Australian Journal of Crop Science AJCS
AJCS 8(8):1168-1174 (2014) ISSN:1835-2707

In vivo bioavailability of essential minerals and phytase activity during soaking and germination in soybean (*Glycine max* L.)

Sweta Kumari†, Veda Krishnan†, Monica Jolly and Archana Sachdev*

Division of Biochemistry, Indian Agricultural Research Institute (IARI), New Delhi - 110012, India

***Corresponding author: arcs_bio@yahoo.com (†Equal contribution by both authors)**

Abstract

Dietary quality is an important limiting factor for proper nutrition in many resource poor settings, with a major concern, micronutrient bioavailability. High content of phytate present in soybean (*Glycine max.* L) chelates divalent essential mineral ions and reduces its bioavailability and thus dictates the nutritional role of this wonder crop. In this study, the changes in phytate, phytase activity and bioavailability of essential minerals (Fe^{2+}, Zn^{2+},Ca^{2+}) were investigated using various colorimetric assays, AAS and functionally validated using an *in vivo* simulation model in two Indian cultivars, Pusa 9712 and Kalitur after soaking and germination. Phytate-mineral ratio, another parameter for bioavailability was also analyzed. Phytate content was reduced to 10-13% after 12 hrs of soaking and a further reduction to 46-65% was observed after 72 hrs of germination in Kalitur and Pusa 9712, respectively. Phytate being mainly hydrolyzed by phytase, we observed a six-fold increase in its activity after 72 hrs of germination, compared to control. Considering the dietary role, using an *in vivo* mimicking approach, a significant increase in the bioavailability of Fe^{2+}(8-21%), Zn^{2+} (7-18.5%) and Ca^{2+} (8-24%) in Kalitur and 9-27% (Fe^{2+}), 9-25% (Zn^{2+}) and 9-23% (Ca^{2+}) in Pusa 9712, respectively was observed after 72 hrs of germination. A negative correlation ($p \leq 0.05$) was observed between phytate and mineral bioavailability in sprouted soybean seeds. Thus soybean sprouts enhance the nutritional quality by lowering the anti-nutrient contents and increasing the bioavailability of minerals.

Keywords: endogenous phytase; *in vivo* – simulation; molar ratios; phytic acid.

Abbreviations: AAS_Atomic Absorption Spectrometry; DW_Dry Weight; PU_Phytase Unit.

Introduction

In resource-poor communities it has become clear that, malnutrition is attributable not solely to insufficient amounts of food but also due to poor nutritional quality of the available food supply particularly among plant-based diets as it contains only very small amounts of bioavailable micronutrients (Hotz et al., 2007). Legumes are an inexpensive and nutrient rich source of various low glycemic index carbohydrates, macronutrients (proteins, carbohydrates) and micronutrients (Fe^{2+}, $Zn^{2+}$and Ca^{2+}) and hence preferred by both vegetarians and non-vegetarians. Among them, soybean (*Glycine max* L.) is the richest source of protein, essential amino acids and various dietary components and is suitable for all ages, infants to the elderly. Soybeans have attracted the most scientific interest, mainly because they are a unique source of phytoestrogens like isoflavones and have been related to lowering the cholesterol levels, anti-cancerous properties and reducing risk for cardiac diseases (He et al., 2013; Murphy et al., 2003). Unfortunately from a dietary perspective, soybean is nutritionally dictated mainly due to poor bioavailability due to the presence of anti-nutritional factors such as phytic acid. These mineral deficiencies are major public health threat worldwide and especially in developing countries like India. Phytate is a principal storage form of phosphate, ubiquitously distributed in plants, particularly in legumes and cereals. The effects of phytate in human and animal nutrition are related to the interaction of phytate with proteins, vitamins and several minerals and there by restrict their bioavailability, which is defined as the proportion of the minerals that can be absorbed and utilized within the body (Lestienne-Besancon et al., 2005) Bioavailability of minerals are determined by measuring the molar ratio of phytate/minerals in the food and diet or precisely using *in vivo* studies (Afify et al., 2011). Larsson et al., 1997 reported that solubility of minerals, dietary factors, pH of intestinal lumen and residence time at the absorption site are the various factors affecting the bioavailability of minerals. Household strategies aim to increase the physiochemical accessibility of micronutrients, decrease the content of anti-nutrients such as phytate or increase the content of compounds that improve bioavailability. A combination of strategies is probably required to ensure a positive and significant effect on micronutrient adequacy. In view of tackling these anti-nutritional effects of phytate many attempts were carried out like activating indigenous enzyme phytase, addition of microbial phytase or silencing genes in phytate biosynthetic pathway (Kumari et al., 2012). Soaking usually forms an integral part of processing methods such as germination, fermentation, cooking, dehulling and toasting using different media like water, salts or alkali solutions. Phytate being water soluble, a significant reduction can be realised by discarding the soaked medium. Germination is a widely used process in legumes to increase its palatability and nutritional value. During germination, a marked increase in phytate degrading activity with a concomitant decline in phytate content was also observed (Kumar et al., 2010). Soaking of soybean, millets, maize, mung bean and sorghum at 30^0C for 24 hrs have been reported with a reduction in phytic acid content by 4–51%. Mahgoub and Elhag (1998) observed that soaking sorghum at room temperature for 24 hrs and germinating

Table 1. Effect of soaking and germination methods on phytic acid P and phytic acid content of soybean varieties.

