African Journal of Biotechnology Vol. 10(75), pp. 17095-17105, 28 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2574
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Induction, characterization and genetic analysis of *Aspergillus flavus* resistant mutants in *Arachis hypogaea*

Ahmed Nagib Sharaf[1], Ahmed Gaber[1], Abdelhadi Abdallah Abdelhadi[1] Abdelshafy Ibrahim Ragab[2] and Walid Ahmed Korani[2]*

[1]Department of Genetics, Faculty of Agriculture, Cairo University, Giza, Egypt.
[2]Plant Research Department, Nuclear Research Center, Atomic Energy Authority, Inshas, Egypt.

Accepted 14 November, 2011

The *Aspergillus flavus* infection of peanut (*Arachis hypogaea*) results in the accumulation of aflatoxins in seeds, which are very harmful to humans and animals. Mutation breeding programs are an effective way of inducing resistant mutants. In this study, we induced a genetic variation by using ethyl methanesulfonate (EMS) and gamma rays treatment for four peanut cultivars (32 mutants from Giza 6, 22 mutants from Gregory, 15 mutants from Giza 4 and 15 mutants from Giza 5). The resistant mutants for *A. flavus* were identified by analyzing β-1-3-glucanases activities of the controls and infected mutants using polyacrylamide gel electrophoresis (PAGE). Two, four and four mutants derived from Giza 6, Gregory and Giza 4, respectively, showed high activities of β-1-3-glucanases and therefore more resistant to the infection of *A. flavus*. The genetic similarity of these mutants and their controls was also tested using random amplified polymorphic DNA (RAPD) approach. Although natural polymorphism among peanut cultivars was very low, RAPD patterns showed high polymorphism percentage of DNA fragments (37.13%).

Key words: *Aspergillus flavus*, aflatoxins, *Arachis hypogaea*, peanut, gamma rays, ethyl methanesulfonate (EMS), pathogenesis related (PR) proteins.

INTRODUCTION

Peanut or groundnut (*Arachis hypogaea*) is the fourth world's major source of crop oils after soybean, cottonseed and rapeseed (FAO, 2009). Peanut is rich in its oils and proteins content; its seeds contain about 49% oils and 26% proteins (USDA, 2010a). China and India are the biggest peanut producers as they produce nearly 60% of the world's yield (USDA, 2010b). All parts of the peanut plant can be utilized and it can be used in various types of industries.

Mutations breeding programs are one of the efficient approaches to create a new species in crops by inducing mutants using mutagenesis. Chemical and physical mutagens were used widely for producing mutations to increase genetic variability in target materials. Difficulties of traditional breeding programs in peanut led to using mutation induction as alternative technique. Of more than 265 grain legume cultivars produced using induced mutations in 32 countries, 44 peanut cultivars were developed (Bhatia et al., 2001). Many peanut mutants have been induced by physical mutagenesis (Branch, 2002) and chemical mutagenesis (Rajendraprasad et al., 2000).

However, one of the biggest problems facing the increase of peanut production is the infection by *Aspergillus flavus*. *A. flavus* spores invade the peanut flowers and then travel down the pegs and become established in the developing seeds (Styer et al., 1983). It is an ascomycetous fungus that can infect plants, animals, insects and human (Klich, 2007). It is considered the

*Corresponding author. E-mail: waleedkorany@yahoo.com. Tel: +20225100285. Fax: +20224620812.

All authors contributed equally to this work.

most important pathogen for peanut and many other oil crops; it produces aflatoxins as a secondary metabolite in the seeds (Diener et al., 1987). Aflatoxins are acutely toxic, carcinogenic and immunosuppressive class of mycotoxins to animals and human (Scheidegger and Payne, 2003). They are well known hepatotoxic, hepatocarcinogenic and mutagenic agents. These effects are mainly due to adduct formation with DNA, RNA and protein. In addition, it also causes lipid peroxidation, as well as oxidative damage to DNA (Verma, 2004).

Aflatoxin B1 is the most potent and carcinogenic naturally substance known (Squire, 1981). The extent of contamination varies with geographic location, agricultural and agronomic practices, storage and processing period. In some regions, contamination is predominantly pre-harvest, while in others it is major postharvest (Swindale, 1987; Ahmed et al., 1989). The reduction of aflatoxins in peanut seeds can be achieved by many approaches like, heating, drying and particular agricultural practice, but the development of crop species with high resistance to afla-toxigenic molds is the greatest potential way (Rustom, 1997).

Screening of *A. flavus* resistant mutants in peanut mutation breeding programs is the most challenged step. Many approaches have been studied for this context (Liang, et al., 2006). One of the best ways to achieve the screening is using the Pathogenesis Related (PR) proteins. Infection of plant with pathogens induces the accumulation of a group of proteins collective known as Pathogenesis Related proteins (PR-proteins). The PR-proteins have certain characteristic properties such as being selectively extractable at low pH and highly resistant to proteolytic enzymes (Pierpoint et al., 1981). The infected peanut seeds with *A. flavus* synthesize PR protein. Chitinase and β-l-3-glucanase are the important PR-proteins in defending the plant against pathogens. They can protect the plant from fungal infection by their direct lytic action on fungal cell wall or by releasing oligosaccharide signal molecules that can activate a variety of plant defenses (Nasser et al., 1990 and Boiler, 1985). β-l-3-Glucanase' activity is used to identify the *A. flavus* resistant germplasms in peanut (Liang et al., 2005).

