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Full Length Research Paper

Flavonoids production in *Hydrocotyle bonariensis* callus tissues

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Callus tissue of *Hydrocotyle bonariensis* was initiated from the leaf of *H. bonariensis* treated with 2 mg/L 2,4-D and 1 mg/L kinetin. The culture was kept at 25°C, under light (cool white fluorescent tubes, 1200 lux). The data obtained showed that the best medium for the highest flavonoids production was in DKW basal medium. Fructose as a carbon source increased the flavonoids content by 3%. Callus treated with 2 mg/L 2,4-D in comparison with the other auxins and among the cytokinins, kinetin and thidiazuron (TDZ) at 1 mg/L enhanced the flavonoid accumulation. Higher flavonoid production was obtained in combination with the 2 mg/L 2,4-D and 1 mg/L kinetin, while the pH value which was higher than 5.7 showed inhibitory effect on flavonoids production. It was found that the highest flavonoids were produced at 16 days of culture.

Key words: Flavonoids, cell culture, media composition.

INTRODUCTION

Hydrocotyle bonariensis Comm. ex Lam (Apiacae) locally known as *Pegaga Embun* is a perennial prostrate herb and is found mostly in tropical and subtropical regions of the world. *H. bonariensis* is valued for treating tuberculosis, relieving the pain of rheumatism and arthritis, to increase brain capacity and for longevity (Vimala et al., 2003). Flavonoids are a large family of secondary plant metabolites, and are present in plant tissues in relatively high concentration as sugar conjugates and are common in leaves, flowering tissue and pollens (Anderson and Markham, 2006). It has been reported that the leaves of this plant contain alkaloids, flavonoids, tannins, phenolic compounds and saponins as bioactive components (Ajani et al., 2009). Plant flavonoids are important in the diet because of their beneficial effects on human health (Gebhardt et al., 2005). In addition, they are also reported to act as better antioxidants than the classical ones (Narayan and Venkataraman, 2002). Tissue culture can be used in industrial production of secondary metabolites because of the limitation in natural production (Saxena, 2001). The manipulation of medium component has proven to be an important strategy for the improvement of secondary metabolites yield by callus and cell suspension cultures (Kittipongpatana et al., 1998). Production in cultured cell of plants could further be enhanced by many approaches including nutrient stress and elicitation (Rao and Ravishankar, 2002). In cultured *Nicotiana tabacum* (Taguchi et al., 2001), the growth plant regulators used in the medium greatly influenced secondary metabolite production.

In general, to obtain maximum yield of high flavonoids content, it is necessary to fine-tune the type and level of growth regulators required in the cultured medium. However, the influence of other culture conditions that may support the continuous production of biomass, as well as flavonoids, also need to be established (Luczkiewics and Glod, 2003). The main objective of this study was to optimize media composition to obtain the optimum callus growth and flavonoids production of *H. bonariensis.* In this work, the callus of *H. bonariensis* was established and grown in different media formulation to determine the individual and interaction effect of medium

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Table 1. Effects of different auxins on callus induction in leaf explants of *Hydrocotyle bonariensis* after 35 days of culture on MS medium supplemented with B5 vitamins at 25±2°C with photoperiod of 16 h light.

Concentration (Auxin 1 mg/L)	Days of callus formation	Callus induction (Cip %)	Callus morphology	Biomass growth after 35 days (g DW/culture)	Score
MS 0	-	0	-	0	0
2,4-D	28±2	90	Friable, pale yellow	0.10±0.01	3
Picloram	29±2	88	Friable, green	0.09±0.03	3
Dicamba	31±2	85	Friable, green	0.07±0.01	2
IBA	-	0	Root formation	0	0
NAA	-	0	Root formation	0	0

Callus scores: 0 = no callus formed, 1 = little callus formed, 2 = callus formed along the margin of leaf, 3 = callus formed covering the whole leaf. Cip: Callus induction percentage.

component, media strength, plant growth regulators, various carbon sources and different pH values on the growth of callus cultures and flavonoids production.

MATERIALS AND METHODS

Plant materials

H. bonariensis was collected from greenhouse at Universiti Putra Malaysia and the surface was disinfected using 70% (v/v) ethanol for 1 min, followed by 15% aqueous sodium hypochlorite solution for 20 min; then, it was rinsed three times in sterile distilled water.

Effect of plant growth regulator on callus induction and callus growth

Leaves of *H. bonariensis* were cut into small pieces (0.5 × 0.5 cm) and aseptically placed on MS medium (Murashige and Skoog, 1962) supplemented with B5 vitamins, gelrite (2.75% w/v), 3% (w/v) sucrose and with different concentrations of auxins (0 to 3 mg/L) and cytokinins (0 to 1.5 mg/L). The pH of the medium was adjusted to 5.7 with NaOH or HCl before autoclaving at 121°C for 15 min. The explants were incubated under 16 h photoperiod of 1200 lux at 25±2°C, until callus was induced. The callus tissues, formed from the leaf explants, were subcultured onto fresh medium every three weeks. Three different kinds of auxins (2,4-D, picloram and NAA) at the range of 1 to 4 mg/L (w/v) and cytokinins, kinetin, benzylaminopurin (BAP) and thidiazuron (TDZ) were studied at the range of 1 to 3 mg/L. The best auxin and cytokinin combination were tested at the optimal concentration.

Effect of media composition and media strength

Growth and production of flavonoids from callus tissues were measured in different media including MS (Murashige and Skoog, 1962), B5 (Gamborg et al., 1968), SH (Schenk and Hildebrandt, 1972), DKW (Driver and Kuniyuki, 1998) and NN (Nitsch and Nitsch, 1969), supplemented with 3% of sucrose, 2 mg/L of 2,4-D and 1 mg/L kinetin.

