Ceylon Journal of Science (*Bio. Sci.*) 41 (1): 19-26, 2012

Mineral bioavailability in three locally consumed pulses processed using popular methods: interpreted using molar ratios with phytic acid

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Accepted 19 April 2012

ABSTRACT

Pulses, an essential component of the Sri Lankan diet, have high levels of phytic acid (PA) which chelate essential mineral nutrients limiting their bioavailability. Three locally grown pulses; mung beans, black gram and soya bean were investigated to determine the effects of popular processing methods on their Zn, Fe and Ca bioavailability as affected mainly by phytic acid (PA). Processed mung beans (*Vigna radiata var*. MI 6) and black gram (*Vigna mungo var.* MI 1) had very high levels of PA. In mung beans, with increasing germination time, a significant decrease (P=0.05) in PA from 2517 to 1998 mg per 100g (dry weight basis) was observed but PA:Zn molar ratio did not improve. Natural fermentation of black gram, did not affect PA level effectively, but levels decreased when inoculated with a lactic acid bacterium or with *Pantoea agglomerans*. In soya bean (*Glycine max* var. *Pb*), significant (P=0.05) reduction in PA was observed in tempeh (487 mg/100 g dry weight). Overall, in soya products, the Zn bioavailability has increased from low to moderate levels in both tofu and tempeh. The molar ratio of PA:Fe was lower in all soya products. Although, PA:Zn molar ratio has decreased in germinated mung beans, the Ca x PA:Zn ratio exceeded 0.5 indicating that Ca influences Zn bioavailability.

Key words: zinc, iron, calcium, *Vigna radiata*, *Vigna mungo*, *Glycine max*

INTRODUCTION

In Sri Lanka, like in most other Asian countries the diet is mainly composed of cereals and pulses. In view of the fact that animal protein intake is minimal, pulses play an important role by providing the much needed proteins in the diet. However, seeds of legumes (pulses) have an inherent draw back due to the presence of a metal chelating agent named as phytic acid (PA) limiting absorption of certain essential mineral nutrients such as Fe, Zn and Ca (Gibson and Ferguson, 1998). It should be noted that the beneficial effects of PA also have attracted attention pertaining to certain dietary patterns (Kumar *et al*., 2010) although the number of publications on adverse effects of PA outweighs that of its beneficial effects. In the present context however, the attention will be on its adverse effect of chelating metal micronutrients.

As in other rice eating countries in the region, in Sri Lanka too, mineral nutritional deficiencies in local populations are of concern. In this scenario, increasing the bioavailability of mineral levels present in the food is of utmost importance. In fact in a previous study (Karunaratne *et al.*, 2008) the levels of PA were found to be unacceptably high in some meals that contained pulses. Several molar ratios have been developed to predict bioavailability. For instance, the World Health Organization has worked out a molar ratio of PA to Zn, to determine the influence of PA on Zn bioavailability (W.H.O., 1996). In the study by Karunaratne *et al.* (2008), PA to Zn molar ratios were found to be between 5 and 43 and according to W.H.O. specifications these diets have moderate to low bioavailability of Zn. More recently in a radio-isotope study conducted on humans, a dose dependent inhibitory effect of PA has been shown with respect to retention of Ca, and on the absorption of Zn, where the retention of Ca was at lower molar ratios than for Zn (Fredlund *et al*., 2006). Considering the above findings, it appears that the interference by Ca on bioavailability of Zn needs to be accounted for. It has been noted that high Ca levels in foods can promote the PA induced decrease in Zn bioavailability when the Ca x PA:Zn ratio exceeds 0.5 (Umeta *et al*, 2005; Cossack and Prasad, 1983).

Similarly, a molar ratio has been calculated to determine the influence of PA on Fe.

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Although not as widely used as the PA:Zn molar ratio, absorption of radiolabelled iron has been shown to decrease significantly in rats when the PA:Fe molar ratios were above 14 in wheat-flour-based diets (Luo *et al*., 2008; Saha *et al*., 1994). It has also been noted that Ca:PA molar ratios above 6 are unfavourable for Ca absorption (Umeta *et al.*, 2005). The present study focuses on the effect of processing methods of three specific pulses commonly consumed in Sri Lanka; green gram also known as mung bean (*Vigna radiata*), black gram (*Vigna mungo*) and soya bean (*Glycine max*) to determine their effects on PA, and on the molar ratios of PA:Zn, PA:Fe, Ca:PA and Ca x PA:Zn.

