Iron-fortification of soybean seeds - effect on phenolic and phtic acid and phosphorous contents and germination rates in soybean sprouts

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ABSTRACT

Background: Iron (Fe) deficiency is considered the most deleterious micronutrient deficiency to plants and human beings especially in developing countries like Cameroon. Fortification of food products is the most effective strategy to combat micronutrient deficiencies in local populations. Soybean (*Glycine max* (L) Merr) is an agronomic crop with high protein and fatty acid contents utilized all over the world. It is the best vehicle of iron-fortification. Successful fortification of soy seedlings with iron has been reported by many authors. Increase loading of iron into these seeds might alert changes in the seed physiology during germination, disruption of the antioxidant system and changes in the synthesis of other biomolecules. The objective of this study was to determine the effects of iron-fortification of soybean seeds on its early germination, growth, phenolic, and flavonoids phytic acid and phosphorus contents.

Materials and method: Soybean seeds were pre-treated with different iron salt concentrations [water (control), 100 and 500mg/l] solution and allowed to grow in the dark , followed by evaluation of changes on germination and growth. Methanolic extract of the seedlings were evaluated for phenolic comtents using Folin-Ciocalteu. flavonoids were measured using iron trichloride colorimetric assay, while phytic acid and inorganic Phosphorus contents were quantified using published spectrophotometric assays.

Results: Pretreatment of soybean with Fe did not affect the growth rates of seedlings. However, seedlings treated with 500mg/L Fe enhanced the germination rate after 48 and 72h (16 and 12%, respectively). the phenolic content of sprouted seeds was significantly increased in response to pretreatment with Iron at concentration of 100mg/L after 72 h. However, Fe-supplemented seedlings did not show any significant influence on the flavonoid contents. In addition, Phytic acid contents were decreased in seedlings treated with 500mg/L Fe after 48h germination period. Supplemented seedlings showed an increase in phosphorus contents throughout the germination period in response to iron at concentrations of 500mg/L . Therefore, an incubation of seeds in Fe solution at concentrations of 500 mg/L is a promising method of reducing the phytate, and improving the Phosphorus contents in soybean sprouts.

Conclusion: The study suggests that preincubation of soy seeds in iron could increase its germination rate, phenolic and phosphorus contents. Iron at concentrations of 100 mg/L is ideal for the increase of phenolic compounds in soybean seedlings. Iron at concentration of 500mg/L can be recommended as the ideal dose to pre-treat seeds prior to sprouting due to its ability not just to promote seed germination and phosphorus synthesis but also concomitantly reduces the phytic acid contents of soybean seedlings. Therefore, fortifying soybeans with iron solution could be a promising strategy of obtaining enriched sprouts with potentials as a source of natural antioxidants and antiphytates in functional food.

Key words; Iron-fortification, soybeans, germination rate, phenolics, phytic acid, phosphorus compounds.

1. Introduction

Iron (Fe) deficiency is considered the most deleterious MND posing a major constraint for both agriculture and human health [1]. Iron is an essential mineral element necessary for proper functioning of tissues in living organisms: it is a component of hemoglobin, myoglobin and other hem- andnonheme compounds. It plays vital roles in oxygen storage and transport, transfer of electrons in electron transfer chains and involves in redox reactions. Reduced Fe levels in human organisms are associated with the development of iron dependent anemia (IDA), alerted immune responses, reduced cognitive ability, hampered physical activity and a general increase in mortality rate[2]. Introduction of supplements, industrial fortification of food products or biofortification of crops are strategies for prevention of mineral deficiency in humans [3,4,5]. As such, fortification of soy foods has been proposed as one of the most cost-effective of most health interventions[6]. Soybean seeds possesses the highest ability to accumulate supplied iron minerals [7]. Iron-fortified soybean sprouts have been proposed as a supplement of this element for iron-deficient animals [8]. Extremely high content of iron has been obtained in soyseeds sprouted in the Ferric sulphate ($FeSO_4$) solutions [9]. Ferric sulphate has been accepted both as a food ingredient (used supplement and food fortificant) and as a component of edible plant fertilizers [8]. Therefore, it could be expected that fortification of soybean sprouts preparation should be absolutely safe for the consumer. However, the extraneous introduction of a new source of iron to the human diet always raises serious concerns about the iron toxicity, especially oxidative stress. Treatment of seedlings with iron solution might increase the production of reactive oxygen species which might destabilize seedling antioxidant machinery [10] thus, encouraging synthesis of important biomolecules [11].However, germination of seeds in iron medium could additionally improve the nutritional value of soybean [12] and can reduce the potentials of iron deficiencies [13]. It has been suggested that, 1 g of iron-fortified sprouts covers 25 or 60 % RDA established for healthy adult woman or man, respectively [14]. Pre-treatment of soybeans seeds with higher concentrations of iron solution might lead to undesirably changes in the seed physiology and biochemistry. Hence, the aim of the present study was to investigate the effects of iron-fortification on germination, growth, phenolic, flavonoids, phytic acid and phosphorus contents of soybean sprouts.

