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Mutagenic Action of Sodium Azide on Phytic Acid and Cooking Time in the First Mutant Generation M_1 of Mangu Beans *(Phaseolus vulgaris* L.*)*

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Abstract: *Phytic acid is a potent anti-nutrient;it reduces the bioavailability of certain nutrients and inhibits enzyme activity. This can impact negatively on metabolic pathways that require these nutrients and lead to deficiency diseases. A study was carried out to access the mutagenic action of sodium azide on the expression of phytic acid and cooking time. These were assessed by the seed analysis of the first mutant generation in pinto, red kidney and navy genotypes of Phaseolus vulgaris. Four doses of the mutagen were applied in concentrations of 0.1M, 0.04M, 0.03M and 0.02M for each genotype and planted in a randomized complete block design. The results showed that phytic acid decreased in the pinto for all the doses except at 0.1M, where it showed an increase (17.56mg/100g) of 60.21%. Phytic acid also showed a decrease (3.9mg/100g, 61.15%) at the highest dose of sodium azide (0.1M) in the red kidney. Phytic acid content in the seeds of navy showed a decrease at all the doses. The lowest phytic acid value (2.74mg/100g, 85.47%) was induced in the navy at dose 0.04M. All the sodium azide doses used in the study significantly ($p<0.05$) increased cooking time in red kidney and navy. In the pinto, higher doses of the mutagen (0.1M and 0.04M) were more effective in reducing cooking time. Breeding programmes can utilize sodium azide to improve the nutritional capacity of Phaseolus vulgaris by reducing phytic acid. A wider range of sodium azide doses which may reduce cooking time in the red kidney and navy are recommended for further study.*

Key words: *Sodium azide, phytic acid, cooking time, Phaseolus vulgaris*

I. Introduction

Phaseolus vulgaris contain a reasonable amount of anti-nutritional substances and this has led to their gross under use (Lyimo *et. al.,* 1992). These substances include chymotrypsin and alpha-amylase inhibitors, lectins, saponin, phytic acid and flatulence factors (Lyimo, *et. al.,* 1992). Phytic acid is the hexaphosphate derivative of inositol (Shaahu, 2011) and is the main storage form of phosphorus in grains and oil seeds (Jacela *et al.,* 2010). It is not digestible in humans because of their inability to produce its digestive enzyme - Phytase. Research has shown feacal phytic acid was strongly correlated with the daily excretion of calcium and magnesium (Owen, 1996). Phytic acid removal or degradation significantly improved iron absorption from soy protein and pea isolates (Hurrel, 2003). After 6 months of storage, there was an approximate reduction of 21% in the phytic acid content of common beans as observed by Nyankuni *et al.,* 2008). A decrease in phytic acid was correlated positively and significantly with a decrease in seed hardness in *Cicer arietinum* (Chickpea) [Reyes-Moreno *et al.,* 2000]. These researchers suggested that in chickpeas, the susceptibility to the development of the hard to cook phenomenon or condition may be related to phytic acid levels in the cotyledon. This view was corroborated by (Gupta, 2011) when he reported that susceptibility to the hard to cook defect could be attributed to a phytic acid interaction with proteins, carbohydrates and small seed grain size of the *Phaseolus* bean species. Storage of beans at temperatures higher than 25^0c and relative humidity greater than 65% can lead to the development of the hard to cook (HTC) phenomena resulting in significantly increased in cooking time, fuel consumption and water use impacting negatively on the acceptability and utilization of the beans (Balamaze *et al.,* 2008). The purpose of this study was to investigate the effect of the mutagen sodium azide on phytic acid and cooking time of *P. vulgaris.* The high nutritive value of *P. vulgaris* and its underuse especially in West Africa where quality nutrition and diversity in diet is a matter of concern prompted this study.

