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Production of Flavonoids and Phenolic Compounds by Elicitation of *Iphiona mucronata* (Forssk.) Asch. & Schweinf (Asteraceae) Callus and Suspension Cultures

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ABSTRACT

Callus and suspension cultures of *Iphiona mucronata* grown on Murashige and Skoog medium, supplemented with 0.1 mg/l kinetin (Kn), 0.1 mg/l naphthalene acetic acid (NAA) and 5 mg/l ascorbic acid were elicited with different elicitors. Total flavonoids and phenolics were determined by aluminum chloride - potassium acetate and Folin-Ciocalteu colorimetric methods respectively. When callus and suspension cultures elicited with 10 mg/l yeast extract (Y), it showed an increase of 2.8 and 3.7 folds for flavonoids production respectively**.** When callus cultures treated with 0.001 mM phenyl alanine (Ph), a slight increase in flavonoids content reached 1.17 folds, while treated suspension reached maximum level after 60 hours. Methyl jasmonate (MJ; 150 µM) increased flavonoids concentration 4 times for callus and 2.3 times for suspension. Determination of phenolics for elicited suspension showed an increase of 2.3 (Y), 2.5 (Ph) and 1.3 (MJ) times. The obtained results favored the use of yeast extract for high productivity and viability of cell lines.

Keywords- Callus and suspension cultures, elicitors, flavonoids, *Iphiona mucronata*, methyl jasmonate, phenolics, yeast.

1. INTRODUCTION

Flavonoids are of great economic functions as potential drugs, food nutraceuticals and industrial materials. Although their importance is manifested by the larger demands, their low yields in natural plants and the remarkable complexity for chemical synthesis lead to shortage of supply. So, an alternative and effective strategy is focused on elicitation treatment, which furthermore is considered as a useful experimental system and a promising production mode for the active constituents from medicinal plants [1]. A review for *in vitro* production of flavonoids is recently published [2]. Application of different strategies *in vitro* culture as media manipulation, phytohormone regulation, precursor feeding, plant cell immobilization, biotransformation and genetically modified cell can be used to obtain good yield of secondary metabolites in tissue culture. Elicitation of tissue culture was found to be more economic [3].

Biotic and abiotic elicitors can enhance secondary metabolites production. The stimuli are perceived by receptors activating secondary messengers. These transmit signals into the cell through signal transduction pathways leading to gene expression and biochemical changes resulting in secondary metabolites formation [4, 5]. The basis for successful elicitation of secondary metabolites is the choice of suitable elicitor, its concentration, and optimal time of treatment. Methyl jasmonate activates a multitude of jasmonate induced proteins which are associated with accumulation of secondary metabolites [3]. Methyl jasmonate [6], yeast [7] and phenyl alanine [8,9] have been used for flavonoids and phenolic elicitation in family Asteraceae, however no records for elicitation have been performed in genus Iphiona.

Iphiona mucronata **(**Forssk**)** Asch. & Schweinf [Astraceae (Compositae)] is an endangered plant growing in Egyptian deserts containing polysulphated flavonoids as major constituents [10]. In a previous study [11], we reported the best hormonal combination for induction and maintenance of callus culture of *I. mucronata* by using Murashige and Skoog (MS) medium [12], to get the highest growth index (GI) and flavonoids contents compared with other tested phytohormones. Moreover, we reported a protocol for somatic embryogenesis and plant regeneration from callus and suspension cultures of *I. mucronata* [13]. In the current study, yeast, methyl jasmonate and phenyl alanine were tried in different concentrations to increase the productivity of total flavonoids and phenolic compounds and vitality of callus and suspension cultures.

2. EXPERIMENTAL

2.1 Plant material

Aerial parts and seeds of *Iphiona mucronata* (Forssk.) Asch. & Schweinf family Asteraceae (Compositae) were collected from Wadi Hogool area and Al-Sokhna-Katamia road, Egypt. The plant was identified by Dr. A. Mary, Faculty of Science, El Azhar University. A voucher sample (AR-2007) was kept in Pharmacognosy Department, Faculty of Pharmacy, MSA University, Egypt.

2.2 Elicitation of callus culture

Callus was induced using seedling explant grown on MS medium supplemented with 0.1 mg /l NAA, 0.1 mg /l Kn, 5 mg /l ascorbic acid, 30 g/l sucrose and solidified with 10 g/l agar. Media were adjusted to pH 5.8 using 1 N NaOH or 1 N HCl, autoclaved at 121 °C for 20 min and incubated at 25 °C in the dark as previously reported [11]. Known weight of well grown, friable callus (3 g) was sub-cultured to fresh media supported with elicitors. Fresh and dry weights in addition to flavonoids contents were estimated at zero time, day 5, 21 and 31. The used elicitors were dried yeast, methyl jasmonate in addition to a feeding precursor (phenyl alanine) [6], [14], [15]. All results were recorded in triplicate.

Yeast (Y): Different concentrations of yeast Y1 **(**10 mg/l), Y2 (20 mg/l), Y3 (50 mg/l) and Y4 (100 mg/l) were added at zero time to callus cultures. Control media was prepared by substituting the yeast extract with distilled water. Fresh and dry weights of callus elicited with yeast were recorded to determine the effect of yeast on growth.

