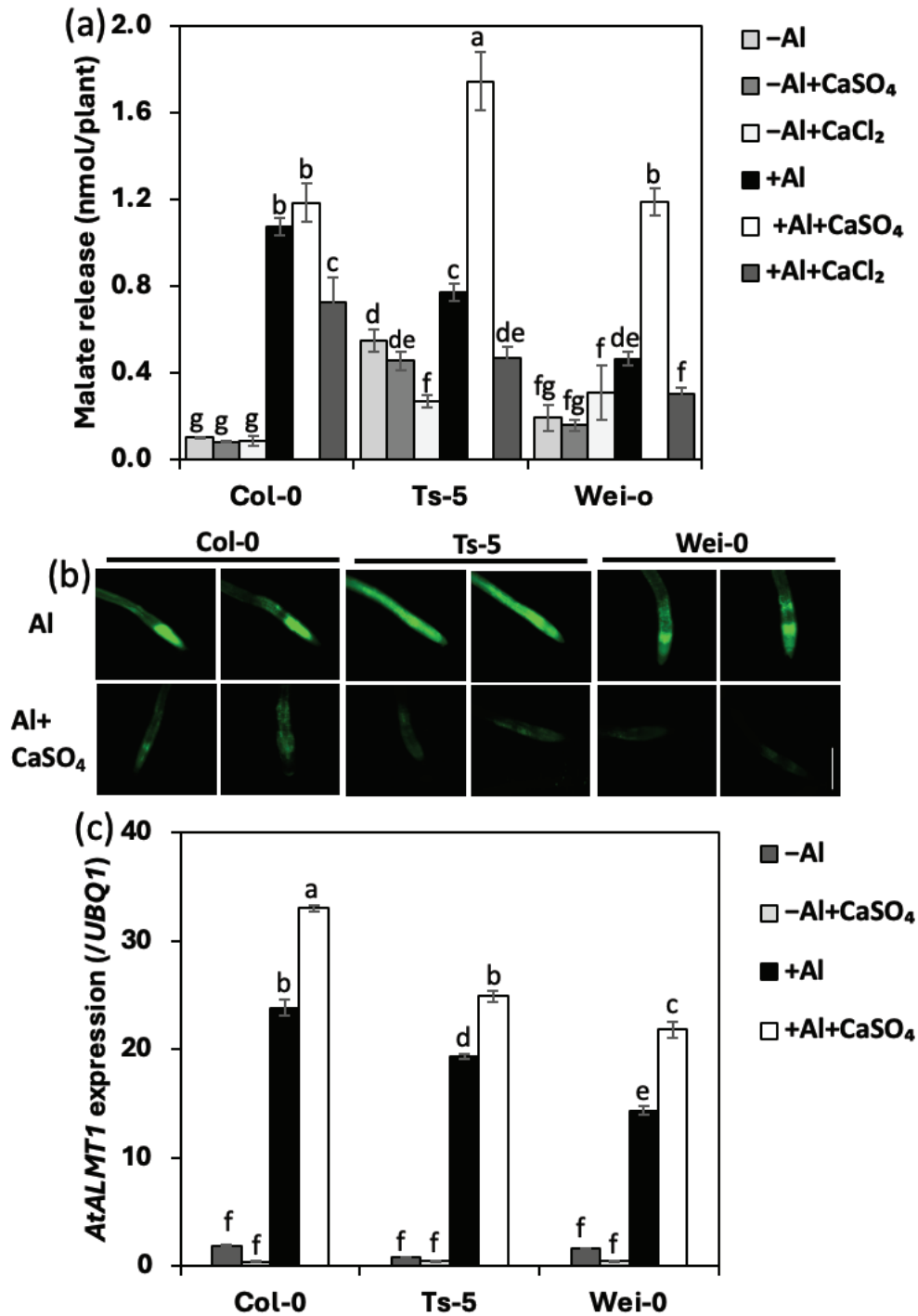
Figure 2.3 Effect of CaSO$_4$ and CaCl$_2$ on malate release from Arabidopsis root (a). Malate release from Arabidopsis (Col-0, Ts-5, Wei-0) was measured. After 5 days of growth in aseptic conditions, the seedlings were soaked in treatment medium under ± 10 μM Al with ± 600 μM CaSO$_4$ and CaCl$_2$ at pH 5.0. for 12 h. (b) Morin staining of Col-0, Ts-5, and Wei-0 treated with 5 μM AlCl$_3$ ± 600 μM CaSO$_4$. Fluorescence indicates Al^{3+} in the root. Scale indicates 20 μm. (c) *AtALMT1* expression level in the root quantified by Real Time-PCR at 24 h in the presence or absence of 10 μM Al and 600 μM CaSO$_4$ treatments. Expression levels were normalized to that of *UBQ1*. The means and SE values are shown (n = 3). Different letter indicates a significant difference ($P < 0.05$, Tukey's test).

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2.3.3. *SULTR* gene expression in the accessions under Al stress with CaSO₄

