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Research Article

Phytochemical compounds of *in vivo* and *in vitro* organs of peanut (*Arachis hypogeae* L.)

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Abstract

In this study, peanut explants of (*Arachis hypogaea* L.) were cultured with hormones to obtain regenerated organs. Oil and protein from organs were extracted with organic solution and buffer. Polyphenol oxidase and peroxidase activities were measured at 510 and 420 nm respectively. Fatty acids (FAs) were analyzed by GC-MS. Regenerated organs contained 10.5–41% of oil and 0.96- 16.9% of protein. The shoots and seeds contained 15 and 57% of oil and 18.1 and 26.1% of protein. Peroxidase activity of the seeds and shoots was 11 and 27.4 mg (gp.min)$^{-1}$. Peroxidase activity of regenerated organs was 73.6- 397.5 mg (gp.min)$^{-1}$. Polyphenol oxidase activity of seeds and shoots was between 3.4 and 8.7 mg (gp.min)$^{-1}$. Polyphenol oxidase activity of regenerated organs were 9.6- 72.6 4 mg (gp.min)$^{-1}$. In the all samples were found 2- 13 FAs. Oleic was main FA of seeds and some regenerated organs. In the shoots, roots and regenerated shoots, main FA was linoleic acid. The hormones have shown positive effects on enhancing the production of certain fatty acids. The results showed that hormones including cytokinin (BAP) along with some auxins (IAA, NAA and 2, 4-D) at concentrations of 0.25–0.5 mg/L affect the organogenesis and the phytochemical composition of peanut.

Keywords: Oil, Organogenesis, Peroxidase, Polyphenol oxidase, Hormone

Introduction

Peanut (*Arachis hypogeae* L.) is an important crop belonging legumes family (Gohari and Niyakised, 2010). Peanut is a cheap source of protein and oil, a good source of essential fatty acids, vitamins and minerals, and a component of many food products (Chowdhury *et al*., 2015). *In vitro* culture, cotyledons and nodes explants of peanut produced a good quality callus, somatic embryos, shoot and roots in MS (Murshige and Skooge) medium containing BAP (Benzyl Amino Purine) supplemented with IAA (Indole Acetic Acid) or NAA (Naphtalen Acetic Acid) and 2,4-D (2,4-Dichlorophenoxy Acetic Acid) (Dolce *et al*., 2018). *In vitro* culture of peanut seed in MS medium showed that the transcripts of the AhGPAT9 gene were involved in lipid synthesis and accumulation in the peanut tissues. This gene, which accumulates in various peanut tissues, is highly expressed during seed development and is flowed by leaves (Shen *et al.*, 2023). Peroxidase and polyphenol oxidase are among the enzymes that play an important role in plant stress. They play a crucial roles in the plant's life cycle. Peroxidase has a specific role in lignification and strengthening the plant cell wall, so that is highly resistant to biodegradation. Polyphenol oxidase also contributes to lignification. The peroxidases in plants such as sweet potato, tobacco, peanut, soybean etc. have been also explored for their applications as biosensors (Barros *et al.*, 2015). The activity of antioxidant enzymes activity such as polyphenol oxidase, peroxidase and catalase increased with stresses (Purohit Harsh *et al*., 2020). Peanut oil including both saturated and unsaturated fatty acids (FAs). Oleic and linoleic are two important unsaturated FAs that together comprised about 80% of the FAs composition (Kokkiligadda *et al.*, 2017). The levels of saturated and unsaturated FAs from a variety of peanut cultivars have been reported to range from 10.92- 17.47% and 81.13- 94.81%, respectively. The oleic content of peanut genotypes can vary from 21–85% and the linoleic content from 2 to 43%. Several factors, such as variety, climatic and growing conditions, and maturity affect the FAs content in

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peanut oil (Chaiyadee *et al.*, 2013). We have not seen any reports on the effects of plant hormones and organogenesis on the biochemistry of regenerated organs in peanuts grown *in vitro*. Therefore, the aim of this work was to determine the effects of some plant hormones (growth regulators) on organogenesis and the interactions of phytohormones in regenerated organs with protein and oil content, peroxidase and polyphenol oxidase activities, and fatty acids from peanuts.

