Chapter 1

Mutagenesis: A Useful Tool for the Genetic Improvement of the Cultivated Peanut *(Arachis hypogaea L.)*

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1. Introduction

Ranking 4th among world oilseed crops and 13th among food crops, the cultivated peanut (*Arachis hypogaea* L.) is an important cash crop currently grown in over eighty countries/regions from 40°N to 40°S across tropical and warm temperate regions [1,2]. Its seeds contain about 50% oil and 25% protein, and the crop is thus deemed as a rich source of edible oil and dietary protein. In developing countries, a large portion of peanuts are crushed for edible oil [3]. Food uses of peanut are predominant in developed nations, where high oleate, high protein and reduced fat peanuts are most preferred, as high oleate not only means better keeping quality, but also brings about multiple health benefits, for example, reduced risk of cardiovascular disease, increased sensitivity to insulin, preventative effects on tumorigenesis, and amelioration of some inflammatory diseases [3,4].

Breeding for high yield has been and will continue to be one of the most important objectives of any peanut genetic improvement programs. For quite a long time, intraspecific hybridization (IH) has been the major breeding method of peanut, through which a sizable number of peanut cultivars with high yields have been released. The narrow gene base of the peanut cultigen caused by reproductive isolation from wild *Arachis* relatives has rendered the genetic improvement of the cultivated peanut through IH more and more difficult. To obtain fertile interspecific hybrids, in most cases special technical measures have to be taken to cope with incompatible obstacles and/or ploidy difference in wide crosses [5,6]. Furthermore, backcrossing is needed to break undesirable linkages. Wide segregation range in peanut interspecific derivatives and linkage drags make the breeding

process using wild *Arachis* species lengthy and tedious. In contrast, traits in mutagenized populations tend to stabilize easily. Recent years, in peanut breeding, much attention has therefore been paid to mutagenesis.

Developing peanut cultivars with improved quality has long been proposed as a major breeding objective of the crop. With the depletion of fossil fuel, interest in peanut as a source of renewable energy is growing. Peanut genotypes with both high oil and high oleate are considered most suitable for biodiesel production. Yet little progress had been made in peanut quality improvement, especially for oil and protein content during the past several decades, largely due to the limited genetic variability within the cultivated peanut gene pool as well as unavailability of simple, rapid and cost-effective selection techniques. Conventional analytical procedures for oil, protein and fatty acid determination are destructive, costly and time-consuming, unsuitable for handling large samples frequently encountered in the process of breeding [7]. Fortunately, the stagnant situation is being changed as the wide application of mutagenesis in peanut breeding and the development of near infrared spectroscopy calibration equations for peanut by several authors [8-14].

Using sun-dried peanut seed we have developed NIRS (near infrared reflectance spectroscopy)calibration equations predictive of main quality characters of bulk seed samples and intact single seeds, making it possible to screen a large number of peanut seeds for multiple quality traits rapidly, simultaneously and non-destructively [13,14].

This chapter summarized the recent progress in peanut mutagenesis for yield and quality made by scientists from our research group.

2. Development of high-yielding peanut mutants through chemical mutagenesis

2.1. Flower injection of ethyl methane sulfonate (EMS)

Through injection of 0.3% EMS into flowers of Huayu 16 at 9:00-9:30 a.m. and subsequent selection, we were able to develop a high-yielding peanut cultivar - Huayu 40 [15,16](Table 1).

Huayu 40 has an erect growth habit and sequential branching pattern. As compared with its wild type (Huayu 16), Huayu 40 possesses faster growing and darker green foliage [16]. A study conducted at flowering and pegging stage in 2009 showed that leaf water content, chlorophyll a and b content of Huayu 40 were significantly higher than those of Huayu 16 [15]. IT-ISJ (Intron-Targeted Intron-Exon Splice Conjunction) profiling using 7 primer pairs resulted in 8 bands that could differentiate Huayu 40 and Huayu 16 [15,17]. While Huayu 16 has white and yellow inner seed coat color, Huayu 40 only has white inner seed coat color. At Shandong Peanut Research Institute (SPRI), Huayu 40 showed a yield increase of about 5% over Huayu 16 [15] (Table 1). Basal pod setting around central axes and high peg strength minimizes pod losses at harvest [15,16].