This study aimed at enhancing genetic variations in four peanut cultivars (Giza 6, Gregory, Giza 4 and Giza 5) using gamma rays and ethyl methanesulfonate (EMS). In addition, we isolated different mutants from M_2, screened them for the *A. flavus* resistance under artificial infection using PR proteins and characterized them under molecular level using RAPD approach.

MATERIALS AND METHODS

Plant and fungus materials

In this study, we used two peanut cultivars (Giza 6 and Gregory) and two bulks mutants derived from Giza 4 and Giza 5 cultivars selected by Ragab et al. (2008). Aflatoxins (B_1 and B_2 groups) producer strain of *A. flavus* Link anamorph obtained from Egyptian Microbial Collection, MIRCN, Cairo, Egypt was used in artificial infection for peanut seeds.

Chemicals and mutagenesis

Ethyl methanesulfonate (EMS), 2,3,5 triphenyltetrazolium chloride, acrylamide, N,N' methylenebisacrylamide, ammonium per sulphate and N,N,N',N' tetramethylenediamine (TEMED) were purchased from Sigma Chemical Company (St. Louis). Go-Taq DNA polymerase was purchased from Promega Company (Madison, USA). Other chemicals were of the highest purity grade commercially available. Gamma rays were from Co^{60} source at Nuclear Research Center, Egyptian Atomic Energy Authority.

Filed experiment

Seeds of Giza 6 and Gregory cultivars were treated by two concentrations of EMS (0.2 and 0.3%) and irradiated by two doses of gamma rays (150 and 200 Gy). Seeds of mutant bulks derived from Giza 4 and Giza 5 cultivars were treated by 0.2% EMS and 200 Gy of gamma rays. The treated seeds and controls were grown in experimental field of Nuclear Research Center, Egyptian Atomic Energy Authority in split plot design with three replications for two generations (M_1 and M_2) during a two successive peanut growing seasons (2008 and 2009). All the optical agricultural practices were applied and the plants were left for the natural pollination. The selection for new mutants was achieved for M_2 plants depending on the morphological and economical characters; 32 mutants were isolated from Giza 6, 22 mutants were isolated in Gregory, 15 mutants were isolated from Giza 4 and 15 mutants were isolated from Giza 5. The yield components (plant height (cm), number of branches/plant, number of pods/plant, weight of pods/plant (g), number of seeds/plant, weight of seeds/plant (g), shelling (%), weight of 100-seeds (g), protein content (%) and oil content (%) were measured for all isolated mutants.

Estimation of seed protein and oil content

Estimation of seed protein content and oil content for M_1 and M_2 generations was performed by Instalab 600 Near InfraRed Product Analyzer.

Artificial infection

Seeds of control and mutants were surface sterilized by using 12% hypochloride for 10 min and washed three times by sterilized distilled water. Seeds were exposed to *A. flavus* L. suspension (6 spore/ml), then incubated in potato dextrose agar media (PDA) which consists of 100 g potato, 20 g dextrose, up to 1000 ml distilled water and 15 g agar at 26°C for two weeks.

Screening for resistance to *A. flavus*

Pathogenesis related protein (β-1-3 glucanase and its isoforms) were isolated and screened by polyacrylamide gel electrophoresis (PAGE) under native conditions according to the method of Pan et al. (1991) and the modification of Liang et al. (2005). Gels were incubated in a solution containing 1% laminarin for 90 min at 40°C. β-1-3-Glucanase activity in the gels were visualized by staining the gels for 10 min and boiling in a 1 M NaOH solution containing 0.3% (wt/vol) 2,3,5-triphenyl-tetrazolium chloride. After staining, gels were placed in 7.5% acetic acid and stored at 4°C.

Table 1. List of RAPD primers and their nucleotide sequences.

Primer (OP-)	Sequence (5'-3')	Primer (OP-)	Sequence (5'-3')
A01	CAGGCCCTTC	B15	CCACAGCAGT
A02	TGCCGAGCTG	C13	TGCGTGCTTG
A03	AGTCAGCCAC	C18	TGAGTGGGTG
A07	GAAACGGGTC	E02	GGTGCGGGAA
A08	GTGACGTAGG	E16	GGTGACTGTG
A09	GGGTAACGCC	G04	AGCGTGTCTG
A14	TCTGTGCTGG	G05	CTGAGACGGA
A19	CAAACGTCGG	G06	GTGCCTAACC
A20	GTTGCGATCC	G07	GAACCTGCGG
B01	GTTTCGCTCC	G08	TCACGTCCAC
B02	TGATCCCTGG	G16	AGCGTCCTCC
B07	GGTGACGCAG	O02	ACGTAGCGTC
B08	GTCCACACGG	O03	CTGTTGCTAC
B11	GTAGACCCGT		

Polymerase chain reaction with random primers

DNA was extracted according to the method of Dellaprota et al. (1983). Polymerase chain reactions (PCR) was performed in 30 µL volumes tubes according to Williams et al. (1990). 27 decamer oligonucleotide primers of arbitrary sequences (Operon Technologies, Inc) were used in this study (Table 1). The PCR were carried out in 20 µL volume containing 50 ng of genomic DNA template, 2.0 µM primers, 2.0 µM each of dNTPs mix, 2.0 mM $MgCl_2$, 1x buffer and 2 units of Taq DNA polymerase. The reaction mixture was incubated in thermocycler (MWG-BIOTECH Primus) programmed as follows: an initial strand separation at 94°C (5 min) followed by 40 cycles with the following temperature profile: 94°C (30 s), 35°C (1 min), 72°C (2 min) and then a final extension cycle at 72°C (5 min) was done. Amplification products were analyzed by electrophoresis in 1% agarose gels stained with ethidium bromide and photographed using gel documentation system (UV transilluminator). DNA fragments were determined using 1 kb Ladder marker.

