STIMULATION OF GERMINATION BY GIBBERELLIC ACID AND *BACILLUS SUBTILIS* ON COMMON BEAN, MAIZE, WHEAT AND OKRA EXPOSED TO ACID MINE WATER AT DIFFERENT TEMPERATURE REGIMES

by

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DECLARATION

I declare that the work presented in this dissertation titled “Stimulation of germination by gibberellic acid and *Bacillus subtilis* on common bean, maize, wheat and okra exposed to acid mine water at different temperature regimes.” is to the best of my knowledge and belief original expect as acknowledge in the text and that the material has not been submitted, either in whole or in part, for a degree at this or other universities. I also certify that I have complied with the rules, requirements, procedures, and policies of the university.

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Daniel 2:23

> *"I thank and praise you, God of my ancestors: you have given me wisdom and power, you have made known to me what we asked of you, you have made known to us the dream of the king".*

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LIST OF ACRONYMS AND ABBREVIATIONS

°C	Degree Celsius
ACC	1-aminocyclopropane-1-carboxylate deaminase
AMD	Acid mine drainage
ANOVA	Analysis of Variance
ARC	Agriculture Research Council
B. subtilis	*Bacillus subtilis*
CEC	Crop Estimates Committee
CFU	Colony Forming Units
CKs	Cytokinins
CRD	Complete Random Design
DALRRD	Department of Agriculture, Land Reform and Rural Development
DMRT	Duncan's Multiple Range Test
DO	Dissolved oxygen
EC	Electrical conductivity,
ERI	Emergence rate index
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FGP	Final germination percentage
GA_3	Gibberellic Acid
GS	Germination speed
ha	Hectare
IAA	Indoleacetic acid
IFPRI	International Food Policy Research Institute
LB	Luria-Bertani
µS/m	microSiemens per metre
m	metre
mg/L	Milligrams per litre
mM	Millimolar
NaClO	Sodium hypochlorite solution
PABRA	African Bean Research Alliance

PGPR	Plant growth-promoting rhizobacteria
ROS	Reactive oxygen species
rpm	Revolutions per minute
RSA	Republic of South Africa
SA	Salicylic acid
SACEC	South Africa’s Crop Estimates Committee
SAGL	Southern African Grain Laboratory
STATS SA	Statistics South Africa
TDS	Total dissolved solute
t	Tons
t/ha	Ton per hectare
UN	United Nations
UNWR	United Nations Water Research
VI	Vigour index
VOCs	Volatile organic compounds
WHO	World health Organizations

ABSTRACT

Seed quality management is considered the key to boosting agricultural production, which may be quickly done using seed improvement technologies. Comprehensive seed priming techniques is one of the tools that enhance fast germination and improve plant yields. On the other hand, access to heavy metals from acid mine drainage water can cause germination to be delayed and impair its pace and uniformity, resulting in poor crop production. Plants’ diverse biochemical processes such as enzyme and antioxidant production, protein mobilization, and photosynthesis may be disturbed. Herein, sowing common bean, maize, wheat, and okra treated with substantial acid mine drainage water at different concentrations (0 %, 25 %, 50 %, 75 %, 100 %) at 25 °C and 35 °C was carried out to assess the effects of gibberellic acid and *B. subtilis* BD234 on seed germination and seedling growth, for better seedling establishment. Seeds that had not been primed were also exposed to various levels of AMD and used as controls. The results indicated that seed germination parameters (Final germination percentage; germination speed; emergence rate index, vigour index) were reduced with higher acid mine drainage (AMD) concentrations (75 % and 100 %) compared with low AMD concentrations (0 %, 25 % and 50 %) for common bean, maize, wheat, and okra. In contrast, the germination indices were improved by applying gibberellic acid and *B. subtilis*, while increase in AMD concentrations had adverse effects on root and shoot length. The results also showed that temperature influenced all crops' germination speed and percentage. Overall, the results suggest that gibberellic acid and *B. subtilis* BD234 priming agents can be a practical approach to improve seed germination and seedling growth in bean, maize, wheat, and okra under acid mine water drainage contaminated environments.

CHAPTER 1

BACKGROUND OF THE STUDY

1.1 Descriptive project title

Determining the enhancement of gibberellic acid (GA_3) and *B. subtilis* BD234 on the germination response of common bean (*Phaseolus vulgaris* L.), maize (*Zea mays* L.), wheat (*Triticum aestivum* L.) and okra (*Abelmoschus esculentus* L.) exposed to different acid mine water concentrations and temperature regimes.

1.2 Introduction and problem statement

Germination is the first crucial phase in every plant life cycle, in which the plant’s seedlings, root system, and adaptability to the environment are regulated (Donohue *et al*., 2010; Yakoubi *et al*., 2019). Seed germinations compromise the plant's morphological, physiological, and biochemical developments entirely by initiating the imbibition phase and radicle appearance from the seed's coat (Bewley *et al*., 2012). Hence the germination process is critical for the plant's survival, longevity, and the persistence of plant populations (Donohue *et al*., 2010; Willis *et al*., 2014). Furthermore, vigorous seed germination is significant in crop production increasing crop yields (Zhang, 2021). However, plants face multiple environmental stresses throughout their lifespan that reduce their growth and negatively affect crop productivity (Srivastava *et al*., 2021). Abiotic factors such as soil quality, extreme salinity, acidity, drought, and pollution often disrupt the germination process, causing substantial redundancy (Srivastava *et al*., 2021).

Exacerbating anthropogenic activities had played a significant role in destroying agricultural soil, so industries post-impacts like uncontrollable acid mine water are putting agriculture water use under strain. Sulfur-bearing materials, for example, are the most prevalent ions in the groundwater of South Africa and the East African Rift, contaminating rivers and streams (Abiye *et al*., 2019; Mengistu *et al*., 2019). The acid mine water affects the nutrient supply for crops and disrupts the hormonal balance, weakening the seed germination and emergence in plants (Rambabu *et al*., 2020);

therefore, the quality and quantity of plant yield development is also affected (Singh *et al*., 2010).

Mercury (Hg), zinc (Zn), and iron (Fe) lead (Pb) in acid mine water decreases the species diversity of both plants and microorganisms. According to Jaishankar (2014), the cells of the plants degenerate by inducing the formation of hydroxyl free radicals. The excess of heavy metals in the environment is a dangerous threat even to the health of human beings (Liu *et al*., 2013; Abiye *et al*., 2018; Guo *et al*., 2020). In addition to acid mine water, due to climate change, land degradation and desertification are also likely to affect agricultural productivity, compromising food production in Africa (Hummel, 2016).

Recently, the habitable environment has been experiencing an increase in global temperature and fluctuations in precipitation, a physical impact of the changing climate (Mersha and Leta, 2019). The water shortage has been associated with drought and degradation by several kinds of pollution, and the mining sector has already polluted the water into incompatible for use (Du Plessis, 2019). Suppose the impact of the changing climate leading to drought keeps on escalating. The agricultural sector may have no other option than to use contaminated water such as acid mine water for crop irrigation. According to United Nations-Water Research (2018), water deficit negatively impacts crop yield, and the agricultural sector may experience a loss of revenue. When the crop is under environmental stress, the production and quality are also affected (Kajla *et al*., 2015). Hence most farmers use chemical fertilizers and pesticides for growing crops and managing diseases to reach the goal of the required crop production.

The use of fertilizers can increase the production and quality of crops. However, African countries are not resourceful in producing fertilizers (African Fertilizer Financing Mechanism, 2018). Hence the use of chemical fertilizers is not economically friendly to African farmers, and the adverse effects of chemical fertilizers on the plant environment and ecosystem cannot be ignored. According to Drobek *et al*. (2019), chemical fertilizers are the leading cause of eutrophication, a worldwide main toxic waste challenge (Howarth *et al*., 2002). However, plant hormones and microbes as seed pre-treatment for pest and disease resistance are environmentally friendly and beneficial to agroecology agriculture (Finkel et al., 2017, Mitra et al., 2020).

The beneficial impacts of plant seeds pre-treatment are reflected in the outcome of the germination process under standard culture conditions and an appropriate means for the plant to successfully overcome the action of stressors (Delian *et al*., 2017). *Bacillus subtilis* (*B. subtilis*) is a gram-positive bacterium commercially marketed as a biopesticide and biofertilizer (Guo *et al*., 2014). The primary function of *B. subtilis* is to suppress the soil-borne diseases that inhibit pathogens' growth through antibiotic production (Dong *et al*., 2020; Romano *et al.,* 2020). *B. subtilis* stabilizes the pathogens and promotes plant growth by secreting growth hormones such as IAA and GA_3, fixed with increased nutrient availability (Chowdappa, 2013).

GA_3 is among the phytohormones that are positive stimuli of plant development by increasing the plant's length and the number of cells (Oral *et al*., 2019). It is commonly used as a growth regulator for effective physiological dormancy-breaking and increasing germination efficiency and photosynthetic activity (Rout *et al*., 2017; Oral *et al*., 2019). Also, It promotes seed germination by activating hydrolases in the seed and stimulating hydrolysis from starch to glucose (Richards *et al*., 2001; Shekafandeh *et al*., 2017). Furthermore, Willis *et al*., (2014) stated that gibberellins could significantly improve seed germination in many species. Primarily over the activation of embryo growth, mobilization of reserves, and weakening of the endosperm layer (Baskin and Baskin, 2014). *B. subtilis and* GA_3 are efficient natural biocontrol agents; thus, they can stimulate the germination on significant crops such as maize, wheat, common bean, and okra to boost productivity, minimizing the profit losses that could be initiated by acid mine water.

Plant hormones and rhizobacteria, have seed priming components that induce the seed germination process under stressful conditions. For example, Safari *et al*. (2018) reported the effects of seed priming with ABA and salicylic acid on seed germination and seedling growth of sesame under saline conditions. In addition, the study by De Lima *et al*. (2019) showed the effects of *B. subtilis* on maize and common bean performance under water deficit conditions. However, no study has been reported to investigate the stimulation of germination using *B. subtilis* and GA_3 as pre-treatment germination stimulants on significant crops such as common bean maize, okra, and wheat exposed to acid mine water.

The present study studied the growth-regulating effect of GA_3 and *B. subtilis* on common bean, maize, wheat, and okra seeds, exposed to varied AMD concentrations. To better understand the results, non-primed and primed seeds of common bean, maize, wheat, and okra were used to measure germination and seedling growth in relation to physiological changes after seed priming at different temperatures.

1.3 Aim

To determine the effects of GA_3 and *B. subtilis* doses on the germination of common bean (*P. vulgaris*), maize (*Z. mays)*, wheat (*T. aestivum*) and okra (*A. esculentus*) exposed to various acid mine water concentrations at different temperature regimes.

1.4 Objectives

- To determine the stimulated germination growth response of common bean, maize, wheat and okra treated with GA_3 and *B. subtilis*, exposed to various concentrations of acid mine water at 25 °C and 35 °C.
- To evaluate the effects of different acid mine water concentrations on seed germination of common bean, maize, wheat and okra primed by *B. subtilis* and GA_3 at different temperature regimes.
- To determine the interactive effects of acid mine water concentrations, *B. subtilis*, GA_3, and temperature between the treated seeds.

1.5 Outline of the study

The dissertation is divided into six chapters and presented in that order. The project's background, problem statement, justification for the study, aim, and objectives are all introduced in the first chapter. The literature review on common bean maize, wheat and okra forms part of chapter 2; the chapter also consists of the in-depth literature about acid mine drainage water, GA_3 and *B. subtilis.* The third chapter is comprised of detailed materials and methods followed in the study. The findings of a study on the effects of seed priming with GA_3 and *B. subtilis* BD234 on seed germination and early seedling growth exposed to acid mine drainage water was divided into two chapters, the cereal crops (maize and wheat) results and discussions from chapter 4 whereas the legumes (bean and okra) were presented on chapter 5. Then, Chapter 6 present the general discussion and conclusion with recommendations of the study.

CHAPTER 2

LITERATURE REVIEW

2.1 Common bean (*Phaseolus vulgaris* L.)

The common bean is a legume plant belonging to the Fabaceae family (Integrated Taxonomic Information System, 2014). The crop originates from Latin America and is widely distributed from Northern Mexico to North-Western Argentina (HernándezLópez, 2013). Common bean is also known as navy, pinto, red kidney, or French beans (Yang *et al*., 2018), and is one of the world's most valuable food sources, especially in developing countries, in terms of food energy and nutrients (Namugwanya *et al*., 2014; McClean *et al*., 2017). Sub-Saharan Africa has the world's largest per capita pulse consumption of common bean, and the most considerable proportion are people living in abject poverty (Larochelle *et al*., 2015; Pan African Bean Research Alliance (PABRA), 2019). As a result, the common bean is a critical crop for food security and a cost-effective way to improve the diets of low-income households in Sub-Saharan Africa (Fetahu *et al*., 2013). The kilojoules generated by common bean are precisely the same as the energy food offered by red meats; thus, they are nicknamed "poor man's meat" (Mendes *et al*., 2018).

Common bean is an annual and self-pollinated crop with some progression of outcrossing to increase genetic diversity (Ferreira *et al.,* 2000; Bareke *et al.,* 2018). The crop consists of lateral and adventitious roots, stem up to 3 m long which can be sharp or cylindrical (Ferreira *et al.,* 2000). Fruits are presented in a linear pod up to 20 cm long, straight or curved with a prominent beak (DALRRD, 2010). Common bean flowers are arranged in pairs or single along the rachis, with attractive white to purple colour (Fourie, 2014; Kim *et al.,* 2014; Bareke *et al.,* 2018). After pollination, each flower produces one open pod around 1-1.5 cm broad and up to 20 cm long and contains 4 to 12 seeds in each pod (Wortmann, 2006). The grain legumes are kidney-shaped, ellipsoid, or oblong and can reach 1.5 cm long (DALRRD, 2010).

The germination period of the common bean is approximately 7-10 days depending on growing conditions (Raveneau *et al*., 2011; Gerhardt, 2017). The first growth stage starts when the bean seed absorbs water through micropyle (Ali and Elozeiri, 2017), a small stoma located on the seed coat (Fig 2.1a). The seeds continue to expand until the seed coat ultimately breaks, allowing the radicle to emerge (Fig 2.1b and c). The radicle is the plant's embryonic root, which emerges and grows into the soil. The root cap, which is the tip of the radicle, protects the root as it pushes its way through the soil (Ali and Elozeiri, 2017). The root system begins to form once the radicle emerges, and the bean plant has a fibrous root system, which means it has numerous branches (Fig 2.1d and e). Finally, the epicotyl forms between the cotyledons during this growth period, and the first seed leaves activate adult leaves (Fig 2.1f). Green bean seeds are dicotyledons because they produce two seed leaves within the cotyledons. The cotyledons are established above the earth during this green bean germination process, also known as hypogeal germination (Gerhardt, 2017). The plant will continue to develop for roughly six weeks after the first leaves appear. About that time, the plant will enter the reproductive stage and begin with the flowering process (Amstutz, 2015).

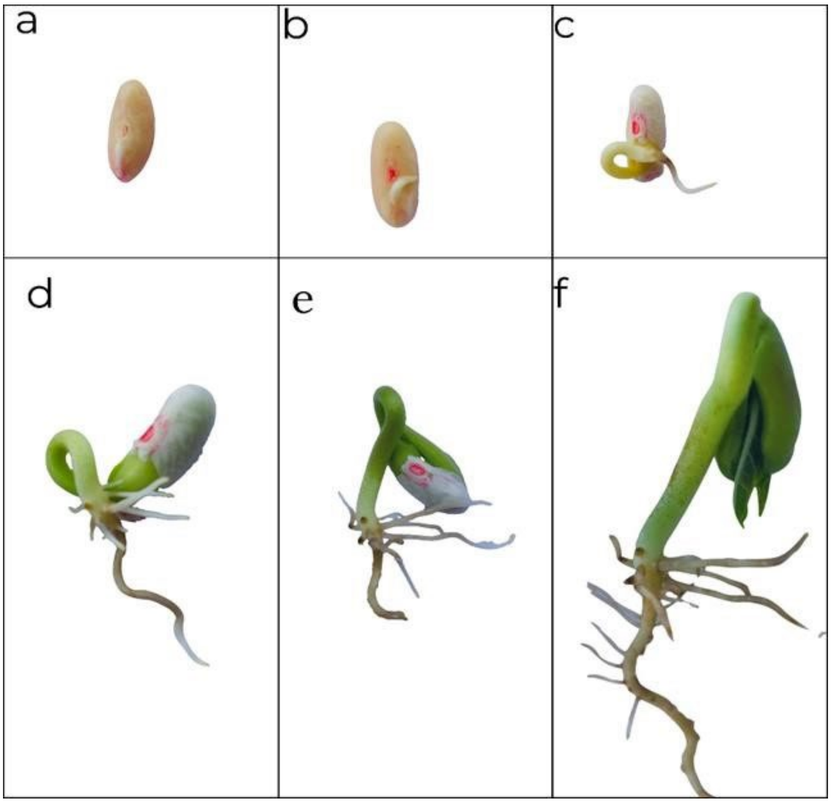

Figure 2.1: A schematic representation of the germination process of a common bean seed. Each alphabet (a-f) represents the germination process until completion.

Common bean distribution and cultivation in Africa is widespread, yielding about 80 % on the continent. (FAO, 2017). The annual global production of common bean is estimated to be around 12 million tons (Nkhata *et al*., 2020). Around 2.5 million tons are produced annually in the East and Southern African regions (Petry *et al*., 2015). In Sub-Saharan Africa, farmers cultivate 7.7 million hectares of common bean (Larochelle *et al*., 2015; FAO, 2019). Approximately 40 % of Africa's production is sold for around 450 million dollars in the United States (Alemu *et al*., 2017).

In the Republic of South Africa (SA), the average common bean production recorded over the previous 5 production seasons was 59 5000 tons, while the local consumption demand is 137 712 tons, representing the deficit of 78 212 tons of the common bean production (Grain SA, 2018). Approximately 4 378 tons on average of common bean is exported per annum, mainly to neighbouring countries such as Zimbabwe, Angola, Mozambique, and Swaziland (FAO, 2019). South Africa imports about 79 000 tons of common bean per annum, which is extremely low compared to the amounts that have been imported previously. According to DALRRD (2018), the annual crop report on common bean imports by South Africa has been declining between the years 2012 and 2017, with significant trade originating from Asia. Furthermore, from 2008 the annual common bean imports statistics showed that Asia is responsible for 75 % of common bean, while China alone accounts for about 98 % imported by South Africa and other regions throughout Africa. Americas and Europe contributed only 25 % towards the total common bean imported by South Africa. According to DALRRD (2018), 1200 farmers produce an average of 65 000 tons of common bean per annum. The area under common bean cultivation in South Africa is declining, negatively impacting the production volumes of the crop (Table 2.1). Land planted for common beans in the country fluctuated between 40 and 64 hectares between 2008 and 2017 (Table 2.1).

Table 2.1: Total cultivated production statistics of common bean in South Africa from 2008-2018.

Production Year	Area Planted (1000 ha)	Total Production (1000 tons)
2008/09	44	74
2009/10	44	57
2010/11	42	46
2011/12	40	52
2012/13	44	66
2013/14	56	90
2014/15	64	88
2015/16	34	39
2016/17	46	71
2017/18	45	75

Source: DALRRD Statistics and Economic Analysis, (2018).

Common bean strategically addresses malnutrition and food insecurity challenges in Africa, ranking among the top five foods with the highest micronutrient to price concentration ratio (Katungi *et al*., 2009; Drewnowski, 2010). This crop also provides an income source for millions of smallholder farmers in Africa, who market about 40 % of their production to domestic and export markets (Larochelle *et al*., 2015). Therefore, the common bean is one of the essential efficient vegetable crops in the socioeconomic farming systems of Africa (Monda *et al*., 2003; Fetahu *et al*., 2013).

2.2 Maize (*Zea mays* L.)

Maize belongs to the tribe of Maydae, from the Poaceae family, and is considered to originate from Mexico and Central America (Hossain *et al*., 2016). The genus *Zea* comprises four species, of which *Zea mays* is economically cultivated. According to Grassini *et al*. (2013), maize is a dominant crop throughout Africa and Latin America, usually recognized in most English-speaking nations as ‘corn’ which denotes the ‘local staple’ (Sinyolo *et al*., 2016; Matlou *et al*., 2017). Maize processing and consumption differ widely from country to country, with maize flour and meal being two of the most common products. Furthermore, 63 % of maize is used for livestock feed worldwide (Shiferaw *et al*., 2011), for ethanol fuel, in alcoholic beverages, and a significant source of oil, starch, and biofuel (Prasanna *et al*., 2020). Maize can also be used in combinations with other ingredients, such as corn-soy blend, for new-borns and food-aid feeding (Ranum *et al*., 2014).

