Original Research Article

Priming with Methylene Blue Enhances the Antioxidant Properties and Germination Power of Cowpea, Millet and Sorghum Seeds

Abstract

Seed priming is the preparation of plants to overcome stress with the aim of improving the efficiency and rate of germination.The effect of priming cowpea, millet and sorghum seeds with methylene blue (MB) on germination power (GP) was investigated in this work. The seeds were primed by steeping for 6 h in different concentrations of MB solution, germinated over 48 h on wet cotton and the GP determined. Further, soluble protein and total phenolic contents, and the antioxidant properties [scavenging ability for hydrogen peroxide, nitric oxide, hydroxyl radical, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) and iron chelation ability] of the seeds steeped in MB that gave the highest GP were determined. The highest GP was observed for cowpea and sorghum seeds steeped in 80 µM MB solution, and 60 µM MB solution for millet seeds. At these MB concentrations, a faster rate of germination coupled with an increase in soluble protein and total phenolic content was observed. The increase in germination rate was also characterized by enhanced antioxidant properties of the seeds. The results of this study show that priming with MB can be used to improve the germination of these seeds.

Keywords: Antioxidant capacity, *Eleusinecoracana*, Germination power, Methylene blue, *Sorghum bicolor*, *Vignaunguiculata*,

1. Introduction

Seed priming is the preparation of plants to overcome both biotic and abiotic stress during germination by increasing or boosting the plants defense system. Priming strategies such as hydropriming and osmo-priming have been employed for long periods to improve the germination of seeds [1]. In mungbeans (*Vignaradiata*), a faster and more efficient germination was observed upon hydro- and osmo-priming [2]. Similar increases in germination rates have also been observed in canola [3]and rice [4-6] seeds upon priming.

In addition to hydro- and osmo-priming, other priming strategies such solid matrix priming, hormonal priming and biopriming have also been employed to improve seed germination [1]. Chemical priming which involves the treatment of seeds with natural or synthetic compounds is also lately gaining much attention. The use of β-amino butyric acid on *V. radiata* with the aim of increasing tolerance to drought and salt stress has been reported [7]. Growth regulators and salt have also been used to enhance the germination of wheat seed [8]. Other chemical seed priming agents such as ascorbate to improve the drought and salt resistance of wheat have been reported [9-10]. Chemicals such as methylene blue, owing to the presence of the free radical scavenging serial conjugate bonds in its structure, may have the ability to act as a favourable seed priming agent.

Methylene blue (MB), a dye which dissolves in water to produce a deep blue colour, has been shown to have antioxidant properties. In mammalian cells, MB was shown to delay senescence by increasing mitochondrial respiration [11]. This effect was attributed to the ability of MB to scavenge for reactive oxygen species (ROS) [12], thereby reducing oxidative damage to cells [11]. The ability of MB to scavenge for ROS might have a beneficial effect on plants.

Seed germination is accompanied by increased production of ROS as a result of increased respiration during germination [12]. Thus, in this work, it was postulated that through its role in reducing oxidative damage, MB might have a beneficial effect on seed germination. Therefore, the objective of this study was to investigate the effect of steeping seeds in MB solution on the germination power of cowpea, millet and sorghum. Additionally,the effect of steeping in MB solution on the antioxidant properties of the seeds was investigated.

2. Materials and method

2.1 Sample collection and preparation

Certified cowpea (*Vignaunguiculata*), millet (*Eleusinecoracana*, Finger millet) and sorghum (*Sorghum bicolor*) seeds were obtained from a registered Agro-Chemical shop in Cape Coast, Ghana. The seeds were briefly disinfected in 1% hypochlorite solution and washed with distilled water.

Priming was carried out by steeping 500 seeds in different concentrations of methylene blue solution (0, 10, 20, 40, 60, 80, 100 and 150µM) for 6h. The methylene blue solution was then discarded, the seeds germinated on wet cotton and the germination power of the seeds determined after 48h. Three replicate experiments per condition were carried out and the seeds which were not steeped in MB (steeped only in water) used as the control.

