

INFLUENCE OF DIFFERENT PLANT GROWTH REGULATORS ON CALLUS INDUCTION AND FLAVONOID CONTENTS IN ALFALFA (*Medicago sativa* L.).

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ABSTRACT

Alfalfa contains many important substances including flavonoids which play many diverse roles in plant. Alfalfa callus was induced on media containing 6- benzyl aminopurin (BA), α - Naphthalene acetic acid (NAA), 2, 4 Dichloro phenoxy acetic acid (2, 4-D) and kinetin. The best callus formation was obtained from the medium of 2 mg^l⁻¹ 2, 4- D + 1.5 mg^l⁻¹ Kinetin; leaf was the best organ of alfalfa in production of fresh weight. The 2nd subculture was higher in growth characters and flavonoids production as compared to the 1st subculture. Flavonoid concentration was higher in shoot tip of 1st subculture and leaves in the 2nd subculture.

Keyword: Alfalfa, callus, plant growth regulators, flavonoid.

INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a leguminous plant species that originated in different places at the world. This plant species has been grown for a variety of purposes such as soil improvement, animal feed, medicinal uses and suitable foliage (Stepppler, 1987). Alfalfa had consisted on major constituents as coumarines, alkaloids, vitamins, porphyrines, stachydrine, homostachydrine and flavonoids (Duke, 1985; Rogers, 1997). Flavonoids are plant secondary metabolites found in most terrestrial vascular plants. They belong to a group of natural phenolic substances with variable chemical structures and in plants more than 6000 different flavonoids have been identified, many of them are responsible for the attractive colors of flowers, fruits and leaves (Nijveldt *et al.*, 2001). The interesting biological activities of flavonoids have prompted the intensive research on physiological properties of these compounds and their effects on human health. Their wide occurrence, complex diversity and manifold functions have made flavonoids a very attractive system for research on a molecular-biological level. Up to date, vast amount of knowledge on flavonoids has been accumulated. This has provided the tools and the know-how for successful metabolite engineering of the flavonoid pathway (Forkmann and Martens, 2001). Flavonoids possess significant antihepatotoxic, antiallergic (Dicarlo *et al.*, 1999), anti-inflammatory, antiosteoporotic, and antiatherogenic effects (Ishimi *et al.*, 1999), antitumor,

antiproliferative, and anticancer activities (Kuo, 1996; Birt *et al.*, 2001; Manthey and Guthrie, 2002); as well as antioxidant (Hollman and Katan, 1999; Havsteen, 2002), antiviral (Jassim and Naji, 2003) and inhibition activity against many mammalian enzyme systems *in vitro* (Middleton *et al.*, 2000).

Thus, in the present experiment there were attempts to cultivate whole plant organs, *i.e.* shoot tips, roots, leaves and stems under *in vitro* conditions with the aim to produce medicinally important compound. As it was expected, such organ cultures produced similar patterns of secondary metabolites as intact plants.

MATERIALS AND METHODS

These experiments were carried out during the period from Aug. 2006 - Dec. 2007 in Plant Biotechnology Research Laboratory, Faculty of Agriculture, Cairo University, Giza, Egypt.

Seeds of alfalfa received from Ornamental Department, Faculty of Agriculture, Cairo University, Giza, Egypt were used in the present experiments

Seeds were germinated on different tissue culture media under aseptic conditions at the rate of 15 seed / jar.

Experiment treatments

This work included three experiments as follows:

1- Effect of medium types on callus induction.

This experiment consisted of 4 medium types as shown in Table 1.

Table 1: MS medium with different concentrations of growth regulators.

A	MS + BA (1 mg l ⁻¹)+ NAA (0.2 mg l ⁻¹)
B	MS + BA (2 mg l ⁻¹)+ NAA (0.2 mg l ⁻¹)
C	MS + BA (4 mg l ⁻¹)+ NAA (0.2 mg l ⁻¹)
D	MS + 2, 4-D (2 mg l ⁻¹)+ Kinetin (1.5 mg l ⁻¹)

In vitro procedure

Seeds of alfalfa were surface sterilized by using ethanol (70%) for 30 sec., then by 20% commercial bleach (sodium hypochlorite, 0.052%) for 20 min followed by 3-4 times washes with sterile distilled water under aseptic condition to release bleach (Ehsanpour and Amini, 2003). The sterilized seeds were cultured on MS basal medium (Murshige and Skoog, 1962), with 3% sucrose and solidified with 0.7% agar, pH was adjusted to 5.7 before autoclaving at 121 °C for 25 min. Seeds were grown under 16 h photoperiod at 1250 lux of cool white light and incubated at 24 °C for 7-10 days in growth chamber.