Maize is a thick-stemmed annual tall grass with intersecting sheaths and extensive leaves perpendicular to each side of an axis (Kumar *et al*., 2012). The single stem develops typically up to 4 cm in thickness, reaching 4 m in height (Paliwal, 2000; Dowswell *et al*., 2019). The root system of the maize crop is formed of varying numbers of roots; the seminal roots, the nodal roots coming from below the stem, and the brace roots (Kumar *et al*., 2017; Gao and Lynch, 2016). The maize grain is botanically a caryopsis, meaning that a dried fruit bearing a single seed in which the ovary wall is linked to the inner tissues of the seed coat (Farnham *et al*., 2003; Kumar *et al*., 2012).

Germination of a maize seed starts with the appearance of the radical shoots, which is maize's initial growth phase (Fig 2.2b), whilst the radicle root emerges near the end of the maize's tip. The seminal lateral roots emerge and initially stretch towards the dent end of the maize seed (Fig 2.2c). As the germination process continues, the growth of the coleoptile becomes visible (Fig 2.2d), which protects four or five leaves folded up inside each other to establish a plumule known as the embryonic shoot of the plant (Kumar *et al*., 2012). The growth of the seminal root slightly declines, and nodal roots are initiated at the crown (Fig 2.2e), and finally, the formation of the leaves rapidly develops and grows through the coleoptile tip (Fig 2.2f).

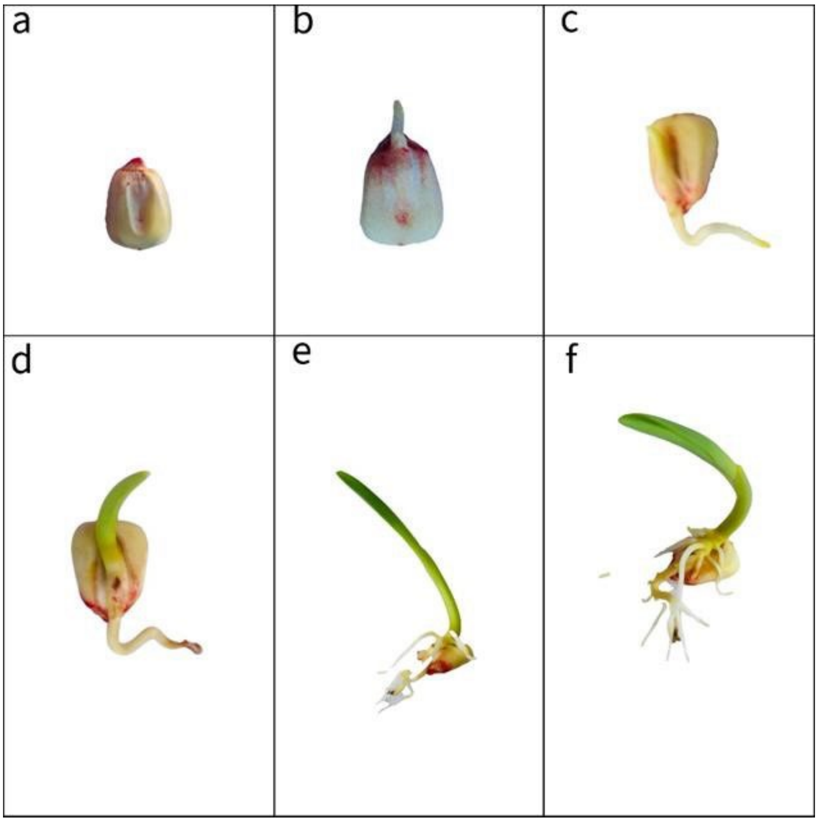

Figure 2.2: A schematic representation of the germination process of a maize seed. Each alphabet (a-f) represents the germination process until completion.

Maize is a primarily warm-weather crop grown in a wide range of climatic conditions and can successfully be grown in areas receiving an annual rainfall of 600 mm (Birch *et al*., 2008). The crop is mainly grown using the traditional tillage approach of ploughing (Zhou *et al*., 2019). The production system in South Africa is dominated by small-scale farmers and emerging commercial farmers (Iortyom *et al.,* 2018). Currently, 79 % of the world's maize is produced in the United States, Brazil, Mexico, Argentina, India, France, Indonesia, RSA, and Italy (FAO, 2019). Alberts *et al.* (2019) reported that countries in sub-Saharan Africa required 76 % of maize output in previous years whilst South Asia needed 70 % of yield production. Furthermore, based on the International Food Policy Research Institute (IFPRI) estimate, maize demand was predicted to surpass wheat and rice in 2020.

Approximately 60 % of maize produced in South Africa is white, and 40 % is yellow (DALRRD, 2011). Yellow maize is utilized for animal feed, while white maize is generally consumed by humans (DALRRD, 2018). Maize is produced throughout

South Africa, with Free State, Mpumalanga and Northwest provinces being the leading producers in the country, accounting for around 83 % of total production. Commercial agriculture contributes roughly 98 % of maize in RSA, while the remaining 2 % is generated by emerging farming (DALRRD, 2018). The maize harvest in 2020 was 35 % larger than the 2019 crop production (Figure 2.3).

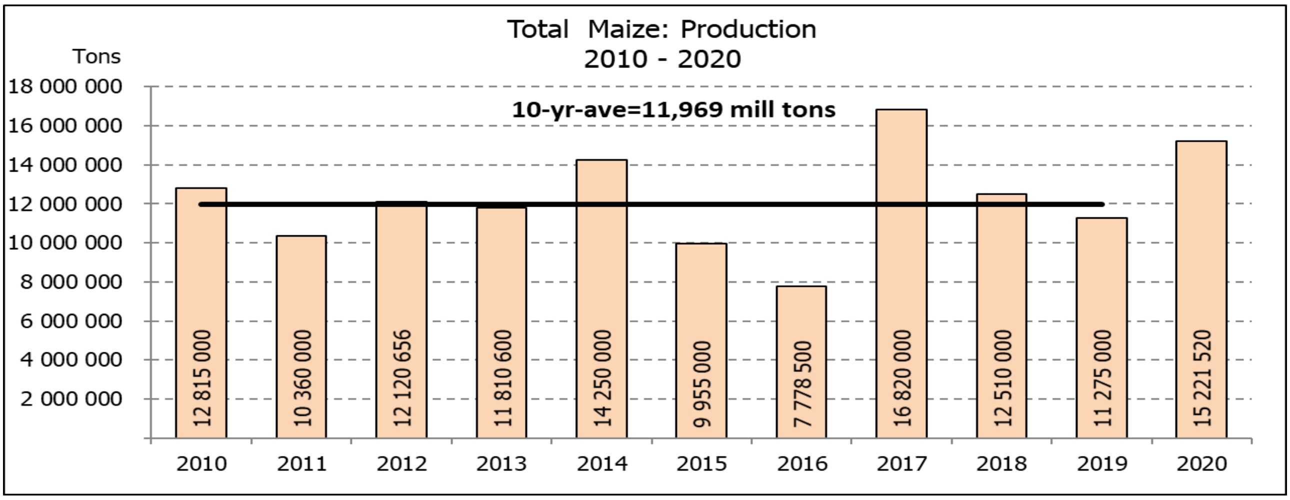

Figure 2.3: Total South African maize production levels for the past 10 years (2010 to 2020). Source: CEC, (2020).

The commercial maize area planted for 2019/20 was predicted to be influenced favourably by relatively high local corn price level (South Africa's Crop Estimates Committee, 2020). Around 2.6 million hectares of corn were planted by commercial farmers in 2019/20 (Table 2.2), 13 % greater than the area planted in 2018/19 (South Africa's Crop Estimates Committee, 2020). Local maize prices traded at reasonably high prices until the following year's harvest season, providing commercial producers with great initiative to plant more maize fields (CEC, 2020).

Table 2.2: Area planted, yield and production statistics of South Africa’s white and yellow maize produced by commercial and subsistence farmers for 2018/19 (actual) and 2019/20 (estimate).

Maize	**Area 1000 ha**	**Yield t/ha**	**Total production 1000 t**	**Area 1000 ha**	**Yield t/ha**	**Total Production 1000 t**
	2018/19			**2019/20**		
Commercial						
White	1.300	3.6	4.700	1.500	4.5	6.70
Yellow	1.000	5.3	5.300	1.100	5.5	6.10
Subsistence						
White	200	1.5	300	200	1.5	300
Yellow	100	2.0	200	100	2.0	200
Total	**2.600**	**4.0**	**10.50**	**2.900**	**4.6**	**13.300**

Source: Crop Estimates Committee, (2020).

Maize is a highly substantial and intensively farmed grain crop, a vital portion of the diet for rural and urban communities in RSA (Agriculture Research Council, 2016; Matlou *et al.*, 2017). The crops’ production provides a staple food and a source of income for many developing countries (Ngoune and Mutengwa, 2020). Meanwhile, its products supplied 30 % of America's food, 38 % of Africa's, and 6.5 % of Asia's (Prasanna *et al.*, 2020). Thus, maize crop production is crucial for food security and economic development, accounting for almost 30 % of the revenue of impoverished farmers (Beyene and Getu, 2020

2.3 Wheat (*Triticum aestivum* L.)

Wheat is believed to have originated in the eastern part of the world, in the areas now occupied by Syria, Turkey, Afghanistan, Iraq and Iran. Grains of domesticated wheat was found in the archaeological remains in Ali Koshi in Iranian Khusistan, dating back to 6 500 BC; and Anatolia in Turkey (National Research Council, 2006). A very likely place of origin is the area known in early historical times as the Fertile Crescent, a region with rich soils in the upper reaches of the Tigris-Euphrates drainage basin (Hemdane *et al.*, 2016). Cultivation of wheat spread from its origin to India, Pakistan, and China in the east, Mediterranean countries in the west and other countries in the north (Hemdane *et al.*, 2016).

Wheat plants grow for about 3 to 10 mm in length, 3 to 5 mm straight, with cylindrical, jointed, and smooth stems (Cossani and Reynolds, 2012). The plants consist of both the root and shoot systems. They have long, slender leaves and hollow stems in most varieties (Setter and Carlton, 2000). The grain consists of a germ, embryo and endosperm and the typical wheat kernel is 3 to 10 mm in length and 3 to 5 mm in diameter (Kumar *et al*., 2017). Germination of wheat begins after a short period of dormancy in the seeds (Kumar *et al*., 2017) because the crop has been associated with low dormancy levels that are easily broken down. Depending on the cultivar, dormancy can last anywhere from 3 to 7 months after the seed coat has been removed (Dane, 2020). When the seed begins to absorb moisture, the first phase begins (Fig 2.3a and b). At a relative humidity of around 97.7 %, a wheat seed must reach a moisture content of around 35 to 45 % of its dry weight to begin germination (Jagdish, 2020). The embryo grows visibly; the radical emerges, followed by other primary roots (Fig 2.3c). The coleoptile appears to protect the emergence of the first leaf (Fig 2.3d). The coleoptile then emerges to the surface, and the first genuine leaf pushes through the tip (Figure 2.3e). The seedling development occurs with the seed embryo consisting of 2 to 3 leaf primordia, and almost half of the leaf primordia are already initiated (Figure 2.3e-f). Plant growth begins, and the young plant is referred to as a seedling.

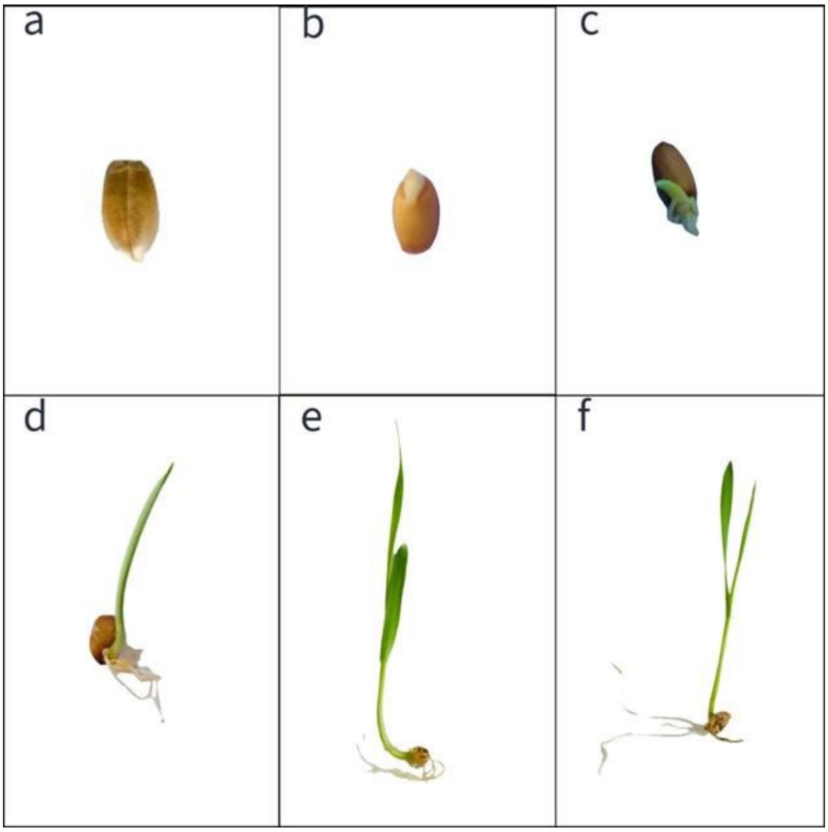

Figure 2.4: A schematic representation of the germination process of a wheat seed. Each alphabet (a-f) represents the germination process until completion.

Wheat is cultivated over a wide range of soil and successfully grows over large land portions (Poole, 2012; Schlatter *et al.*, 2020). Annual rainfall of 254 mm is generally required for the growing stage; however, it can be grown in a wide variety of climates. The crop grows best in cool regions with temperatures between 10 and 24 °C (Poole, 2012), under deep, fertile, well-drained ventilated soil at a pH between 5.5 and 7.5 (Kumar *et al*., 2017). Wheat is an extensively important staple cereal crop that provides a substantial portion of energy to approximately 4 billion people globally (Shiferaw *et al*., 2013; Hemdane *et al*., 2016; Hernandez-Ochoa *et al.,* 2018).

For the past 8000 years, wheat has been the primary staple food of major civilizations of Europe, North Africa, and West Asia, cultivated in 220 million hectares of land, representing 30 % of the world's total cereal production area (Cossani and Reynolds, 2012). In Africa, Egypt is the world's largest wheat producer, producing 9 million tons per annum (pa), followed by Algeria, with 4 million tonnes pa, which is almost half of

Egypt's wheat production (Tadesse *et al*., 2019). Sub-Saharan African countries are also known to import about 17 billion tons of wheat annually.

After maize, wheat is the second most important grain crop produced in South African agriculture (DALRRD, 2015; FAO, 2020). The national wheat yield has decreased by approximately 740 000 tons between 2002 and 2012 (Dube *et al*., 2019), leaving a significant gap of approximately 1 million tonnes annually, which had to be imported (Dube *et al*., 2019). For the past two decades, South Africa's wheat area has deteriorated at around 500,000 hectares, about a million hectares in the 20-year timeline statistics (Figure 2.4).

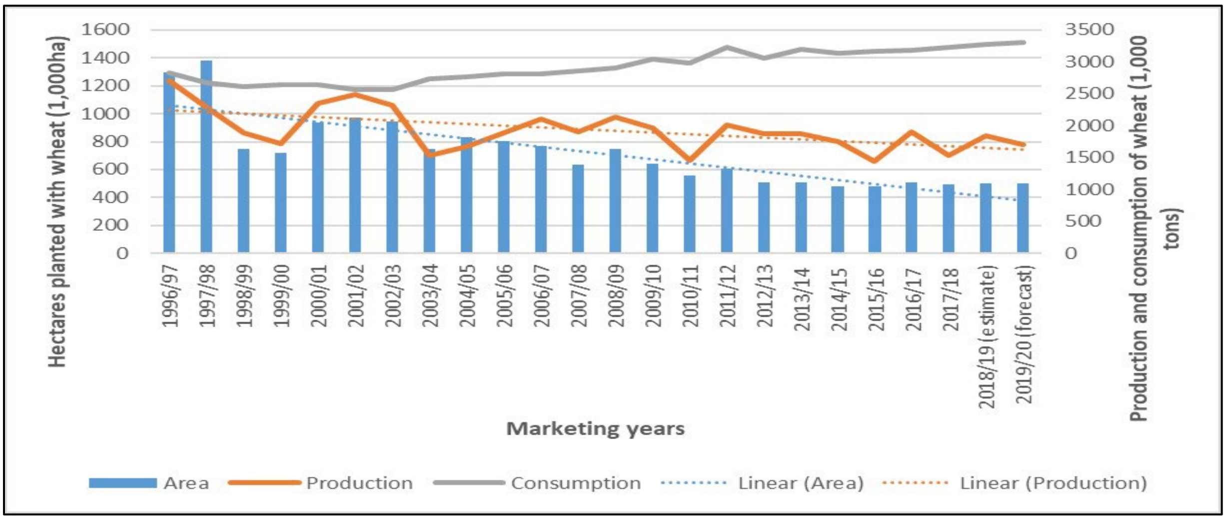

Figure 2.5: Statistical trend of wheat area planted production and consumption in South Africa from 1996 – 2020. Source: USDA Foreign Agriculture Service Report (2020).

Due to extreme drought events, wheat production decreased to 1.5 million tons in 2019, resulting in an almost 50 % loss of production. According to the Southern African Grain Laboratory (SAGL, 2020), Western Cape produced 650 000 tons of wheat in 2020, contributing 42.3 % of the total crop, compared to the 47.7 % of the previous season. The Free State's wheat production of 326 000 tons declined by 15 % year on year is still considered the second-highest provincial production figure (SAGL, 2020). The irrigation areas of the Northern Cape, the third-largest wheat-producing area at 262 500 tons, was 11 % less than the 2018/2019 season. Additionally, the wheat residue produced in Limpopo was 120 000 tons, representing a decrease of 6 %

compared to the 2018/19 season, while North West production decreased by 7 % (SAGL, 2020). The production upturn harvested in July 2020 resulted from favourable weather conditions and an above-average planted area (FAO, 2020). Overall, the 2020 wheat cereal output was forecasted at 18.6 million tons, nearly 30 % higher than the five-year average and the second-largest output on record (FAO, 2020).

According to Nhemachena *et al*. (2017), there is a wheat production crisis in RSA, as the production fails to meet the demand. The country currently produces 1.8 million tons of wheat grain annually and must meet over 40 % of the domestic demand through imports. Nalley *et al.* (2018) reported that to prevent the future food security crisis over potential wheat shortages, a strategic goal for agriculture is to at least double wheat production by 2030. Over the years, South Africa's primary wheat breeding goals have been to improve pest resistance, grain yield, and grain quality. However, more research still has to be done to combat multiple environmental stress in wheat. Much progress has been made regarding grain quality improvement, aiming to increase crop production to sidestep the shortage of wheat and loss of employment in the cereal industry (Mondal *et al*., 2016).

2.4 Okra (*Abelmoschus esculentus* L.)

Okra (*Abelmoschus esculentus* L.) is a vegetable crop belonging to the Malvaceae family, native to Africa and extensively disseminated in tropical and subtropical worldwide (Gido *et al*., 2016; Muli *et al*., 2020; Kang *et al*., 2020). It is a pharmaceutical and nutritious plant that is known by a variety of regional names across the world, including lady's finger in England, gumbo in the United States, guino-gombo in Spanish, guibeiro in Portuguese, and bhindi in India (Benchasri, 2012; Islam, 2019; Daliu *et al*., 2020). The crop is widely considered a vegetable in Africa because its pods, seeds, leaves, shoots, and the outer cover of the flowers are all consumed as cooked greens (Khan *et al*., 2017; Ojiako *et al*., 2018). Okra fruits are also consumed while still immature to be edible (Radovich, 2018). The green fruits can be consumed in salads, soups, and stews, fresh or dried and fried or boiled (Ndunguru and Rajabu, 2004; Khan *et al*., 2019). Matured okra seeds can be dried and used to make vegetable curds or roasted and powdered to serve as a coffee preservative or replacement (Moekchantuk *et al*., 2004).