2. Materials and Methods

2.1 Materials and Chemicals

Soybean seeds [*Glycine max* (L.) Merr] (Fabaceae)were supplied by the Department of Genetics and Plant Breeding, Poznan University of Life Sciences.Folin-Cocalteu reagent was purchased from

Merck KGaA (Darmstadt , Germany), gallic acid, Catechin, phytic acid, ascorbic acid, Dodecasodium, ammonium Molybdate and DPBA (diphenylboricacid-2- amino-ethylester) were obtained from Sigma (Aldrich, Germany), iron chloride, Ferrous sulphate, potassium dihydrogen phosphate, was gotten from Poch Basic (Poland), TritonX-100 purchased from Serva Fein Biochemica (New York, U.S.A) and PBS from Bioshop (Burlington, Canada), Quercitin, gallic acid and were gotten from Griffin and George (Wembly Middlesex, England). Methanol was purchased from LobaChemiePvt. Ltd.107, Woodehouse (Mumbai, India) were obtained from the laboratory of Plant Ecophysiology of the Adam Mickiewicz University of Poznan, Poland. All chemicals used were of high analytical grade. Reagents were stored in the refrigerator at 4 ^{0}C and only removed prior to use.

2.2 Methods

2.2.1 Sprouts Preparation

To avoid contaminations of bacteria and fungi, the soybean seeds were surface-sterilized for 5min with 75% ethanol and afterwards with 20% sodium hypochlorite solution for 10 min. Sterilized Seeds were rinsed under running tap water for 30 minutes. The Fe fortification process was done under laboratory conditions in sterilized Petri dishes using the method described by[9]. Briefly, sterilized seeds were soaked for 2 h in 30 ml of distilled water (control) or $FeSO_4$ with Fe at concentrations 100 and 500 mg/L.Each experimental variant consisted of 50 seeds. Thereafter, the seeds were washed with distilled water and transferred to sterilized glass Petri dishes of 30 cm in diameter lined with two layers of lignin and one layer of blotting paper. The seeds were moisturized with 30ml of dH_2O. The Petri dishes were placed in sealed plastic trays, and the seeds allowed germinating in the dark at stable temperature of 21-22 °C.

2.2.2 Biometric analysis of Soybeans seedlings

Germinated Seedlings were harvested after 24, 48 and 72h and evaluated each time for fresh weights germination rate and root lengths.The germination rate was calculated from the germination percentage (GP) formula [15] :

$$GP = \frac{Number\ of\ sprouted\ seedlings}{Total\ number\ of\ seeds} \times 100$$

The root lengths were measured only after 72 h using a millimeter scale ruler and the values used to determine the growth rate.

2.2.3 Quantification of the total phenolic compounds

The Folin–Ciocalteu method (FCM) was used to determine total phenolic compounds in accordance with previously described procedures [16,17] with slight modifications. Briefly, one seedling each weighing about 300mg was homogenized in 2.5ml of 80% MeOH. The extract was collected in Eppendorf tubes and incubated for 15 min at 70 ^{0}C in a digital dry bath, ACCUBLOCK^{TM}. The crude extract was then allowed to cool and centrifuged for 10 min at 11000rpm using mini spin Eppendorf® centrifuge. The resulting supernatant was transferred to glass tubes and the total volume adjusted to 5 ml with 80% MeOH. The reaction mixture in the test tube contained 3.75 ml H_2O, 250 µl of Follin-Ciocalteu reagent and 250 µl of sample (in the blank, the methanol).The mixture was homogenized in an electronic mini shaker, IKA® for 3 minutes and 750 µl of 20% Na_2CO_3 was added. The mixture was allowed to stand for 2h, then, the absorbance was measured at λ=760 against a blank in a UV-VIS spectrophotometer (model BIOMATE 3S, Labomed Inc., USA). A calibration curve was prepared using a standard solution of Gallic acid (0-150 µg/ml). Results were expressed as mg of Gallic acid equivalents per gram of dry weight (mgGAE/g of extract). All samples were analyzed in sextuplet.