The cultivar, also known as 08-test-A2, was approved for release in Anhui province in 2011 and in Jilin province in 2012 [15,16]. In summer sowing in Anhui province, Huayu 40

matured in 116 days, and produced an average pod yield of 3970.8 kg ha^{-1} at 3 locations, outyielding the local control Luhua 8 by 14.6%, ranking first among the 11 entries in the test [16]. In Jilin provincial peanut cultivar evaluation test conducted in 2010 and 2011, Huayu 40 matured in 124 days. It produced an average pod yield of 3385 kg ha^{-1}, 22.32% over the local check Baisha 1016.

Year	Action
2001	EMS injection and seed harvest
2002	Cultivation and harvest. Extremely poorly performed plants were discarded
2003-2004	Single plant selection
2005	A plant line (E7-2) with more sound mature kernels (SMK) was noticed and selected
2006-2007	Multiplication of E7-2 to obtain adequate seeds for primary yield evaluation
2008-2009	Yield evaluation in Laixi, Shandong, under the name of 08-test-A2. It showed a superiority of 24.93% (kernel yield) over Luhua 11 in 2008, 5.42% and 10.71% over Huayu 16 and Fenghua 1, respectively, in 2009.
2010-present	Continual yield evaluation and seed multiplication in Laixi, Shandong, and in Gongzhuling, Jilin. National peanut primary and secondary yield evaluation test in northern China (2010 and 2011, spring sowing), National peanut pre-release final yield evaluation test in northern China (2012, spring sowing), Jilin provincial peanut cultivar evaluation test (2010 and 2011, spring sowing), Anhui yield evaluation test (2011, summer sowing)

Table 1. Huayu 40: how it was bred

In 2010 Anhui yield evaluation test (2010, summer sowing), Jilin provincial peanut cultivar evaluation test (2010 and 2011, spring sowing), national peanut primary and secondary yield evaluation test in northern China (2010 and 2011, spring sowing), national peanut pre-release final yield evaluation test in northern China (201 2, spring sowing), Huayu 40 produced an average kernel yield of 3057.15 kg ha^{-1}, outyielding the national control Huayu 19 by 8.22%. Having passed the 2 years' regional evaluation test in northern China, Huayu 40 is eligible to enter the national peanut pre-release final yield evaluation test to be performed in May-September, 2012.

In a trial conducted in 2011 to select suitable peanut cultivars for northeast China, Huayu 40 showed high and stable yields across all locations (Prof Hua Yuan Gao, Hong Bo Yu, Shu Li Kang, unpublished data).

2.2. Seed treatment with chemical mutagens

Other lines with high productivity derived from chemical mutagenized peanut cultivars have also been bred. In primary yield evaluation test conducted in 2011 in Laixi, Shandong,

three mutant lines derived from Huayu 22 (a Virginia type peanut cultivar) seeds performed well. 11-L36, a line developed through treatment of Huayu 22 peanut seeds with 0.39% sodium azide (NaN_3), outyielded the local control Fenghua 1 by 27.04% (kernel yield). 11-L39 and 11-L40, both bred through treatment of Huayu 22 peanut seeds with 0.39% diethyl sulphate (DES), had 37.60% and 22.60% more kernel yield than Fenghua 1. These promising lines are to be tested further for yield stability over years and locations.

3. Utility of mutagenesis in peanut breeding for better quality

3.1. NIRS to select quality materials from EMS mutagenized peanut populations

Previously, with the help of NIRS, a peanut plant (M_2) with elevated oleate content was selected from sodium azide mutagenized Huayu 22 seeds [14]. In autumn 2011, single seeds (M_5) with over 70% oleate content was identified by NIRS and further confirmed by GC (gas chromatography).