Phenyl alanine (Ph): It was used in different concentrations; Ph1 (1mM), Ph2 (0.01mM), Ph3 (0.05mM) and Ph4 (0.001mM). Control media was prepared by substituting phenyl alanine with distilled water.

Methyl jasmonate (MJ): MJ1 (50 µg/l), MJ2 (100 µg/l), MJ3 (150 µg/l), MJ4 (200 µg/l), MJ5 (300 µg/l) and MJ6 (400 µg/l) in 95% ethanol were used. Control was made using ethanol 95% instead of methyl jasmonate and also compared with normal blank with distilled water to explore ethanol effect.

2.3 Cell suspension culture and growth parameters

Well grown, friable callus (4-5 g) was planted aseptically to 50 ml of liquid MS medium (250 ml flask) supplemented with 0.1 mg/l Kn, 0.1 mg/l NAA, 5 mg/l ascorbic acid and 3% sucrose. The pH of the media was adjusted to 5.8±0.1 prior to autoclaving. Cultures were kept in an incubator shaker at 100 rpm/min, at 25°C in the dark. The suspension was cultured into fresh medium every two weeks. Different growth parameters were estimated. Growth curves for fresh and dry weight were monitored [16]. Growth index (GI), specific growth rate (µ) and doubling time (dt) were calculated as follow [17]:

GI = (Ge - Gstart)/ Gstart; where Ge = suspension mass at the end of each generation (final fresh weight). Gstart = suspension biomass at zero time (Initial fresh weight).

$\mu = (\ln x - \ln x_o)/t$; where x_o is the initial fresh biomass (or cell density), x is the biomass (or cell density) at time t, and µ is the specific growth rate.

Doubling time (dt) = ln (2)/ µ.

2.4 Elicitation of suspension culture

Successful concentrations of elicitors obtained from callus elicitation were added at 9th day aseptically to suspension culture. Estimation of flavonoids and phenolic contents was performed at different intervals and compared with control. The control samples received equal volumes of sterile water and ethanol 95% in case of MJ.

2.5 Determination of flavonoids contents

One gram of dried callus or suspension biomass was refluxed with 25 ml of 95% hot ethanol for 10 min then left overnight, filtered and adjusted with 80% ethanol (v/v) to 25 ml. 0.5 ml of each sample was mixed with 1.5 ml 95% ethanol (v/v), 0.1 ml 10% aluminium chloride (w/v), 0.1 ml 1 M potassium acetate and 2.8 ml water. Aluminium chloride was substituted by the same volume of distilled water in blank. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 415 nm [18]. For preparation of calibration curve, standard solutions of 12.5, 25, 50, 80 and 100 µg/ml rutin were dissolved in 80% ethanol (v/v). 0.5 ml of each concentration was separately treated as mentioned above. Calibration curve was plotted representing the correlation between absorbance and rutin concentration.

2.6 Determination of phenolic contents

One gram of dried suspension biomass was extracted with 25 ml methanol. 0.5 ml of each sample was added to 0.5 ml of water, 5 mL of 0.2 N Folin-Ciocalteu reagent (S.d fine-chem limited), and 4 mL of saturated sodium carbonate solution (75 g/l).The absorbance was measured at 765 nm with a Shimadzu UV-visible spectrophotometer after incubation for 2 h at room temperature [19]. Different concentrations (100, 200, 300, and 400 mg/l) of gallic acid were prepared for production of calibration curve. Absorbance was plotted against each concentration used. Concentration of phenolic content was expressed as gallic acid equivalent.

3. RESULTS

3.1 Elicitation of callus culture

Yeast: As yeast concentration increased, fresh and dry weight decreased (Figure 1). Y1 (10 mg /l) showed the highest fresh and dry weight and kept the vitality even after 31 days while control became brownish even with more biomass. Y1 resulted in the maximum increase in the flavonoids content after 5 days (2825.8 µg/g.DW) compared with control (1008µg/g.DW). This increase in the flavonoids content decreased gradually till 31 days (1266.8µg/g.DW) which was still higher than control (540.8µg/g.DW) (Figure 2). The other three used concentrations also showed their peak concentration after 5 days, but with different pattern. Y2 and Y3 flavonoids concentration decreased till being equal to control, while Y4 remains higher than the control (1069 µg/g.DW).