Materials and Methods

***In vivo* and *in vitro* culture:** Ten peanut seeds were cultured in 10 pots (30 cm in height and 20 cm diameter) at a spring in a green house. Ten weeks after the seeds germinated, shoots were used for biochemical assays. For *in vitro* culture, we selected some growth regulator treatments according to previous studies to producing some *in vitro* regenerated organs of peanut (*Arachis hypogaea* L.) (Ebrahimzadeh *et al.*, 2001). For this, seeds of the Florispan cultivar of peanut, were provided from the peanut research center of Lashte Neshaa (Iran). The seeds were sterilized by soaking in 0.5% hypochlorite for 30 min. Then several washes were made with sterile water, followed by treatment with 70% for 1 min. After the evaporation of the ethanol, the seeds were peeled, and the root region was cut from the embryos. Embryos without rootlet were used as explants for all treatments. The explants were cultured in full MS medium (Murashige and Skoog, 1962) supplemented with different concentrations of N-6- Benzyl Adenine(BA), Indole Acetic Acid (IAA), Naphthalene Acetic Acid (NAA), and 2,4-Dichlrophenoxi Acetic Acid (2,4-D) (Table 1).

The pH of the medium was adjusted to 5.8, and the samples were held in the dark at 25±1°C for 10 weeks. After 10 weeks, the callus and regenerated organs were used for protein, peroxidase, polyphenol oxidase activity, oil and Fatty Acids (FAs) assays.

Protein: The protein extraction was carried out by the modified methods described by Branlard and Bancel (2007). Seed, shoot and regenerated samples (5 g) were homogenized in a mortar for 1 min with Tris-HCl (pH 6.8) containing ascorbic acid 0.2 g, PVPP 3% and Triton X-100. Protein extract was centrifuged at 13,000 g for 20 min at 4°C by a high-speed centrifuge (Bekman centrifuge). The supernatant was collected and stored at -20 °C. Total proteins were determined according to the method described by Branlard and Bancel (2007) by a spectrophotometer (UV-visible Shimatzu) at 595 nm. For the determination of proteins, a standard curve was made by mixing 100 µl of albumin of known protein concentration with 5 ml of dye (Coomassie Brilliant Blue G-250).

Enzyme activity: Polyphenol oxidase activity of the samples was determined by measuring the absorbance of 0.1 ml of protein solution in 2 ml of catechol and 0.9 ml of sodium acetate buffer (0.2 M) pH 4 at 420 nm by spectrophotometer for 4 minutes by the method of Padanare and Rathod (2018) was determined. For Peroxidase activity determining, 0.2 ml of protein solution added to 2 ml of 0.2 M phosphate buffer containing substrates (saturated aqueous solution of benzidine) with 0.3 ml hydrogen peroxide and measured change in absorbance at 510 nm by spectrophotometer for 4 min.

