

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM CALLUS AND SUSPENSION CULTURES OF *IPHIONA MUCRONATA* (FORSSK)

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Abstract

A protocol was designed for plant regeneration of *Iphiona mucronata* from embryogenic callus via somatic embryogenesis to enable micro propagation of this endangered plant. The embryogenic callus was induced using seedling cultured for nine months on Murashig and Skoog medium (MS) supplemented with 0.1 mg l⁻¹ naphthalene acetic acid (NAA), 0.1 mg l⁻¹ kinetin (Kn) and 5 mg l⁻¹ ascorbic acid and incubated in the dark followed by growing on hormone free medium. Transfer of developed embryos to MS medium supplemented with 0.5 mg l⁻¹ kinetin induced shoot formation. Four treatments were further tried for plant development by using indole acetic acid (IAA) or indole butyric acid (IBA) alone or in combination with kinetin. The results showed that 2 mg l⁻¹ IAA was the best for *in vitro* plantlet regeneration. Embryogenic suspension was induced by transfer of embryogenic callus to liquid medium having the same composition followed by hormone free medium where different stages of embryos were monitored. Shoots were developed upon transfer to liquid medium supplemented with 0.5 mg l⁻¹ Kn. However, no further development appeared upon transfer to semi solid medium containing different phytohormones. Embryogenic callus showed the highest phenolic contents when compared with embryogenic suspension, regenerated plantlets and the parent plant while flavonoids were detected only in embryogenic callus.

Keywords: *Iphiona mucronata*, callus and suspension culture, somatic embryogenesis, plant regeneration

Introduction

Iphiona Cass. is a small genus of about eleven species, which is distributed from North-East Africa to central Asia (Anderberg, 1985). Studies on *Iphiona scabra* and *Iphiona mucronata* which are native in Egyptian deserts (Zahran and Willis, 2009) revealed that polysulphated flavonoids and sesquiterpene glycosides were the major constituents and seem to be characteristic for this genus (Ahmed and Mabry, 1987; Ahmed *et al.*, 1988). *In vitro* propagation was not tried in any of its species, as an endangered plant, somatic embryogenesis would be of value. In a previous work (Al-Gendy *et al.*, 2008), a successful callus cell line was established with high phenolic and considerable production of flavonoids when compared with the parent plant using MS medium (Murashige and Skoog, 1962).

The culture of somatic embryos in a liquid medium has numerous advantages as the swirling medium naturally separates the embryos, which are then easily observed and fractionated according to their stages. They can be obtained in great quantity and used as a basis for a large-scale micropropagation (Monnier, 1990).

The objective of this study is to develop an efficient protocol for micropropagation of *Iphiona mucronata* via somatic embryogenesis to save this plant from eradication. We also investigate the flavonoid and phenolic contents of somatic embryos in callus and suspension culture compared with regenerated plantlets.

Material and methods

Induction of embryogenic callus

Callus was induced using MS medium supplemented with 0.1 mg l⁻¹ NAA, 0.1 mg l⁻¹Kn, 5 mg l⁻¹ ascorbic acid, 30 g l⁻¹ sucrose and solidified with 10 g l⁻¹ agar (MS-1). 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 (Al-Gendy *et al.*, 2008). After nine months of culture, the nodular embryogenic calli were moved to the same medium but without phytohormones (hormone free medium; MS-HF), maintained at 25°C, with 12-h photoperiod (using fluorescent white lamps) and subcultured into fresh medium every 4 weeks for 12-24 weeks.

Somatic embryo formation and development

The well developed embryogenic calli grown on MS-HF were removed to semi solid media supplemented with 0.5 mg l⁻¹Kn, 50 g l⁻¹ sucrose, 5 mg l⁻¹ ascorbic acid and solidified with 8 g l⁻¹ agar (MS-XS) to enhance the development of somatic embryos for 12-24 weeks. Cultures

were maintained at 25°C, with 12-h photoperiod (using fluorescent white lamps). Cultures were routinely examined microscopically at each subculture and photographs were recorded.

Conversion of somatic embryos into plantlets

Cultures grown on semi solid MS-XS were classified into 4 groups as follow; group A cultured on 2 mg l⁻¹ IAA, group B cultured on 2 mg l⁻¹ IAA and 0.5 mg l⁻¹ Kn, group C cultured on 2 mg l⁻¹ IBA, group D cultured on 2 mg l⁻¹ IBA and 0.5 mg l⁻¹ Kn. All cultures were supplemented with 30 g l⁻¹ sucrose, 5 mg l⁻¹ ascorbic acid and incubated at 25±2°C with 16-h light exposure and regularly transferred to fresh medium every 2-4 weeks according to the growth.

Induction and maintenance of embryogenic suspension culture (ESC)

Embryogenic callus grown on MS-1 was transferred to 250 ml Erlenmeyer flask containing 50 ml liquid medium having the same composition as MS-1 except agar, incubated on rotary shaker (120 rpm at 25±2°C in dark) and transferred into fresh medium every two weeks. After 6 weeks in culture, the embryogenic suspension was sub-cultured on hormone free media supplemented with 50 g l⁻¹ sucrose and 5 mg l⁻¹ ascorbic acid and sub-cultured into fresh medium every two weeks for 8 generations. Biomass was separated from liquid medium and examined microscopically. At the 4th generation growth curve and production of phenolic content were studied.