In another experiment, seeds were either steeped in MB solution or water (control) for 6h and GP determined every 12h for a duration of 48h. This was only carried out for the MB solution which concentration gave the highest GP for the different seeds. After 48h, the germinated seeds were homogenized in phosphate buffer, pH 7.0 (1:2, gmL^{-1}). The homogenized samples were

centrifuged at 4 000 rpm for 20 min and the supernatant recovered was aliquoted and stored at 20°C for the determination of soluble protein content, total phenolic content, scavenging ability for hydrogen peroxide, nitric oxide, hydroxyl radical, and 2,2-diphenyl-1-picrylhydrazyl (DPPH) as well as iron chelating ability.

2.2 Determination of soluble protein and total phenolic content

Protein content of the phosphate extract of the seeds was determined based on the Biuret assay using bovine serum albumin as a standard. Biuret reagent was added to the seed extract in a 3:2 ratio, incubated at 37°C for 10min and the absorbance measured at 562nm.

The total phenolic content of the phosphate extract of the seeds was determined based on the FolinCiocalteu assay using gallic acid as a standard [13]. Briefly, 0.5mL of the seed extract was mixed with 2.5mL of 10% Folin-Ciocalteu's reagent and 2.5mL of 7.5% $NaHCO_3$ solution. The mixture was vortexed and incubated at 45°C for 45min and the absorbance measured at 765nm.

2.3 Determination of hydrogen peroxide, nitric oxide and hydroxyl radical scavenging ability

Phosphate buffered hydrogen peroxide solution (40mM) was prepared by diluting 30% aqueous hydrogen peroxide ($w.w^{-1}$) in 100mM phosphate buffer, pH 7.4. The hydrogen peroxide solution (0.6mL) was added to the seed extract to a final volume of 3mL. The mixture was incubated in the dark for 10min and the absorbance measured at 230nm. The absorbance of the buffered hydrogen peroxide solution was also measured and the percent hydrogen peroxide scavenging ability estimated [14].

To determine the nitric oxide radical scavenging ability, 2mL of 10mM freshly prepared sodium nitroprusside in a phosphate buffer was mixed with 0.5mL of the seed extract. The mixture was incubated for 150min at 25°C and 0.5mL aliquot added to 0.5mL of Griess reagent. This mixture was mixed and incubated at room temperature for 30min after which the absorbance was measured at 540nm. Gallic acid was used as the positive control [15].

Hydroxyl radical scavenging ability was determined based on the assay of [16]. To varying concentrations of the seed extract, 1mL of iron-EDTA [sodium iron (Fe^{3+}) ethylenediaminetetraacetic acid] solution was added, followed by 1mL of dimethyl sulfoxide (DMSO) (in phosphate buffer) solution and 0.5mL of ascorbic acid solution. After incubation for 15min at 90C, 1mL trichloroacetic acid (TCA) solution was added. Nash reagent (3mL) was added, the mixture was incubated for 15min at room temperature and the absorbance measured at 412nm.

2.4 Determination of iron chelation ability

The iron chelating ability was determined based on the assay as modified by Chai et al. [16]. To 2mL of the seed extract, 0.10mM ferrous sulfate ($FeSO_4$) and 0.25mM ferrozine was added and vortexed, allowed to react at room temperature for 10min and the absorbance measured at 562nm.

2.5 DPPH scavenging ability

The DPPH scavenging activity was determined by adding 0.5mL of methanolic solution of DPPH to varying volumes of the seed extract. The mixture was vortexed and allowed to stand at room temperature for 30min and the discoloration of the purple color was measured at 517nm. Methanol was used as blank and the methanolic DPPH solution as a positive control [17].

2.6 Statistical analysis

Statistical analysis was carried out using SPSS (IBM, SPSS Statistics 20). The Student's t-test and analysis of variance (ANOVA) were carried out to determine the differences among means at significance level of 0.05. The reported data are the means of three independent determinations along with the standard deviations.

3. Results and Discussion

3.1 Steeping in MB enhances germination of seeds

The effect of steeping for 6 h in different concentrations of MB on germination power is shown in Figure 1. After 48 h, the germination power of the control seeds was 73.75, 69.75 and 71.25 % for cowpea, millet and sorghum seeds, respectively. Steeping in varying concentrations of MB had differential effects on the germination power of the seeds and as well on seed type. For cowpea (Figure 1A), the lowest (62.50 %) and highest (89.75 %) GP were observed for seeds steeped in 10 and 80 µM MB solutions, respectively. These GP were significantly different compared to the control seeds, i.e. when steeping was done in water. Similarly, the lowest (67.25 %) and highest (82.75 %) GP for millet seeds steeped in MB were observed at concentrations of 10 and 60 µM (Figure 1B). Similarly, steeping sorghum seeds in 20 and 80 µM MB resulted in the lowest (67.25 %) and highest (82.75 %) GP (Figure 1C).

