

Full Length Research Paper

Application of marker-assisted selection for *ahFAD2A* and *ahFAD2B* genes governing the high-oleic acid trait in South African groundnut cultivars (*Arachis hypogaea* L.)

Mienie, C. M. S.^{1*} and Pretorius, A. E.²

¹North West University, Private Bag X6001, Potchefstroom, South Africa.

²Agricultural Research Council-Grain Crops Institute, Private Bag X1251, Potchefstroom, South Africa.

Accepted 26 June, 2013

One of the major shortcomings in cultivated groundnut in South Africa is the short shelf-life of the product due to rancidity of the oil rich seeds. Polyunsaturated fatty acids are more susceptible to oxidation than mono-unsaturated residues. Thus, it would be preferable to adjust the oleic acid: linoleic acid ratio to a more favourable one. The high-oleic acid trait in groundnut was reported to be dependent on two homeologous oleoyl-PC desaturase genes, *ahFAD2A* and *ahFAD2B*. Breeding of new cultivars with this characteristic can be time-consuming and expensive when doing fatty acid analysis in every generation for selection of the progeny with the highest oleic acid: linoleic acid ratio. Marker-assisted selection was applied to the local groundnut breeding program with the utilization of real-time polymerase chain reaction (PCR). The inheritance of the high oleic trait was followed in the 4th backcross progeny and revealed that all combinations of the two genes were found, except the *ol₂ol₂* homologous mutant. The highest oleic acid percentage was found in progeny with all four mutant alleles (*ol₁ol₁ol₂ol₂*).

Key words: High-oleic, real-time PCR, oleoyl PC desaturase, marker-assisted selection.

INTRODUCTION

Two genomes, A and B, ($2n = 4x$, AABB) are involved in the genetic make-up of the cultivated allotetraploid groundnut (*Arachis hypogaea* L.). Groundnut is better known worldwide as peanut and to a lesser extent as earthnut, monkey nut and goobers. Groundnut is not indigenous to South Africa as it originated from between Southern Bolivia and Northern Argentina in South America where it was discovered in 1502. From there, traders spread the crop worldwide (Pattee and Stalker, 1995; Seijo et al., 2007; CGIAR, 2010, 2011). Groundnut is among the most widely produced legume crops worldwide (Weiss, 2000; CGIAR, 2010). Groundnuts are used as food (raw, roasted or boiled), animal feed (pods,

seeds and plant material) and for industrial raw material. Groundnut products include flour, oil, peanut butter, confectionary and paste. Optimal kernel quality, such as that present in high-oleic acid kernels, is thus, essential for good nutritional value and shelf life of products. High-oleic governing genes dramatically increase the oleic/linoleic acid ratio (O/L) and therefore shelf life of groundnut (Hinds et al., 1992; Damude and Kinney, 2008; Braddock et al., 2006; Henry, 2009; CGIAR, 2010; Pretorius et al., 2010).

Groundnut represents an important source of protein (20 to 36%), oil (45 to 50%) – containing essential fatty acids, carbohydrates ($\pm 20\%$), fibre, folacin, phosphorus,

*Corresponding author. charlotte.mienie@nwu.ac.za. Tel: +27182992319. Fax: +27182992330.

phases were separated and the organic (top) layer, containing the FAMES was dried under nitrogen gas and analysed with a gas chromatograph. The gas chromatograph was equipped with a split/splitless capillary injector and flame ionisation detector (FID). The gas chromatograph utilised a DB-23 capillary column (90 m × 250 µm × 0.25 µm). Nitrogen was used as carrier gas. The detector and injector temperature was 300°C. The column programme was: 145°C for 5 min, then to 216°C at 5°C/min and held at this temperature for 5 min, then to 240°C at 3.3°C/min and held for 10 min. The profile of fatty acids of all the parents involved in the backcrosses was done using the polymeric triglyceride exclusion HPLC method and measured by a nuclear resonance analyser (Oilseed Board, 1996; ARC-Irene Analytical Services, 2003; PPECB, 2000-2007).

Homogenized samples (100 g) from germplasm entries and all potential high-oleic acid progenies were tested for oleic content by using an Atago Palette digital handheld refractometer (PR-301 alpha) to identify high-, intermediate and low-oleic genotypes. This unit allows the user to input the coefficient into the formula: (concentration = Brix × coefficient) to display the concentrations of the samples. Rancidity (RANCIMAT) tests (to compare oxidative heat stability of groundnut oil at frying temperatures) and oleic- and linoleic acid (O/L) ratios were done (Gertz et al., 2000). The selected genotypes were then planted in the greenhouse to multiply.