Data analysis

The amplified fragments were scored as present (1) or absent (0). Ladder 1 kb marker was used to identify the molecular weights of fragments. Similarity matrix among mutants and their controls was calculated according to Dice (1945) and similarity co-efficient were used to design the phylogenetic relationships according to Sneath and Sokal (1973).

RESULTS

Yield and kernel quality of the selected mutants

The mutation breeding efforts led to the development of 32 (A01-A32), 22 (B01-B22), 15 (C01-C15), 15 (D01-D15) mutants from Giza 6, Gregory, Giza 4 and Giza 5, respectively (Table 2). Recurrent mutagenic treatment as applied to Giza 4 and Giza 5 cultivars on M_4 bulks mutants were characterized with high yield production and sensitive for *A. flavus* infection (Rageb et al., 2008), thus increasing the resistance for the fungus. Grain yield advantage was observed for most of mutants and their quality in terms of protein and oil contents. As shown in Table 2, among the selected mutants, mutant A03 not only had the highest value of weight of pods per plant (220.98 g), but also the highest value of weight of seeds per plant (147.44 g). Concerning 100-seeds weight, mutant C09 showed the highest value than all parents (104.04 g). Four mutants; A15, A19, A20 and A26, were characterized with high frequency of triple pods (pods with three seeds). Meanwhile, eight mutants; A23, A25, B4, B5, B17, C3, C5 and C13, showed high frequency of single pods (pods with one seed). We isolated 15 mutants that had large pod size. In addition, we selected 12 mutants that had small pod size.

Detecting β-1-3-glucanase isozymes in the resistant mutants after the inoculation with *A. flavus*

The activities of β-1-3-glucanase isozymes in resistant and susceptible peanut genotypes were analyzed on the PAGE gels in extracts from infected seed of three control cultivars and their selected mutants (Figure 1). In gel, assays were conducted in native PAGE to detect the isoform patterns of β-1,3-glucanase in resistant and susceptible seeds as a result of infection of *A. flavus* (Figure 1). Five bands indicating different β-1,3-glucanase isoforms were detected in Giza 6, Giza 4 and Gregory mutants and labeled as Glu 1 to 5 (Figure 1). Bands Glu 1 and Glu 5 were present in all samples, indicating constitutive expression of endogenous β-1,3-glucanases. Band Glu 2 might not be of peanut origin because this band was detected and accumulated only

Table 2. Yield component traits of the selected mutants and means of their parents (Giza 6, Gregory, Giza 4 and Giza 5).

Mutant	Height (cm)	Number of branch	Number of pod/ plant	Weight of pod/ plant(g)	Number of seed/ plant	Weight of seeds/ plant (g)	Shelling (%)	100-seeds weight (g)	Protein (%)	Oil (%)
Giza 6	38.30	3.60	58.73	92.52	77.93	58.96	63.66	75.85	29.82	40.44
Gregory	41.81	3.41	46.85	71.49	64.04	44.15	60.99	65.96	31.27	39.03
Giza 4	48.20	3.50	49.26	80.28	59.17	46.11	57.72	77.84	30.14	38.52
Giza 5	40.37	3.47	37.50	51.77	42.10	30.26	57.86	70.89	31.99	39.04
A01	50	3	83	129.31	98.00	76.64	59.27	78.20	32.66	41.75
A02	53	4	137	220.98	145.00	124.68	56.42	85.99	30.96	42.52
A03	51	5	183	240	203.00	147.44	61.43	72.63	30.61	40.85
A04	51	5	90	90.63	83.00	49.87	55.03	60.08	27.87	37.75
A05	49	4	91	151.33	108.00	89.72	59.29	83.07	30.53	43.63
A06	42	4	106	189.72	147.00	120.96	63.76	82.29	30.84	44.11
A07	33	3	74	122.28	76.00	69.43	56.78	91.36	33.47	41.81
A08	38	3	95	170.2	126.00	106.31	62.46	84.37	31.57	43.17
A09	44	3	143	174.75	167.00	104.38	59.73	62.50	31.26	43.59
A10	53	4	88	161.5	112.00	94.44	58.48	84.32	33.74	41.94
A11	42	3	120	149.43	143.00	102.41	68.53	71.62	32.63	44.56
A12	44	3	79	109.78	107.00	77.86	70.92	72.77	30.45	39.59
A13	41	4	55	92.05	68.00	62.15	67.52	91.40	31.33	38.84
A14	48	3	100	168.83	126.00	96.86	57.37	76.87	30.15	35.95
A15	46	4	45	71.6	62.00	48.48	67.71	78.19	31.16	41.54
A16	42	4	81	131.44	102.00	81.79	62.23	80.19	34.39	41.62
A17	43	4	102	157.4	116.00	93.03	59.10	80.20	33.73	42.58
A18	40	4	109	123.7	107.00	63.91	51.67	59.73	35.58	41.81
A19	42	4	80	118.17	95.00	56.92	48.17	59.92	31.26	39.02
A20	53	3	101	167.31	146.00	97.15	58.07	66.54	30.49	40.26
A21	47	3	101	118.14	92.00	55.04	46.59	59.83	30.74	40.88
A22	58	4	86	154.03	113.00	93.78	60.88	82.99	30.30	40.47
A23	61	4	177	210.91	214.00	140.48	66.61	65.64	29.82	41.86
A24	39	3	108	164.59	127.00	99.91	60.70	78.67	31.86	40.65
A25	52	3	128	193.87	129.00	110.63	57.06	85.76	30.32	36.36
A26	44	4	100	157.63	99.00	76.09	48.27	76.86	35.33	40.95
A27	49	4	107	201.8	138.00	121.71	60.31	88.20	32.40	41.98
A28	42	4	63	100.53	71.00	61.00	60.68	85.92	30.32	39.42
A29	41	4	125	181.48	138.00	113.31	62.44	82.11	30.43	39.40
A30	53	5	181	202.49	187.00	115.64	57.11	61.84	29.51	40.95
A31	54	4	116	151.82	131.00	87.31	57.51	66.65	29.26	37.12
A32	35	3	56	90.91	66.00	51.58	56.74	78.15	30.90	36.52
B01	51	4	131	197.52	145.00	124.20	62.88	85.66	32.07	39.16
B02	51	4	78	145.9	121.00	96.94	66.44	80.12	31.73	41.17
B03	53	4	112	207.01	163.00	136.96	66.16	84.02	33.71	37.40
B04	65	4	133	182.43	163.00	110.88	60.78	68.02	33.65	40.83
B05	69	4	106	163.6	137.00	102.21	62.48	74.61	30.98	38.11
B06	56	4	81	141.18	107.00	89.32	63.27	83.48	30.89	42.26
B07	46	4	101	168.77	137.00	108.56	64.32	79.24	32.96	39.90
B08	52	3	61	104.37	83.00	64.00	61.32	77.11	30.91	42.03
B09	57	4	53	90.17	61.00	52.51	58.23	86.08	32.71	40.10
B10	53	3	62	115.07	91.00	73.16	63.58	80.40	32.76	38.73
B11	46	4	99	160.22	141.00	110.61	69.04	78.45	28.72	42.88
B12	66	4	121	216.09	171.00	130.90	60.58	76.55	28.91	39.30