Produced seedlings were separated into different types of organs (leaf, stem, root and shoot tip). Each of these explants was cultured in baby jars (150 ml capacity) containing 30 ml of callus induction media shown in

Table 1. Twenty days after culture, data were taken on the formed callus as following:

- 1- Callus fresh weight (C. F. W.).
- 2- Callus diameter (C. D.).

2- Effect of different 2, 4- D concentrations with or without using kinetin of callus induction.

Medium D was showed the best performance regarding callus induction in the first experiment. It was chosen for this experiment. This medium contained 2 mg l^{-1} 2, 4- D and 1.5 mg l^{-1} Kinetin. Some modifications were made for this medium (Table 2.), and the obtained media were tested on callus induction. All steps regarding callus production were followed as in the first experiment. Moreover, another subculture after 20 days was conducted. Subculture was also done in 150 ml baby jars. Data were taken on produced formed callus of both culture and subculture, as shown in the first experiment.

Table 2: MS medium with different 2, 4- D concentrations with or without using Kinetin.

T1	MS + 2,4-D (1 mg l^{-1})
T2	MS + 2,4-D (2 mg l^{-1})
T3	MS + 2,4-D (3 mg l^{-1})
T4	MS + 2,4-D (4 mg l^{-1})
T5	MS + 2,4-D (1 mg l^{-1}) + Kinetin (1.5 mg l^{-1})
T6	MS + 2,4-D (2 mg l^{-1}) + Kinetin (1.5 mg l^{-1})
T7	MS + 2,4-D (3 mg l^{-1}) + Kinetin (1.5 mg l^{-1})
8	MS + 2,4-D (4 mg l^{-1}) + Kinetin (1.5 mg l^{-1})

3- Response of callus flavonoid content to different explants and subcultures.

Different explants, *i. e.* leaf, stem, root and shoot tip were cultured on medium 6, which consisted of MS + 2, 4- D (2 mg l^{-1}) + kinetin (1.5 mg l^{-1}). Twenty days later subcultures of produced callus were performed as described in experiment 2. Data were recorded on callus produced from explants culture and the subculture as follows:

- 1- Callus fresh weight (C. F. W.).
- 2- Callus diameter (C. D.).
- 3- Flavonoids concentrations ($\mu\text{g/g F.W.}$).

Procedure of flavonoid determination

Sample preparation:

To determine the active constituent flavonoids of the callus produced from different organs *in vitro*. The different calluses were obtained fresh for analysis. One g of callus was used for determination of alfalfa flavonoids (μg flavonoids / g F.W.), a fresh sample of alfalfa callus was weighted and

separately mixed with 6 ml of 80% aqueous methanol by mortar for 2 minute, extracts were obtained under vacuum.

Total flavonoid assay

Total flavonoid concentration was measured by the aluminum chloride colorimetric assay. An aliquot (1 ml) of extracts or standard solution of quercetin (100, 250, 375, 500 µg/ ml) was added to 10 ml volumetric flask containing 4 ml of dd H₂O. To the flask was added 0.3 ml 5% NaNO₂. After 5 min, 0.3 ml 10% AlCl₃ was added. At 6th min, 2 ml 1M NaOH was added and the total volume was made up to 10 ml with dd H₂O. The solution was mixed well and the absorbance was measured against prepared reagent blank at 415 nm. (Marinova *et al.*, 2005).

Data analysis

The experiments were set in three factors completely randomized design (CRD) in and five replicates, each replicate consisted of one jar and each jar contained one explant. The data on different parameters were collected and statistically analyzed. Thereafter, the least significant differences between means of treatments were recorded at 5% level using L.S.D as described by Steel and Torrie (1960).

RESULTS AND DISCUSSION

1- Effect of medium types on callus induction.

In this experiment four different supplementations of growth regulators (2, 4- D, NAA, BA and kinetin) were added to MS medium to obtain the best callus formation (C.F.W and C.D.) from different organs (leaves, stems, roots and shoot tips). Visible callus formation was obtained with in a few days, and data were taken at 20th days of the culture.

a. Effect of medium types on callus fresh weight (C. F. W.).

