Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2019 16(2): 1276-1289. **OPEN ACCESS**

Phytochemical contents of white and pink flowers of marshmallow (*Althaea officinalis* L) plants and their androgenesis potential on anther culture in response to chemical elicitors

Emad A. Ewais*, Mohamed A. Ismail, Mohamed A. Amin and Eslam S. Abd-El-moety

Botany and Microbiology Department, Faculty of Science, Al-Azhar University, Madinet Nasr, 11884 Cairo, **Egypt**.

.

***Correspondence:** ewais_e@yahoo.com **Accepted:** 09 April. 2019 **Published online:** 06 May 2019

The effect of genotype (white and pink flowers) and chemical elicitors (biotin & set of amino acids) on androgenesis potential of marshmallow (*Althaea officinalis*) was investigated using anther culture. Flower buds of marshmallow were collected as explants and cultured on basal Murashige and Skoog (MS) medium for 30 days. Immature anthers selected from developing flowers *in vitro* were isolated and cultured on MS media to induce androgenesis. Part of this work was done to determine some phytochemical constituents according to the color of flowers (white and pink). It was found that pink flower either obtained from *in vivo* or *in vitro* had the great contents of total alkaloids, flavonoid, phenolic acids, saponin, tannins and antioxidants than white flowers. Flowers developed *in vitro* culture contained more secondary metabolites than flowers grown *in vivo*. Haploid callus was induced from anthers at the uninucleate stage and optimized by plant growth regulators. The best combination for callus was on MS medium supplemented with 5.0 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D) and 1.5 mg L^{-1} benzyl adenine (BA). The percentage of callus induction was higher in MS medium treated with set of amino acids (amino18) than in MS treated with biotin. It was also found that haploid callogenesis in *Althaea officinalis* that were directly regenerated from either anther of pink or white flowers showed no significant difference. Moreover, in the anther culture of marshmallow biotin or amino18 treatments, calli embryo-like structures were obtained. The results indicated that the best multicellular and embryo-like structures was obtained with anther isolated from pink flowers and cultured in medium pre-treated with 4.0 mg L^{-1} amino18.

Keywords: Marshmallow, biotin, amino18, anther culture, embryogenesis, phytochemical contents.

INTRODUCTION

Methods of modern biotechnology allow the process of breeding to be accelerated, and haploid production is one of the most widely used biotechnological methods in the breeding of self-pollinating many plants. Since the first haploid plant was obtained through pollen in the anther culture of *Datura innoxia* (Guha and Maheswari, 1964), many studies have been carried out on anther culture in various crop species (Bobkov, 2014, working with pea; Roshany et al, 2013 working on pepper; Kozak et al,2012, working on lupin; Can and Yoshida ,1999, working on sorghum; Asakaviciute, 2008, working on barley and potato). Marshmallow (*Althaea officinalis*), also known as khatma and khatmi in the east, is belonging to the Malvaceae family. It's characterized by pentamerous flowers that vary

from white to pink as well as its grey-brown mucilaginous root used for many medicinal purposes. Marshmallow was traditionally used as vegetable foods. The Romans and Egyptians ate the plant daily as a vegetable and used it as a soothing agent for colds, coughs, and irritated skin. Moreover, the leaves, flowers, and roots possess medicinal properties (Hage-Sleiman et al., 2011). Marshmallow is a perennial species indigenous to Africa, which is used as a medicinal plant and ornamental plant. A confection made from the root since ancient Egyptian time evolved into today's marshmallow treat. For marshmallow, limited reports about the establishment of initial cultures for androgenesis are available. However, In *Althaea officinalis L.* (Marshmallow), some studies on tissue culture using other parts of plants; i.e., root, leaf and shoot apex have been reported (Ionkova and Alfermann, 1994). In addition, calli were established from leaf explants of marshmallow on solidified LS medium (Ghanati et al., 2015).

The anther culture method is commonly applied in inducing androgenesis. It was a powerful method of plant breeding and used in recent years as a tool to fasten plant breeding programs and crop improvement through developing genetic viability. Therefore, anther cultures can provide effective haploid and micro propagation techniques for many plants. Success of the anther culture method depends on plant growth conditions, plant genotype, and choice of growth medium (Caredda et al., 2000). Most scientists using the method of anther culture report that the morphogenetic potential of callus and embryoids is genetically predetermined (Sugiyama, 1999). Many factors influence the triggering of microspore embryogenesis in many plants and are mostly related either to the physiological conditions during the development of the donor plant or the *in vitro* growth conditions at each step of the process. Due to the enhancement of culture techniques, it is now possible to induce anther derived embryoids in many plant species. These factors can be physiological, genetic, chemical, or physical, and cause the microspores to come in a new developmental pathway (Jacquqrd et al., 2006).

Medicinal plants have significance in the international markets for pharmaceutical applications, cosmetics, spices and health industries objectives as a part of the plant species use in the universe. Presently, 80% of developing countries rely on consuming of herbal medicines to obtain good health. Marshmallow (*Althaea officinalis* L.) is widely cultivated in gardens of Egypt as an ornamental plant and it has a great history of folkloric medicinal uses. In the present work, the use of haploid plants is of increasing importance in plant biology and plant breeding. Recently, the application of natural substances on plant tissue cultures has been intensified in order to increase the resistance and improvement the growth of many plants (Pradeep and Ranjitha, 2010; Akhtar et al., 2016; Teixeira et al., 2017). Among these products are included plant biostimulants that may contain vitamins, amino acids, and plant regulators in their composition. However, there is little information on the effect of each of these constituents on anther cultures.

Haploid plants have many uses in basic plant research disciplines such as cytogenetic, molecular genetics, crop evolution, plant biotechnology and traditional plant breeding (Touraev et al., 2009) so, we have been investigated on anther culture of marshmallow and tested the effect of genotype, plant growth regulators, biotin and set of amino acids (amino18) treatments and their effects on androgenizes response and some phytochemical contents of flowers either obtained from *in vivo* or *in vitro*.

MATERIALS AND METHODS

Plant materials

The flower buds of marshmallow (*Althaea officinals* L.) and flowers of both white and pink were collected in flowering season from a Garden of Al-Azhar University, Cairo, Egypt and identified by Botany Department, Al-Azhar University. The flower buds were used as the explants and were first washed with tap water and disinfected sequentially with 70% (v/v) ethyl alcohol for 4 min and 5% (v/v) sodium hypochlorite for 2 min followed by three times rinsing with sterilized double distilled autoclaved water (Fig. 1).

After surface sterilization, the explants (buds without calyces) were placed on the surface of semi-solid basal MS medium (Murashige and Skoog, 1962). The developing flowers (after 30 days) and flowers collected from the field were used for phytochemical studies.