Aluminum-tolerant transcription factor STOP1 regulates the expression of the *SULTR3;5* (Sawaki et al. 2009). *SULTR3;5* co-localizes with *SULTR2;1* in xylem parenchyma and pericycle cells and promotes root-to-shoot sulfate transport, but where *SULTR2;1* contributes when its expression is induced by sulfur limitation (Takahashi et al. 2000; Kataoka et al. 2004). We then measured expression of *SULTR3;5* and *SULTR2;1* to examine their response to CaSO₄ under Al stress as well as *AtALMT1* expression. The expression levels of the genes were measured in accessions that showed a CaSO₄ response under Al stress (Figures 2.2 and 2.3). The expression level of *SULTR2;1* was higher under conditions without CaSO₄ and that was higher in Al-tolerant accession than in sensitive accessions. However, *SULTR2;1* expression was suppressed by CaSO₄ with or without Al (Figure 2.4 (a)). In contrast to the expression pattern of *SULTR2;1*, *SULTR3;5* expression was significantly upregulated by CaSO₄ with or without Al (figure 2.4 (b)). Furthermore, it is also significantly upregulated by Al in sensitive accessions (figure 2.4 (b)).
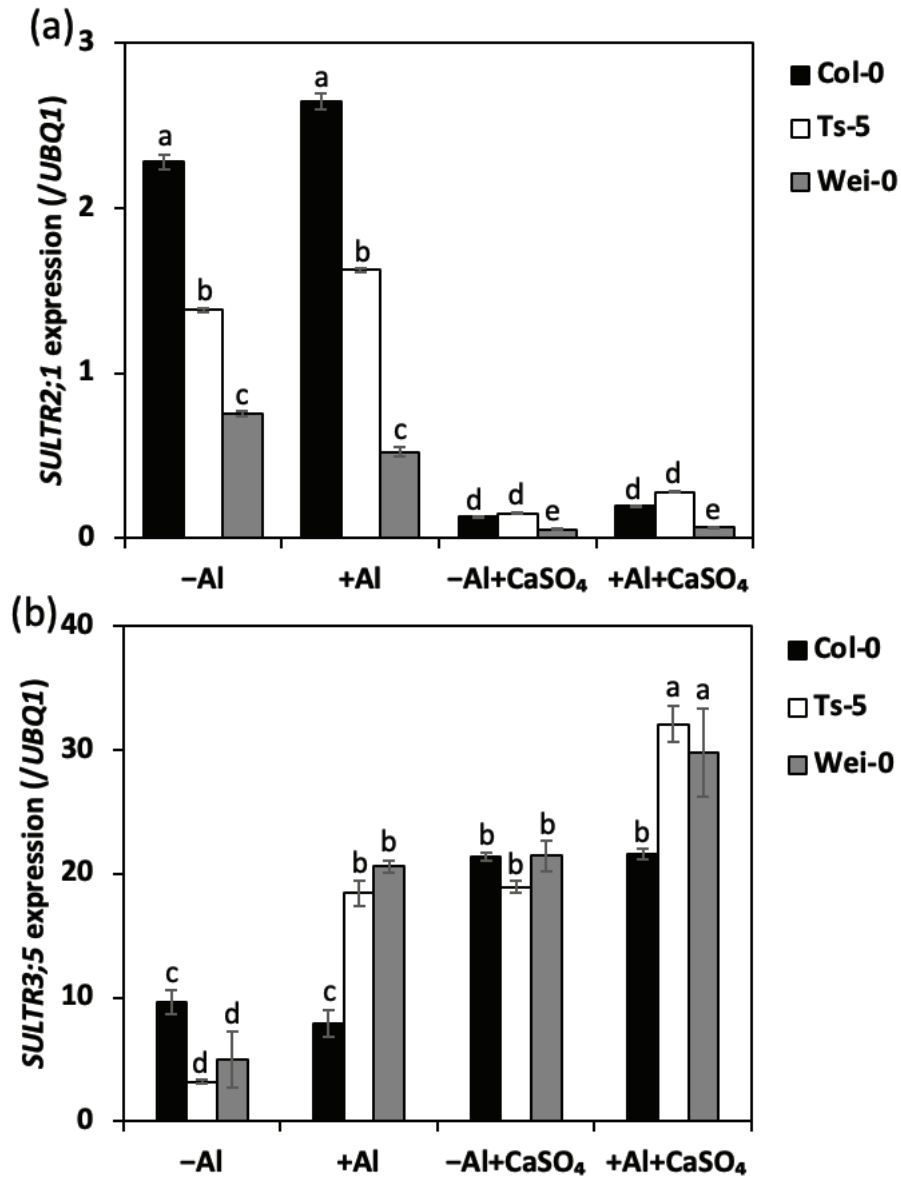
Figure 2.4 Sulfur transporter genes expression level in root of Col-0, Ts-5, and Wei-0. (a) and (b) indicates the expression level of *SULTR2;1* and *SULTR3;5*, respectively, under ± 10 μM Al with ± 600 μM $CaSO_4$ at pH 5.0 for 24 h. The expression levels were quantified by Real Time-PCR and normalized with the expression level of *UBQ1*. The means and SE values are shown (n = 3). Different letter indicates a significant difference ($P < 0.05$, Tukey's test).

2.3.4. Involvement of sulfur transporter genes in Al tolerance

In addition, the effects of Al stress and $CaSO_4$ supply on these sulfur transporter genes using the gene disruption lines, *SULTR2;1*-KO and *SULTR3;5*-KO (Figure 2.1). The RRL (+Al/−Al) without $CaSO_4$ were significantly reduced compared to the wild-type (WT) Col-0, however, the RRL of mutants recovered as well as WT by $CaSO_4$ (Figure 2.5 (a)). These findings suggest that *SULTR2;1* and *SULTR3;5* are involved in Al tolerance with low sulfate. Malate release under the Al stress without $CaSO_4$ of *SULTR2;1*-KO and *SULTR3;5*-KO was lower than that in WT, which was recovered by $CaSO_4$ (Figure 2.5 (b)). Similarly, *AtALMT1* expression in the KO lines was lower than that in WT, which was recovered by $CaSO_4$ under Al stress that *AtALMT1* was induced. (Figure 2.5 (c)). Accordingly, the Al content at the root tip decreased with $CaSO_4$ (Figure 2.5 (d)).
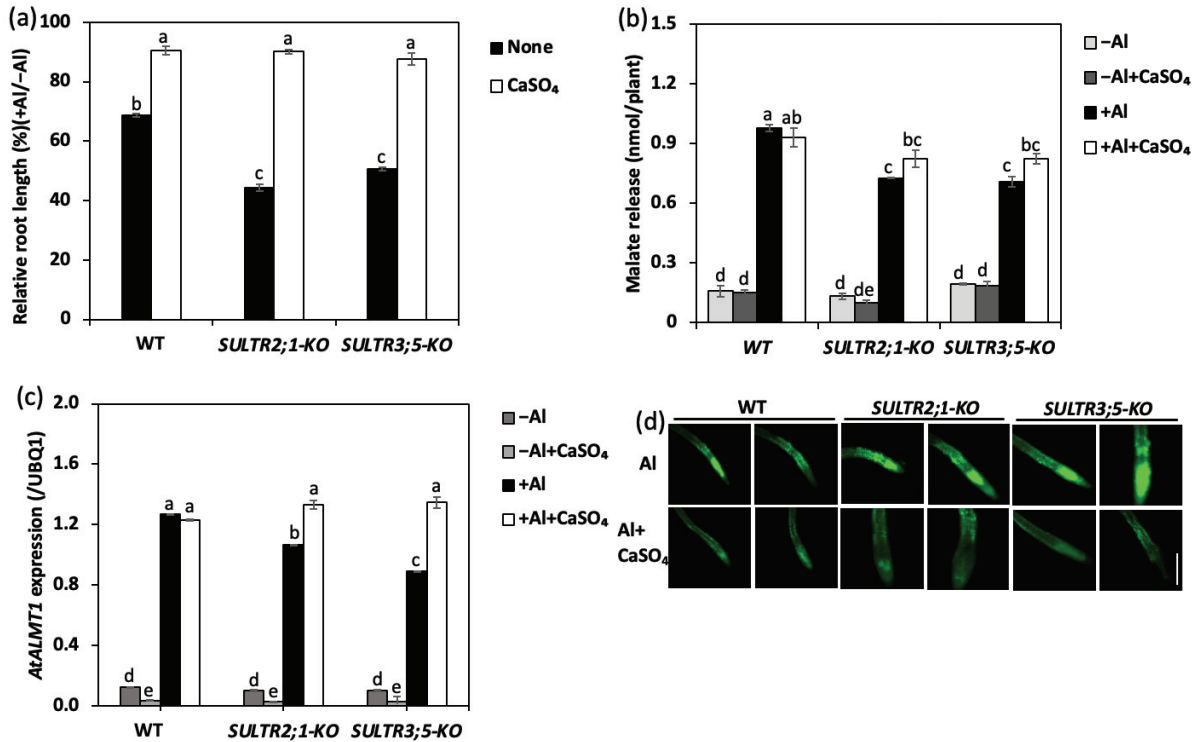
Figure 2.5 Calcium sulfate effect on relative root length (a), malate release (b), *AtALMT1* expression (c) and morin staining (d) of *SULTR2;1*-KO and *SULTR3;5*-KO. (a) Wild-type (Col-0), *SULTR2;1*-KO and *SULTR3;5*-KO were grown for 7 days in the presence or absence of 5 μM Al at pH 5.0 with or without 600 μM $CaSO_4$. (b) Malate release was measured of 5-d-old seedlings soaked in medium ± 10 Al with ± 600 $CaSO_4$ at pH 5.0 for 12 h. (c). *AtALMT1* expression level in the root quantified by Real Time-PCR at 24 h in the presence or absence of 10 Al and 600 $CaSO_4$ treatments. (d) Morin staining of *SULTR2;1*-KO and *SULTR3;5*-KO treated with 5 μM $AlCl_3$ with ± 600 $CaSO_4$ at pH 5.0 for 24 h Fluorescence indicates Al^{3+} in the root. Scale indicates 20 μm.