Phenyl alanine: Ph3 was the best concentration showing the highest fresh and dry weight for callus (Figure 3). It was noticed that phenyl alanine effect was similar to control through the whole culture but slight increase was

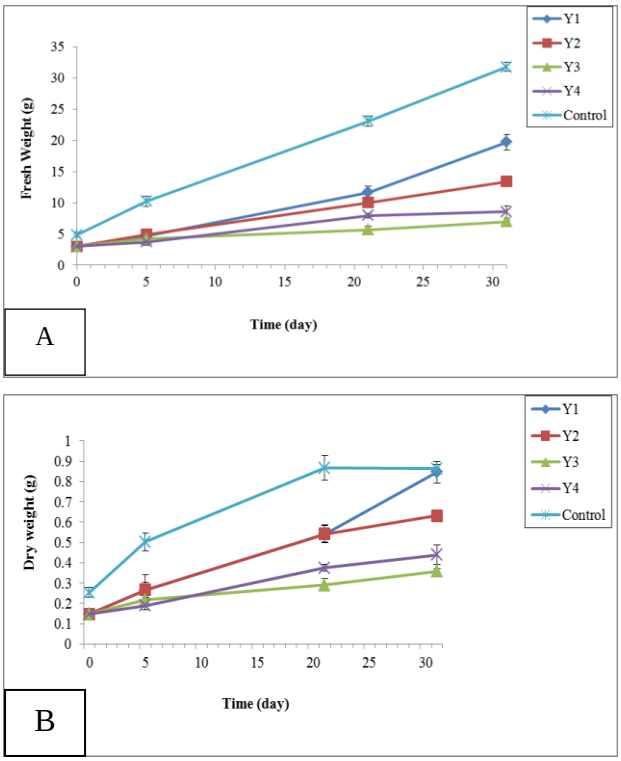

Figure 1 Effect of yeast elicitation on growth of *I. mucronata* callus culture.

A: Fresh weight B: Dry weight

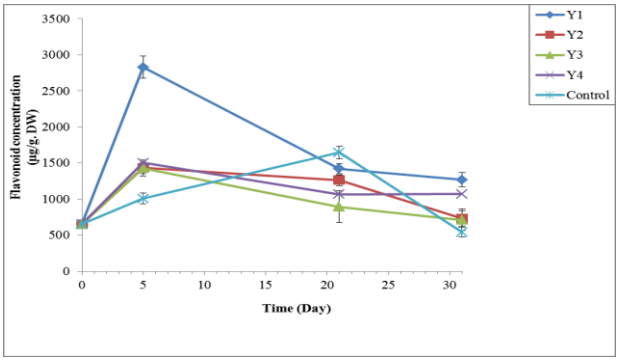

Figure 2 Effect of yeast elicitation on flavonoids contents of *I. mucronata* callus culture.

observed in flavonoids content with Ph3 where its maximum concentration reached 1.17 times control while Ph1 was 1.05 fold the control. Decreasing phenyl alanine concentration resulted in a flavonoids content of 0.71 and 0.11 times the control for Ph2 and Ph4, respectively (Figure 4).

Methyl Jasmonate: MJ3 (150 µM) appeared to be more effective than other concentrations, beginning after 5 days (1595.6 µg/g.DW) compared with control (381µg/g. DW). This increase continued till the 21st day (2193.6µg/g.DW) compared with control (1559 µg/g.DW) representing 40% increase although cells are brownish in color and appeared necrotic but flavonoids production continued (Figure 5).

Through the use of different type of elicitors added at zero time of subculture and through measuring flavonoidal

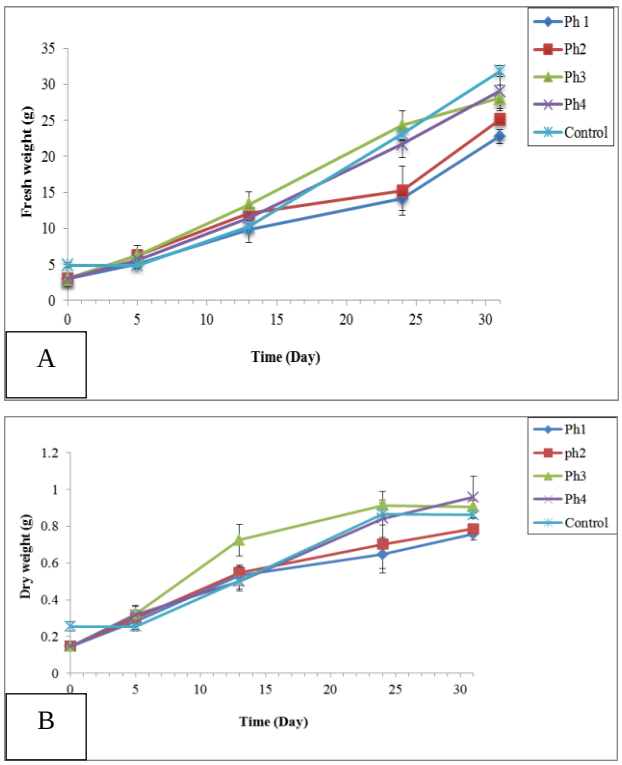

Figure 3 Effect of phenyl alanine elicitation on growth of *I. mucronata* callus culture.

A: Fresh weight B: Dry weight

concentration through the whole subculture, comparison of results was represented in figure 6.

3.2 Suspension culture

The obtained results showed that growth index (GI) is 3.59, specific growth rate (µ) is 0.23; while doubling time (dt) is 3.013. Growth curve is represented in fig. 7. The graph shows relatively slow growth during the lag phase (two days) where a small increase in fresh and dry weight is observed. The exponential phase extends up to 6 days where the culture growth per time unit increases. By the 9th day stationary phase is reached, while after 15 days browning of suspension culture occurred indicating the necessity for subculture every 14 days.