At SPRI, the NIRS calibration equations has also been successfully used to identify individual single peanut seeds with high oleate, high oil or high protein from EMS mutagenized peanut populations. In the experiment, 2 Virginia type peanut cultivars with desirable external traits for export, viz., LF 2 and Huayu 22 (Table 2), were chosen for mutagenic treatment with a hope to develop peanut cultivars with improved quality attributes and comparable or even higher productivity. The seeds in mesh bags (1140 seeds for each genotype) were soaked in tap water for 4 hours. Just prior to EMS treatment, 0.5%, 1.0% and 1.5% EMS solutions were prepared in 0.1 M phosphate buffered saline (PBS) (pH 7.0). The pre-soaked seeds were then treated with EMS (5ml EMS solution per seed) for 2 hours with continuous agitation. After treatment, the seeds were thoroughly washed in running water for 2 hours. Untreated seeds of LF 2 and Huayu 22 were used as controls. After 30 min of air drying, the mutagenized seeds and the untreated controls were sown (one seed per hill) in twin-row seedbeds with 80 cm bed spacing, 30 cm inter-row spacing and 16.67 cm within-row inter-plant spacing under polythene mulch on 4 May 2010 at the Nianzhitou Experimental Plots, Laixi, China. Routine cultural practices were followed [18]. Since rainfall was adequate during the crop season, no irrigation was applied. Peanut was harvested on 12 September 2010.

As shown in Table 2, EMS treatment of peanut seeds significant influenced the percentage of fertile plants, and the productivity of resultant M_1 plants as well (data not shown). With the increase of EMS concentration from 0.5 % to 1.5%, the number of fertile peanut plants harvested generally decreased; so did the number of plants producing adequate seeds as bulk seed samples for NIRS scanning (Table 2).

Oleate, protein and oil contents of sun dried peanut seeds (M_2) from individual single peanut plants (M_1) were predicted by NIRS using the calibration equations for bulk seed samples [13,14], provided that seeds were enough for the rotating sampling cup of the NIRS machine (Matrix-I, Bruker Optics, Germany). Each seed sample was measured once. Only the seeds from individual single plants predicted as with >58% oleate, >55% oil or >28%

protein were used for further analysis. Individual single seeds from selected single plants were then scanned with the same NIRS machine using a small cup for a single seed. Each seed sample was measured 3 times. Oleate, oil and protein contents were predicted by NIRS equations for single intact peanut seeds [13,14]. For comparison, seed samples (at least 30 seed samples from individual single plants and at least 30 intact single seeds for each genotype) of the untreated controls were analyzed with NIRS. The number of M_1 peanut plants with >60% oleate, >55% oil, or >27% protein predicted by NIRS calibration equations for bulk seed samples and the number of single intact M_2 seeds from these plants that had over 58% oleate, >55% oil for LF 2 and >58% oil for Huayu 22, or >28% protein, predicted by NIRS calibration equations for single intact seeds were listed in Table 3 and Table 4. A large number of single seeds selected had quality traits going far beyond the variation scope of the controls (Table 5). There were marked genotypic effects on quality of M_2 peanut seeds. For LF2-derived populations, 8, 118 and 11 M_2 seeds were predicted to have >60% oleate, >55% oil, and >28% protein by NIRS, respectively; of the 3 treatments, 1.0% EMS produced the largest number of quality materials, as far as oleate, oil and protein contents were concerned (Table 3). For Huayu 22, a total of 14, 70 and 23 M_2 seeds were predicted to have >60% oleate, >58% oil, and >28% protein by NIRS, respectively; a large portion of high oleate/ protein seeds (M_2) were from 0.5% EMS treatment, while 1.5% EMS was most suitable for induction of high oil mutations (Table 4). The frequency of high oil single seeds was generally higher than that of high oleate/protein single seeds (Table 3 and Table 4).