1.4. Discussion

In this study, we found that the application of $CaSO_4$ and $CaCl_2$ significantly increased Al tolerance and $CaSO_4$-responsive accessions (Ts-5 and Wei-0) (Figure 2.2). Unlike $CaSO_4$, $CaCl_2$ did not increase malate release, which is a mechanism of Al tolerance (Figure 2.3 (a)). These results indicated that the alleviation of Al-stress in Ts-5 and Wei-0 by $CaCl_2$ in hydroponic cultivation is supported by mechanisms (I) and (II) described below. Ca^{2+} was reported to alleviate Al-induced rhizotoxicity in acid soils in soybean [244], Arabidopsis [34], Wheat [245] throughout mechanisms (I) and (II) described by Kinraide et al. (1998, 2004)[23,246] as follow: Mechanism I; the

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displacement of toxicants, such as Al at plasma membrane (PM) surface and mechanism II; Ca^{2+} restoration at PM surface displaced electrostatically by toxicant.

In addition, the alleviation caused by $CaSO_4$ supply could be due to mechanism (III) (a combination of mechanisms I and II), thus triggering several internal mechanisms, such as internal organic acid activation by sulfur metabolism. $CaSO_4$ can mediate the alleviation of Mn^{2+}[35] and Al^{3+} [188] toxicity and promote plant growth and development via a combination of mechanism I and II of mechanism III. For example, some of these sulfate ligands are less toxic to $AlSO_4^+$ than to Al^{3+} [33,247]. When the solution composition was simulated as described by [34], the bulk activity of Al^{3+} decreased owing to the formation of $AlSO_4^+$, resulting in a slight decrease in $\{Al^{3+}\}PM$ and a slight increase in $\{Ca^{2+}\}PM$ (Figure S3).

In addition, unlike $CaCl_2$, $CaSO_4$ was able to provide tolerance to Al stress by promoting the release of malate, accompanied by enhanced expression of *AtALMT1* (Figure 2.3), suggesting that sulfate is involved in increasing malate release and *AtALMT1* expression during Al stress. Similarly, the application of sulfur under P-limitation increased the content and release of malate and citrate, at which time the citrate transporter gene, *GmMATE*, expression was increased in soybean [230]. This mechanism of organic acid release in response to $CaSO_4$ under stress may be common in plants. The correlation between Al tolerance, release of organic acids and expression of their transporter genes is well established [157,248,249]. Thus, enhancing this mechanism with gypsum ($CaSO_4$) supply is a beneficial strategy for improving Al tolerance.

The *SULTR3;5*-KO lines were sensitive to Al stress without $CaSO_4$, but in contrast, accessions with high expression of *SULTR3;5* showed improved Al tolerance by supplying $CaSO_4$ to Al (Figure 2.4 (b) and 2.5 (a)). This suggests that Al tolerance correlates with the expression level of *SULTR3;5*, which is involved in STOP1 regulation [250], however, the contribution of each *SULTR* to Al tolerance requires further investigation. On the other hand, *SULTR3;5* expression was responsive to both Al and $CaSO_4$ (Figure 3(b)). Furthermore, co-expression of *SULTR3;5* and *SULTR2;1* is involved in promoting sulfate uptake and root-to-shoot transport under sulfur deficiency [37,251] and the KO lines of these genes were Al-sensitive and reduced malate release under Al without $CaSO_4$, suggesting involvement of sulfate transport to the shoot in Al tolerance including malate release (Figure 4). Because *SULTR2;1* is low sulfate responsive [252] and Al responsive gene [250]. However, in the present experiments, $MgSO_4$ in the original nutrient solution was replaced with $MgCl_2$ and without $CaSO_4$ to create S-limiting condition, which resulted in Al induction of *SULTR2;1* was not observed because of its high expression without $CaSO_4$, and its expression was suppressed by $CaSO_4$ supply (Figure 2.4 (a)). Furthermore, the *SULTR2;1*-KO was Al-sensitive under such S-limiting condition that enhanced the ability of *SULTR2;1* [252] (Figure 2.5(a)).