Table 2 Cont

B13	55	5	150	248.83	177.00	142.67	57.34	80.60	29.15	35.90
B14	52	4	72	112.53	83.00	63.34	56.29	76.31	30.53	39.09
B15	67	3	59	91.58	59.00	45.59	49.78	77.27	28.61	39.67
B16	62	5	116	176.19	131.00	98.33	55.81	75.06	28.10	37.40
B17	57	4	113	117.28	78.00	52.31	44.60	67.06	29.81	39.28
B18	57	4	68	102.2	62.00	48.91	47.86	78.89	31.78	40.94
B19	52	4	80	138.32	99.00	84.34	60.97	85.19	32.80	43.96
B20	49	4	106	170.96	144.00	110.17	64.44	76.51	32.01	40.13
B21	57	4	100	167.02	129.00	101.42	60.72	78.62	28.88	36.05
B22	53	4	86	155.69	122.00	104.53	67.14	85.68	30.35	44.14
C01	35	4	86	126.86	107.00	77.21	60.86	72.16	32.15	38.38
C02	47	3	63	103.23	77.00	66.99	64.89	87.00	30.40	40.48
C03	40	4	63	75.23	56.00	45.96	61.09	82.07	31.53	44.85
C04	41	4	98	175.33	101.00	89.69	51.15	88.80	29.87	42.09
C05	35	3	62	53.97	43.00	25.62	47.47	59.58	33.65	38.54
C06	45	3	48	86.47	61.00	57.90	32.42	94.92	31.56	40.44
C07	47	3	77	125.2	99.00	81.98	66.46	82.81	33.71	40.69
C08	49	4	107	178.59	120.00	101.49	56.83	84.58	34.07	40.07
C09	56	4	66	123.35	76.00	79.07	65.54	104.04	34.66	46.47
C10	52	3	90	158.14	108.00	94.03	65.88	87.06	33.31	40.04
C11	39	3	67	120.65	81.00	79.92	104.36	98.67	34.22	42.13
C12	48	4	108	142.73	116.00	89.06	102.97	76.78	32.39	39.25
C13	37	3	56	76.58	16.00	47.20	33.15	295.00	32.04	39.74
C14	41	3	56	86.49	66.00	53.38	61.72	80.88	30.63	40.00
C15	43	3	85	142.38	114.00	93.79	65.87	82.27	32.11	42.49
D01	47	3	58	86.4	62.00	48.52	28.85	78.26	30.80	37.73
D02	49	4	77	118.1	88.00	67.14	52.78	76.30	30.89	39.39
D03	53	4	96	168.16	112.00	108.04	64.88	96.46	31.78	39.41
D04	46	4	81	127.2	89.00	76.88	60.44	86.38	32.56	34.38
D05	46	4	106	166.53	109.00	99.02	59.46	90.84	32.93	39.60
D06	51	4	104	190.43	121.00	116.36	61.10	96.17	31.79	38.16
D07	52	4	112	189.98	145.00	105.73	55.65	72.92	31.94	39.69
D08	54	4	92	177.32	123.00	105.31	59.39	85.62	32.15	38.52
D09	53	4	83	167.41	126.00	111.32	66.50	88.35	29.40	42.64
D10	52	4	86	137.52	101.00	84.14	60.46	83.31	31.95	39.75
D11	36	4	114	121.85	131.00	78.41	140.02	59.85	33.06	41.43
D12	43	4	83	139.16	123.00	87.28	53.89	70.96	31.89	39.65
D13	38	3	35	56	56.00	42.80	76.43	76.43	32.56	41.53
D14	42	4	105	161.95	136.00	100.92	62.32	74.21	30.45	40.67
D15	49	4	98	167.5	122.00	108.72	64.91	89.11	29.84	42.31

on the *A. flavus* susceptible than resistant seeds (Figure 1). Two isoform bands (designated as Glu 3 and Glu 4) on the gel seem specifically associated with *A. flavus* resistant seeds.

