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RESEARCH ARTICLE

Callus cultivation and determination of some flavonoids by HPLC from some species of cucumber

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Abstract

The objectives of the current study were to evaluate the best medium for callus induction and regeneration of two species of *Cucumis sativus* L. (Waffir F1 and Fares). Evaluation of some flavonoids in tissue culture calli and tissue culture regenerated plantlets. Four explants (leaves, shoot tips, nodes and internodes) of cucumber cultivars (Waffir F1 and Fares) cultured five weeks on MS medium supplemented with different combinations of two auxins (2,4-D and NAA) and two cytokinins (BA and Kin). Both of callus and regenerated plantlets of Waffir F1 cultivar was the most valuable and suitable for production of a great amounts of flavonoids through indirect regeneration systems.

Introduction

The genus Cucumis contains 52 species; of which cucumber and melon are the two most economically important food crops (Ghebretinsae et al., 2007). The cucumber is thought to have been first domesticated in central Asia (Harlan, 1971). Cucumber is cultivated for its edible fruits which also have high medicinal values (Ugandhar et al., 2013).The cucurbitaceae family is the second larger family of cucurbitales that includes 2 subfamilies and 8 tribes (Jeffrey, 1990). Callus usually can be produced from any differentiated structure (e.g., leaf, stem, root … etc.) by placing explants on media containing relatively high level of auxin. Once produced, the callus can be grown either as large, multicellular masses on solid media or as small cell aggregates in rotated liquid media (El-Bahr et al., 2001). Kim et al. (1988) obtained the callus of ten cultivars of cucumber on Murashige and Skoog (MS) medium (1962) supplemented with 2,4-D and BA. *In vitro* regeneration of cucumber is possible using various culture techniques (Malepszy, 1988). A variety of explants have been used for plant regeneration from cucumber via organogenesis viz., cotyledons (Chee, 1990), primary leaves (Seo et al., 2000), petioles (Punja et al., 1990) and hypocotyls (Selvaraj et al., 2006). Plant cell and organ culture is fast developing in the field of secondary metabolism regulation, with the development of molecular biology, deepen understanding of the biosynthetic pathways of natural products and newly developed treatment strategies. More and more medicinal herbs have been used to establish different culture systems, which will facilitate preserving the natural resources and improve the possibilities of producing compounds of interest at industrial level (Gaosheng and Jingming, 2012). Marais et al. (2006) stated that the term "flavonoid" is generally used to describe a broad collection of natural products that include a C6-C3-C6 carbon framework, or more specifically phenylbenzopyran functionality. Flavonoids represent one of the largest groups of secondary metabolites, with more than 8000 different compounds described in the literature (Harborne and Williams, 2000; Ververidis et al., 2007). Flavonoids are not synthesized in animal cells, thus their detection in animal tissues is indicative of plant ingestion (Mennen et al., 2008). Flavonoid compounds have been implicated in several biological processes and some of their functions include the attraction of pollinating agents via pigmentation of floral organs (Huits et al., 1994). They also have roles in human being as antioxidants (Williams et al., 2004; Scalbert et al., 2005), anti-inflammatories (Li et al., 2001), anti-carcinogenics (Lu et al., 2004) and protective agents against coronary disease (Bagchi et al., 2003). Jian and Wei (2005) reported that leaves of cucumber are rich of flavonoids compounds. Flavonoids in leaves were determined by spectrometric method with rutin as a standard. Ibrahim et al. (2010) stated that cucumber is more active antioxidant than some cucurbits

cultivated in Egypt (*Citrullus colocynthis* L. and *Momordic acharantia* L.). McNally et al. (2003) reported that cucumber produce some active flavonoids with higher quantities to induce resistance against powdery mildew fungi (acting as phytoalexins) such as cucumerin A, cucumerin B, vitexin, isovitexin, orientin, isoorientin, p-coumaric acid and p-came. Mukherjee et al. (2013) succeeded in isolating some bioactive compounds from cucumber fruits such as cucumerin A and B, vitexin, orientin, isoscoparin 2″-O-(6‴-(E)-p-coumaroyl) glucoside, apigenin 7-O-(6″-O-p-coumaroylglucoside).

The aim of study is to determine the best explant type and effective plant growth regulators (PGRs) in development of callus and plantlets. Evaluation of some flavonoids in tissue culture calli and tissue culture regenerated plantlets of two cultivars of cucumber qualitatively and quantitatively using HPLC.

Material and Methods

Plant material

Seeds of two cultivars of *Cucumis sativus* L. (Waffir F1 and Fares) were obtained from the Agricultural Research Center, Ministry of Agriculture, Dokki, Giza, Egypt. The seeds where sterilized and germinated in MS medium free from growth regulators to regeneration.