In the current study, we conducted an experimental measurement of sulfur content under Al with or without $CaSO_4$ in Ts-5, which showed high malate release and expression of *SULTR3;5* in response to $CaSO_4$ (Figure 2.3 and 2.4). We observed an increase in sulfur content by $CaSO_4$ supply in roots and shoots, although we also noticed a decrease in Al content in the shoot (Figure S4 (b)(e)(d)). In case of root, expression of the *AtALMT1* was enhanced by $CaSO_4$, resulting in lower Al accumulation in root

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tips due to malate release at root tips. (Figure 2.3). However, the Al content of the entire root due to long-term Al exposure was not significantly altered by $CaSO_4$ (Figure S4(a)). These observations suggest that $CaSO_4$ plays a role in alleviating Al toxicity in plants, by increasing the expression of *AtALMT1*, but also by promoting growth through increased S translocation with decreased Al translocation to the shoot.

Sulfate is absorbed from the root, translocated to the shoot by the SULTR transporter, and metabolized into various substances via the sulfur assimilation pathway. For example, cysteine, which is synthesized by the assimilation of sulfate ions, is reduced and degraded to produce H_2S, an environmental stress response signal [253]. In soybeans, H_2S induces citrate transporter gene expression and citrate release via an Al tolerance mechanism [111]. H_2S applied to rice induced expression of the citrate efflux transporter gene *OsFRDL4* as a signaling molecule, which promotes citrate secretion, reduces Al toxicity, increases expression of *OsSTAR1* and *OsSTAR2* genes that reduce cell wall Al content, and decreases expression of *OsNRAT1* that reduce Al uptake, suggesting that the Al signal contains H_2S signals [254]. Since the transcription factor STOP1, which regulates *AtALMT1* expression, is subject to stabilizing regulation by TRX1 that reduces disulfide bridges, the regulation of *AtALMT1* expression may involve a redox-dependent mechanism regulated by H_2S in STOP1 [255,256]. Taken together, the enhancement of H_2S signaling via the sulfur assimilation pathway by the addition of $CaSO_4$ may also be involved in the increased expression of *AtALMT1* and malate release under Al stress. Additionally, the redox state of cysteine-containing thioredoxin regulates photosynthesis [257], and photosynthetic products affect malate metabolism in the TCA cycle via glycolysis, suggesting that sulfur metabolism may affect the increased malate

release observed with $CaSO_4$ supply (Figure 2.3 (a)).Finally, because the responses to $CaSO_4$ observed in this study differ among accessions (Figures 2.2 and 2.3), a comprehensive survey of genetic factors deployed across many accessions, such as a genome-wide association study [157,258] would be important in future studies investigating the mechanisms of the relationship between sulfur metabolism/signal and Al tolerance

2.5. Conclusion

This study indicated the inhibition of root length by Al was gradually alleviated with the addition of $CaSO_4$. $CaSO_4$ treatment was found to promote root growth more effectively than $CaCl_2$ under Al stress, indicating a sulfate-specific effect. Additionally, $CaSO_4$ treatment increased aluminum-induced malate release and the expression of *AtALMT1*, suggesting its involvement in mitigating Al stress effects. Moreover, the expression patterns of sulfur transporter genes *SULTR2;1* and *SULTR3;5* revealed their roles in Al tolerance and sulfate transport, with $CaSO_4$ positively regulating *SULTR3;5* expression. Lastly, studies with *SULTR2;1-KO* and *SULTR3;5-KO* lines confirmed their involvement in Al tolerance and malate release, which were recovered by $CaSO_4$ supplementation. These findings collectively suggest that $CaSO_4$ plays a significant role in alleviating Al stress in Arabidopsis, potentially through the regulation of sulfate transporters and malate release mechanisms which need further investigation.

Acknowledgments

Disclosure statement

Funding

Effectiveness of gypsum application on soil acidity

3.1. Introduction

Acidity occurs in most tropical and subtropical countries and produces complex interactions of plant growth limiting constraints involving physical, chemical, and biological properties of soil [235]. It affects approximately 60% of the soil of the area under the tropics and subtropics [8]. The major physical constraints in these areas are dominated by soil erosion and low water-holding capacity [25]. Because most of the developing countries especially in Sub-Saharan Africa countries are located where acid soil resides, Al toxicity and P, Ca deficiency are the major chemical stresses that hinder the crop yield and adversely increase food insecurity and poverty [8] Furthermore, managing soil acidity is very difficult for farmers because of the complexities of its constraints, the lack of knowledge, and the high cost of chemical fertilizer. Therefore, searching for an alternative solution like soil organic amendment or combining inorganic and organic could be an efficient solution to increase farmers' crop yield and income under the tropics.

Lime generally called the foundation of crop production is widely applied to acid soils to counteract Al toxicity. The application of lime increases soil pH, P, and cation exchange capacity availability due to the Al and Fe precipitation [25] However, liming is not effective for subsoil acidity (75% worldwide) because of its low solubility [259] Therefore, Gypsum is applied to reduce the detrimental effects of subsoil acidity [260]. Two mechanisms of this alleviation were earlier proposed. Ligand exchangeable from SO_4^{2-} for OH^- and from Fe or Al oxide mineral and the formation of a less toxic $AlSO_4^+$ ion pair [261]. In the Northeast of Brazil, the use of Gypsum in irrigation water improved soil physical and chemical properties and mitigated sodic soil stress [29]. The effect of lime and gypsum on acid Andosol properties like pH, Al saturation, plant-availability of Sulfur (S) and phosphorus (P) showed a slight rise of soil pH, a decrease of Al, an increase of plant available S, P, Calcium (Ca) in the soil [27]. It was found that the positive effect

of lime and gypsum application on soil physical and chemical properties and cassava growth could be attributed to the Ca^{2+} [122]. The objective of this study is to assess the effect of gypsum on acid soil

3.2. Material and methods

3.2.1. Soil culture

The soil (acid andosol) containing a large amount of organic matter and Al was collected in minokamo and kept at a room temperature of 4°C. The properties of these soils are well characterized and are frequently used for physiological experiments of Al and proton toxicities [34]. The soil pH and exchangeable Al were quantified according to the method of Toda et al [262]. The soil was fertilized with both macro- and micronutrients as described by Kobayashi et al. [249] 250 mg NaH2PO4, 48 mg KCl, 36 mg MgSO4, 132 mg (NH4)2SO4 per 100 g of soil which was used as the basal test soil. Different treatments of $CaCO_3$ (0,20,40,60,120,170,250mg/100g soil) and $CaSO_4$ (0,20,40,60,120,170,250 mg/100g soil) were added to find the optimum concentration. Soybean (*G. max*) and Wheat (*T. aestivum*) were grown in individual plastic pots filled with 500 g of soil (dry weight). After sowing, the plants were grown in individual plastic pots filled with 500 g of soil for three weeks at the same temperature and light/dark regime as in hydroponics. Throughout the whole experiment, the plants were irrigated everyday with de-ionized water to maintain soil moisture.