Figure 2 shows the GP as a function of time determined for the different seeds based on the concentration of MB that resulted in the highest GP. It can be observed that the MB primed seeds always had a higher GP than the control seeds at the different time points, although significant differences were observed only beyond 24 h. In cowpea seeds, the control seeds had GP of 39.50

and 73.25 % compared to GP of 51.25 and 91.25 % for the MB primed seeds after 24 and 48 h of germination, respectively. In both millet and sorghum seeds, higher GP was observed for seeds steeped in MB compared to the control at the different time points. This demonstrates that priming with appropriate concentrations of MB can be useful in enhancing the GP of seeds.

Seed priming has been used to enhance the germination of several seeds [1], including rice [5,6,9] and wheat [4,6]. Among other functions, it is possible that MB priming better enhances the ability of seed embryos to absorb water to help improve germination. Also, MB priming enhanced the breakdown of dormancy leading to improved germination of seedby acceleratingthe release of metabolites such as sugars, amino acids, organic acids and ions, as well as repair of genetic materials, resulting in earlier and faster synthesis of DNA, RNA and proteins [18].

3.2 Effect on steeping in MB on soluble protein and total phenolic content

The effect of MB priming on soluble protein and total phenolic content is shown in Figure 3. The protein content of the control seeds was 18.03, 6.23 and 8.76 g/100 g DW for cowpea, millet and sorghum seeds, respectively (Figure 3A). MB priming resulted in an increase in the protein content for all seeds compared to the controls (Figure 3A). The increased soluble protein content of MB primed seeds can be attributed to increase in enzyme levels as a result of protein synthesis during germination. This increase in soluble protein shows an improved nitrogen metabolism involving the activity of proteolytic and recycling enzymes. MB priming can also ensure proper hydration, which may have resulted in enhanced activity of enzymes that play essential role in germination such as amylases [2].

The total phenolic content of the control seeds was 293.09, 205.06 and 607.59 mg GAE /100 g

DW for cowpea, millet and sorghum seeds, respectively (Figure 3B). Compared to the controls, MB priming resulted in a significant increase in the total phenolic content of all the seeds (Figure 3B). An increase in phenolic and flavonoids content as well as several photosynthetic pigments was observed following priming of rice seeds [19]. An increased protein and phenolic content was also observed in rice seeds primed with selenite [20], and primed*Moringaoleifera* seeds [21].

Germination is an oxidative stress process that is generally associated with ROS production [1]. Thus, the increased phenolic content can be due to the fact that MB priming causes an increase in the production of compounds, such as phenols, with antioxidant properties to help mitigate the oxidative effects of ROS produced during germination. According to [22], the increase and possible accumulation of phenolic compounds could be due to both their ROS scavenging ability and the potential to inhibit lipid peroxidation caused by the an increased in respiration and other metabolic activities due to the germination of seed.

3.3 Steeping in MB enhances the antioxidant properties of seeds

The effect of MB priming on the antioxidant properties of the seeds is shown in Figures 4 and 5. There was a significant increase in the hydrogen peroxide scavenging ability of the germinated seeds following MB priming (Figure 4A). In cowpea seeds, MB priming increased the hydrogen peroxide scavenging ability by about 38 %, while an increase of about 47 and 54 % was observed in millet and sorghum seeds, respectively (Figure 4A).

Nitric oxide scavenging ability of the seeds was affected by MB priming. A significant increase of about 31 % was observed in MB primed cowpea seeds relative to the control. In millet and sorghum seeds, an increase of 13 and 17 % was observed, respectively (Figure 4B). Hydroxyl radical scavenging ability of the seeds also increased upon MB priming (Figure 4C). In cowpea

seeds, MB priming significantly increased the hydroxyl radical scavenging ability by about 44 %, while an increase of 8 and 14 % was observed in millet and sorghum seeds, respectively.