Calli production

One month old plantlets at the physiological age were used as a source of explants. Different explants including shoot tip, nodal segment, leaf and internode (3-4mm) of each plant parts were exiced from sterilized plantlets then, transferred to 150 ml glass jars of MS basal media. Different combinations of plant growth regulator (auxins and cytokinins) were added to the basal media. Each solidified basal MS medium was supplemented with the following combinations of different auxins and /or cytokinins as the following:-

1- Basal MS medium
2. 0.5 mg/l 2,4-D
3. 1.0 mg/l 2,4-D
4. 0.5 mg/l NAA
5. 1.0 mg/l NAA
6. 0.5 mg/l BA
7. 1.0 mg/l BA
8. 0.5 mg/l Kin
9. 1.0 mg/l Kin
10. 0.5 mg/l 2,4-D + 0.5 mg/l BA
11. 1.0 mg/l 2,4-D + 1.0 mg/l BA
12. 0.5 mg/l 2,4-D + 0.5 mg/l kin
13. 1.0 mg/l 2,4-D + 1.0 mg/l kin
14. 0.5 mg/l NAA + 0.5 mg/l BA
15. 1.0 mg/l NAA + 1.0 mg/l BA
16. 0.5 mg/l NAA + 0.5 mg/l kin
17. 1.0 mg/l NAA + 1.0 mg/l kin

Shootlets formation

The effect of different concentrations of BA as a cytokine on number of shootlets formation, shootlet length and number of leaves was studied. Consequently, calli cultures were transferred to solidified MS medium supplemented with (0.5, 1, 1.5, 2 mg/l) of BA. This experiment included 5 treatments as follows:

1- MS free PGR.
2- MS + 0.5 mg/l BA.
3- MS + 1 mg/l BA.
4- MS + 1.5 mg/l BA.
5- MS + 2 mg/l BA.

All calli cultures were examined after 5 weeks of incubation under a 16hours photoperiod at 1500 lux of cool fluorescent lamp at 26±2°C .

Estimation of some flavonoids using HPLC

The leaves of *in vivo* plants, calli and leaves of regenerated plantlets were collected separately from three cucumber cultivars (Waffir F1, Fares and Beith Alpha "Lashin and Mamdouh 2014") for qualitative and quantitative determination of some flavonoids using High Performance Liquid Chromatography (HPLC) according to the method of Mattila et al. (2000).

Five grams dry weight of each sample were mixed with methanol and centrifuged at 10000 rpm for 10 min and the supernatant was filtered through 0.2 µm Millipore membrane filter then 1–3 ml was collected in a vial for injection into HPLC (Agilent 1200 series) equipped with auto-sampling injector, solvent degasser, ultraviolet (UV) detector set at 254 nm and quarter HP pump. The column temperature was maintained at 35°C.

Statistical analysis

Data collected in callus induction and plantlets regeneration experiments was performed by analysis of variance using Sigma Plot software version 12, followed by a comparison of means test at 5% of probability by Holm-Sidak method. The statistical analysis of the data of molecular characterization experiments was performed considering a binary matrix that was constructed using the value '1' to indicate band present and '0' to indicate band absent.

Result and Discussion

Callus production

This part of study aimed to record the various responses for callus formation using four explants (leaves, shoot tips, nodes and internodes) of cucumber cultivars (Waffir F1and Fares) cultured five weeks on MS medium supplemented with different combinations of two auxins (2,4-D and NAA) and two cytokinins (BA and Kin).

The obtained results revealed that callus formation were varied depending on the type of cucumber cultivar, type 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 three cucumber cultivars.

Percentage of callus formation

Data shown in Table (1) and Fig.1 (A) represent the percentage of callus formed from four explants (leaf, shoot tip, node and internode) of cucumber Waffir F1 cv. after four weeks of cultivation. The results reveal that shoot tip and node explants gave higher percentage of callus followed by leaf and internode explants in most of plant growth regulators treatments. It is important to mention that leaf and node explants gave callus in all MS media containing growth regulators while shoot tip explants didn't give callus in MS medium supplemented with 0.5 and 1 mg/l Kin. The obtained results are in agreement with those obtained by Turhan et al., (2009) on callus induction in *Linum usitatitum* L. and *Lactuca sativa* L. (Mohebodini et al., 2011) concluded that the explant type has a direct effect on callus formation frequency. Furthermore, study by Shasthree et al. (2012) on *Citrullus colocynthis* showed the effect of different explants (stem, leaf and cotyledon) used to induce callus and found that the best explant in callus production was stem.

Data tabulated in Table (2) and Fig.1 (B) show the percentages of callus formed using four explants of cucumber Fares cv. after 4 weeks of cultivation on different concentrations of plant growth regulators. The obtained results revealed that all concentrations of plant growth regulators gave the highest percentage of calli formed (100%) from leaf explants except 0.5 mg/l 2,4-D and 1 mg/l NAA which gave 75% followed by shoot tip then node and internode explants. This results are in agreement with the studies of Ewais (1995) used 2,4-D and Kinetin for mass production of cucumber callus. Seo et al. (2000) obtained callus using MS medium supplemented with 5+5 µM of NAA+BA.

Table 1: The percentage and morphological characters of calli formed in cucumber Waffir F1 cultivar.