3.2.2. Growth biomass and mineral content

Three sets of plants were grown in both acidic and then the shoots and roots were excised and rinsed with de-ionized water. Growth was measured in terms of shoot, root fresh weight, and shoot root dry weight. After a three-week growth period, the plants shoots and roots were separated from the soil. The wheat and Soybean's fresh root and shoot were immediately collected and weighed. Then they were kept in the oven at 60°C for three days and removed for dry weight. The

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samples were collected in three biological replicates and then mineral content was measured as described by Watanabe et al. [240].

3.2.3. Statistical analysis

The experimental design was completely randomized; all data are expressed as the mean of three biological replicates (3 plants per replicate) and three technical replicates for each biological replicate, ± standard error. The differences between the parameters were evaluated using the Student's t-test and turkey with Microsoft Excel 2010, and P values ≤0.05 were considered as statistically significant.

3.3. Results

3.3.1. Lime and Gypsum promote plant growth in acid soil

To evaluate the effectiveness of gypsum ($CaSO_4$) and calcium carbonate ($CaCO_3$) on Al stress, wheat, and Soybean were grown under the treatments of $CaSO_4$ and $CaCO_3$. The growth was gradually increased when $CaSO_4$ and $CaCO_3$ were added to the soil compared to the control (without $CaSO_4$ and $CaCO_3$) (Figure 3.1a). Soybean and wheat responsiveness of the treatment by Lime and gypsum at 170mg/100g soil was significantly high compared to the control condition (Figure 3.1 (b,c)). Meanwhile, to examine the effect of sulfate, $CaSO_4$ treatment was compared to $CaCO_3$. Interestingly, the $CaSO_4$ addition showed a high responsiveness in wheat vis-à-vis $CaCO_3$ treatment (Figure 3.1(b)). This response was 2 and 5 times respectively for fresh and dry weight with gypsum amendment if we compared to the control and 1 and 3 times if we compared to $CaCO_3$ treatment. However, that of $CaCO_3$ treatment was 1 and 3 times for fresh and dry weight respectively. By contrast, gypsum effects on soybean did not show a high difference compared to $CaCO_3$ for both (fresh and dry weight) (Figure 3.1 (c)). These results suggest that the sulfate contained in gypsum could play a supplementary key

role in the alleviation of Al toxicity thus reinforcing the Ca-promoted growth. Therefore, we decided to pursue the experiment by analyzing the mineral content of wheat and the Al-sensitive Ts-5.

	g/100g soil			
CaCO$_3$	0.0	0.17	0	0.24
CaSO$_4$	0.0	0.0	0.17	0.24
pH(H$_2$O)	4.8	5.5	4.7	5.7
Al exch. (meq. Kg^{-1}) soil	100 ±0.5	24 ± 0.5	88 ± 0.9	4 ± 0.2
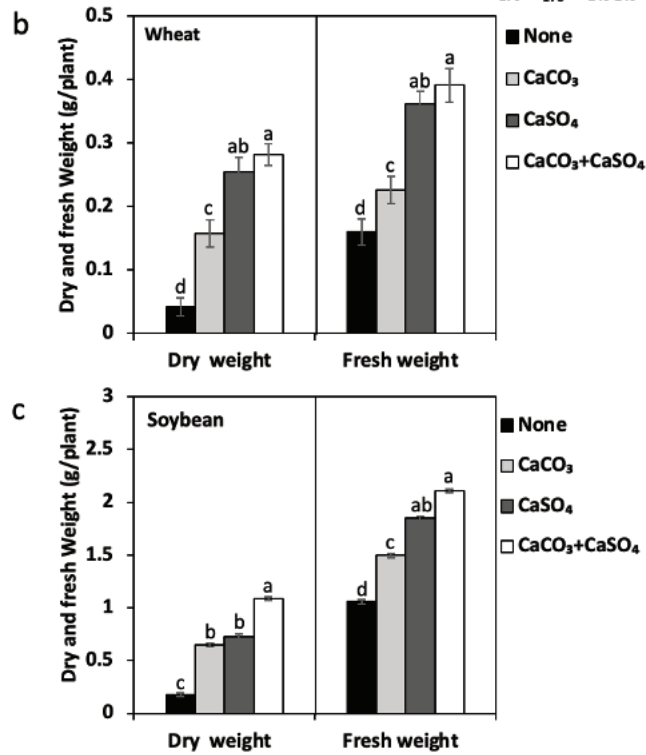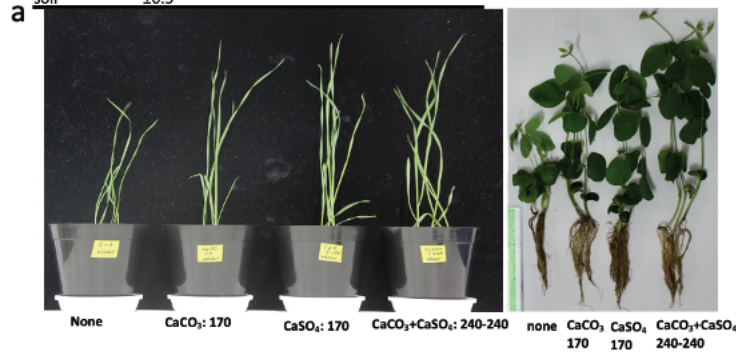
Figure 3.1 Wheat and Soybean growth in response to the treatment of CaSO$_4$ and CaCO$_3$ on acid soil(a-c). Plants were grown for 4 weeks and fresh and dry weight were plotted for Wheat (b) and for Soybean (c) respectively in response to the treatment of 170mg/100g soil. Mean values ± SE are shown (n = 5 for wheat and n=3 for soybean). Bar :1 cm= 5cm (a). Different letter indicates a significant difference (P < 0.05, Tukey's test).