3.2.1 Flavonoids and phenolic contents in suspension culture

Maximum production of flavonoids and phenolic accumulation was observed at the 9th day where phenolic contents reached 6591.7µg/g.DW while flavonoids content was 1100.7 µg/g.DW, then productivity declined to its initial value (Figure 8).

3.3 Elicitation of suspension culture

The most successful concentrations of elicitors and precursor applied for callus culture were used in suspension culture.

Yeast: The effect of yeast (SY1) began after 3 hours but the maximum flavonoids content was reached after 12 hr

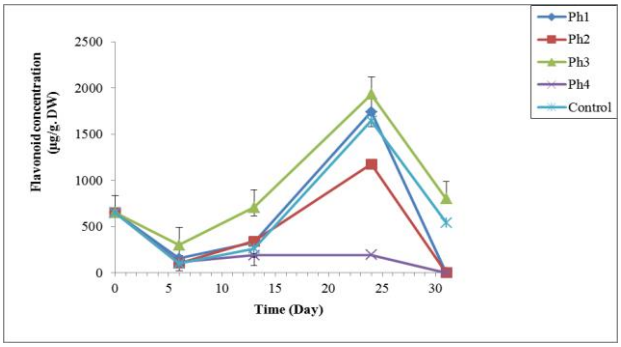

Figure 4 Effect of phenyl alanine elicitation on flavonoids contents of *I. mucronata* callus culture

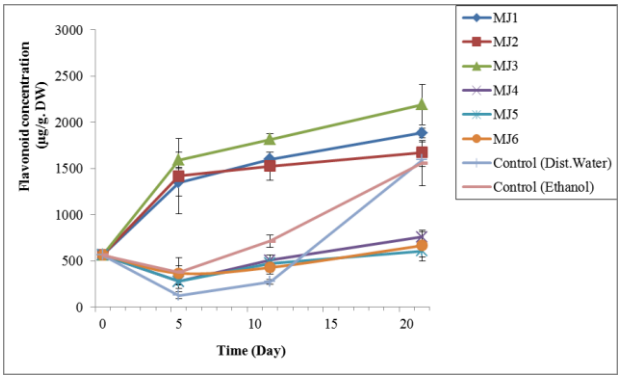

Figure 5 Effect of methyl jasmonate elicitation on flavonoids contents of *I. mucronata* callus culture.

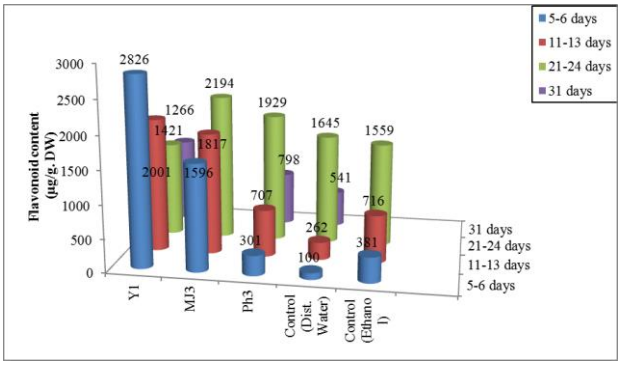

Figure 6 Effect of different elicitors on flavonoids contents of *I. mucronata* callus cultures

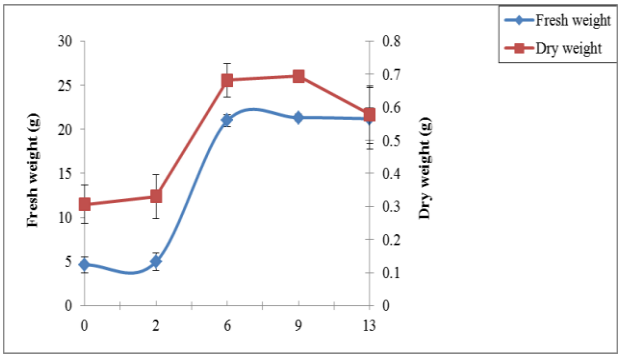

Figure 7 Growth curves of suspension culture of *I. mucronata*

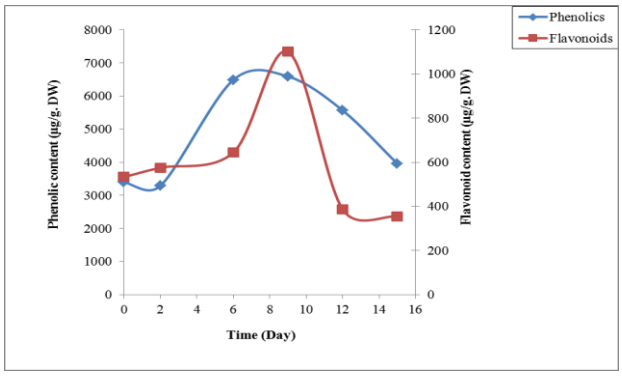

Figure 8 Flavonoids and phenolic contents of *I. mucronata* suspension culture.

of elicitation reaching 2036 µg/g.DW comparing with control reaching 545.4 µg/g.DW (Figure 9A). Yeast resulted in elicitation of phenolic contents reaching 6132 µg/g.DW compared to 2719 µg/g.DW for control after 12 h and even after its decrease, it will return higher than control after 48 h (Figure 9B).