Effect of carbon sources

Different carbon sources (sucrose, fructose, glucose, galactose, mannitol and sorbitol), each at 3 and 6% also were added to the medium to determine its respective flavonoid production. The selected carbon source was individually tested at a concentration ranging from 1 to 7%.

Effect of pH value

To optimize the medium pH for flavonoids accumulation, the pH of the media was adjusted at the range of 4.5 to 7 (prior to autoclaving), using either NaOH or HCL.

Extraction and determination of flavonoids

Extraction of flavonoids from the dried callus, were performed as described by Jia et al. (1999). Quantitative determination of flavonoids was achieved with $AlNO_3$ reagent as described by Zhang et al. (1992) and Liu et al. (2002). The absorbance was noticed at 510 nm using quercetin at the standard. Each treatment was replicated three times.

Statistical analysis

The experiments were independently repeated three times under the same conditions and concentrations and all analyses were performed in triplicate. Results are expressed as the mg/g DW for flavonoid accumulation in treated *in vitro* cultures when compared to untreated samples. Graph showing the flavonoid accumulation was plotted using Microsoft® Excel. Error bars of graphs show the standard error of mean value (±S.E.M.). The data were analyzed using one-way ANOVA followed by Duncan's multiple range test for mean comparison at P = 0.05.

RESULTS AND DISCUSSION

Effects of hormone on callus induction

Tables 1, 2 and 3 show the response of *H. bonariensis* leaf explants callus formation. Callus was formed within five weeks of culture in treatments containing 2,4-D which was supplied individually or in combination with kinetin or BAP. Table 1 and Figure 1 showed the response of callus formation on MS basal medium containing 1 mg/L concentration of auxins supplemented individually (2,4-D,

Table 2. Effect of various concentrations of 2,4-D on callus induction in leaf explants of *Hydrocotyle bonariensis* after 35 days of culture on MS medium supplemented with B5 vitamin at 25±2°C with photoperiod of 16 h light.

Concentration (mg/L)	Days of callus formation	Callus induction (Cip %)	Callus morphology	Biomass growth after 35 days (g DW/culture)	Score
0	-	0	-	0	0
0.5	30±2	90	Friable, pale yellow	0.10±0.01	2
1	27±2	85	Friable, pale yellow	0.11±0.02	2
1.5	24±2	75	Friable, pale yellow	0.12±0.04	3
2	21±2	88	Friable, pale yellow	0.14±0.01	3
2.5	22±2	85	Friable, pale yellow	0.11±0.01	2
3	25±2	84	Friable, pale yellow	0.09±0.03	2

Callus scores: 0 = no callus formed, 1 = little callus formed, 2 = callus formed along the margin of leaf, 3 = callus formed covering the whole leaf. Cip: Callus induction percentage.

Table 3. Effect of 2 mg/ L 2,4-D in combination with kinetin and BAP on callus induction in leaf explants of *Hydrocotyle bonariensis* after 35 days of culture on MS medium supplemented with B5 vitamins at 25±2°C with photoperiod of 16 h light.

Concentration (mg/L)	Days of callus formation	Callus induction (Cip %)	Callus morphology	Biomass growth after 35 days (g DW/culture)	Score
2 (2,4-D)	21±2	88	Friable, pale yellow	0.14±0.01	3
0.5 (Kin)	19±2	95	Friable, yellow	0.13±0.04	2
1 (Kin)	16±2	98	Friable, yellow	0.15±0.01	3
1.5 (Kin)	18±2	96	Friable, yellow	0.14±0.01	3
0.5 (BAP)	20±2	85	Friable, pale yellow	0.12±0.02	2
1 (BAP)	17±2	91	Friable, pale yellow	0.12±0.03	3
1.5 (BAP)	19±2	87	Friable, pale yellow	0.13±0.03	2

Callus scores: 0 = no callus formed, 1 = little callus formed, 2 = callus formed along the margin of leaf, 3 = callus formed covering the whole leaf. Cip: Callus induction percentage.

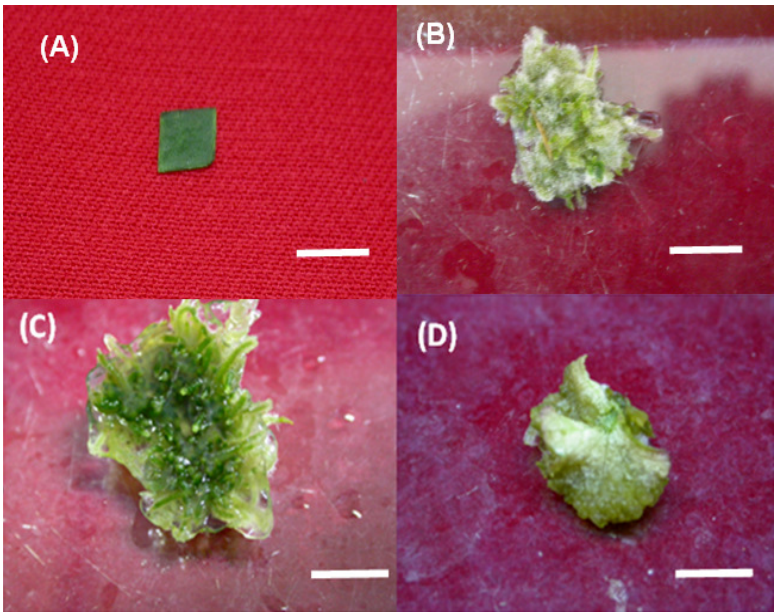

Figure 1. Response of *H. bonariensis* leaf explant on MS medium supplemented with B5 vitamins and 3% sucrose. A: Leaf segmented; B: 1 mg/L IBA; C: 1 mg/L NAA; D: 1 mg/L picloram. The bar is 5 mm.