3.3.2. Mineral content

The dry weight of wheat (root and shoot) and arabidopsis Al-sensitive, Ts-5 (shoot) were analyzed to determine the mineral contents, especially Al, Ca, and S content. In wheat, both root and shoot Al and Fe content significantly decreased with $CaCO_3$ and $CaSO_4$ addition. More importantly, Al content in the root of wheat drastically reduced by 57.49% and 81% respectively for $CaCO_3$ and $CaSO_4$ at 0.17 g.Kg^{-1} compared to the control (none $CaCO_3$ and $CaSO_4$) (Figure 3.2 (a),(b)). In Ts-5 shoot it decreases at about 60% as well for both treatments (Figure 3.2 (c)). In wheat root, Al significantly decreased in the presence of gypsum amendment than liming. This result may be one of the reasons for the increase in wheat fresh and dry weight (Figure 3.1 (b)). This result brings a second proof of the high responsiveness of wheat to gypsum and the effect of the sulfur contained in gypsum-induced Al tolerance. Ca and S content significantly decreased in the presence of $CaSO_4$ or combined $CaSO_4$ and $CaCO_3$ in wheat root and shoot (Figure 3.2 (d),(g)). However in the shoot, Ca content increased only with $CaCO_3$ addition whereas S and Ca content decreased with $CaSO_4$ application (Figure 3.2 (e),(h)). One reason for these results could be the competing effects of nutrient uptake in the presence of gypsum. The second reason could be due to the different mechanisms involved in Al tolerance. For example, once Ca^{2+} and SO_4^{2-} get into plant cell, the two compounds can be complexed or Al can complex SO_4^{2-} to form an internal less toxic compound like $Al(SO_4)^+$. In the Ts-5 shoot, Ca and S significantly increased in the presence of $CaCO_3$ and $CaSO_4$ Figure 2 (f),(i)). Furthermore, S content was higher under gypsum treatment than liming (Figure 3.2 (i)). Both Ca^{2+} and SO_4^{2-} have ameliorative effects on arabidopsis Ts-5 growth but gypsum is more efficient than lime. These results support the data obtained in the hydroponic culture thus indicating that gypsum alleviation Al toxicity by decreasing the concentration of Al at the plasma membrane surface of the root and in plant tissue.
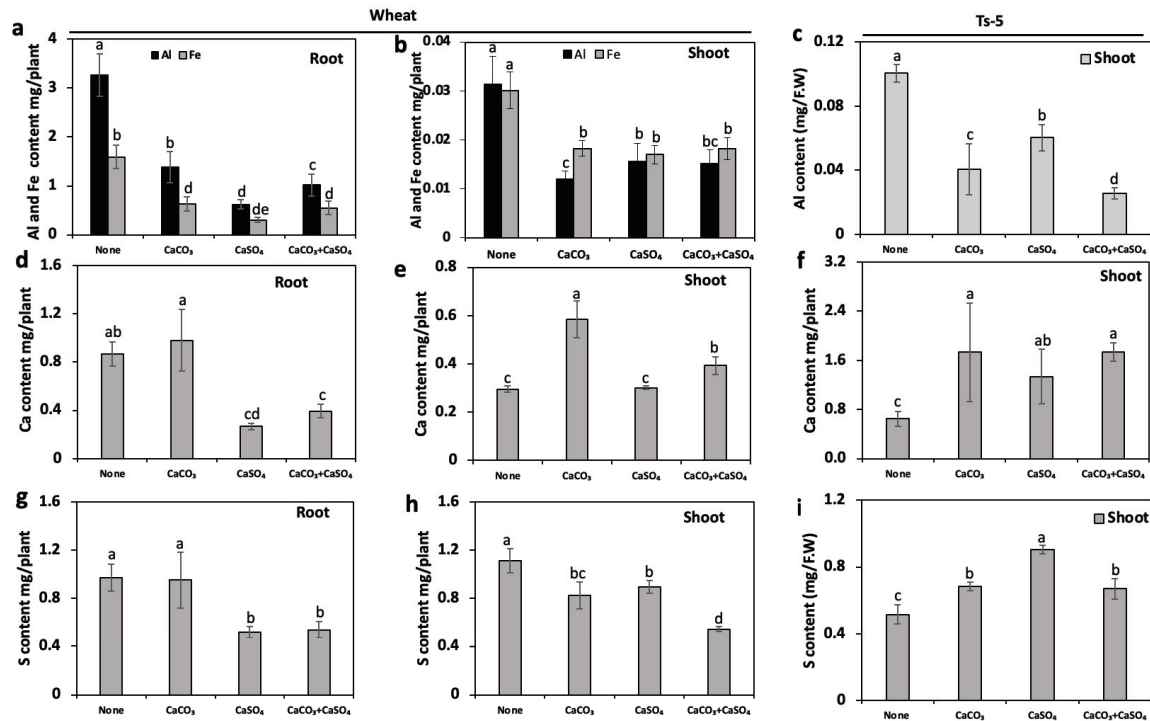
Figure 3.2 Mineral content of wheat (root and shoot) and Al-sensitive Ts-5 (shoot) in the presence of $CaCO_3$ (0.17g.Kg^{-1}), $CaSO_4$ (0.17g.Kg^{-1}), and $CaCO_3$ + $CaSO_4$ + (0.24g.Kg^{-1}+0.24g.Kg^{-1}). Figures a, b, represent Al and Fe content for wheat root and shoot respectively, c represent Al treatment for Ts-5 shoot, d, e, f, are Ca content g, h, and I represent S content. Mean values ± SE are shown ($n = 5$ for wheat and n=3 for Ts-5). The different letter indicates a significant difference ($P < 0.05$, Tukey's test).

3.4. Discussion

In this study, we found that $CaCO_3$ and $CaSO_4$ treatments at 170mg/100g soil significantly promoted wheat, soybean, and Ts-5 growth compared to the control (without any treatment). Wheat fresh increased by 41.25% and 126.25% for $CaCO_3$ and $CaSO_4$ respectively whereas that of soybean was at about 28% and 29% (Figure 3.1). Lime and gypsum application in acid soils (Oxisols) increased *Eucalyptus* stem wood volume by up to 60% [263,264]. Similar patterns were found by Carducci et al. [265]. In addition, the increase in plant fresh weight is due to the decrease in the exchangeable Al [259]. Lime and gypsum reduce Exchangeable Al [266]. Furthermore, Gypsum responsiveness in wheat was 3 times higher than in lime suggesting the involvement of crop specificity response in nutrient uptake [27]. These findings explained how corn and soybean crops respond to gypsum may be linked to differences in their capacity for

calcium ion uptake, influenced by Cation Exchange Capacity Ratio (CECR), which appears to be lower in corn compared to soybean.