II. Materials And Methods

2.1 Location Of The Experimental Site, Treatment of P. vulgaris Seed And Cultural Practices

This study was conducted in Jos, at the Mista-ali Fadama experimental site on the Jos Plateau of Plateau State, Nigeria. The seeds used for this study were genotypes of *P. vulgaris* identified with respect to their colour, shape and size. The designation of the genotypes were as follows: pinto, red kidney and navy. The seeds were pre-soaked in distilled water up to ten times their volume, for six hours and air dried for 20 minutes then soaked in the mutagen solution for one and a half hours. Four doses of the mutagen with concentrations of 0.1M, 0.04M, 0.03M, 0.02M were used to treat seeds of each genotype. The seeds were washed under running water for 30 minutes and immediately taken to the field for planting. The field experimental site measured 14.8m X 8.5m, was divided into three blocks measuring 14.8m x 2m with spacing of 0.4m between the blocks. Each block was divided into 15 plots measuring 2m x 0.98m each. The seeds were sown 15 per plot and to a depth of 2.5cm (Purseglove, 1974) with 0.3m spacing between columns and rows. Weeding was done manually using the small African hand hoe on the 4^{th} and 8^{th} Weeks After Planting (WAP). Insecticides were sprayed on the plants to kill insect pests on the 6^{th} Week After Planting (DAP). The plants were watered three times weekly till harvest. The pods were harvested at maturity, shelled manually and packed according to the treatments for further analysis.

2.2Determination Of Cooking Time of The Seeds ofP. vulgaris

The measuring cylinder was used to measure 1,600ml of water which was poured into a pressure pot. The pot was covered and put on a lit gas burner which provided a constant source of heat over the cooking time. The pot was opened after 10 minutes and the temperature of the water was taken to ensure the water was at boiling point (100^{o}c). Fifty randomly selected seeds of one genotype were then put into the pot, a timer was immediately started and the was lid replaced. After 40 minutes, one bean seed was randomly removed from the pot every three minutes to check if the cooking time has been reached. Once the first cooked seed was verified, the interval was reduced to fourty seconds until the first five bean seeds were verified to be cooked. The mean cooking time for the five verified cooked seeds was recorded. The beans were considered cooked if a single seed removed from the pot and placed on a white tile was completely mashed when a 1kg steel weight was very gently placed over it. The time taken from the point of introduction of the seeds into the boiling water, to the point where the last of the five bean seeds were verified to be cooked was considered to be the cooking time of the seeds.

Assessment of the softness of the seeds or their suitability for eating at the point when cooking time was said to be reached was done by fifteen trained individuals who tasted the seeds for each treatment and indicated the perceived level of softness of the seeds, rating it on a scale of 1 to 5.One (1) meaning extremely hard and five (5) meaning very soft while laboratory determination of phytic acid content of the seed was done using the method of Wheeler and Ferrel (1971). The data generated was analysed using Analysis of Variance (ANOVA).

III. Results

3.1 Phytic Acid Content of M_1 P. vulgaris Seeds

Table 1: Phytic acid (mg/100g) in M_1 pinto (*P. vulgaris*) bean seeds

Dose of Sodium azide	Phytic acid (mg/100g)	Percentage increase(%)	Percentage decrease(%)
Control	10.96	----	----
0.02M	10.04	----	8.39
0.03M	8.22	----	25
0.04M	6.58	----	39.96
0.1M	17.56	60.21	----

Table 2: Phytic acid (mg/100g) in M_1red kidney (*P. vulgaris)* bean seeds

Dose of Sodium azide	Phytic acid (mg/100g)	Percentage increase(%)	Percentage decrease(%)
Control	10.04	----	----
0.02M	10.04	No change	No change
0.03M	48.16	379.68	----
0.04M	11.89	18.42	----
0.1M	3.9	----	61.15

Table 3: Phytic acid (mg/100g) in M_1 navy (*P. vulgaris)* bean seeds

Dose of Sodium azide	Phytic acid (mg/100g)	Percentage increase(%)	Percentage decrease(%)
control	18.87	----	----
0.02M	6.62	----	64.91
0.03M	7.08	----	62.64
0.04M	2.74	----	85.47
0.1M	8.90	----	52.83