The remarkable difference in response to *A. flavus* infection between the resistant and susceptible genotypes was due to the induction of these two isoforms of β-1,3-glucanase (Glu 3 and Glu 4). The resistant genotypes expressed these two isoforms of β-1,3-glucanase and a quicker response to inoculation of *A. flavus* than did the susceptible genotypes (Figure 1). Mutant A10 and A25 of Giza 6 and C01, C03, C04 and

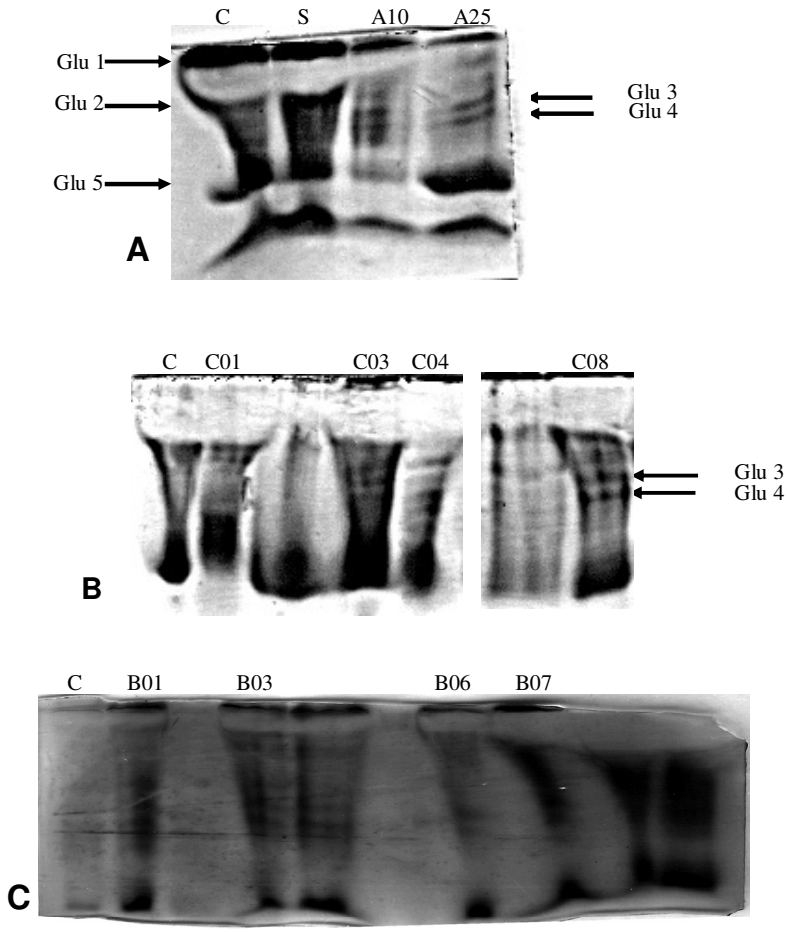


Figure 1. β-1,3-Glucanase activities on a native polyacrylamide gel (PAGE) of *Aspergillus flavus* infected seeds of Giza 6 (A), Giza 4 (B) and gregory (C) cultivars and mutants.

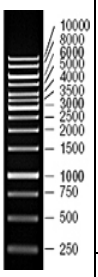
C08 of Giza 4 showed high activities of β-1-3-glucanase than the control one (Figure 1A and B). Moreover, the Gregory mutants B01, B03, B06 and B07 showed high activities of β-1-3-glucanases than the control one (Figure 1C). On the other hand, all Giza 5 mutants did not show any β-1-3-glucanases activities over the control one (data not shown).

Molecular identification of different peanut cultivars and their selected mutants

The DNAs of the three cultivars (Giza 6, Gregory and Giza 4) and selected resistant mutants were extracted and amplified using 27 decamer primers to estimate the genetic similarity and variability among them. All primers were successfully used as a fingerprinting tool and reproducibility was confirmed for each primer before gel documentation scanning (Figure 2). Fragments variations based on RAPD polymorphism for the three cultivars and selected resistant mutants are shown in Table 3. 21 of the 27 used primers showed polymorphism among mutants and their controls. The total amplified fragments were 186, and 67 of them were polymorphic and the others were not. The polymorphism percentage reached 37.13%, with primer OP-B08 showing the highest percentage of polymorphism (87.5%). 11 unique bands appeared; OP-B08 showed three unique bands, OP-B02, OP-G06 and OP-O03 had two and finally, OP-B01 and OP-C13 primers showed one unique bands. OP-C13 showed the highest number of bands (12) followed by OP-A14 (11).