Okra plants are tall shrubby annual crops with scratchy hairs, cultivated during warm seasons, typically reaching 2 m in height. However, some African varieties may grow up to 5 m tall, with a base stem of 10 cm in diameter (Nimona, 2019). The leaves are polymorphous, with the bottom being roundish-angled and the upper being palmately 3-5-lobed; and leafstalks of up to 15 cm long (Moosavi *et al*., 2018). The flowers are large, axillary, golden, and have red centres (Islam, 2019). The flowers also have seeds and mucilage and a gently spherical capsule with six chambers and a fibrous texture (Ahiakpa *et al*., 2014).

The seed of an okra plant is tiny, round, smooth in texture, and green to dark green (Fig 2.6a). The seed generally germinates from day 2 to day 12 (Jagdish, 2019), whereby the visibility of the radical is evidence that the germination process is taking place (Fig 2.6b). Therefore, the coleoptile continues growing into roots, and small green leaves become noticeable (Fig 2.6c and d). More green leaves appear with a more vital stem and roots (Fig 2e and f). Okra then starts producing flowers from week 12 or 16 weeks (Rockets gardens, 2019).

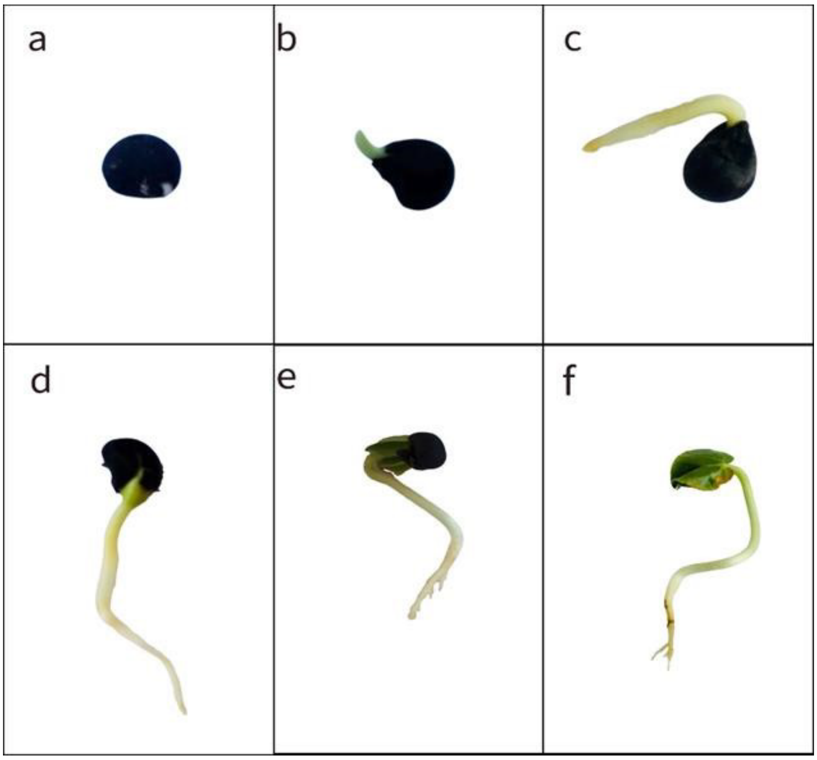

Figure 2.6: A schematic representation of the germination process of an okra seed. Each alphabet (a-f) represents the germination process until completion.

A warm-season annual crop such as okra grows well in a wide range of soils and climates, can withstand a wide range of rainfall, and is suited to the lowland wet tropics (National Research Council, 2006). Germination requires a minimum soil temperature of 16 °C, with an average temperature of 20-30 °C ideal for growth, flowering, and pod development (Akande *et al*., 2010; El-Kader *et al*., 2010). Although okra cultivars respond swiftly to persistently high temperatures, they thrive in well-drained, rich soils with sufficient organic matter and a surplus of essential components (Moosavi *et al*., 2018). In addition, okra seeds favour well-drained soils and can survive a pH range from 5.5 to 8 (El-Kader *et al*., 2010; Jain *et al*., 2012).

According to Gemede *et al*. (2015), okra is a multifunctional crop due to its numerous pods applications, fresh leaves, buds, flowers, stems, and seeds. As a result, it is regarded as a high-value crop since it contains nutrients essential to human health (Asare *et al*., 2016; Kang *et al*., 2020). Previous studies have revealed various health benefits of okra, including anti-adhesive, antioxidant, anti-hyperglycemic, immunomodulatory, and anti-fatigue qualities (Hu *et al*., 2014; Xia *et al*., 2015; Chen *et al*., 2016; Zhu *et al*., 2020). In medicine, for example, okra has been utilised as a transfusion replacement or vascular resistance expansion (Kumar *al et*., 2009). A study in China reported using ethanol extracts from okra leaves in neutralizing oxidative stress, relieving renal tubular-interstitial disorders, minimizing proteinuria, and improving kidney function (Kumar *et al*., 2010). Furthermore, the antioxidant found in okra aids in the proper functioning of the digestive tract by promoting healthy mucous membranes (Georgiadisa *et al*., 2011).

A recent study emphasises the possible application of okra antidiabetic characteristics and nutraceutical future (Durazzo *et al*., 2019). The okra plant has scientifically established that positive health qualities can be utilised to prevent and even cure various pathologic diseases (Santini *et al*., 2017; Santini and Novellino; 2018); and progressive metabolic diseases such as diabetes *mellitus* (Daliu *et al*., 2020). Furthermore, the future use of okra extracts in food or food supplements might propose the development of a sustainable and new nutraceutical food that takes advantage of the health benefits of okra (Santini *et al*., 2013). The excessive natural random coil polysaccharide (mucilage) in okra comprises galactose, rhamnose, and galacturonic acid (Zaharuddin *et al*., 2014; Zhang *et al*., 2018). The usage of mucilage benefits synthetic polymers due to the reduced procurement cost and biodegradability (Araújo *et al*., 2020). It is also utilized in the food and pharmaceutical sectors as a thickening, emulsion stabiliser, suspending agent, and binder (Archana *et al*., 2013; Raj *et al*., 2020).

Okra has excellent potential as an industrial/commercial crop. Regardless, okra is only recognised as a garden crop in rural RSA, and its production market is very restricted. In contrast, the crop is seldom produced in some parts of RSA due to poor yielding indigenous landraces and insufficient agronomic management methods (Agricultural Research Council, 2014). The worldwide production statistics of okra in 2013 was expected to be 8.947 million tons in total, grown on an area of 1.126 million hectares (Babalola *et al*., 2020), predominantly high production coming from Asia and Africa. According to 2021 statistics predicted by Atlasbig, India remains the leading okra producing country standing on 6,126,000 tons of production yearly, followed by Nigeria, making it the largest okra producer in Africa, producing 2,033,129 tons annually. In addition, African countries such as Sudan produced 304,712 tons, Mali: 241,033 tons, Cote d' Ivoire: 112,966 tons, and Ghana produced 66,360 tons of okra annually (Atlasbig, 2021). To this day, these countries grow okra primarily for human consumption.

Oka might be a traditional crop with little agronomic operational needs but has essential qualities such as proteins, carbohydrates, and vitamins that play a substantial role in developing countries' food security (Sathish and Eswar, 2013; Agricultural Research Council, 2014; Sami *et al*., 2019). In a study conducted by Nurmas *et al*. (2021), the authors reported its importance as protective food for maintaining health and preventing illness. Although okra is underutilised, it can contribute to the world's rapidly growing population with food, nutrition, and welfare (Gerrano, 2018). It also contributes significantly to revenue generation and poverty alleviation (Gerrano, 2018).

2.5 Factors affecting the production of common bean, maize, wheat and okra

Although the cultivation and utilization of common bean, maize, wheat and okra in RSA have been successful for decades, their products have been hindered by environmental stresses such as the mine residues. Mining is an important commodity in SA; however, its residues are harmful to the environment. Residues from the mine tailings dam, such as acid mine drainage (AMD) water, are among those that release heavy metals onto the environment, contaminating soil and water. AMD results from the exposure of sulfide minerals to oxygen, water, and microorganisms (Rodríguez-Galán *et al.*, 2019). AMD occurs when ‘‘fool’s gold’’ (FeS^2) or iron pyrite found in mined rock oxidizes. When pyrite (FeS^2) is exposed to oxygen and water, it becomes oxidized, resulting in hydrogen ion release-acidity, sulfate ions, and soluble metal cations, creating a very high run-off in sulphates (McCarthy, 2010). Pyrite (FeS^2) is responsible for starting acid generation and metals dissolution in coal and complex rock sites. This oxidation process occurs in uninterrupted rocks but at a slow rate, and the water can shield the acid generated (Akcil and Koldas, 2006; Fig 2.7).

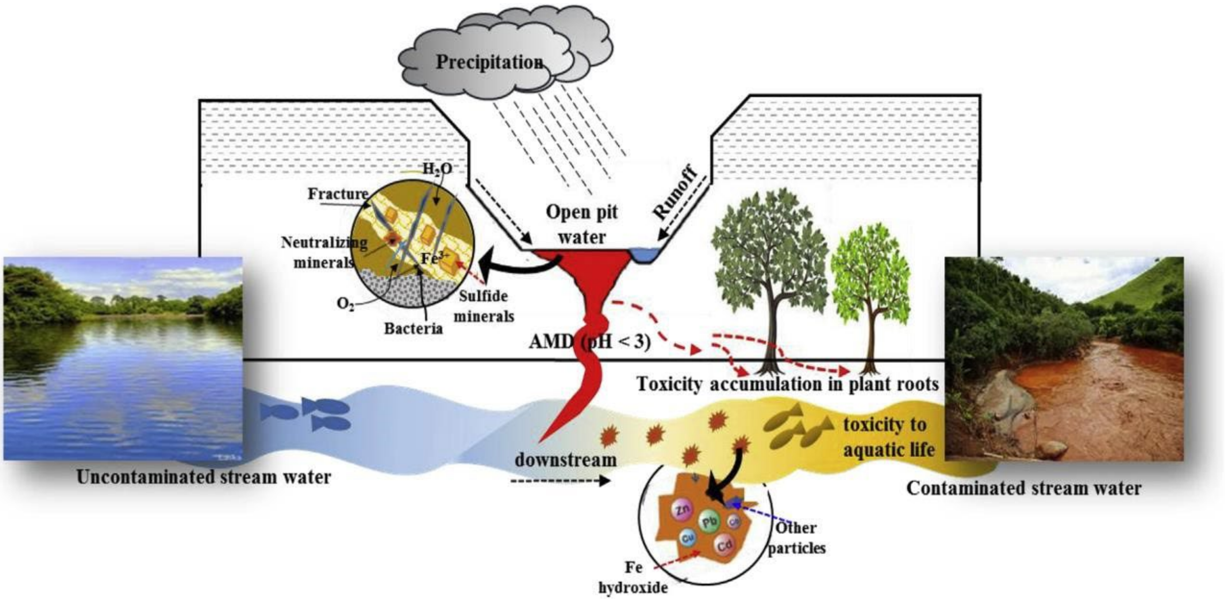

Figure 2.7: AMD generation and related contamination pathways. Source: Naidu et al., 2019.

Mining activities hasten the formation of AMD, producing severe environmental consequences on soil and water (Ríos *et al.,* 2008; Colvin and Burns; 2011; Kefeni *et al.*, 2017). AMD may alter soil pH, diminish soil organic carbon, and influence the soil microbial population, all of which can harm nutrient intake and plant development (Fig 2.8; Quadros *et al.*, 2016). Furthermore, due to its low pH, it enhances the solubility and toxicity of different metals such as cadmium (Cd), copper (Cu), lead (Pb), and nickel (Ni) which compete with critical soil nutrients (Dong *et al.,* 2018).

Figure 2.8: Example of the AMD released from an old metalliferous mine (A) and an old colliery mine (B), South Africa. Source: Akcil and Koldas, 2006.

High quantities of heavy metals present in AMD are hazardous and cause plant seed germination to be delayed (Wang *et al*., 2011; Sethy and Ghosh 2013). It lowers the soil pH, which causes a lack of critical nutrients to plants (Dean *et al*., 2019). Therefore, the breakdown of organic matter decreases due to insufficient microbial activity (Simate and Ndlovu, 2014). According to Rodríguez-Galán *et al*. (2019), when the pH of the soil is low, nitrogen (N), phosphorus (P) and potassium (K) are restrained in the soil, and the calcium and magnesium content is limited; therefore, seeds fail to germinate due to a lack of required nutrients for survival. Furthermore, heavy metal enrichment in organisms via the food chain might threaten people and ecosystems in the long run (Zhang *et al*., 2015; Lei *et al*., 2016). Consuming heavy metal contaminated crops can drastically reduce iron stores in the body and other malnutrition-related deficits (Iyengar and Nair, 2000).

2.6 Priming seeds for crop production

Seeds play a major role in cultivating and producing crops of economic importance. Hence, priming seeds with plant growth-promoting bacteria and phytohormones are important for crop production, especially in RSA, where mining activities contaminate the environment. Plant growth-promoting bacteria such as *B. subtilis* and GA_3 are among priming agents that have been reported to aid in combating environmental stress and enhancing seed germination in plants (Hu *et al*., 2019; Jabborova *et al*., 2020; Wang *et al*., 2020).

2.6.1 ***Bacillus subtilis*** **on seed germination**

Seed germination is dependent on the embryo's viability and the breaking of dormancy induced by environmental conditions (Cabra Cendales *et al*., 2017). The inoculation of *B. subtilis* has been an essential seed stimulus in controlled and field tests to combat the influence of unfavourable environmental conditions (de Andrade *et al*., 2020). Beneficial microorganisms colonize the plant rhizosphere and induce stress tolerance by producing exopolysaccharides, plant hormones, 1-aminocyclopropane-1carboxylate deaminase, organic substances, osmotic adjustment accumulation, phytonutrients, increased or decreased stress expression levels, and morphological changes in the roots (Vurukonda *et al*., 2016).

Luna-Martnez *et al*. (2013) showed that inoculating tomato seeds with *Bacillus* strains boosted germination percentages by 5 % to 6 %. Similarly, Lazzaretti and Melo (2005) also reported the effect of inoculating beans with *B. subtilis* as a potential strategy for boosting root nodulation and promoting bean plant growth. Thus, the germination and growth of different plants can be enhanced by priming with *B. subtilis*. In addition, the production of plant hormones such as salicylic acid (SA), gibberellins (GA), cytokinins (CKs) and IAA secreted by *B. subtilis* in response to unfavourable conditions can stimulate seed germination and enhance plant growth (Table 2.3; Singh *et al*., 2008; Chowdappa *et al*., 2013; Pereira *et al.* 2020).

Table 2.3: Plant growth stimulators secreted by *B. subtilis* to enhance seed germination and plant growth.

Source	Crop	Treated Respond	References
N_2-fixation	Maize	Increased seed germination, root and shoot system	Szilagyi-Zecchin *et al.,* (2014)
P-solubilization	Cucumber	Increased germination rate and the total accumulation of P uptake.	Garcia-Lopez *and* Delgado (2016)
IAA, GA	Tomato	Enhanced seed germination, seedling growth, vigour index and leaf area increase the level of plant hormones.	Chowdappa *et al.* (2013)
CKs	Lettuce	Increased plant shoots and root weight.	Arkhipova *et al.* (2005)
IAA, CKs, GAs, ABA	Soybean	Enhance the growth and increase proline contents	Xu *et al.* (2014)

2.6.2 Plant hormones on seed germination

Plant hormones belong to the class of plant growth regulators that are natural or synthetic and can regulate plant development (Sajjad *et al.,* 2017). They contain molecules produced in low concentrations but can control various cellular activities in plants (Voß *et al*., 2014). The molecules work as chemical messengers to interconnect cellular activities in plants (Voß *et al*., 2014). Plant growth, reproduction, and survival mechanisms are regulated by cross-communication and signal-transduction pathways controlled by plant these hormones (Verhage *et al*., 2010; De Vleesschauwer *et al*., 2013 Kazan, 2015). Significantly, plant hormones play essential roles in seed germination and resistance against biotic and abiotic stress. (Colebrook *et al*., 2014; Xu *et al*., 2016; Bücker-Neto *et al*., 2017).

Although plant response to stresses depends on various factors, phytohormones are considered the most critical endogenous substances for modulating physiological and molecular responses, a crucial requirement for the plant's survival (Fahad *et al.,* 2015). Various plant hormones, including cytokinins, abscisic acid and gibberellins, among others, have shown positive plant-protective functions by playing an influential role during several plant physiological processes and developmental stages, including seed dormancy, stomatal opening, embryo morphogenesis, and synthesis of storage proteins and lipids (Table 2.4). Therefore, phytohormones have proven to enhance plants' growth and yield under diverse environmental conditions.

Table 2.4: Plant hormones and their functions on the development and growth of the plant.

Hormones	Function in Plants	References
Brassinosteroids	(BRs) Control of division; growth by elongation; differentiation of the vascular system; inhibiting root growth; fertility; increase the growth rate	Hussain *et al*., 2020; Nolan *et al*., 2020.
Cytokinin	Control of cell division; bud development; development of the leaf blade; senescence retardation; promote shoot initiation; increase the growth rate; control of stomata apparatus function; growth inhibition; seed dormancy; inhibits shoot growth; induces storage protein synthesis in the seed.	Masood *et al*., 2012; Nishiyama *et al*., 2011. Kieber *et al*., 2018.
Abscisic acid	Control of stomata apparatus function; growth inhibition; seed dormancy; inhibits shoot growth; induces storage protein synthesis in the seed.	Nishiyama *et al*., 2011; Alazem, 2017; Chen *et al*., 2020.
Auxin	Induction of elongation growth and stem growth; stimulates cell division; differentiation of phloem elements; apical dominance; tropisms; initiation of root formation.	Piotrowska-Niczyporuk *et al*., 2014; Camacho-Cristóbal *et al*., 2015;
Ethylene	Senescence induction; initiation of defensive responses; decrease elongation; leaf and fruit abscission.	M cmanus, 2012; Chang *et al.,* 2013; Yang *et al*., 2019; Binder *et al*., 2020.
Gibberellins	Stem elongation; initiation of seed germination; cell division and elongation; enzyme production during germination.	Gupta and Chakrabarty, 2013; Dilip *et al*., 2017

2.6.2.1 Gibberellic acid (GA_3)

Plants respond to exogenous application of phytohormones, and among the plant hormones, GA_3 is a vital growth hormone in plants (Rafique *et al*., 2021). GA_3 is the most used growth regulator for dormancy-breaking, increasing germination efficiency within a brief treatment period (Kwon, 2020). GA_3 have been reported to be the most vigorous to weaken the inhibitory effects of salinity and drought for germination to occur through increasing nutrient uptake, dry weight, and seedlings growth (Tsegay & Andargie, 2018; Sabagh *et al.,* 2021). Literature has also shown that the growth of wheat, rice, cotton has been significantly enhanced in the presence of GA_3 under stressful conditions such as salinity (Iqbal & Ashraf, 2012, Colebrook *et al*., 2014; Chen *et al*., 2020).

Gibberellins play essential roles in many plants growth and development processes, including seed germination, stem elongation, leaf expansion, flower and fruit development, and floral transition (Baliyan *et al*., 2021). They can significantly improve seed germination by activating embryo growth, mobilizing reserves, and weakening the endosperm layer (Pallaoro *et al*., 2016). For example, Sinha and Kumar (2021) reported the effects of GA_3 as a growth modulator that was proficient in breaking dormancy and improving germination percentage. In addition, GA_3 can promote seed germination by activating hydrolases in the seed, stimulating starch's hydrolysis to glucose (Richards *et al*., 2001). For instance, the α-amylase secretion produced by the GA_3 induced the formation of plant embryos in the endosperm and increased the germination rate in a study by Kwon *et al*. (2020).

Despite various other physio-biochemical processes, GA_3 has also been shown to improve photosynthetic activity, plant development, and plants' source-sink relationship (Kaya *et al*., 2020). In a previous study, GA_3 has been reported to be effective in mitigating different stresses such as cadmium contamination (Masood *et al*., 2016), salt stress (Ghodrat and Rousta, 2012; Chauhan *et al*., 2019), and chilling stress (Hu *et al*., 2018). Furthermore, GA_3 supports the development of plant adaptation and resistance to various biotic and abiotic stresses and protects the plant against the toxicity of heavy metals (Maggio *et al*., 2010).