The obtained results showed that there were significant differences between treatments; the highest C.F.W from all explants was observed on MS supplemented with 2 mg 2, 4- D + 1.5 mg kinetin.

Data indicated that medium A produced alfalfa callus tissue from both roots and leaves and hardly from stems. Only callus tissues were produced from leaves in B and C media. However, the best medium was D which successfully produced callus tissue from all organs of alfalfa plant, where C.F.W obtained from leaf explants on medium D was about of 6.61 times that of medium A; 7.43 times that of medium B and 5.68 times that of medium C. Stems and roots gave a higher C.F.W on medium D with 28.33 and 11.82, times respectively, than that produced on medium A, while they didn't produce callus on other media. Shoot tips produced callus only on medium D (Figure 1.).

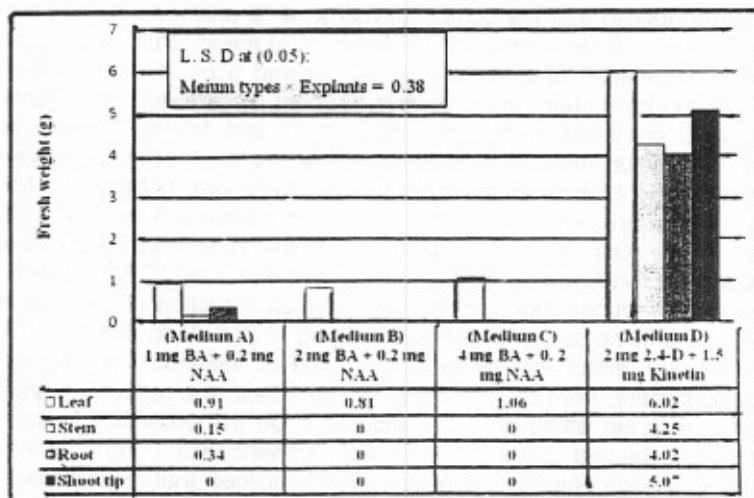


Figure 1: Effect of medium types on callus fresh weight.

b. Effect of medium types on callus diameter (C. D.).

Data indicate that there were significant differences among treatments on C.D. which produced from the different media. The highest callus volume was observed on MS supplemented with 2 mg l^{-1} 2, 4-D + 1.5 mg l^{-1} kinetin (Figure 2).

Medium A produced alfalfa callus tissue from both roots and leaves and hardly from stems. Only callus tissues were produced from leaves in B and C media. However, the best medium was D which successfully produced callus tissue from all organs of alfalfa plant. The highest value of callus diameter (C. D.) produced from leaves was obtained on medium D which was about 1.99 times that of medium A; 2.78 times that of medium B and 2.23 times that of medium C. Stems and roots showed a higher C.D. on medium D (11.03 and 2.95 times, respectively than medium A), while they didn't produce any callus on others media. Shoot tips produced callus only on medium D.

Thus, it could be concluded from this experiment that leaf explant was the best part for induction of callus formation and the treatment that contained 2 mg l^{-1} 2, 4- D + 1.5 mg l^{-1} kinetin was chosen be used in the next experiment. In previous studies on explants of alfalfa callus production, it was stated that 2, 4 - D and Kinetin could be necessary for efficient callus formation from all explants, e. g. leaf, stem, root and shoot tip. Similar data were obtained by Ehsanpour and Taheri (2005) who found that alfalfa callus could production on MS medium containing 2, 4- D, Kinetin and NAA. These results are in agreement with Simovic (1985) and Rudus *et al.* (2002) who noticed that callus of alfalfa was induced from stem explants on MS basal