The M_1 generation of the *P. vulgaris* genotypes showed variation in the phytic acid content of the seeds across the different sodium azide treatments. The pinto showed reduced phytic acid for all the doses except at 0.1M, where it showed an increase (60.21%). It was also observed that at dose 0.04M, phytic acid was lowest (6.85mg/100g, 39.96% decrease) for the pinto treatments. On the other hand, the highest dose (0.1M) in the pinto almost doubled the phytic acid content when compared to the pinto control. Red kidney showed a tremendous increase in the phytic acid content of the seeds at dose 0.03M (379.68%) and much less at 0.04M (18.42%) while the highest dose (0.1M) induced the lowest quantity of phytic acid (61.15%). There was no change in the phytic acid content of the seeds at 0.02M as compared to the control. It was noted that the same treatment dose that induced the highest amount of phytic acid in the Pinto (0.1M) induced the lowest amount of phytic acid in the red kidney. The 0.1M treatment induced more than five times less phytic acid in the red kidney than the amount found to be in the pinto at the same dose. The sodium azide treatments for the navy all induced reductions of phytic acid. Sodium azide was most effective in inducing lower phytic acid in the navy genotype as dose 0.04M induced the lowest phytic acid (2.74mg/100g, 85.47%) not just among the navy treatments but among all the *P. vulgaris* treatments combined.

3.2 Cooking Time of First Mutant Generation (M_1) Treatments of P. vulgaris

Table 4: Analysis of variance for cooking time in pinto, red kidney and navy genotypes of *P. vulgaris*

Source of variation	Degree of freedom	Sum of squares	Mean squares	F calc	F tab
Doses	4	386,946.13	96,736.53	9,117.48	2.71*
Genotype	2	210,816.17	105.408.08	9,934.78	3.34*
Dose x Genotype	7	278,207.89	39,743.98	3,745.89	2.44*
Error	28	297.2	10.61		
Total	44	319,412.4			

Table 5: Cooking Time (M) In M_1 Pinto (*P. Vulgaris)* Bean Seeds

Dose of Sodium azide	Cooking time(M)	Percentage increase(%)	Percentage decrease(%)
0.1M	61.00[a]	----	3.67
0.04M	63.00[ab]	----	0.52
Control	63.33[ab]	----	----
0.03M	65.00[ab]	2.63	----
0.02M	67.66[b]	6.83	----

LSD=5.44 * Means with the same letters are not significantly different from each other

Table 6: Cooking time (M) in M_1red kidney (*P. vulgaris)* bean seeds

Dose of Sodium azide	Cooking time(M)	Percentage increase(%)	Percentage decrease(%)
Control	47.66[a]	----	----
0.02M	48.00[a]	0.71	----
0.04M	48.66[a]	2.09	----
0.1M	51.00[a]	7.0	----
0.03M	51.00[a]	7.0	----

LSD=5.44 * Means with the same letters are not significantly different from each other

Table 7: Cooking time (M) in M_1 navy (*P. vulgaris)* bean seeds

Dose of Sodium azide	Cooking time (M)	Percentage increase(%)	Percentage decrease(%)
Control	60.00[a]	----	----
0.04M	60.66[a]	1.09	----
0.03M	67.00[b]	11.66	----
0.1M	70.33[bc]	17.21	----
0.02M	72.66[c]	1.09	----

LSD=5.44 * Means with the same letters are not significantly different from each other

Table 8: Assessment of softness by 15 trained individuals on a scale of 1-5 (1 meaning extremely hard and 5 meaning very soft)

Genotype	Scale	Number of individuals	Percentage
pinto	1	Nill	0
	2	Nill	0
	3	Nill	0
	4	Nill	0
	5	15	100
red kidney	1	Nill	0
	2	Nill	0
	3	Nill	0
	4	Nill	0
	5	15	100
navy	1	Nill	0
	2	Nill	0
	3	Nill	0
	4	1	6.67
	5	14	93.33