Genetic distances among different peanut cultivars and their selected mutants

Similarity matrix of the three cultivars and selected mutants based on RAPD-PCR are presented in Table 4. C08 showed the highest similarity with B07 (0.972), while Giza 6 control showed the lowest similarity with C04 (0.869). The mean of similarities was 0.927. Cluster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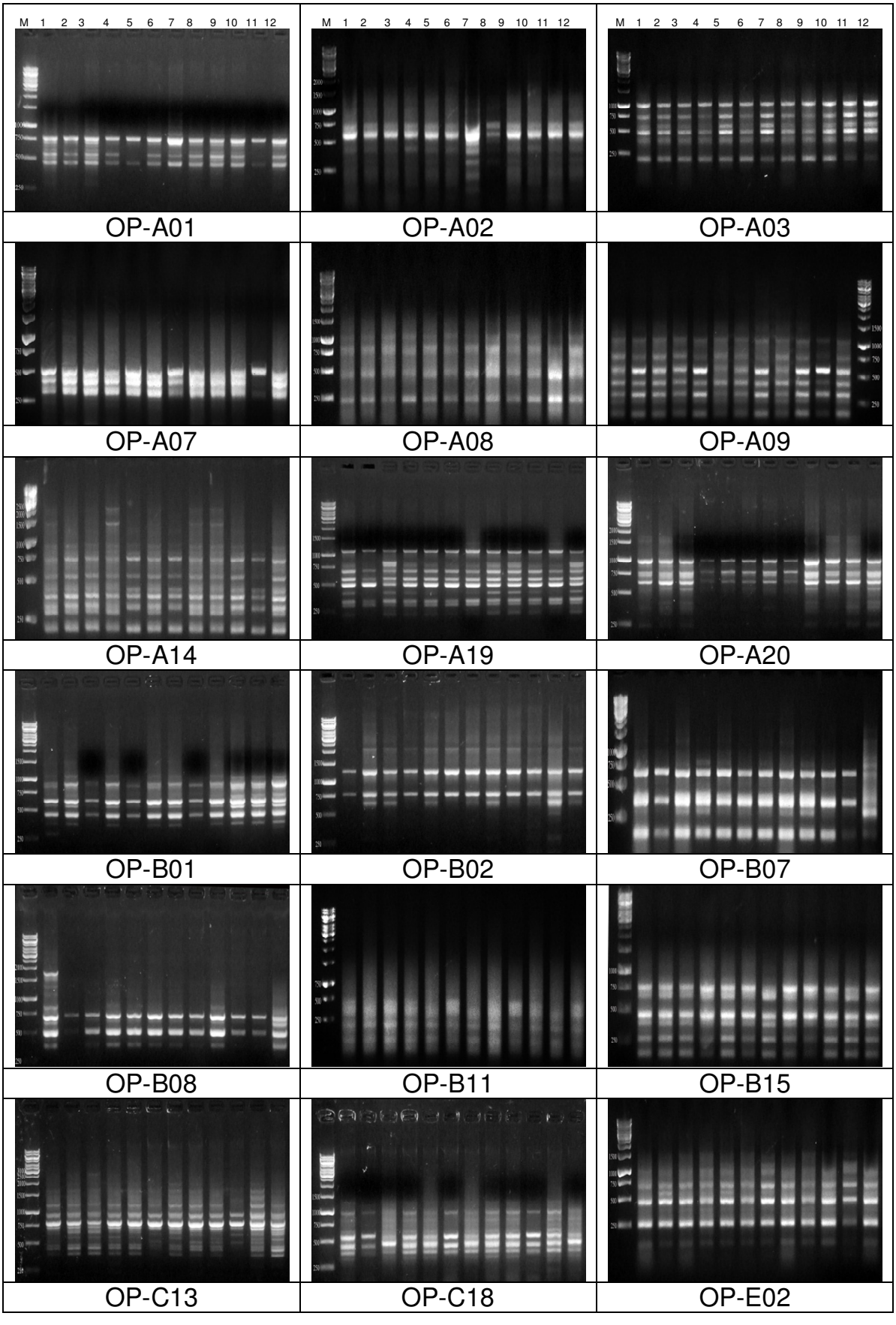
6000
5000
4000
3500
3000
2500
2000
1500
1000
750
500
250


M 1 2 3 4 5 6 7 8 9 10 11 12
OP-A01
OP-A02
OP-A03
OP-A07
OP-A08
OP-A09
OP-A14
OP-A19
OP-A20
OP-B01
OP-B02
OP-B07
OP-B08
OP-B11
OP-B15
OP-C13
OP-C18
OP-E02

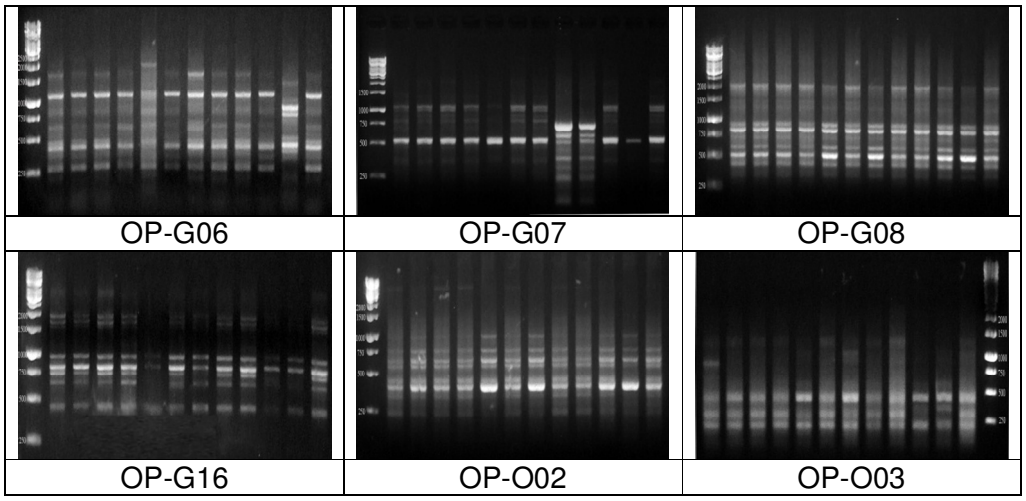


Figure 2. RAPD-PCR for the selected mutants of the three cultivars.1, Giza 6 control;, 2, mutant A10; 3, Gregory control; 4, B01, 5, B03; 6, B06; 7, B07; 8, Giza4 control; 9 ,C01; 10, C03; 11, C04; 12, C08.