CHAPTER 3

GENERAL MATERIALS AND METHODS

3.1 Seed material

Seeds of common bean (Lazy housewife), maize (Zama star), and okra (Clemson Spineless) produced by Starke Ayres (Johannesburg, RSA); and wheat (Morocco), an international check were selected for the experiment. The seeds were carefully hand-picked from the packet to avoid malformed seeds throughout the experiment, and surface sterilized by repeated dipping in 3.5 % commercial Sodium hypochlorite solution (NaClO) and 70 % ethanol. Seeds were rinsed thoroughly three times with double-distilled water to eliminate treated chemical residues prior to the start of the experiment. The seed was imbibed for 24hrs in distilled water to break dormancy. The research was carried out in a laboratory setting at the University of South Africa (UNISA, Florida Science Campus). Each crop germination trial was tested at a different period: maize (04 April 2021), wheat (15 April 2021), okra (02 April 2021).and common bean (24 May 2021).

3.2 Physicochemical parameters of acid mine water samples

Acid mine water used in the experiment was collected from the Sibanye Gold Mine Randfontein, located 34 kilometres west of Johannesburg in the Gauteng Province of South Africa. Physico-chemical properties of the water, namely: pH, temperature, electrical conductivity, total dissolved solute, nitrate and dissolved oxygen, were measured on-site using the H19828 multi-parameter ion-specific meter (Hanna Instruments (Pty) Ltd, Bedfordview, South Africa). Further analysis of the water was done in the laboratory and the results are presented in Table 1. The measured parameters were pH: 4.2, Electrical conductivity: 3351.33 *µS/m*, Total dissolved solute: 4874.00 *mg/L*, Nitrate: 3.17 *mg/L*, which were all were beyond the permitted limit set by the RSA and the World Health Organization (WHO). Meanwhile, dissolved oxygen: 20.09 of the sampled AMD water was within the limit. Levels of heavy metals such as sulphate oxide, Cadmium, Copper, Nickel, and Zinc were also beyond the limits established by the South African National Standards for irrigation, as well as the WHO water quality recommendations data for agricultural use (Table 1).

Table 3.1: Summary of the mean physicochemical parameters of sampled acid mine drainage water, tap water heavy metal content of the water samples.

Physicochemical parameters	**Sampled acid mine drainage water**	**Tap water**	**South African Standard**	**WHO Standard**
pH	4.2	8.4	5.0-9.7	6.5-8.5
Temperature (˚*C*)	29	21.24	-	-
EC (*µS/m*)	3351.33	68.98	250	300
TDS (*mg/L*)	4874.00	145.35	-	80
NO_3 (*mg/L)*	3.17	7.54	-	-
DO	20.09	8.54	95	150
Metals		**Levels of heavy metals (mg/L)**		
SO_4	18515.33	244.55	-	-
Cd (*mg/L*)	0.18	0.01	0.01-0.05	0.01
Cr (*mg/L*)	5.87	0.05	0.10-1.0	0.05
Cu (*mg/L*)	0.95	0.12	0.1-1.0	0.005
Ni (*mg/L*)	10.42	0.04	0.20-2.0	0.02
Zn (*mg/L*)	55.47	0.92	1.0-5.0	5.00

*EC- electrical conductivity, TDS- total dissolved solute, NO_3 - Nitrate, DO- Dissolved oxygen

3.3 Preparation of *B. subtilis* and inoculation method

B. subtilis BD234 was acquired from the Agricultural Research Council - Plant Protection Research Institute at Roodeplaat. The *B. subtilis* strain BD234 previously cultivated on Luria-Bertani (LB) liquid medium was sub-cultured aseptically in the microbiological safety cabinet, grown overnight on LB medium at 37 °C. The culture was harvested by centrifugation and a 1×10^8 CFU/mL solution of bacteria was prepared in distilled water.

3.4 Preparation of Gibberellic Acid Solution (GA_3)

Bio-Reagent with ≥90 % gibberellin A_3 basis (total gibberellins) was prepared afresh before the soaking phase. The powder of GA_3 (0.069 g) was dissolved in 1000 ml of double distilled water to prepare the 0.2 mM of GA_3 pre-treatment solution. The pre-treatment solution was stored in a cool environment and shaken well before use.

3.5 Seed priming

A priming technique by Safari *et al*. (2018) was used in this study, whereby seeds were soaked inside the germination stimulants (GA_3 and *B. subtilis*). Based on the preliminary trials, 0.2 mM GA_3 and *B. subtilis* solutions were used for priming, and different AMD solutions were used for irrigating seeds. The seeds were primed by soaking with 0.2 mM GA_3 and *B. subtilis* BD234 solution for 6 hours at room temperature. Seeds were then treated with 0 %, 25 %, 50 %, 75 %, and 100 % AMD concentrations. Seeds were left to dry between sterilized filter papers. Control seeds (non-primed) were soaked in water for 6 hours, dried and then sown on moistened cotton wool plated in petri dishes. All the experiments were replicated three times.

3.6 Experimental Design

A complete randomized design (CRD) was used to determine the effects of seed priming with GA3 and *B. subtilis* BD234 on germination indices of the common bean, maize, wheat and okra exposed to AMD water stress at different temperature regimes. The experiment was in a factorial design with three factors: AMD water at different concentrations, GA_3 and *B. subtilis* priming agents with 2 levels (non-primed and primed seeds), and different temperature regimes (25 °C and 35 °C). Petri dishes (90x15 mm) were sterilized with 70 % ethanol for 5 minutes, washed with distilled water, and labelled for various treatments. Forty-five Petri dishes per temperature regime were prepared for each crop, including replicates. Sterilised cotton wool was then placed in each petri dish, and 5ml distilled water was inoculated to moisten the cotton wool, which served as the standard growth media. Twenty seeds of each crop were sown in each petri dish and treated with different concentrations of AMD solution (0 %, 25 %, 50 %, 75 %, and 100 %) instantly after sowing. Seeds were incubated in a controlled environment at 25 °C and 35 °C, respectively, in a Nüve growth chamber test cabinet (Model: TK120, Turkey). Seed germination parameters were recorded every day for 10 days. A seed was germinated when the radicle emerged by about 2 mm in length (Ahmad *et al*., 2012). Regardless of the possible cumulative effect of

AMD water, each petri dish was supplied with equal volumes (10 ml) of the different AMD concentrations (0 %, 25 %, 50 %, 75 %, and 100 %) per day for both primed and nonprimed experiment. Samples were monitored daily after the initiation of the experiment at a constant temperature for any unusual growth seedling until the maximum seed germination period. Physiological parameters such as seed germination percentage and mean germination time were calculated. All seedlings' shoot and root length were measured using a transparent ruler.

3.7 Measurements of germination indices

3.7.1 Calculations of germination parameters

Germination percentage (GP), which measures a seed population's vitality, was determined using the formula described by Czabator's index (1962), whilst germination speed (GS), which measures the time course of seed germination, was calculated as described by Damalas *et al*. (2019). The emergence rate index (ERI), defined as the time taken by the seeds to emerge into a seedling, was calculated using a formula described by Fakorede and Agbana (1983).

The parameters were calculated as follows:

The parameters were calculated as follows:

(i) $Final\ germination\ percentage\ (\%) = \frac{Total\ number\ of\ seeds\ germinated}{Total\ number\ of\ seeds\ in\ a\ replicate} x\ 100$

(ii) $Germination\ speed = \frac{\sum nt}{\sum n}$

also called rate of Maguire, where n = number of normal germinated seeds at a time

(iii) $Emergence\ rate\ index = \frac{G1}{1} + \frac{G2}{2} + \cdots \frac{Gi}{i}$

: G1 is the germination percentage on day 1, G2 is the germination parentage on day 2, until infinity.

3.7.2 Growth response analysis of germination

Germination trials were retained and monitored in the growth chamber for three days to obtain data on seedling growth. Normal seedlings were selected randomly in each treatment from each replicate whereby the shoot length was measured from the base of the primary leaf to the base of the hypocotyls, and the root length was measured from the tip of the primary root to the base of the hypocotyls as described by Amarnath *et al*. (2015). Vigour index was calculated using a formula by Abdul-Baki and Anderson (1973) to assess whether GA_3 and *B. subtilis* induce a sustained vigour response on common bean, maize, wheat and okra and seedlings under AMD. The formula for vigour index is as follows:

iv. $Vigour\ Index = Germination(\%)\ x\ Seeedling\ length(root + shoot\ )$

3.8 Data Analysis

Analysis of variance (ANOVA) was performed using STATISTICA software version 10 (StatSoft Inc., Tulsa, OK, USA). The Duncan's Multiple Range Test (DMRT) was used to differentiate and compare the mean values for statistical significance levels ($p<0.05$).

CHAPTER 4

EFFECTS OF GIBBERELLIC ACID AND *BACILLUS SUBTILIS* ON SEED GERMINATION OF MAIZE AND WHEAT EXPOSED TO ACID MINE DRAINAGE WATER AND DIFFERENT TEMPERATURE REGIMES

Crop exposure to heavy metals from acid mine drainage (AMD) water can hinder germination resulting in poor uniformity, poor stand establishment and eventually poor crop production. Herein, the effects of seed priming with gibberellic acid (GA_3) and *Bacillus subtilis* on maize and wheat seed exposed to AMD water at different temperatures were examined. A completely randomized design experiment was conducted to compare germination indices between primed and non-primed seeds. The results showed that seed germination parameters (final germination percentage; germination speed; emergence rate index and vigour index) were reduced with higher AMD concentrations (50%-100%) compared with the lower AMD concentrations (0%-25%) for both maize and wheat. In contrast, the germination indices (final germination percentage; germination speed; emergence rate index, vigour index) were improved by applying GA_3 and *B. subtilis*, while increases in AMD concentration, had negative effects on root and shoot length. The results also showed that temperature influences germination speed and percentage of both crops. Overall, the results suggest that GA_3 and *B. subtilis* priming agents can be a practical approach to improve seed germination and seedling growth in maize and wheat in AMD water contaminated environments.

4.1 Introduction

Acid mine drainage (AMD) caused by mining is a significant contamination of crops in areas close to the mines. Some of its effects include impairment of germination which directly results in reduced stand establishment and poor crop production (Daraz *et al.*, 2021). Although there are various chemical treatments to restore AMD water, none of them are entirely effective (Gouda *et al.*, 2018). Some farming communities experience seepage of AMD water from mines and therefore crops become disposed to this abominable hazard. Moreover, water scarcity, forces many farmers in underdeveloped nations to utilize wastewater such as AMD water (Rehman *et al.*, 2020). The significant features of AMD water are low pH, excessive salinity, high quantities of heavy metals, and sulphate, all of which are toxic to plants (Kefeni *et al*., 2017). The drainage water's acidity and quantity of hazardous heavy metals surpass water supply regulations, rendering it unsuitable for human consumption (Nevhulaudzi *et al*., 2020) and agricultural purposes such as crop irrigation (Carlson *et al*., 2002).

In plants, excessive exposure to heavy metals disrupts metabolism by upsetting oxidation reduction processes (Rehman *et al*., 2020). The toxicity of heavy metals in soil poses a threat to the long-term sustainability of human health. Frequently, the root absorption systems of the cereal crop cultivars can accumulate and transfer more than 40 % of heavy metals throughout the plant (Retamal-Salgado *et al*., 2017). Among other crops, maize (*Zea mays*) and wheat (*Triticum aestivum*) cultivation are key sources of revenue in agriculture’s economy (Godfray *et al.*, 2010), which are affected by AMD contamination, especially in RSA. Hence, there is a need to develop methods for providing enough crop production while protecting natural resources and the environment (Brilli *et al*., 2019). The alternative method would be more appropriate when it is straightforward, cost-effective, and simple for farmers to implement without having issues while also successfully demonstrating tolerance towards environmental stress (Jisha *et al*., 2013). The use of phytohormones and plant growth-promoting rhizobacteria (PGPR) in seed priming could be a comprehensive solution.

Priming seed has emerged as a potential method for conventional stress management, allowing the plant to limit pathogenic and pesticide damage without using chemicals (Boukari *et al*., 2019). Imbibing seeds in various solutions for a specific amount of time under controlled conditions, then drying them back to their average moisture content, prevents radicles from forming prior to seed planting (Pawar and Laware, 2018). During seed imbibition, partial germination occurs, triggering numerous physiological and biochemical processes such as cell repair and protein synthesis in seeds, which is stopped before plumule and radical development (Sarkar *et al*., 2018; Singh *et al*., 2020). This helps to guarantee that seeds germinate and ensure no delay in breaking the seeds dormancy (Conrath *et al*., 2015; Mauch-Mani *et al*., 2017).

Standard models utilized in this research include seeds hormonal priming (treating seeds with plant growth hormones) and bio-priming (treatment of seeds with biological -stimulants). Because of their proven advantages on stressed crops, GA_3 and *B. subtilis* were chosen for this study. Taken together a systemic stress factor inducer, such as GA_3 is well-known in plant-hormones studies (Werle *et al*., 2020), whereas *B. subtilis* strains have remarkable plant growth-promoting properties (Ruzzi *et al*., 2015) and their use as crop inoculants is well-established (Chandra *et al*., 2018; Bulgari *et al*., 2019).

Previous research has primarily examined the positive benefits of seed priming under abiotic stress, such as drought (Khan *et al*., 2020), salt (Zhu *et al*., 2019), low osmatic (Ghosh *et al*., 2019), fire (Rimpika *et al*., 2017). So far, no study has investigated the effects of seed priming on maize and wheat seed germination under AMD water. Thus, the overall aim of this study was to assess the effects of GA_3 and *B. subtilis* BD234 on seed germination of maize and wheat under different levels of AMD conditions at different temperatures. A theoretical framework was essential for understanding maize and wheat resistance development mechanisms under AMD concentrations and the impact of GA_3 and *B. subtilis* BD234.

4.2 Results

4.2.1 Evaluation of maize and wheat seeds post priming treatment

Non-primed (controls) and primed seeds of maize and wheat were sown and watered with different concentrations of AMD solutions to assess the response in laboratory settings. The findings of the analysis were obtained by measuring various germination parameters. Analysis of variance (ANOVA; Table 4.1) showed that treatments of maize and wheat respectively, with both the stimulants GA_3 and *B. subtilis* BD234 under acid mine conditions, at different temperatures, were significant at $p<0.05$ for all the measured parameters. Temperature, stimulants, and AMD concentrations substantially affected all maize and wheat seed indices (final germination percentage, germination speed, emergence rate index, shoot and root length and vigour index (Table 4.1). Furthermore, the effects of interactions between experimental factors on these variables were significant. Table 4.1 further reveals that the effects of temperature on various wheat germination indices were not statistically significant at $p<0.05$.

Table 4.1: Output of the factorial ANOVA analysis of the effect of temperature, stimulants, and acid mine drainage on germination indices of maize and wheat seed.

	df	FGP	F-stats	GS	F-stats	ERI	F-stats	SH	F-stats	RTL	F-stats	VI	F-stats
Sources of variation													
						Maize							
T	1	13225.3*	1170.53	13.53*	460.74	715.45*	102.60	111.77*	66.76	185.47*	63.59	370216*	12.08
S	2	1585.8*	140.35	1.12*	38.44	209.21*	30.00	11.63*	6.95	11.56*	3.97	286637*	9.35
AMD con.	4	1687.3*	149.33	1.41*	48.28	163.55*	23.45	28.45*	17.00	42.27*	14.49	1380019*	45.02
T x S	2	806.8*	71.40	0.29*	10.08	174.26*	24.99	6.19*	3.70	19.80*	6.79	492115*	16.05
T X AMD conc.	4	61.9*	5.48	0.17*	5.92	14.71^{ns}	2.11	6.71*	4.01	5.26^{ns}	1.80	75792^{ns}	2.47
S X AMD conc.	8	124.2*	11.00	0.14*	5.06	18.62*	2.67	7.86*	4.70	7.40*	2.54	171232*	5.59
Temp x S X AMD	8	187.0*	16.55	0.10*	3.72	25.17*	3.61	4.53*	2.71	10.96*	3.76	122085*	3.98
Error	60	11.3		0.02		6.97		1.67		2.91		30656	
						Wheat							
T	1	100.3 ^{ns}	2.188	0.0819 ^{ns}	2.13	8.40 ns	2.55	229.76*	154.57	22.201*	35.982	727201*	57.22
S	2	460.3*	10.04	0.3757*	9.80	24.39*	7.41	4.52^{ns}	3.04	13.450*	21.799	351741*	27.67
AMD concentrations	4	918.5*	20.03	0.6942*	18.11	28.63*	8.70	13.49*	9.07	7.066*	11.453	377183*	29.68
T x S	2	1048.6*	22.87	0.8560*	22.33	55.26*	16.79	5.48*	3.69	0.030 ^{ns}	0.049	119965*	9.44
T X AMD	4	80.1^{ns}	1.74	0.0484^{ns}	1.26	2.10 ns	0.63	8.67*	5.83	5.210*	8.444	128702*	10.12
S X AMD	8	102.6*	2.23	0.0793^{ns}	2.06	4.25 ns	1.29	2.07 ns	1.39	1.756*	2.847	47233*	3.71
Temp x S X AMD	8	163.9*	3.57	0.1270*	3.31	5.32 ns	1.61	3.92*	2.64	0.803 ^{ns}	1.302	26685*	2.10
Error	60	45.8		0.0383		3.29		1.48		0.617		12708	

Mean values significantly different at ∗ $p<0.05$ and ns = not significant

4.2.2 Effects of Gibberellic acid (GA_3) on maize and wheat seeds exposed to acid mine water at different temperature regimes

4.2.2.1 Final germination percentage (FGP)

Increasing acid mine water concentrations in the germination media decreased the germination percentage of maize and wheat (Figure 4.1a, b and Figure 4.2a, b). Maize and wheat FGPs were generally higher at 25 °C than 35 °C under various AMD concentrations and GA_3 priming (Figure 4.1a and Figure 4.2a). The FGP process was delayed under AMD concentrations, where the reduction was significantly more pronounced in the non-primed controls than in the GA_3 primed seeds of maize and wheat (Figure 4.1a, b and Figure 4.2a, b). Furthermore, reduction of FGP was more evident at the highest concentrations of AMD (75 % and 100 %). The results showed that the FGP of maize seeds primed with GA_3 was significantly increased by 20 % under 100 % AMD than the control at 25 °C (Figure 4.1a). However, along with increasing AMD concentrations, germination of the primed maize stayed essentially constant or slightly altered. Meanwhile, at 35 °C, the FGP of GA_3 primed maize seeds gradually increased by 77.6 % under 25 % AMD concentrations (Figure 4.1b). At the concentration of 25 % AMD, no significant difference was observed in FGP of wheat primed seeds and control (Figure 4.2a). GA_3 primed wheat seeds exposed to 50 % AMD concentration at 25 °C showed a higher germination percentage of up to 90 % (Figure 4.2a). The same trend was observed at 35 °C with an improved FGP of 83.33 % and 80 % under 0 % and 50 % (Figure 4.2b).