2.2.4 Quantification of flavonoids

Total flavonoids content was determined using Aluminium chloride colorimetric method [18,19]. Briefly, 300 mg of sample was homogenized in 2.5 ml of 80% methanol using porcelain mortars. The mixture was transferred to Eppendorf tubes and incubated in a digital dry bath at 37°C for 2h with mixing (by inversion) at 30 min intervals. The mixture was centrifuged for 10 minutes using an Eppendorf centrifuge (model 5415R) at 11 ^{0}C at 12,000 rpm and the resultant supernatant was used to prepare the reaction mixture. To this end in a glass tube, 250 µlof the methanol extract was diluted with 1.25ml of distilled water and 75 µl of 5% $NaNO_2$ solution was added. The mixture was allowed to stand at room temperature for 6 min. Then, 150 µl of a 10% $AlCl_3.6H_2O$ solution was added and allowed to stand for a further 5min after which 0.5ml of a 1M NaOH solution was added. The contents were homogenized in an electronic mini shaker and the absorbance measured immediately against the prepared blank (containing all reagents except for sample replaced with 80% methanol) at 510nm using a UV-VIS spectrophotometer (model BIOMATE 3S). The total flavonoid content was determined using a standard curve with Catechin (0–150 µg/ml) as the standard. TF amounts were expressed as milligrams of Catechin equivalents per 1gram of dry matter of extract (mg/g of extract) from calibration curve. All samples were analyzed in sextuplet.

2.3 Statistical analyses

Results were presented as means ± standard error of Means (SEM) of replicates derived from four (4) independent experiments. The significant differences in relation to the group treated with water (control) were calculated with the use of One factor analysis of variance (ANOVA) by $p < 0.05$ (differences marked with **) and $p < 0.1$ (differences marked with *) with the help of Graphpad Instat software Version 3.

2.4 Results

2.4.1 Germination and seedlings growth Rates

The impact of iron pre-treatment on the germination and growth rates of soybean seedlings is shown in Figure 1. The seedlings pre-treated treated with 500mg/l Fe showed a significant increase in the germination rate at $p<0.05$ after 48 and 72h germination periods (Figure 1a).However, iron-treated seedlings did not influence the growth rate as indicated by a non-significant increase in root lengths throughout the examined germination period (Figure 1b).

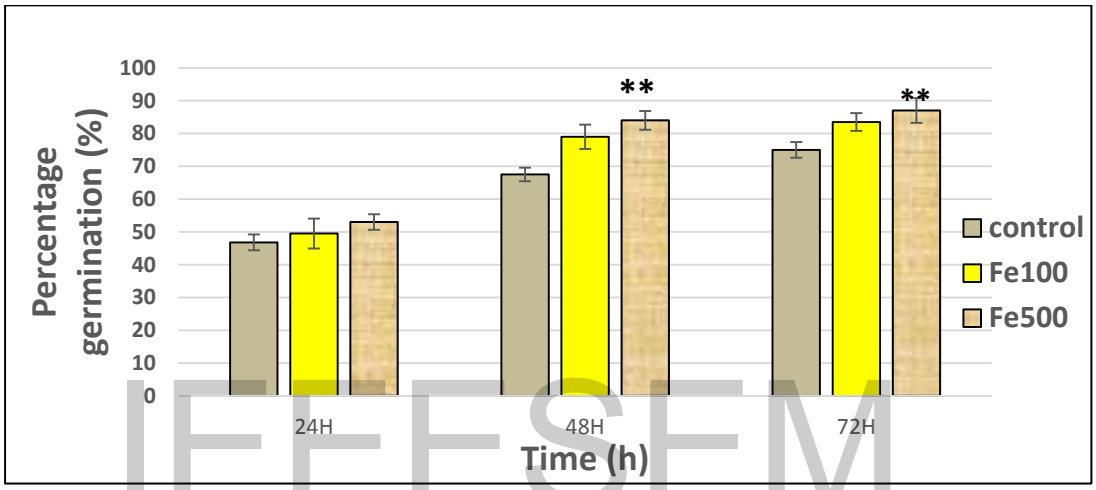

Figure 1a: Germination rate of seeds exposed to FeSO4 solutions with Fe at 100 and 500 mg/L) measured after 24, 48 and 72 h