Phytochemical studies:

The tissue developed *in vitro* from flower buds after 30 days cultured on MS media and flowers which collected from the field was dried in room temperature. Then, 100 mg of dried tissue was homogenized in 100 ml of respective solvent

(ethanol) and extractions were carried in an orbital shaker (REMI, India) with constant stirring at 150 rpm for 24 h. The mixtures were centrifuged at 10,000 rpm for 10 min and the supernatant was filtered through Whatman filter paper (No. 1). Measurements of biochemical parameters were taken on a Lambda-35 double beam spectrophotometer (Perkin-Elmer, USA). Each of the sample of the regenerated flowers was subjected to the following analysis:

Determination of total Alkaloids: The method used for determination of alkaloids was according to Woo et al., (1977).

Determination of total flavonoids: Total flavonoid content was determined by using the aluminum chloride colorimetric method with minor modifications (Chang et al., 2002).

Determination of total phenolic acids: The methods used for determination of total phenolic acid was according to Chun et al., (2013).

Determination of total saponins: The saponins content was calculated in percentage according to Obadoni and Ochuko (2001).

Determination of total tannins: This method depends on quantitative precipitation of tannin with copper acetate solution, igniting the copper tannate to copper oxide and weighing the residual copper oxide (Ali et al., 2011).

Determination of total antioxidant: The anti-oxidant activity of the extracts of regenerated samples was analyzed according to (Bhatt et al. 2012).

Culture media

MS medium (Duchefa, Netherlands) was used for micropropagation of *Althaea officinalis L*, supplemented with 30 g L^{-1} sucrose and 100 mg/L myo-inositol. PGRs (Duchefa, Netherlands); NAA, BA and 2,4 D were added independently or in combinations at different concentrations for the micropropagation stages of *Althaea officinalis L*. The pH of the media was adjusted to 5.7-5.8 before gelling with 2.7 g L^{-1} phytagel. Fifteen ml volumes of media were dispensed into 25×150 mm culture tubes or 50 ml volumes into 350 ml large jars. Then, closed with polypropylene caps and autoclaved at 121°C at a pressure of 1.1 kg/cm² for 20 minutes, then left to cool.

Culture conditions

The sterilized explants were cultured on the prepared media under complete aseptic conditions in the laminar air flow hood. Tissue cultured jars were incubated in an air-conditioned incubation room at a temperature of 26±2°C and 70±10% relative humidity, under a photoperiod of 16 hours with a light intensity of 2 klux, provided by cool white light fluorescent tubes (F 140t9d/38, Toshiba). All observation was recorded based on degree of callusing, morphology of callus, the color of callus, and the percentage of callus induction.

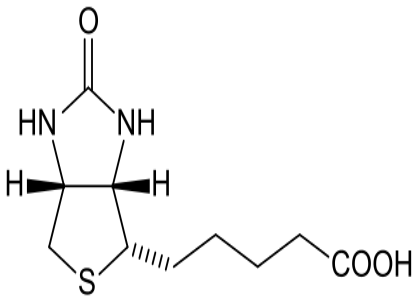
Effect of different growth regulators at different concentrations for haploid callus initiation of *Althaea officinalis* in uninucleate stage.

This experiment was designed to study the effect of MS medium (Murashige and Skoog, 1962) fortified with some growth regulators i.e. BA, naphthalene acetic acid (NAA) and 2,4 D at different concentrations and combination for the *in vitro* establishment. MS medium was supplemented with different concentrations BA (0.5; 1.0 and 1.5 mg L^{-1}), NAA (2.5 and 5.0 mg L^{-1}) and 2,4-D (2.5 and 5.0 mg L^{-1}) individually, in addition to the control treatment (MS nutrient medium without plant growth regulators). Each treatment was represented in 5 replicates.

Effect of two chemical activators biotin and amino18 at different concentrations on haploid callus fresh weight and dry weight. This experiment was designed to study the effect of two active elicitors, biotin and set of amino acids (amino18).

Chemicals used:

A. Biotin:



Chemical formula $C_{10}H_{16}N_2O_3S$

Biotin is a water-soluble B vitamin, also called vitamin B7 and formerly known as vitamin H or coenzyme R.

b. Amino 18:

Pharmaceutical product each capsule contains 400 mg each capsule contains mixture of amino acids (essential and non-essential amino acids) as followed:

- Essential amino acids: L-Isoleucine 4.83 mg, I-leucine 9.73 mg, L-lysine 12.86 mg, hydroxy lysine 2.81 mg, L-methionine 2.55 mg, L-phenylalanine 6.5 mg, L threonine 6.4 mg and L-valine 7.65 mg.
- Non-essential amino acids: L- Arginine 25.47 mg, L- histidine 2.25 mg, L- glycine 68.24 mg, L-alanine 28.66 mg, L- serine 11.40 mg, L-tyrosine 0.85 mg, L-aspartic acid 18.91 mg, L- glutamic acid 32.66 mg, L- proline 45.02 mg and hydroxyproline 39.73 mg.

MS medium (Murashige and Skoog, 1962) fortified with 1.5 mg L^{-1} BA, and 5.0 mg L^{-1} 2,4-D and supplemented with two chemical activators, biotin (1.0, 2.0 and 4.0 mg L^{-1}) and amino18 (1.0, 2.0 and 4.0 mg L^{-1}) for the in vitro establishment, in addition to the control. Each treatment was represented in 10 replicates. The ability percentage and morphological characters were measured and were recorded after six weeks of culture. We began the experiment by certain and equal weights of callus. Fresh weights were measured and recorded after six weeks of culture. Then we left the callus to dry in air for three days and dry weights were measured and recorded.

-Effect of biotin and mino18 on androgenesis stages

For further multiplication, the explants were sub-cultured two successive subcultures on the best medium MS supplemented with (4.0 mg L^{-1} biotin or amino18) to obtain stock materials to be recorded embryo-like structure and androgenesis response.

Experimental design and statistical analysis

The experiments were subjected to completely randomized design. Each experiment was repeated twice, and treatments consisted of at least 10 replicates. Variance analysis of data was carried out using ANOVA program for statistical analysis. Data were statistically analyzed in a Completely Randomized Design (CRD). Mean values were compared using Least Significant Difference (LSD) test at 0.05 (SAS, 2001).

RESULTS

Developing marshmallow flowers *in vitro* culture

The flower buds were used as the explants to develop two genotypes of white and pink flowers (Fig. 2). The flower culture of marshmallow was established from buds without calyces (pink and white). Buds were growing on semisolid MS basal medium (Fig. 3). After three weeks the developing flowers (Fig. 4) were taken for derived flowers *in vitro***.**

Phytochemical studies:

Qualitative phytochemical analysis

The aqueous extract of the developing flowers either *in vivo* and *in vitro* of marshmallow plants *Althaea officinalis* L**.**(white flowers and pink flowers) were subjected to phytochemical examination for alkaloids, flavonoids, glycosides, steroids, saponins and tannins as described by using standard procedures.