Maize

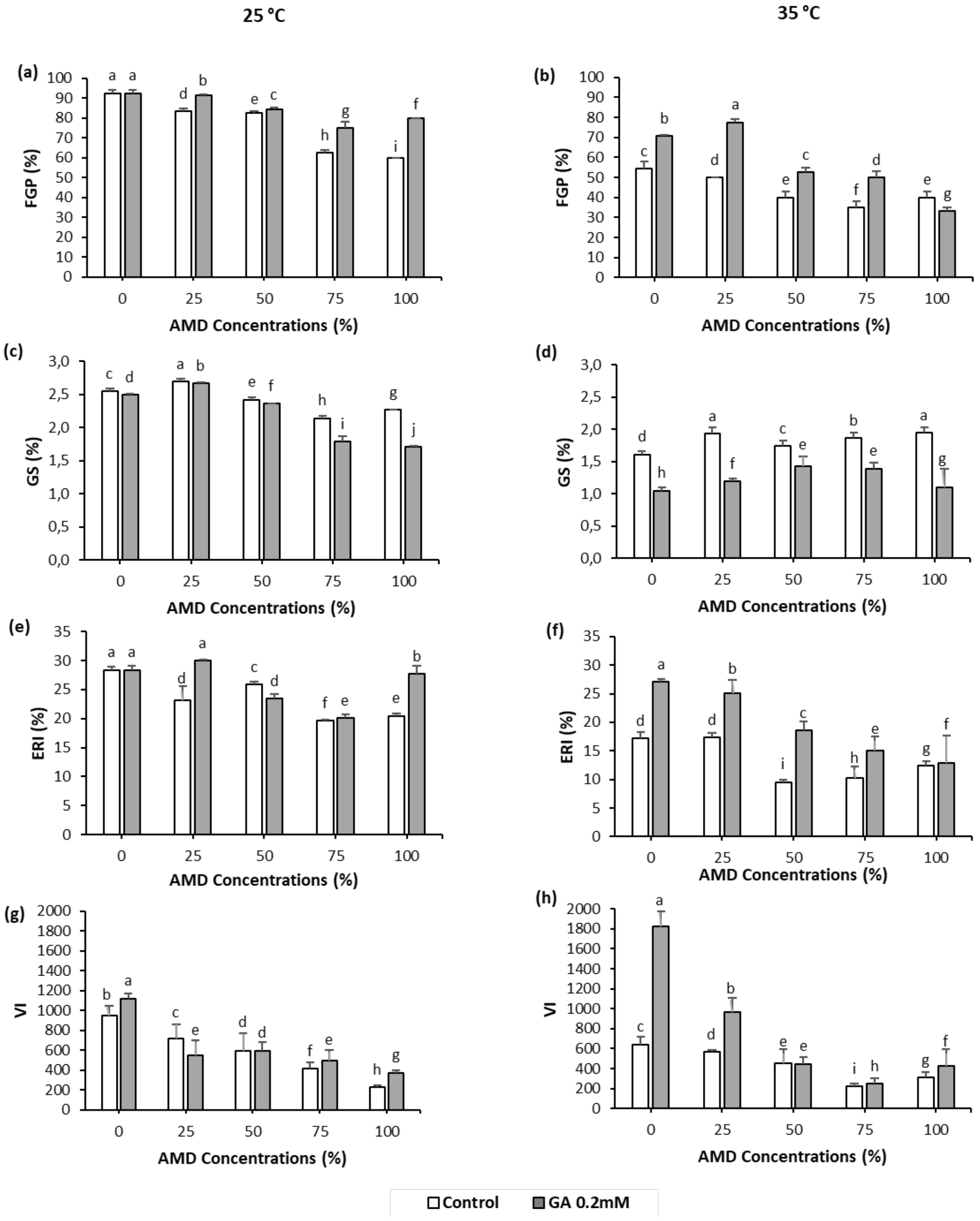

Figure 4.1: Interaction of non-primed (control) and primed (GA_3) maize seeds on final germination percentage (FGP), germination speed (GS), emergence rate index (ERI) and vigour index (VI) exposed to different acid mine concentrations at two temperature regimes. Bars with a different letter(s) show significant differences ($p < 0.05$) between the samples based on Duncan's Multiple Range Test (DMRT).

Wheat

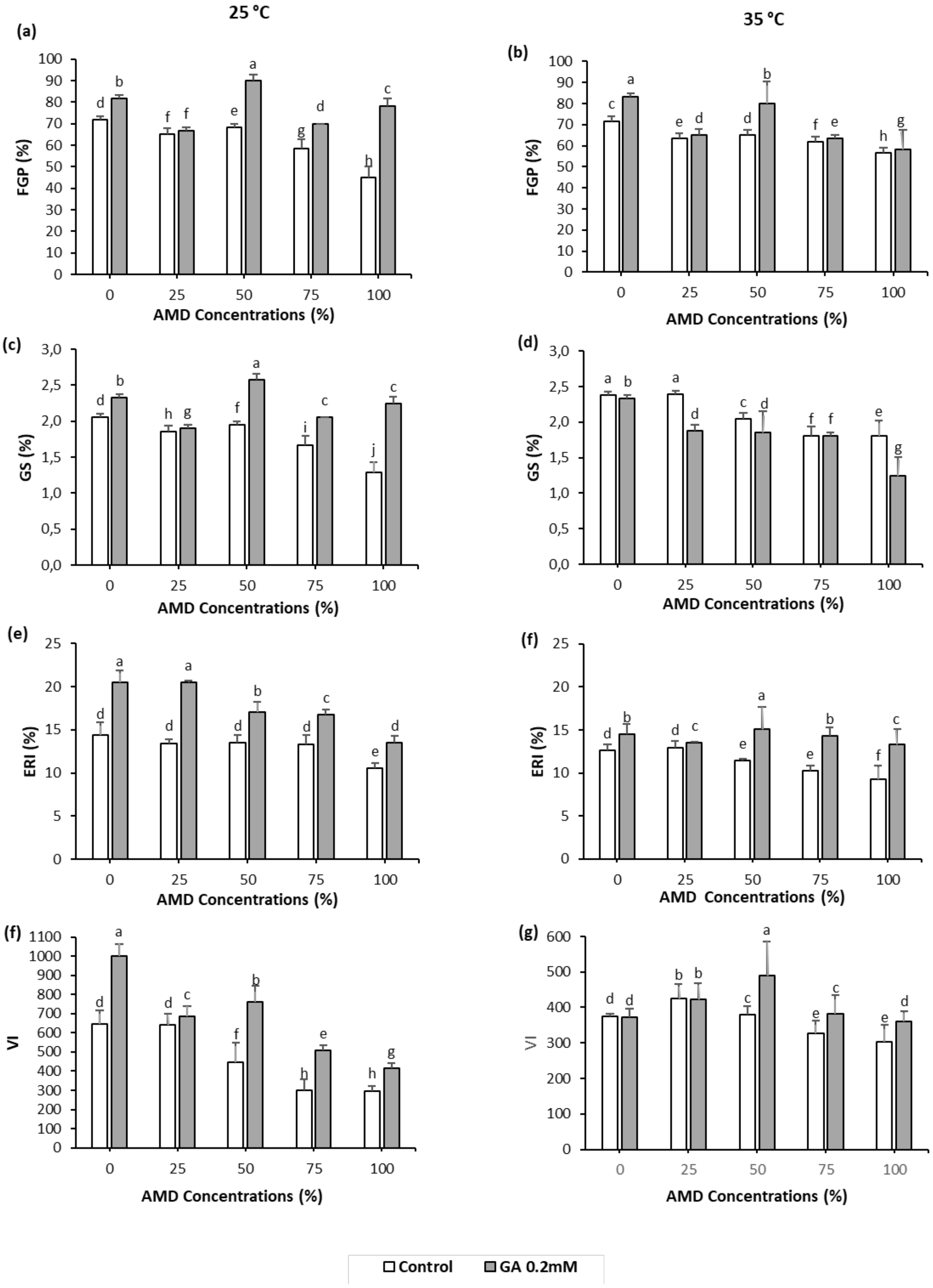

Figure 4.2: Interaction of non-primed (control) and primed (GA_3) wheat seeds on final germination percentage (FGP), germination speed (GS), emergence rate index (ERI) and vigour index (VI) exposed to different acid mine concentrations at two temperature regimes. Bars with a different letter(s) show significant differences ($p < 0.05$) between the samples based on Duncan's Multiple Range Test (DMRT)

4.2.2.2 Germination speed (GS)

The germination speed of the non-primed controls and the GA_3 primed maize (Figure 4.1c, d) and wheat (Figure 4.2c, d) seeds were assessed to measure the maximum number of seeds germinated per day at different temperature regimes. Non-primed controls and GA_3 primed maize germinated faster at 25 °C (Figure 4.1c) than the seeds treated at 35 °C (Figure 4.1d). Conversely, at both 25 °C and 35 °C, GA_3 primed maize seeds under 0-100 % AMD concentrations germinated slower than the nonprimed controls maize seeds (Figure 4.1c, d). The germination speed of wheat results recorded at 35 °C showed similar trends as those recorded on the maize crop experiments. However, at 25 °C, the germination rate of wheat primed seeds showed an interesting trend. The highest germination speed was observed on wheat seeds primed with GA_3 under 50 % AMD concentrations at 25 °C by 2.57 % (Figure 4.2c). Furthermore, the germination speed of primed wheat seeds was improved by 0.95 under 100 % AMD (Figure 4.1c).

4.2.2.3 Emergence rate index (ERI)

Gibberellic acid priming of maize and wheat seeds had effects on the ERI under different AMD concentrations and temperature regimes (Figure 4.1e, f and Figure 4.2 e, f). The ERI of both maize and wheat germinated seeds was higher at 25 °C, compared to 35 °C (Figure 4.1e, f and Figure 4.2e, f). However, there was no significant difference between the control and the GA_3 primed seeds on the ERI of maize under 0 % at 25 °C (Figure 4.1e). The highest ERI of 29.99 % on maize was enhanced by GA_3 under 25 % at 25 °C (Figure 4.1e). Furthermore, there was a gradual increase in the ERI of maize seeds primed with GA_3 under 100 % by 7.27 % compared to control at 25 °C (Figure 4.1e). Even though the GA_3 improved the ERI of the maize at 35 °C, as the AMD concentrations increased, lower ERI was observed on non-primed controls and primed seeds (Figure 4.1f). The study also revealed that GA_3 significantly improved the ERI of maize by 9.86 % and 7.64 %, respectively, compared to the non-primed controls when seeds were treated with 0 % and 25 % AMD concentrations at 35 °C (Figure 4.1f).

On the other hand, the effects of priming wheat seeds with GA_3 at 25 °C showed a higher ERI (Figure 4.2e). The results showed a significant increase of the ERI from

20.50 %, 20.48 %, 17.08 %, 16.77 % and 13.50 % on samples treated under 0 % - 100 % At 35 °C, the high ERI was observed on wheat seeds primed with 25 % of GA_3, compared to other AMD concentrations (Figure 4.2f). Overall, the results suggest that an increase in AMD concentration from 0-100 % decreased the ERI on both the control and primed wheat seeds.

4.2.2.4 Vigour index (VI)

VI results of this study showed the viability and ability of the maize and wheat seeds to emerge and survive under AMD contamination. Reductions in seedling performance were seen on all tested seedling performance traits (Figure 4.1g, h & Figure 4.2g, h). Results showed that the VI of maize and wheat was significantly affected by GA_3, AMD concentration and temperature (Figure 4.1g, h & Figure 4.2g, h). The VI of maize and wheat significantly declined by increasing AMD concentrations. Priming with GA_3 on maize seeds under 25 % AMD concentration significantly decreased the VI compared to non-primed control at 25 °C (Figure 4.1g). Respectively, under 0 % and 25 % AMD concentrations, the results showed no statistical difference between the primed and non-primed controls of wheat seeds at 35 °C (Figure 4.1g). Although certain changes between AMD concentrations were not significant, this study demonstrates that GA_3 might significantly maintain seed viability even in moderately severe AMD concentrations.

4.2.2.5 Seedling growth

In terms of seedling growth, a significant increase in shoot and root length of maize and wheat on primed seeds was observed. However, the exposure of germinated seeds to higher AMD concentration caused stress and decreased shoot and root length on non-primed controls of both crops. The GA_3 primed shoot and root lengths of maize exposed to 100 % AMD concentration at 25 °C was 1.83 cm and 3.17 cm, which were longer than the non-primed controls (Table 4.2). Meanwhile, at 35 °C, the shoot length was 3.30 cm over the respective controls (Table 4.2). The root length of GA_3 primed seeds on maize at 35 °C showed a slight decrease from 50 % to 100 % (Table 4.2). The results of shoot and root lengths of wheat resemble the maize's measured parameters. The shoot length of the wheat at 25 °C and 35 °C under 100

% AMD concentrations was 1.43 cm and 1.19 cm, respectively, which was higher than the nonprimed controls (Table 4.2). The root length of wheat was also slightly increased by GA_3 priming under 100 % AMD concentrations compared to the control, which was 2.17 cm and 3.03 cm at 25 °C and 35 °C respectively, (Table 4.2).

Table 4.2: Mean comparison of 10-day seedling growth parameters of maize and wheat seeds primed with GA_3 and non-primed (control) exposed to varying AMD concentrations and temperatures.

conc.	**Shoot length (cm)**				**Root length (cm)**			
AMD (%)	**Maize**		**Wheat**		**Maize**		**Wheat**	
	Control	**GA_3**	**Control**	**GA_3**	**Control**	**GA_3**	**Control**	**GA_3**
				25 °C				
0 %	5.60±0.82 b	6.53±0.83 a	5.60±0.82 b	6.53±0.83 a	5.50±1.32 b	5.70±0.61 a	3.93±0.67 b	4.07±0.55 a
25 %	4.10±0.75 c	2.57±0.81 h	4.10±0.75 c	2.57±0.81 h	4.73±1.22 c	3.47±1.55 h	3.43±0.90 d	3.50±0.70 c
50 %	3.13±1.90 e	3.73±1.30 d	3.13±1.90 e	3.73±1.30 d	4.47±1.05 d	3.63±1.46 g	2.33±0.96 g	3.27±0.47 e
75 %	2.60±1.11 g	3.10±1.05 f	2.60±1.11 g	3.10±1.05 f	3.80±1.06 e	3.77±1.61 f	2.13±0.57 i	2.87±0.71 f
100 %	1.30±0.36 j	1.83±0.65 i	1.30±0.36 j	1.43±0.65 i	3.07±0.42 j	3.17±0.65 i	2.07±0.38 j	2.17±0.31 h
				35 °C				
0 %	5.83±2.71 d	12.80±3.68 a	2.47±0.30 e	2.70±0.26 a	6.67±0.15 d	12.80±1.31a	4.17±0.59 f	4.53±0.21 d
25 %	5.80±1.13 e	6.83±0.65 b	2.12±1.01 f	2.63±0.67 b	6.70±0.44 c	8.47±1.16 b	4.70±0.46 b	4.87±0.67 a
50 %	4.63±1.16 f	4.20±0.60 g	2.03±0.44 g	2.57±0.47 c	5.20±2.43 e	4.67±0.32 f	4.50±0.26 e	4.70±0.84 b
75 %	3.30±1.25 h	2.97±0.78 i	2.00±1.78 h	2.53±0.49 d	4.50±2.20 g	3.27±0.61 j	2.97±0.72 h	4.60±0.85 c
100 %	2.97±1.33 i	6.27±3.17 c	1.17±0.15 j	1.19±0.36 i	4.40±0.10 h	4.37±2.73 i	2.37±0.25 i	3.03±1.72 g

Means with the same letters in each column have no significant difference at $p < 0.05$ (M±SD).

4.2.3 Effects of *B. subtilis* BD234 on maize and wheat seeds exposed to acid mine water at different temperature regimes

4.2.3.1 Final germination percentage (FGP)

The effect of *B. subtilis* BD234 priming on germination percentage of maize and wheat under five levels of AMD at 25 °C and 35 °C were presented in Figure 4.3a, b and Figure 4.4a, b. The FGP of maize at 25 °C showed no significant differences between the primed and non-primed treatments exposed to 0 % - 50 % AMD concentrations. Under 75 % and 100 % AMD, the *B. subtilis BD234* primed seeds increased 9 % and 13.66 %, respectively, compared to the non-primed controls at 25 °C (Figure 4.3a). Even though the FGP at 35 °C was lower than at 25 °C, *B. subtilis* significantly improved the FGP at 35 °C temperature from 0 % to 100 % (Figure 4.3b).

Due to *B. subtilis* BD234 seeds priming, the FGP of wheat varied widely. Wheat seeds primed with *B. subtilis* BD234 under 100 % at 25 °C gradually increased to 76.66 % compared to 45 % FGP on non-primed control (Figure 4.4a). Moreover, at 35 °C, the FGP under 0 % was 88.33 %, which increased by 16.67 % compared to the non-primed control, while under 100 %, *B. subtilis* BD234 slightly increased the FGP of wheat by 8.33 % compared to the non-primed control (Figure 4.4b).

Maize

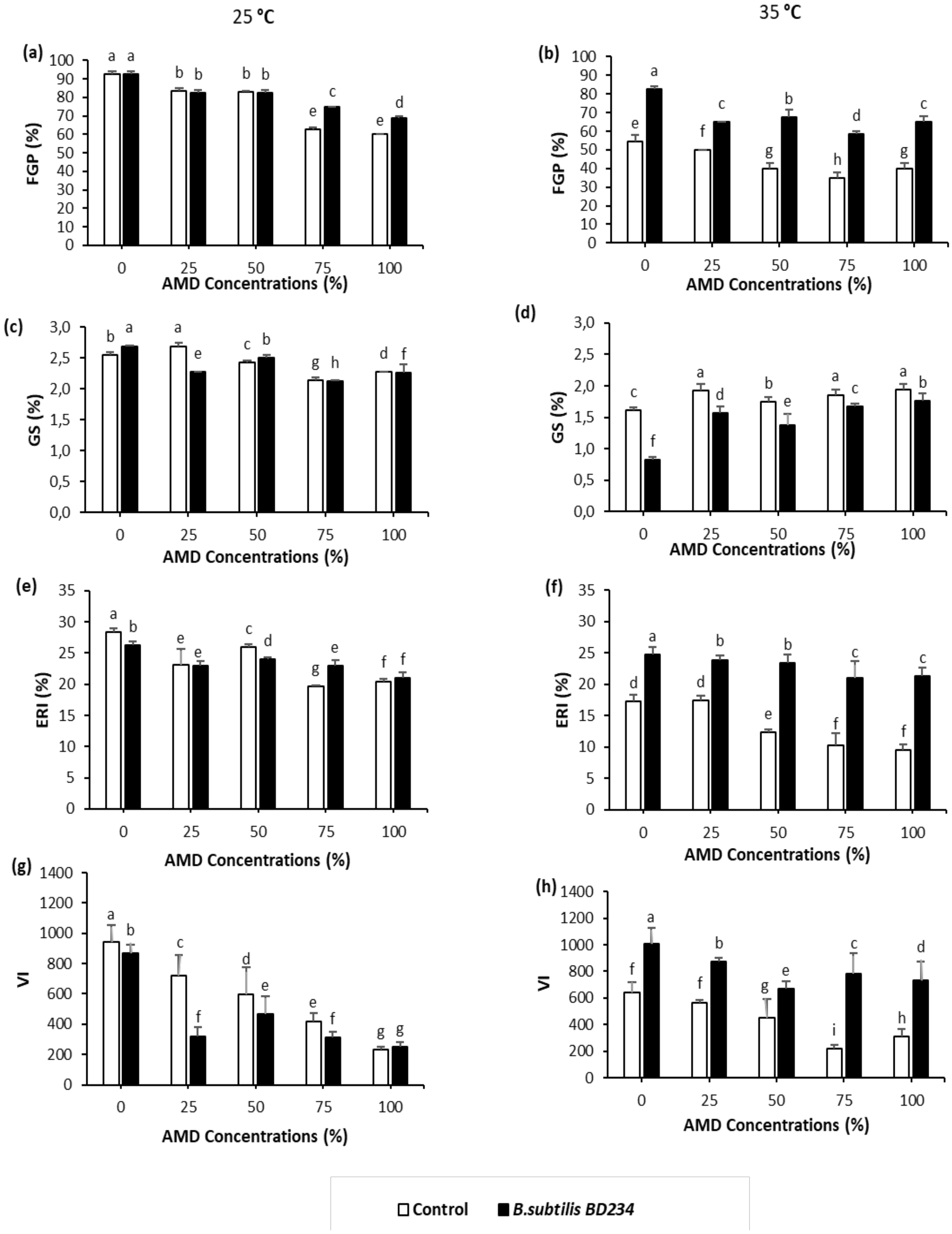

Figure 4.3: Interaction of non-primed (control) and primed (*B. subtilis* BD234) maize seeds on final germination percentage (FGP), germination speed (GS), emergence rate index (ERI) and vigour index (VI) exposed to different acid mine concentrations at two temperature regimes. Bars with a different letter(s) show significant differences ($p < 0.05$) between the samples based on Duncan's Multiple Range Test (DMRT).