Samples	Phytic acid P content ($mgKg^{-1}$)	Inorganic phosphorus ($mgKg^{-1}$)	Phytic acid content ($mgKg^{-1}$)	Phytic acid (%)
Kalitur				
Control (Raw dry)	$8.14^{a} \times 10^{-3} \pm 0.05$	$0.90^{a} \times 10^{-3} \pm 0.02$	$28.90^{a} \times 10^{-3} \pm 0.06$	$2.89^{a} \pm 0.06$
12 h. soaked	$7.10^{b} \times 10^{-3} \pm 0.04$	$1.01^{b} \times 10^{-3} \pm 0.01$	$25.20^{b} \times 10^{-3} \pm 0.05$ (-13)	$2.52^{b} \pm 0.05$
Germination				
24 h.	$6.06^{c} \times 10^{-3} \pm 0.06$	$1.13^{c} \times 10^{-3} \pm 0.03$	$21.50^{c} \times 10^{-3} \pm 0.06$ (-26)	$2.15^{c} \pm 0.08$
48 h.	$4.96^{d} \times 10^{-3} \pm 0.03$	$1.25^{d} \times 10^{-3} \pm 0.04$	$17.60^{d} \times 10^{-3} \pm 0.04$ (-39)	$1.76^{d} \pm 0.05$
72 h.	$2.82^{e} \times 10^{-3} \pm 0.04$	$1.43^{e} \times 10^{-3} \pm 0.02$	$10.03^{e} \times 10^{-3} \pm 0.04$ (-65)	$1.00^{e} \pm 0.04$
Pusa 9712				
Control (Raw dry)	$6.34^{a} \times 10^{-3} \pm 0.4$	$1.07^{a} \times 10^{-3} \pm 0.02$	$22.50^{a} \times 10^{-3} \pm 0.04$	$2.25^{a} \pm 0.05$
12 h. soaked	$5.72^{b} \times 10^{-3} \pm 0.05$	$1.17^{b} \times 10^{-3} \pm 0.05$	$20.30^{b} \times 10^{-3} \pm 0.05$ (-10)	$2.03^{b} \pm 0.05$
Germination				
24 h.	$4.87^{c} \times 10^{-3} \pm 0.03$	$1.34^{c} \times 10^{-3} \pm 0.03$	$17.30^{c} \times 10^{-3} \pm 0.08$ (-23)	$1.73^{c} \pm 0.08$
48 h.	$4.08^{d} \times 10^{-3} \pm 0.02$	$1.43^{d} \times 10^{-3} \pm 0.02$	$14.50^{d} \times 10^{-3} \pm 0.09$ (-36)	$1.45^{d} \pm 0.09$
72 h.	$3.21^{e} \times 10^{-3} \pm 0.04$	$1.52^{e} \times 10^{-3} \pm 0.01$	$11.40^{e} \times 10^{-3} \pm 0.08$ (-49)	$1.14^{e} \pm 0.09$

*Values are mean ± SD of three determinations. Different superscripts in the same column among Kalitur and Pusa 9712 with different letters are significantly ($P < 0.05$) different. Figures in parentheses indicate percent decrease (-) over control values.

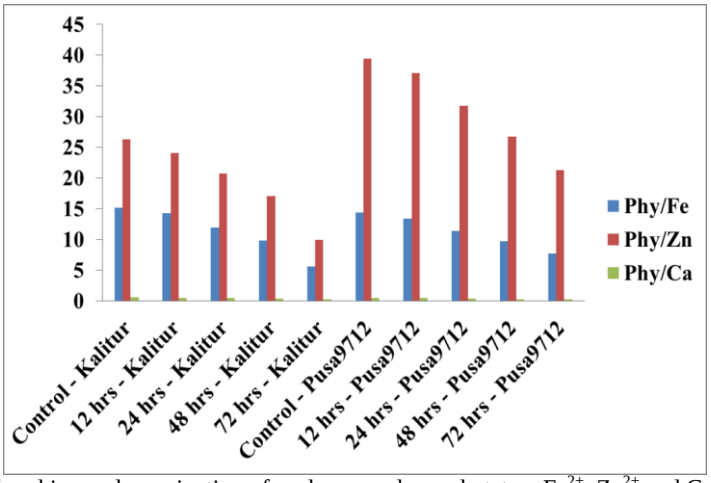


Fig 1. Effect of soaking and germination of soybean seeds on phytate – Fe^{2+}, Zn^{2+} and Ca^{2+} molar ratios.

further for 4 days reduced the phytic acid levels by 16–21% and 68–87% respectively. Studies conducted by Hotz et al. (2001) showed a reduction in phytate content by 51% after soaking for 1 hr at room temperature. Badau et al. (2005) reported that, with 96 hrs of germination time, HCl-extractability of Ca^{2+}, Fe^{2+} and Zn^{2+} in pearl millet increased by 2–16%, 15–45% and 12–25%, respectively.

Considering the prevalence of malnutrition in India, there is a need for developing cost effective, simple and less time consuming processes to utilize crops like soybean, which are a potential source of proteins, carbohydrates, minerals and various nutraceuticals. In contradiction, the other side of the coin says 'India is gaining weight', and thus raw foods and sprouts are also major attractions in the grocery shelves. Several studies have reported that there is an increased demand for sprouts with lowered levels of anti-nutrients and enhanced antioxidants in the current diet conscious society (Sokrab et al., 2012). Since the health benefits of improving the bioavailability of minerals in soybean are considerable, we have undertaken the present study to estimate the changes in the levels of phytate, phytase activity, total mineral content, phytate-mineral molar ratios and also the an *in vivo* bioavailability of Ca^{2+}, Fe^{2+} and Zn^{2+} ions in soybean as a functional study during soaking and germination at various time intervals up to 72hrs.