PGR (mg/l)	Callus formation (%)				Morphological characters (color; texture)			
	Leaf	Shoot tip	Node	Internode	Leaf	Shoot tip	Node	Internode
Free PGR	0	0	0	0	-------	----------	-------	-------
0.5 2,4-D	83.33	100	100	50	Cr to LG; C	LG; C	Cr to LG; C	Cr to LG; L
1.0 2,4-D	83.33	66.67	100	50	Cr to W; L	LG; C	Cr; C	Cr; F
0.5 NAA	58.33	100	66.67	0	Cr to W; L	Cr to LG; C	Cr; F	-------
1.0 NAA	66.67	33.33	100	25	LG; C	Cr; C	Cr; F	Cr; L
0.5 BA	25	41.67	33.33	0	Cr; C	Cr; C	Cr; C	-------
1.0 BA	100	100	41.67	25	Cr to G; C	B to W ; C	Cr to G; C	Cr; L
0.5 Kin	50	0	25	0	Cr; C	----------	Cr; C	-------
1.0 Kin	100	0	16.67	0	Cr to G; C	-------	Cr to W; C	-------
0.5 2,4-D+0.5 BA	100	100	100	25	LG; C	LG; C	Cr to W; F	Cr; L
1.0 2,4-D+1.0 BA	66.67	58.33	66.67	100	Cr to LG; C	LG; C	Cr to W; F	Cr; F
0.5 2,4-D+0.5 Kin	100	100	100	50	Cr to B; C	Cr to W; C	Cr to W; C	Cr to W; C
1.0 2,4-D+1.0 Kin	75	100	50	33.33	Cr to G; C	Cr; C	Cr; C	Cr to W; L
0.5 NAA+0.5 BA	83.33	100	100	50	Cr to LG; C	Cr; C	LG; F	Cr to W; L
1.0 NAA+1.0 BA	41.67	100	100	50	Cr; F	Cr; C	Cr; F	Cr; C
0.5 NAA+0.5 Kin	100	58.33	100	58.33	Cr; L	Cr; C	Cr; C	Cr; C
1.0 NAA+1.0 Kin	66.67	100	100	41.67	Cr to W; C	Cr to W; C	Cr; C	Cr; L

G: green, LG: light green, Cr: cream, W: white, C: compact, F: friable, L: loose

Table 2: The percentage and morphological characters of calli formed in cucumber Fares cultivar.

PGR (mg/l)	Callus formation (%)				Morphological characters (color; texture)			
	Leaf	Shoot tip	Node	Internode	Leaf	Shoot tip	Node	Internode
Free PGR	0	0	0	0	-------	-------	-------	-------
0.5 2,4-D	75	100	75	58.33	Cr; C	Cr to W; C	Cr to W; C	Cr; L
1.0 2,4-D	100	100	75	33.33	Cr to W; C	Cr; C	Cr to W; C	Cr to W; L
0.5 NAA	100	0	41.67	41.67	Cr; C	-------	Cr; C	Cr; L
1.0 NAA	75	0	66.67	41.67	Cr; C	-------	Cr; C	Cr; L
0.5 BA	100	66.67	83.33	50	Cr; C	Cr to LG; C	Cr to W; C	Cr to W; C
1.0 BA	100	100	66.67	58.33	Cr; C	Cr; C	Cr; C	Cr; L
0.5 Kin	100	0	33.33	41.67	Cr; C	-------	Cr; C	Cr; L
1.0 Kin	100	0	50	33.33	Cr to W; C	-------	LG; C	Cr to W; L
0.5 2,4-D+0.5 BA	100	83.33	66.67	58.33	Cr; C	Cr; C	Cr; C	Cr to LG; L
1.0 2,4-D+1.0 BA	100	91.67	100	41.67	Cr; C	Cr to LG; C	Cr to LG; C	Cr; L
0.5 2,4-D+0.5 Kin	100	100	75	58.33	Cr to W; C	Cr; C	Cr to W; C	Cr; C
1.0 2,4-D+1.0 Kin	100	100	83.33	41.67	Cr; C	LG to W; C	Cr; C	Cr; C
0.5 NAA+0.5 BA	100	100	83.33	75	Cr; C	Cr to LG; C	Cr; C	Cr; C
1.0 NAA+1.0 BA	100	100	100	66.67	Cr to W; C	Cr; C	Cr to LG; C	Cr; C
0.5 NAA+0.5 Kin	100	91.67	75	50	Cr; C	Cr to W; C	Cr; C	Cr to W; C
1.0 NAA+1.0 Kin	100	100	91.67	66.67	Cr; C	Cr; C	Cr; C	Cr; L