Table 3. DNA fragment variations based on RAPD polymorphism for the three cultivars and selected mutants based on RAPD-PCR.

OP-	Monomorphic band	Polymorphic (without Unique)	Unique band	Polymorphic (with Unique)	Total number of band	Polymorphism (%)	Mean of band frequency
A01	2	2	0	2	4	50.00	0.896
A02	3	2	0	2	5	40.00	0.950
A03	5	1	0	1	6	16.70	0.972
A07	3	0	0	0	3	0.00	1.000
A08	4	0	0	0	4	0.00	1.000
A09	7	0	0	0	7	0.00	1.000
A14	8	3	0	3	11	27.30	0.856
A19	6	4	0	4	10	40.00	0.883
A20	3	7	0	7	10	70.00	0.733
B01	2	5	1	6	8	75.00	0.740
B02	2	2	2	4	6	66.70	0.639
B07	3	3	0	3	6	50.00	0.819
B08	1	4	3	7	8	87.50	0.427
B11	3	1	0	1	4	25.00	0.938
B15	6	0	0	0	6	0.00	1.000
C13	5	6	1	7	12	58.30	0.799
C18	4	1	0	1	5	20.00	0.833
E02	5	1	0	1	6	16.70	0.903
E16	5	0	0	0	5	0.00	1.000
G04	4	1	0	1	5	20.00	0.967
G05	5	4	0	4	9	44.40	0.954
G06	4	3	2	5	9	55.60	0.750
G07	1	6	0	6	7	85.70	0.691
G08	8	0	0	0	8	0.00	1.000
G16	3	4	0	4	7	57.10	0.941
O02	6	2	0	2	8	25.00	0.979
O03	2	3	2	5	7	71.40	0.571
Total	110	65	11	76	186	37.13	23

analysis using the RAPD data (Figure 2) for the three cultivars and selected mutants is presented in Figure 3. The selected mutants and their controls were divided into two main groups. The first group (A) contained only C04 mutant with 8% similarity with the rest of the all cultivars, while the other group (B) contained the others genotypes. The latter was sub-grouped into two clusters, the first (cluster C) included B03 mutant with a similarity index over 32% among them. The second cluster (cluster D) included the rest genotypes with a similarity index of 52% or more. Furthermore, cluster D can be resolved in two sub-clusters. Sub-cluster E contained Giza 6 control and sub-cluster F contained the rest genotypes. Some genotypes come together in closer cluster (B07 and C08 mutants, Giza 4 and C01 mutant and finally B06 and C03 mutants). The shortest distance was between B07 and C08 mutants. Of remarkable notice was the fact that there were no trends for distribution of each cultivar and its mutants among other mutants.

DISCUSSION

Radiation and chemical mutagenesis were used widely for producing useful mutants with improved characteristics in peanut and many crops (Rehman et al., 1987; Javed et al., 2000). In this study, Gamma rays and EMS increased the genetic variations in the four peanut cultivars (Giza6, Greogy, Giza 4 and Giza 5), which led to obtaining a large scale of mutants in M_2. Gamma rays are known to influence plant growth and development by inducing cytological, genetical, biochemical, physiological and morphogenetic changes in cells and tissues (Gunckel and Sparrow, 1961). EMS is a nearly ideal mutagen, producing G/C-to-A/T transitions (Greene et al., 2003). Knauft and Wynne (1995) observed negative correlations between disease resistance and yield, so we isolated our mutants depending on production traits and other traits in positive and negative direction. Some of our mutants' attributes agreed with previous isolated mutants. Hussein et al. (1991) isolated some mutants with higher yielding ability from Giza 4 cultivar. Branch (2001) released peanut mutants line (Georgia Valencia) that has a large pod size with 25% more size than its parent (Georgia Red). These results show that mutation breeding is an effective approach for introducing new varieties of peanut. In addition, it avoids the difficulties of classical breeding strategies that depend on crosses.

Plant β-1-3-glucanases comprises of large and highly complex gene families involved in pathogen defense, as well as a wide range of normal developmental processes. In this study, we presented evidence that peanut has β-1,3-glucanase PR proteins. The isoforms of β-1,3-glucanase were revealed on native PAGE differently in different genotypes as a result of infection of *A. flavus*. In the seed inoculated with *A. flavus*, the activities of β-1,3-glucanase were increased significantly in the resistant genotypes in comparison with the susceptible genotypes. Five isoforms were detected in an in-gel (native PAGE) assay and labeled as Glu 1 to 5, consecutively. Although Glu 1 was expressed constitutively, Glu 3 and 4 were expressed in response to the resistance to *A. flavus*, and Glu 2 may be produced by the fungus itself. Liang et al. (2005) found that he activities of β-1-3-glucanases increased significantly in the *A. flavus* resistant genotypes of peanut after inoculation in comparison with the susceptible genotypes and they identified eight isoforms of β-1-3-glucanases; Glu 1-6 (expressed in response to the infection), Glu 7 (produced by the fungus) and Glu 8 (expressed constitutively) that led us to use the activity of this enzyme for detection of the *A. flavus* resistant mutants. Some mutants (A10, B01, B03, B06, B07, C01, C03, C04 and C08) increased the activity of β-1-3-glucanases that may lead to hydrolyte β-1-3-glucans in the cell wall of the fungus so that the cell lyses and fungus death occurred. These results agree with some researchers' results; for example Adrienne and Barbara (2006) stated that pathogenesis related can increase resistance of plant against a pathogenic attack. Borad and Sriram (2008) stated that β-1-3-glucanases comprises of large and highly complex gene families involved in pathogen defense; these enzymes are found in wide variety of plants and having resistivity against various fungi. Bartnicki-Garcia (1969) also found that the major compound of fungi cell wall are β-1-3-glucans, and Simmons (1994) suggested that β-1-3-glucanases enzymes are involved in hydrolytic cleavage of the 1-3- β-D-glucosidic linkage in β-1-3-glucans.