The effect of MB priming on iron chelation ability of the seeds is shown in Figure 5A. An iron chelation ability of 24.75 % was observed for the control cowpea seeds. However, upon MB priming, there was an increase of about 6.25% to an overall total of 31 %. Increases of about 22 and 24 % were observed between the control and the MB primed seeds of millet and sorghum, respectively (Figure 5A). The DPPH radical scavenging activity of all the seeds was affected by MB priming (Figure 5B). In cowpea seeds, a DPPH radical scavenging activity of 52.75 and 61.25 % was observed for the control and MB primed seeds, respectively. Comparing millet and sorghum seeds, an increase of about 11 and 16 %, respectively, was observed upon MB priming.

An essential aspect of seed priming is the management of oxidative stress during germination [1]. Germination triggers a host of metabolic processes such as mitochondrial respiration and β-oxidation, which lead to increased production of ROS. The accumulation of ROS may affect the germination process and, therefore, increasing the action of antioxidant systems that control ROS levels may help promote germination [1].

Methylene blue has serial conjugate bonds in its structure and so can have the ability to scavenge ROS thereby possessing antioxidant properties. This attribute of MB has been reported [11], and may help increase the rate at which ROS are scavenged in seeds, particularly during germination. In tomato, a partial protection against abiotic stress root damage was observed following treatment with MB. This protection was attributed to reductions in hydrogen peroxide and malonyldialdehyde levels in the roots [12]. MB has also been shown to negate the effect of nitric oxide in maize roots [23], and can scavenge nitric oxide radicals. Stohr and Ullrich[24]

demonstrated that MB may have an apoplastic location, where it scavenges NO radicals. In sweet potato tissues, growth stimulation was observed following treatment with MB [25].

4. Conclusions

In this study cowpea, millet and sorghum were used to demonstrate that priming with the appropriate concentration of MB can enhance the germination power of seeds. This increase in germination power is accompanied by an increase in soluble protein content that apparently reflect enhanced synthesis of relevant proteins for the needed metabolic efficiency of the emerging plant. ROS are generated as a result of the enhanced metabolic and respiratory activity of the growing plant, but the oxidizing effects are mitigated through accompanying increase in total phenolic content as well as the general antioxidant properties of the germinating seeds.