G: green, LG: light green, Cr: cream, W: white, C: compact, F: friable, L: loose

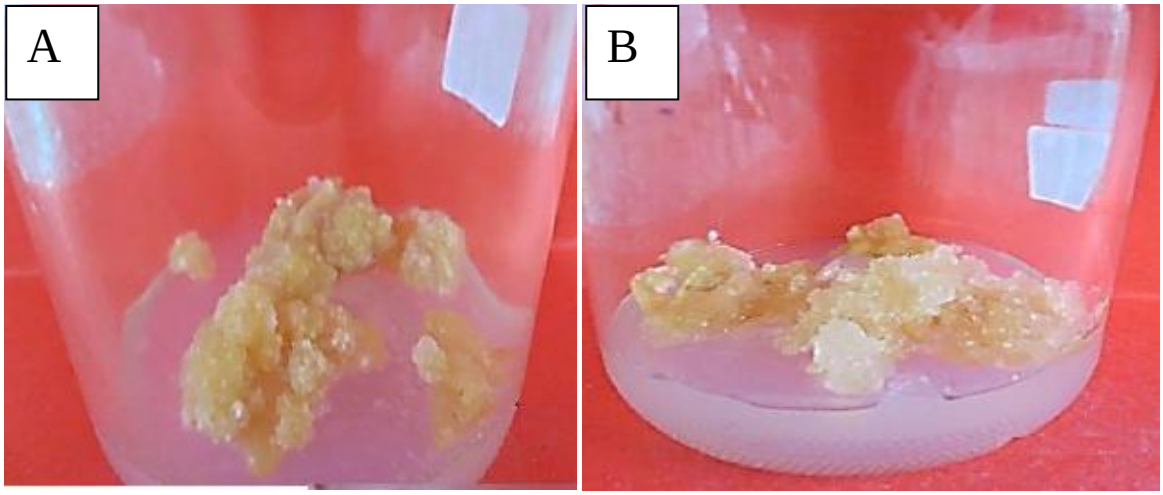


Figure 1: Initiated calli of cucumber after five weeks of cultivation: (A) Waffir F1 callus, (B) Fares callus.

Shootlets regeneration

In this part of study, leaflet calli (calli originated from leaves) of different cucumber cultivars (Waffir F1 and Fares) were used to enhance and produce shootlets. Results of this experiment showed the effect of MS medium supplemented with different concentrations of BA (in addition to MS free PGR as control) on number of shoots, number of nodes, number of leaves, and shoot length of regenerated shootlets. It was noticed that no response obtained in MS medium free of growth regulators in two cucumber cultivars.

The obtained results of cucumber Waffir F1 cv. in Table (3) and Figure (2) showed the highest number of shootlets and leaves 5.667, 18.333 respectively were obtained from callus cultured on MS medium supplemented with 2 mg/l BA. While the highest number of nodes (4.667) was obtained from media containing 0.5 mg/l BA. Finally, the longest regenerated shootlets (4.7 cm) were recorded in shootlets regenerated on media containing 1.5 mg/l BA.

Although, MS medium supplemented with 0.5 mg/l BA showed the highest number of nodes, it recorded the lowest number of shootlets (2.667) and shootlet length (2.633 cm). However MS medium containing 1.5 mg/l BA gave the highest length of shootlets, it showed the lowest number of leaves (10.667). The lowest number of nodes (2.667) was recorded in media containing 1 mg/l BA.

Table 3: Effect of different concentrations of BA on number of shootlets, nodes, leaves and shootlets length produced from calli in Waffir F1 cultivar.

PGRs (mg/l)	Shootlets Length (cm)	No. of leaves	No. of nodes	No. of shootlets
Free PGR	0±0.00b	0±0.00c	0±0.00b	0±0.00c
0.5 BA	2.63±0.15c	12.33±1.76ab	4.67±0.88a	2.67±0.67b
1 BA	3.1±0.17ac	13.33±1.45ab	2.67±0.33a	3.67±0.33b
1.5 BA	4.7±1.36a	10.67±2.96b	4±0.577a	3.67±0.88b
2 BA	4.17±0.37ac	18.33±1.45a	3.67±0.88a	5.67±0.33a

All data expressed in Mean ± SE. Pairwise comparison between concentrations of BA was performed with Holm-Sidak method

Table 4: Effect of different concentrations of BA on number of shootlets, nodes, leaves and shootlets length produced from calli in Fares cultivar.

PGRs (mg/l)	Shootlets Length (cm)	No. of leaves	No. of nodes	No. of shootlets
Free PGR	0±0.00b	0±0.00b	0±0.00b	0±0.00c
0.5 BA	2.23±0.52a	6.67±0.88ab	2±0.58a	2±0.577b
1 BA	3±0.46a	9.67±2.03a	3±0.58a	2.33±0.33ab
1.5 BA	3.1±0.15a	9.67±1.20a	1.667±0.33a	3.33±0.33ab
2 BA	4±0.32a	13.67±4.06a	3±0.00a	4±0.58a

All data expressed in Mean ± SE. Pairwise comparison between concentrations of BA was performed with Holm-Sidak method

Figure 2: Shoot initiation from calli of cucumber Waffir F1 cultivar after five weeks of cultivation on MS medium containing BA.

The obtained results of cucumber Fares cv. showed in Table (4) revealing that 2 mg/l BA gave the highest number of shootlets (4.03), nodes (3.05), leaves (13.667) and longest shootlets (4.03 cm). It is important to indicate that the highest number of nodes (3) was reached also byusing 1 mg/l BA. On the other hand, 0.5 mg/l BA gave the lowest number of shootlets (2), number of leaves (6.667), and shortest shootlets (2.233 cm) while the lowest number of nodes (1.667) was reached on MS medium containing 1.5 mg/l BA (Figure 3).