In this study, Al contents drastically decreased in wheat (root and shoot) and Ts-5 (shoot) with gypsum and lime amendment compared to the control (Figure 3.2 (a), (c)). In Brazilian Oxisol, It was found that the decrease in Al and Fe contents by liming is due to the increase of soil pH [25]. An experiment conducted by Fageria et al. [235] in acid soil on soybeans indicated that gypsum application decreases Al content. Ca and S contents significantly decreased in wheat root in the presence of $CaSO_4$ and $CaCO_3+CaSO_4$ compared to those in $CaCO_3$ and the control condition where they increased their contents in the tissue (Figure 3.2 (d), (g)). Wheat root fresh and dry weight increases with $CaSO_4$ and $CaCO_3+CaSO_4$ addition (Figure 3.1 (b)). However, Ts-5 shoot Ca and S content significantly increases in the presence of $CaSO_4$ compared to the control and the result found in the hydroponic culture previously. If the nutrient concentration surpasses the critical value necessary for crop growth, enhancing it further can increase plant growth, especially when gypsum is added to boost the plant's uptake of the limiting nutrient [123]. For years, gypsum application failed to elevate the S concentration in crop species' leaf tissues due to remobilization compared to the control group, [267]. The contrast change in nutrient content may be due to the plant life stage or the remobilization of macronutrients (Ca, S). The crop species cultivated in identical soil demonstrated that both *T. aestivum* and *H. vulgare* efficiently remobilized all macronutrients at a rate nearly approaching -80% [267]. Sulfur application can modify soil microbial communities, impacting the sulfur metabolic pathway and thereby affecting sulfur availability for plant uptake [268].

3.5. Conclusion

In conclusion, the study evaluated the effectiveness of gypsum (CaSO4) and calcium carbonate ($CaCO_3$) in alleviating aluminum (Al) stress in wheat and soybean. The results demonstrated that both $CaSO_4$ and $CaCO_3$ amendments led to increased growth compared to the control, with $CaSO_4$ showing higher responsiveness in wheat. Additionally, mineral analysis revealed significant decreases in Al content in the presence of gypsum, particularly in wheat roots, further supporting its efficacy in mitigating Al toxicity. These findings underscore the potential of gypsum as a promising strategy for improving plant growth in Al-contaminated soils. Further investigation about plant internal metabolism needs to be done to clarify the mechanism of Al tolerance in crop and Arabidopsis induced by the S-contained in gypsum.

General Discussion

This research conducted in Chapter 2 revealed significant improvements in aluminum (Al) tolerance and responses in $CaSO_4$-treated accessions (Ts-5 and Wei-0) (refer to Figure 2.2). Unlike $CaSO_4$, the application of $CaCl_2$ did not lead to an increase in malate release, which is a known mechanism for enhancing Al tolerance (see Figure 2.3 (a)). In the acid soil experiment (Chapter. 3), Wheat fresh increased by 41.25% and 126.25% for $CaCO_3$ and $CaSO_4$ respectively whereas that of soybean was at about 28% and 29% (Figure 3.1(b), (c)). These findings suggest that Ca^{2+}-contained in Calcium carbonate, lime, and gypsum can alleviate Al stress in Ts-5 and Wei-0 during hydroponic cultivation and in wheat and soybean in soil culture. Studies have indicated that Ca^{2+} can mitigate aluminum-induced rhizotoxicity in soybean [244], Arabidopsis [34], and wheat [245] grown in acid soils. This effect is attributed to two mechanisms as outlined by Kinraide et al. (1998, 2004) [23,246] : Mechanism I involves the displacement of toxicants, such as aluminum, from the plasma membrane (PM) surface, while Mechanism II entails the restoration of Ca^{2+} at the PM surface, which has been displaced electrostatically by the toxicant. Furthermore, the increase in plant wheat and soybean fresh weight is due to the increase in soil pH by lime [26] resulting in a decrease in the exchangeable Al [259]. Gypsum reduces exchangeable Al [266] by complexing with SO_4^{2-} to form a less toxic compound aluminum sulfate ($AlSO_4^+$) [261]. Additionally, the relief brought about by the supply of $CaSO_4$ in soil and hydroponic culture may stem from mechanism (III), which is a fusion of mechanisms I and II. This triggers various internal processes, such as the activation of internal organic acids through sulfur metabolism. $CaSO_4$ facilitates the mitigation of Mn^{2+} [35] and Al^{3+} [188] toxicity, while also enhancing plant growth and development through the combined action of mechanism I and II within mechanism III. For instance, certain sulfate ligands exhibit lower toxicity towards $AlSO_4^+$ compared to Al^{3+}. Through simulations described by Kinraide [33], the composition of the solution led to a decrease in the bulk activity of Al^{3+}

due to the formation of $AlSO_4^+$, resulting in a slight decrease in $\{Al^{3+}\}PM$ and a slight increase in $\{Ca^{2+}\}PM$ (refer to Figure S3).

Furthermore, in contrast to $CaCl_2$, $CaSO_4$ demonstrated the capacity to confer tolerance to aluminum stress by facilitating the liberation of malate, concomitant with the heightened expression of *AtALMT1* (refer to Figure 2.3, Chapter 2). This implies the involvement of sulfate in augmenting malate release and *AtALMT1* expression under aluminum stress conditions. Similarly, sulfur supplementation under phosphorus limitation led to elevated levels and discharge of malate and citrate. During this phase, the expression of the citrate transporter gene, *GmMATE*, was upregulated in soybean [230]. The well-established correlation among aluminum tolerance, the release of organic acids, and the expression of their transporter genes has been documented in several studies [14,249] . Therefore, supplementing with gypsum ($CaSO_4$) to enhance this mechanism represents a beneficial strategy for enhancing aluminum tolerance. This may be one of the reasons for the increasing fresh weight of wheat under $CaSO_4$ at about 126 % compared to 41,25% for $CaCO_3$ treatment (Figure 2.2 (b), Chapter 2). Investigated on sulfur transporters have shown that the *SULTR3;5-KO* lines exhibited sensitivity to aluminum stress in the absence of $CaSO_4$. However, accessions displaying elevated *SULTR3;5* expression demonstrated enhanced aluminum tolerance when provided with $CaSO_4$, as depicted in Figures 2.4 (b) and 2.5 (a) (refer chapter 2). This indicates a correlation between aluminum tolerance and the expression level of *SULTR3;5*, a gene implicated in the regulation by STOP1 [250]. Nevertheless, deeper examination is needed to ascertain the specific contribution of individual SULTR genes to aluminum tolerance. On the contrary, the expression of *SULTR3;5* was found to be influenced by both aluminum (Al) and calcium sulfate ($CaSO_4$) (see Figure 2.4(b)). Moreover, the concurrent expression of *SULTR3;5* and *SULTR2;1* plays a crucial role in facilitating sulfate uptake and its transportation