DNA analysis

The parental as well as the BC's genotypes were tested for the high-oleic molecular markers. Young leaves were collected from single plants, freeze-dried and DNA was extracted using a modified cetyltrimethylammoniumbromide (CTAB) method (Singsit et al., 1997). A multiplex Real-Time PCR assay developed by Barkley et al. (2009, 2011) was used to detect wild type and mutant alleles of the *FAD2A* mutation G448A (Barkley et al., 2011). The sequences of forward and reverse primers were 5'-GCC GCC ACC ACT CCA ACA-3' and 5'-GTT ATA CCA TGA TAC CTT TGA TTT TGG TTT TG-3', respectively. Two TaqMan probes targeted the wild type allele (VIC) and mutant allele (6FAM), with 5' reporter fluorophores, 3' minor groove binders (MGB) and 3' non-fluorescent quenchers (NFQ), namely 5'-VIC CCT CGA CCG CGA CG MGBNFQ-3' (*Ol₁*) and 5'-6FAM CCT CGA CCG CAA CG MGBNFQ-3' (*ol₁*), as synthesised by Applied Biosystems (London, UK). PCR reactions were carried out in 25 µl containing 0.4 ng/µl genomic DNA, 1 × SensiMix II Probe (Bioline, Celtic Molecular Diagnostics, South Africa), 0.16 µM of forward and reverse primers, 0.4 µM VIC probe and 0.3 µM 6FAM probe. The reaction conditions consisted of 1 cycle of 95°C for 10 min, followed by 50 cycles of 95°C for 15 s and 65°C for 1 min, with a final cycle of 60°C for 30 s. All Real-Time PCR was carried out in a BioRad CFX96 cycler.

The *FAD2B* mutation was detected by the same forward primer as for *FAD2A* and reverse primer 5'-TGG TTT CGG GAC AAA CAC TTC-3' (Barkley et al., 2009). Two TaqMan probes were synthesised as aforementioned with the sequences 5'-VIC ACA GGT TCC CTC GAC MGBNFQ-3' and 5'-6FAM ACA GGT TCC CTC AGA C MGBNFQ-3'. The PCR reaction was carried out as aforementioned. Each PCR reaction included duplicate non-template controls as well as positive (UF85) and negative (Akwa) controls.

Statistical analysis

The Real-Time PCR data were subjected to an allele discriminant analysis using the CFX Manager™ software from Bio-Rad. Unknown samples were assigned to homozygote or heterozygote groups using the RFU of positive and negative control samples.

RESULTS AND DISCUSSION

Real-Time PCR was used for detection of *FAD2* alleles on both the A and B genome and to test the heredity in BC4 progenies according to Barkley et al. (2009, 2011). Mono- and heterozygotes for both alleles belonging to the two genes could be distinguished in the allele discriminant analysis (Figure 2). Parents of the backcross progenies as well as 500 individual BC4 plants were subjected to Real-Time PCR. UF85 was used as positive control and was homozygous for the double mutant *ol₁ol₁ol₂ol₂*, whereas, Akwa (negative control) was homozygous for the wildtype *OL₁OL₁OL₂OL₂*. Results show that both SA Juweel and ARC-Oleic2 were homozygous for the mutant alleles, *ol₁ol₁ol₂ol₂*. Both these high oleic acid cultivars displayed an increase in fluorescence with the 6FAM-A probe for the *FAD2A* mutant allele (Figure 2a), as well as with the 6FAMinsA for the *FAD2B* mutant allele (Figure 2b). The low oleic acid kultivar, Akwa, displayed strong fluorescence with both the VIC-probes for the wild type alleles of *FAD2A* and *FAD2B* (Figure 2e and f). Heterozygous progeny displayed high fluorescence for both alleles for these genes (Figure 2e and f). A distinctive grouping of samples could be identified with the allele discriminant analysis (Figure 2g and h) for both genes. Progeny containing allele 1 or allele 2 of the *FAD2A* gene could be clearly distinguished from heterozygous samples displaying equal fluorescence for both alleles. The same tendency was observed for progeny carrying the *FAD2B* mutant and/or wild type allele. The technique is easily applicable to large numbers of samples and very quick to perform.