NAA, IBA, picloram and dicamba). Among the different auxins tested, 1 mg/L of 2,4-D was found to give the highest callus induction percentage (90%) when compared to other auxins. The result showed that leaf explant did not produce callus on MS medium containing 1 mg/L of IBA and NAA. In terms of callus formation period, 1 mg/L of 2,4-D only needed 28 ± 2 days for callus induction and achieved a dry weight of 0.1 g DW/culture after 35 days of culture. Martin (2004) also reported that callus from *Centella asiatica* was successfully induced with 2,4-D. The presence of 2,4-D at concentrations 0.5 to 1.5 mg/L in the culture medium resulted in callus induction from *Z. officinale* explant (Ma and Gang, 2006). According to Shohael et al. (2008), 1 mg/L of 2,4-D was sufficient to induce and maintain the callus from *E. sessiliflorus*.

Although, callus was successfully obtained from picloram and dicamba treatments, it required a longer period for induction. According to Kiong et al. (2008), 4 mg/L picloram induced callus from *Cycas revolute*. The callus initiated in 2,4-D was pale yellow, while picloram and dicamba treatment was a green color callus. The highest callus growth gained was only 0.09 and 0.07 mg DW /culture after 35 days of culture and was obtained in the media containing 1 mg/L of picloram and dicamba, respectively. In the present study, 2,4-D was more effective for callus induction than NAA and IBA, which enhanced callus induction and growth. This was in agreement with Wu's opinion, who reported that 2,4-D is usually more efficient than other phytohormones in the induction of callus from plant explants (Wu et al., 2003). Callus from *Gymnema sylvestre* had been successfully induced when supplemented with 0.5 mg/L 2,4-D (Gopi and Vatsala, 2006).

The leaf explants started to swell in all treatments containing 2,4-D after 14 days. The callus induction occurred after 28 days of incubation where the callus arose from the entire surface of the explants (Table 2). In terms of the period needed for callus formation, as well as the growth, 2 mg/L of 2,4-D only needed 21 ± 2 days for callus induction and achieved a dry weight of 0.14 g DW/culture after five weeks, whereas 0.5 mg/L of 2,4-D only formed callus after 30 ± 2 days with 0.1 g DW /culture after five weeks. This study revealed that a 2,4-D concentration of 0.5 mg/L was found to be inadequate for the optimal callus induction from young leaf explants of *H. bonariensis*, whilst concentrations higher than 2 mg/L showed an inhibitory effect on callus induction. The callus which is obtained in the medium containing different concentrations of 2,4-D appeared pale yellow in color and it has friable texture.

It is reported that callus was successfully induced from leaf explants of *E. sessiliflorus* cultured on Murashige and Skoog (MS) basal medium supplemented with 1 mg/L 2,4-D (Shohael et al., 2005). A combination of 2,4-D and NAA in *M. malabathricum* callus induction indicated that 0.5 mg/L NAA or 2 mg/L 2,4-D alone are sufficient in inducing callus. Generally, most herbaceous plants require only one type of callus induction from explant (Chan et al., 2008).

The addition of kinetin significantly increase callus weight in treatments containing 2,4-D, which means kinetin could promote the growth of the callus tissue. With the addition of kinetin to the 2,4-D medium, a shorter period of 16 to 19 days was required for callus induction (Table 3). Differences in physical appearance of callus were also detected, and it was observed that the callus initiated in the (2 mg/L 2,4-D + 0.5 mg/L kinetin) medium was yellow. All the treatments gave friable callus and no organogenesis was observed. In fact, the addition of kinetin (up to 1.5 mg/L) to the medium containing 2 mg/L 2,4-D leads to the production of callus which is friable.

The combination of BAP with 2,4-D was described in Table 3. The result showed that MS containing 2 mg/L of 2,4-D with 1 mg/L BAP combination produce high capacity of callus when compared to all media containing different concentrations of 2,4-D and BAP. Therefore, it could be concluded that a combination of 2,4-D with BAP or kinetin is suitable for induction of callus. Combination of 0.5 mg/L 2,4-D and 0.05 mg/L kinetin was used to induce callus form *Arabidopsis thaliana* in Gamborg's B5 basal medium (Kai et al., 2006). According to Trejo-Tapia et al. (2008), callus was induced from *Beta vulgaris*, supplemented with 0.1 mg/L kinetin and 0.5 mg/L 2,4-D adequate for hypocotyls, whereas 0.2 mg/L kinetin and 1 mg/L 2,4-D was adequate for leaf explant. Callus cultures of *Rudbeckia hirta* were initiated by placing sterile cotyledon sections (a total of 2 mm in length) on Schenk and Hildebrandt (SH) basal medium supplemented with 5 mg/L 2,4-D, and 5 mg/L kinetin (Luczkiewicz et al., 2002). The aforementioned references supported the fact that the combination of 2,4-D and kinetin was best suitable for callus induction in different types of plants, and in *H. bonariensis* as well.

Effect of plant growth hormones

The type and concentration of auxin and cytokinin, either alone or in combination, has been known to strongly influence growth as well as the secondary metabolites in tissue culture. The cell line in the present study was first tested for short- term effect of different auxins, and their levels on flavonoids production are presented in Figure 2. Generally, flavonoids production in the present study is not growth- associated. All growth regulars tested have shown a steady enhancement of biomass up to a certain level of each auxin and further, they have shown a higher level of suppressed biomass and flavonoids content in each set of treatment. The productivity varied with a maximum of flavonoids (8.29 mg/g DW) in the presence of 2,4-D medium followed by picloram (7.77 mg/g DW) of flavonoids with a further low productivity under the influence of NAA. It should be noted that while the