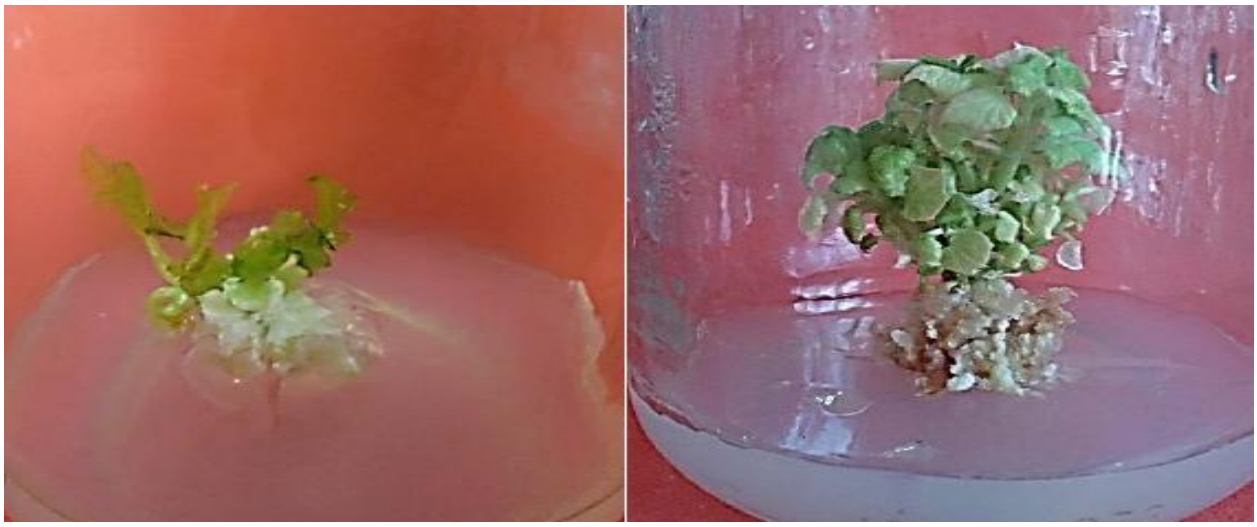
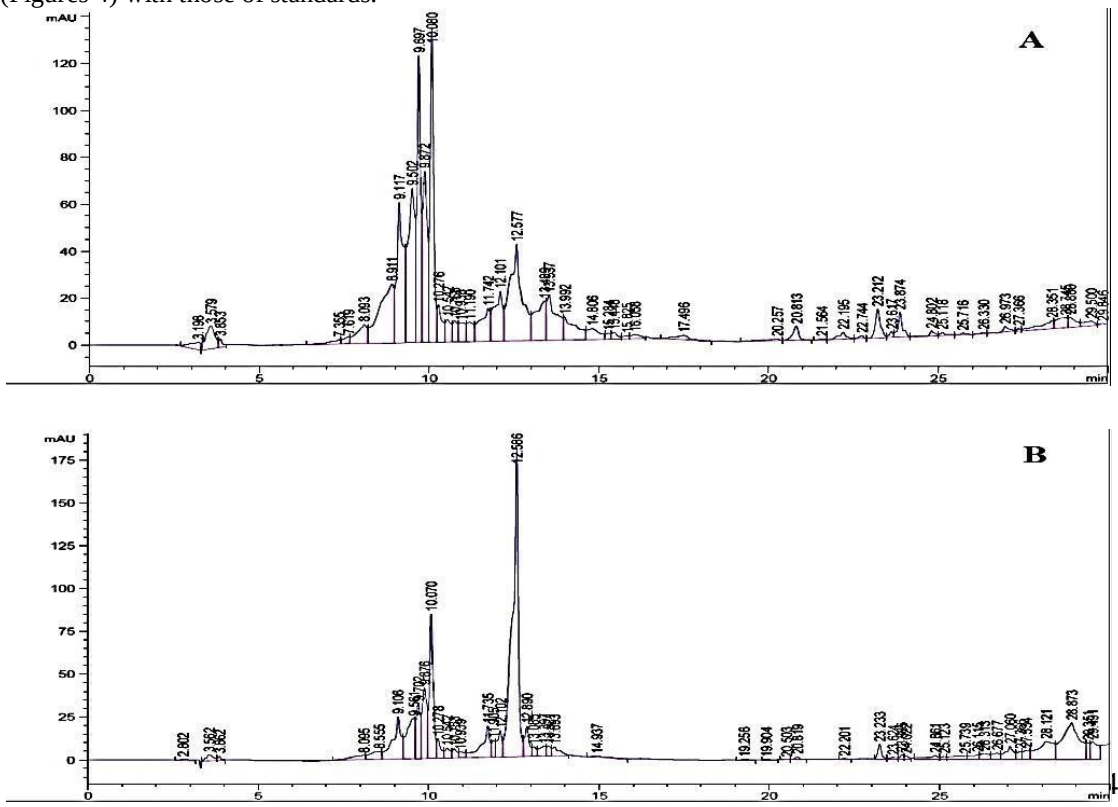