medium supplemented with 2, 4- D and Kinetin. In contrast, Zabata *et al.* (2003) noticed that the combination of 2, 4- D and Kinetin gave no response for callus induction from leaf and root of turmeric (*Curcuma longa* L.) while the combination of 2, 4- D and BA showed a positive response for callus induction from stem. In our results the best medium for callus formation was 2, 4- D combined with Kinetin at 2 mg l⁻¹ and 1.5 mg l⁻¹ respectively. The same results were recorded by Moursey *et al.* (1999). However, in other studies the highest callus induction of alfalfa was obtained in MS medium supplemented with 2, 4- D at 2 mg l⁻¹ and Kinetin 0.5 mg l⁻¹ (Pushpalatha *et al.*, 2005; Kaur *et al.*, 2005; Huling *et al.*, 2006 and Lanas *et al.*, 2006). Moreover, Mofidabadi *et al.* (2001) noticed that 2 mg l⁻¹ 2, 4- D with 0.5 mg l⁻¹ Kinetin produced high percentage of callus induction of *Populus euphratica* oliv. and increasing of 2, 4- D from 2 mg l⁻¹ 2, 4- D to 3 mg l⁻¹ 2, 4- D didn't have any effect on percentage of callus initiation but reducing kinetin concentration from 0.5 mg l⁻¹ to 0.1 mg l⁻¹ with the same amount of 2, 4- D showed great effect on callus induction. On the other hand, Rui Yun *et al.* (2004) achieved callus induction from turmeric with 1 mg l⁻¹ 2, 4- D and 16 mg l⁻¹ Kinetin. Amoo and Ayisire (2005) noticed that the fact of callus was induced by 2, 4- D but not by NAA suggests that cotyledon explants of *P. biglobosa* are auxin specific. Zafar *et al.* (1995) also reported that callus induction from cotyledon, hypocotyls and root explants of *Medicago littoralis* in the presence of 2, 4- D alone and when it was replaced with NAA, these results were in agreement with Jeeva *et al.* (2005) who noticed that callus induction from leaf of gerbera cultivars on the treatment 2, 4- D (2 mg l⁻¹) with BA (1 mg l⁻¹) recorded the highest percentage of callus production, while the combination between BA with NAA showed the lowest percentage of callus production.

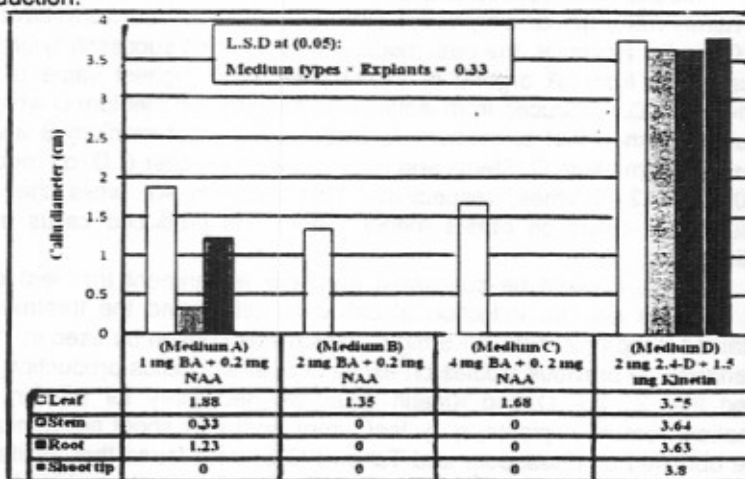


Figure 2: Effect of medium types on callus diameter.

2- Effect of different 2, 4- D concentrations with or without using kinetin of callus induction.

The present data showed that leaf was the best explants in producing fresh weight as compared to stem, root and shoot tip of *Medicago sativa* in vitro callus on both 1st and 2nd subculture. The 2nd subculture produced 3.52 times greater callus fresh weight than the 1st subculture. Thus, the leaf was chosen as the best organ for callus production in the following experiments (Table 3).