KEY to the scale:
1- Extremely hard
2- Very hard
3- Hard
4- Soft
5- Very Soft

In the pinto treatments, there were statistically significant ($p<0.05$) reductions in the cooking time at 0.1M (61minutes, 3.67%) and 0.04M (63minutes, 0.52%), while the two lowest doses, 0.03M (65minutes, 2.63%) and 0.02M (67.66minutes, 6.83%) indicated a significant ($P<0.05$) increase in their cooking time (Table 4). All the treatments of the red kidney showed significantly ($p<0.05$) higher cooking time than the control

(Table 5). This trend was also observed in the navy treatments as all of them showed significant ($p<0.05$) increments in the time the seeds took to cook (Table 6). In the navy treatments, all the treatment doses increased the cooking time significantly ($p<0.05$) except at 0.04M where the increment was not significant. Generally, higher doses of the mutagen reduced cooking time only in pinto and increased them in red kidney and navy. The results of the assessment of softness of the seeds showed that 100 percent of the trained individuals that participated in the assessment considered the pinto and red kidney beans to be very well cooked at the point the cooking method verified the seeds to be cooked while 93.33% of the individuals considered the navy beans to be very well cooked at the same point.

3.3 Interaction Between Dose Of Sodium Azide And Genotype In The Cooking Time OfP. vulgaris Seeds.

Table 9: Table of interaction for three genotypes of *P. vulgaris* treated with five doses of sodium azide.

	pinto	**red kidney**	**navy**	**Mean**
Control	63.33	47.66	60.66	57.2
0.02M	67.66	48.00	72.66	62.7
0.03M	65.00	51.00	67.00	61.00
0.04M	63.00	48.66	60.60	57.4
0.1M	61.00	51.33	70.33	60.8
Mean	63.99	49.31	66.26	

A significant ($p<0.05$) interaction between the independent variables used in the study (sodium azide doses x genotype) indicated that both variables are responsible for the effect that resulted in the cooking time. The treatment combinations showed navy 0.02M ashaving the overall highest cooking time at 72.66 minutes while red kidney control showed the lowest time at 47.66 minutes. Irrespective of the doses of sodium azide, the

red kidney showed the lowest mean cooking time at 49.31 minutes while navy was the highest at 66.26 minutes. Irrespective of the genotypes, the least dose of sodium azide cooked in a mean time of 57.4 minutes.

IV. Discussion

Myo-inositol is the six carbon ring that acts as the phytic acid backbone. Its synthesis is catalyzed by the enzyme D-*myo*-inositol-3-phosphate synthase (MIPS) and it is the sole source of *myo*-inositol which when disrupted, phytic acid cannot be produced. Jinrui *et al.,* (2005) found that in the low phytic acid mutant 3 (lpa 3) of maize that there are multiple phosphorylation routes to phytic acid from *myo*-inositol and that these phosphorylation routes interact. This implies that a phytic acid mutant can by a combination of phosphorylation routes and possibly the creation of new ones double or even triple phytic acid production as observed in the sodium azide dose 0.1M of the pinto treatments which induced a 60.21% increase in phytic acid. Dose 0.03M of the red kidney also induced an unusual 379.68% increase in phytic acid where it more than tripled the amount found in the control. This kind of behavior is often attributed to the quantitative nature of a trait, which effect cannot be determined by single gene action but a corresponding number of biochemical pathways. Consequently, gene modifications resulting from treatments with sodium azide is able to alter the phytic acid amounts in the resulting mutant generation in different ways, either increasing or reducing the amounts of the chemical found in the seeds. Generally, reduction in the production of phytic acid in the seeds is more desirable as it would make free phosphorus more available which could in no small way improve the nutritive potential of the plant (Krishnan, 2009).

In Adesoye and Ojobo (2012) the Nei's genetic similarity indicated that *P. vulgaris* genotypes have a wide genomic base despite their genetic similarity. The study indicated that the high polymorphism observed in the plant could be as a result of the variations in genes that are responsible for the expression of the same character in the different genotypes studied. The different expressions of *P. vulgaris* genotypes used in this study with respect to phytic acid gives credence to variations in the genes controlling the same character in the various genotypes thus resulting in the unique expressions of phytic acid observed in each of the genotypes. Pinto had increased phytic acid in the seed at dose 0.1M, the highest amount for that genotype (17mg/100g) including the control. Red kidney at the same dose (0.1M) had reduced phytic acid (3.9mg/100g, 61.15%), the lowest among the red kidney treatments. This variation of phytic acid in all the three genotypes of *Phaseolus* used in the study establishes that sodium azide can vary the amount of phytic acid produced in each of the three varieties at the different doses of the mutagen used as also reported by Wakentin *et al.,* (2011), when he reported low phytic acid in sodium azide induced mutants in pea.