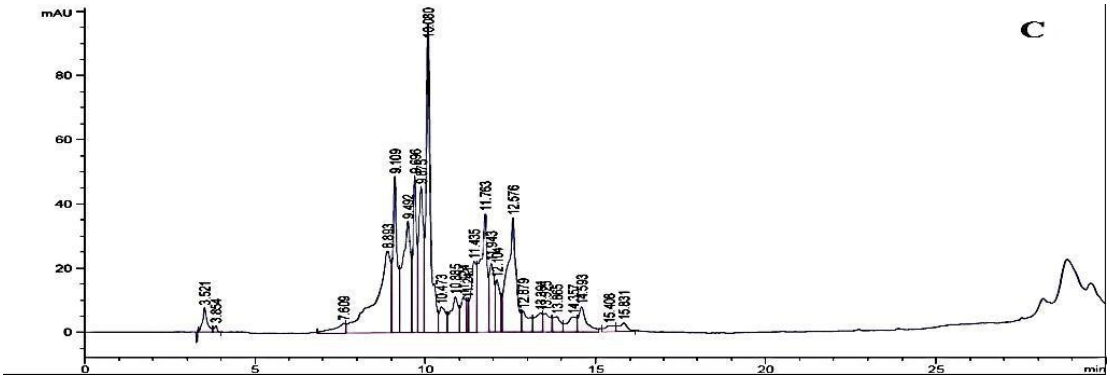
Figure 3: Shoot initiation from calli of cucumber Fares cultivar after five weeks of cultivation on MS medium containing BA.

Generally, higher concentrations of BA 1.5-2 mg/l is preferred for obtaining regenerated shootlets from cucumber calli with high length, numbers of leaves and nodes. Our findings regarding shoot regeneration on BA are in conformity with report of Han et al., (2004) who concluded BA as an essential factor for shoot regeneration in bottle gourd (*Lagenaria siceraria* Standl.). Similar conclusion has been drawn in another study on bottle gourd stating BA essential for shoot bud formation (Saha et al., 2007).

Detection of flavonoids in tissue-culture raised plants

The leaves of in *vivo* plants, calli and leaves of regenerated plantlets of three cucumber cultivars (Waffir F1, Fares and Beith Alpha) were collected separately for qualitative and quantitative analysis of some flavonoids. Naringin, rutin, hesperidin, rosmarinic acid, quercetrin, quercetin, luteolin, naringenin, kaempferol, apigenin, 7-hydroxy flavone and hesperetin standards were used for determining their presence and quantity in each specimen using High Performance Liquid Chromatography (HPLC). Separated flavonoid peaks were initially identified by direct comparison between retention times detected in *vivo* plants, calli and regenerated plantlets of three cucumber cultivars (Figures 4) with those of standards.





Figures 4: Retention times and peaks recorded in vivo plants of Waffir (A), Beith Alpha (B) and Fares (C).