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Figures

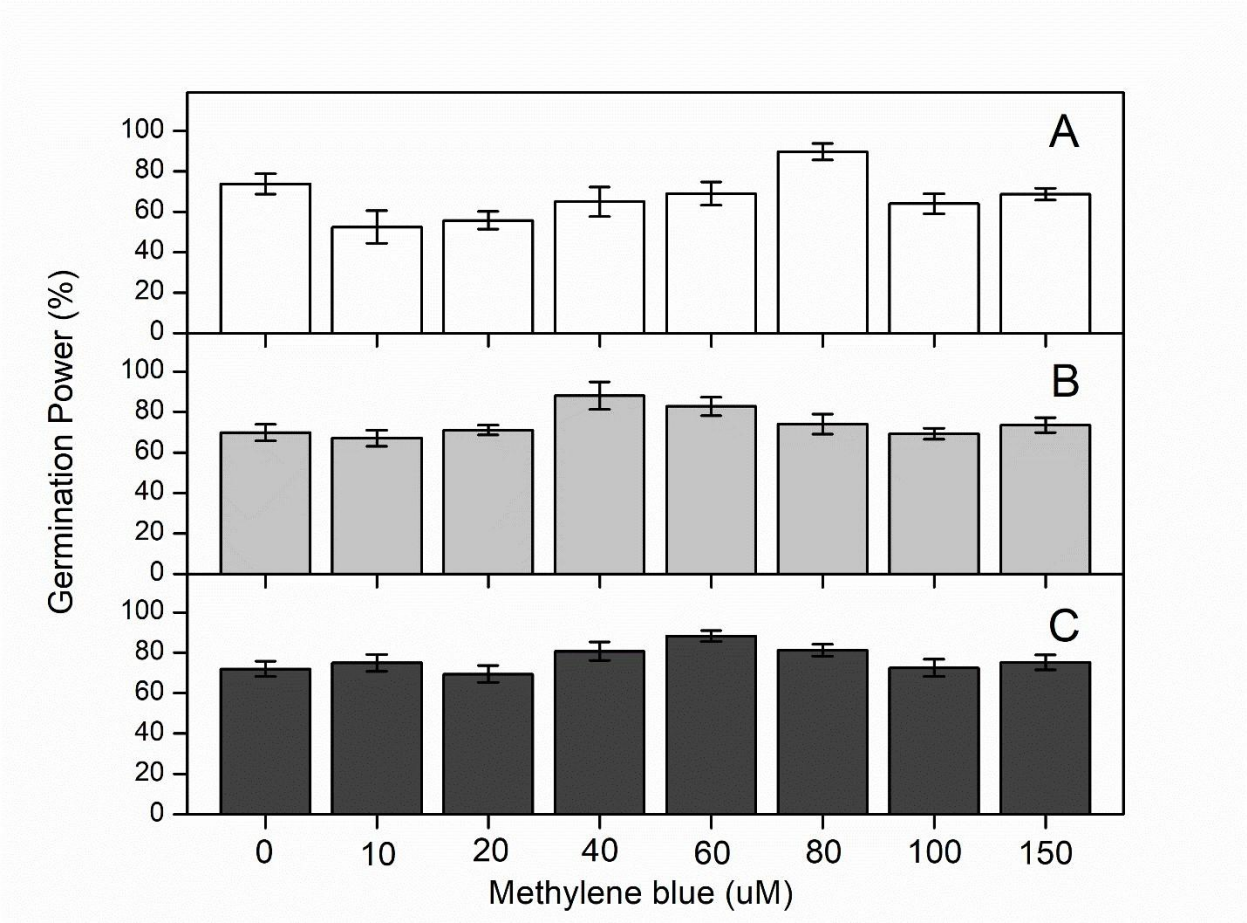

Figure 1: Germination power of cowpea (A), millet (B) and sorghum (C) seeds after 48 h, having previously primed for 6 h in different concentrations of MB.