The locally popular processing methods of the above three pulses are as follows. Mung beans are consumed after boiling, or of late as sprouted beans. Fermented black gram is made into Thosai (referred to as `Dosa' in India), a fermented pancake consumed as a main meal for breakfast or dinner. Preparations of soya bean are available as fresh soya bean, soya powder, soya-tofu and as soya-tempeh (locally referred to as `Soya-Karawala').

In a previous study (Karunaratne *et al.*, 2008), food samples prepared from the selected pulses were obtained for investigation from households and retail outlets, as the focus was on the nutritional intake of preschool children. For the present study locally grown pulses of known varieties were obtained directly from the field. However, two popular commercial soya preparations, soya powder and soya tofu were included for comparison. Commonly used processing methods at household level were determined, so that future studies could concentrate on introducing those processing methods that are beneficial in increasing bioavailability of metal micronutrients in these pulses. Therefore, the objectives of the present investigation were two fold. Firstly to determine the levels of PA, as well as Zn, Fe and Ca, in specific local varieties of mung, black gram and soya bean. Thereafter, to predict levels of bioavailability when subjected to locally used processing methods, by interpreting results using previously recorded cut-off levels of specific molar ratios with PA. Additionally, to investigate the ability of three microorganisms individually to reduce PA levels in black gram. Also, to determine the acceptability of 'Thosai' prepared by inoculating with a microorganism, in place of traditional method of preparation.

MATERIALS AND METHODS

Materials

Fresh seeds of mung beans (*Vigna radiata*) *var*. MI 6, black gram (*Vigna mungo*) *var.* MI 1 and soya bean (*Glycine max*) var. *Pb* were obtained directly from the Government Field Crops Research and Development Institute, Maha Illuppallama, Sri Lanka. Soya tempeh was prepared by the Food Research Unit of the Department of Agriculture, on request using the above seeds and fermenting with *Rhizopus oligosporus*. Soya tofu and soya powder were purchased from a grocery store and used immediately for analysis.

Sample preparation

Fresh mung beans (100 g) were soaked in 500 ml of distilled water in a beaker for 24 h in the dark at room temperature (*ca* 28 ^{o}C). The excess water was drained and seeds were divided into three portions and germinated for 24 h, 48 h, and 72 h, in the dark in plastic containers covered with a wet cloth. The seed coats were removed from germinated seeds before analysis.

Powders for analyses were prepared by grinding fresh seeds (50 g), tofu (200 g) and tempeh (500 g) as described by Mohamed *et al.* (1986). Fermented black gram was prepared by mixing freshly ground powder (20 g) with distilled water (25 ml) into a slurry and allowing to ferment overnight.

Determination of Zn, Fe, Ca and PA

Moisture content of samples was determined using a standard method (A.O.A.C., 1990). Wet ashing, was carried out using methods described by Alcock (1987). Each triplicate sample was digested using H_2O_2 in a conventional oven at 50 – 80 ^{0}C over a period of 8 weeks. All ashed samples were dissolved in 0.5 N HNO_3 (6–26 ml). Zn, Fe and Ca levels were determined using Flame Atomic Absorption Spectrophotometry (BUCK, 200-A).

For PA analysis, the following method described by Mohamed *et al.* (1986) was used. The samples in triplicate (0.5 g) were treated with trichloroacetic acid and defatted with hexane. PA was precipitated in the form of ferric phytate and subjected to a chromogenic reaction and quantified by determining absorbance at 830 nm using a spectrophotometer. All glassware used in the assay was cleaned using 0.5 N HNO_3. All disposable plastic-ware was used once. For PA analysis, each time a batch of measurements were made, at least 5 standards of known concentrations of PA were assayed and the standard curve was drawn for each batch.

In experiments where molar ratios were calculated, at least two independent samples per assay category were investigated, each in triplicate. Data were subjected to analysis of variance followed by Tukey's test for mean separation.