As for the media type effects on callus fresh weight, media T6 (2 mg l⁻¹ 2, 4- D + 1.5 mg l⁻¹ Kinetin) produced the highest mean value of F.W 6.15 g followed by the media T1 (1 mg 2, 4- D) with the mean value of 3.99 g F.W. Hence, the best medium for *Medicago sativa* callus production was 2 mg l⁻¹ 2, 4- D + 1.5 mg l⁻¹ kinetin. Jarszina *et al.* (2005) observed that the efficiency of callus production for *Cannabis sativa* L. obtained on MS supplemented with 1 mg l⁻¹ Kinetin + 0.5 mg l⁻¹ NAA, 2 mg l⁻¹ Kinetin + 0.5 mg l⁻¹ NAA or 2 mg l⁻¹ 2, 4- D was highly only for some explants source and cultivars. The highest induction callus was from leaves and petiole. Martin (2004) showed that 2, 4- D was the best for the callus formation of *Andrographis Paniculata* (Burm. F.), while the combination of 2, 4- D and Kinetin was superior. These results are also in agreement with Al- Bahrany and Al- Khayri (2002) who stated that the highest callus induction of "Hassawi" rice occurred on 2.5 mg l⁻¹ 2, 4- D alone; however, greatest callus weight was obtained on 1.5 mg l⁻¹ 2, 4- D combined with 2 mg l⁻¹ Kinetin. Kumar and Kanwar (2006, 2007) and Kumar *et al.* (2004) observed that the maximum callus induction and growth in petal explants of *Gerbera jamesonii* was obtained on MS medium supplemented with 1.5 and 2 mg l⁻¹ 2, 4- D in leaf and with 3 mg l⁻¹ BA in petiole explants. Tam and lang. (2003) found that MS medium with 2 mg l⁻¹ 2, 4- D is concenter as a medium suitable for callus induction of rice cultivars. Jain *et al.* (2005) reported that 4 mg l⁻¹ 2, 4- D was found to yield maximum callus induction of hybrid bermudagrass Tifton 85 with 88 (± 13 %) of the explants responding, followed by 66 (± 11 %) on 3 mg l⁻¹ 2, 4- D. Callusing response was lower by 73, 34, and 39 % on 1, 2 and 6 mg l⁻¹ 2, 4- D, respectively, as opposed to 4 mg l⁻¹ 2, 4- D. Ibrahim *et al.* (2007) observed that MS medium supplemented with 1 mg l⁻¹ 2, 4- D is quite suitable for callus induction from hypocotyl and cotyledon explants of Sunflower. In the contrast, Mohammad *et al.* (2003) mentioned that cytokinins like Kinetin and BA were used alone at 3 and 4 mg l⁻¹, respectively, good callus was observed on MS Medium containing BA at 4 mg l⁻¹. On the other hand, Hoque *et al.* (2007) who works on Water Chestnut (*Trapa* sp.) showed that no callus was induced in 2, 4- D containing media.

Table 3: Effect of different 2, 4- D concentrations with or without using kinetin of callus induction.

Subculture (A)	Explants (B)	Media (C)								Mean
		T1	T2	T3	T4	T5	T6	T7	T8	
1 st	Leaves	2.90	2.35	3.19	0.84	0.82	3.06	0.50	0.41	1.76
	Stems	1.22	0.90	1.07	0.35	1.23	2.84	0.22	0.26	1.01
	Roots	0.66	0.61	0.39	0.24	0.84	3.73	1.04	0.49	1.00
	Shoot tips	2.22	1.19	1.04	0.69	1.07	1.94	0.81	0.37	1.14
Mean		1.75	1.26	1.42	0.53	0.99	2.89	0.59	0.38	1.23
2 nd	Leaves	6.73	6.23	6.28	2.60	5.16	10.74	2.61	2.43	5.35
	Stems	3.97	2.05	3.76	1.33	8.32	9.76	1.08	2.65	4.12
	Roots	5.55	1.67	1.47	0.55	5.23	8.96	1.24	4.46	3.64
	Shoot tips	8.69	2.60	1.80	0.62	5.05	8.13	3.86	2.80	4.19
Mean		6.23	3.14	3.33	1.28	5.94	9.40	2.20	3.08	4.33
General mean of explants	Leaves	4.82	4.29	4.74	1.72	2.99	6.90	1.56	1.42	3.55
	Stems	2.60	1.47	2.42	0.84	4.77	6.30	0.65	1.46	2.56
	Roots	3.11	1.14	0.93	0.40	3.04	6.34	1.14	2.47	2.32
	Shoot tips	5.46	1.90	1.42	0.66	3.06	5.04	2.23	1.58	2.67
Mean		3.99	2.20	2.38	0.90	3.47	6.15	1.40	1.73	
L.S.D at 0.05 for	A		0.13							
	B		0.24							
	C		0.17							
	A×B		0.34							
	B×C		0.48							
	A×C		0.24							
	A×B×C		0.67							

3- Response of callus flavonoid content to different explants and subcultures.

a. Effect of subcultures and explant types on fresh weight of alfalfa culture

Data indicate that there were significant differences between explants and subcultures on fresh weight. Figure 3 showed that all explants callus was increased at 2nd sub culture compared with 1st subculture. Leaf fresh weight in the 1st subculture gave the highest value of C.F.W; then shoot tip; following by stem and finally root, while gave the lowest F.W of callus. On the other hand in the 2nd subculture leaf gave the highest weight of callus; then stem following by root and finally shoot tip which gave the lowest weight callus.