The parents as well as the end products of the breeding program, the cultivars SA Juweel and ARC-Oleic2, were subjected to gas chromatography to determine the fatty acid composition and verify the results (Table 1). Using a constructed scale of low-oleic (0 to 49% oleic acid); intermediate-oleic (50 to 69%) and high-oleic (> 70%), the backcrosses were grouped into seven possible allele-groups (Figure 3) according to the amount of mutant alleles present as identified with Real-Time PCR.

It is interesting to observe that with only one mutant allele, *ol₁* or *ol₂* present (heterozygote), the percentage low to intermediate offspring was almost the same, but with two mutant alleles from *FAD2A*, *ol₁ol₁*, a much higher percentage low-oleic offspring than intermediate-oleic offspring occurred. Inheritance of both mutant alleles, *ol₁ol₁ol₂ol₂*, led to the highest number of offspring with high oleic acid. Only single plants were found with intermediate oleic acid, with no plants having low oleic acid content. Individual BC4 plants that were homozygous for the mutant alleles of both SNPs were selected for use in the breeding program and further advanced to develop the two cultivars, SA Juweel and ARC-Oleic2. It appeared that the MITE described by Patel et al. (2004) was not present in any of the parents involved in the backcrosses (results not shown).

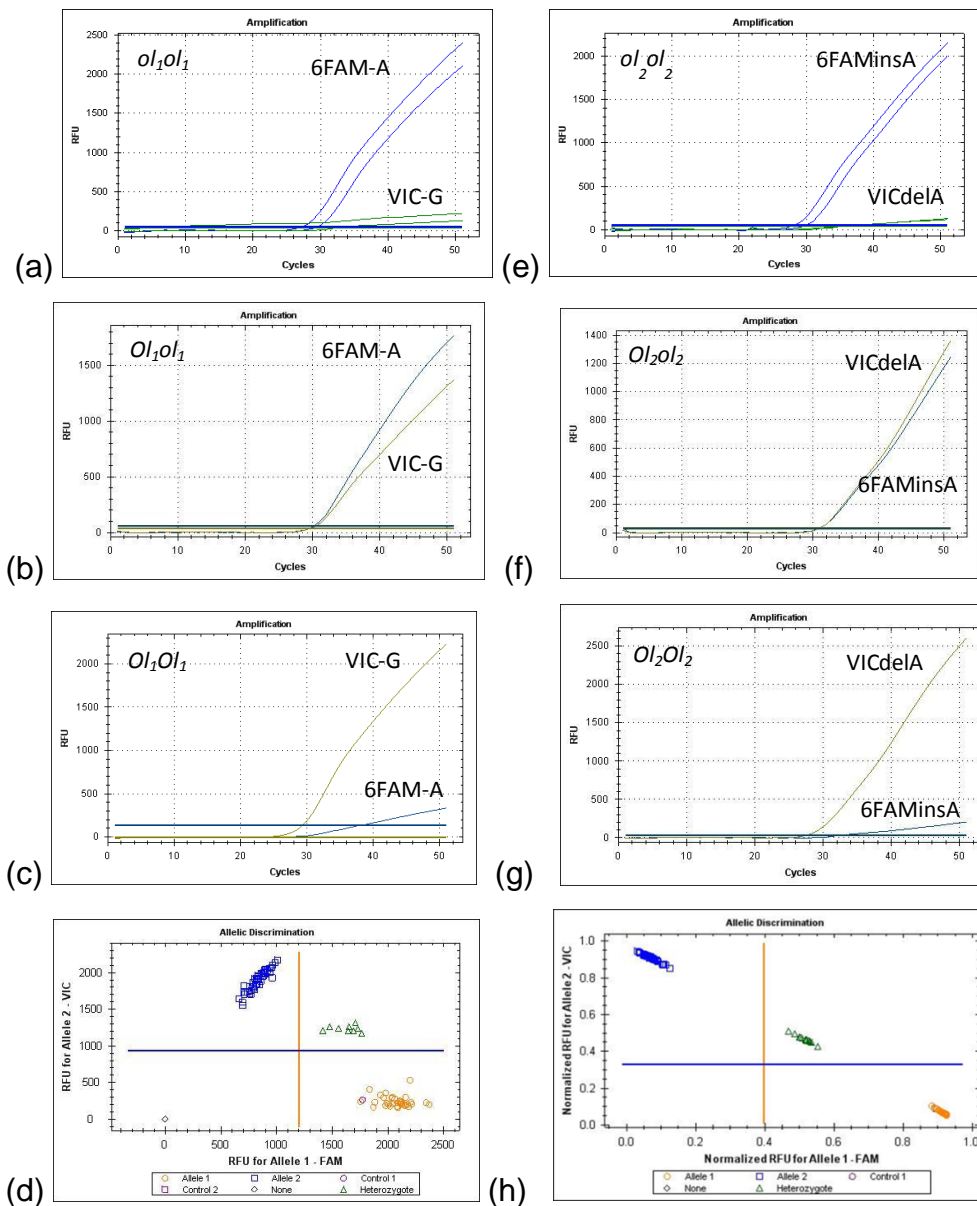


Figure 2 Real-Time PCR profiles of (a) and (e) SA Juweel and ARC-Oleic2; (b) and (f) heterozygote; (c) and (g) Akwa with TaqMan probes for *ahFAD2A* and *ahFAD2B* respectively. (d) Allele discrimination test *ahFAD2A* and (h) *ahFAD2B* of backcross individuals.