Phenyl alanine: Flavonoids content exceeded control after 48 hours (414.3 µg/g.DW) and reached maximum level after 60 hours (613.7 µg/g.DW), where flavonoids were nearly depleted in control cells; then flavonoids decreased gradually and there was complete depletion after 6 days at the time the control cells regain their values (Figure 10A). Phenolic contents were increased to 2.5 times control (3361 µg/g.DW while control 1353 µg/g.DW) after 48 hours but then began to decrease compared to control till being depleted after 6 days of exposure which denoted the inhibitory effect of phenylalanine which may be due to accumulation of harmful intermediates (Figure 10B).

Methyl Jasmonate: Elicitation of suspension by SMJ3 (150 µM) increased flavonoids and phenolic contents as shown in figure 11. Flavonoids increased gradually compared to control suspension (ethanol) and reached maximum level after 6 hours (3585 µg/g.DW) compared to control (1610 µg/g.DW) then decreased gradually, but flavonoids disappeared after 36 hours where cells were darker in color and necrotic. Similar pattern was observed for control, which denoted the solvent effect. Upon the use of SMJ3, slight increase in phenolic contents over control occurred after 6 hours but declined gradually till complete depletion after 60 hours.

4. DISCUSSION

4.1 Elicitation of callus culture

The elicited calli showed different responses towards the applied elicitors and their concentrations. Y1 appeared to be the best concentration for callus growth and vitality. It is the lowest concentration resulted in the maximum increase in the flavonoids content after 5 days, which is 2.8 times the control. Although this increase in the flavonoids content decreased gradually till 31 days, it is 2.3 times the control in addition to keeping vitality of cells.

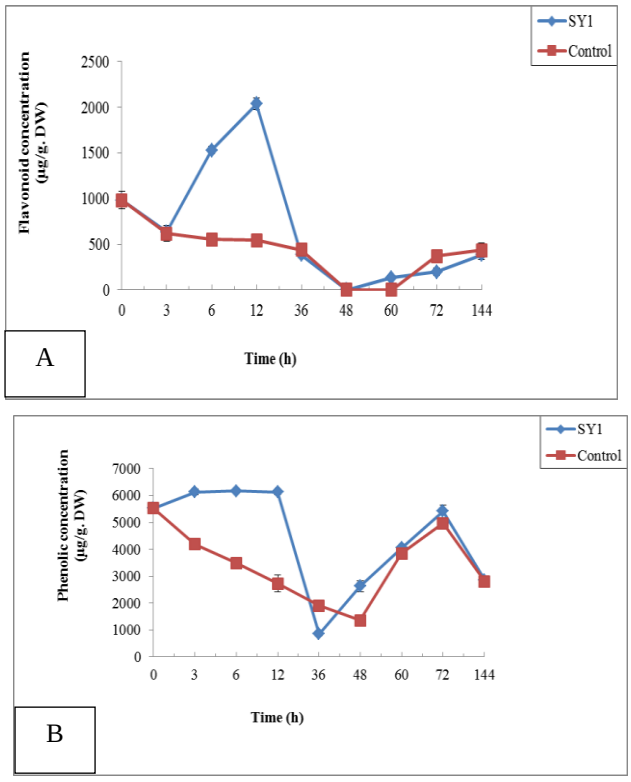

Fig.9. Flavonoids and phenolic contents of *I. mucronata* suspension culture elicited with yeast extract.

A: Flavonoids B: Phenolics

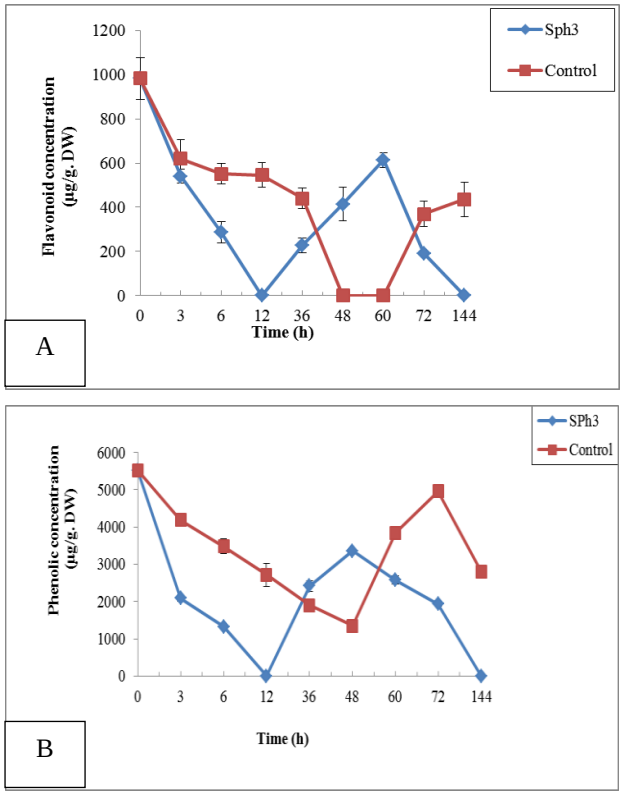

Fig.10. Flavonoids and phenolic contents of *I. mucronata* suspension culture elicited with phenyl alanine extract.