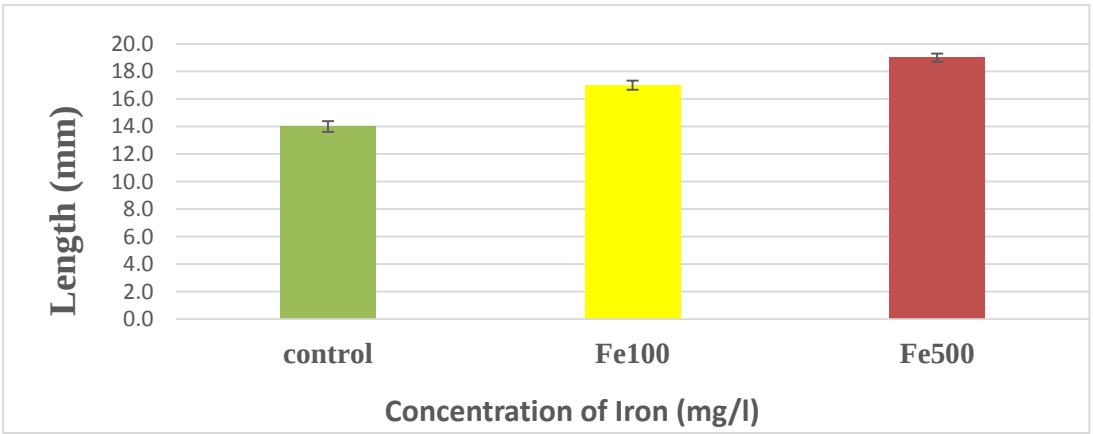

Figure 1b: Root lengths of seedlings exposed to FeSO4 solutions with Fe at 100 and 500 mg/L) measured after 24, 48 and 72 h

2.4.2 Total phenolic and flavonoid contents

The content of Total phenols and flavonoids quantified in the methanol extracts of *Glycine max* (L) Merr whole seedlings are summarized in Figures 2a and 2b, respectively. The extract of soybeans showed a significant increase of phenolic compounds at $p<0.05$ in response to iron at a concentration of 500 mg/L solution (Figure 2a). However, either concentrations of iron did not show any significant difference in the flavonoid contents throughout the examination periods (Figure 2b).

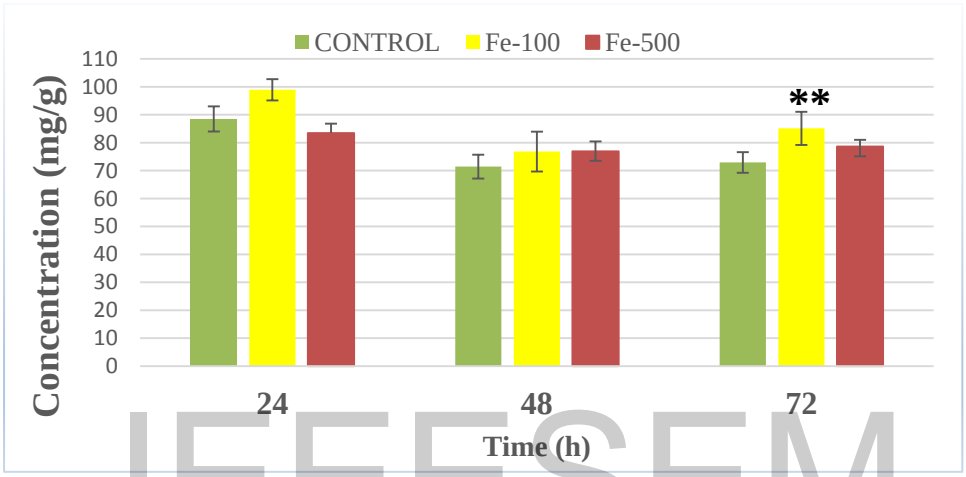

Figure 2a: Total phenolic content of soybeans exposed to FeSO4 solutions with Fe at 100 and 500 mg/L) measured after 24, 48 and 72 h