Phytic acid is produced by different biochemical pathways (Jinrui *et al.,* 2005) and the processes which are not fully understood are under study. But the mutagenic action of sodium azide which is known for point mutations (Khan *et al.,* 2009) can disrupt some of these processes such as mutating a gene responsible for the production of an enzyme that catalyses a step in one of the pathways thus inhibiting the formation of phytic acid through that pathway, and thereby reducing the overall amount that could be produced. The low phytic acid (lpa) gene in a rice genotype was found to encode for 2-phosphoglycerate kinase (2-PGA Kinase) (Krishnan, 2009) which is involved in the elaborate phosphorylation process leading to the production of phytic acid. Sodium azide can effect point mutations and mutate single genes such as the one responsible for 2-PGA Kinase. This action may reduce the amount of phytic acid formed in a mutant such as is observed in the pinto at 0.02M where only a slight decrease in the phytic acid was observed.

The navy variety showed a decrease in the phytic acid content of its seeds at all the doses. Sodium azide was therefore most effective in reducing phytic acid in navy. This observation indicates a high susceptibility of genes that influence the character to sodium azide attack in a beneficial way.

From the proximate analysis of the untreated seeds, phytic acid levels in the control pinto and red kidney genotypes stood at 10.98mg/100g and 10.04mg/100g respectively. The corresponding value for navy was found to be higher at 18.87mg/100g. After the treatment, much lower phytic acid levels were found for navy (2.74mg/100g) at sodium azide dose 0.04M. This is an 85% reduction in the phytic acid levels in the seeds. Lower phytic acid could possibly be induced at this dose (0.04M) for the navy. Sodium azide dose 0.1M also induced a 61% reduction (from 10.04mg/100g to 3.9g/100g) in the red kidney. Dose 0.1M of sodium azide could be an optimum dose for reducing phytic acid in the red kidney. The lowest phytic acid level in the pinto was also recorded at dose 0.04M. This has shown that higher doses of sodium azide are more useful in inducing lower phytic acid levels in the seeds of *P. vulgaris*.

4.1 Cooking Time of First Mutant Generation (M_1) Treatments Of P. vulgaris

The seed coat (testa) constitutes about ten percent of the seed weight (Blair, *et al.,* 2013) and is a factor in cooking time consideration. The formation of lignin in the cell wall of the testa and cotyledon can occur through enzyme mediated pathways. Laffont *et al.,* (2010) reported that the down-regulated O-Methyl Transferase enzyme involved in the bio-synthesis of lignin in the model legume-*Mendicago truncaluta* is found

to be encoded by a mutant gene. The reduction in the production of the enzyme had a direct and corresponding effect on the production of lignin, drastically reducing the amount produced. Thus the down-regulation of an enzyme may reduce the quantity of the final product produced through that process. The two highest doses of sodium azide (0.1M and 0.04M) used in the pinto treatments reduced the cooking time of the seeds. The reduction of cooking time only at the highest doses in the pinto indicates that this can take place only at higher doses of sodium azide by possible interruptions from the down-regulation or total elimination of an enzyme in the pathway for lignin formation. Lignification will mean a mechanically harder and impermeable seed coat which will then translate into less water available for the gelatinization of starch and protein swelling (Irwing and Singh, 2010), processes necessary for proper cooking. The potency of this process could be enhanced by higher doses of sodium azide as observed in the study especially in the pinto.

