International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2019; 7(2): 270-274

Received: 14-01-2019
Accepted: 17-02-2019

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Effect of dominant seed-borne Mycoflora on the quality of different cultivars of groundnut seeds

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Abstract
Groundnut or peanut (*Arachis hypogaea* L.) is a crop of global importance, also known as "King of oilseeds". It is widely grown in the tropical and subtropical regions of the world. Quality of seeds play a very important role for the production of healthy crop. A seed-borne pathogen present externally or internally or associated with the seed as contaminant may caused seed abortion, seed rot, seed necrosis, reduction or elimination of germination capacity as well as caused biochemical changes in infected seeds. Over all, six fungal species belonging to three genera *viz.*, *Aspergillus niger*, *Aspergillus flavus*, *Fusarium oxysporum*, *Rhizopus* sp., *Aspergillus fumigatus* and *Aspergillus terreus* were detected as seed mycoflora in groundnut by agar plate and blotter paper method. *Aspergillus flavus* Link and *Aspergillus niger* van Tieghem were found dominant seed mycoflora in groundnut in all ten cultivars both in agar plate and blotter paper method. Seeds of groundnut cultivars, which were inoculated with two dominant fungi *viz.*, *Aspergillus niger* van Tieghem and *Aspergillus flavus* Link contained low oil (37.89%, 35.07%) and crude protein (21.20%, 21.85%) as compared to control (46.03%, 26.37%), respectively. While free fatty acid content increased (19.25%, 19.14%) in most of the tested cultivars as compared to control (17.63%), respectively.

Keywords: groundnut, seed mycoflora, *Aspergillus flavus*, *Aspergillus niger*, agar plate method, blotter paper method

Introduction
Groundnut or peanut (*Arachis hypogaea* L.) is a crop of global importance, also known as "King of oilseeds". It is widely grown in the tropical and subtropical regions of the world. The crop is believed to have originated in Brazil. It is an important crop to both small holder and large commercial producers. It is classified both as a grain legume and an oilseed crop. It plays a major role in bridging the vegetable oil deficit in the country. It contains 48-50 per cent oil, 26-28 per cent protein and is rich in dietary fibers, minerals and vitamins (Rani, 2014) [14]. Groundnut belongs to the botanical family: Fabaceae (Leguminosae). The specific name *hypogaea* means "under the earth" because groundnut pods develop underground a feature known as geocarpy. Like most other legumes, groundnut harbour symbiotic nitrogen-fixing bacteria in root nodules. This capacity to fix nitrogen implies groundnut require less nitrogen-containing fertilizer and improve soil fertility, thus are valuable in crop rotations. Total production of groundnut in the world is reported to be 43.9 million tons (Anon., 2014) [5]. It is mainly grown in China, India, Nigeria, USA, Sudan, Burma, Indonesia, Argentina, Tanzania and Senegal. India is the second largest producer of groundnut in the world, which is grown in an area of 4.8 million hectares with an annual production of 74.20 lakh tons (Anon., 2015) [6]. It is mainly grown in the states of Andhra Pradesh, Gujarat, Karnataka, Maharashtra, Madhya Pradesh, Tamil Nadu, Rajasthan and Uttar Pradesh among which, Gujarat leads in area and production. Annually, 40.65 lakh tons of groundnut are exported mainly to South-East Asian countries, namely, Indonesia, Malaysia, Vietnam and also to neighbouring countries like Pakistan, Sri Lanka and Nepal (Anon., 2013) [4]. In Gujarat, it is mainly grown in districts *viz.*, Junagadh, Jamnagar, Rajkot, Porbandar, Amreli, Bhavnagar, Kutch, Sabarkantha and Banaskantha, *etc*. with a total area of 14.01 lakh hectares with annual production of 30.18 lakh tons (Anon., 2015) [6].
Two different most common methods, blotter paper and agar plate method were used to detect seed-borne mycoflora. Fungi like *Aspergillus niger*, *Aspergillus flavus*, *Alternaria dianthicola*, *Curvularia lunata*, *Curvularia pallescens*, *Fusarium oxysporum*, *Fusarium equiseti*, *Macrophomina phaseolina*, *Rhizopus stolonifer*, *Penicillium digitatum* and *Penicillium*