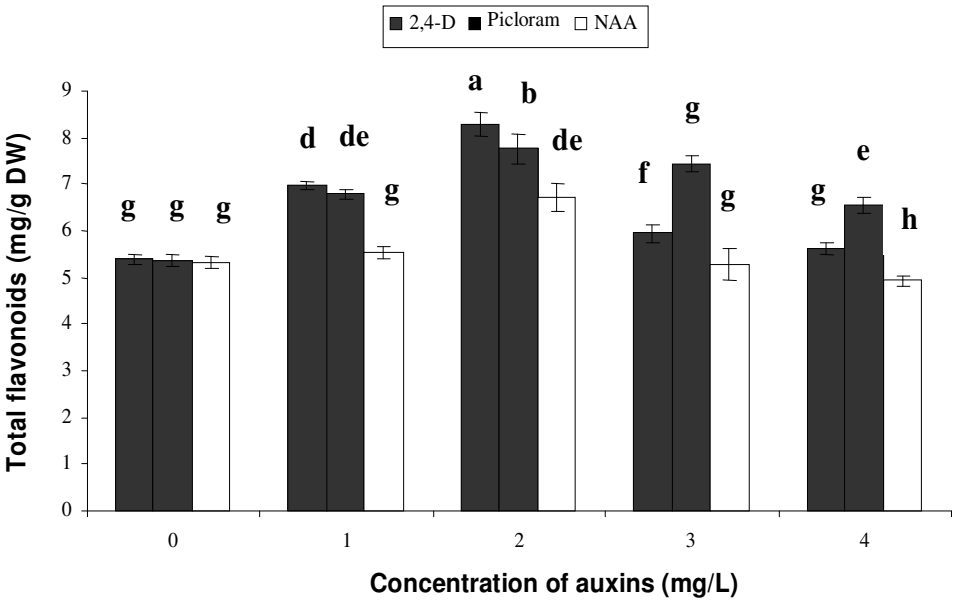

Figure 2. Effects of the different auxins at various concentrations on the total flavonoids accumulation of *H. bonariensis* callus culture. Values are mean ± S.E.M. of three experiments. Means followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

flavonoids content decreased with increase of 2,4-D, the higher level of biomass output accounted for higher flavonoids productivity. Similar results have been recorded for other secondary metabolites (Whitmer et al., 1998). It should be noted that the control medium in the present study (without any auxin) itself, supported a fair amount of callus growth that is most likely due to a carry over effect of 2,4-D, whereas transferring it to the NAA medium drastically supported further formation of flavonoids even though biomass accumulation was enhanced. In order to evaluate the effect of 2,4-D on pigment composition, the *Ipomoea batas* suspension culture was exposed to 2,4-D levels of 0 to 4 mg/L for a period of two weeks. With an increase in the auxin level, a gradual decrease of the relative concentration of anthocyanin has been monitored, from 29 to 14.1% at 0 and 4 mg/L 2,4-D, respectively (Konczaka et al., 2005).

The influence of three cytokinins, benzylaminopurin (BAP), kinetin and thidiazuron (TDZ) were tested. While 3 mg/L BAP showed the lowest level of (5.34 mg/g DW) flavonoids, 1 mg/L of TDZ and kinetin showed a very high productivity of flavonoids (Figure 3), and 1 mg/L kinetin showed a beneficial effect for biomass, as well as flavonoids production. Comparison between NAA and IBA in *Artemisia absinthium* revealed that 2 mg/L NAA promoted artemisinin production in callus culture (Zia et al., 2007).

Thus, 2,4-D showed a maximum beneficial productivity of flavonoids among auxin and kinetin, together with cytokinin. The variation in this combination was re-assessed in the culture, where the interaction of 2,4-D and kinetin at varying concentration was tested. The result presented in Figure 4 showed that the combination of 2 mg/L 2,4-D and 0.5 mg/L kinetin was best for biomass accumulation, but the most suitable combination for flavonoids production was 2 mg/L 2,4-D:1 mg/L kinetin. Addition of 2,4-D and kinetin into the media was also found to enhance the flavonoids production in *Genista tinctoria* (Luczkiewicz and Glod, 2003). According to Maurmann et al. (2006), the presence of kinetin in combination with 2,4-D was beneficial to valeportiate accumulation in *Valeriana gelechomifolia* callus culture.

Effect of media composition and media strength

A stable and high yield cell line is an important component for scaling up a process. The pattern of flavonoids production (Figure 5) was tested on different basal media with 2,4-D (2 mg/l) and kinetin (1 mg/l) in the medium (Figure 6). Macro and micronutrients have been reported to have considerable influence on growth and secondary metabolite synthesis in cultured plant cell (Luczkiewics and Glod, 2003). In most cases, increased level of nitrate, potassium, ammonium and phosphate tend to support rapid cell growth, whereas the depletion (from normal level) of some of the nutrient lead to growth limitation with a concomitant enhancement of secondary metabolite (Narayan et al., 2005). The result showed that