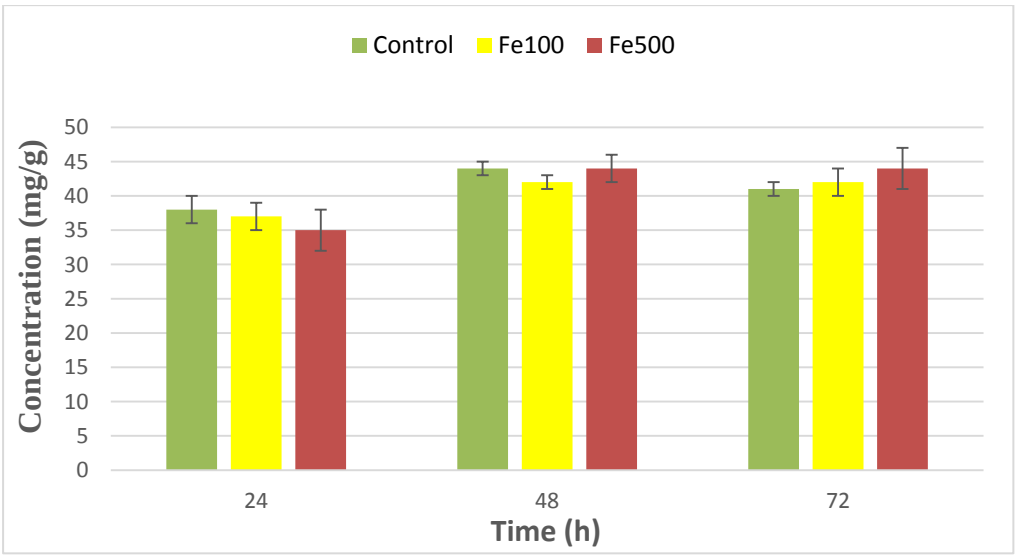

Figure 2b: Total flavonoid content of soybeans exposed to FeSO4 solution with Fe at concentrations of 100 and 500 mg/L) measured after 24, 48 and 72 h

2.4.3 Total phytate and phosphorus compounds

The effect of iron-pre-treatment on the phytate and phosphorus contents is summarized in Figure 3a and 3b. In Fe-supplemented seedlings, the phytate and phosphorus contents show time- and dose-dependent effects. The solution of 100 mg/L Fe shows a decrease in both compounds after 24 h germination time. This effect was reversed after 48h for both and 72h only in the case of phytic acid However, the solution of 500mg/l Fe reduced non-significantly the phytic acid content after 48h, with no effect on phosphorus throughout the germination period. Water tends to decrease periodically the phytic acid content.

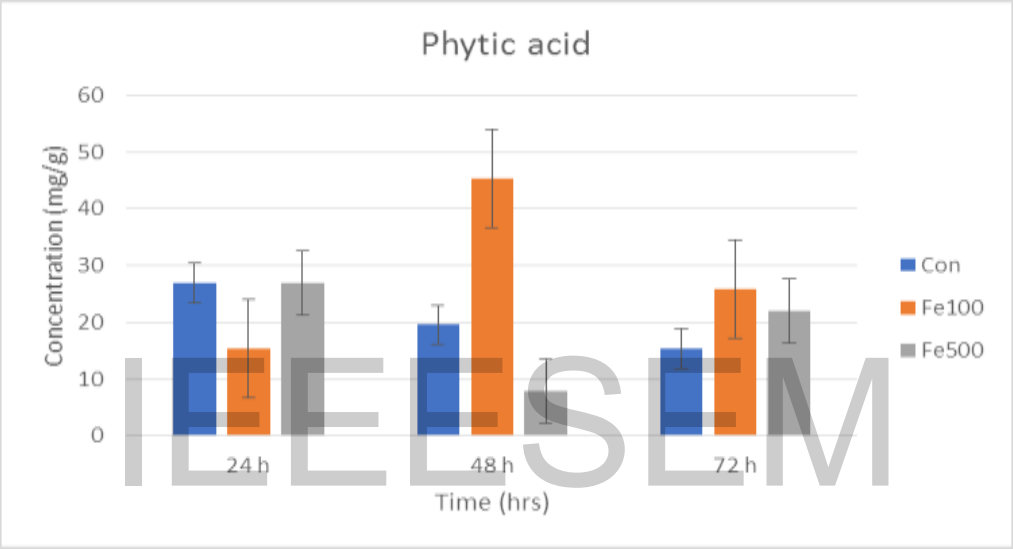

Figure 3a: Effect of iron pre-treatment on the phytic acid content of soybean seedlings.