Fermentation of black gram by inoculating with microorganisms

Powdered black gram was dried in an oven at 60^{o}C for two days. Three samples of weighed (5 g each) black gram were placed in separate vials and handled aseptically. Into each vial was separately added, known amounts of microbes from the culture collection of the Department of Botany [lactic acid bacterium-LAB (Gram positive cocci), isolated from a yogurt culture, a biocontrol bacterium *Pantoea agglomerans* (Karunaratne, 2011; Karunaratne and Gunasinghe, 2011) *Rhizopus oligosporus* from tempeh] and mixed well with sterile water to make a paste. Thereafter, they were incubated in the dark for 24 h at room temperature. Colony counts were obtained by conducting standard pour plate techniques. The PA levels were determined using the procedure described above.

Preparation of Thosai and sensory evaluation

Two sets of dough were made (each from 50 g of powdered black gram), one set using the traditional method, and the other set using an inoculant of LAB (1.4×10^{7} Colony forming units per gram). Into each dough, 25 g of wheat flour was added and mixed with appropriate amount of water to reach the correct consistency. The two sets of dough were well covered and placed in the dark for 24 h. Thereafter, equal amounts of coconut milk were added to each dough to prepare Thosai. Thosais prepared from each dough were kept separately in labelled containers for sensory evaluation. Sensory evaluation by 20 untrained panellists was conducted according to paired difference test (Jellinek, 1985).

RESULTS AND DISCUSSION

Known varieties of pulses prepared according to several methods (except for soya powder and soya tofu, which are commercially available and are not commonly prepared at a house hold level) were used in the investigations. The results indicated that the bioavailability levels of minerals are unacceptably low in the pulses. It is of concern that the local varieties of mung bean and black gram used in the present study had unusually high levels of PA. In an earlier investigation carried out in the same laboratory using unknown varieties of mung beans consumed by local pre-school children (Karunaratne *et al.*, 2008) the PA level (56 mg 100 g^{-1}) and Zn (0.44 mg 100 g^{-1}) as well as Fe (3.45 mg 100 g^{-1}) were lower. For soya beans, the PA values were comparable in the present and previous studies (970 mg 100 g^{-1} for textured vegetable protein from soya) but the Zn and Fe levels were lower (2.48 and 1.25 mg 100 g^{-1} respectively) .

According to the cut off levels suggested by the W.H.O. (1996), the PA:Zn molar ratio is within the low bioavailable category (>15) in all except in tofu and tempeh which are within moderately bioavailable (5–15) category (Table 1). Using the cut-off level of PA:Fe molar ratio used by Luo *et al.* (2008), fresh soya bean and all soya products are below the cut-off of 14. This suggests that the influence of PA on Fe absorption in 'those' is not of much concern, >14 ratios in mung and black gram preparations tested in this study, indicate that bioavailability of Fe is compromised in them. Additionally, Ca:PA molar ratios above 6 are recorded for both tofu and tempeh, and this ratio is higher for all soya bean preparations indicating that bioavailability of Ca (based on the critical ratio reported by Umeta *et al*., 2005), is low in both mung and black gram preparations. In all black gram and mung bean preparations the PA:Zn molar ratios are in the low bioavailable category. Ironically, although PA:Zn molar ratio has decreased in germinated mung beans, the Ca x PA:Zn ratio exceeded 0.5 even after 72 h of germination (Table 1) suggesting that the Ca has promoted phytate induced decrease in Zn bioavailability (Umeta *et al*., 2005).

With increasing time of germination of mung beans, there was an increase in moisture content which would have facilitated the metabolic breakdown of PA, evident by the corresponding decrease in PA (Table 2). Metabolism related to germination is known to activate enzymes such as phytase (Frias *et al*., 2003). After 48 h of germination, there was an increase in Zn while Fe and Ca levels have not changed. A possible conjecture is that the germinating seedlings have picked up the Zn from the surroundings. As the seeds were not sterile, the presence of metal chelating bacteria cannot be ruled out (Kira *et al.*, 2002). After 48 h, and after 72 h of germination, PA content has reduced significantly (P=0.05) but this reduction in PA level has not been sufficient to increase the Zn bioavailability from the low bioavailability level using the molar ratio of PA:Zn (cut-off suggested by the W.H.O., 1996), in spite of the recorded increase in Zn levels.