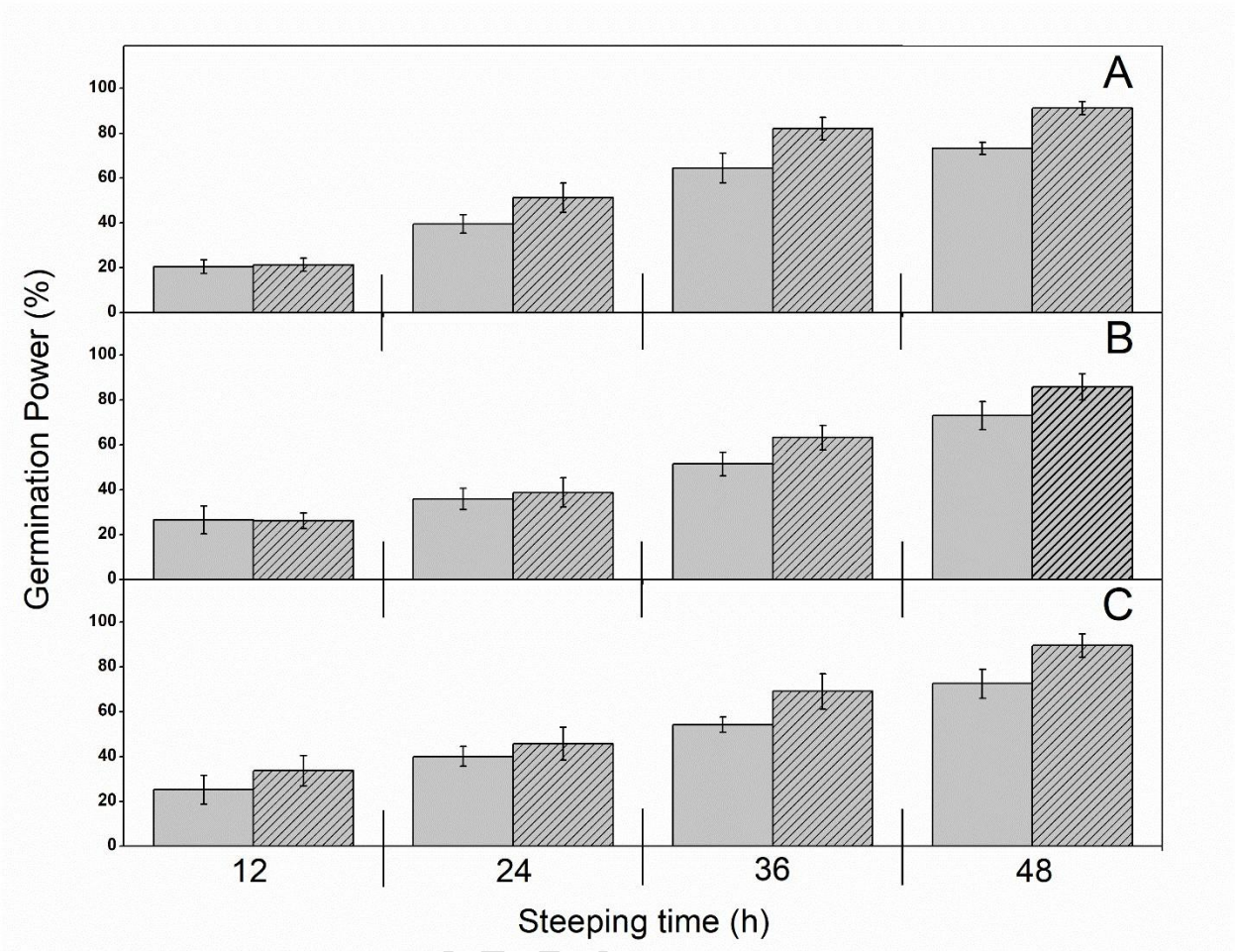

Figure 2: Germination rates of cowpea (A), millet (B) and sorghum (C) seeds primed in MB. The clear bars represent the control seed and the striped bars represent seeds primed in MB solutions that gave the highest germination power.

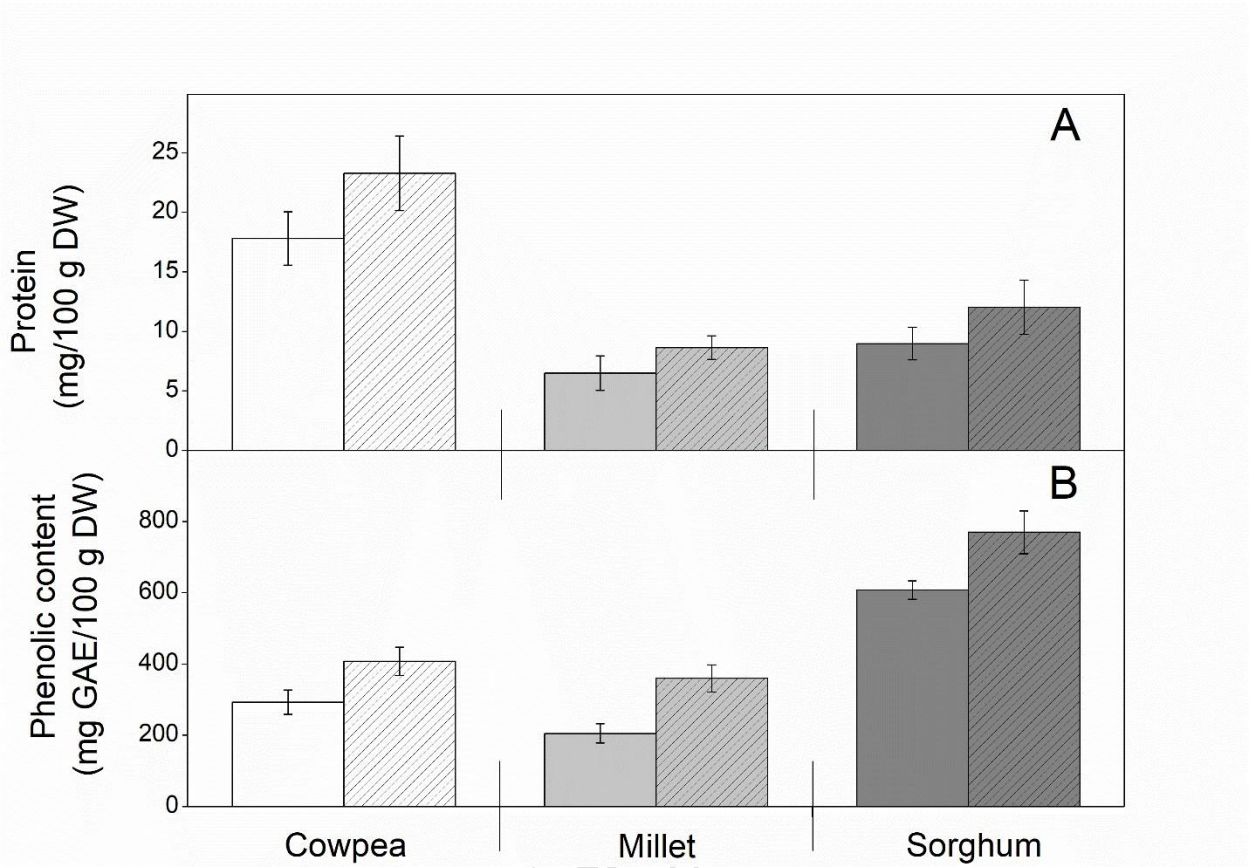

Figure 3: Protein (A) and total phenolic content (B) of germinated cowpea, millet and sorghum seeds. The clear bars represent the control seeds and the striped bars represent seeds primed in MB solutions that gave the highest germination power.