Wheat

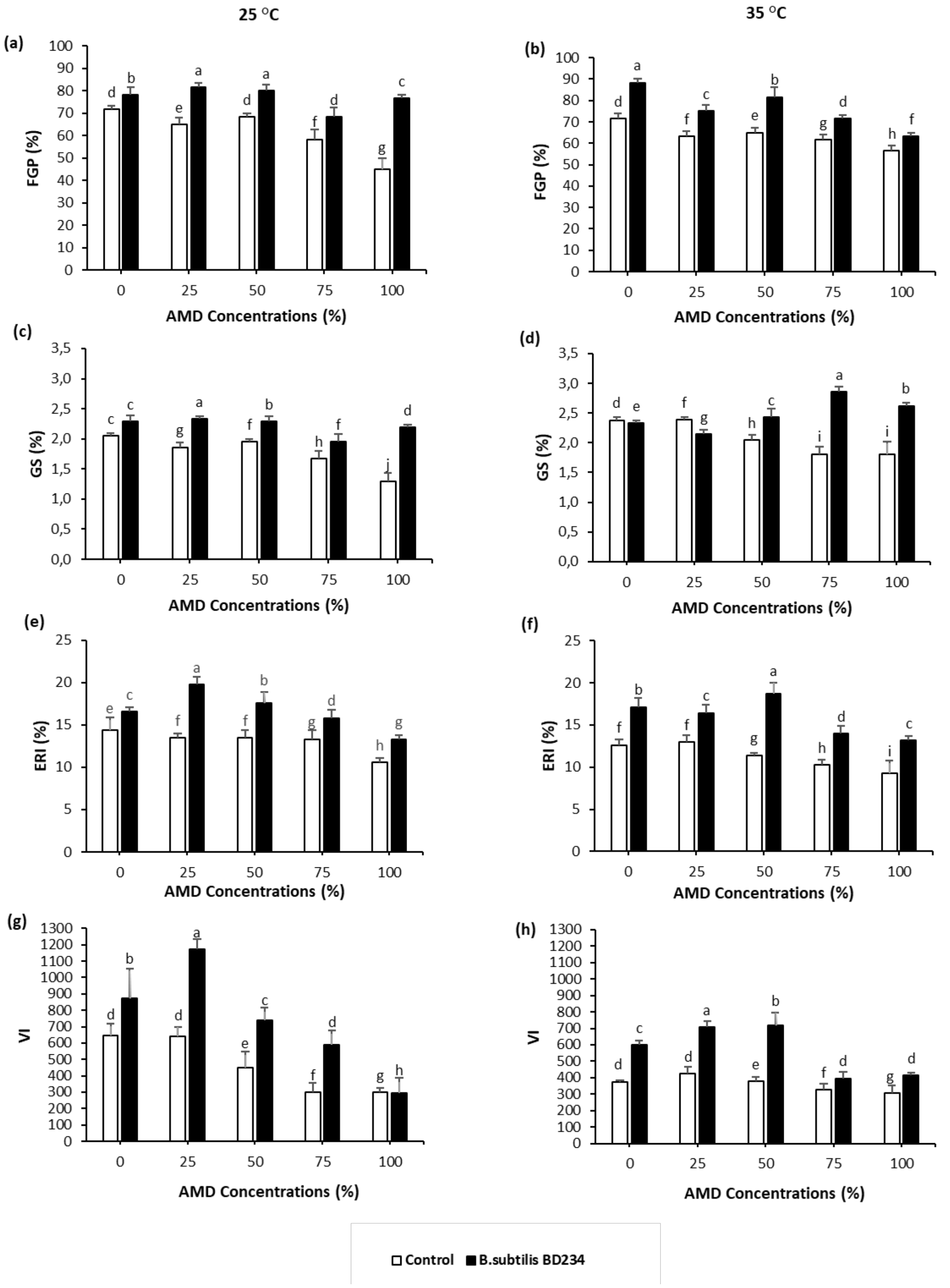

Figure 4.4: Interaction of non-primed (control) and primed (*B. subtilis* BD234) wheat seeds on final germination percentage (FGP), germination speed (GS), emergence rate index (ERI) and vigour index (VI) exposed to different acid mine concentrations at two temperature regimes. Bars with a different letter(s) show significant differences ($p < 0.05$) between the samples based on Duncan's Multiple Range Test (DMRT).

4.2.3.2 Germination speed (GS)

A slight increase in GS of 0.12 % and 0.8 % on maize seeds primed with *B. subtilis* BD234 under 0 % and 50 % at 25 °C was observed (Figure 4.3c). Intriguingly, the GS of maize primed with *B. subtilis* BD234 at 35 °C showed a similar trend to that of the seeds primed with GA_3 (Figure 4.3d). The positive effects of *B. subtilis* BD234 were more evident on wheat seeds. This is because, under 75 % and 100 % AMD, *B. subtilis* BD234 significantly increased germination speed by 2.86 % and 2.62 %, respectively, compared to the control at 25 °C (Figure 4.3c). The germination speed of wheat under 0 % and 25 % slowly decreased by 0.5 % and 0.26 % at 35 °C on seeds primed with *B. subtilis* BD234 (Figure 4.4d). The results also showed that *B. subtilis* BD234 gradually increased the GS of wheat seeds under high AMD concentrations at a high temperature (Figure 4.4d).

4.2.3.3 Emergence rate index (ERI)

Emergence rate index results on primed maize seeds showed an increase under 75 % and 100 % AMD concentrations at 25 °C by 3.32 % and 0.23 %, compared to non-primed controls (Figure 4.3e). Furthermore, there were no significant differences between the nonprimed control and *B. subtilis* BD234 primed maize seeds under 25 % and 100 % AMD concentrations (Figure 4.3e). Interestingly, at 35 °C, there was a significant decrease of ERI on control as AMD concentration increases, while priming with *B. subtilis* BD234 dramatically increased the ERI (Figure 4.3f). On the other hand, Wheat ERI was greatly improved, and higher ERI was observed at 0 % - 100 % AMD concentrations compared to the non-primed controls at both 25 °C and 35 °C (Figure 4.4e, f). However, priming with *B. subtilis* BD234 under 25 % AMD concentration showed to be a more favourable condition with a higher ERI of 19.6 % at 25 °C (Figure 4.4e). Meanwhile, at 35 °C, the higher ERI 18.79 % on wheat was recorded under 50 % AMD concentration (Figure 4.4f).

4.2.3.4 Vigour index (VI)

The lowest value of seedling VI was shown in non-primed controls and *B. subtilis* BD234 primed seeds exposed to 100 % AMD concentration on maize (Figure 4.3g, h) and wheat (Figure 4.4g, h) treatments. At 25 °C, *B. subtilis* BD234 did not enhance the VI of maize throughout all the AMD concentrations. Furthermore, no significant difference was observed between the non-primed and primed seeds under 100 % AMD concentrations (Figure 4.3g). Priming wheat seeds with *B. subtilis* BD234 significantly increased VI on all AMD concentrations at both temperatures compared to the non-primed control (Figure 4.4g, h). The VI highest value of 1173.5 was recorded in wheat at 25 °C, exposed to 25 % AMD concentration (Figure 4.4g). Furthermore, at 35 °C, the lowest VI from primed maize seeds was recorded under 50 % AMD (Figure 4.3h).

4.2.3.5 Seedling growth

Based on the data presented in Table 4.3, an increase in AMD concentrations resulted in a significant reduction in shoot length on maize at 25 °C. Under 75 % and 100 % AMD concentrations, the shoot of maize decreased by 0.89 cm and 0.87 cm respectively on *B. subtilis* BD234 primed seedlings compared to the non-primed controls. The reduction in seedling growth traits of maize was observed when the AMD concentrations increase, and the *B. subtilis* BD234 could not reverse the effect when the stress became severe (75 % and above). Surprisingly, the root length of the maize at 25 °C and 35 °C significantly increased on all primed and non-primed control exposed to 0 %-100 % AMD concentrations.

B. subtilis BD234 priming on wheat seeds showed interesting results at 25 °C, whereby the shoot and root length were improved respectively under all AMD concentrations. Interestingly, the drastic improvement was highly shown at 25 % of AMD concentration, increasing by 2.47 cm and 2.57 cm in shoot and root length. The improvement of shoot and root length of wheat seedlings were also observed at 35 °C under 100 % and were found to be increased to 4.90 cm and 2.43 cm, respectively. Nonetheless, priming with *B. subtilis* BD234 had a major stimulatory effect on all physiological traits over non-primed controls up to a certain AMD level (75 %).

Table 4.3: Mean comparison of 10-day seedling growth parameters of maize and wheat seeds primed with *B. subtilis* BD234 with non-primed (control) exposed to varying AMD concentrations and temperatures.

AMD conc. (%)	**Shoot length (cm)**				**Root length (cm)**			
	Maize		**wheat**		**Maize**		**Wheat**	
	Control	***B. subtilis BD234***	**Control**	***B. subtilis BD234***	**Control**	***B. subtilis BD234***	**Control**	***B. subtilis BD234***
				25 °C				
0 %	5.50±1.32a	3.43±1.05 e	5.63±1.24d	5.77±3.05 c	5.60±0.82d	6.47±0.35 b	3.93±0.67c	5.37±1.11 b
25 %	4.73±1.22b	2.10±0.90 i	5.90±1.61 b	8.37±0.74 a	4.10±0.75 f	6.87±0.67 a	3.43±0.90 f	6.00±0.61 a
50 %	4.47±1.05c	3.00±1.13f g	4.87±2.15 g	5.43±0.71 e	3.13±1.90 g	5.57±1.70 e	2.33±0.96 h	3.73±0.49 e
75 %	3.80±1.06d	2.93±0.42 h	4.20±2.23 h	5.03±1.99 f	2.60±1.11 h	5.30±0.80 f	2.13±0.57 i	3.83±1.40 d
100 %	3.07±0.42 f	2.20±0.61 i	3.03±1.37 j	3.30±0.82 i	1.30±0.36 i	6.30±0.80 c	2.07±0.38 j	2.57±1.33 g
				35 °C				
0 %	6.67±0.15d	6.80±0.10 b	8.10±0.30 d	8.37±0.25 c	5.83±2.71 d	5.90±3.42 c	4.17±0.59 e	4.43±0.32 d
25 %	6.70±0.44 c	6.96±1.01 a	9.03±1.01 a	8.87±1.18 b	5.80±1.13 e	8.73±1.17 a	4.70±0.46 b	5.63±0.85 a
50 %	5.20±2.43 g	5.63±1.89 e	7.00±0.44 e	6.43±1.85 f	4.63±1.16 f	6.13±2.67 b	4.50±0.26 c	4.50±0.36 c
75 %	4.50±2.20 g	5.07±2.45 f	5.47±1.78 g	5.17±0.35h	3.03±1.25 i	3.23±2.76 h	2.97±0.72 g	3.27±1.27 f
100 %	4.40±0.10 i	4.41±0.56 h	4.17±0.15 j	4.90±0.72 i	2.97±1.33 j	3.47±2.42 g	2.37±0.25 i	2.43±0.23 h

Means with the same letters in each column have no significant difference at $p< 0.05$ (M±SD)

4.3 Discussion

Priming agents are one of the most important methods to protect and improve seed and plant growth under contaminated environments. The adjustment occurs when agents such as GA_3 and *B. subtilis* BD234 inhibit pollutants to impact or induce seed germination and seedling growth (Tammam *et al*., 2021; Umar *et al*., 2021). Although priming agents have improved seed and plant growth, their success is not well documented, especially in crops of economic importance. This chapter aimed at determining the effects of seed priming on maize and wheat seed germination indices exposed to acid mine drainage water at different temperatures.

The germination rate of both maize and wheat seeds was substantially reduced when seeds were exposed to increasing AMD concentrations. However, the effect of AMD on seed germination was mitigated when the seeds were primed with 0.2 mM GA_3 (Figure 4.1a, c and Figure 4.2a, c). Compared to primed seeds, decreased germination process and seedling growth quality were observed on non-primed seeds. Temperature also played an important role during the germination and growth stages of the seeds. These results coincided with the results of wheat (Ulfat *et al*., 2017), rice (Chunthaburee *et al*., 2014), and corn (Pallaoro *et al*., 2016) grown under contaminated environments. Seed priming with GA_3 has been shown to stimulate plant development and yield in a range of crops. Similarly, favourable priming effects with GA_3 were observed on maize and wheat's germination and seedling growth parameters (Figure 4.1 and Figure 4.2; Table 4.2).

Gibberellins modulate various physiological and developmental processes in plants (Khandaker *et al*., 2018; Banerjee and Roychoudhury, 2020). Aside from that, GA_3 can also boost the hydrolytic enzymes involved in converting starch into sugar (Tripathi *et al*., 2018). Ardebili *et al*. (2019) showed that seed deterioration lowered germination index and germination percentage compared to non-deteriorated seeds, whereas GA_3 priming enhanced germination index and germination percentage deteriorated seeds. Similar findings were observed in this study on maize and wheat primed with GA_3 postexposure to different AMD concentrations. The priming agent GA_3 enhanced the seeds to overcome contamination and increased the root and shoot length. Moreover, this enhancement could also be related to the absorbed GA_3 activating specific genes for amylase mRNA transcription. Amylase accelerates starch breakdown in the cotyledons and makes accessible monosaccharides to the newly sprouting embryo (Tsegay and Andargie, 2018).

The GA_3 hormone can also activate several additional genes required to produce important enzymes in the germination process. Enzymes like proteases and, in some situations, lipases play essential roles in the embryo's early growth and development (Nawaz *et al.*, 2013). Any increase in these enzymes activity could lead to early, strong germination and quality crop establishment (Tripathi *et al*., 2018). According to Taiz *et al*. (2015), acid phosphatase and ribonucleic acid (RNA) synthesis may rise significantly in embryonic axes and cotyledons of the primed seeds compared to nonprimed seeds. Furthermore, GA_3 signalling pathways can increase seed germination by releasing seed dormancy and counteracting the function of abscisic

acid (ABA), which inhibits embryo development during stressful situations by weakening endosperm and expanding embryo cells (Miransari and Smith, 2014).
On the other hand, *B. subtilis* BD234 strain was also used to prime maize and wheat seeds to enhance seed germination. FGP, GS, ERI, SG, and VI were significantly enhanced by *B. subtilis* BD234 compared to non-primed controls post-exposure to different AMD concentrations (Figure 4.3, Figure 4.4, and Table 4.3). The emergence of early seedling parameters of maize and wheat also revealed that *B. subtilis* BD234 could stimulate seedling and early plant growth. In support of this research, Lastochkina *et al*. (2017; 2020) reported the effects of *B. subtilis* 10^{-4} in boosting plant growth and biomass under saline and *Fusarium culmorum* contaminated environments.

When maize and wheat seeds were exposed to AMD stress, *B. subtilis* BD234 primed seeds had greater root biomass, which might partially explain the observed higher conductance than the non-primed controls. The current findings were consistent with those of Khatri *et al.* (2020), who indicated that *B. subtilis* priming increased seed germination percentage in two wheat cultivars exposed to Cd-contaminated soil. Moreover, Ferreira *et al*. (2018) reported similar findings whereby *B. subtilis* priming promoted maize growth under salinity stress. Our findings are also in line with a study on the potential of *Bacillus* species to boost maize plant output and tolerance to the environment (Junges *et al*., 2013; Nevhulaudzi *et al*., 2020).

An increase in total growth biomass under stress conditions is the most reported plant response mediated by *B. subtilis* inoculation in various crops (Kasim *et al*., 2013). The current study revealed the potential of *B. subtilis* BD234 in alleviating AMD stress in the seedling growth of maize and wheat (Table 4.3). These observations agree with previous reports on the potential of endophytic bacteria in improving plant productivity and enhancing drought tolerance (Mei and Flinn, 2010).

CHAPTER 5

EFFECTS OF GIBBERELLIC ACID AND *BACILLUS SUBTILIS* ON SEED GERMINATION OF COMMON BEAN AND OKRA EXPOSED TO ACID MINE DRAINAGE WATER AND DIFFERENT TEMPERATURE REGIMES

Abstract

South Africa's water supplies are compromised by present and historical mining practices, such as abandoned and closed mines. As a result, Acid mine drainage (AMD) is one of the country's most controversial environmental issues, with unsustainable polluting effects on the water for agriculture. The extent to which it is possible to supplement the agronomic quality of bean and okra production with pesticides and fertilisers without negatively impacting agricultural land is lacking. Consequently, the main objective of this study was to explore the potential of GA_3 and *B. subtilis* application and their mechanisms for enhancing bean and okra seed germination under varied AMD concentrations at 25°C and 35°C. An experiment with a completely random design was done to compare the germination variables of primed and control seedlings. Results indicated that seed germination variables (final germination percentage, germination speed, emergence rate index, and vigour index) were modified. The results demonstrated that seed germination can be influenced by temperature throughout seed development. Germination parameters for common bean and okra were lowered when AMD concentrations were 100 % than when AMD concentrations were 0 % to 75 %. A higher concentration of AMD had detrimental effects on seedling growth while GA_3 *and B. subtilis* had positive effects on all the other germination indicators (final germination percentage; germination speed; emergent rate index; vigour index). Overall, the findings show that priming agents derived from GA_3 and *B. subtilis* could be useful in assisting bean and okra seeds to

5.1 Introduction

Agriculture in Sub-Saharan Africa generates most economic revenue and acts as a foundation for smallholder farmers, generating household income and food (Vidigal *et al*., 2019). The study of crops subjected to acid mine drainage conditions, such as common bean and okra, will be a critical tool for adaptability to environmental pollution. Thus, there are tremendous scientific efforts to strengthen agricultural output under multiple environmental challenges to deal with increasing global food demands (Alshaal *et al*., 2017; Elhawat *et al*., 2018). Acid mine water is one key water stress that triggers plant metabolic and growth abnormalities, severely affecting productivity (Shirinbayan *et al*., 2019). Furthermore, plant development and productivity are impaired dramatically, providing major hurdles to achieving food security.

Throughout this perspective, the use of inoculants such as seed priming needs to be rigorously reviewed. Seed priming is a pre-sowing procedure effectively utilized to promote seed germination (Lechowska *et al*., 2019); and it includes soaking seeds prior to sowing, adding to the hydroponic solution, irrigating, or spraying with plant inoculant solutions (Hasanuzzaman *et al*., 2019). Those strategies have been shown to protect diverse plant species against unfavourable conditions by activating a wide range of activities involved in stress tolerance mechanisms (Horvath *et al*., 2007; Jisha *et al*., 2013). An alternate strategy is necessary to increase plant production high in micronutrients, protein, fibre and antioxidants to promote food and nutrition security (Schreinemachers *et al*., 2017).

Many crops such as common bean and okra are water-stressed because they are grown under AMD contaminated environments (Siddique and Kumar, 2018). Common bean is one of the world's most widely grown crop species, and its high protein content makes it an essential human diet (Barbosa and Gonzaga, 2012). Like some other legumes, the common bean can fix atmospheric nitrogen through a symbiotic interaction with bacteria in the root nodules (Machiani *et al*., 2019). In addition, okra is an alternative vegetable crop that provides high protein, carbs, minerals, vitamins and antioxidants (Petropoulos *et al*., 2018). Apart from gastronomic uses, okra has been linked to various health benefits, including preventing severe diseases like cancer (Adetuyi and Osagie, 2011, Arlai *et al*., 2012, Ghori *et al*., 2014). With changing lifestyles and dietary preferences, producing demand-driven crops is a potential choice for farmers' economic upliftment because they will provide guaranteed markets and reasonable export prices (Sarkar *et al*., 2019).

Understanding how acid mine drainage water impacts seed germination and average crop yields is crucial. There is, however, very little published research on the effects of GA_3 and *B. subtilis* as a seed priming on common bean and okra when exposed to varied acid mine drainage concentrations and temperatures. In light of the importance of common bean and okra seed quality and the influence of seed priming, the objective of this chapter was to evaluate the seed germination and seedling emergence of common bean and okra primed with GA3 and B. subtilis under different AMD concentrations.

5.2 Results

5.2.1 Evaluation of common bean and okra seeds post priming treatment

Germination parameters of primed and non-primed common bean and okra seeds were assessed using ANOVA (Table 5.1). The results showed that AMD concentrations and priming with germination stimulants significantly affected the final germination percentage and seedling growth of common bean and okra (Table 5.1). Interaction of crop type, AMD concentration, stimulants treatment, and temperature also significantly affected seedling growth ($p< 0.05$).

Table 5.1: Output of the factorial ANOVA analysis of the effect of temperature, stimulants, and acid mine drainage on germination indices of maize and wheat seed.