Predicting shelf life of a product is easily linkable, percentage wise to Rancimat values. Rancidity (Rancimat) tests are used to compare oxidative heat stability of groundnut oil at frying temperatures (Gertz et al., 2000: 546-551; PPECB, 2003-2007). If the Rancimat value of Akwa and Kwarts is 3.53 h and the shelf life of products hypothetically equals 100%, then products made from SA Juweel with a Rancimat value of 20.3 h will have a shelf life percentage of 575%. So SA Juweel has potentially a ± 5 times longer shelf life than Akwa and Kwarts (PPECB 2000-2007). Where only one or two of the alleles were inherited by the offspring, they had low to

intermediate values of oleic acid. Plants with two *ol2* alleles and one *ol1* allele had higher oleic acid values than plants with two *ol1* alleles and one *ol2* allele (Figure 3). There must be other factors such as the linoleic acid content; besides these two genes influencing the offspring's oleic percentage. The O/L ratio will in turn influence the rate that products will become rancid. Almost 100% of the offspring with the double mutant allele (*ol1ol1ol2ol2*) had a high-oleic acid content. Again, there must be other factors present influencing the expression of the *ol1ol1ol2ol2* as some offspring showed intermediate-oleic percentages. Wherever heterozygotes of the allele

Table 1. Genotypes of parentage involved in the development of commercial cultivars.

Cultivar	<i>FAD2A</i>	<i>FAD2B</i>	% Oleic	O/L
Akwa	<i>Ol₁Ol₁</i>	Wildtype	40.73	1.09
Harts	<i>Ol₁Ol₁</i>	Wildtype	35.10	0.81
Kwarts	<i>Ol₁Ol₁</i>	Wildtype	39.31	1.11
Namark	<i>Ol₁Ol₁</i>	Wildtype	41.08	1.01
Natal Common	<i>Ol₁Ol₁</i>	Wildtype	43.01	1.10
ARC-Opal	<i>Ol₁Ol₁</i>	Wildtype	39.07	1.02
Sellie	<i>Ol₁Ol₁</i>	Wildtype	40.18	1.01
Atete	<i>Ol₁ol₁</i>	heterozygote	54.01	2.15
Guat	<i>Ol₁ol₁</i>	heterozygote	53.65	2.09
Tufa	<i>ol₁ol₁</i>	mutant	54.56	2.21
UF85-1241	<i>ol₁ol₁</i>	mutant	77.5	16.94
SA Juweel	<i>ol₁ol₁</i>	mutant	78.76	12.22
ARC-Oleic2	<i>ol₁ol₁</i>	mutant	78.8	19.96

*O/L: Oleic/linoleic ratio. *Low-oleic acid content (0 to 49%); *Intermediate-oleic acid content (50 to 69%); *High-oleic acid content (>70%).