RAPD approach has been used widely in the detection of the genetic variation in many crops. We used this approach to identify the polymorphism among the mutants and their control, and we found that the polymorphism percentage reached 37.13%. It is well known that a low level of variation has been observed for cultivated peanut at the DNA level using RAPD technique (Halward et al., 1991), and this is because cultivated peanut has narrow genetic base which originated from a single and recent polyploidization event (Young et al., 1996).

However, our data does not agree with these reports and this may be because mutants derived by chemical and physical mutagenesis have increased variability than the cultivated peanut (controls). Hence, in our study, the polymorphism percentage increased compared to Dwivedi et al. (2001) who found about 18.74% of polymorphism among selected peanut cultivars using same RAPD technique. Moreover, the dendrogram matrix showed that there were interactions in the distributions of the cultivated peanut and the mutants; for example, Giza 6 control was separated by it mutants with five genotypes. It may be that the similarities among cultivated peanut are high so that when the mutants occurred, the similarity between it and its control may be less than the similarity among the cultivars themselves.

Table 4. Similarity matrix of the three cultivars and selected mutants based on RAPD-PCR.

G6	A10	Gr	B01	B03	B06	B07	G4	C01	C03	C04	C08	G6
G6	1.000											
A10	0.916	1.000										
Gr	0.926	0.953	1.000									
B01	0.934	0.924	0.940	1.000								
B03	0.879	0.907	0.917	0.913	1.000							
B06	0.923	0.932	0.935	0.951	0.907	1.000						
B07	0.913	0.953	0.956	0.946	0.924	0.942	1.000					
G4	0.906	0.914	0.956	0.952	0.910	0.928	0.937	1.000				
C01	0.934	0.942	0.958	0.948	0.887	0.931	0.939	0.957	1.000			
C03	0.921	0.949	0.946	0.929	0.892	0.957	0.939	0.913	0.935	1.000		
C04	0.869	0.935	0.907	0.882	0.870	0.883	0.913	0.873	0.903	0.908	1.000	
C08	0.931	0.957	0.960	0.951	0.910	0.940	0.972	0.935	0.961	0.944	0.906	1.000

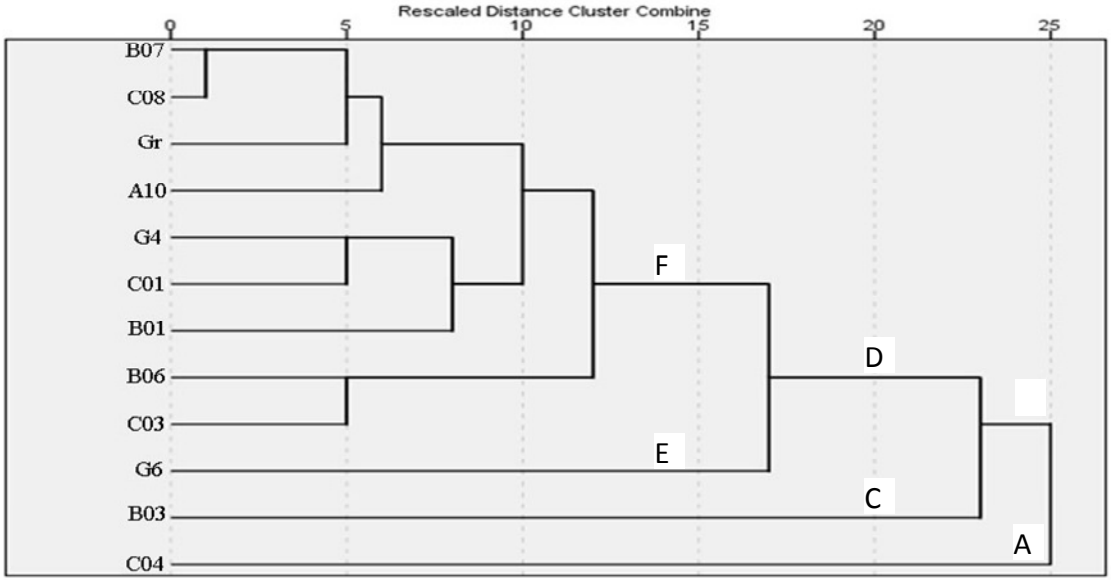


Figure 3. Dendrogram tree based on similarity matrix for the three cultivars and selected mutants based on RAPD-PCR.

Conclusion

Gamma rays and EMS can be used sufficiently to induce mutants in peanut; some of these mutants may have more activities of the β-1-3-glucanases enzyme. This enzyme has a role in the defense of peanut against the infection by *A. flavus*. So, these mutants have the ability to reduce the aflatoxins accumulation. RAPD-PCR showed pattern can be used as marker assisted selection (MAS) for the resistance of the fungus.

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