chrysogenum causes discoloration, rotting, shrinking, seed necrosis, loss in germination capacity and toxification to oilseeds (Chavan and Kakde, 2008) [9]. *Aspergillus flavus* was found more pathogenic and cause more deterioration due to production of amylase. Amylase can convert starch in to glucose and glucose is easily absorbed by fungi and seeds become more viable and showing high percentage of germination inhibition. Maximum loss in protein and starch was recorded at 45°C due to *Aspergillus flavus* (Khairnar, 2015) [12]. Fungi growing on stored seeds, can reduced the germination rate along with loss in the quantum of carbohydrate, protein and total oil content, induces increased moisture content**,** free fatty acid content and enhancing other biochemical changes. Deterioration of groundnut seeds due to fungal activity is normally associated with the production of off-colours flavours and rancidity. The discoloration of seeds due to seed mycoflora caused effect on yield and quality of oil, loss of seed viability and formation of mycotoxins (Twiddy, 1994) [16]. Adiver and Kumar (2015) [3] reported that seed mycoflora reduced seed quality of groundnut. The infection lead to the reduction in sugars (reducing, non-reducing and total sugars), proteins, oil content and seed germination. The oil extracted from diseased kernel contain high amount of fatty acids as compared to healthy seeds (Begum *et al*., 2013) [7].

Materials and methods

Preparation of Spore Suspension: Spore suspension of dominant seed-borne fungi (*Aspergillus flavus*, *Aspergillus niger*) of groundnut were prepared separately by adding 10 ml distilled water into the sporulating pure cultures of seed-borne fungi of groundnut and maintain on PDA slant for seven days at room temperature. The slants were shaken and content were filtered through muslin cloth to separate mycelium and spore. The filtrate thus obtained was used as spore suspension.

***In vitro* Inoculation of Dominant Seed Mycoflora on Seeds of Groundnut Cultivars:** In order to study the bio deterioration of oil, crude protein and free fatty acid content, the seeds were surface sterilized separately with 1 per cent sodium hypochlorite (NaOCl) and subsequently washed repeatedly with distilled water to remove traces of disinfectant. The seeds were placed in pre-sterilized 100 ml conical flask (50 g seeds/ flask) and separately inoculated with 2 ml of spore suspension of their dominant seed-borne fungi *i.e. Aspergillus flavus* and *Aspergillus niger*. These flasks were incubated for 10 days at room temperature. After the incubation period, the seeds were washed repeatedly under running tap water to remove mycelial growth from their surface. These seeds then dried in oven for 48 hours at 60°C. These oven dried seeds were used for estimation of oil, crude protein and free fatty acid. The seeds were incubated in a similar manner without inoculation with spore suspension of fungi served as control.

Oil Content (%): The oil content of different groundnut cultivars were analyzed through procedure as described by Mehta and Lodha (1979). The total oil was extracted by soxhlet extraction using organic solvents like hexane or petroleum ether (60-80°C).

Apparatus: 1. Soxhlet extract or assembly 2. Absorbent cotton 3. Vacuum oven

Reagents/ Glassware's: 1. Hexane or Petroleum ether 2. 150 ml round flat bottom flask 3. Thimble 4. Mortar and pestle

Procedure: Take 7-8 g seeds in a mortar and crushed it in fine powder with pestle. Five gram quantity from this, weighed accurately and rolled in a piece of filter paper and make into a sample packet. Put this packet into thimble and covered it with absorbent cotton. The thimble was then placed into extractor flask of soxhlet apparatus. Add organic solvent one and half times the capacity of the extractor and extract oils in a pre-weighed flask for a period of six hours at a condensation rate of five to six drops per second or for a period of 8 hours at two to three drops per second. Put-off the heaters and removed thimble from extractor. Washed the extractor one after heating the solvent and then distilled out the solvents. The flask was then transfer into vacuum oven maintained at 55°C for 24 hours. Removed the flask from oven and put into dessicator until it cames to room temperature. Weighed the flask and calculate percentage oil in samples.