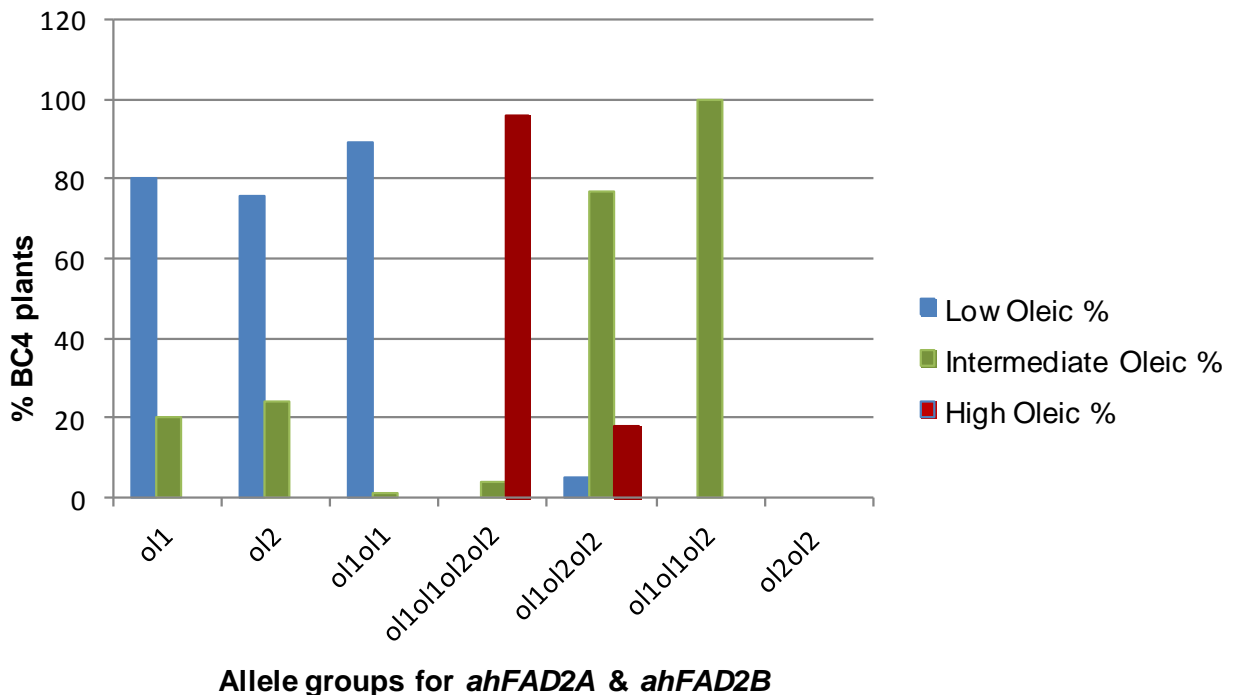


Figure 3. Frequency of allele groups transferred to backcross individuals as identified by Real-Time PCR. Bars indicate % of plants within the specific allele group.

were present, the offspring showed segregating oleic percentages; although, the *ol₁ol₁ol₂OL₂* heterozygote offspring was 100% intermediate-oleic types. It was also interesting that not one of the BC4 offspring was homozygous for the *ol₂*, mutant alleles. This is in accordance with the study of Chen et al. (2010) who also noted that the genotype *OL₁OL₁ol₂ol₂* has not actually been de-

tected from natural populations and thus far has only been found in segregating progenies from controlled crosses.

Other crops planted in South Africa include pumpkin, dry bean, soybean, canola, olive, sunflower and maize. The fatty acid analysis for these crops in comparison with groundnut is summarized in Figure 4 (Norden et al., 1987;