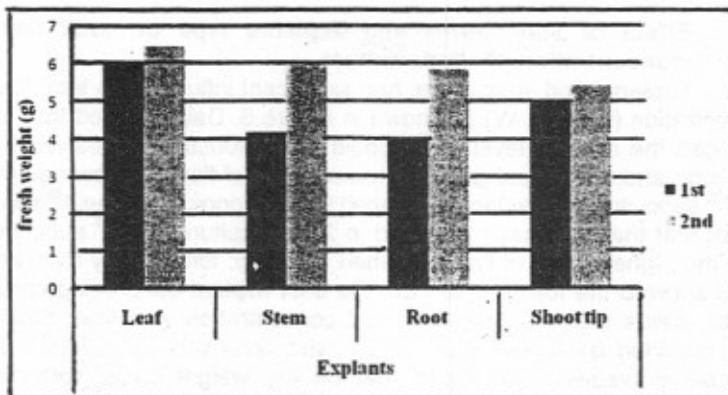


Figure 3: Effect of subcultures and explant types on fresh weight of alfalfa culture.

b- Effect of subcultures and explants type on callus diameter of alfalfa culture.

Data indicate that explants and subcultures had effect on callus diameter. Figure (4) showed that all explants callus diameter was increased at 2nd sub culture compared with 1st subculture. Shoot tip in the 1st subculture exhibited the highest C.D.; followed by root, then stem and finally leaf that gave the lowest C. D. of callus, while in the 2nd subculture leaf gave the highest value of callus; then shoot tip following by stem and root.

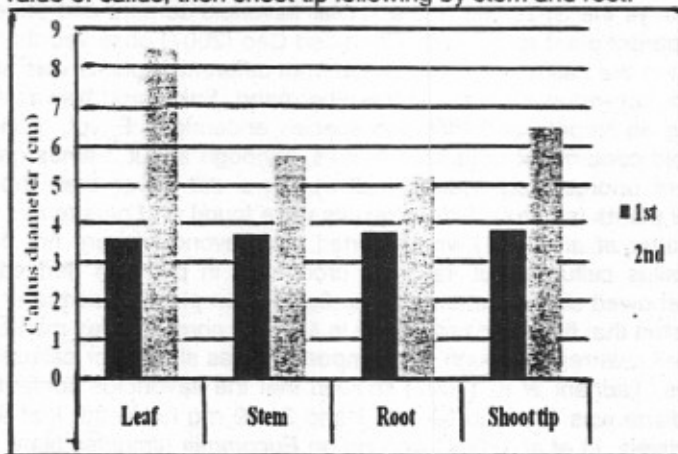


Figure 4: Effect of subcultures and explant types on fresh volume of alfalfa culture.

c. Effect of subcultures and explants type on total flavonoids concentration of alfalfa culture.