EMS (%)	LF2			Huayu 22		
	NST	NFPH	NPS(%)	NST	NFPH	NPS
0.5	1140	717(62.89)*	517(45.35)	1140	839(73.60)	523(45.88)
1.0	1140	740(64.91)	413(36.23)	1140	588(51.58)	488(42.81)
1.5	1140	425(37.28)	231(20.26)	1140	422(37.02)	324(28.41)

*Values in parenthesis are percentages of NST.

Table 2. No. of seeds treated with EMS (NST), no. of fertile plants harvested (NFPH) and no. of plants scanned by NIRS (NPS)

EMS (%)	No. of quality plants predicted by NIRS			No. of quality seeds predicted by NIRS		
	Oleate (>58%)	Oil (>55)	Protein (>27%)	Oleate (>60%)	Oil (>55%)	Protein (>28%)
0.5	6	2	1	1	33	5
1.0	7	7	13	6	70	6
1.5	4	1	1	1	15	0

Table 3. No. of quality plants (M_1)/seeds (M_2) identified by NIRS in mutagenized LF 2 populations

EMS (%)	No. of quality plants predicted by NIRS			No. of quality seeds predicted by NIRS		
	Oleate (>58%)	Oil (>55%)	Protein (>27%)	Oleate (>60%)	Oil (>58%)	Protein (>28%)
0.5	4	2	1	11	15	19
1.0	6	4	5	2	14	2
1.5	10	4	6	1	41	2

Table 4. No. of quality plants (M_1)/seeds (M_2) identified by NIRS in mutagenized Huayu 22 populations

Untreated control		Single plant			Single seed		
		Oleate (%)	Oil (%)	Protein (%)	Oleate (%)	Oil (%)	Protein (%)
LF 2	Min.	39.80	47.76	24.10	29.26	47.41	20.94
	Max.	51.53	51.46	26.52	39.70	53.19	27.70
	Mean	50.54	49.30	24.56	38.96	50.00	21.00
Huayu 22	Min.	49.28	47.58	23.20	30.96	51.63	20.75
	Max.	56.86	52.24	26.77	52.25	56.99	26.63
	Mean	50.12	49.97	25.90	50.30	55.50	23.82

Table 5. Variation ranges and averages of oleate, oil and protein content in bulk seeds from single plants and in single seeds of two peanut cultivars as predicted by NIRS

Some seeds (M_2) with high oleate, oil or protein content predicted by NIRS were sampled and sent to Food Supervising and Testing Centre (Wuhan), China to analyze their quality by standard methods. For quality analysis by conventional means, a small seed portion of selected single seeds, distal to embry end, weighing no less than 100 mg, was cut off and sent. Oleate, oil and protein contents were determined using GC for determination of fatty acids, Soxlet oil extraction method and Kjeldhal nitrogen determination procedure, respectively. A conversion factor of 5.46 was used to convert the amount of nitrogen to amount of proteins in the samples.

High oleate trait of 5 M_2 seeds selected by NIRS were confirmed by GC analysis; oleate content in these seeds was not less than 60.0%, significantly higher than that in untreated seeds (ck) (Table 6 and Table 5). Similarly, several high oil/protein M_2 seeds were also analyzed with conventional methods, and all of them were found to contain over 60% oil or over 29% protein, much higher than untreated controls (Table 7 and Table 8).

EMS (%)	Cultivar	Seed serial no.	NIRS	GC
0 (ck)	LF 2	LF 2-1-15	38.1	44.2
1.0	LF 2	E2-4-256-24	60.5	62.9
1.0	LF 2	E2-4-178-18	62.4	66.4
1.0	LF 2	E2-4-83-12	63.6	64.3
0(ck)	Huayu 22	HY22-2-10	34.9	47.3
0.5	Huayu 22	E1-3-173-10	63.8	67.3

Table 6. Oleate content (%) in selected single peanut seeds (M_2)

EMS (%)	Cultivar	Seed serial no.	NIRS	Soxhlet extraction
0(ck)	Huayu 22	HY22-2-10	55.5	52.82
0.5	Huayu 22	E1-3-343-24	60.2	61.49
0.5	Huayu 22	E1-3-343-25	60.8	62.38