Calculation

$$\text{Oil percentage} = \frac{\text{Weight of flask (g)} + \text{oil} - \text{Weight of flask (g)}}{\text{Weight of sample (g)}} \times 100$$

Crude Protein (%)

The crude protein (%) content of different groundnut cultivars were analyzed by micro-kjeldahl method described by A.O.A.C. (1965). In crude protein determination first the nitrogen content was determined by micro-kjeldahl method and then protein per cent was calculated by using the graph factor 5.46 for groundnut.

Equipments and Glassware's: 1. One micro-kjeldahl digestion unit 2. One micro-kjeldahl distillation equipment 3. 12 micro-kjeldahl digestion flask of 30 ml capacity 4. Two pipettes each of 1 ml, 2 ml and 10 ml 5. Two burettes of 10 ml capacity 6. One measuring cylinder of 10 ml capacity 7. Three volumetric flask of 100 ml capacity 8. Wash bottle

Reagents: 1. Sulphuric acid sp.gr. 1.84, N-free 2. Catalyst mixture: Grind together in a mortar 99.0 g of K_2SO_4, 4.1 g of HgO and 0.8 g of $CuSO_4$ 3. Sodium hydroxide sodium thiosulphate solution: Dissolved 50 g NaOH and 5 g $Na_2SO_3.5H_2O$ in distilled water and diluted to 100 ml 4. Boric acid solution: Dissolved 4 g in warm water and diluted to 100 ml 5. Hydrochloric acid solution: 0.02 N 6. Methyl red-bromocresol green indicator solution: Mixed one part 0.2% methyl red in ethanol with 5 parts 0.2% bromocresol green in ethanol

Procedure: Weighed 40 mg sample and transfer it to a digestion flask. Add 1 g of catalyst mixture and 2 ml of concentrated sulphuric acid (H_2SO_4). Digest until the solution was colorless (approx. 40 min. at 370°C). After cooling add minimum quantity of water to dissolve solids and allowed to cool. Pipette 10 ml of boric acid solution into a 100 ml erlenmeyer flask. Add 2-3 drops of indicator solution and place the flask under condenser extending below surface of the solution. Transfer digest to distillation apparatus and rinsed the flask four times with 2-3 ml portions of distilled water. Add 10 ml sodium hydroxide-sodium thiosulphate solution to still and steam distilled until about 20 ml of distillate collected (20 min.). Lower the receiving flask and

continue distillation one more minute. Wash the tip of the condenser with a few drops of water. Removed the receiving flask. Titrate contents of receiving flask to grey end point or first appearance of violet colour. Make blank determination (without sample) using same quantity of reagents and same digestion and distillation period as for sample determination. Include one standard check of tyrosine by taking 10 mg of it.

Calculation

$$\% N = \frac{(\text{ml } H_2SO_4 \text{ in determination- ml blank}) \times \text{Normality} \times 100 \times 14.007}{\text{Weight of sample (mg)}}$$

% Protein = % N x 5.46 (factor for a groundnut)

Free Fatty Acid (%): The estimation of free fatty acid content from different groundnut cultivars were analyzed through procedure as described by Cox and Pearson (1962).

Materials: 1. Phenolphthalein (1%) in 95 per cent ethanol 2. 0.1N potassium hydroxide 3. Neutral solvent: Mix 25 ml ether, 25 ml of 95 per cent alcohol and 1ml of 1 per cent phenolphthalein solution and neutralize with N/10 alkali.

Procedure: Dissolved 1-10 g of oil or melted fat in 50 ml of the neutral solvent in a 250 ml conical flask. Add a few drops of one per cent phenolphthalein. Titrate the contents against 0.1N potassium hydroxide. Shake constantly until a pink colour which persists for fifteen seconds was obtained.