A: Flavonoids B: Phenolics

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Y2 and Y3 highest flavonoid concentration were also observed after 5 days but decreased till being equal to control, while Y4 was higher than the control representing 1.9 times.

These results go with the belief that the stronger stimulation of secondary metabolites by fungal elicitor often occurs in the late exponential growth stage and early stationary phase [20]. In a report dealing with silymarin biosynthesis in milk thistle hairy root culture the results of yeast elicitation correlating culture time with biosynthesis reached a maximum by 72 h after culture (2-fold higher than the control) [7]. However, the use of 16 mg/l yeast extract inhibited prenylated flavanones in addition to growth in *Sophora flavescens* (Fabaceae) callus culture [21].

The effect of phenyl alanine appeared mainly to cell viability**.** When callus cultures treated with 0.001 mM, they appeared more viable and yellowish in color even after 31 days. Different reports showed variable effects for phenylalanine. Although it did not affect flavonoids production in transformed *Scutellaria baicalensis* roots [22], it increased productivity of anthocyanins in callus culture of *Catharanthus roseus* which was explained by the intracellular L-phenylalanine accumulated at high levels which was used as a biosynthetic precursor material for anthocyanin and related flavonoids. Moreover, it had a function as a kind of signal that promoted the transcription of the genes on the anthocyanin biosynthetic pathway. Phenylalanine supplementation is expected to increase metabolic flux through phenyl-propanoid biosynthetic pathway and elevated level of target compound [23]. Although its effect was weaker when compared with yeast, but it resembled yeast in its ability to keep the vitality of cells till the last day of subculture.

Methyl jasmonate: MJ3 (150 µM) appeared to be more effective than other concentrations which represents 4 times increase in flavonoids concentration after 5 days. Opposite to yeast effect, flavonoids concentration increased in presence of MJ till 21 days (1.4 times ethanolic control) but browning of cells appeared which may be due to detrimental effect of MJ occurred. In this case the excretion of metabolites can be explained by leakage or cell lysis [24]. Ethanol control exhibited a solvent effect increasing flavonoids concentration when compared with distilled water so ethanol is considered partially as elicitor. When MJ was used for 7 days for flavonoids elicitation in *Passiflora quadrangularis* callus cultures, MJ strongly induced orientin production (up to 300 µg/g.DW), and to a lesser extent, isovitexin and vitexin, but had no effect on isoorientin, relative to untreated controls [25].

4.2 Suspension culture

The difference in growth rate and doubling time of suspension (0.23, 3.013) compared to those previously reported for callus culture of *I. mucronata* (0.09, 7.17) represents the main advantage of suspension culture, which is higher growth rate [11]. The growth of plant cells

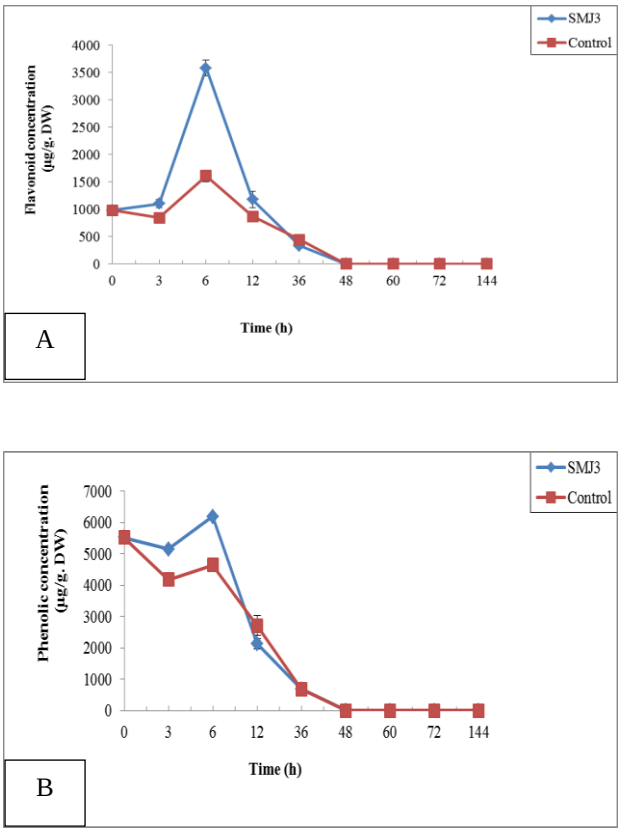

Figure 11 Flavonoids and phenolic contents of *I. mucronata* suspension culture elicited with methyl jasmonate.