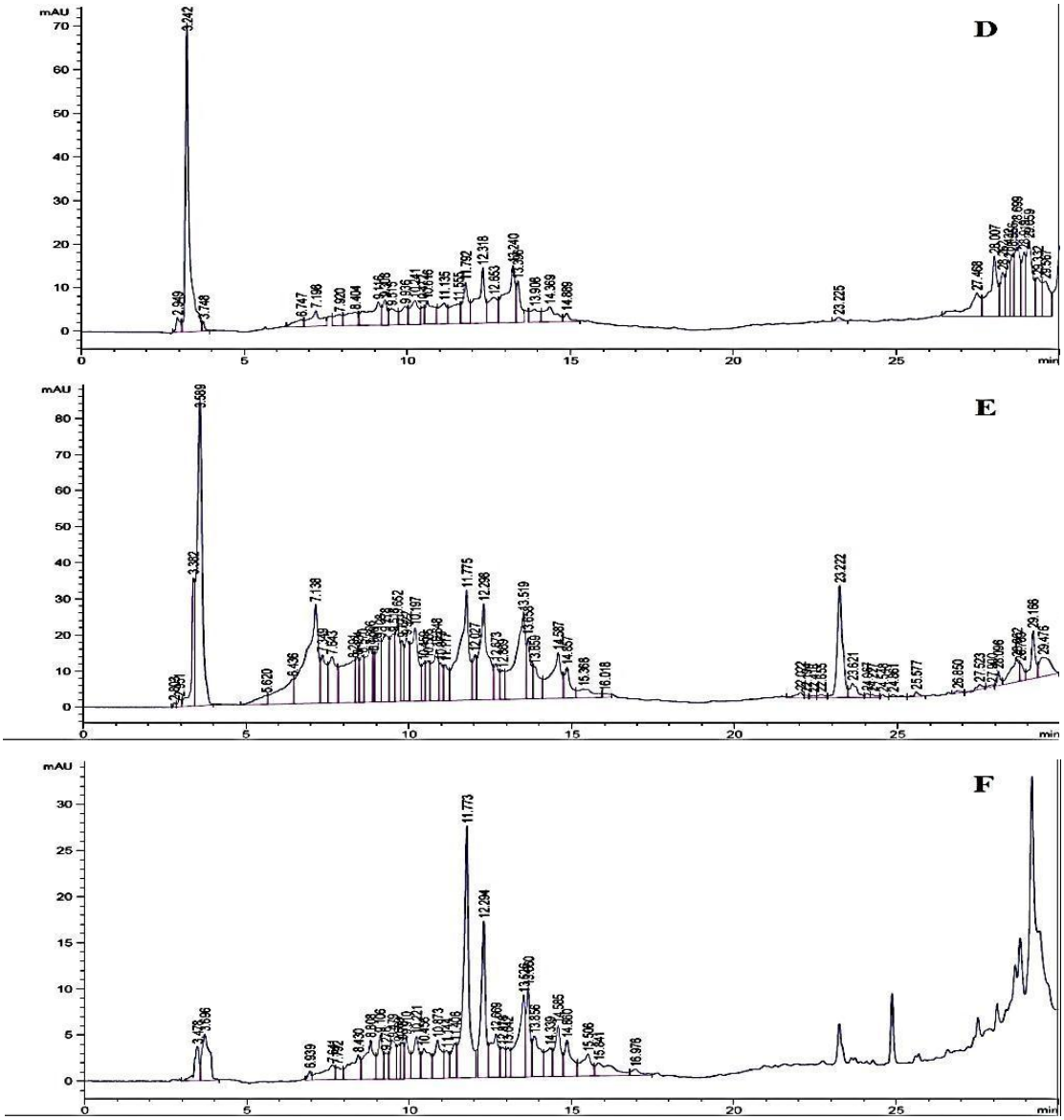


Figure 4(Continued): Retention times and peaks recorded in calli of Waffir (D), Beith Alpha (E) and Fares (F).

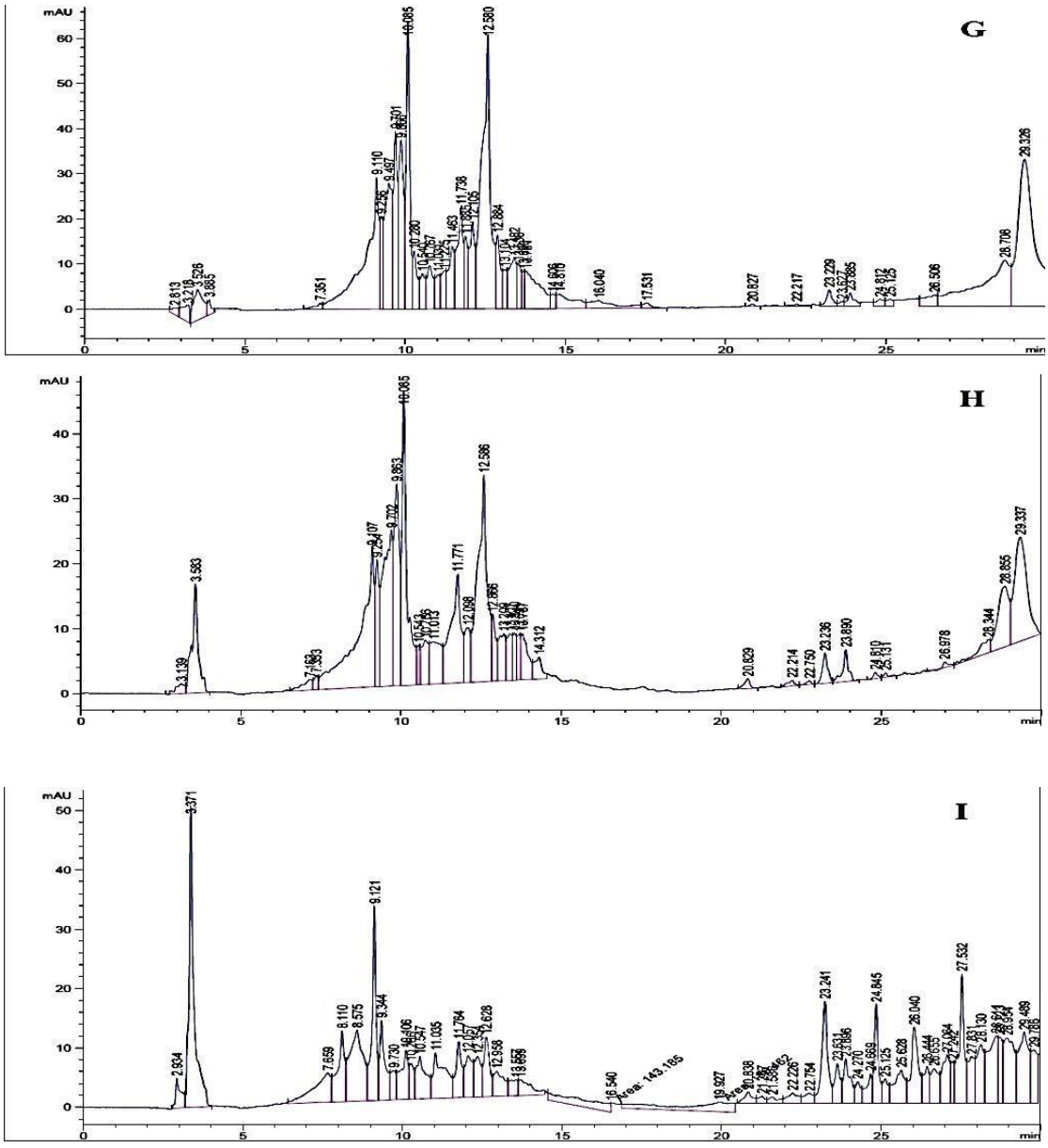


Figure 4 (Continued): Retention times and peaks recorded in regenerated plantlets of Waffir (G), Beith Alpha (H) and Fares (I).

The data demonstrated in Table (5) showed that a great amount of naringin (35147.31 µg/100 gm DWt) was observed in regenerated plantlets of Waffir cultivar with more than three times increasing than that in *vivo* plants (11148.36 µg/100 gm DWt). However, the absence of naringin in *vivo* plants of Beith Alpha cultivar but relatively high amount of naringin (10853.41 µg/100 gm DWt) was detected in its regenerated plantlets.