Results and Discussion

Effect of soaking and germination on phytic acid and phytase activity

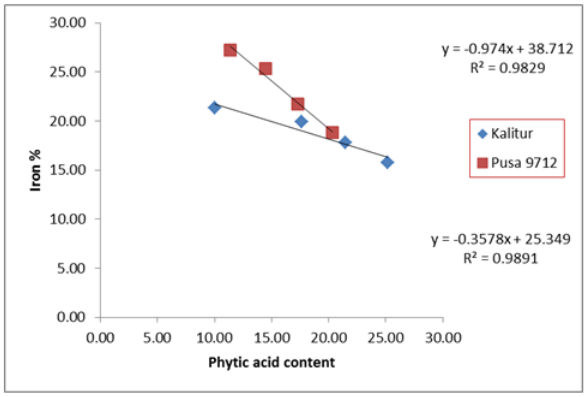
Phytic acid and phytic acid phosphorus contents were analyzed in raw dry soybean seeds, 12 hrs soaked seeds and in germinated (24 hrs, 48 hrs and 72 hrs) soybean (Table 1). Phytic acid and phytic acid phosphorus contents were higher in Kalitur (0.0289 $mgKg^{-1}$ and 0.0081 $mgKg^{-1}$) as compared with Pusa 9712 (0.022 $mgKg^{-1}$and 0.00634 $mgKg^{-1}$). The values of the phytate content before and after treatment shown in (Table 1) are close to those reviewed by Raboy and coworkers (1984). Depending on the amount of plant derived foods in the diet and the grade of food processing, the daily intake of phytate can be as high as 4500 mg. On an average, daily intake of phytate was estimated to be 2000-2600 mg for vegetarian diets as well as diets of inhabitants of rural areas in developing countries and 150-1400 mg for mixed diets (Reddy et al., 2002). Soaking and germination being integral parts of traditional methods of processing legume seeds in India before their consumption offer dual advantage of saving energy costs by shortening cooking time as well as by removing certain anti-nutritional factors like phytic acid, tannin, trypsin inhibitor and so on.

Table 2. Effect of soaking and germination methods on phytase activity in soybean varieties.

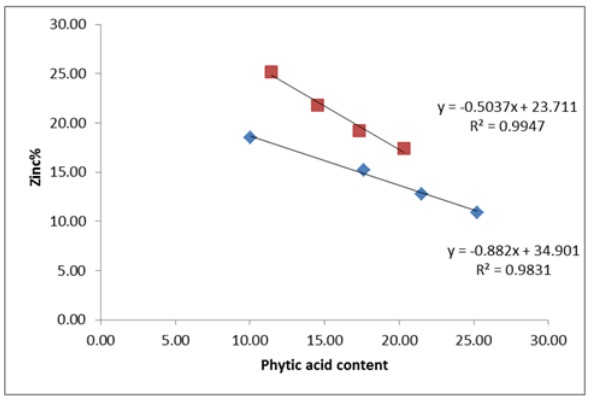
Varieties	Control (Raw dry)	12 hrs Soaked	Germination 24 hrs	48 hrs	72 hrs
Kalitur	ND	$0.22^{d} \pm 0.02$	$0.43^{c} \pm 0.02$	$0.58^{b} \pm 0.02$	$0.73^{a} \pm 0.04$
Pusa 9712	ND	$0.18^{d} \pm 0.03$	$0.38^{c} \pm 0.03$	$0.52^{b} \pm 0.02$	$0.65^{a} \pm 0.03$

** Values are mean ± SD of three determinations. All mean scores bearing different superscripts in rows are significantly different ($P< 0.05$).
* 1 Phytase unit (PU) is equivalent to the enzymatic activity which liberates 1 µmol inorganic phosphorous min^{-1}. (ND- Not Detected)