from roots to shoots when subjected to sulfur deficiency [37,251,37]. Knockout (KO) lines of these genes exhibited sensitivity to aluminum (Al) and a decrease in malate release under Al stress in the absence of $CaSO_4$, indicating the involvement of sulfate transport to the shoot in conferring Al tolerance, including malate release (refer to Figure 2.5). Notably, while *SULTR2;1* is known to be responsive to low sulfate conditions [252] and exhibits an Al-responsive nature [250], in the conducted experiments, magnesium sulfate ($MgSO_4$) in the original nutrient solution was substituted with $MgCl_2$, and $CaSO_4$ was excluded to induce sulfur limitation. Consequently, the induction of *SULTR2;1* by Al was not observed due to its already elevated expression in the absence of $CaSO_4$, with its expression being repressed upon $CaSO_4$ supplementation (see Figure 2.4 (a)). Additionally, under such sulfur-limiting conditions, the *SULTR2;1* knockout was found to be sensitive to aluminum (Al), which underscores the significance of *SULTR2;1* in such conditions [252] (refer to Figure 2.5(a)).

In the current study, we conducted an experimental measurement of sulfur content under Al with or without $CaSO_4$ in Ts-5 in hydroponic. Overall, sulfur content by $CaSO_4$ supply in Ts-5 shoot increases, although we also noticed a decrease in Al content in the shoot and in wheat (root and shoot). In soil experimental conditions, under Al with or without $CaSO_4$ or $CaCO_3$ in Ts-5, wheat, and soybean, similar results were found. These findings indicate that calcium sulfate ($CaSO_4$) contributes to mitigating aluminum (Al) toxicity in plants. A study conducted on Brazilian Oxisol revealed that the reduction in aluminum (Al) and iron (Fe) levels resulting from liming is attributed to the rise in soil pH [25]. Furthermore, research by Fageria et al. [235] demonstrated that gypsum application decreases Al content in acidic soil when applied to soybeans. The calcium (Ca) and sulfur (S) contents in wheat roots notably declined when exposed to $CaSO_4$ or

$CaCO_3$. Gypsum application over several years did not increase sulfur concentration in crop species' leaf tissues compared to the control group, likely due to remobilization [267]. On the other hand, the addition of sulfur can alter soil microbial communities, influencing the sulfur metabolic pathway and consequently impacting the availability of sulfur for plants to uptake [268]. The elucidation of the decreasing of Ca and S in the presence of gypsum or lime requires further investigation. Overall, $CaSO_4$ plays a role in alleviating Al toxicity in plants. This is achieved through two mechanisms: firstly, by enhancing the expression of *AtALMT1*, and secondly, by facilitating growth via enhanced sulfur (S) translocation while reducing the translocation of aluminum to the shoot.

Sulfate is absorbed from the root, translocated to the shoot by the SULTR transporter, and metabolized into various substances via the sulfur assimilation pathway. For instance, cysteine, generated through the assimilation of sulfate ions, undergoes reduction and degradation, yielding H_2S, a signal for environmental stress response [253]. H_2S applied to rice induced expression of the citrate efflux transporter gene *OsFRDL4* as a signaling molecule, which promotes citrate secretion, reduces Al toxicity [269], increases expression of *OsSTAR1* and *OsSTAR2* genes that reduce cell wall Al content, and decreases expression of *OsNRAT1* that reduce Al uptake, suggesting that the Al signal contains H_2S signals [254]. Given that the transcription factor STOP1 governs the expression of *AtALMT1* and is under the stabilizing influence of TRX1, which mitigates disulfide bridges, it suggests a redox-dependent mechanism in the regulation of *AtALMT1* expression mediated by H_2S in STOP1 [255,256]. Moreover, the augmentation of H_2S signaling through the sulfur assimilation pathway facilitated by

CaSO$_4$ supplementation might contribute to heightened *AtALMT1* expression and subsequent malate release in response to aluminum stress.

Moreover, the oxidation-reduction status of cysteine-containing thioredoxin plays a regulatory role in photosynthesis [257]. Furthermore, the products of photosynthesis impact malate metabolism within the tricarboxylic acid (TCA) cycle through glycolysis, indicating that sulfur metabolism might contribute to the observe Ultimately, given the varied responses to CaSO4 observed in this study across different accessions, it is imperative for future research investigating the connection between sulfur metabolism/signal and aluminum tolerance to conduct an extensive examination of genetic factors. A thorough survey encompassing numerous accessions, such as a genome-wide association study [157,258], would be crucial for elucidating the underlying mechanisms of elevation in malate release with the provision of CaSO$_4$.

General conclusion

In conclusion, the intricate dynamics influencing global crop production, particularly environmental stresses like soil acidity, Al, drought, and heat stress, have been thoroughly examined. The profound impact of soil acidity dominated by Al toxicity, nutrient balance and crop yield losses, especially in tropical regions, underscores the urgent necessity for innovative strategies to tackle these challenges. Integration of organic and inorganic fertilizers, tailored to specific regions such as through ISFM in SSA, emerges as a pivotal solution to enhance soil health and alleviate environmental stress. In this way, our study explored the efficacy of gypsum ($CaSO_4$) in mitigating aluminum (Al) stress in wheat and soybean plants. It was found that $CaSO_4$ supplementation gradually alleviated Al-induced inhibition of root length, with a sulfate-specific effect that promoted root growth more effectively than $CaCl_2$ under Al stress. Moreover, $CaSO_4$ treatment increased aluminum-induced malate release and the expression of *AtALMT1*, known to be one the most important mechanisms of Al tolerance evolved by plants to mitigate Al stress effects. Additionally, the study revealed the involvement of sulfur transporter genes *SULTR2;1* and *SULTR3;5* in Al tolerance and sulfate transport, with $CaSO_4$ positively regulating *SULTR3;5* expression. Furthermore, experiments with *SULTR2;1-KO* and *SULTR3;5-KO* lines confirmed their roles in Al tolerance and malate release, which were restored by $CaSO_4$ supplementation. These findings collectively highlight the significant potential of gypsum as a soil amendment strategy for improving plant growth in acid soils, while also indicating the need for further investigation into the mechanisms underlying Al tolerance induced by sulfate-containing compounds in plants.

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