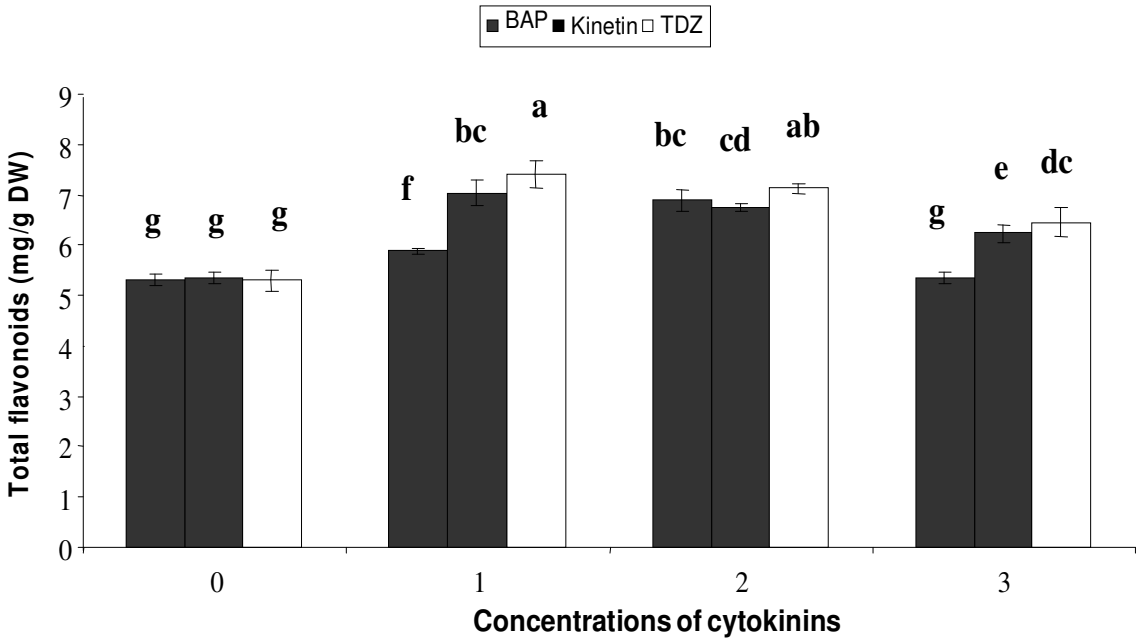

Figure 3. Effects of the different cytokinins at various concentrations on the total flavonoids accumulation of *H. bonariensis* callus culture. Values are mean ± S.E.M. of three experiments. Means followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

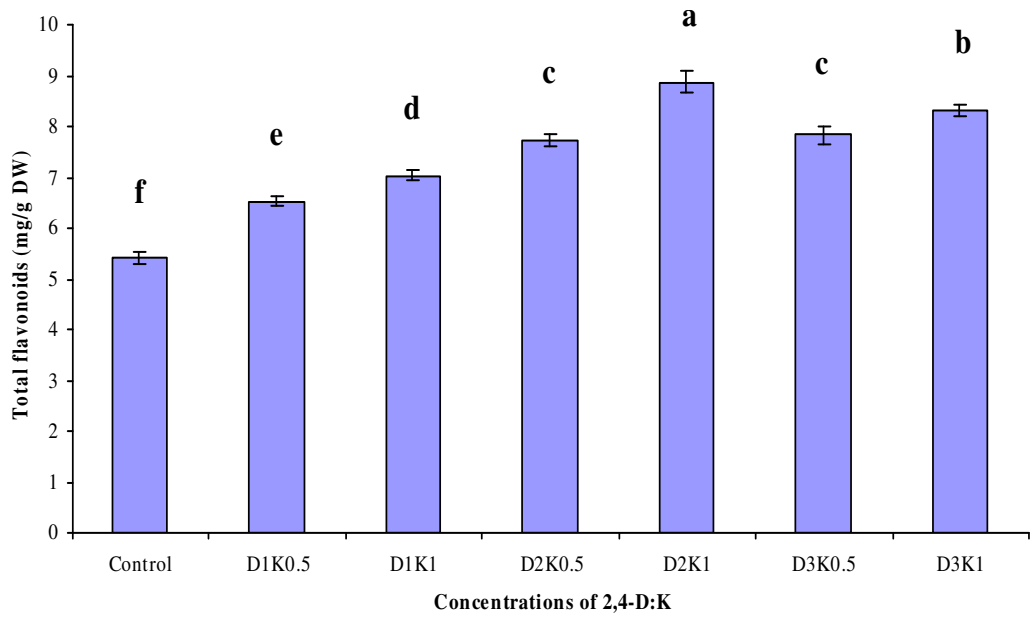

Figure 4. Effects of 2,4-D (D) and kinetin (K) combination at various concentrations on the total flavonoids accumulation of *H. bonariensis* callus culture. Values are mean ± S.E.M. of three experiments. Means followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

the effect of different media formulation on flavonoids production in callus culture was significantly different. Maximum flavonoids synthesis (8.32 mg/g DW) was observed in DKW and NN medium, respectively. In other media, there was no significant difference in flavonoids production. The DKW medium was used successfully for increased secondary metabolite production. Effectiveness of DKW in promoting secondary metabolite