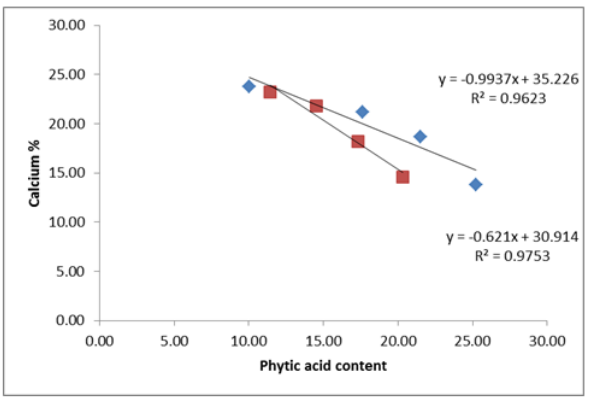
In house hold situations legumes are typically soaked overnight in water at room temperature. In the present study it was observed that phytic acid content in both Kalitur and Pusa 9712 decreased by 10-13% during the 12 hrs soaking period and further down to levels which were 49-65% of the basal levels of dry seeds after 72 hrs of germination. These findings are in range with those reported in the previous studies, which found that soaking, germination, mashing, boiling and fermentation strongly reduced the phytate content (Allen et al., 1997). Significant reductions($p \leq 0.05$) in the concentrations of phytic acid after 72 hrs of germination were high in Kalitur (65%) as compared with Pusa 9712 (49%). In general the decrease in phytic acid and phytic acid P showed a consistent trend, 72 hrs sprouts > 48 hrs sprouts > 24 hrs sprouts > 12 hrs soaked seeds > raw dry soybean seeds (Table 1). A decrease in phytate levels by 8% and 31% was observed in mung bean when soaked for 6 hrs and 18 hrs respectively (Kataria et al., 1989). Grewal and Jood, (2006) also reported a reduction of approximately 19% in phytic acid levels in green gram seeds when soaked for 12 hrs. The magnitude of reduction induced by soaking in this study can be explained by its leaching out into the soaking medium under a concentration gradient or by partial hydrolysis by endogenous phytase (Shimelis and Rakshit, 2007). The reduction in phytate caused by soaking could also be due to water solubilization of some phytic acid salts. During germination, phytins are generally broken down by endogenous phytase activity, releasing their P, myo-inositol and minerals for use by the seedlings (Raboy et al., 1990). Various studies in soybean and other legumes also implicated the reduction in phytate levels due to enzymatic changes during seed germination (Ibrahim et al., 2002; Khattak et al., 2007). Soaking of soybean seeds for 12 hrs and their germination for 24 hrs, 48 hrs and 72 hrs was carried out with an aim to study the changes in the total phytase activity in the seeds of Kalitur and Pusa 9712 (Table 2). Phytase activity increased significantly ($p \leq 0.05$) on soaking and germination. It was found to be absent in the unsoaked seeds but in 12 hrs soaked cotyledons an activity of 0.11 PU/g DW and 0.14 PU/g DW was observed in Pusa 9712 and Kalitur respectively. Mandal and Biswas, 1972 also reported the absence of phytase activity in the unsoaked raw seeds of mung bean. The appearance of the activity in 12 hrs soaked seeds was followed by an increase, reaching its maximum at 72 hrs. During germination a significant increase ($p \leq 0.05$) in the phytase activity 0.65 PU/g DW and 0.73 PU/g DW was observed in Pusa 9712 and Kalitur, respectively, after 72 hrs of germinations. Increased phytase activity in soybean and white bean at 72 hrs of germination was also reported by Egli et al., 2002. The observations of the present study reinforce the previous results observed by Gibson and Ullah (1998). The inverse relationship between phytase activity and phytic acid hydrolysis was thus confirmed in the germinating soybean seeds in this study and the observations were in agreement with the findings of Marero et al, 1991 who had reported the degradation of phytate in foods by the addition of phytases or by the activation of endogenous phytase by a combination of processes like soaking, germination and fermentation. Since humans have negligible intestinal phytase



(A)



(B)



(C)

Fig 2. Each Fig must have a general legend and the (A), (B), (C) etc. be explained separately in the legends. Fig legends must be more informative (A-C). A significant ($P<0.05$) negative correlation of phytic acid with *in-vivo* bioavailability of Fe^{2+}, Zn^{2+} and Ca^{2+} was observed.

Table 3. Effect of soaking and germination methods on total Fe^{2+}, Zn^{2+} and Ca^{2+}.

Samples	Fe^{2+} (mg Kg^{-1})	Zn^{2+} (mg Kg^{-1})	Ca^{2+} (mg Kg^{-1})
Kalitur			
Control (Raw dry)	$1.620^{a} \pm 0.6$	$1.080^{a} \pm 0.6$	$29^{a} \pm 1$
12 hrs. soaked	$1.540^{b} \pm 0.4$ (-5)	$1.030^{b} \pm 0.7$ (-5)	$27.8^{b} \pm 2$ (-4)
Germination			
24 hrs.	$1.536^{b} \pm 0.8$(-5)	$1.028^{b} \pm 0.6$ (-5)	$27.6^{b} \pm 1$ (-5)
48 hrs.	$1.520^{b} \pm 0.2$ (-6)	$1.018^{b} \pm 0.8$ (-6)	$27.2^{b} \pm 2$ (-6)
72 hrs.	$1.502^{c} \pm 0.3$ (-7)	$0.993^{c} \pm 0.6$ (-8)	$26.8^{c} \pm 3$ (-8)
Pusa 9712			
Control (Raw dry)	$1.330^{a} \pm 0.4$	$0.562^{a} \pm 0.5$	$28.2^{a} \pm 2$
12 hrs. soaked	$1.286^{b} \pm 0.6$ (-3)	$0.539^{b} \pm 0.6$ (-4)	$27.3^{b} \pm 1$ (-3)
Germination			
24 hrs.	$1.278^{b} \pm 0.5$ (-4)	$0.537^{b} \pm 0.4$ (-4)	$27.1^{b} \pm 1$ (-4)
48 hrs.	$1.258b^{c} \pm 0.8$ (-5)	$0.534^{b} \pm 0.3$ (-5)	$26.7^{b} \pm 2$ (-5)
72 hrs.	$1.244^{c} \pm 0.6$ (-6)	$0.526^{c} \pm 0.4$ (-6)	$26.3^{c} \pm 2$ (-6)

*Values are mean ± SD of three determinations. Different superscripts in the same column among Kalitur and Pusa 9712 with different letters are significantly ($P< 0.05$) different. Figures in parentheses indicate percent decrease (-) over control values.

Table 4. Effect of soaking and germination methods on *in-vivo* bioavailability of Fe^{2+}, Zn^{2+} and Ca^{2+}.