Oil extraction: Five grams of fresh weight (FW) of peanut samples were ground and oil was extracted by a 100 ml mixture of chloroform, methanol, and distilled water (3:1:1, V/V/V), at 4 °C. The chloroform phase containing oil was collected (3 repeated). The chloroform was accumulated and concentrated at 60 °C by a vacuum rotary evaporator. Oil percentages were determined by the weight of the residue. For the esterification procedure; methanol- potassium solution 1N (KOH in methanol 80%) was added to the glass tube containing the 5 ml of oil in chloroform. The tube was then capped tightly, vortexed, and heated on a heater for 1 h to oil saponification. After samples were cooled to room temperature, they were mixed with 30 ml distilled water and petroleum ether (1:1) and shaken with repeated inversions. The petroleum ether was decanted. This procedure was repeated twice and the solvent was pooled. The aquatic saponification phase containing FAs was collected and mixed with 40 ml 4N hydrochloride acid (HCl) and 30 ml petroleum ether. The petroleum ether phase containing FAs was concentrated at 60 °C by vacuum rotary evaporator and collected in a vial. For methylation, FAs methyl esters were prepared using sulfuric acid (H_2SO_4) in methanol (Takadas and Dokar, 2017). 5 ml of sulfuric acid (0.1 M) in absolute methanol was added to the FAs vial, and the tubes were heated to 100 °C for 60 min. 5 ml ethyl ether and 5 ml of water were added after getting cold solution. FAs methyl esters were separated by ethylic ether. The ethylic ether containing FAs methyl esters was transferred to a 1.5-ml screw-cap glass vial fitted and stored in a freezer (-70 °C) for GC-MS analysis (Seppanen- Laakso *et al*., 2002).

Gas chromatography-mass spectrophotometry (GC-MS): An Agilent gas chromatograph from Hewlet-Packard (6890), equipped with an HP 5971 MS detector, was used for the determination of FAs composition (in Islamic Azad University of Science and Research). Separations were carried out on an Agilent (Hewlett-Packard) HP-5 fused silica capillary column (30 m × 0.25 mm I.D., 0.25 µm film thickness). The GC-MS interface temperature was maintained at 300°C. 1 µl of an unheated sample was injected manually in split-less mode with the injector port temperature at 200°C. The helium carrier gas flow rate was 1 ml min^{-1}. The column temperature program was as follows: 90°C, held for 1 min; 12 °C min^{-1} to 150 °C, held for 1 min; 2°C min^{-1} to 210 °C, held for 3 min, and 10 ºC min^{-1} to 300 °C, held for 30 min. The selective ion mode was used in the analysis. The retention time (RT) and abundance of confirmation ions relative to those of quantification ions are used as criteria for identification. The start button and the injection of a sample are

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Table 1. Plant regulator concentration (mg/L)

Treats	BAP	IAA	2,4-D	NAA
T1	0.25	5	-	-
T2	5	0.25	-	-
T3	0.25	-	-	5
T4	0.25	-	-	5
T5	0.25	-	5	-
T6	5	-	0.25	-
T7	0.25	-	0.25	-
T8	0.5	-	0.5	-

synchronized to have consistent resistant temperature (RT) values. An oven temperature program is used to maintain the temperature at 50 °C–300 °C.

Statistical analysis: All experiments (except GC-MS) were performed with at least 3 replicates. Analysis of variance and comparison of mean averages were done using SPSS software and Duncan's test, respectively.

Results and discussion

***In vitro* culture:** After 10 weeks of incubation, regenerated plantlets (R-pl), shoots (R-sh), roots (R-ro), multiple shoots (mu-sh) and callus were produced by peanut explants in different plant regulator treatments (T1-T8) and some samples with different organogenesis and phytohormone concentrations were used for biochemical assays (Table 2). Hoa *et al*. (2021) stated that the embryonic cotyledon of peanut is a suitable explant for shoot multiplication on MS medium containing 4 mg/L BAP and the regenerated shoots produced numerous roots on MS medium supplemented with 0.5 mg/L NAA. In the literature, numerous studies on *in vitro* cultures of peanut were reported previously, such as organogenesis, micropropagation, somatic embryogenesis and callus from embryos and cotyledons explants in MS medium supplemented with NAA and BAP (Limbua *et al*., 2019). Some organs derived from embryonic explants, concurrent R-Pl in T1 (BAP/IAA) and T5 (BAP/2,4- D); R-sh in T2 (BAP/IAA) and T8 (BAP/2,4-D); fleshy shoot (R-fsh) in T6 (BAP/2,4-D), Mu-sh in T7 (BAP/2,4-D); R-ro in T3 (BAP/NAA); and callus (Ca) in T4 (BAP/NAA) (Table 1 and Figure 1). Organogenesis of peanut explants in this study was according to Ebrahimzadeh *et al.* (2001) and Dolce *et al.* (2018). Cultivation of peanut cotyledon explants in the culture medium containing thidiazurone (TDZ) led to shoot regeneration, and transferring the shoots to the medium containing NAA led to root formation (Akasaka *et al*., 2000).