The preliminary phytochemical screening of two extract of *A. officinalis* genotypes showed the presence of alkaloids, flavonoids, saponins and tannins in all cases of both pink and white flowers, while steroids were absence in both genotypes either from *in vivo* or *in vitro* (Table 1). On the other hand, glycosides were absence in case of white flowers obtained from *in vivo*.

Quantitative phytochemical analysis

Results in fig. (5a.) indicated that total alkaloid recorded higher value in pink flowers 3.51 % and 2.55% than white flowers which gave 2.43 % and 2.11%, in *vitro* and *in vivo*, respectively. Fig. (5b.) indicated that, pink flowers gave the high value of total flavonoid 242 and 233 mg/eq.gm rutin then white flowers which gave the low value of total flavonoid 180 and 176 mg/eq.gm rutin in *vitro* and *in vivo*, respectively. Regarding to total phenolic acids determination**,** results in fig (5c.) indicated that total phenolic acid were higher value in pink flowers than white flowers. Data (Fig. 5d&e) demonstrated that pink flower had the great percentage value of total saponin and tannins than white flowers. Moreover, pink flowers recorded higher content of antioxidants than white

flowers (Fig. 5f.). This is valid in both cases in *vitro* and *in vivo*.

Among medicinal plants, extract from the marshmallow flowers is a source of antocyanides and flavonoids. Marshmallow is a species for which there is no efficient method of haploid plant recovery yet.

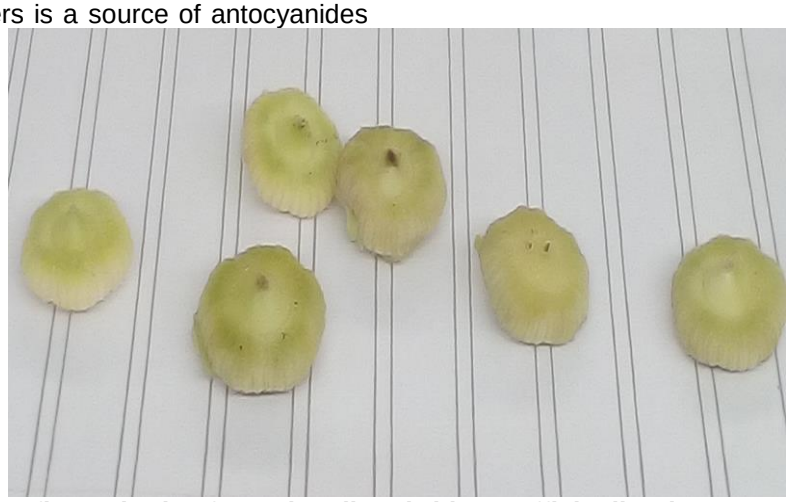
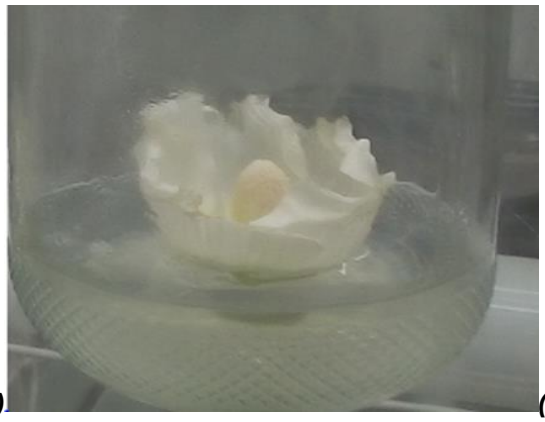


Figure. 2. Immature flower buds of marshmallow (*Althaea officinalis* L.) were used as explants for derived flowers *in vitro.*

Figure. 3. Flowers buds (without calyces) of marshmallow were cultured on basal MS media.

(a)



(b)

Figure. 4. Development of flowers in culture of marshmallow (*Althaea officinalis* L.) (a, pink flowers; b, white flower) *in vitro* having anthers at the uninucleate stage were used for anther cultures.

Table 1: Phytochemical analysis of *A. officinalis* extracts (white flowers and pink flowers).

Source	Flower	Alkaloids	Flavonoids	Glycosides	Steroids	Saponins	Tannins
In vivo	Pink	+	+	+	-	+	+
	White	**+**	**+**	-	**-**	**+**	**+**
In vitro	Pink	**+**	**+**	**+**	**-**	**+**	**+**
	White	+	+	+	-	+	+