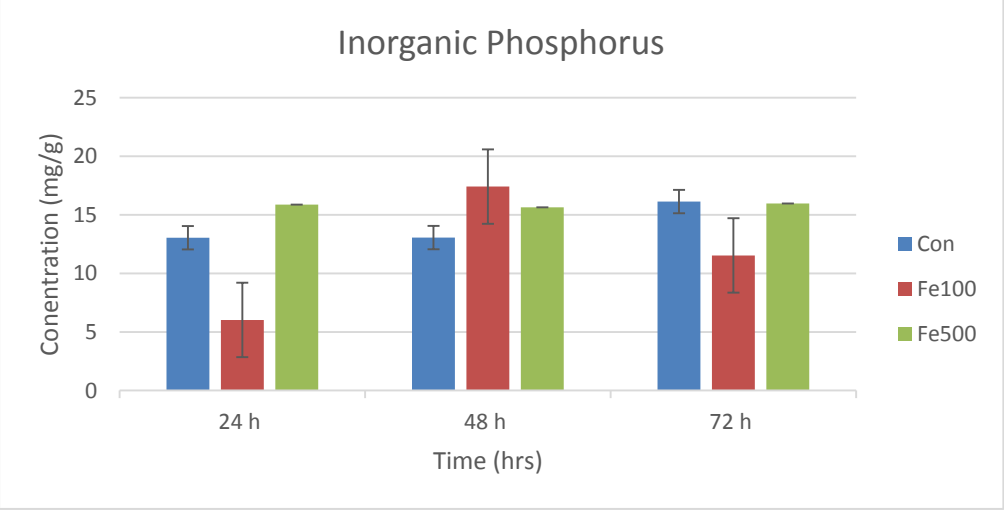

Figure3b: Effect of iron pre-treatment on inorganic phosphorus content of soybean seedlings.

Discussion

Micronutrient malnutrition, which is also known as “hidden hunger”, is a major public health issue in most parts of the world and affects more than two billion people particularly in developing countries.[20,21]. More than 60% of the world’s population is Fe deficient [22]. Developing countries most affected by MNDs including Cameroon often have a high reliance on a plant based diet, with the consumption of meat and dairy products limited in availability [23]. This lack of dietary diversity can often lead to an insufficient intake of Fe [24].

Soybeans biofortification

There are three key strategies which can be used for prevention of mineral deficiency in humans including introduction of supplements, industrial fortification of food products or biofortification of crops [3,4,5]. In Cameroon as in most developing countries food supplements strategies are currently being introduced in public policy while fortified food is yet to be implemented. As an attempt to address the gaps related to the use of ion supplement against ion-deficiency, the present study performed soybeans biofortification which is considered to be the most reliable source for ion against ion-deficiency anaemia. Due to the relatively low cost and potential for wide distribution, food fortification has been proposed over supplementation as one of the most cost-effective of all health interventions [6]. Fortification of foods with iron compounds in soy foods can add nutritional value and can reduce the potential of anaemia [13]. Because of the high prevalence of deficiencies associated with this micronutrient in the developing world, the research to develop fortified foods is on the rise and has largely focused on increasing the Fe content of the world’s most important staple food crops [25-29]

Two different Fe concentrations 100 and 500 mg/L were used in this study as an attempt to investigate which of the two will have a direct impact on germination of seedlings and their phytochemical contents. This approach is relevant in public health because there is an urgent need to improve the Fe concentrations in food crops to minimize Fe deficiency-related health problems in human populations. In order to eliminate the subjective interpretation of visual-color comparison in this study, total phenolic compounds were quantified using iron trichloride colorimetric assay to measure color changes, while phytic acid and inorganic Phosphorus contents were quantified using published spectrophotometric assays [30]. The colorimeter and spectrophotometer both works on the basis of Beer-Lambert's law, which states that the absorption of light transmitted through a medium is directly proportional to the concentration of the medium.

The choice of soybean in this study is because it is one of the most widely consumed crops of the world due to its high content of easily digested proteins, unsaturated fatty acids including omega-3 and numerous other biologically active compounds such as flavonoids and isoflavones (genistein, daidzein, glycitein) [29,31] and it’s extremely high iron content sprouted in the FeSO4 solutions[9].