Total oil and protein: The oil contents of the seeds and shoots were determined to be 57% and 15%, respectively (Table 1). The total oil of other plant shoots had been reported to range from 4.9% to 7.1%, and for peanut seeds to range from 43% to 56% (Jemieson *et al.*, 1921). The total oil of the plantlets from T1 with 0.25 mg. L^{-1} BAP and 5 mg. L^{-1} IAA was highest at 41% but was lower than that of the seeds. The protein content ranged from 0.96 to 16.9% in all the treated samples, but was lower than the seeds. The peanut seed is rich in monounsaturated (oleic acid) and polyunsaturated (linoleic acid) fatty acids which are affected by genotype, year and their interaction (Sahin *et al*., 2022). In some Indian genotype of peanut, Oleic acid (C18:1) varied between 33.57 and 68.30%, whereas linoleic (C18:2) ranged from 8.05% to 45.46% (Sahin *et al*., 2022). The results of the study of Sahin *et al.* (2022) showed that the oil contents of Indian lines of peanut varied between 41.26 and 52.16%.

Enzyme activity: Peroxidase and polyphenol oxidase activities of seeds and shoots were very low (11 and 27.4 $mg(gp.min)^{-1}$; and 3.4 and 8.7 $mg(gp.min)^{-1}$) compared to growth regulators treatments of T1-T8 (73.6-397.5 and 9.3-72.6 $mg(gp.min)^{-1}$) and regenerated organs (Table 2). Increasing growth regulators cause stress to plants. Artificially, polyphenol oxidase and peroxidase activities can be elevated by increasing stresses (Nabity *et al.*, 2006). The increased activities of these enzymes in a plant are considered a resistance state of the plant to the stress (Barros *et al*., 2015). Antioxidant enzyme activity was affected by salinity stress in peanuts, but the application of gibberellic acid (GA3) reduced the activity of polyphenol oxidase and peroxidase (Purohit Harsh *et al*., 2020). Peroxidase and polyphenol oxidase activity in multiple shoots (Mu-sh) in 2, 4-D 0.25 mg/L with BAP 0.25 mg/L were higher than other treatments (Tables 1 and 2). These results are similar to the reports that, with any organogenesis caused by the effect of plant growth regulators, the overall activity of peroxidase and polyphenol oxidase increases (Barros *et al.*, 2015).

Regenerated plants and shoots of T1 and T2 with BAP and IAA and regenerated roots (R-ro) of T3 with BAP and NAA had less peroxidase and polyphenol oxidase activities (Table 2). After multiple shoots (in 2, 4-D 0.25 mg/L with BAP 0.25 mg/L), Polyphenol oxidase activity of callus (Ca) was higher than other treatments (Table 2), and the peroxidase activity was high. This is different from the report of Barros *et al.* (2015). In the sample in the growth regulator-free medium, organogenesis occurred (single shoot development and rhizogenesis), but there was no increase in total peroxidase and polyphenol oxidase activities. Each form of organogenesis is associated with an alteration of peroxidase activity. Some studies reported that there were no differences in polyphenol oxidase and peroxidase activities between some non-

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Table 2. Regenerated plantlet (R-pl), shoot (R-sh), roots (R-ro), multiple shoot (Mu-sh) and callus (Ca) production in different growth regulator (mg/L) treatments (T1-T8), total oil and protein (%), and peroxidase and polyphenol oxidase activities mg (gp.min)$^{-1}$ (milligrams substrate reaction/grams protein. minute).