Sources of stats	**df**	**FGP**	**F-stats GS**	**F-**	**ERI**	**F-**	**SH**	**F-**	**RTL**	**F-stats VI**	**F-stats variation**	**stats**	**stats**
						Common bean							
T	1	12.35 ^{ns}	1170.53	0.01 ns	460.74	0.49 ns	102.60	124.40*	66.76	269.71*	63.59	2714618*	12.08
S	2	312.59 ^{ns}	140.35	0.14 ns	38.44	12.62 ^{ns}	30.00	131.14*	6.95	147.39*	3.97	2691826*	9.35
AMD con.	4	998.77*	149.33	0.46*	48.28	37.36*	23.45	195.59*	17.00	44.28*	14.49	2720867*	45.02
T x S	2	542.72*	71.40	0.25*	10.08	16.84 ^{ns}	24.99	152.70*	3.70	16.60*	6.79	1298815*	16.05
T X AMD conc.	4	45.68 ^{ns}	5.48	0.02 ns	5.92	1.89 ns	2.11	48.47*	4.01	32.95*	1.80	523891*	2.47
S X AMD conc.	8	98.40 ^{ns}	11.00	0.05 ns	5.06	3.47 ns	2.67	13.39*	4.70	10.23*	2.54	123701*	5.59
Temp x S X AMD	8	196.42 ^{ns}	16.55	0.09 ns	3.72	8.43 ns	3.61	22.36*	2.71	18.17*	3.76	220703*	3.98
Error	60	161.98		0.02		0.07		1.67		6.58		30656	
						Okra							
T	1	17.63 ^{ns}	2.188	0.01 ns	2.13	21.85*	2.55	0.32 ns	154.57	21.85*	35.982	165009*	57.22
S	2	678.21*	10.04	0.34*	9.80	0.38 ns	7.41	7.34*	3.04	0.38 ns	21.799	52310*	27.67
AMD conc.	4	319.16*	20.03	0.18*	18.11	0.68*	8.70	0.69*	9.07	0.68*	11.453	35052*	29.68
T x S	2	94.39 ^{ns}	22.87	0.19*	22.33	1.45*	16.79	10.16*	3.69	1.45*	0.049	24220*	9.44
T X AMD	4	126.51*	1.74	0.03 ns	1.26	1.30*	0.63	1.44*	5.83	1.30*	8.444	29054*	10.12
S X AMD	8	159.64*	2.23	0.13*	2.06	0.50*	1.29	0.53*	1.39	0.50*	2.847	19537*	3.71
Temp x S X AMD	8	50.90 ^{ns}	3.57	0.03 ns	3.31	0.32 ns	1.61	0.49*	2.64	0.32 ns	1.302	7706 ns	2.10
Error	60	17.63 ^{ns}		0.0383		0.05		1.48		0.17		12708	

Mean values significantly different at $*$ $p<0.05$ and ns = not significant

5.2.1 Effects of GA_3 on common bean and okra seeds exposed to acid mine water at different temperature regimes

5.2.1.1 Final germination percentage (FGP)

The FGP of common bean and okra results are presented in Figure 5.1a, b and Figure 5.2a, b. Concerning seed germination percentage, priming seed with GA_3 under different AMD concentrations at different temperatures showed significant treatment differences. Increasing AMD concentrations in the germination media decreased FGP in both common bean and okra seeds. FGP reduction was highly observed under 100 % AMD concentration on non-primed control treatments of both common bean and okra compared to primed treatments. A higher germination percentage was observed at 25 °C in the common bean exposed to 25 % AMD concentration. This was significantly higher than non-primed control by 26.67 % (Figure 5.1a). Meanwhile, there was no significant difference between GA_3 primed and non-primed common bean seeds under 0 % AMD at 35 °C (Figure 5.1b). Contrastingly, GA_3 primed okra seeds were significantly enhanced on all the AMD concentrations at 25 °C compared to nonprimed seeds (Figure 5.2a). Similar trends were observed in treatments exposed to 0 %, 50 %, 75 % and 100 % under 35 °C, except for the treatment under 25 % AMD concentrations, whereby there was no statistical difference between the primed and non-primed (Figure 5.2b).

Common bean

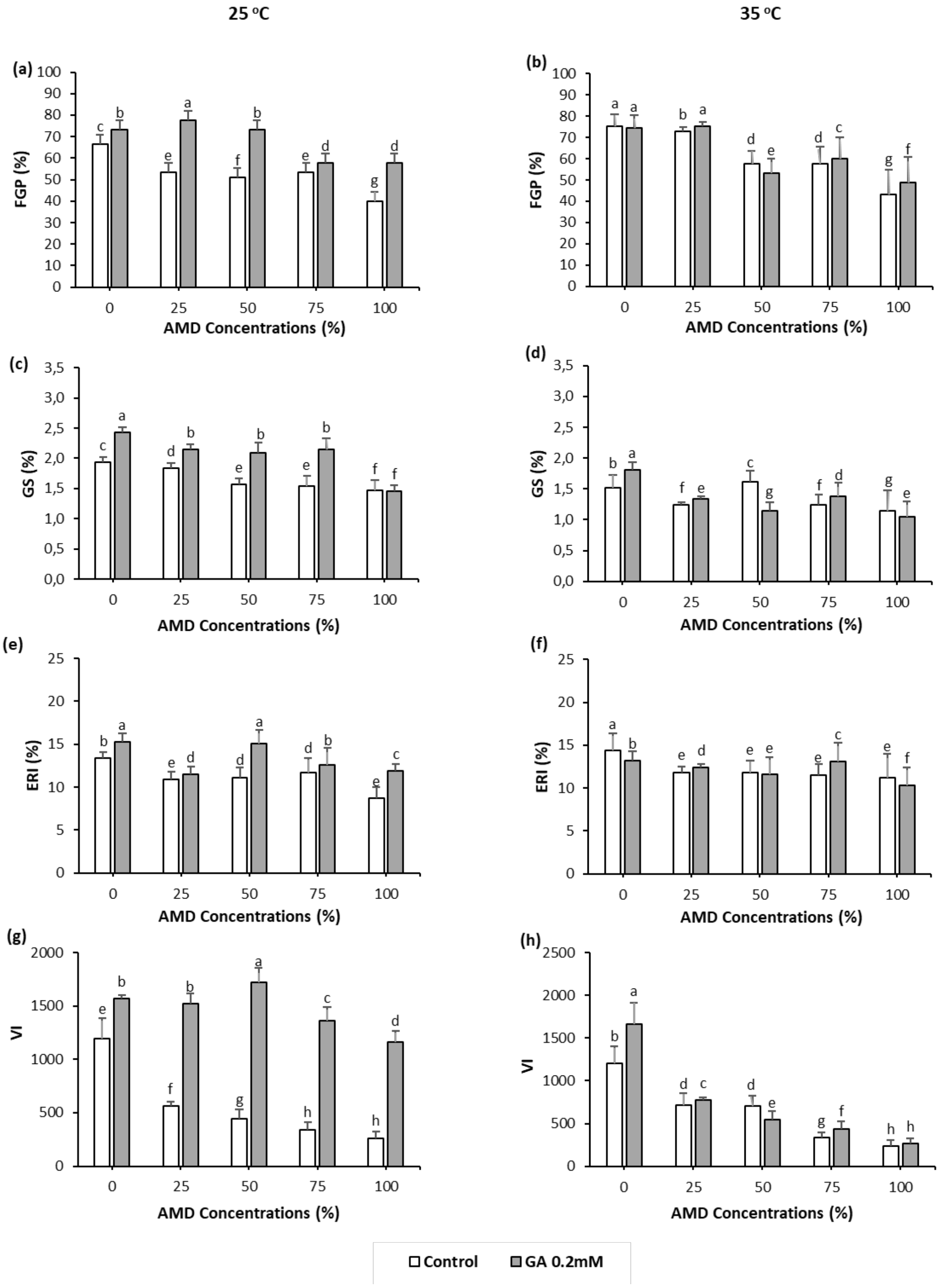

Figure 5.1: Interaction of non-primed (control) and primed (GA_3) common bean seeds on final germination percentage (FGP), germination speed (GS), emergence rate index (ERI) and vigour index (VI) exposed to different acid mine concentrations at two temperature regimes. Bars with a different letter(s) show significant differences ($p < 0.05$) between the samples based on Duncan's Multiple Range Test (DMRT).

Okra

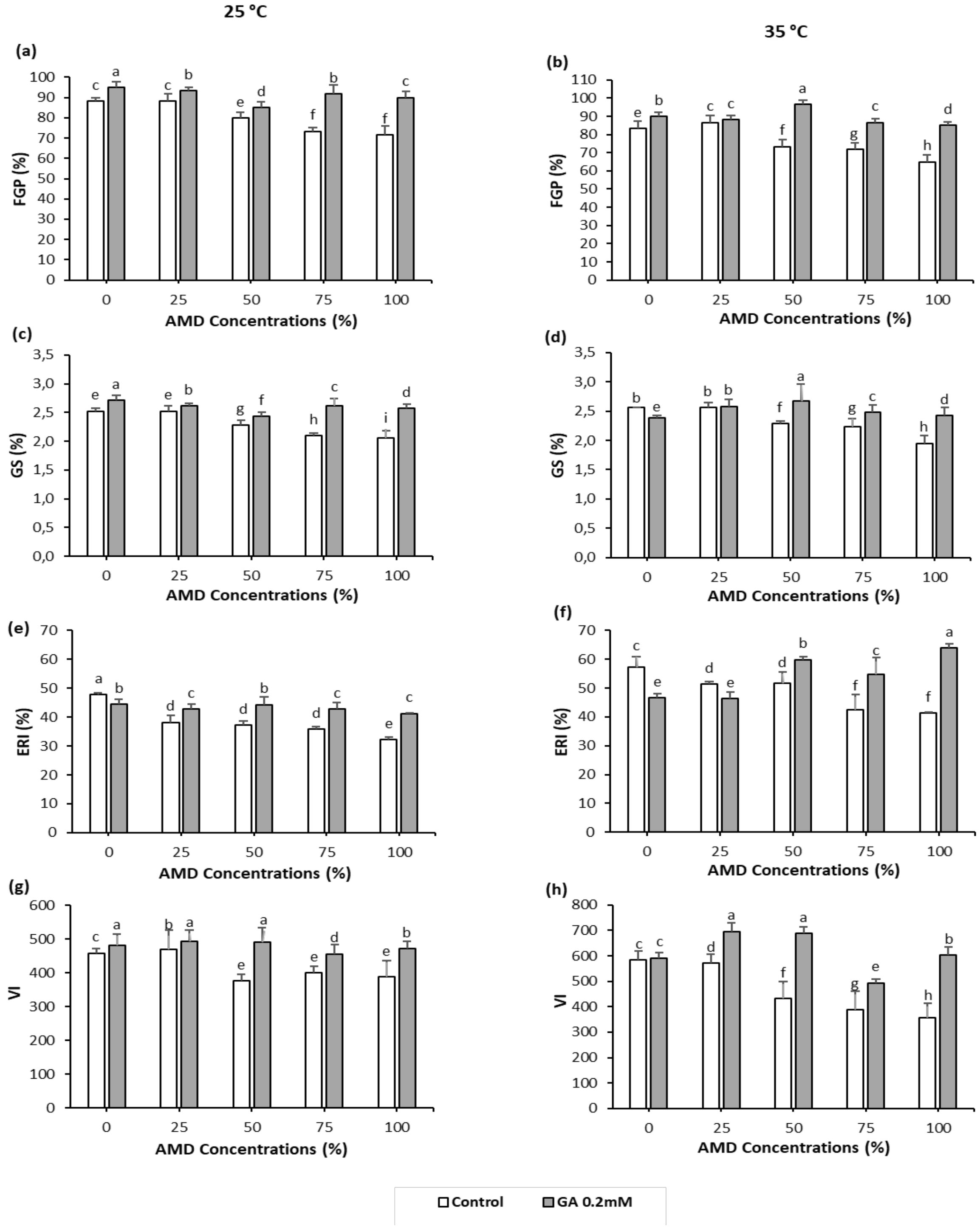

Figure 5.2: Interaction of non-primed (control) and primed (GA_3) okra seeds on final germination percentage (FGP), germination speed (GS), emergence rate index (ERI) and vigour index (VI) exposed to different acid mine concentrations at two temperature regimes. Bars with a different letter(s) show significant differences ($p < 0.05$) between the samples based on Duncan's Multiple Range Test (DMRT).

5.2.1.2 Germination speed (GS)

Results on common bean (Figure 5.1c, d) and okra (Figure 5.2c, d) GS parameters indicated significant differences at different AMD concentrations and temperatures. The progressive decrease in seed germination rate was observed at 0 % to 100 % AMD concentrations on non-primed control seeds of common bean and okra (Figure 5.1c, d and Figure 5.2c, d). A slight increase in germination rate was observed in the primed treatments on all the AMD concentrations. The treated common bean seeds at 35 °C only increased the GP under 0 %, 25 % and 75 % by 1.80 %, 1.33 % and 1.38 %. At the same time, a slight decrease in germination rate of 0.47 % under 50 % AMD and 0.1 % under 100 % AMD was observed (Figure 5.1d).

In okra, seeds showed a significant increase in germination rate at 25 °C compared to non-primed seeds. GS increased in seeds treated with GA_3 exposed to all the AMD concentrations to 2.71 %, 2.61 %, 2.43 %, 2.62 % and 2.57 % compared to the nonprimed which were 2.52 %, 2.52 %, 2.28 %, 2.09 % and 2.05 % (Figure 5.2c). Okra seeds treated with GA_3 at 35 °C geminated faster than the non-primed under 50 %, 75 %, and 100 % AMD concentrations. At the same time, there was no significant difference between the primed and non-primed seeds exposed to 25 % AMD concentration (Figure 5.2d).

5.2.1.3 Emergence rate index (ERI)

In terms of ERI, GA_3 primed common bean seeds showed an ERI maximum of 15.29 % and 15.03 % under 0 % and 50 % AMD concentration at 25 °C (Figure 5.1e). Under 100 % AMD concentration, the primed common bean seeds increased the ERI by 3.15 % at 25 °C (Figure 5.1e). However, ERI decreased by 0.89 % at 35 °C was observed (Figure 5.1f). In okra, a significant increase in ERI was observed in GA_3 primed seeds exposed to 25 %, 50 %, 75 % and 100 % AMD concentrations at 25 °C, yet the ERI on non-primed control was higher than the GA_3 primed seed by 3.37 % under 0 % AMD concentration (Figure 5.2e). Additionally, a decline in ERI was observed on primed seeds of okra exposed to 0 % and 25 % at 35 °C. However, the results showed a significant increase in ERI at 50 %, 75 % and 100 % AMD concentrations in the GA_3

primed seeds, with 50 % and 100 % being the highest recorded ERI increase compared to the non-primed (Figures 5.2f).

5.2.1.4 Vigour index (VI)

The results showed that the VI was higher at 25 °C compared to 35 °C on common bean (Figure 5.1g, h). VI of common bean ranged between 1162.22 -1722.44 at 25 °C (Figure 5.1g). The highest VI was recorded on seeds exposed to 50 % AMD concentration at 25 °C (1722.44) (Figure 5.1g). However, the VI declined significantly by 160.22 at 35 °C on GA_3 primed seeds compared to the non-primed under 50 % AMD concentration (Figure 5.1h). Similarly, GA_3 okra primed seeds increased vigour index at 25 °C and 35 °C exposed to all the AMD concentrations compared to the non-primed control (Fig. 5.1g, h). Nonetheless, 50 % and 100 % AMD seeds showed the highest VI at 35 °C.

5.2.1.5 Seedling growth (SG)

The shoot and root length data of GA_3 primed and non-primed common bean and okra seeds is presented in Table 5.2. The results showed a significantly increased shoot and root length in GA_3 primed common bean and okra seeds compared to the nonprimed control on both temperatures (Table 5.2). There was a drastic increase in shoot and root length of common bean seeds exposed to all AMD concentrations compared to control at 25 °C. Common bean's highest recorded shoot and root length was 11.43 and 15.35 cm exposed to 25 % AMD at 25 °C. At 35 °C, the highest shoot and root length was observed in seeds exposed to 0 % AMD (Table 5.1). On okra, there was a significant increase in shoot length exposed to 0 %, 25 %, 50 % and 100 % AMD; and root length exposed to 0 %, 50 %, 75 % and 100 % AMD at 25 °C (Table 5.1). Furthermore, an increase in root and shoot length was also observed in okra exposed to all AMD concentration, except for shoot length at 35 °C with a slight decrease.

Table 5.2: Mean comparison of 10-day seedling growth parameters of okra and common bean seeds primed with GA_3 with non-primed (control) exposed to varying AMD concentrations and temperatures.

AMD conc. (%)	Shoot length (cm)				Root length (cm)			
	Common bean		Okra		Common bean		Okra	
	Control	GA_3	Control	GA_3	Control	GA_3	Control	GA_3
				25 °C				
0 %	5.63±0.71 f	8.20±0.40 d	2.03±0.10 e	2.50±0.15 a	12.07±0.59 e	13.26±0.93 d	3.43±0.21 e	2.83±0.25 j
25 %	5.90±0.92 e	11.43±0.59 a	2.13±0.20 c	2.23±0.26 b	4.70±0.84 g	15.36±0.90 a	3.90±0.56 a	3.40±0.30 f
50 %	4.20±0.28 g	10.20±0.37 b	2.03±0.20 f	2.07±0.23 d	4.70±0.89 g	13.43±0.66 c	3.50±0.20 c	3.63±0.21 b
75 %	3.03±0.78 i	10.73±0.92 c	1.67±0.15 g	1.63±0.15 h	3.30±0.43 h	13.46±0.63 b	3.23±0.15 g	3.47±0.25 d
100 %	3.87±0.33 h	8.17±0.38 e	1.27±0.15 j	1.50±0.31 i	2.73±0.56 i	11.93±0.43 f	3.13±0.10 i	3.23±0.87 h
				35 °C				
0 %	2.47±0.89 d	10.03±0.17 a	2.43±0.21 d	3.50±0.15a	14.73±0.55 b	15.57±0.73 a	4.43±0.21 d	4.60±0.10 c
25 %	2.03±0.81 g	5.97±0.58 b	2.37±0.15 e	3.17±0.15 b	5.50±0.44 g	11.47±0.11 c	3.10±0.52 f	5.10±0.36 a
50 %	2.00±0.49 h	3.23±0.25 c	2.33±0.44 f	2.43±0.21 d	6.80±0.67 e	7.87±0.55 d	2.13±0.57 h	4.63±0.57 b
75 %	2.10±0.28 e	1.53±0.02 i	2.30±0.10 g	2.53±0.25 c	3.40±0.11 h	5.73±0.08 f	1.90±0.13 i	3.00±0.72 g
100 %	1.17±0.43 j	2.07±0.08 f	2.17±0.10 i	2.27±0.10 h	3.33±0.58 i	3.40±0.11 h	1.83±0.15 j	3.97±0.55 e

*Means with the same letters in each column have no significance difference at $p < 0.05$ (M±SD).

5.2.2 Effects of *B. subtilis* BD234 on common bean and okra seeds exposed to acid mine water at different temperature regimes

5.2.2.1 Final germination percentage (FGP)

The application of *B. subtilis* on common bean and okra improved the FGP compared to the control under different AMD concentrations at both temperatures (Figure 5.3a, b and Figure 5.4a, b). The highest germination percentage on common bean was observed under 25 % AMD concentration at 25 °C (Figure 5.3a). Similar results were recognized on okra seeds primed with *B. subtilis* at 25 °C, under 0 % and 25 % AMD concentrations compared to non-primed seeds (Figure 5.4a). At 35 °C under 0 % AMD, non-primed common bean seeds had higher FGP than seeds primed with *B. subtilis* (Figure 5.3b). Okra seeds were also greatly improved by priming with *B. subtilis* under

100 % AMD with final germination of 90 % compared to 65 % on non-primed (Figure 5.4b).