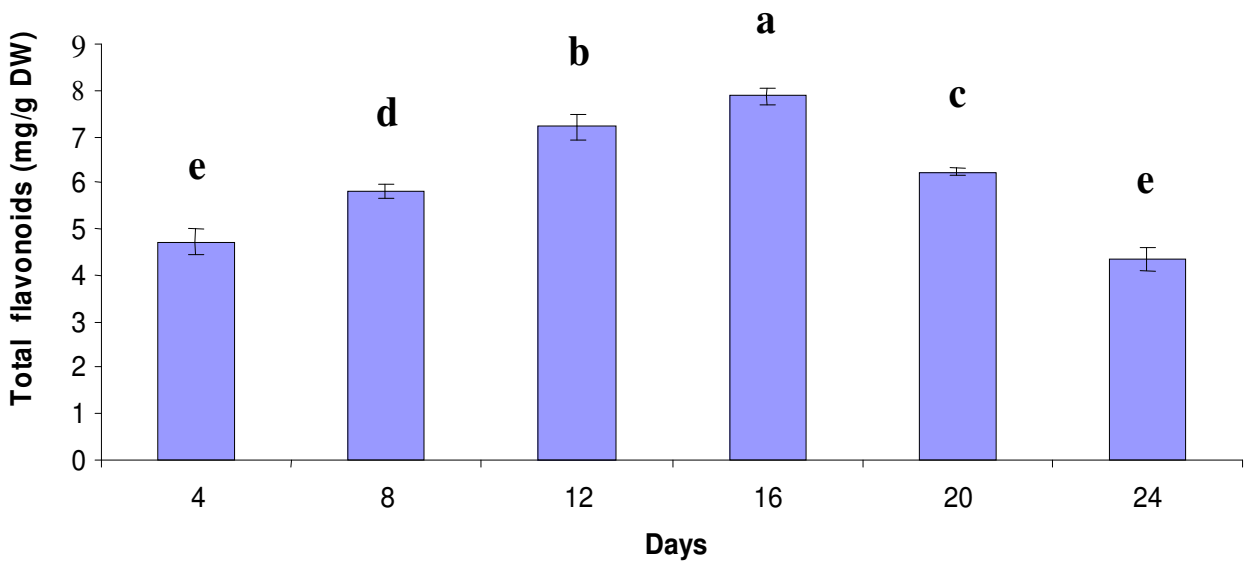

Figure 5. The total flavonoids accumulation of *H. bonariensis* callus culture on basal DKW in different days. Values are mean ± S.E.M. of three experiments. Means followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

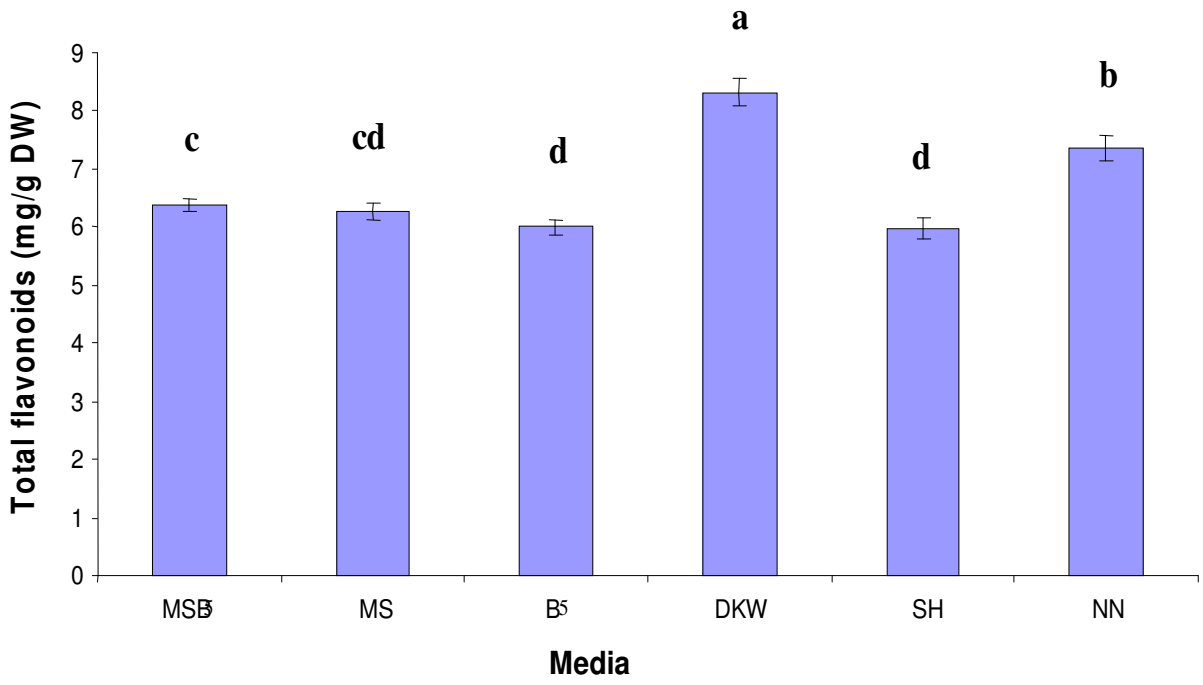

Figure 6. Effects of the different basal media on the total flavonoids accumulation of *H. bonariensis* callus culture. Values are mean ± S.E.M. of three experiments. Means followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test. Note: The MSB5: MS medium is supplemented with B5 vitamins.

could be partly due to its high phosphate concentration compared to that of MS and NN media (Rao and Ravishankar, 2002). Full strength of the DKW medium, was the most suitable for flavonoids production (Figure 7). However, a low production of flavonoids was obtained with increased strength of DKW. Even though in half strength DKW medium callus, growth was lower than other media strength, the flavonoids production was high.