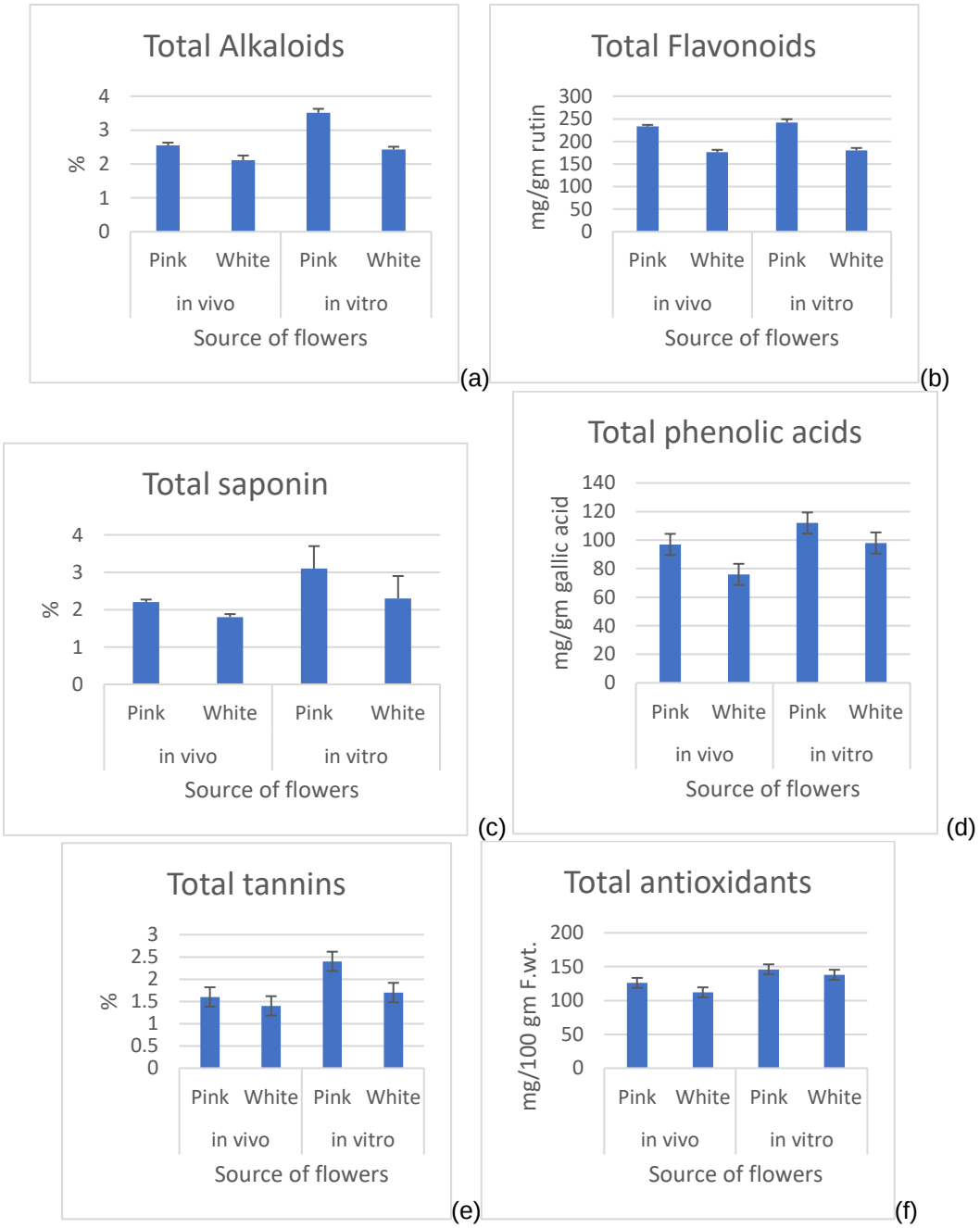


Figure. 5. Total active constituents of *A. officinalis* flowers (white and pink) obtained from *in vivo* and *in vitro*. Each value is a mean of 3 replicates ± Standard error.

The aim of the current research was to investigate the influence of two genotypes, biotin, and amino18 treatments on callogenesis, embryogenesis and secondary metabolites from anther cultures of marshmallow. In our study, we used two explants, anthers of both white and pink flowers.

Haploid callus induction through anther culture

This part of study aimed to record the various responses for callus induction using two explants (anthers of both white and pink flowers fig. 6 a1 & a2) of marshmallow plants cultured four weeks on MS medium supplemented with different combinations of two auxins (2,4-D and NAA) and one cytokinin (BA).

The obtained results revealed that the callus induction was varied depending on the source of explant, type and concentration of growth regulators used. The obtained data show that no callus was observed with any explants cultured on MS medium free of growth regulators in the two marshmallow genotypes.

Table (2) shows the frequency of callus formation in anthers of *Althaea officinalis* cultured on MS medium. The MS medium supplemented with 5.0 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BA induced callus formation in 100% of both anthers (white and pink flowers) and callus creation was creamy to white in color and compact in texture.

It is important to notice that both sources of anthers explants gave callus in all MS media containing growth regulators.

Fresh weights of calli developed from two types of explants (anther of both white and pink flowers) of marshmallow were recorded after five weeks of cultivation. It was observed (Table 3) that MS medium supplemented with 5.0 mg L^{-1} 2,4-D + 1.5 mg L^{-1} BA gave the highest fresh weight of callus originated from anther of pink flowers (2.31 gm) followed by anther of white flowers (2.10 gm). On the other hand, the lowest (0.86 gm) was obtained in MS medium containing 2.5 mg L^{-1} NAA + 0.5 mg L^{-1} BA with the anther of white flowers. With respect to dry weight, it was found that MS medium containing 5.0 mg L^{-1} 2,4-D + 1.5 mg L^{-1} BA recorded the highest values 0.192 and 0.173 gm with pink and white flowers, respectively. Generally, anther of pink flowers showed slightly better callus than white anther.

Effect of genotypes, biotin and amino18 on anther derived callus

The results indicated that the optimal supplement for callus formation was MS medium containing 5.0 mg L^{-1} 2,4-D + 1.5 mg L^{-1} BA, so the calli were transplanted to that medium supplemented with different concentration from biotin and some amino acids (amino18). The second experiment was performed to study the effect of biotin and amino18 on morphological characters of callus. So, three levels (1.0, 2.0 and 4.0 mg L^{-1}) of two elicitors (biotin & amino18) were tested on the same MS medium containing with the best concentration of 2,4-D (5.0 mg L^{-1}) and BA (1.5 mg L^{-1}) to compare their ability to induce uninucleate stage of callus and an androgenesis potential from eight-week-old callus. Anthers of both white and pink was evaluated as starting material and cultures were sub-cultured after 4 weeks. The results therefore showed that in the absence of biotin and amino18, callus possessed low growth and lower percentage of callus induction, 32.3 and 30.6 in white and pink anthers, respectively as compared to biotin or amino18 treatment (Tables 4&5). It was observed that type and concentration of elicitors used in the medium had significant effect on the induction of haploid callus (Figure 1). Percentages of callus induction were higher either in the presence of biotin or amino18 up to 85% as compared to control 30%. Amino18 at 4.0 mg L^{-1} promoted maximum callus induction with anther of white (85.6%) and pink (84.3%), while biotin at high concentration induced 74.7% and 73.4% with white and pink anthers, respectively. Callus growth index was found high at high concentration of either biotin or amim18 which increased fresh and dry weights of derived callus with increased elicitors concentration (Table 5). Amino18 at high concentration was superior to other treatments in fresh and dry weight of pink and white calli. It was also found that both biotin and amino18 significantly promoted haploid callus of marshmallow, especially at 4.0 mg L^{-1} concentration.

Effect of genotypes, biotin and amino18 on androgenesis potential

After the second subculture, haploid calli of both white and pink anthers were transferred to a similar medium composition containing 4.0mg/l.

Table 2: The percentage and morphological characters of calli formed on anthers culture of white and pink flower of *Althaea officinalis* L.

PGR (mg L^{-1})	Callus formation (%)		Morphological characters (texture; color)	
	White	Pink	White	Pink
0.0	0	0	0	0
2.5 2,4-D + 0.5 BA	63.3	70.5	Cr; F	Cr to W; F
5.0 2,4-D + 0.5 BA	61.6	66.6	Cr to W; F	Cr; L
2.5 2,4-D + 1.0 BA	70.5	80.6	Cr, F	Cr to W; C
5.0 2,4-D + 1.0 BA	80.6	83.6	Cr to W; C	Cr to W; C
2.5 2,4-D + 1.5 BA	85.0	90.6	Cr to W; C	Cr; C
5.0 2,4-D + 1.5 BA	100	100	Cr to W, C	Cr to W; C
2.5 NAA + 0.5 BA	58.6	70.6	Cr; C	Cr to W; C
5.0 NAA + 0.5 BA	65.6	66.5	Cr; F	Cr; C
2.5 NAA + 1.0 BA	85.7	70.5	Cr to W; F	Cr to W; F
5.0 NAA + 1.0 BA	90.6	88.6	Cr; L	Cr; C
2.5 NAA + 1.5 BA	88.6	75.6	Cr; L	Cr; F
5.0 NAA + 1.5 BA	85.6	70.6	Cr; F	CR; F

Cr: cream, W: white, C: compact, F: friable, L: loose

Table 3: The effect of different plant growth regulators on fresh and dry weights of calli induced on anthers culture of white and pink flowers of *Althaea officinalis* L.