Ferric sulphate has been accepted both as a food ingredient (used as a supplement and food fortificant) and as a component of edible plant fertilizers [32].The method of seed pre-incubation is cheap, fast and easy in application. Additionally, studies of fortification with nine elements of garden cress, sunflower, mungbean, soybean and lentil demonstrated that soybeans and lentils possess the highest ability to accumulate supplied minerals. Other studies have shown that pre-incubation of soy seeds in Fe solution resulted in higher amount of these minerals in the seedlings[7,9]

Germination and seedlings growth Rates

Germination has been identified as an effective technology that improves the nutritional value of grain legumes [33]. Exposure of seeds to metals and other elements might alert the growth and germination rates of seedlings [34]. In this present research, preincubation of soybean seeds in 100mg/mL Fe solution did not affect the growth rate as indicated by root lengths consistent with previous finding[9] revealing that enrichment of soybean with iron solution does not affect its growth rates. However, iron at concentration of 500 mg/L showed a significant increase in the germination rates after 48 and 72h incubation. Thus it can be concluded that incubation of seeds in Fe solutions will influence in part the productivity of soybean sprouts. In contrast, previous studies revealed that exposure to high Fe concentrations resulted in decrease in germination rate of wheat [35].It has been shown that Phenolic compounds such as flavonoid-related compounds synthesized during germination can impair auxin (plant growth hormone) transport hence, inhibiting seedling growth [36].The result of this study is not consistent with previous reports [37] stating that preincubation of seeds in Fe solution had neither inhibitory nor stimulatory influence on germination of soybean seedlings. Nevertheless, this difference might be due to variations in season, sampling methods and metal tolerance between strains of seeds

Impact of fortification on phenolic compounds

In this study the extract of soybeans showed a significant increase of phenolic compounds at $p<0.05$ in response to iron at a concentration of 100 mg/L solution after 72 h, revealing how germination could modify the phenolic composition of legumes. It has been revealed that treatment of seeds with minerals might also affect plants antioxidant status [38]. Iron pretreatment may lead to high Fe^{2+} uptake by roots. These ions may damage membranes, DNA and proteins due to the production of free radicals thus disputing the antioxidant system [39,40]. Flavonoids are a group of multifunctional plant secondary metabolites [41] that play a key role in protecting plants against UV radiation, pathogens, and abiotic stresses [42]. They determine flower, fruit, and seed colors [41] and are also responsible for allelopathy, plant bacteria symbiosis [42], and control of plant growth and development through inhibition of auxin transport [43,44,45].However, in this study, iron pretreatment did not influence the level of flavonoids in all the treatment options consistent with

previous studies [36,46].This suggests that the increase in total phenolics might be due to increase synthesis of other subclasses of phenolic compounds other than flavonoids, such as tannins, coumarins, lignans, stilbens, quinones, etc. These results are in accordance with previous studies [47], revealing how germination could modify the phenolic composition of legumes. It has been shown that iron-fortification of soybeans with higher concentrations could reduce the antioxidant capacity[9]. This could be the counter effects of antioxidants phenolic against potential free radicals generated during iron sequestration into seeds.

It has been proven that supplementing the living system with exogenous antioxidants or stimulating the endogenous antioxidant defences of the body enables alleviation of the oxidative damages induced by ROS. In this line plants have been shown to possess a wide variety of antioxidants capable of attenuating ROS- induced oxidative damages [48,49].

Plant derived bioactive compounds with antioxidant properties include polyphenols, flavonoids, vitamin C, etc. Flavonoids comprise the most studied group of polyphenols. This group has a common basic structure consisting of two aromatic rings bound together by three carbon atoms that form an oxygenated heterocycle. Quercetin, myricetin, catechins etc., are some most common flavonoids. Polyphenols are effective ROS scavengers and metal chelators due to the presence of multiple hydroxyl groups. They therefore offer protection against development of many diseases [49,50].