The extremely high levels of PA present in locally grown mung and black gram (Tables 2-3) is of concern. A study done in India (Kakati *et al*., 2010) using different cultivars of black gram and mung beans reported levels as high as 1147 and 1124 mg/100g in two black gram genotypes SBC 40 and SBC 47, and levels of 692 mg/100 g and 664 mg/100 g in two mung bean genotypes SGC 20 and SGC 16 respectively. For the study, both black gram (MI 1) and mung bean (MI 6) were obtained directly from the same source (Field Crop Research & Development Institute, Maha Iluppallama) during the same period of time. Soya beans were obtained at least a year later from the same source. It is likely that the level of PA in a plant may be affected by environmental and soil factors. It has been reported that the biosynthesis of PA is linked to several metabolic pathways in a plant; its precursor being D-glucose-6-P (Loewus *et al*., 2000), a common intermediate in a multitude of metabolic pathways. However, whether the high levels of PA in some plants is an adaptive effect of chelating excessive metals, due to prevailing environment factors or due to a varietal effect need to be determined in a long term study. This phenomenon needs to be viewed in the light of the recent concern over possible heavy metal content in soil of the North Central Province of Sri Lanka.

Table 1. Molar ratios of phytic acid to iron (PA:Fe), phytic acid to Zn (PA:Zn), Ca to phytic acid (Ca:PA) and calcium x phytic acid/ Zn (Ca*PA:Zn) in preparations of black gram, mung bean and soya bean.

Ratio / Food Item	PA:Fe	PA:Zn	Ca:PA	Ca*PA:Zn
Fresh Black gram	22	59	0.7	0.1
Fermented black gram	21	55	0.7	0.1
Germinated Mung bean 24 h	41	91	0.4	0.4
Germinated Mung bean 48 h	40	80	0.5	0.5
Germinated Mung bean 72 h	29	66	0.6	0.6
Fresh Soya bean	13	22	5.7	0.1
Soya powder	12	18	5.4	0.1
Tofu	6	13	7.8	0.04
Tempeh	5	10	5.9	0.04

Table 2. Moisture content and levels of PA, Zn, Fe, and Ca in untreated and germinated mung bean (*Vigna radiata).*

Sample	Moisture (g / 100g)	PA (mg / 100g dry weight)	Zn (mg / 100g dry weight)	Fe (mg / 100g dry weight)	Ca (mg / 100g dry weight)
Germinated 24 h	65.9 ±0.9 [a]	2517 ± 62 [c]	2.75±0.0 [a]	5.18±0.1 [a]	66.02±1.3 [a]
Germinated 48 h	68.2 ±0.2 [b]	2296 ± 58 [b]	2.84±0.0 [ab]	4.84±0.0 [a]	70.07±0.3 [a]
Germinated 72 h	72.4 ±0.2 [c]	1998 ± 32 [a]	2.99±0.0 [b]	5.83±0.2 [a]	67.67±0.7 [a]

Means followed by the same letter in a column are not significantly different ($P<0.05$)

Fermentation is reported as an efficient method of reducing the effect of PA (Chaoui *et al*., 2003; Eltayeb *et al*., 2007) and therefore the increase in bioavailability metal nutrients in tempeh could be attributed to the microbial action. However, fermentation of black gram by the traditional method of activating indigenous microflora, did not result in a significant reduction of PA (Table 2). There was only an approximate reduction of PA by about 7% but this reduction has not been sufficient to increase the bioavailability levels of metal nutrients. Inoculation of black gram with different microorganisms caused reduction in PA to different levels (Table 4), the highest reduction being recorded when inoculated with *P. agglomerans*. As the sample tested with LAB showed reduction in PA by about 34% and as the bacteria were isolated from yoghurt and are widely used in fermentation of food products it was decided to make Thosai by inoculating the dough with LAB.

Thosai prepared using LAB inoculation was preferred by 70% of panelists (Table 5). It was also noted for its softer texture compared to the traditionally prepared Thosai which was acceptable to all the panelists but the intense sour taste associated with traditional Thosai was an acceptable attribute to all the panelists.

In investigations carried out by Chaoui *et al*. (2003), >75% reduction of PA has been reported in black gram inoculated with *Lactobacillus plantarum*. Based on the above observations, it appears that when fermenting black gram for various food applications, inoculation of seeds with edible bacteria known to be capable of reducing PA levels, would be a means of improving bioavailability of trace minerals without compromising traditional food preparation practices. *Bifidobacterium* strains which produce phytase have been tried out on bread to reduce PA (Sanz-Penella *et al*., 2009). As more and more Sri Lankans depend on instant mixtures for Thosai, our preliminary experiments show the use of a phytase producing microbial inoculant to promote its fermentation may be a feasible method of improving its micronutrient bioavailability.