A: Flavonoids B: Phenolics

is more rapid in suspension than in callus culture and is also more readily controlled because the culture medium can be easily amended or changed [26]. Specific growth rate was similar to that obtained by *Euphorbia characias* L. (Euphorbiaceae) suspension culture [27] with (µ) equal to 0.256 d^{-1}. *Hyoscyamus muticus* (Solanaceae) also showed a growth rate of 0.25 d^{-1} [28]. Upon elicitation of suspension culture by yeast (SY1), an increase of 3.7 and 2.3 folds of flavonoids and phenolic contents were reached after 12 hr of elicitation. Phenolics involve beside flavonoids, lignans, phenolic acids and hydroxycinnamic acid. So, even in absence of flavonoids the phenolic contents will remain high**.** When suspension cultures of *Silybum marianum* (L.) treated with 50 µg/ml medium of yeast extract, an increase in silymarin accumulation was noticed after 12 h of treatment reaching a maximum between 24 and 48 h and declining drastically afterwards.

It was found that yeast extract caused 50% and 200% increase in total silymarin accumulation in suspension cells and medium respectively. It was suggested that yeast caused a complex stress response in the cultures in addition to accumulation of flavonolignans, as for example lipid peroxidation, and/or activation of other metabolic pathways [6]. Results of phenyl alanine elicitation were non significant due to the rapid decrease followed by an increase in flavonoid and phenolic contents which appeared unexplained but finally complete cell death occurred compared with control which is probably due to accumulation of toxic metabolites.

Although methyl Jasmonate **(**SMJ3) elicitation for suspension increased flavonoids level by 2.3 times and phenolics by 1.3 times after 6 h, depletion occurred after 36h and 60h respectively. This would denote that methyl jasmonate effect was higher on flavonoids than phenolic contents. Addition of MJ to a broad range of plant-cell suspensions resulted in higher accumulation or *de novo* synthesis of secondary metabolites. Jasmonates are expected to be the chemical signal compounds in the process of elicitation leading to *de novo* gene transcription, and finally, the biosynthesis of natural products in cultured plant cells [29]. In a recent report, MEJA induced enhancement in phenolic and flavonoid accumulation in suspension culture of *Artemesia absinthium* L [30].

Cell cultures of *Saussurea medusa* (Asteraceae) were challenged by MJ. The highest jaceosidin and hispidulin concentrations were achieved being 2.2-fold and 4.2-fold, respectively, higher than those from controls. L-phenylalanine ammonia- lyase (PAL) activities were transiently increased after treatment with MJ, which suggested that this elicitor modified jaceosidin and hispidulin production by regulating the phenylpropanoid pathway [31].

Elicitation of *I. mucronata* suspension by MJ suppressed cell growth where cells appeared darker in color and necrotic. Methyl jasmonate treatment led to a clear repression of cell growth in suspension culture of *Ginkgo biloba* and induced cell browning as well [32]. In view of the inverse relationship between the production of biomass and the accumulation of secondary metabolites, the cell growth inhibition elicited by MJ treatment may favour the synthesis of secondary metabolites. It was reported that increased concentrations of MJ (5 - 500 µM) was toxic to cells and did not induce the phenylpropanoid/flavonoid pathway genes [33].

5. CONCLUSIONS

Elicitation of *Iphiona mucronata* (Asteraceae) callus and suspension cultures using yeast, phenylalanine and methyl jasmonate increased the flavonoids and phenolic production. The results favored the use of yeast extract compared with methyl jasmonate and phenylalanine for the high productivity and keeping viability of cell lines. To the best of our knowledge, there is no report for elicitation in genus iphiona.

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REFERENCES

[1] Giri, A., and Narasu, L. (2000). Transformed hairy roots: Recent trends and applications. Biotechnol. Adv., 18, 1-22.

[2] Bharati, A. J., and Bansal, Y. K. (2014). *In vitro* production of flavonoids: A review. WJPPS, 3 (6), 508-533.

[3] Sharma, M., et al., (2011). Enhancement of secondary metabolites in cultured cells through stress stimulus. American J. Plant Physiol., 6, 50-71.

[4] Sudha, G., and Ravishankar, G. A. (2002). Involvement and interaction of various signaling compounds on the plant metabolic events during defense response, resistance to stress factors, formation of secondary metabolites and their molecular aspects. Plant Cell Tiss. Org. Cult., 71, 181-212.

[5] Kákoniová, D., et al., (2009). The possibility to enhance flavonoids production in *Rubia tinctorum* l. callus cultures. Nova Biotechnologica, 9(2), 191-197.

[6] Sánchez –Sampedro, M. A., et al., (2005). Yeast extract and methyl jasmonate-induced silymarin production in cell cultures of *Silybum marianum* (L.) Gaertn. J. Biotechnol., 119, 60–69

[7] Hasanloo, H., et al., (2009). Evaluation of the yeast-extract signaling pathway leading to silymarin biosynthesis in milk thistle hairy root culture. World J. Microbiol. Biotechnol. 25 (11), 1901-1909.

[8] Arya, D. (2007). *In vitro* propagation and biochemical studies in *Puchea lanceolata* Oliver & Hiern: An important medicinal herb. PhD thesis, University of Rajasthan, Jaipur.