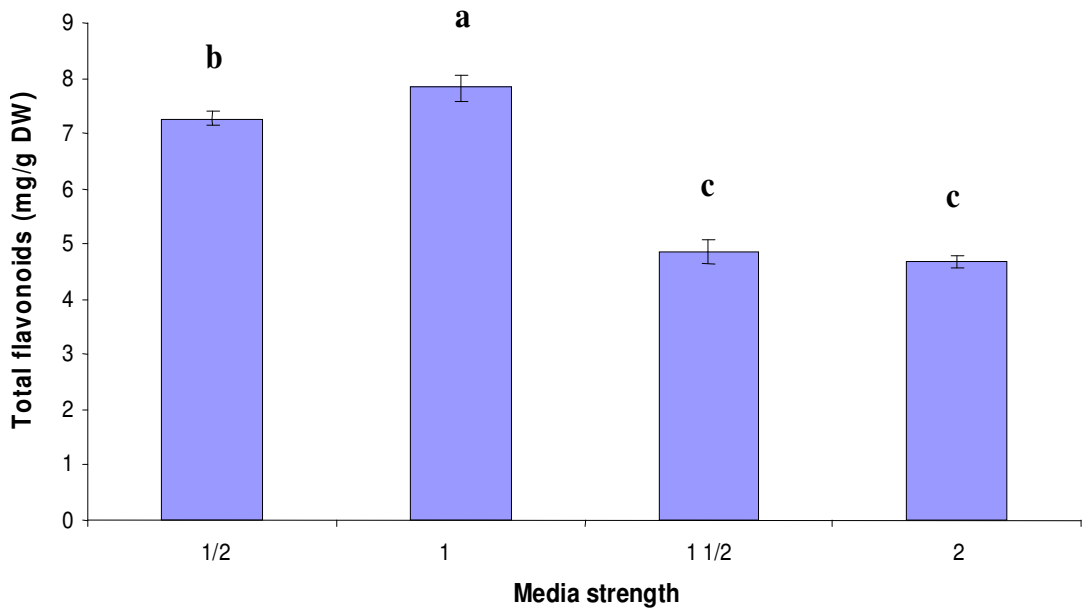

Figure 7. Effects of the DKW media strength on the total flavonoids accumulation of *H. bonariensis* callus culture. Values are mean ± S.E.M. of three experiments. Means followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

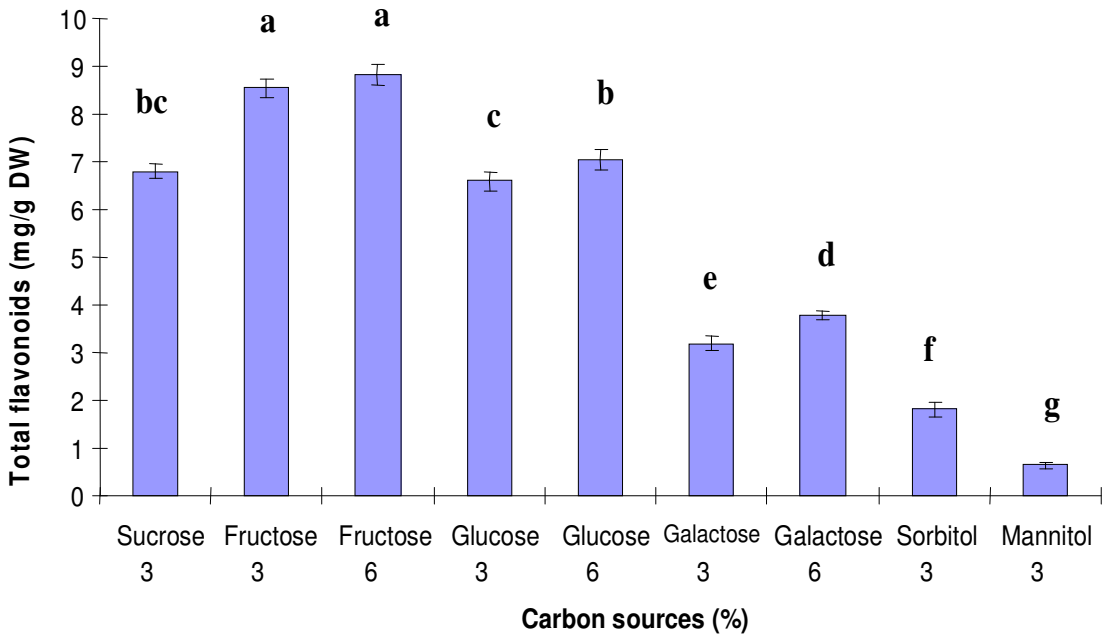

Figure 8. Effects of the different carbon sources on the total flavonoids accumulation of *H. bonariensis* callus culture. Values are mean ± S.E.M of three experiments. Means followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

In addition, full strength of the MS medium increased biomass production and antioxidant activity of *Origanum vulgare* shoot culture when compared with half strength (Lattanzio et al., 2009).

Effect of carbon source and fructose concentration

Figure 8 shows the flavonoids production pattern following the use of different carbohydrates as carbon

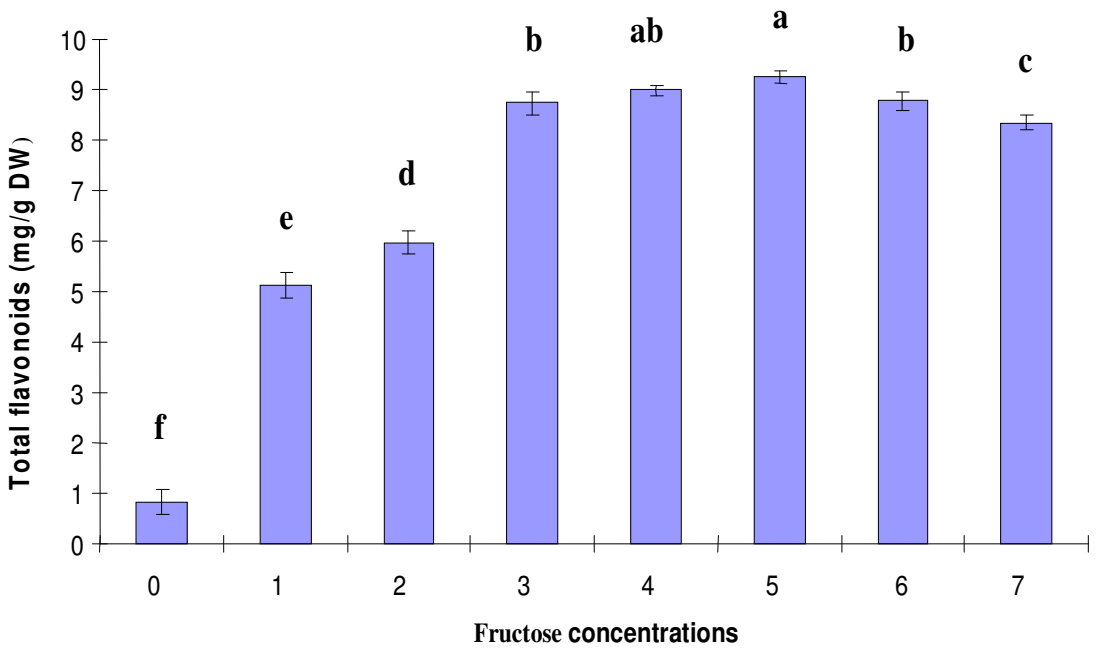

Figure 9. Effects of the different fructose concentrations on the total flavonoids accumulation of *H. bonariensis* callus culture. Values are mean ± S.E.M. of three experiments. Means followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