In the present investigation on soya products, soya textured vegetable protein (TVP) was not included as the respective levels of PA, Zn and Fe were reported in a previous investigation (Karunaratne *et al*., 2008). The similarity of PA values recorded previously (970 mg g^{-1}, moisture 9%, for PA on soya textured vegetable protein) showed that in soya unlike in mung and black gram the PA levels did not deviate highly (Table 6). When the fermented soya product tempeh, was compared with levels in fresh soya beans, a significant reduction in PA was observed. In tofu, the reduction of PA resulted in a shift of the PA:Zn molar ratio to moderate bioavailability category. Therefore, in soya products, the Zn bioavailability has increased from low bioavailability category to moderate bioavailability category in both tofu and tempeh.

Due to the large difference in PA content of mung bean in a previous (Karunaratne *et al*., 2008) and the present investigations, a comparison of the processing methods cannot be made. In the previous investigation besides the mung beans of unknown varieties, obtained from retailers, their processing methods were different as they were boiled before analysis and the seed coats were intact. In the present study, the seed coats were removed and the seeds were not cooked before analysis, which would also have helped in the retention of PA. Additionally, removal of seed coat in mung bean has resulted in higher levels of PA according to a previous report as well (Hemalatha *et al.,* 2007).

Table 3. Moisture content and levels of PA, Zn, Fe, and Ca in fresh and fermented ground black gram *(Vigna mungo*).

Sample	**Moisture (g/100g)**	**PA dry weight (mg/100g)**	**Zn**	**Fe**	**Ca**
Fresh	10.1 ± 0.1 [b]	1580 ± 45.82 [a]	2.63 ± 0.0 [a]	5.90 ± 0.2 [a]	62.8 ± 1.1 [a]
Fermented	62.0 ± 5.4 [a]	1463 ± 32.82 [a]	2.65 ± 0.5 [a]	5.82 ± 0.1 [a]	64.5 ± 0.9 [a]

Means followed by the same letter in a column are not significantly different (P<0.05)

Table 4. The extent of reduction of PA in black gram inoculated with three different microorganisms.

Inoculant/ microorganism	Microbial count of inoculant	% Reduction of PA
P. agglomerans	8×10^7 Colony forming units per gram	68 ± 6 %
Lactic acid bacterium (Gram positive, coccus) from a yogurt culture	1.4×10^7 Colony forming units per gram	34 ± 13 %
Rhizopus oligosporus	1.5×10^6 spores ml^{-1}	6 ± 2 %

Table 5. Acceptability of Thosai prepared by inoculating lactic acid bacterium and by traditional method.

Properties	Thosai Preparation Method	Acceptability	
		High	Low
Presence of sour taste	Tr	100 %	-
	LAB	-	100 %
Softer texture	Tr	-	100 %
	LAB	100 %	-
General Preference	Tr	30 %	70 %
	LAB	70 %	30 %

Tr = Traditional method LAB = Thosai dough inoculated with LAB

Table 6. Moisture content and levels of PA, Zn, Fe, and Ca in fresh soya bean, soya powder, tofu and tempeh.

Sample of food item	Moisture (g / 100g)	PA (mg / 100g dry weight)	Zn (mg / 100g dry weight)	Fe (mg / 100g dry weight)	Ca (mg/ 100g dry weight
Fresh Soya bean	11.9±0	1223±7[a]	5.54±0.3[a]	8.24±1.3[a]	175±1.2[b]
Soya Powder	4.1±0.6	994±6[a]	5.40±0.2[a]	7.29±0.0[a]	186±1.5[c]
Tofu	75±7	730±4[ab]	5.40±0.5[a]	9.9±1.2[a]	128±6.0[a]
Tempeh	6.6±3	487±8[b]	4.79±0.1[a]	7.80±1.3[a]	169±5.1[b]

Means followed by the same letter in a column are not significantly different (P<0.05)

In the present investigation, in black gram, the seed coat was kept intact. However, in certain food preparations, the seed coat of black gram is removed. Black gram without seed coats were not investigated because, at the beginning of the investigation removal of seed coat from mung bean, and leaving them in the dark for germination were precautions taken to prevent any influence of photosynthesis that may occur on the green seed coat. However, it is reported that decortication has an influence on mineral contents (Hemalatha *et al*., 2007).