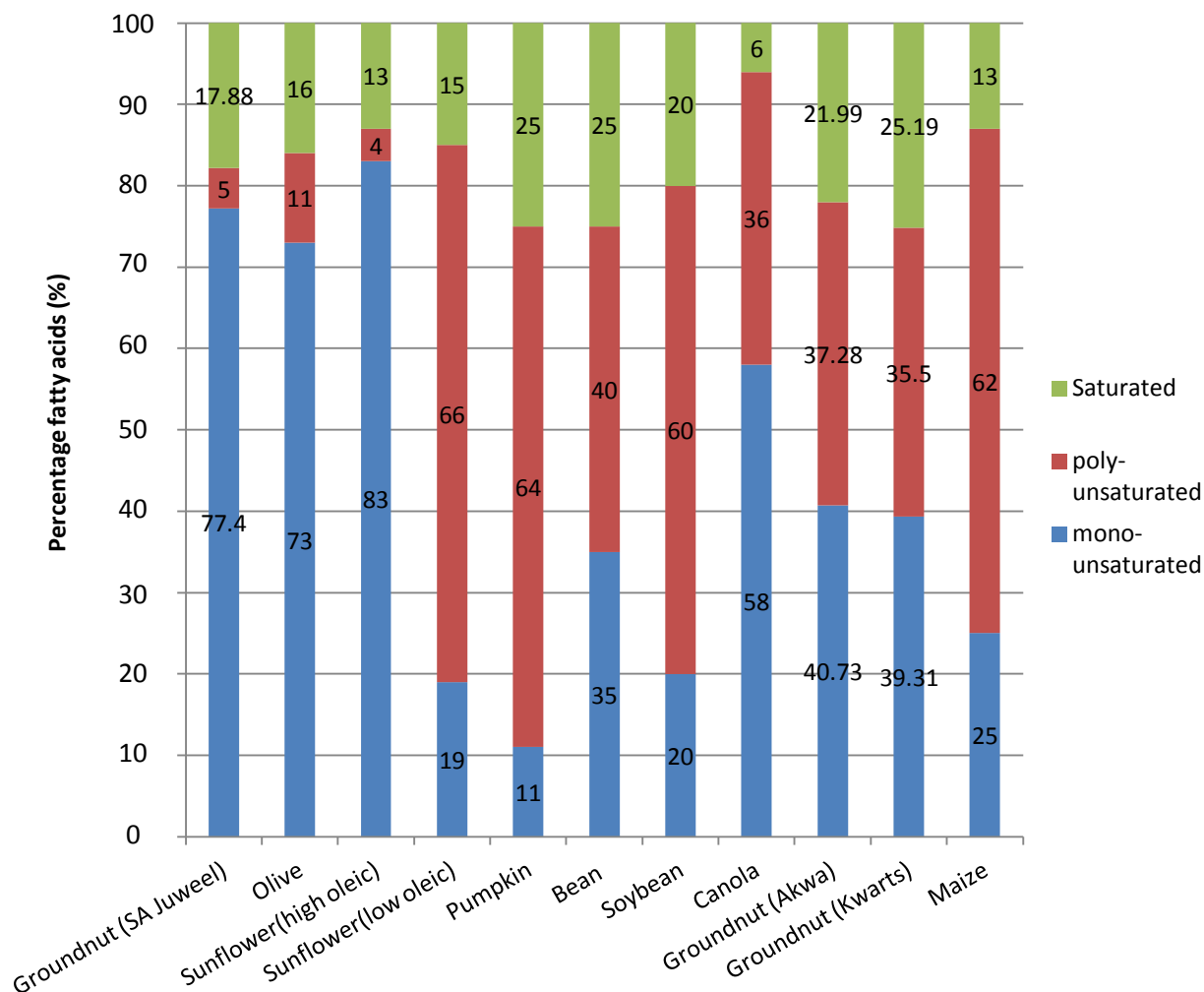


Figure 4. Comparison of fatty acid percentages in the oil (per 100 g sample) of different crops.

Gertz et al., 2000; Özcan and Seven, 2003; Sanders et al., 2003; Anon, 2005; Stevenson et al., 2007; Farooqui, 2010; Shin et al., 2010). It is clear that SA Juweel compares very well with olive and high oleic sunflower, which places it in a very competitive position considering health purposes and shelf life. Rancidity is most prevalent in oils with high levels of polyunsaturated fats (Moore and Knauff, 1989); thus, with the low percentage of linoleic acid present; this cultivar will provide long-term oxidative stability and be a valuable commodity for human consumption.

Conclusion

The Real-Time PCR assays as developed by Barkley et al. (2009, 2011) were successfully applied in the local groundnut breeding program. Molecular marker-assisted breeding programs make it possible to follow the inheritance of the oleic acid trait and provide a huge

benefit for the selection of superior cultivars as less time and thus less finance is needed. Instead of low-cost energy-dense food containing a high percentage saturated fatty acids, rural and urban people can plant the easily cultivated groundnut (use a small space, so it can even be planted in house gardens), containing a high percentage mono- and poly-unsaturated fatty acids. A diet high in MUFA's and PUFA's can combat obesity, resulting in the occurrence of a smaller percentage of cardiovascular diseases.

ACKNOWLEDGEMENTS

Laboratories used in analysis of unsaturated fatty acids in groundnut kernels included ARC-Irene Analytical Services, P/B X2, Irene, Pretoria; Oilseed Board, Oilseeds Building, Arcadia, Pretoria; Perishable Products Export Control Board, Silverton, Pretoria. Genotypes included in the parentage were a kind gift from the

University of Florida (Dr Gorbet) and ICRISAT. The ARC – GCI expresses their appreciation for funding provided by the Oil and Protein Seed Development Trust (OPDT), South Africa.