PGR (mg L^{-1})	Fresh Wt. (g/jar)		Dry Wt. (g/jar)	
	White	Pink	White	Pink
0.0	0	0	0	0
2.5 2,4-D + 0.5 BA	0.96±0.03	0.75±0.06	0.085±0.01	0.061±0.01
5.0 2,4-D + 0.5 BA	1.36±0.04	1.26±0.04	0.102±0.02	0.096±0.01
2.5 2,4-D + 1.0 BA	1.47±0.05	0.86±0.03	0.104±0.02	0.057±0.02
5.0 2,4-D + 1.0 BA	1.80±0.04	1.83±0.06	0.157±0.01	0.179±0.02
2.5 2,4-D + 1.5 BA	1.56±0.05	1.80±0.05	0.123±0.01	0.180±0.01
5.0 2,4-D + 1.5 BA	2.10±0.03	2.31±0.03	0.173±0.01	0.192±0.03
2.5 NAA + 0.5 BA	0.86±0.05	1.68±0.02	0.116±0.02	0.131±0.01
5.0 NAA + 0.5 BA	0.95±0.04	0.98±0.03	0.120±0.04	0.128±0.02
2.5 NAA + 1.0 BA	1.67±0.06	1.72±0.04	0.132±0.01	0.130±0.03
5.0 NAA + 1.0 BA	2.02±0.12	0.86±0.06	0.142±0.02	0.062±0.01
2.5 NAA + 1.5 BA	1.34±0.07	0.66±0.04	0.105±0.01	0.042±0.02
5.0 NAA + 1.5 BA	1.81±0.06	1.73±0.05	0.142±0.01	0.138±0.01
L.S.D at 0.05	0.27	0.12	0.31	0.15

Table (4): Effect of biotin and amino18 at different concentrations (mg L^{-1}) on anther callus induction (%) and morphological characters of marshmallow (*Althaea officinalis* L.) flowers (white and pink) cultured on MS medium supplemented with 5 mg L^{-1} 2,4-D + 1.5 mg L^{-1} BA.

Treatment Mg/l	Callus induction (%)		Morphological characters (texture; color)	
	White	Pink	White	Pink
MS	32.3	30.6	W, F.	W, F.
MS+Biotin1.0	58.6	56.3	W, C.	W, F
MS+Biotin2.0	66.4	67.6	W to B; C	W to Cr; C
MS+Biotin4.0	74.7	73.4	W to Cr; C	W to B; C
MS+Amino1.0	65,6	66.4	W to Cr; C	W to B; C
MS+Amino2.0	72.3	71.5	W to Cr; C	W to Cr; C
MS+Amino4.0	85.6	84.3	W to Cr; C	W to Cr; C

Cr: cream, W: white, B: brown; C: compact, F: friable.

Table (5) Effect of biotin and amino18 on dry weight and fresh weight (mg/jar) of anther derived callus of white flowers and pink flowers cultured on MS medium supplemented with 5 mg L^{-1} 2,4-D + 1.5 mg L^{-1} BA.

Treatment Mg/l	white		pink	
	Fresh weight	Dry weight	Fresh weight	Dry weight
MS	8.6±0.92	0.65+0.04	9.5+0.29	0.76+0.4
MS+Biotin1.0	12.3±0.86	0.85+0.03	13.6+0.42	1.15+0.02
MS+Biotin2.0	13.2±0.53	1.12+0.4	14.2+0.38	1.32+0.04
MS+Biotin4.0	14.1±0.51	1.07+0.4	15.3+0.62	1.42+0.02
MS+Amino1.0	13.4±0.56	1.02+0.3	14.1+0.34	0.92+0.02
MS+Amino2.0	15.7±0.60	1.230.06	17.6+0.51	1.37+0.03
MS+Amino4.0	18.5±0.38	1.56+0.4	19.8+0.49	1.65+0.027
L.S.D at 0.05	1.33	0.15	1.45	0.14

Table (6): Androgenic response of white and pink marshmallow (*Althaea officinalis* L.) anthers incubated on MS medium with biotin or amino18, after the 3rd subculture.

Genotype	Treatment 4.0 mg/l.	Number of anthers cultured	Embryogenic anthers (%)	Embryogenic response
White flowers	Control	60	8.33	+
	Biotin	70	42.85	++
	Amino18	60	70.00	+++
Pink flowers	Control	50	12.00	+
	Biotin	70	47.14	++
	Amino18	80	86.25	+++

+ = medium; ++ = Good; +++ = Excellent


(a1)

(a2)