Although we opted to interpret our results based on molar ratios, it was not easy to make a logical decision on the best choice of cut-offs of various molar ratios recorded in the literature. Our ideas on this aspect are discussed below briefly: It is noteworthy that molar ratios are worked out based on previously known interactions for specific food items and several reports have relied on molar ratios in interpreting data, such as Umeta *et al.,* 2005, Karunaratne *et al.,* 2008 and Luo *et al.,* 2009. However, in a food matrix the partial influences of different molecules which finally have a cumulative effect on the bioavailability of a nutrient, will be ignored when relying on molar ratios. The different inhibitors in a food matrix (Hemalatha *et al*., 2007) may have synergistic effects or antagonistic effects and therefore working out molar ratios based on a few micronutrients may not give a good prediction of the bioavailability. Besides, the use of cut-offs for various molar ratios are sometimes arbitrary, with no rationale. For instance Umeta *et al.* (2005) have reported a cut-off of >0.15 for PA:Fe as indicative of poor Fe bioavailability. However, this cut-off seems unrealistic considering the high values obtained for PA:Fe in the present study for all foods, as well as those reported by Umeta *et al.* (2005) in their own study. Therefore, we used the cut off of 14 reported elsewhere (Luo *et al*., 2009; Saha *et al*., 1994). Among the other cut-offs used are PA:Fe >1, PA:Ca >0.24, PA×Ca:Zn >200 (Ma, 2011).

Although the use of molar ratios serve as a convenient alternative to studies involving human subjects to determine bioavailability of nutrients it may not be a reliable means specially for testing bioavailability in Sri Lankan dishes (and those in the region) which are more complex in nature. For instance, the general accompaniments to rice, known popularly as curries are prepared with spices in the form of dried powders (such as tumeric, chilli-powder and coriander) and flavourings added in the form of leaves (such as curry leaves and lemon grass and Pandan leaves) or as whole spices (such as garlic, ginger, fenugreek, cinnamon, cloves, cardamoms, mustard seeds and cumin seeds). As these are added in minute quantities their effects tend to be ignored, but their role probably cannot be ignored considering the fact that they are added regularly in varying proportions to all the curries.

In conclusion, the PA levels in locally grown variety of mung bean (*Vigna radiata var*. MI 6) were unusually high and that of black gram (*Vigna mungo var.* MI 1), were also higher compared to previously recorded levels for both crops. However, the PA levels of locally grown soya bean (*Glycine max*) var. *Pb* were comparable to the levels recorded earlier. Differences were also observed in the Fe and Zn levels. The results interpreted based on molar ratios show that the improvement of bioavailability of the minerals was from low to moderate levels. Soya bean preparations had the best bioavailability levels for Zn, Fe and Ca. Of the different processing methods practiced locally, the fermented product soya-tempeh had the highest bioavailability of Zn (moderately bioavailable). The study points toward the fact that germination of mung beans reduces PA levels significantly ($P<0.05$).

While in fermented soya product tempeh, the PA levels were reduced significantly ($P<0.05$), natural fermentation of black gram was not helpful in reducing PA. However, as the preliminary investigations using microbial inoculants to ferment Thosai gave encouraging results, such practices could be intervened with scientific knowledge to reduce PA levels without compromising traditional practices. Therefore, harnessing the activity of phytase rich microbes in fermented food preparations may be a way to improve bioavailability of metal nutrients in pulses which should be the future direction of research. Additionally as a postscript, having interpreted the results based on pre-defined cut-offs of molar ratios, the need to develop an efficient means to determine micronutrient bioavailability is felt.

ACKNOWLEDGEMENTS

We acknowledge funds received from University of Peradeniya (Research Grant No. RG 2006/51/S). Receipt of seeds from Field Crop Research and Development Institute, Maha Iluppallama, Sri Lanka and help extended by the Food Research Unit, of the Department of Agriculture, Sri Lanka are gratefully acknowledged. Technical assistance provided by Ms. Shiromi Rajapura is much appreciated.

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