Samples Kalitur	Fe^{2+} (%)	Zn^{2+} (%)	Ca^{2+} (%)
Control (Raw dry)	$8.03^{e} \pm 0.08$	$7.32^{e} \pm 0.07$	$8.1^{e} \pm 2$
12 hrs. soaked	$15.8^{d} \pm 0.06$(+3)	$10.9^{d} \pm 0.08$(+5)	$13.8^{d} \pm 1$(+4)
Germination			
24 hrs.	$17.8^{c} \pm 0.05$(+19)	$12.8^{c} \pm 0.04$(+22)	$18.7^{c} \pm 2$(+12)
48 hrs.	$19.9^{b} \pm 0.07$(+27)	$15.2^{b} \pm 0.10$(+28)	$21.2^{b} \pm 1$(+19)
72 hrs.	$21.3^{a} \pm 0.13$(+33)	$18.5^{a} \pm 0.12$(+36)	$23.8^{a} \pm 3$(+26)
Pusa 9712			
Control (Raw dry)	$9.1^{e} \pm 0.11$	$8.9^{e} \pm 0.08$	$9.13^{e} \pm 3$
12 hrs. soaked	$18.8^{d} \pm 0.09$ (+3)	$17.4^{d} \pm 0.09$(+2)	$14.6^{d} \pm 2$(+4)
Germination			
24 hrs.	$21.7^{c} \pm 0.06$(+16)	$19.2^{c} \pm 0.11$(+12)	$18.2^{c} \pm 1$(+11)
48 hrs.	$25.3^{b} \pm 0.08$(+24)	$21.8^{b} \pm 0.06$(+19)	$21.8^{b} \pm 2$(+18)
72 hrs.	$27.2^{a} \pm 0.05$(+29)	$25.2^{a} \pm 0.15$(+28)	$23.2^{a} \pm 2$(+25)

* Values are mean ± SD of three determinations. Different superscripts in the same column among Kalitur and Pusa 9712 with different letters are significantly ($P< 0.05$) different. Figures in parentheses indicate percent increase (+) over control values.

activity (Iqbal et al., 1994), though they usually consume high phytate diets, it becomes important to recognize and account not only for the phytase activity, but also for activities of other phosphatases present in the plant material in order to understand the phytate hydrolysis in their gut.

Effect of soaking and germination on total mineral (Fe^{2+}, Zn^{2+} and Ca^{2+}) content and phytate-mineral molar ratios

Soaking of raw dry soybean for 12 hrs, resulted in a decrease of 3-5%, 4-5% and 3-4% respectively in the levels of Fe^{2+}, Zn^{2+} and Ca^{2+} (Table 3). A further significant reduction ($P< 0.05$) in the levels of the three minerals under study was observed after germination of the soaked seeds (Table 3)**.** The loss in the mineral content on soaking may be attributed to leaching of these minerals into the soaking medium (Kumar et al., 1978). The reduction was however less after 72 hrs of germination in the Fe^{2+}, Zn^{2+} and Ca^{2+} contents (5- 6%, 6-8% and 7-8% respectively) in soaked-germinated soybean seeds. Germination itself as well as the period of germination had no significant effect on the loss of total contents of Fe^{2+}, Zn^{2+} and Ca^{2+} as the percent loss observed in all these was almost constant with the increase in the period of germination (Table 3). Hence, the loss observed may be due to soaking and washing during germination and not due to the germination period. The values obtained in the present study were consistent with those reported earlier in bean bulgar (Ertas et al., 2012). The effect of soaking and germination on phytate/ Fe^{2+}, phytate/Zn^{2+} and Phytate/Ca^{2+} molar ratios in both varieties of soybean were determined (Figure 1). The phytate-mineral molar ratio is assumed to be as a critical indicator for mineral bioavailability. Phytate/Fe^{2+}, phytate/ Zn^{2+} and phytate/Ca^{2+} molar ratios were associated with their absorption capacities. It could be noticed that the phytate/Fe^{2+}, phytate/Zn^{2+} and phytate/Ca^{2+} molar ratios in control seeds of Kalitur were 15.1, 26.3 and 0.59, respectively, and Pusa 9712 were 14.3, 39.4 and 0.48, respectively. Molar ratios of these essential minerals after soaking and germination decreased upto 63% (Fe^{2+}), 62% (Zn^{2+}) and 63% (Ca^{2+}) in Kalitur and upto 45.85% (Fe^{2+}), 45% (Zn^{2+}), and 45.8% (Ca^{2+}) in Pusa9712. Hence the ratio entirely depends on the phytate content, an obvious maximum decrease was observed in black than yellow soybean after germination. Kayode et al. (2006) calculated the phytate/Fe^{2+} and phytate/Zn^{2+} molar ratios as an index for the potential mineral bioavailability. Also, phytate was hydrolyzed during germination, so that Fe^{2+} solubility under simulated physiological conditions was greatly increased. It is somewhat difficult to predict the overall impact of soaking or germination on Fe^{2+} solubility. Soaking or germination might be effective in reducing the phytate content of which showed the decrease in molar ratios. Even though molar ratios are an indirect way of analyzing mineral bioavailability, *in vivo* studies are necessary to get a clear picture.

Effect of soaking and germination on bioavailable essential minerals (Fe^{2+}, Zn^{2+} and Ca^{2+}) using in vivo simulation model