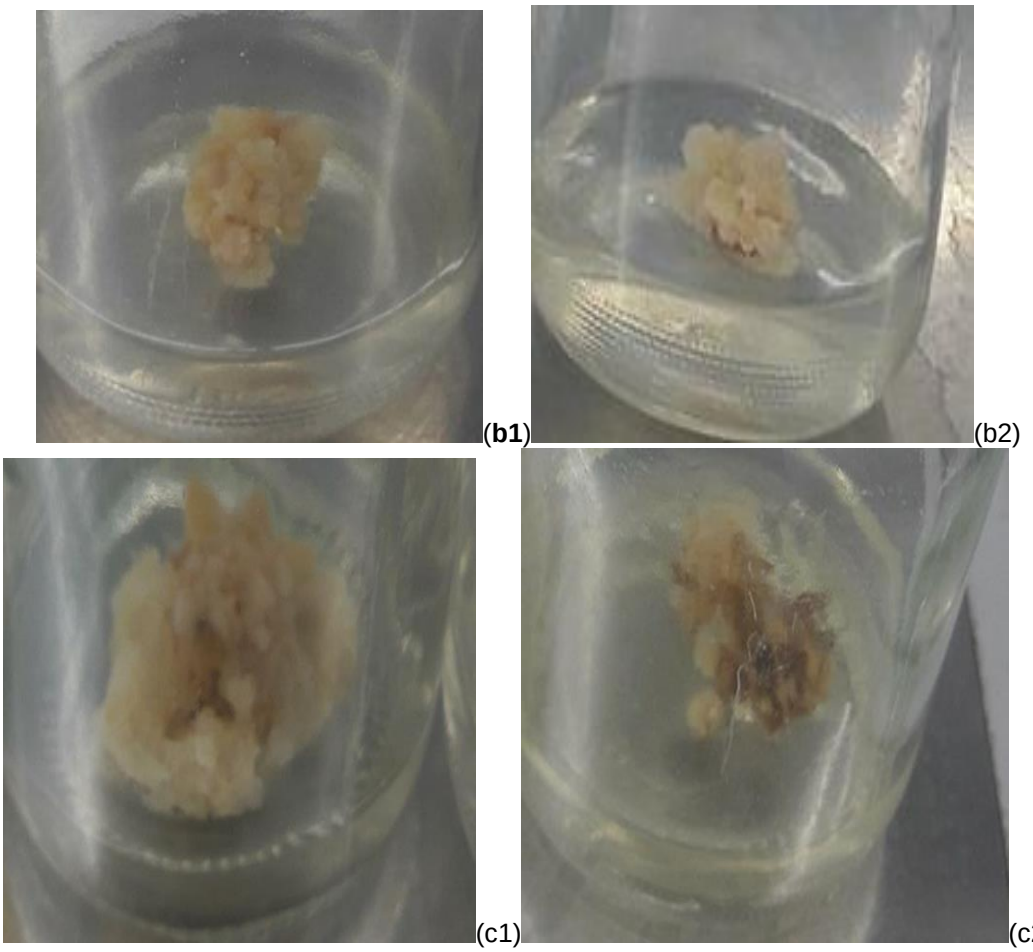


Figure. 6. Androgenesis induction through anther culture of marshmallow (*Althaea officinalis* L.)
a- Anther of uninucleate stage.
b-Callus from anther tissue after 4-week culture on callus-induction medium.
c-An embryogenic callus with embryo like structure after 2 months of culture on MS medium without any phytohormone. (a1, b1, c1 derived from pink flowers; a2, b2, c2 derived from white flowers).

biotin or amino18 for further androgenesis induction. One piece of callus fifty days old per jar were subjected to each treatment. After the 3rd subculture, embryogenic anthers (%) and embryogenic response were calculated (Table 6). In the present study, amino18 with pink anthers derived calli (Fig. 6 c1 & 2) surpassed all other treatments in embryogenesis (86.25%), followed by amino18 with calli of white anther (70%). On the other hand, biotin showed 42.85% and 47.14% embryogenic anthers percentage with white and pink anthers derived calli, respectively. In case of untreated calli (control), formation of multicellular and embryo-like structures was lowest than treated culture. These results indicate the efficacy of amino acids and biotin in stimulating androgenizes from haploid callus and may be suitable for future use in genetic transformation studies to enhance regeneration of transgenic sugarcane plants.

DISCUSSION

Phytochemical studies

Althaea officinalis L. (marsh-mallow) belonging to the Malvaceae family, is a medicinal plant that contains a variety of economic phytocompounds including asparagine, pectin, flavonoids, polyphenolic acid, and scopoletin. The antioxidant activity of various parts of this plant has also been demonstrated in several other studies (Elmastas et al., 2004; Sadhighara et al., 2012). In this study, we use flowers of two genotypes of *A. officinalis* obtained from *in vivo* and from *in vitro* culture to determine present or

absent of alkaloids, glycosides, flavonoid, steroids, saponin, tannins. The flowers from *in vitro* culture showed better result for preliminary phytochemical screening than the flowers obtained from the field. Preliminary phytochemical screening of plant extract has been reported in several medicinal plants **(**Arokiyaraj et al., 2007).All the phytochemical components detected were known to support bioactive activities in medicinal plants (Sirigiri et al 2014).The chemical compounds produced by plants are collectively referred to as phytochemicals. Biotechnologists have special interest in plant tissue culture for the large-scale production of commercially important compounds. These include pharmaceuticals, flavours, fragrances, cosmetics, food additives, feed stocks and antimicrobials. Many researchers used *in vitro* culture of medicinal plants to produce secondary metabolites (Hussein et al., 2010, Cheng et al., 2014; Tiwari and Rana, 2015, Scossa et al., 2018). With respect to the phytochemical contents of the developing flowers either from *in vivo* or *in vitro*, the obtained results (Fig 2) showed increasing of the amount of total active constituents of *A. officinalis* flowers (white and pink) obtained from *in vitro* culture than flowers grown *in vivo.* These results agreed with the study of Lopez-Laredo et al., (2009) who found the concentration of metabolite levels accumulated in the cultured tissue was higher in the callus of *Tecoma stans* (L.). The changes in concentrations of secondary metabolites contents in flowers of *A. officinalis* derived *in vitro* culture than in flowers obtained from *in vivo* may be a result of nutritive and regulators substances treatments of *in vitro* cultures which differ from field conditions.It was also observed, in the present study, that pink flowers in all cases (*in vivo* and in *vitro*) contained higher phytochemical contents than white flowers. In this respect, Sadighara et al., (2012) examined three colors of petals of *Althaea officinalis* flowers, i.e., pink, reddish pink, and white were examined for total antioxidant activity. The authors found that the reddish pink flowers of *A. officinalis* have more antioxidant activity and the power of antioxidant activity was reddish pink > pink > white.

Callus induction through anther culture

Haploid plants are of special benefit to plant breeder because by chromosome doubling completely homozygous diploid plants can be obtained from haploid plants. Completely homozygous plants with various combinations of genes may be obtained in a short period of time. Haploid callus is a powerful method of plant breeding. Recently, the production of haploid plants from anther culture was reported in many plants (Bobkov, 2014; Roshany,et al., 2013; Kozak et al.,2012). Marshmallow is a species for which there is no efficient method of haploid plant recovery yet. So, the aim of the current research was to investigate the influence of two genotypes, biotin and amino18 treatments on callogenesis and embryogenesis from anther cultures of marshmallow**.**

In current research nutrient media (MS) with deferent concentrations of auxins (2,4-D & NAA) and cytokinin (BA) showed differences in the efficiency of morphogenic haploid calli formation. It was also observed that on anther culture of marshmallow, good calli were predominantly produced on media with 5.0 mg L^{-1} 2,4-D and 1.5 mg L^{-1} BA. With respect with marshmallow, Mujib et al., (2017) studied *in vitro* propagation of *Althaea officinalis*. They developed callus induction and multiple shoot regeneration protocols from another explants, i.e., roots, nodes, and leaves, developed compact white or yellow calli in a medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D), which grew vigorously.