[9] Arya, D., et al., (2008). *In vitro* propagation and quercetin quantification in callus cultures of Rasna (*Puchea lanceolata* Oliver & Hiern) Indian j. Biotechnol., 7, 383-387

[10] Ahmed, A. A., et al., (1988). Polysulphated flavonoids from *Iphiona mucronata*. Rev. Latinoamer Quim., 19 (3), 107-109.

[11] Al-Gendy, A. A., et al., (2008). Production of flavonoids in callus cultures of *Iphiona mucronata*, Astraceae. Egypt. J. Biomed. Sci., 28, 142-150

[12] Murashige, T, and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant., 15, 473-497.

[13] Al-Gendy, A. A., et al., (2013). Somatic embryogenesis and plant regeneration from callus and suspension cultures of *Iphiona mucronata* (Forssk). European Scientific Journal, 9 (27), 37-49

[14] Shohael, A. M., et al., (2007). Methyl jasmonate induced overproduction of eleutherosides in somatic embryos of *Eleutherococcus senticosus* cultured in bioreactors. Electron. J. Biotechnol**.,** 10 (4), 633-637.

[15] Edahiro, J., et al., (2005). Enhanced accumulation of anthocyanin in cultured strawberry cells by repetitive feeding of L-phenylalanine into the medium. J Biosci. Bioeng., 99 (1), 43–47.

[16] Godoy-Hernández, G. and Vázquez-Flota, F. (2006). Growth measurements estimation of cell division and cell expansion. In: Loyola-Vargas VM, Vázquez-Flota F (ed) "Plant cell culture protocols". 2nd edn. Humana Press Inc.

[17] Verpoorte, R, et al., (1998). Metabolic engineering for the improvement of plant secondary metabolite production. Plant Tiss. Cult. Biotechnol. 4, 3 -19.

[18] Kosalec, I., et al., (2004). Quantitative analysis of the flavonoids in raw propolis from northern Croatia. Acta pharma Zagreb., 54, 65-72.

[19] Sellappan, S. and Akoh, C. C. (2002). Flavonoids and antioxidant capacity of Georgia-Grown Vidalia Onions. J. Agric. Food Chem., 50, 5338-5342.

[20] Kitamura, Y., et al., (1998). Induction of furanocoumarin biosynthesis in *Glehnia littoralis* cell suspension cultures by elicitor treatment. Phytochemistry, 48, 113–117.

[21] Yamamoto, H, et al., (1995). Stimulation of prenylated flavanone production by mannans and acidic polysaccharides in callus culture of *Sophora flavescens.* Phytochemistry, 40 (1), 77-81.

[22] Kuzovkina, I. N., et al., (2001). Flavonoid production in transformed *Scutellaria baicalensis* roots and ways of its regulation. Russ. J. Plant Physiol., 48 (4), 448-452.

[23] Taha, H. S., et al., (2008). Successful application for enhancement and production of anthocyanin pigment from calli cultures of some ornamental plants. Aust. J. Basic & Appl. Sci., 2 (4), 1148-1156.

[24] Vasconsuelo, A. and Boland, R. (2007). Molecular aspects of the early stages of elicitation of secondary metabolites in plants. Plant Sci., 172 (5), 861–875

[25] Antognoni, F., et al., (2007). Induction of flavonoid production by UV-B radiation in *Passiflora quadrangularis* callus cultures. Fitoterapia, 78, 345–352

[26] George, E. F. (2008). Plant tissue culture procedure – Background. In :(George, E.F, Michael, A.H and De Klerk, G-J.) plant propagation by tissue culture 3rd Edition. Volume 1. The Background

[27] Fernandes-Ferreira, M., et al., (1989). Calli and suspension cultures for biomass production of *Euphorbia characias* L. subsp. *characias*. Biotechnol. Lett., 11, 259-264.

[28] Carvalho, E. B. and Curtis, W. R. (1999). The effect of inoculum size on the growth of cell and root cultures of *Hyoscyamus muticus:* Implications for reactor inoculation. Biotechnol. Bioprocess Eng., 4, 287-293.

[29] Sudha, G. and Ravishankar, G. A. (2003). Elicitation of anthocyanin production in callus cultures *of Daucus carota* and the involvement of methyl jasmonate and salicylic acid. Acta Physiol. Plant., 25 (3), 249-256.

[30] Ali, M. et al., (2014). Elicitation of antioxidant secondary metabolites with jasmonates and gibberellic acid in cell suspension cultures of *Artemesia absinthium* L. Plant Cell Tiss. Organ Cult. DOI 10.1007/s11240-014-0666-2

[31] Fu, C. X., et al., (2006). Methyl jasmonate stimulates jaceosidin and hispidulin production in cell cultures of *Saussurea medusa*. Appl. Biochem. Biotechnol., 134 (1), 89-96

[32] Kang, S. M., et al., (2006). Effects of methyl jasmonate and salicylic acid on the production of biloalide and ginkgolides in cell cultures of *Ginkgo biloba*. In Vitro Cell. Dev. Biol.-plant, 42, 44-49

[33] Suzuki, H., et al., (2005). Methyl jasmonate and yeast elicitor induce differential transcriptional and metabolic re-programming in cell suspension cultures of the model legume *Medicago truncatula*. Planta, 220, 696–707.