source. The maximum value of flavonoids was obtained in treated media containing 3 and 6% fructose. Manitol produced the lowest flavonoids when compared with other carbon sources. For fructose, glucose and galactose, the flavonoids production values increased when the sugar concentration was increased from 3 to 6%. Fructose can increase the production of other secondary metabolites (Oksman-Caldentey et al., 1994). Thus, 60 g/L glucose was the best carbon source in order of biomass and podophyllotoxin production in *Podophyllum hexandrum* (Chattopadhyay et al., 2003). Figure 9 shows the effect of fructose concentration on the production of flavonoids by *H. bonariensis* calli grown on DKW medium supplemented with 2 mg/L 2,4-D and 1 mg/L kinetin. The increase in fructose concentration, increased flavonoids production, but the flavonoids content has no significant difference between 3 and 7% fructose. The result indicates that the increase of fructose is ineffective, due to the fact that the main concern of the study is an increase in the overall production of flavonoids by *in-vitro* culture. Limited in signification, flavonoid accumulation at higher fructose concentration could be a result of higher osmotic strength that probably affects the water content of the cells negatively. Both biomass and flavonoids yields declined with the increasing osmotic stress in the medium (Kim et al., 2001). In addition, Luo and He (2004) reported that optimum concentration for taxol production in *Taxus chinensis* was 20 g/L sucrose. The culture growth of *Azadirachta indica* decreased 3.5 times with increasing glucose concentrations from 30 to 120 g/L (Prakash and Srivastava, 2006).

Effect of pH value

Medium pH is extremely important as it influences the uptake of nutrient and plant growth regulator by regulating their solubility in the culture medium (Bhatia and Ashwath, 2005). It also regulates a wide range of biochemical reaction occurring in plant tissue. A pH higher than 6 gave a fairly hard medium, while a pH found below 5 did not allow satisfactory gelling.

The medium pH had no statistically significant effect on biomass and flavonoids production; however, a trend was seen such that the better flavonoids production occurred at the acidic pH rather than at the alkaline pH (Figure 10). The optimal pH was 5.7 at 8.24 mg/g DW flavonoids production. Statistically, no significant effect of pH was observed for flavonoids and growth of calli at pH 5.7 to 7. These results suggested that growth of the cells in a wide range of pH may be due to the high buffering capacity of the cells to adjust their intracellular pH condition or to adapt to their extracellular pH within the tested range. Ouyang et al. (2002) also reported that pH 5.8 enhanced flavonoid production in the transformed hairy root culture of *Glycyrrhiza uralensis*. The initial medium pH range of 5.0 to 6.0 was best for promoting the growth of *Echinacea angustifolia* roots and their accumulation of phenols and flavonoids, but growth was inhibited when

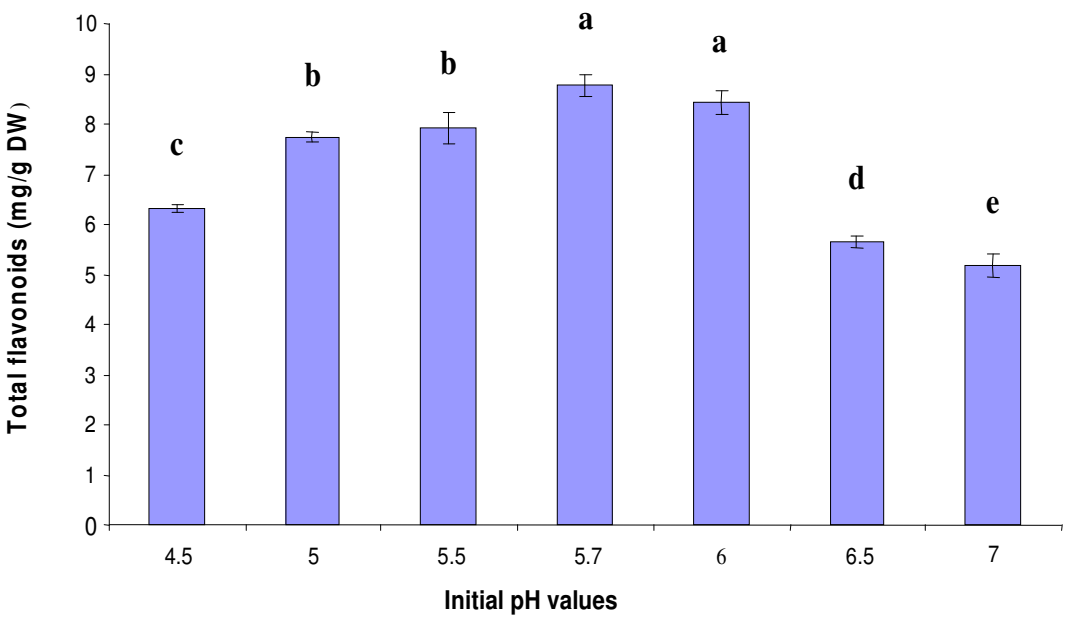

Figure 10. Effects of the different initial pH values on the total flavonoids accumulation of *H. bonariensis* callus culture. Values are mean ± S.E.M. of three experiments. Means followed by the same letter are not significantly different at P = 0.05 according to Duncan's multiple range test.

the initial pH was maintained either below 5.0 or above 6.0 (Wu et al., 2006).

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