Common bean

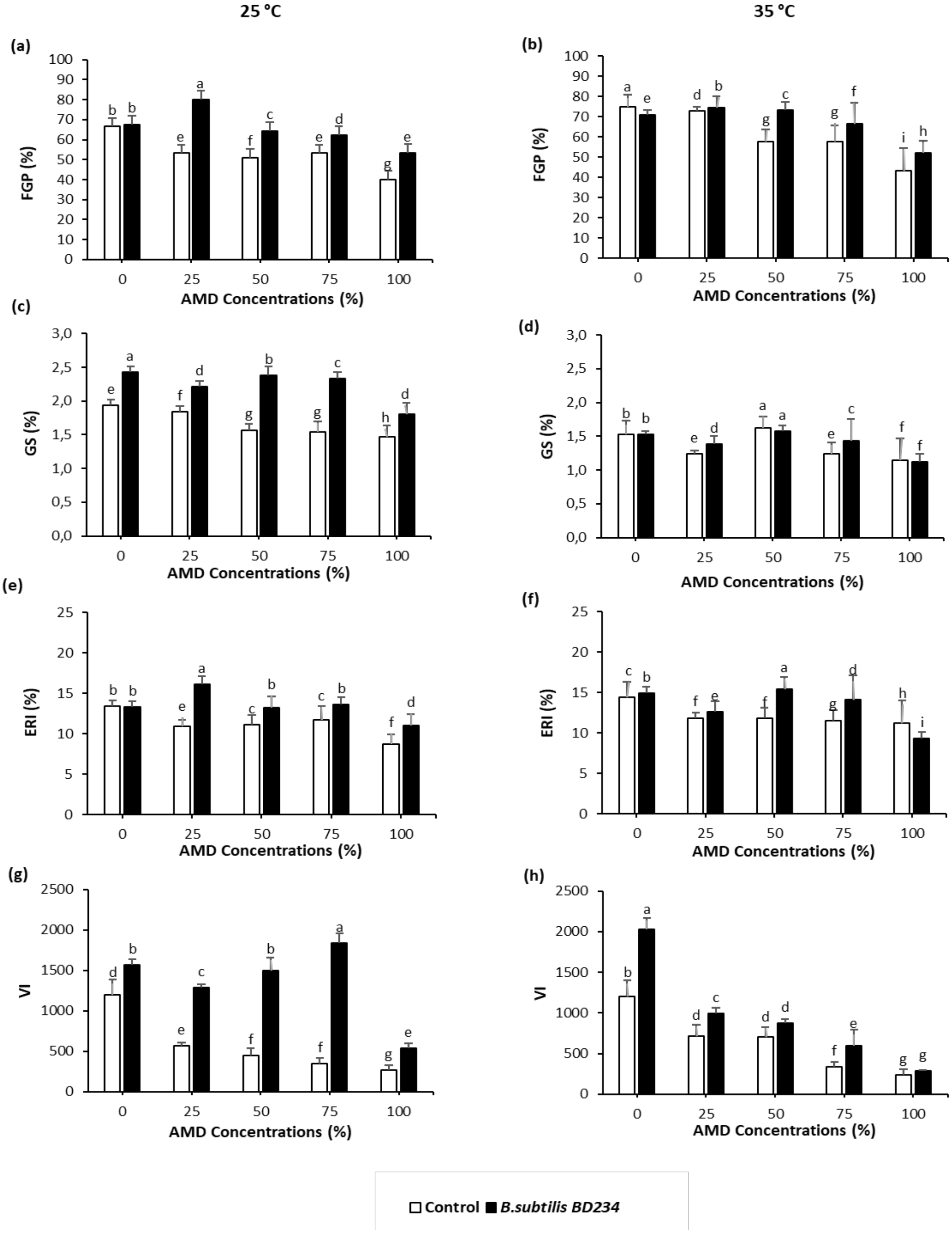

Figure 5.3: Interaction of non-primed (control) and primed (B. subtilis BD234) common bean seeds on final germination percentage (FGP), germination speed (GS), emergence rate index (ERI) and vigour index (VI) exposed to different acid mine concentrations at two temperature regimes. Bars with a different letter(s) show

significant differences ($p < 0.05$) between the samples based on Duncan's Multiple Range Test (DMRT).

Okra

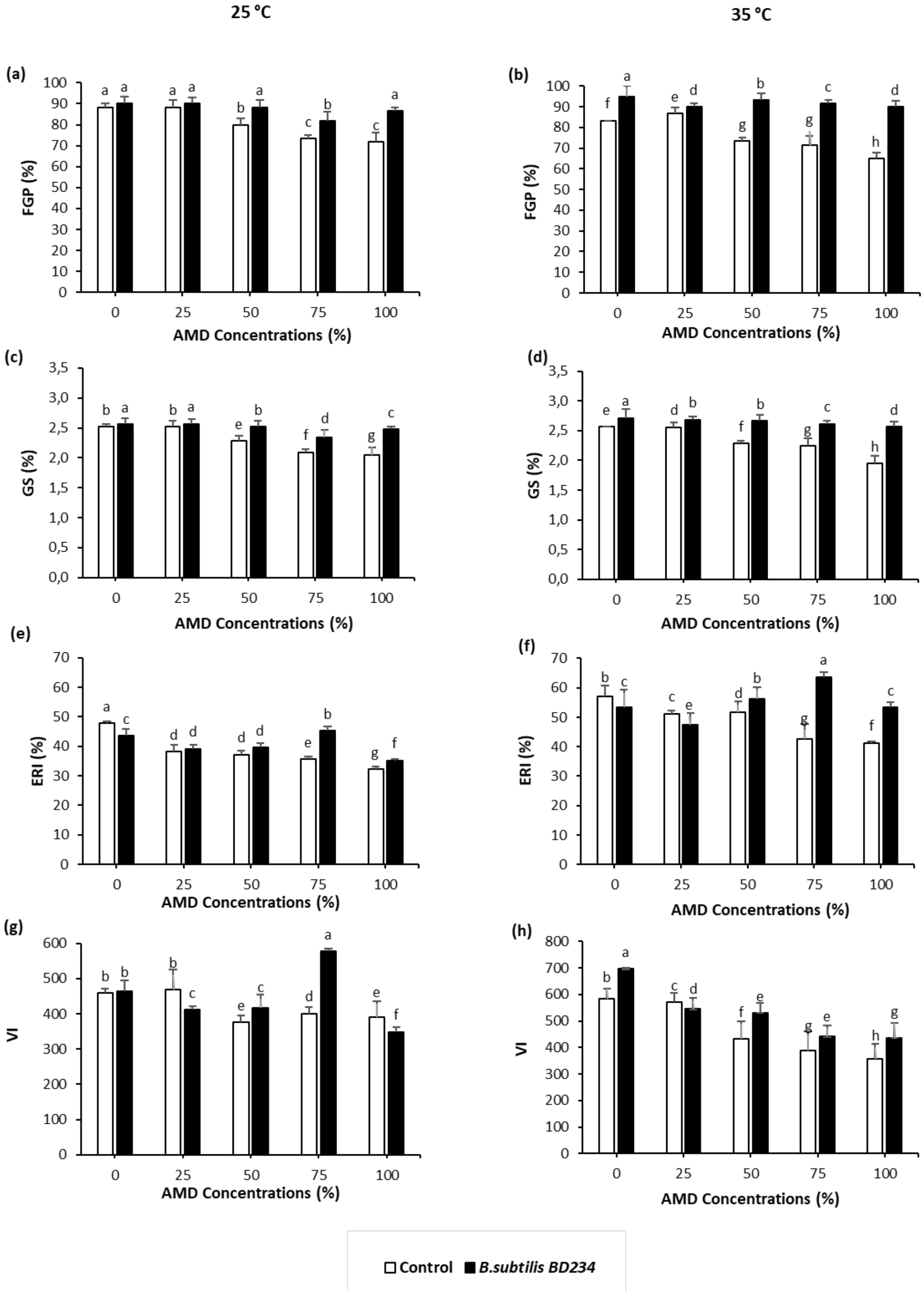

Figure 5.4: Interaction of non-primed (control) and primed (B. subtilis BD234) okra seeds on final germination percentage (FGP), germination speed (GS), emergence rate index (ERI) and vigour index (VI) exposed to different acid mine concentrations at two temperature regimes. Bars with a different letter(s) show significant differences ($p < 0.05$) between the samples based on Duncan's Multiple Range Test (DMRT).

5.2.2.2 Germination speed (GS)

The results of *B. subtilis* application showed significant variations in GS under AMD concentrations on treated common bean and okra seeds at 25 °C (Figure 5.3c and Figure 5.4c). The lower GS of common bean was observed at 35 °C ranging from 1.57 to 1.12 % (primed) and 1.52 to 1.14 (non-primed) compared to the 25 °C germination speed, which ranged from 1.80 % - 2.48 % (primed) and 1.9 % -1.14 % (non-primed). Yet *B. subtilis* improved the germination speed of common bean under all the AMD concentrations compared to the non-primed (Figure 5.3c, d). Similar results were recorded on okra seeds at 35 °C, which showed significantly lower values than 25 °C (Figure 5.4d). Okra primed seeds exposed to low AMD concentration germinated faster than others at both temperatures. (Figure 5.4c, d), while there was no significant germination difference between primed and non-primed seeds exposed to 25 % of AMD at both temperatures at 35 °C (Figure 5.4d).

5.2.2.3 Emergence rate index (ERI)

Common bean seeds primed with *B. subtilis* improved ERI from 25 % to 100 % AMD compared to the non-primed at 25 °C (Figure 5.3e). The highest ERI of 16.11 % was recorded under 25 % compared to the non-primed at 10.88 % (Figure 5.3e). Furthermore, no significant difference was observed at 0 % AMD at both 25 and 35 °C, (Figure 5.3e, f). The results further showed that under 100 % at 35 °C, the non-primed common bean seeds slightly showed an increase of 1.89 % ERI, higher than the seeds primed with *B. subtilis* BD234 (Figure 5.3f). In contrast, the ERI of okra was higher at 25 and 35 °C exposed to 0 % AMD in non-primed control seeds compared to the primed seeds (Figure 5.4e, f), while there were no significant differences between the nonprimed and primed seeds at 25 % and 50 %, at 25 °C respectively (Figure 5.4e). Furthermore, there was a decline in okra ERI under 0 % and 25 % AMD concentrations by 3.91 % and 3.67 %, at 35 °C, respectively (Figure 5.4f). The highest ERI of 63.68 % was recorded under 75 % AMD concentration on okra seeds treated with *B. subtilis* compared to the non-primed of 42.53 % at 35 °C (Figure 5.4f).

5.2.2.4 Vigour index (VI)

Seeds exposed to 0 %; 25 %; 50 %; 75 %, and 100 % respectively and primed with *B. subtilis* significantly improved the VI by 370.67; 722.89; 1053.76; 1493.33 and 276.89 compared to the non-primed at 25 °C (Figure 5.3g). Similar trends were observed at 35 °C, whereby *B. subtilis* increased VI under all the studied AMD concentrations compared to the non-primed (Figure 5.3h). Okra VI under 25 % and 100 % decreased by 58.5 and 41.16 at 25 °C (Figure 5.4g). At 35 °C, the *B. subtilis* improved the VI of the okra under 0 %, 50 %, 75 % and 100 % compared to the non-primed (Figure 5.4h).

5.2.3.5 Seedling growth (SG)

Priming seeds using *B. subtilis* significantly enhanced the root length up to 7.93 cm on common bean and 3.93 cm on okra under 100 % AMD at 25 °C (Table 5.3). Similar trends were observed under 35 °C with a slight increase of 0.9 cm on common bean and 2.9 cm on okra under 100 % AMD compared to the non-primed, respectively. The shoot and root length were enhanced on all AMD concentrations at both temperatures, where the higher the AMD, the lower the shoot length recorded on both crops (Table 5.3). At 25 °C, the lowest shoot length was recorded under 100 % AMD of common bean, which was 6.30 cm on *B. subtilis* BD234 primed seed and 3.03 cm on non-primed seeds, and at 35 °C, it was 2.90 cm on primed seeds and 1.17 cm on non-primed. Meanwhile, on okra, the lower shoot length (1.83 cm) was recorded under 100 % AMD concentrations on primed seed.

Table 5.3: Mean comparison of 10-day seedling growth parameters of okra and common bean seeds primed with *B. subtilis* BD234 with non-primed (control) exposed to varying AMD concentrations and temperatures.

AMD conc. (%)	Shoot length (cm)				Root length (cm)			
	Common bean		Okra		Common bean		Okra	
	Control	*B. subtilis* BD234	Control	*B. subtilis* BD234	Control	*B. subtilis* BD234	Control	*B. subtilis* BD234
				25 °C				
0 %	5.63±0.71 g	8.10±1.27 d	2.03±0.10 e	2.13±0.22 d	12.07±1.59 d	15.37±1.29 b	3.43±0.21 e	3.43±0.43 e
25 %	5.90±0.92 f	8.37±0.42 c	2.23±0.20 b	2.10±0.29 d	4.70±0.89 e	7.80±0.75 d	3.38±0.56 f	3.47±0.06 c
50 %	4.20±0.28 h	10.10±0.72 b	2.27±0.20 c	2.23±0.46 a	4.70±0.89 e	13.20±0.90 c	3.30±0.20 f	3.40±0.36 d
75 %	3.83±0.78 i	12.70±1.30 a	1.67±0.15 g	2.23±0.15 a	3.30±0.43 f	17.03±1.12 a	3.23±0.15 g	3.67±0.23 b
100 %	3.03±0.33 i	6.30±0.47 e	1.27±0.15 h	1.73±0.15 f	2.73±0.56 g	7.93±0.94 d	3.13±0.35 h	3.93±0.15 a
				35 °C				
0 %	2.10±0.17 g	7.03±0.34 a	2.43±0.34 f	3.60±0.21 a	14.73±1.55 b	16.47±1.32 a	4.43±0.21 f	5.10±0.21 a
25 %	2.03±0.58 h	5.53±0.98 b	2.37±0.15 g	3.07±0.12 d	5.50±0.44 e	10.07±1.20 b	3.10±0.52 g	7.16±0.26 b
50 %	2.00±0.25 i	4.77±0.27 c	2.33±0.44 h	3.47±0.15 b	6.80±1.67 d	7.17±0.40 c	2.13±0.57 h	6.40±0.34 c
75 %	2.47±1.02 f	3.17±0.84 d	2.30±0.10 i	2.80±036 e	3.40±0.11 h	5.40±0.55 f	1.90±0.13 i	4.85±0.65 d
100 %	1.17±0.88 j	2.90±0.41 e	2.17±0.10 j	3.00±0.15 c	3.33±0.58 i	4.13±1.13 g	1.83±0.15 j	4.73±0.23 e

*Means with the same letters in each column have no significant difference at $p < 0.05$ (M±SD).

5.3 Discussion

This chapter established the response effects of seed priming on the germination indices of common bean and okra exposed to acid mine drainage water at different temperature regimes. Seeds of common bean and okra germinated even in the presence of high concentrations of AMD, with significant differences among the five concentrations on non-primed and primed seeds. The germination was enhanced by priming with GA_3 and *B. subtilis* BD234, respectively. Our results partially correlate with Saderi and Zarinkamar (2012) study, who reported that high concentrations affect germination while low concentrations show no significant effect.

Based on the reports in the literature, previous studies have shown that the excess of GA_3 in plants induces better and quicker germination (Pawar and Laware, 2018; Guijarro-Real *et al*., 2020; Singh *et al*., 2020; Sappalani *et al*., 2021). For example, the efficacy of GA_3 in enhancing germination and seedling elongation was also demonstrated in chickpea (Mazid, 2014), castor bean (Jiao *et al*., 2019) and mung bean (Navya *et al*., 2021). This study reported that GA_3 had positive effects on the seeds and can regulate plant growth and the development of common bean and okra plants. Massoud *et al*. (2018) concluded that GA_3 had shown promising effect in breaking seed dormancy with accelerated seed germination and seedling growth.

The present study showed that increased AMD concentrations decreased seed germination, and AMD stress-induced conquest of seed germination was alleviated by GA_3 (Figure 5.1a, b and Figure 5.2a, b). A study by Dilip *et al*. (2017) reported that GA_3 could enhance seed germination and the growth of legume seeds under stressful environments, which supports our study on common bean and okra. Chauhan *et al*. (2019) also reported a similar increase in germination percentage of oat cultivars in response to GA_3 treatment under salinity stress. Priming with a suitable concentration of GA_3 played an essential role in inducing tolerance to AMD stress in this study.

The role of GA_3 application in alleviating the adverse effects of stress, including heavy metals, was also suggested by Gangwar and Singh (2011) and Gangwar *et al*. (2011). Such effects include the decrease in the level of ROS and the increase in seed germination rate, growth and nitrogen metabolism. The data provided in this study proved that the supplementation of GA_3 enhanced germination parameters of the common bean and okra seeds exposed to various AMD concentrations. These findings are in line with the literature that reported the involvement of GA_3 in increasing plant tolerance against environmental stress (Chauhan *et al*., 2018; Thongsri *et al*., 2021), to promote plant growth and development, as well as to alleviate growth inhibition under adverse environmental conditions (Rady *et al*., 2019; Abbasi *et al*., 2019).

Furthermore, an increase in shoot and root length was observed on common bean and okra primed seeds during germination (Table 5.2). The findings agree with Noor *et al*. (2017), who found that priming with GA_3 enhanced seedling elongation and dry weight accumulation on French beans. The favourable effects of GA_3 on seedling growth of common bean and okra may be due to increased auxin levels in the roots, which stimulate more nutrient uptake and root cell elongation, and that increase seedling growth, according to Massoud *et al*. (2018).

B. subtilis on the other hand, has gotten much attention because of its catabolic plasticity and capacity to colonize roots and its ability to create a variety of enzymes and metabolites that can help plants thrive under biotic and abiotic stress (GagnéBourque *et al*. 2016). The germination percentage of primed common bean and okra seeds was higher under medium AMD concentrations (25 % and 50 %). Furthermore, common bean and okra germination process was more favourable at 25 °C temperature. Hence, a considerable increase in germination percentage in *B. subtilis* primed common bean, and okra seed was seen in this study (Figure 5.1 and Figure 5.2), which suggested the possible direct influence of *B. subtilis* on seed germination. The current results correlated with Walia *et al*. (2014), who observed an increase in germination and dramatically improved plant height and root length of tomato seeds in soil inoculated with *B. subtilis* strain CKT1. The present study also revealed that *B. subtilis* BD234 decreased the AMD concentrations on common bean and okra seeds. This is consistent with Abd_Allah *et al*. (2018), who showed that inoculation with *B. subtilis* ameliorated the NaCl-induced adverse effects on plant growth of chickpea. Overall, *B. subtilis* has shown its beneficial effects in enhancing germination and seedling growth on common bean and okra seeds, suggesting its potential in plant production and seed establishment.

CHAPTER 6

GENERAL DISCUSSION AND RECOMMENDATIONS

AMD contamination is a global environmental issue that significantly impacts plant growth and production and seed management. However, priming agents such as GA_3 and *B. subtilis* could be a novel strategy to improve AMD-contaminated plants. Previous studies have reported the beneficial effects of GA_3 and *B. subtilis* in enhancing and protecting plants from different environmental stresses (Walia *et al*., 2014; Abd_Allah *et al*., 2018; Chauhan *et al*., 2018; Abbasi *et al*., 2019; Rady *et al*., 2019; Thongsri *et al*., 2021). However, little or no studies have been reported in common bean, maize, wheat and okra exposed to AMD contamination.

In this study, GA_3 and *B. subtilis* priming agents were utilized because they have been reported to enhance seed germination and protect against stressful environments. This study showed that seed priming could help mitigate the adverse effects of AMD water contamination on germination characteristics and seedling growth of common bean, maize, wheat and okra. The use of chemical priming agents has also been reported to be effective (Ashraf *et al*., 2018). However, they have harmful effects on the environment. These results have proven that plant growth-promoting bacteria and phytohormones are important in plant protection against environmental contaminants and seed establishment.

The study further assessed GP, GS, ERI, VI and SG of primed and non-primed common bean, maize, wheat, and okra seeds post-exposure to AMD concentrations. GP is used to assess germination speed and uniformity. The higher the value, the stronger the germination potential, which is one of the critical indicators of seed quality. Post priming with GA_3 and *B. subtilis* BD234, the GP of common bean, maize, wheat, and okra treated seeds improved compared with the non-primed controls, with the primed seeds performing significantly better. However, the enhancement is more visible on seeds primed with lower AMD concentrations than higher concentrations. These results agree with other studies with different contaminants on different hosts (Bose *et al*., 2018; Tsegay *et al*., 2018; Rhaman *et al*., 202; Youssef *et al*., 2021).

Additionally, germination speed and percentage were measured to analyse the seed quality. The non-primed and primed seeds exhibited species-specific responses to various AMD concentrations and temperatures, although with minor variances. Seed priming with GA_3 and *B. subtilis* BD234 considerably increased the germination and seedling growth of these crops. The results also demonstrated that seeds might experience metabolic arrest at unfavourable temperatures, emphasising the importance of required specific climate conditions during seed germination. Up to 100 % AMD concentration, priming relieved the inhibitory effect of AMD stress on seedling growth of the examined crops.

At the highest levels of AMD, the inhibition of germination percentage, the delay in the germination speed, emergence rate index and the loss in all other seedling performances were greatly exacerbated. GA_3 and *B. subtilis* BD234 pre-treatment increased the overall final germination, germination speed, emergence rate index, seedling length, and vigour index of common bean, maize, wheat and okra. As a result, this study demonstrated the usefulness of seed priming strategies in AMD contaminated environments to alleviate the adverse effects of acid mine drainage water.

This study validates the use of priming agents such as GA_3 and *B. subtilis* BD234 to enhance seed germination and protecting plants against environmental contaminants. However, to understand the role of these stimulants in plant response to AMD water contamination, the cell contents of some oxidative stress indicators and the antioxidant enzyme activities in germinating seeds need to be determined. Further research is required to investigate the effects of seed priming with GA_3 and *B. subtilis BD234* in cell division during the seed germination on seeds exposed to AMD contamination.

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