Pectin-cation cross linking is associated with the degradation of phytate especially in the presence of moisture. This degradation takes place during a resulting calcium- magnesium cation flux which then cross links with pectin to form insoluble pectates (Siddiq and Uebersax, 2010). These insoluble pectates are partly responsible for the difficulty in cooking the seeds. The action of sodium azide at doses 0.04M of the pinto could be explained by the reduction in the formation of phytates which degradation feeds the formation of insoluble pectates. In this study, the dose 0.04M of sodium azide that reduced phytic acid by almost 40% also reduced cooking time in the pinto treatments. The reduction of phytates which in association with pectin fuel the production of insoluble pectates led to a corresponding decrease in cooking time. This phenomenon can also be observed by the drop in the phytate content of *Phaseolus* seeds in storage which have developed the Hard-To-Cook problem. This agrees with the finding of Gupta (2011) when he reported that the hard to cook problem in *P. vulgaris* could be as a result of phytic acid interaction with proteins and carbohydrates in the seeds.

The cooking time trait has also been found to be genetically controlled in cowpea (Nielson, *et al.,* 1993) and Common bean (Jacinto-Hernandez *et al.,* 2003). Whereas, pinto performed differently from red kidney and navy while red kidney and navy had similar responses to sodium azide especially with respect to cooking time.

4.2 Interaction Between Dose And Genotype In The Cooking Time Of P. vulgaris Genotypes

A joint effect of the two independent variables revealed that irrespective of the genotypes, 0.04M is the best performing dose with respect to cooking time even though it cooked at almost the same time as the control.This interaction shows that sodium azide cannot induce appreciably lower cooking time at the doses and in the genotypes used. Other doses performed way above the control. Subsequent studies could incorporate a few more doses of the mutagen while spreading the range to much higher concentrations. This could increase the variability of the results as more acute mutations would be effected.On the other hand, irrespectiveof the doses of the mutagen, the red kidney had the shortest mean cooking time of 49.31 minutes while navy had the highest at 66.26 minutes. It would be recalled that the navy had the highest phytic acid content in the control (18,87mg/100g) among the genotypes used, which could have led to the increase in cooking time. This is corroborated by Reyes-Moreno *et al*., (2000) when they reported that the development of the Hard-to Cook phenomenon in the seeds is due to phytic acid interactions with proteins and carbohydrates. It is noteworthy that the navy which all showed increase in phytic acid also showed a corresponding increase in cooking time. This trend could arise from the formation of insoluble pectates between phytic acid and pectin, fueled by the increase in the amount of phytic acid in the seeds (Siddiq and Uebersax, 2010). If phytic acid and cooking time are indeed positively correlated, finding doses at which the traits are simultaneously reduced would be advantageous for breeding. Thus the genes which control cooking time actually control certain traits in different genotypes which influence it. Polymorphism in the expression of the trait across different genotypes of the same plant could be attributed to variations in the genes which influence these traits. Thus an attack on the nature and stability of a gene by a mutagen can alter the behavior of the gene and also by extention the characters they influence.

V. Conclusion

From the findings of this study, sodium azide has the ability to vary the phytic acid content and cooking time of the seeds of *P. vulgaris* genotypes. Red kidney and navy have similar responses to sodium azide with respect to cooking time as opposed to pinto and red kidney, navy. This observation asserts the polymorphic similarities between red kidney and navy and differences between pinto and red kidney, navy.

The first mutant generation of *P. vulgaris* treated with sodium azide showed both simultaneous increments and reductions in phytic acid and cooking time when observed together at certain doses of treatment. A decrease in both phytic acid and cooking time were observed at treatment dose 0.04M in the pinto and at 0.04 and 0.03M in the red kidney. Since phytic acid and cooking time are both agronomic traits which breeders seek to reduce, a sodium azide dose that reduces both traits will make the task easier as indirect breeding can be used to reduce the one while originally working to reduce the other as well as utilize sodium azide directly to improve

the nutritional capacity of *Phaseolus vulgaris* by reducing phytic acid. It will be interesting to find out if these simultaneous increase or decrease in the traits observed have genetic stability and can be inherited in the following mutant generations.The interaction showed that the doses used do not appreciably reduce cooking time thus a wider range of sodium azide doses which may reduce cooking time in the red kidney and navy are recommended for further study

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