Explants and subcultures had significant influence on total flavonoids concentration ($\mu\text{g/g}$, F. W) as shown in Figure 6. Data showed that shoot tip produced the highest level of flavonoid in 1st subculture followed by stem; then root; and at last leaf gave the lowest level of flavonoid concentration. In the 2nd subculture all explants produced less flavonoid than the 1st subculture, except leaf that increased flavonoid in 2nd subculture. In 2nd subculture leaf gave the highest level of flavonoid then shoot tip; followed by root; and stem which showed the lowest level. So, the best explant concerning callus fresh weight, callus diameter and flavonoid concentration was leaf. Similar data were obtained by Cheel *et al.* (2007) who was working in vitro culture of *Sanicula graveolens* and found that on dry weight basis, total flavonoid content ranged from 1.23% to 2.23% and was lower for the root culture. Chen *et al.* (2006) found that the callus growth quantity in *Cyclocarya paliurus* [*Pterocarya* sp.] was higher in stem than that in the leaf, but the flavonoid content in the leaf was higher than that in the stem and the optimum plant growth regulator combination promoting the callus growth and flavonoid content accumulation was 1.0 mg l^{-1} kinetin + 0.5 mg l^{-1} 2, 4- D + 0.3 mg l^{-1} NAA. These results are in agreement with Yamamoto *et al.* (1986) who mentioned that the growth and flavonoid (baicalin, baicalein, wogonin and wogonin-7-0-glucuronide) content of the St-20 line of *Scutellaria baicalensis* callus were best on a medium containing 10-7 to 10-5M kinetin. After culture for 70 days the St-20 line had a similar flavonoid content and pattern to that of the parent plant roots. Also, Chen and Cao (2007) observed that flavonoid content in the callus of *Ginkgo biloba* from different explants was high in root > leaf > cotyledon > stem. On the other hand, Saker and Kawashity (1998), working on *Nepeta* and *Plantago* species endemic in Egypt, found that the flavonoid contents of organized tissues, although about 3-times greater than those of unorganized tissues (callus), were still lower than those of the original plants (control). Similar results were found on *Lotus tenuis* Waldst by Strittmatter *et al.* (1991) who reported that flavonoids were not detected in vitro callus cultures, but flavonoid production in plantlets derived from the callus showed similar patterns to the field-grown plants. Pasqua *et al.* (1991) suggested that flavonoid production in *Maclura pomifera* was markedly higher from cell cultures, although the composition was similar for calluses and cell cultures. Tadhani *et al.* (2007) showed that the flavonoids content in *Stevia rebaudiana* was found to be 21.73 and 31.99 mg / g in the leaf and callus, respectively. Li *et al.* (2004) working on *Eucommia ulmoides* plant found that flavonoid contents was highest in hypocotyl calluses. El-Kazzaz and Nazif (2000) working on mulberry plant found that growth regulators IBA and NAA seemed to play an important role in regulating the flavonoid content in callus or in vitro plantlets, with increasing concentrations in the growth medium increasing the flavonoid content. This effect was greater with NAA than with IBA.

In conclusion, the best media for callus weight and volume was 2 mg 2, 4- D + 1.5 mg Kinetin. Leaf produced the highest callus fresh weight on

this media. As for effect of 2, 4- D concentration, medium T6 produced the highest callus F.W. and volume in both 1st and 2nd subculture. Leaf was best organ for callus production.

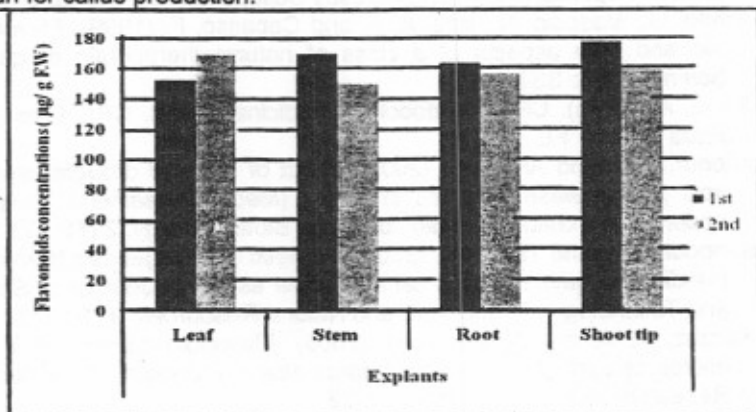


Figure 5: Effect of subcultures and explant types on total flavonoid concentration of alfalfa culture.

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تأثير منظمات النمو النباتية المختلفة على انتاج الكالس والفلافونويد من البرسيم الحجازى.

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أجريت ثلاث تجارب لدراسة تأثير منظمات النمو المختلفة مثل BA, NAA, 2,4-D, Kinetin على انتاج الكالس من البرسيم الحجازى ومعرفة محتوى الكالس الناتج من الأجزاء النباتية المختلفة للبرسيم الحجازى مثل القمة النامية، الأوراق، السيقان والجذور من الفلافونويد،

وقد أظهرت النتائج ما يلى:

- 1- أن المعاملة التى تحتوى على 2 مجم (2,4-D) بالإضافة إلى 1.0 مجم (Kinetin) أعطت أعلى نسبة فى انتاج الكالس من حيث وزن وقطر الكالس.
- 2- تعتبر الأوراق أفضل الأجزاء النباتية فى انتاج الكالس.
- 3- ال subculture الثانية أفضل من حيث الكالس الناتج وكذلك انتاج الفلافونويد.
- 4- كان تركيز الفلافونويد على فى القمة النامية خلال ال subculture الأولى ولكن أعطت الأوراق نسبة أعلى خلال ال subculture الثانية.