In vivo Fe^{2+}, Zn^{2+} and Ca^{2+} bioavailability before and after soaking and germination are shown in Table 4. The Fe^{2+}, Zn^{2+} and Ca^{2+} bioavailability ranged between 8.03-9.1%, 7.32-8.9% and 8.1-9.13% respectively for raw soybean seeds. The *in vitro* Fe^{2+} bioavailability after soaking increased to 15.8% and increased further during germination to 17.8% (24 hrs), 19.9% (48 hrs) and 21.3% (72 hrs) in Kalitur. A similar increasing trend was also observed in Pusa 9712 with an initial of 9.1% Fe^{2+} bioavailability in raw form, to about 18.8% in 12 hrs soaked seeds, 21.7% (24 hrs), 25.3% (48 hrs) and a maximum of 27.2% after 72 hrs of germination. The improved bioavailability of Fe^{2+} after soaking and germination are in agreement with the findings of Henriksen et al, (1985) who reported that food processing treatments such as heating, baking, fermentation, soaking and milling may enhance Fe^{2+} availability. Also, the phytase enzyme breaks down inositol hexa and penta-phosphates, which inhibit Fe^{2+} absorption, to smaller inositol phosphates and inorganic phosphate, which do not have any effect on Fe^{2+} absorption. Two common inhibitors of Fe^{2+} absorption are tannins and phytates. Certain tannins and other polyphenols may also reduce during germination process as a result of formation of polyphenol complexes with proteins and also cause gradual degradation of oligosaccharides. Such reduction in polyphenols may facilitate Fe^{2+} absorption. Processing techniques have been found to reduce significant levels of phytates and tannins by exogenous and endogenous enzymes formed during processing. The bioavailability% of Zn^{2+} increased from 7.32% (raw form), to 10.9% (12 hrs soaking) and to 12.8% and 15.2% after 24 hrs, 48 hrs of germination respectively. A maximum of 18.5% was however observed after 72 hrs of germination in Kalitur. A similar pattern of increase in Zn^{2+} bioavailability% was seen in Pusa 9712. An initial of 8.9% bioavailability was observed in the raw seeds, which increased to 17.4% after 12 hrs soaking and further scaled upto 19.2% (24 hrs), 21.8% (48 hrs) and 25.2% after 72 hrs of germination. The dietary mineral, Zn^{2+} also gets chelated by the anti-nutritional factors reducing its bioaccessability and bioavailability. Vegetarian meals have poor bioavailability of Zn^{2+}. Further, in Indian cooking processes, the main inhibitory factor of Zn^{2+} bioavailability, phytate, gets partially degraded and may not remain as a strong inhibitor (Agte et al., 1999). Among these divalent ions, *in vitro* and animal experiments have implicated Ca^{2+} as a potentiating factor because it reacts with phytate, and further binding of Zn^{2+} ions to these complexes causes precipitation. The bioavailability % of Ca^{2+} increased from 13.8% (12 hrs soaked) to 18.7% after 24 hrs of germination, which further improved to 21.2% (48 hrs) and 23.8% (72 hrs) after extended germination periods in Kalitur. Pusa 9712, following a similar trend showed an initial rise of 14.6% after 12 hrs of soaking and further rose to 18.2% (24 hrs), 21.8% (48 hrs) and 23.2% (72 hrs) in the subsequent germination periods. Previously assumed that fiber negatively affects the Ca^{2+} balance by physical entrapment or by cationic binding, but it is more likely that the phytate associated with fiber rich products probably affects Ca^{2+} absorption. Studies conducted focusing on improving the protein quality of legumes by soaking also showed that pH of the soaking solution favors Ca^{2+} absorption (Teresa et al., 2003). We observed that germination had a beneficial effect on the bioavailability of Fe^{2+}, Zn^{2+} and Ca^{2+} and followed a consistent trend during the germination periods. The low extractability of divalent cations in raw seeds can be attributed to their covalent association with phytic acid which constitutes about 2.2 to 2.8% of the total dry wt. of the soybean seeds. Decrease in the levels of phytic acid by soaking and germination as reported by previous workers (Salunkhe et al., 1986) as well as in the present study, may possibly release these metallic ions in the free form and account for their increased bioavailability. As Kumari et al. (2014) also find a significant and a negative correlation between PA and iron (r= 0.8) in the low phytic acid mutant lines. A significant ($P< 0.05$) negative correlation of phytic acid, with the bioavailability of Fe^{2+}, Zn^{2+} and Ca^{2+} observed in the present study strengthens our findings and clearly underlines the role of phytic acid in lowering the extractability of divalent cations (Figure 2A - C).

Materials and Methods

Materials

Seeds of soybean varieties, Kalitur and Pusa 9712 were obtained from the Pulse Laboratory, Division of Genetics, IARI, New Delhi. The seeds were carefully cleaned and freed from broken seeds and extraneous matter. Pepsin, pancreatin and lipase were purchased from Sigma – Aldrich Chemical Co. (St. Louis, USA) and bile extracts from Win Lab laboratory chemicals reagents (Mumbai, India). All other chemicals used were of analytical reagent grade.

Soaking of seeds

Seeds were soaked in distilled water for 12 hrs at room temperature (28°C± 2^0C) with a ratio 1:10 w/v and the soaked water changed twice for 12 hrs. The soaked seeds were drained and dried at 45±5°C. Dried seeds were milled in a laboratory mill to obtain fine flour and kept at -20°C until analysis. Un-soaked (Raw dry) seeds were used as control.

Germination of seeds

To correlate the role of phytase in hydrolysing phytic acid, soaked seeds were germinated (sprouting) by placing between thick layers of cotton cloth at room temperature 28±2°C for a period of 24 hrs, 48 hrs and 72 hrs. Germinated seeds were frozen for 12 hrs to stop the germination process. After thawing at room temperature, the seeds were dried in an electric hot air oven at 45±5°C and were milled to obtain fine flour and stored at -20°C until analysis. Un-soaked (Raw dry) seeds were used as control.

Estimation of phytic acid

Phytic acid (phytate) was measured by an assay procedure specific for the measurement of phosphorus released, based on the available phosphorus from phytic acid, *myo*-inositol (phosphate)n and monophosphate esters by phytase and alkaline phosphatase using the Phytic Acid/ Total Phosphorus Assay Kit (Megazyme, Ireland), (detail description is given as supplementary).

Phytase activity assay

Phytase was extracted from seed samples using 0.2M citrate buffer (pH 5.5), centrifuged and filtered. 0.2 ml of filtered extract was incubated with 1% sodium phytate for 15 min at 37ºC. Reaction was diluted, mixed with colour reagent for determining free-phosphorous concentration. 1 Phytase Unit

(PU) was defined as the activity that releases 1 µmol of inorganic phosphorous from sodium phytate per min.