According to our findings, a comparison of the haploid callus induction stage showed that there is no significant different between the anthers isolated from the white or pink flower buds. Induction of callus from anther culture is also reported by Can and Yoshida (1999) who observed callus induction of sorghum anther culture significantly affected by genotypes. In addition, Kozak et al., (2012) working on three genotypes of *Lupinus angustifolius.* The authors found that, for all genotypes, callus formation was only observed from few anthers on the MS medium supplemented with 2.0 mg L^{-1} 2,4-D and 0.5 mg L^{-1} Kin**.**

Effect of genotypes, biotin and amino18 on androgenesis:

The results of the present study indicated that type and concentration of elicitors used in MS medium had significant effect on induction of haploid callus. Although, genotypes (white and pink flowers) slightly effect on haploid callus induction. It was found in the present study that

amino 18 at high concentration (4.0 mg L^{-1}) induced maximum callus induction with anther of white (85.6%) and pink (84.3%).However, the best embryogenic callus formation was observed from calli growing on MS media containing 4.0mg/l. amino18 (Figure 3 and 4). One of the most vital parameters for androgenesis is the selection of the active elicitors such as amino18. Such results indicated that the success of the callogenesis depended on the active elicitors. The obtained results indicated that in case of biotin or amino18 pre-treated derived calli, in comparison to untreated calli, a remarkable increase in multicellular and embryo-like structures was observed. Anthers isolated from pink has better embryo formation than from anthers isolated from the white flower buds. Despite the knowledge about the positive effect of amino acid application on plants, most of the studies were carried out with intact plants using products composed of a set of amino acids (Popko et al.,2014 , and Mohga et al., 2015) there is little information regarding the isolated effect of these amino acids on developing tissue *in vitro* (Khan et al., 2009; Colla et al., 2014). Recently, Al-Jibouri et al., (2016) cultured explants of *V. thapsus* (leaves and petioles) *in vitro* on Murashige and Skoog (MS) medium for shoot proliferation using MS medium supplemented with combination of Benzyl adenine (BA) and Naphthalene acetic acid (NAA) for callus induction. The authors observed that the addition of different concentrations of amino acids as a precursor adding separately to the tissue culture medium led to raise the accumulation levels of secondary metabolites in callus tissue. Generally, the enhancement of accumulation depended on the type of amino acids and their concentration. Biotin is a water-soluble, called vitamin B and formerly known as vitamin H or coenzyme R. It is involved in a wide range of metabolic processes in living cells of organisms, primarily related to the utilization of fats, carbohydrates, and amino acids. It was reported that both thiamine and biotin significantly affected callus growth of date palm through increasing callus weight, embryo number; embryo length (Abrahamian and Kantharajah, 2011; Al-Khayri, 2001). Moreover, increasing biotin from 0 to 1 mg L^{-1} gave a maximum callus weight like thiamine (Drew and Smith 1986**)**

CONCLUSION

It could be concluded from this investigation that both genotypes and exogenous basal MS medium could increase secondary metabolites, total alkaloids, flavonoid, phenolic acids, saponin, tannins and antioxidants, in the developing flowers of *A. officinalis*. In addition, genotypes and active elicitors biotin and amino18 could effect on anther derived uninucleate stage of callus and induced multicellular structures and embryo-like structures, especially amino 18 using anther of pink flowers *in vitro*. Moreover, the increase in secondary metabolites, particularly by pink flowers derived *in vitro*, seemed likely to be correlated of high androgenesis potential.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ACKNOWLEGEMENT

The authors are grateful to staff of Botany and Microbiology Dept., Faculty of Science (Boys), Al-Azhar University.

AUTHOR CONTRIBUTIONS

Author contributions MI, MA and EA performed the anther experiments and quantified the data. MI and MA wrote the paper. EA performed phytochemical analysis. EE designed and completed data interpretation and edited the manuscript.

REFERENCES

Abrahamian P and Kantharajah A (2011): Effect of vitamins on *In Vitro* organogenesis of plant. American Journal of Plant Sciences, 2011, 2, 669-674.

Ali M.; Husain shahnaz S. and Ansari shahid H. (2011): Composition of the volatile oil of Zingiber officinale roscoe rhizomes and effects of physical factors on the oil.

International research journal of pharmacy, 2(9), 173-177.

Al-Jibouri, A.M.J., Abed, A.S., Ali, A.-J.A. and Majeed, D.M. (2016) Improvement of Phenols Production by Amino Acids in Callus Cultures of *Verbascum thapsus* L. *American Journal of Plant Sciences*, 7, 84-91. http://dx.doi.org/10.4236/ajps.2016.71010

Al-Khayri, J. M. (2001): "Optimization of Biotin and Thiamine Requirements for Somatic Embryogenesis of Date Palm (*Phoenix dactylifera* L.)," *In Vitro* Cellular & Developmental Biology—Plant, Vol. 37, No. 4, pp. 453- 456.

Akhtar, G.; Jaskani, M.J.; Sajjad, Y. and Akram, A. (2015) Effect of Antioxidants, Amino Acids and Plant Growth Regulators on in vitro Propagation of Rosa centifolia. Iran J Biotech. March;14(1): e1152.

Arokiyaraj S.; Perinbam K.; Agastian P. and Balaraju K. (2007): Immunosuppressive effect of medicinal plants of Kolli hills on mitogen-stimulated proliferation of the human peripheral blood mononuclear cells in vitro. *Indian Journal of Pharmacology*, 39, 180-183.

Asakaviciute, R. 2008: Androgenesis in Anther Culture of Lithuanian Spring Barley (Hordeum vulgare L.) and Potato (Solanum tuberosum L.) Cultivars. Turk J Biol, 32 (2008) 155-160.

Bhatt ID, Dauthal P, Rawat S, Gaira K, Jugran A, Rawal RS, Dhar U (2012) Characterization of essential oil composition, phenolic content and antioxidant properties in wild and planted individuals of Valeriana jatamansi Jones. Sci Hortic 136:61–68.

Bobkov S. (2014): Obtaining calli and regenerated plants in anther cultures of pea. Czech J. Genet. Plant Breed., 50: 123–129.

Can, N.D. and Yoshida, T. (1999): Combining Ability of Callus Induction and Plant Regeneration in Sorghum Anther Culture, Plant Production Science, 2:2, 125-128, DOI: 10.1626/pps.2.125.

Caredda S, Doncoeur C, Devaux P 2000. Plastid differentiation during androgenesis in albino and non-albino producing cultivars of barley (Hordeum vulgare L.). Sex Plant Reprod 13: 95-104.

Chang, C.C., Yang, M.H., Wen, H.M., Chern, J.C., 2002. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J. Food Drug Anal. 10, 178–182.

Cheng S.; Zhang W.; Sun N.; Xu F.; Li L.; Liao Y. and Cheng H. (2014). Production of flavonoids and terpene lactones from optimized *Ginkgo biloba* tissue culture. *Notulae Botanicae Horti Agrobotanici*, 42(1): 88–93.

Chun K.; Kim D.; and Lee C.Y. (2013): Journal of Agriculture and Food chemistry, 51: 8067-8072.

Colla, G., Rouphael, Y., Canaguier, R., Svecova, E., and Cardarelli, M. (2014). Biostimulant action of a plant-derived protein hydrolysate produced through enzymatic hydrolysis. Front. Plant Sci. 5:448. doi: 10.3389/fpls.2014.00448

Drew R. A. and N. G. Smith, N.G. (1986): "Growth of Apical and Lateral Buds of Papaya (Carica papaya L.) as Affected by Nutritional and Hormonal Factors," Journal of Horticultural Science, Vol. 61, No. 1, pp. 535-543.