Calculation

$$\text{Acid value (mg KOH/g)} = \frac{\text{Titrate value} \times \text{Normality of KOH} \times 56.1}{\text{Weight of the sample (g)}}$$

Free fatty acid (%) = Acid value x 0.503

Results and discussion

Oil Content (%): Result on influence of seed mycoflora load on oil content of groundnut cultivars presented in Table 1. Result showed that seeds which were inoculated with two dominant fungi *viz*., *Aspergillus niger* (37.89%) and *Aspergillus flavus* (35.07%) contained average low oil percentage as compared to control (46.03%, without inoculation of fungi) in all the ten cultivars. Overall maximum oil per cent reduction found in *Aspergillus flavus* inoculated seeds (35.07%), which was followed by *Aspergillus niger* inoculated seeds (37.89%). Maximum loss of oil content was found in cultivar GG 3, *Aspergillus flavus* inoculated seeds (29.50%), which was followed by GG 4, *Aspergillus flavus* inoculated seeds (32.10%) and GG 7, *Aspergillus niger* inoculated seeds (32.50%).Minimum loss of oil content was found in cultivar GG 13, *Aspergillus niger* inoculated seeds (43.50%), which was followed by GG 8, *Aspergillus niger* inoculated seeds (41.50%) and GG 2, *Aspergillus flavus* inoculated seeds (41%). Abbas *et al.* (2013) [2] studied effect of seed mycoflora on the quality of three genotypes of groundnut *viz.*, Chandra, Indori and Rajasthani and reported that infection of *A. flavus* caused both quantitative and qualitative damage to the seeds and resulted in characteristic decreased in sugar and oil quantity of seeds.

Table 1: Influence of seed mycoflora load on oil content of groundnut cultivars

S. No.	Oil content in seeds (%)			
	Cultivars	***Aspergillus niger* inoculated seeds**	***Aspergillus flavus* inoculated seeds**	**Control (without inoculation of fungi)**
1	GG 2	37.50	41.00	45.40
2	GG 3	39.00	29.50	45.00
3	GG 4	36.20	32.10	49.50
4	GG 5	33.20	39.50	47.40
5	GG 7	32.50	33.60	39.10
6	GG 8	41.50	35.90	46.90
7	GAUG 10	35.50	30.00	47.50
8	GG 11	39.50	36.10	42.00
9	GG 13	43.50	34.50	46.60
10	GG 20	40.5	38.50	47.80
	Mean	37.89	35.07	46.03

Crude Protein (%): Result on influence of seed mycoflora load on crude protein content of groundnut cultivars presented in Table 2. Result revealed that seeds which were inoculated with two dominant fungi *viz*., *Aspergillus niger* (21.20%) and *Aspergillus flavus* (21.85%) contained low crude protein as compared to control (26.37%, without inoculation of fungi) in most of cultivars, except GG 11 in which crude protein content increased due to inoculation of two dominant fungi. Maximum crude protein content was reduced in cultivar GG 3, *Aspergillus niger* inoculated seeds (17.50%), followed by GG 4 and GG 8, *Aspergillus flavus* inoculated seeds (17.58%). Minimum crude protein content was reduced in cultivar GG 7 and GG 11, *Aspergillus flavus* inoculated seeds (25.23%), which was followed by GG 2, *Aspergillus flavus* (24.09%) and GG 11, *Aspergillus niger* inoculated seeds (24.09%). The present findings are tallied with the similar studies carried out by Bhattacharya and Raha (2002) [8], who reported that protein content reduced in the seeds of maize, groundnut and soybean due to infection of seed mycoflora *viz.*, *Aspergillus*, *Rhizopus*, *Penicillium*, *Curvularia*, *Fusarium* and *Alternaria*. Kandhare (2016) [11] reported dominant seed mycoflora of pulses *viz.*, *Aspergillus flavus*, *A. niger*, *A. fumigatus*, *Drechslera tetramera*, *Fusarium moniliforme* and *Rhizopus stolonifer* caused considerable reduction in protein content of the test pulses. Adiver and Kumar (2015) [3] observed that *A. flavus* inoculated seeds lead to the reduction in sugars (reducing, non-reducing and total sugars), proteins, oil content and seed germination. The present results are also in conformity with the above scientists.