Total Fe^{2+}, Zn^{2+} and Ca^{2+} determination

One gram of ground sample was mixed with 25 ml diacid mixture (HNO_3:$HClO_4$; 9:4 v/v) and incubated overnight. The next day mixture was digested by heating till clear white precipitate settled down at the bottom. The crystals were dissolved by diluting in double distilled water. The contents were filtered through Whatman # 42 filter paper. The filtrate was made up to 50 ml with double distilled water and was used for determination of total Fe^{2+}, Zn^{2+} and Ca^{2+}. The samples, which were digested in a diacid solution of HNO_3 and $HClO_4$, were passed through the Atomic Absorption Spectrometry (ECIL, AAS 4141) system and calibrated for Fe^{2+}, Zn^{2+} and Ca^{2+}.

Phytate- Mineral molar ratios determination

The mole of phytate and minerals was determined by dividing the weight of phytate and minerals with its atomic weight according to Afify et al, 2011.

Bioavailable Fe^{2+}, Zn^{2+} and Ca^{2+} determination using in vivo simulation model

The bioavailability of Fe^{2+}, Zn^{2+} and Ca^{2+} were determined by the *in vitro* digestion method described by Kiers et al, (2000). Triplicate samples (5g) were suspended in 30 ml distilled water and digested under simulated gastrointestinal conditions, subsequently using alpha amylase solution, stomach medium consisting lipase and pepsin, and pancreatic solution consisting of pancreatin and bile. After digestion, the suspension was centrifuged at 3600 g for 15 min. The supernatant was decanted and the pellet was discarded. The supernatants were pooled and filtered through a 0.45 mm filter. A blank was included consisting of 30 ml distilled water digested and filtered as described above. Both filtered supernatants from sample and blank were analyzed for Fe^{2+}, Zn^{2+} and Ca^{2+}. Samples were corrected for added reagents/ water by subtracting Fe^{2+}, Zn^{2+} and Ca^{2+} contents of the blank from that of supernatants from samples. Fe^{2+}, Zn^{2+} and Ca^{2+}content were measured by using the Atomic Absorption Spectrophotometer. The amounts of Fe^{2+}, Zn^{2+} and Ca^{2+} (in supernatant) were regarded as soluble minerals. Percentage of soluble mineral was calculated as bioavailability %.
Bioavailability % = Amount of Fe^{2+}, Zn^{2+} and Ca^{2+} (supernatant) – amount of Fe^{2+}, Zn^{2+} and Ca^{2+} (blank) / amount of Fe^{2+}, Zn^{2+} and Ca^{2+}in undigested sample X 100.

Statistical analysis

Each experiment was carried out with three experimental as well as three technical templates. For the analytical data, mean values and standard deviations are reported. The data was subjected to t-test analysis using SAS software program and difference between the means was compared by the Duncan's Multiple Range Test at ($p \leq 0.05$).

Conclusion

Soaking and germination methods can thus prove beneficial in lowering the phytic acid content and improving the bioavailability of dietary essential minerals in soybean. The reduction in phytic acid content in soaked and germinated soybean seeds has been found to be associated with an increase in the endogenous phytase activity. Once the phytate content is significantly reduced by these simple and traditional processes, soybean would become a potential source of proteins, carbohydrates and minerals. Hence, for proper utilization of soybean, especially in developing countries, these methods should be followed, as they not only save time, energy and fuel consumption but also enhance the nutritional quality of the soybean by lowering the content of anti-nutrients and increasing the bioavailability of the minerals. To be sustainable, a variety of interventions like these are appropriate for rural and low income groups and need to be considered to overcome the existing limitations.

Acknowledgments

The author gratefully acknowledges for the very capable technical assistance at the division of biochemistry and the financial support provided by Post graduate School, IARI, New Delhi**.**

Disclosure of Potential Conflicts of Interest

First and the second author equally contributed to this manuscript. The authors declare no conflicts of interest.

References

Afify AMR, Belgati HS, Salam SM and Omran AA (2011) Bioavailability of iron, zinc, phytate and phytase activity during soaking and germination of white sorghum varieties. Plos one: 6(10): e25512. doi:10.1371.

Agte VV, Tarwadi KV and Chiplonkar SA (1999) The influence of various food ingredients and their combinations on *in vitro* availability of Fe^{2+} and Zn^{2+} in cereal – based vegetarian meals. In: Roussel AM, Anderson RA, Favier AE, ed. Trace elements in man and animals. New York: Kluwer Academic/Plenum Publishers, 261 – 265.

Allen LH and Ahluwalia N (1997) Improving Fe^{2+} status through diet – the application of knowledge concerning dietary Fe^{2+} bioavailability in human populations. John Snow, Inc. /OMNI Project, 1-81

Badau MH, Nkama I and Jideani A (2005) Phytic acid content and hydrochloric acid extractability of minerals in pearl millet as affected by germination time and cultivar. Food Chem. 92: 425–435.

Egli I Davidsson L, Juillerat MA, Barclay D and Hurrell RF (2002) The influence of soaking and germination on the phytase activity and phytic acid content of grains and seeds potentially useful for complementary feeding. J Food Sci. 67 (9): 3484-3488.

Ertas N and Turker S (2012) The role of soaking process on mineral HCl- extractabilities, phytic acid content and trypsin inhibitor activity of common bean bulgur. Food Sci Tech Res. 18 (3): 445-453.

Grewal A and Jood S (2006) Effect of processing treatments on nutritional anti-nutritional contents of green gram. J Food Biochem. 30: 535-546.

Gibson DM and Ullah AHJ (1998) Purification and characterization of phytase from germinating soybean seeds. Arch Biochem Biophys. 260: 503-513.