Treats	Result	Oil %	Pro %	Peroxidase mg(gp.min)$^{-1}$	Poly pheoxidase mg(gp.min)$^{-1}$
T1	R-pl	41b	0.96f	76.8e	9.6e
T2	R-sh	20d	15.1b	80.3e	9.3e
T3	R-ro	24c	9.2d	73.6e	10.6e
T4	Ca	15.5e	12.1c	205.7b	72.6a
T5	R-pl	26c	4.9e	98d	38.4c
T6	R-fsh	16e	13.3c	159.6c	39.9c
T7	Mu-sh	10.5f	16.9b	397.5a	49.5b
T8	R-sh	18.5d	7.3d	145.7c	36.5c
Seeds	-	57a	26.1a	11g	3.4f
Shoots	-	15e	18.1b	27.4f	8.7e

Different letters show significant different

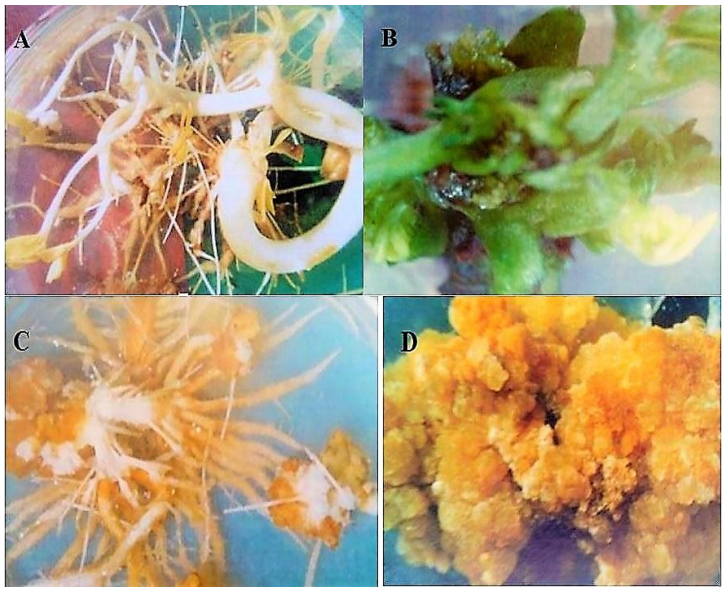


Figure 1. *In vitro* regenerated organs of peanuts are: A-regenerated plantlet (R-pl) in T1, B- Multishoot plantlet (Mu-pl) in T7 (BAP/2,4-D); C-Regenerated roots (R-ro) in T3, and D-callus (Ca) in T4 from peanut explants.

stressed and stressed cereal genotypes. The biochemical response during plant-stress interaction might be specific, either increased or decreased, depending on the plant or stress (Purohit Harsh *et al*., 2020). The response of enzymes can be specific, increased or reduced by interaction with plant growth regulators. These responses depend on the type of plant organism, the type of plant growth regulator, or the concentration of the growth regulator (Panadare and Rathod, 2018).

GC-MS analysis results: In previous reports, peanuts contained 13 different fatty acids (palmitic, palmitotic, heptradecyclic, heptadecenoic, stearic, oleic, linoleic, arachidic, ecoseonic, behenic, nervonic and lignocerc) (Ahmed and Young, 1982) but our results showed 14 fatty acids (Table 2). Oleic acid and linoleic acid in seed oil were 46.84 and 20.26%, respectively. Multiple shoot (Mu-sh) in T7 had the highest Oleic acid (53.13%) and linoleic acid (28.65%) (Table 2). Peanut oil have 39.80-81.13% oleic acid (Gulluoglu *et al.*, 2016). Alabama cultivars were found to have low levels of behenic and a high oleic and linoleic ratio (Berry, 1982). FAs composition of different cultivars of peanut have been reported to contain 37.7-82.2% oleic, 2.9-41.5% linoleic, 9.6-13.2% palmitic, 1.6-3.7% stearic, 1.2-1.7% arachidic, and 1.2-3.5% behenic acid (Chaiyadee *et al.*, 2013). All the FAs of the seeds of the cultivar, Florispan were found to be higher in this study than in other reports and behenic acid was not found (Table 2). The FAs of the oil from the shoots were different from those in the seed. Palmitic acid was the only saturated FAs in shoots. Poly unsaturated FAs dominated in plants shoots (Jemieson *et al.*, 1921). Levels of the FAs in the roots have been reported to be intermediate between the seeds and shoots (Oo and Stumpf, 1983). Hassan *et al*. (2005) stated that fatty acid compositions were affected by environmental conditions such as temperature, moisture, hormonal induction, etc.