Table 7. Oil content (%) in selected single peanut seeds (M_2)

EMS (%)	Cultivar	Seed serial no.	NIRS	Kjeldahl nitrogen determination
0 (ck)	Huayu 22	HY22-2-10	20.8	19.5
0.5	Huayu 22	E1-3-180-6	29.0	31.35
0.5	Huayu 22	E1-3-180-10	28.6	30.49

Table 8. Protein content (%) in selected single peanut seeds (M_2)

M_2 seeds that were identified as with high oil, protein or oleate were sown and resultant M_3 seeds were harvested. NIRS analysis of seeds (M_3) from some of the single plants showed that a portion of the single plants kept the quality trait(s), while others did not. For example, Q3-5-23 (plant no. of 2011) was grown from an M_2 seed (seed no. of 2010: E1-3-343-8) with 57.78% oil, and 23 seeds of the plant were analyzed by NIRS, of which 18 contained higher than 55% oil, with the highest being 58.71%. Q3-12-24 (plant no. of 2011), grown from a seed with 62.97% oleate (seed no. of 2010: E1-2-11-16), still had 60.55% oleate in M_3 generation.

To summarize, the present study demonstrated the successful use of NIRS in selecting a limited number of peanut breeding materials with desired altered quality characters from large populations of M_2. Some of the single seeds with high oil, protein or oleate content lost the quality trait(s) in subsequent generation; these quality traits appeared to be caused by physiological abnormalities rather than genetic changes. However, there were other M_3 seeds with quality traits inherited from previous generation, which could be ascribed to mutations in related genes. We have reported a high oleate EMS mutant of LF 2 (2010 seed no.: E2-4-83-12), an output of the present study, that had dysfunctional mutated *FAD2A* (G448A in the coding region) and *FAD2B* (C313T in the coding region) [4]. Despite the uncertainty in quality traits, selection in M_2 seeds is still necessary as it helps to reduce the population size of M_2 plant and hence M_3 seed generations.

In a separate study, we identified several peanut induced/natural mutants with over 70% oleate content, and used them in a hybridization program [3]. Thus far, a great number of high oleate lines including those with double "high"- high oleate and high yield, or high oleate and high shelling percentage, have been tentatively developed. Notably, some of the large seeded lines had a high shelling outturn when planted in Sanya, Hainan province (located in tropical zone), and some lines consistently exhibited high yield when planted in Laixi (located in temperate zone) and Sanya.

3.2. A bold-seeded peanut natural mutant of *A. duranensis* with high oil content

Breeding high oil peanut cultivars especially those with large seeds is most challenging, and variations in oil between years and locations may be quite high. In fact, in China, several high oil peanut cultivars have been released in Hebei, Hubei and Henan, but all of these

peanut cultivars were found to only contain less than 54% seed oil when planted in Shandong peninsula. Availability of high oil genetic resources is of vital importance to the genetic improvement of the cultivated peanut in this region and the like. High oil peanut wild species have been reported by several research groups, but none of them possessed large seeds. A variant of a wild peanut species, *Arachis duranensis* PI 262133, was identified for the first time as with high oil content and large seeds at SPRI.

Cloning and sequencing of the rDNA ITS internal transcribed spacer (ITS) sequences from *A. duranensis* and the bold-seeded genotype detected no difference in their ITS sequences, suggesting that the variant was unlikely to be a result of natural hybridization.

The mutant was identified in a separate square cement block allotted to *A. duranensis* in the wild peanut nursery at SPRI Experiment Station, Laixi. The plant was grown from a seed of similar size to that of *A. duranensis*, but was found to possess thicker branches, and larger leaflets, pods and seeds (Figure 1, Table 9). The size and weight of pods and seeds of wild and mutant type *A. duranensis* significantly differed ($P<0.01$).