He F and Chen J (2013) Consumption of soybean, soy foods, soy isoflavones and breast cancer incidence: Differences between Chinese women and women in Western countries and possible mechanisms. Food Sci Hum Well. 2(3-4): 146-161.

Henriksen LK, Mahalko JR, Johnson LAK (1985) Canned foods: Appropriate in trace element studies? J Am Dietet Assoc. 85: 563-568.

Hotz C and Gibson R (2007) Traditional Food-Processing and Preparation Practices to Enhance the Bioavailability of Micronutrients in Plant-Based Diets. J Nutr. 137(4): 1097-1100

Hotz C, Gibson RS and Temple L (2001) A home-based method to reduce phytate content and increase bioavailability in maize-based complementary diets. Int J Food Sci Nutr. 52: 133–142.

Ibrahim SS, Habiba RA, Shatta AA and Embaby HE (2002) Effect of soaking, germination, cooking and fermentation on anti-nutritional factors in cowpeas. Food/Nahrung. 46: 92–95.

Iqbal TH, Lewis KO and Cooper BT (1994) Phytase activity in human and rat small intestine. Gut. 35: 1233-1236.

Kataria A, Chauhan BM and Punia D (1989) Anti-nutrients and protein digestibility (*in vitro*) of mung bean as affected by domestic processing and cooking. Food Chem. 32: 9-17.

Kayode PAP, Linnemann AR, Nout MJR, Hounhouigan DJ and Stomph TJ (2006) Diversity and food quality properties of farmer's varieties of sorghum from Berlin. J Sci Food Agri. 86: 1032-1039.

Khattak AB, Zeb A, Bibi N, Khalil SA and Khattak MS (2007) Influence of germination techniques on phytic acid and polyphenols content of chickpea (*Cicerarietinum* L.) sprouts. Food Chem. 104: 1074-1079.

Kumar KG, Venkataraman LV, Jaya TV and Krishnamurthy KS (1978) Cooking characteristics of some germinated legumes: changes in phytins, Ca^{++}, Mg^{++} and pectins. J Food Sci. 43: 85-89.

Kumar V, Sinha A, Makkar H, and Becker K (2010) Dietary roles of phytate and phytase in human nutrition: A review. Food Chem. 120: 945-959.

Kumari S, Jolly M, Krishnan V, Dahuja A and Sachdev A (2012) Spatial and temporal expression analysis of D-myo-inositol 3-phosphate synthase (MIPS) gene family in *Glycine max.* Afr J Biot. 11(98): 16443-16454.

Kumari S, Lal SK and Sachdev A (2014) Identification of putative low phytic acid mutants and assessment of the total P, phytate P, protein and divalent cations in mutant populations of soybean. Aust J Crop Sci. 8(3):435-441.

Larsson M, Minekus M and Havenaar R (1997) Estimation of the bioavailability of Fe^{2+} and phosphorus in cereals using a dynamic *in vitro* gastrointestinal model. J Sci Food Agri. 74: 99-106.

Lestienne I, Besancon P, Caporiccio B, Lullien-Pellerin V and Treche S (2005) Fe^{2+} and Zn^{2+} *in vitro* availability in pearl millet flours (*Pennisetumglaucum)* with varying phytate, tannin, and fiber contents. J Agri Food Chem. 53(8): 3240–3247.

Mahgoub SEO and Elhag SA (1998) Effect of milling, soaking, malting, heat-treatment and fermentation on phytate level of four Sudanese sorghum cultivars. Food Chem. 61: 77-80.

Mandal NC, Burman S and Biswas BB (1972) Isolation, purification and characterization of phytase from germinating mung beans. Phytochem. 11: 495-502.

Marero LM, Payumo EM, Aguinaldo AR, Matsumoto I and Homma S (1991) The anti-nutritional factors in weaning foods prepared from germinated legumes and cereals. Lebensmittelwissenschaft Tech- Food Sci Technol. 24: 177-181.

Murphy S and Allen L (2003) Nutritional importance of animal source foods. J Nutr. 133 (11): 3932S-3935S.

Raboy V (1990) The biochemistry and genetics of phytic acid synthesis. In: Morre D, Boss W, Loewus F, eds. Inositol metabolism in plants. New York: Wiley-Liss. 55-76.

Raboy V, Dickinson DB and Below FE (1984) Variation in seed total phosphorus, phytic acid, Zn^{2+}, Ca^{2+}, magnesium, and protein among lines of *Glycine max* and *G. soja.* Crop Sci. 24: 431–434.

Reddy NR (2002) Occurrence, distribution, content and dietary intake of phytate. In: Reddy NR, Sathe SK, eds. Food phytates. Boca Raton, FL, USA: CRC Press. 25-51.

Salunkhe DK Chavan DK and Kadam SS (1986) Pigeon pea as an important food source. CRC Crit Rev Food Sci. 23: 103–145.

Shimelis EA and Rakshit SK (2007) Effect of processing on anti-nutrients and *in vitro* protein digestibility of kidney bean (*Phaseolus vulgaris* L.) varieties grown in East Africa. Food Chem. 103: 161-172.

Sokrab A, Ahmed I, and Babiker E (2012) Effect of germination on antinutritional factors, total, and extractable minerals of high and low phytate corn (*Zea mays* L.) genotypes. J Saudi Soci Agricult Sci. 11(2): 123 – 128.

Teresa N, Mercedes B, Magdalena L, Concepcion V and Glorio U (2003) Effect of different soaking solutions on nutritive utilization of minerals (Ca, P and Mg) from cooked beans in growing rats. J Agricult Food Chem. 51: 515-520.