REFERENCES

- Anon (2005). National Sunflower Association: Health and Nutrition. British Pharmacopoeia Commission. "Ph Eur monograph 1371". British Pharmacopoeia. Norwich, England. ISBN 0-11-322682-9.
- Anyasor GN, Ogunwenmo KO, Oyelana OA, Ajayi D, Dangana J (2009). Chemical analysis of groundnut (*Arachis hypogaea*) oil. Pak. J. Nutr. 8(3):269-272.
- Barkley NA, Chamberlin KDC, Wang ML, Pittman RN (2009). Development of a real-time PCR genotyping assay to identify high oleic acid peanuts (*Arachis hypogaea* L.). Mol Breeding published online 15 September 2009.
- Barkley NA, Wang ML, Pittman RN (2011). A real-time PCR genotyping assay to detect *FAD2A* SNPs in peanuts (*Arachis hypogaea* L.). Electron J. Biotech. ISSN: 0717-3458 <http://www.ejbiotechnology.com>.
- Braddock JC, Sims CA, O'Keefe SF (2006). Flavor and oxidative stability of roasted high-oleic acid peanuts. J. Food Sci. 60(3):489-493. <http://www3.interscience.wiley.com>.
- CGIAR (Consultative Group of International Agricultural Research). (2010). World Geography of the Peanut. <http://lanra.anthro.uga.edu/peanut/database>.
- CGIAR (Consultative Group of International Agricultural Research). (2011). Agriculture for improved health and nutrition. www.cgiar.org
- Chen Z, Wang ML, Barkley N, Pittman RN (2010). A simple allele-specific PCR assay for detecting *FAD2* alleles in both A and B genomes of the cultivated peanut for high-oleate trait selection. Plant Mol. Biol. Rep. 28:542-548.
- Chu Y, Holbrook C, Ozias-Akins P (2009). Two alleles of *ahFAD2B* control the high oleic acid trait in cultivated peanut. Crop Sci. 49:2029-2036.
- Chu Y, Ramos L, Holbrook CC, Ozias-Akins P (2007). Frequency of a loss-of-function mutation in Oleoyl-PC Desaturase (*ahFAD2A*) in the mini-core of the U.S. peanut germplasm collection. Crop Sci. 47:2372-2378.
- Damude HG, Kinney AJ (2008). Enhancing plant seed oils for human nutrition. Plant Physiol. 147:962-968.
- Elmadfa I, Kornsteiner M (2009). Fats and fatty acid requirements for adults. Ann. Nutr. Metab. 55:56-75.
- FAO (Food and Agriculture Organization of the United States). (2010). World Hunger and poverty facts 2010. <http://www.fao.org/hunger/en/>
- Farooqui T (2010). The beneficial effects of olive oil. The Science Advisory Board. <http://webcache.googleusercontent.com/search?q=cache:EqeWjq8CVmJ:www.scienceboard.net/community/perspectives.219.html+list+of+human+diseases+beneficially+affected+by+oleic+acid&cd=2&hl=en&ct=clnk&gl=za>.
- Galli C, Calder PC (2009). Effects of fat and fatty acid Intake on inflammatory and immune responses: A critical review. Ann. Nutr. Metab. 55:123-139.
- Gerber M (2009). Background review paper on total fat, fatty acid intake and cancers. Ann. Nutr. Metab. 55:140-161.
- Gertz C, Klostermann S, Parkash Kochhar S (2000). Testing and comparing oxidative stability of vegetable oils and fats at frying temperature. Eur. J. Lipid Sci. Tech. 102(8-9):543-551.
- Henry J (2009). Processing, manufacturing, uses and labelling of fats in the food supply. Ann. Nutr. Metab. 55:273-300.
- Hinds MJ, Singh B, Anderson JC (1992). Determination of pod and crop maturity for peanuts using percent pod-fill. Can. J. Plant Sci. 72:1057-1065.
- Jenkins WA (1938). Two fungi causing leaf spot of peanut. J. Agr. Res. 56:317-322.
- Mazhar H, Basha SM (2003). Immunochemical characterisation of a methionine-rich protein from peanut. Austr. J. Exp. Agr. 44(12):1221-1224.
- Melanson EL, Astrup A, Donahoo WT (2009). The relationship between dietary fat and fatty acid intake and body weight, diabetes, and the metabolic syndrome. Ann. Nutr. Metab. 55:229-243.
- Moore KM, Knauff DA (1989). The inheritance of high oleic acid in peanut. J. Hered. 80(3):252-253.