Figure 1. Pods and seeds of mutant type (top 2 rows) and wild type (bottom 2 rows) of *A. duranensis*

Trait		Mean	SD
Pod weight (g)	Mutant type	0.93	0.24
	Wild type	0.24	0.05
Pod length (cm)	Mutant type	2.12	0.23
	Wild type	1.28	0.10
Pod thickness (cm)	Mutant type	1.30	0.16
	Wild type	0.75	0.06
Pod width (cm)	Mutant type	1.11	0.12
	Wild type	0.70	0.07
Seed weight (g)	Mutant type	0.67	0.17
	Wild type	0.19	0.05
Seed length (cm)	Mutant type	1.67	0.20
	Wild type	1.03	0.09
Seed thickness (cm)	Mutant type	0.92	0.13
	Wild type	0.57	0.07
Seed width (cm)	Mutant type	0.70	0.08
	Wild type	0.55	0.06

Table 9. Difference in pod and seed size and weight between wild and mutant type *A. duranensis*

Totally 110 seeds were analyzed by NIRS to predict their oil content (Figure 2). Of them, 40 contained more than 55% oil, with 60.33% being the highest. The average oil content of the mutant was 54.23%, as against 52.62% in the wild type *A. duranensis*.

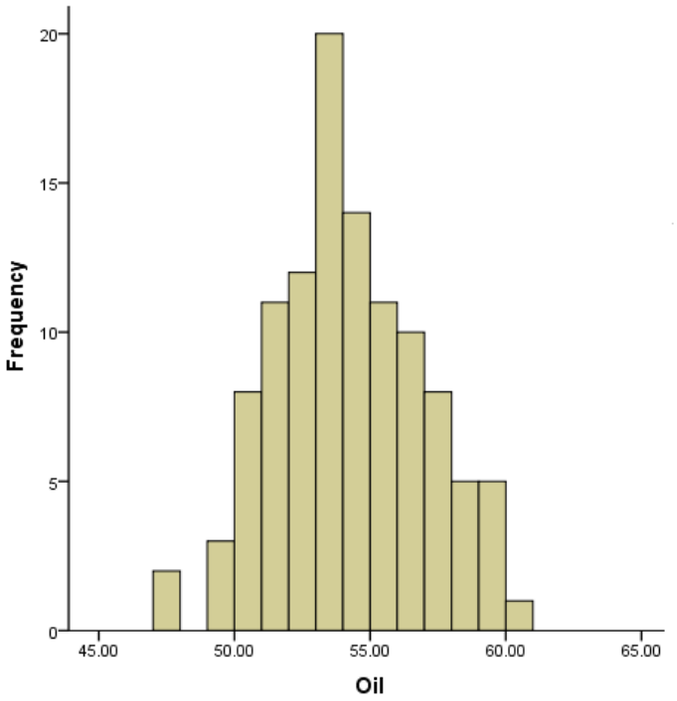

Figure 2. Frequency distribution of seed oil content (%) in 110 seeds of the *A. duranensis* mutant

According to latest classification proposed by Krapovickas and Gregory (1994), the genus *Arachis* is devided into 9 sections, consisting of more than 80 species [19]. Of them, *A. hypogaea* L. is widely cultivated for oil extraction and food uses. Other *Arachis* species of economic importance include *A. glabrata, A. pintoi,* and *A. duranensis*, which are being utilized as forage and/or ground cover [20]. Wild species have higher genetic diversity than the cultivated peanut, providing a desirable source for stress resistance, high oil, protein, oleate, amino acid content as well as high yield factors [20].

Whether the mutant was resulting from chromosome doubling or gene mutation is still unknown. Chromosome counting is absolutely necessary. Anyhow, the mutant reported here is of relevance both to the genetic improvement of the cultivated peanut and to its direct utilization as forage and/or ground cover.

4. Conclusion

Our study on peanut mutagenesis clearly demonstrated that it was possible to induce mutations in productivity, oleate, oil and protein content in the cultivated peanut. NIRS may facilitate identification of induced/natural mutants with improved quality characters. Although not all of the variations in quality traits of M_2 seeds are inheritable, selection in this generation may help to reduce the size of M_3 seed population.

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