Table 2: Influence of seed mycoflora load on crude protein content of groundnut cultivars

S. No.	Crude protein content in seeds (%)			
	Cultivars	*Aspergillus niger* inoculated seeds	*Aspergillus flavus* inoculated seeds	Control (without inoculation of fungi)
1	GG 2	22.20	24.09	24.85
2	GG 3	17.50	21.41	25.62
3	GG 4	21.41	17.58	26.76
4	GG 5	20.75	19.80	25.53
5	GG 7	18.35	25.23	28.00
6	GG 8	20.64	17.58	28.29
7	GAUG 10	23.70	21.41	24.09
8	GG 11	24.09	25.23	23.70
9	GG 13	19.88	23.70	28.29
10	GG 20	23.52	22.50	27.53
	Mean	21.20	21.85	26.37

Free Fatty Acid (%): Result on influence of seed mycoflora load on free fatty acid content of groundnut cultivars presented in Table 3. Result revealed that seeds which were inoculated with two dominant fungi *viz*., *Aspergillus niger* (19.25%) and *Aspergillus flavus* (19.14%) were contained high free fatty acid as compared to control (17.63%, without inoculation of fungi) in most of cultivars except, GG 2 and GG 11 in which free fatty acid content reduced due to inoculation of two dominant fungi. Overall maximum free fatty acid per cent increased in *Aspergillus niger* (19.25%) inoculated seeds, which was followed by *Aspergillus flavus* inoculated seeds (19.14%). Maximum increased in free fatty acid content recorded in cultivar GG 13, *Aspergillus flavus* inoculated seeds (22.01%), which was followed by GG 7, GG 8, *Aspergillus flavus* inoculated seeds (21.44%) and GG 5, *Aspergillus niger* inoculated seeds (21.44%). According to report of Saxena *et al.* (2015) [15], there was gradual decrease in germination percentage, moisture content, protein, total oil, and increase in free fatty acids in soybean seeds due to infection of seed mycoflora. The present findings are also in conformity with this report. The present findings are also tallied with the similar studies carried out by Abbas *et al.* (2013) [2]. They reported oil extracted from diseased kernel contain high amount of fatty acids as compared to healthy seeds in all the three genotypes of groundnut.

Table 3: Influence of seed mycoflora load on free fatty acid content of groundnut cultivars

S. No.	Free fatty acid content in oil (%)			
	Cultivars	*Aspergillus niger* inoculated seeds	*Aspergillus flavus* inoculated seeds	Control (without inoculation of fungi)
1	GG 2	16.36	15.23	17.49
2	GG 3	18.05	16.36	15.52
3	GG 4	20.80	20.30	20.31
4	GG 5	21.44	20.80	15.23
5	GG 7	19.18	21.44	16.93
6	GG 8	20.31	21.44	19.18
7	GAUG 10	20.80	16.36	18.34
8	GG 11	15.23	19.18	21.16
9	GG 13	19.18	22.01	15.80
10	GG 20	21.16	18.34	16.36
	Mean	19.25	19.14	17.63

Conclusion

Seeds of groundnut cultivars which were inoculated with two dominant fungi *viz*., *A. niger* (37.89%) and *A. flavus* (35.07%) contained low oil percentage as compared to control (46.03%, without inoculation of fungi) in all ten cultivars. Maximum loss of oil content was found in cultivar GG 3, *A. flavus* inoculated seeds (29.50%), which was followed by GG 4, *A. flavus* inoculated seeds (32.10%) and GG 7, *A. niger* inoculated seeds (32.50%).Seeds which were inoculated with two dominant fungi *viz*. *A. niger* (21.20%) and *A. flavus* (21.85%) contained low crude protein as compared to control (26.37%, without inoculation of fungus) in most of cultivars, except GG 11. Maximum crude protein content reduced in cultivar GG 3, *A. niger* inoculated seeds (17.50%), followed by GG 4 and GG 8, *A. flavus* inoculated seeds (17.58%). Seeds which were inoculated with two dominant fungi *viz*., *A. niger* (19.25%) and *A. flavus* (19.14%) contained high free fatty acid as compared to control (17.63%, without inoculation of fungi) in most of cultivars, except GG 2 and GG 11. Maximum increased in free fatty acid recorded in cultivar GG 13, *A. flavus* inoculated seeds (22.01%), which was followed by GG 7, GG 8, *A. flavus* inoculated seeds (21.44%) and GG 5, *A. niger* inoculated seeds (21.44%).

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