T1, T2, T3, T4, T5, T6, T7 and T8 oil had 9, 5, 5, 4, 2, 3, 6 and 7 FAs respectively (Table 2). FAs of T1 were similar to the seeds. All the FAs common to oil seeds, except for nonadecylic acid, were observed in T1

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(Table 2). 13- octadecenoic (petroselinic 29.32%) and docosanoic (behenic 2.56%) of T1 were not found in the seeds. The levels of all the FAs in the treated samples were lower than in other reports. Some FAs were absent and the FAs found have not been reported elsewhere (Table 2). For example, Nonadecylic (4.31%) in T6 (except in the seed) and margaric acid (0.48%), linolenic (0.83%) and linolelaidic (1.65%) in T7, petroselinic (29.32%) and behenic (2.56%) in T1 had new present. Gondoic, nonadecylic and arachidic in seed, linolenic in shoot, arachidic, petroselinic and behenic of T1, nonadecylic of T6, margaric of T7; and linolenic and linolelaidic FAs in T8 were special of those treatments. Behenic was not observed in the seeds oil but it was only detected in the T1 sample. This data shows that Florispan cultivar is capable to producing these FAs, but under high concentration of IAA. FAs of Tainang 7, Kidang and Unsaturated and saturated FAs and other FAs were observed in the oil of the seeds, shoots and regenerated organs (T1-T8) by GC-MS analysis. Unsaturated FAs levels increased in all samples except for the seeds at T4 and T8 (Table 2). A total of 4 saturated FAs were found in seeds and at T1, T5 and T8 had 5 different unsaturated FAs. The ratio of unsaturated FAs to saturated FAs increased in the shoots and all treatments except for T8 with 0.5 mg/L BAP and 0.5 mg/L 2,4-D (Tables 1 and 2). A higher concentration of 2, 4-D seems to reduce unsaturated FAs than saturated FAs. The ratios in the seeds and T8 were 3.22% and 1.95%, respectively. Peanut oil normally contains about 80% unsaturated FAs and 20% saturated FAs (Stormer *et al.*, 2022). Environment affects oil levels and FAs in plants (Dybing and Zimermman, 1996). Our results also indicated that plant growth regulators *in vitro* culture, in addition to guiding organogenesis differentiation, had effects on protein contents, peroxidase and polyphenol oxidase, oil and FAs of peanut organs.

Conclusion

Our results showed peroxidase activity in peanuts was greater than the activity of polyphenol oxidase. Plant growth regulators affect the activity of both enzymes. Organogenesis has an effect on the activity of the enzyme. In regenerated plants, the activity of both enzymes was low, but in callus and shoots, the activity of both enzymes was high. 13 types of FAs are synthesized in peanut, but some of these FAs are made under certain conditions, depending on the type and concentration of plant growth regulator and the type of organogenesis. The plant growth regulators have shown some positive effects on enhancing the production of certain FAs in peanuts. Saturated or unsaturated FAs levels depend on the type of organogenesis and the type of plant growth regulator. In general, the results of this research showed that both the growth regulator and the organogenesis affect the activity of the enzymes, total protein, lipids, and fatty acids in peanuts.

Abbreviation

Fatty acids (FAs), Benzyl Amino Purine (BAP), Indole Acetic Acid (IAA), Naphtaline Acetic Acid (NAA), 2,4-Dichlrophenoxy Acetic Acid (2.4-D).

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