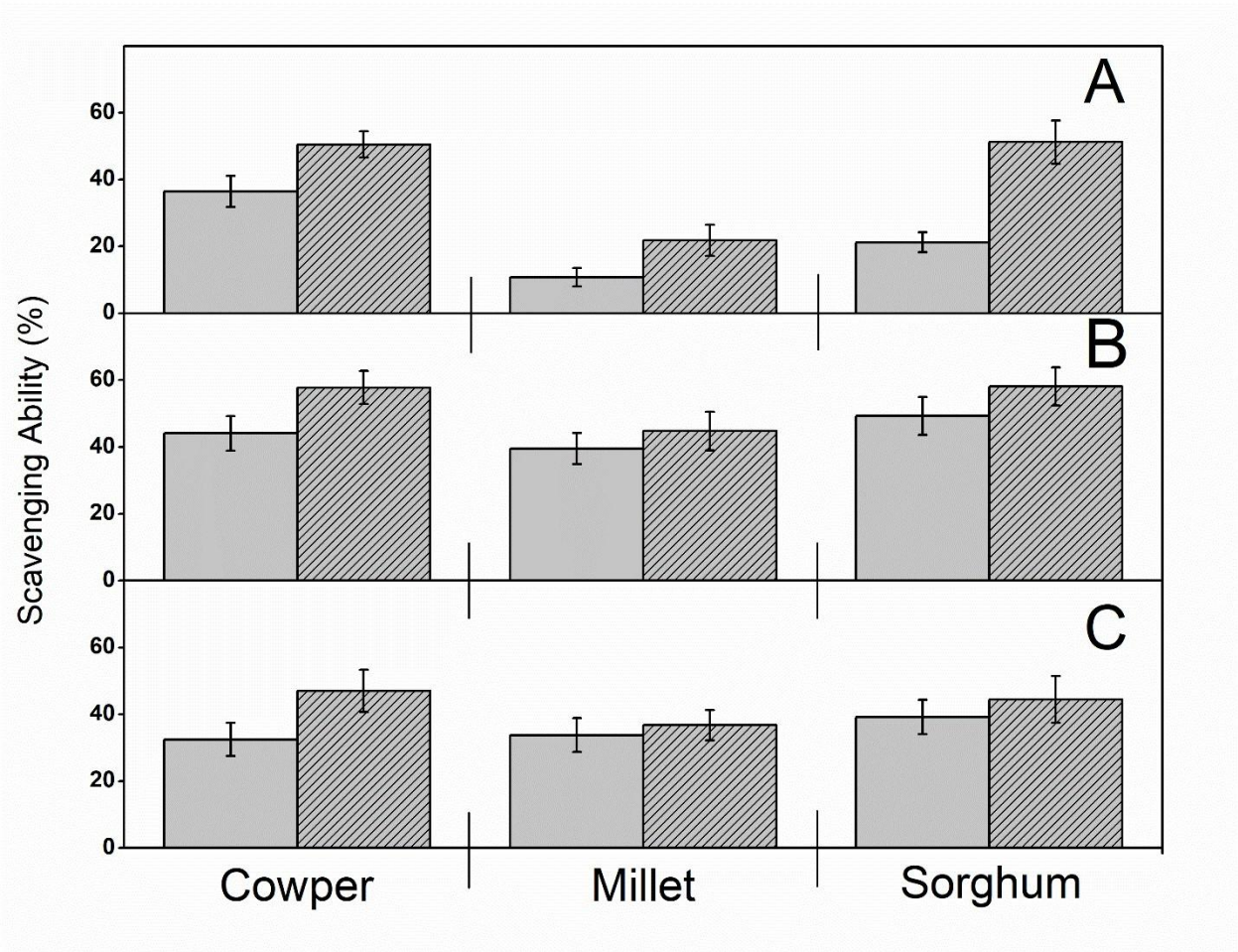

Figure 4: Hydrogen peroxide (A), nitric oxide (B) and hydroxyl radical (C) scavenging ability of germinated cowpea, millet and sorghum seeds primed in MB. The clear bars represent the control seeds and the striped bars represent seeds primed in MB solutions with optimum concentration for the specific seeds.