The effect of ion-fortification on phytic acid content

From these findings, seedlings pretreated with iron showed a reduction in the phytic acid content. However, the effects were dose and time dependent. That is with respect to the control group, seedlings pretreated with 100 mg/L and 500 mg/L showed a decrease in phytic acid content after 24 h and 48 h, respectively. With respect to the contents on first day of germination, the phytic acid contents decreases with increasing germination time, in the case of water and 500 mg/L-treated seeds. However, seedlings pretreated with 100 mg/L tend to reverse this trend. Phytic acid is often present in seeds, serving as a storage for *myo*-inositol and phosphorus, which is utilized during seed germination and seedling growth [51].Measurement of phytic acid in foodstuffs is an important consideration to improve population estimates for mineral deficiency in combination with direct human biomonitoring, FBS, food composition data and better understanding of the spatial controls on their soil-to-crop transfer [52]. Pre-germination of grains in mineral solution has been reported to lower their phytic acid contents [53].In this study, seedlings showed a reduced level of phytic acid after 48h treatment with 500mg/l iron . However, iron at concentrations of 100 mg/L non-significantly reversed this trend during 48 and 72h treatment. Phytic acid is a strong chelator of Fe^{2+}*in-vivo* and poses a major risk of anti-nutrient deficiency throughout Africa and worldwide [24,54], limiting the bioavailability of these essential minerals from an already

deficient dietary intake. Thus, there has been some interest in reducing the phytate content of legumes by soaking or germination (which activate endogenous phytase), or by adding a commercial phytase enzyme. Soaking under optimal conditions activates naturally occurring phytases in cereals and results in varying degrees of phytate hydrolysis depending on the kind of legumes [55]. Cereal grains and oil seeds have particularly high concentrations of phytate[56], which is known to negatively impact the environment as well as human and animal nutrition.

The role of ion-fortification on phosphorous content of soybeans

From this study, it is clear that pre-treatment of soy seeds with 100mg/l of iron non-significantly increased the inorganic Phosphorus contents only after 48h germination period. In contras**t**, iron at concentration of 500mg/L showed a non-significant increase in phosphorus contents throughout the germination time.. Water also increased the Phosphorus content with increasing germination time. This could be due the concomitant decrease of the chelator of P (phytic acid) during the germination. Phosphorus bound in phytates is nutritionally unavailable to monogastic animals such as swine and poultry. These animals lack the enzyme phytase, as such leading to a deficiency of Phosphorus mineral [57]. Acidic soil also reduces seedling root growth, which is critical to Phosphorus uptake. Soil pH values below 5.5 and between 7.5 and 8.5 limit phosphate availability to plants [58]. Pre-germination of grains in mineral solution has been reported to increase inorganic phosphorus contents in these grains [59]. This study showed that pre-treatment of soy seeds with 500mg/l of iron non-significantly increased the inorganic Phosphorus contents during 24 and 48h incubation period. Iron at concentration of 100mg/l showed an increase in Phosphorus only during 48h incubation, and exerts opposite effects during 24 and 48h treatment. The increase in inorganic Phosphorus content might be due to exchangeable Fe^{2+} ions with the bound phosphates in the phosphorus-phytate complex [60].

1 Conclusions

The present study shows that short term incubation of soybean seeds in Fe solution results in important physiological and biochemical changes in the seedlings. Iron fortification significantly increases the germination rate seedlings. However, iron-supplementation of seedlings does not affects its growth rates. Seedlings pre-treated with iron at concentration of 100 mg/L increased the total phenolic contents in soybean seedlings after 72h germination. Importantly, iron-fortification of soybean seeds has neither inhibitory nor stimulatory influence on its flavonoid contents. Moreover, Soybeans seedlings showed a decrease of phytic acid compounds 48 in response to iron at a concentration 500 mg/L solution. Seedlings supplemented with iron at concentration of 100 mg/L showed an elevated amount of inorganic phosphorus after 48 h. In addition, seeds exposed to iron at 500 mg/L Fe solution

showed a slight increase in the phosphorus contents throughout the germination period. It can be recommended that iron at 100 mg/L be used to cultivate seedlings with improved phenolic contents. Therefore, it can be concluded that soybean seeds pre-treated with iron at concentration of 500 mg/L is ideal to obtain high yield sprouts with reduced phytate and elevated phosphorus contents Finally, pre-incubation of soybean seeds in Fe solution could be a promising strategy of obtaining enriched soybean sprouts and maybe recommended for use as a source of natural antioxidants and antiphytates in functional foods for the treatment of anaemia and other ailments. Another study is needed to determine the exact concentration of iron to be used and duration of germination for the production of sprouts that are safer for human consumption.

Competing interests

All authors declared no conflict of interest.

Authors' contributions

MTN, WTN, PFG, and CBJ designed and organized the study. MTN and CBJ supervised the study. WTN, MTN, and CBJ carried out the field and laboratory work and preliminary data analysis. MTN,PFG and WTN drafted and wrote the manuscript. All authors read and approved the final manuscript.

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