On the other hand, in *vivo* plants of Fares cultivar showed higher quantity of naringin (4732.56 µg/100 gm DWt) than its calli (216.49 µg/100 gm DWt) and regenerated plantlets (103.71 µg/100 gm DWt).

The obtained results in Table (5) showed increasing of the amount of rutin in plantlets (1876.12 µg/100 gm DWt) than in *vivo* plants (586.27 µg/100 gm DWt) of Waffir by more than three times but the highest amount of rutin (10844.08 µg/100 gm DWt) was recorded in *vivo* plants of Beith Alpha cultivar however rutin was absent in its

regenerated plantlets. The yield of rutin in Fares cultivar showed decreased amount in regenerated plantlets (85.1 µg/100 gm DWt) relative to the amount detected in *vivo* plants (992.07 µg/100 gm DWt).

The results also showed that the largest amount of hesperidin (9736.67 µg/100 gm DWt) was detected in regenerated plantlets of Waffir cultivar with more than double of the amount detected in its in *vivo* plants (4440.07 µg/100 gm DWt) and calli (4858.07 µg/100 gm DWt). The quantity of hesperidin found in *vivo* plants of Beith Alpha (8162.71 µg/100 gm DWt) was higher than its calli (6247.98 µg/100 gm DWt) and regenerated plantlets (5069.02 µg/100 gm DWt) while the amounts of hesperidin in Fares cultivar were lower than of Waffir and Beith Alpha.

The obtained results showed that the highest quantities of rosmarinic acid and quercetin were obtained in *vivo* plants of Beith Alpha cultivar (2791.63 and 796.44 µg/100 gm DWt, respectively) although the highest amount of quercetrin (2558.81 µg/100 gm DWt) was obtained in its calli. It is important to notice that regenerated plantlets of Waffir cultivar contain higher quantities of rosmarinic acid, quercetrin and quercetin (2623.98, 1939.49 and 484.78 µg/100 gm DWt, respectively) than those of in *vivo* plants and calli. On the other hand, low quantities of these flavonoids detected in Fares cultivar. These results were in agreement with the study of Agarwal and Kamal (2013) on regeneration and phytochemical screening of bitter melon. They suggested that level of plant growth regulators and developmental stages of differentiated organs were main reasons for increasing production of quercetin and luteolin in regenerated multiple shoots of bitter melon.

The obtained results reveal that the largest amount of luteolin (1276.34 µg/100 gm DWt) was recorded in *in vivo*plants of Beith Alpha cultivar followed by regenerated plantlets of Waffir cultivar (1077.45 µg/100 gm DWt).

Naringenin, kaempferol, apigenin, 7-hydroxy flavone and hesperetin were identified in some specimens of three cucumber cultivars but with small amounts.

In general, a profound difference of the compounds was observed between the different samples. Some compounds are found in the mother plants and not detected in the regenerated plantlets and callus raised from tissue culture. The regenerated plantlets of cucumber cultivar Waffir F1 contained large amounts of most of flavonoids tested. These results were in agreement with Jian and Wei (2005) which stated that leaves of cucumber were rich of flavonoid compounds determined by spectrometric method.

Cucumber Waffir F1 cultivar was more suitable for applying regeneration systems than other two cultivars for production of flavonoids. For this reason, this cultivar was the most valuable and suitable for further studies on production of secondary metabolites and pathogen resistance.

Table 5: Amounts of bioflavonoid compounds found in *vivo* plants, calli and regenerated plantlets of three cucumber cultivars determined using HPLC.

Flavonoids	Test results of flavonoids (µg/100 gm dry weight)								
	Waffir F1 cv.			Beith Alpha cv.			Fares cv.		
	In vivo	Calli	Plantlets	*In vivo*	Calli	Plantlets	*In vivo*	Calli	Plantlets
Naringin	11148.36	532.67	35147.31	--	2897.01	10853.41	4732.56	216.49	103.71
Rutin	586.27	--	1876.12	10844.08	1509.71	--	992.07	75.11	85.10
Hesperidin	4440.07	4858.07	9736.67	8162.71	6247.98	5069.02	3719.30	1950.96	1045.28
Rosmarinic	1207.45	149.87	2623.98	2791.63	866.27	908.51	398.65	149.02	24.28
Quercetrin	1703.53	311.30	1939.49	1706.44	2558.81	897.66	549.22	453.33	106.06
Quercetin	236.85	124.05	484.78	796.44	461.74	83.38	166.25	74.84	--
Luteolin	--	--	1077.45	1276.34	--	93.27	--	64.75	19.66
Naringenin	127.29	38.23	--	--	197.30	22.03	--	55.65	3.81
Kaempferol	113.85	51.36	--	231.74	404.11	15.43	--	66.13	15.69
Apigenin	22.57	5.29	103.22	202.78	20.17	0.49	29.54	4.72	2.31
7-hydroxy	--	3.87	49.44	115.17	57.81	27.13	6.12	16.96	--
Hesperetin	--	--	253.43	--	--	33.90	--	13.08	6.88

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