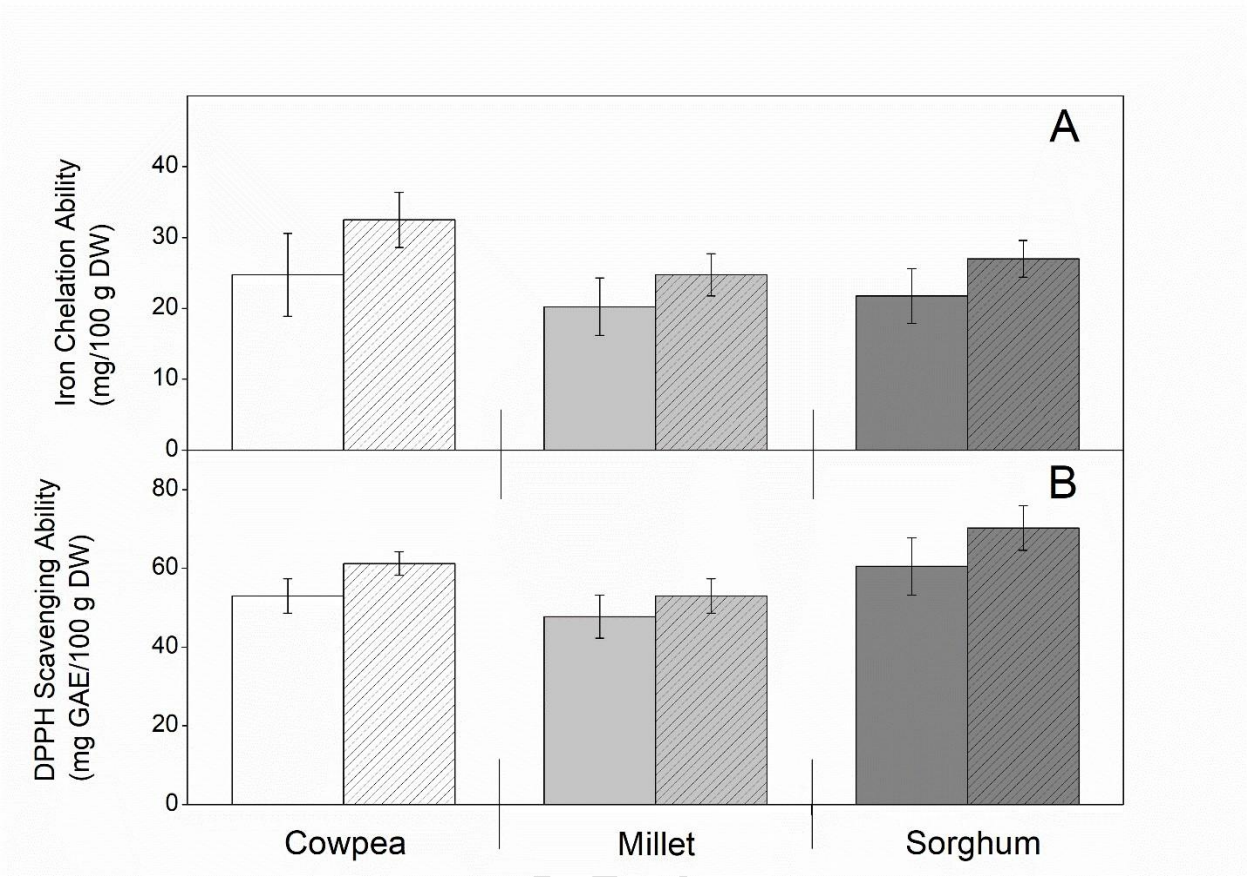

Figure 5: Iron chelation ability (A) and DPPH scavenging ability (B) of germinated cowpea, millet and sorghum seeds primed in MB. The clear bars represent the control seeds and the striped bars represent seeds primed in MB solutions with optimum concentrations for the specific seeds.