- Munné-Bosch S (2005). The role of alpha-tocopherol in plant stress tolerance. J. Plant Physiol. 162(7):743-748.
- Norden AJ, Gorbet DW, Knauff DA, Young CT (1987). Variability in Oil Quality Among Peanut Genotypes in the Florida Breeding Program. Peanut Sci. 14:7-11.
- Özcan M, Seven S (2003). Physical and chemical analysis and fatty acid composition of peanut, peanut oil and peanut butter from ÇOM and NC-7 cultivars. Grasas y Aceites, 54(1):12-18.
- Packer L, Weber SU, Rimbach G (2001). Molecular aspects of α -Tocotrienol antioxidant action and cell signalling. J. Nutr. 131:369-373.
- Pala V, Krogh V, Muti P, Chajès V, Riboli E, Micheli A, Saadatian M, Sieri S, Berrino F (2001). Erythrocyte membrane fatty acids and subsequent breast cancer: a prospective Italian study. J. Natl. Cancer I, 93(14):1088-1095.
- Patel M, Jung S, Moore K, Powell G, Ainsworth C, Abbott A (2004). High-oleate peanut mutants result from a MITE insertion into the *FAD2* gene. Theor. Appl. Genet. 108(8):1492-1502.
- Pattee HE, Stalker HT (1995). Advances in Peanut Science. American Peanut Research and Education Society, Inc. Yoakum, Texas, USA. p. 619.
- Pretorius AE (2006). Evaluation of groundnut (*Arachis hypogaea* L.) germplasm for resistance to leaf diseases and related cytoplasmic factors, testa colour and cup leaf. Department of Plant Sciences, Faculty of Natural and Agricultural Sciences, University of the Free State. (MSc (Agric.) dissertation).
- Pretorius AE, Dreyer J, Salomon L (2010). Release of SA Juweel – a new South African groundnut cultivar with high oleic acid. ejournal.icrisat.org, 8:1-3. www.SAT.eJournal
- Sanders TAB (2009). Fat and fatty acid intake and metabolic effects in the human body. Ann. Nutr. Metab. 55:162-172.
- Sanders TH, Caballero B, Trugo L, Finglas C (2003). Groundnut oil. Encyclopedia of Food and Nutrition: pp.2967-2974.
- Seijo G, Lavia GI, Fernández A, Krapovickas A, Ducasse DA, Bertoli DJ, Moscone EA (2007). Genomic relationships between the cultivated peanut (*Arachis hypogaea*, Leguminosae) and its close relatives revealed by double GISH. Am. J. Bot. 94:1963-1971.
- Shin EC, Pegg RB, Phillips RD, Eitenmiller RR (2010). Commercial runner peanut cultivars in the USA: Fatty acid composition. Eur. J. Lipid Sci. Tech. 112:195-207.
- Shute EV (1957). Alpha tocopherol in cardiovascular disease. Oxford University Medical Gazette, 9:96.
- Singham N, Jogloy S, Kesmla T, Swatsitang P, Jaisil P, Puppala N, Patanothai A (2010). Estimation of heritability by parent-offspring regression for high-oleic acid in peanut. Asian J. Plant Sci. 9(6):358-363.
- Singsit C, Adang MJ, Lynch RE, Anderson WF, Wang A, Cardineau G, Ozias-Akins P (1997). Expression of a *Bacillus thuringiensis* cryIA(c) gene in transgenic peanut plants and its efficacy against lesser cornstork borer. Transgenic Res. 6:169-176.
- Skeaff CM, MILLER J (2009). Dietary fat and coronary heart disease: Summary of evidence from prospective cohort and randomised controlled trials. Ann. Nutr. Metab. 55:173-201.
- Stevenson DG, Eller FJ, Wang L, Jane J, Wang T, Inglett GE (2007). Oil and Tocopherol content and Composition of Pumpkin Seed Oil in 12 Cultivars. J. Agr. Food Chem. (55):4005-4013.
- Vassiliou EK, Gonzalez A, Garcia C, Tadros JH, Chakraborty G, Toney JH (2009). Oleic acid and peanut oil high in oleic acid reverse the inhibitory effect of insulin production of the inflammatory cytokine TNF- α both *in vitro* and *in vivo* systems. Lipids in Health and Disease, 8:25.
- Vorster HH, Kruger A (2007). Poverty, malnutrition, underdevelopment and cardiovascular disease: a South African perspective. African Cardiovasc J, 18(5):321-324.
- Weiss EA (2000). Oilseed Crops. London: Blackwell Science.