Elmastas M., Ozturk L., Gokce I., Erenler R., Aboul-Enein H.Y. (2004) Determination of antioxidant activity of marshmallow flower (Althaea officinalis). Anal. Lett. 37: 1859-1869.

Ghanati, F, Moallemi, R. and Aghdasi, M. (2015): Boron effects on wall polysaccharide composition of marshmallow cells. Iranian Journal of Science & Technology, 39A4: 497-501.

Guha, S. and Maheswari, S.C. 1964. In vitro production of embryos from anthers of *Datura*. Nature 204: 407.

Hage-Sleiman R., Mroueh M., Daher C.R. (2011) Pharmacological evaluation of aqueous extract of Althaea officinalis flower grown in Lebanon. Pharm. Biol. 49: 327-333.

Hussein E.A.; Taj-Eldeen A.M.; Al-Zubairi A.S.; Elhakimi A.S. and Al-Dubaie A.R. (2010): Phytochemical screening, total phenolics and antioxidant and antibacterial activities of callus from *Brassica nigra* L. hypocotyl explants. Int. J. Pharmacol.; 6: 464-471.

Ionkova I. and Alfermann A.W. (1994) *Althaea officinalis* L. (Marshmallow): In Vitro Culture and the Production of Biologically Active Compounds. In: Bajaj Y.P.S. (eds) Medicinal and Aromatic Plants VII. Biotechnology in Agriculture and Forestry, vol 28. Springer, Berlin, Heid.

Lopez-Laredo A.R.; Ramirez-Flores F.D.; Sepulveda-Jimenez G. and Trejo-Tapia G. (2009): Comparison of metabolite levels in callus of *Tecoma stans* (L.) Juss. ex Kunth.

cultured in photoperiod and darkness. In Vitro Cell Dev Biol – Plant.; 45: 550–558

Jacquard C, Asakaviciute R, Hamalian AM (2006). Barley anther culture: effects of annual cycle and spike position on microspore embryogenesis and albinism. Plant Cell Rep 25: 375-381.

Khan, W., Rayirath, U. P., Subramanian, S., Jithesh, M. N., Rayorath, P., Hodges, D. M., et al. (2009). Seaweed extracts as biostimulants of plant growth and development. J. Plant Growth Regul. 28, 386–399. doi: 10.1007/s00344-009-9103-x.

Kozak, K; Renata Galek , R; Waheed, M.T. and Sienkiewicz, E.S. (2012): Anther culture of Lupinus angustifolius: callus formation and the development of multicellular and embryo-like structures. Plant Growth Regul (2012) 66:145–153.DOI 10.1007/s10725-011-9638-

Mohga,K.; Hosni A.M.; Ibrahim A.K. and Hewidy M. (2016): Effect of amino acids application on the growth and quality of octopus tree (SCHEFFLERA ACTINOPHYLLAL.). J.Biol.Chem.Environ.Sci.,11 (2), 283-293

Murashige T, Skoog F. (1962): A revised medium for rapid growth and bioassay with tobacco tissue culture. *Plant Physiol*.; 15:473-497. DOI: 10.1111/j.1399-3054.1962. tb08052.x elberg.

Mujib, A. Pipal, T., Ali, M. Tonk, D. Zafar, N., Gulzar, B. (2017): In vitro propagation of Althaea officinalis: the role of plant growth regulators in morphogenesis. Journal of Biotechnology, Computational Biology and Bionanotechnology. vol. 98(3) pp. 167-173.

Obadoni B.O. and Ochuko P.O. (2001): Phytochemical studies and comparative efficacy of the crude extracts of some homeostatic plants from Edo and Delta States of Nigeria. Global Journal of Pure and Applied Sciences,8: 203-208.

Pradeep K., S. and Ranjitha K., B.D., 2010. Effect of Amino Acids and Growth Regulators on Indirect Organogenesis in *Artemisia vulgaris* L.. *Asian Journal of Biotechnology, 2: 37-45.*

Popko, M.; Wilk, R.; Górecki, H.(2014): New amino acid biostimulators based on protein hydrolysate of keratin. Przem. Chem. 93, 1012–1015.

Roshany, G, Kalantarai, S, Naderi, R, Hassani, M.E.(2013): Callus Formation via Anther Culture in Capsicum annum L. with Differences in Genotypes, Media and Incubation Temperature.Tech J Engin & App Sci., 3 (S): 3847-3853, 2013.

Sadighara P., Gharibi S., Jafari A.M., Khaniki G.J., Salari S. (2012) The antioxidant and flavonoids contents of Althaea officinalis L. flowers based on their color. Avicenna J. Phytomed. 2(3): 113-117.

SAS.(2001): SAS Guid for Personal Computers. Release6.12.SAS institute inc., Cary, NC.USA.

Scossa F., Benina M., Alseekh S., Zhang Y. and Fernie A.R. (2018): The Integration of Metabolomics and Next-Generation Sequencing Data to Elucidate the Pathways of Natural Product Metabolism in Medicinal Plants. Panta Med. ;84(12-13):855-873. doi: 10.1055/a-0630-1899. Epub 2018 May 29.

Sirigiri C.K.; Kokkanti M. Patchala A. (2014): An efficient protocol devised for rapid callus induction from leaf explants of *Biophytum sensitivum* (Linn) DC. Int J Phytopharm.;4(1):20-4.

Sugiyama M.(1999): Organogenesis in vitro. Curr. Opin. Plant. Biol. 2: 61-64.

Tang Y., Huanxiu L., Liu J., Liu B. and Luo H. (2009): Callus formation from anther culture in balsam pear (*Momordica charantia* L.), American-Eurasian J. Agric. & Environ. Sci. 6(3):308-312.

Teixeira, W, Fagan, E.B., Soares, L.H. Umburanas, R.C., Reichardt, K. and Neto, D.D. (2017) Foliar and seed application of amino acids affects the antioxidant metabolism of the soybean crop. Front. Plant Sci. 8:327. doi: 10.3389/fpls.2017.00327| www.frontiersin.org

Tiwari, R. and Rana C.S. (2015): Plant secondary metabolites: a review. International Journal of Engineering Research and General Science Volume 3, Issue 5, ISSN 2091-2730 661 www.ijergs.org

Touraev A, Forester BP and Jain SM. 2009. Advances in haploid production in higher plants. Springer Science + Business media B. V, The Netherlands, 208 pp.

Woo W.S.; Chi H.J. Yun and Hye S. (1977): Alkaloid screening of some Saudi Arabian plants. SaengyakHakhoe Chi (HangukSaengyaKHakhoe), 8(3): 109-113.