ESC was transferred to another liquid media supplemented with 0.5 mg l⁻¹Kn, 5 mg l⁻¹ ascorbic acid and 50 g l⁻¹ sucrose in a trial for embryo germination. Developed shoots were transferred to semi solid media supplemented with 2 mg l⁻¹ IAA, 5 mg l⁻¹ ascorbic acid, 50 g l⁻¹ sucrose and solidified with 8 g l⁻¹ agar.

Growth dynamics in ESC

Growth curve: Samples were taken with intervals of 3 days up to fourteen days in suspension where fresh and dry weights were determined (Godoy-Hernández and Vázquez-Flota, 2006).

Growth index (GI) = (Ge - Gstart)/ Gstart (Verpoorte *et al.*, 1998)

Where Ge = Weight of biomass at the end of generation (final dry weight).

Gstart = Weight of biomass at zero time (Initial dry weight).

Relative growth rate (RGR) was measured on fresh weight basis using the following formula:

$$RGR = 3(Wf^{1/3} - Wi^{1/3}) / (tf - ti) \text{ (Parsaeimehr } et al., 2010)$$

ti: Beginning of the experiment, tf: Last day of subculture, after 14 days

Wi: Weight of initial biomass (at ti), Wf: Final biomass weight (at tf), tf-ti = 14 days of subculture.

Specific growth rate (μ) : $\mu = (\ln x - \ln x_0)/t$

Where x₀ is the initial dry biomass and x is the biomass at time t (14 days) (Godoy-Hernández and Vázquez-Flota, 2006).

Doubling time which is the time required for the biomass of a population of cells to double is denoted as (dt). $dt = \ln(2) / \mu$

Determination of total flavonoids and phenolic contents

Total flavonoids: One gram of each of the dried embryogenic callus, embryogenic suspension biomass and regenerated plantlets was extracted with 25 ml of hot 95% ethanol (v/v) overnight at 37 °C and the filtrate was adjusted with 80% ethanol (v/v) to 25 ml. Total flavonoid content was estimated colorimetrically as reported by Kosalec *et al.* (2004) and used previously for non embryogenic callus (Al-Gendy *et al.* 2008). Quantitation was done based on the standard curve generated with rutin (12.5, 25, 50, 80 and 100 $\mu\text{g ml}^{-1}$) measured at 415 nm.

Total phenolics: Dried embryogenic callus, suspension biomass, regenerated plantlets and parent plant (1 g each) were extracted with 25 ml methanol. Total polyphenols were estimated colorimetrically using the Folin-Ciocalteu method as reported (Sellappan and Akoh, 2002). The absorbance was measured at 765 nm using a Shimadzu UV-visible spectrophotometer (1800-UV probe) after incubation for 2 h at room temperature. Quantification was done based on the standard curve generated with gallic acid (10, 20, 40, 60, 80 and 100 $\mu\text{g ml}^{-1}$).

Results

Embryogenic callus

After nine months of culture in the dark, pockets of embryogenic calli with nodular structures appeared on the surface of the non embryogenic callus. These calli tend to be light greenish yellow in color which is differentiated from the non embryogenic callus. When proliferated calli were moved to hormonal free medium, they kept the embryogenic potential and showed further embryo development.

Somatic embryo formation and development

Globular-staged (G) embryos (75-150 μm in diameter, Fig. 1a), heart shaped (H) embryos (75-200 μm x 75-250 μm , Fig. 1b) and torpedo-shaped (T) embryos (200-350 μm , Fig. 1c) were monitored. Mature torpedo shaped embryos successfully germinated into cotyledonary embryo (Fig. 1d) which further developed into cotyledonary leaves (Fig. 1e) after the fourth generation. A heterogeneous population of somatic embryos appears, showing non synchronous culture (Fig. 1f).

A fraction of somatic embryos differentiated directly into plantlets, while the others produced secondary embryos after each subculture in a repetitive way. The embryogenic callus retained its ability to grow and produce somatic embryos for about a year.

Conversion of somatic embryos into plantlets

When the developed embryos were transferred to MS-XS, shoot formation appeared, the length of the shoot was 0.5 cm after the third subculture (Fig. 2a), and no further elongation appeared.

When regenerated shoots were transferred to several media supplemented with various concentrations of IBA and IAA alone or in combination with Kn (group A-D) for 3 generations, normal shoot length increases to about 3cm (Fig. 2b). However, group A of regenerated plantlets was the most successful. Shoot reached about 4.5 cm in length with green, alternate acicular leaves and root was about 2 cm in length after 6 subcultures (Fig. 2c, d). When Kn was added (group B), roots begin to appear at the first generation but was depleted at the second. Roots were observed for group B, C and D regenerated plantlets at the first generation but no further development occurred afterwards.

Abnormalities of *I. mucronata* embryogenic callus

Some of the embryos that had developed beyond the globular stage were fused in pairs (Fig. 3a), early and late torpedo stage (Fig. 3b, c). Other forms of anomalies may be present (Fig. 3d, e). Certain abnormality appeared in some plantlets which seems dwarfed (Fig. 3f).

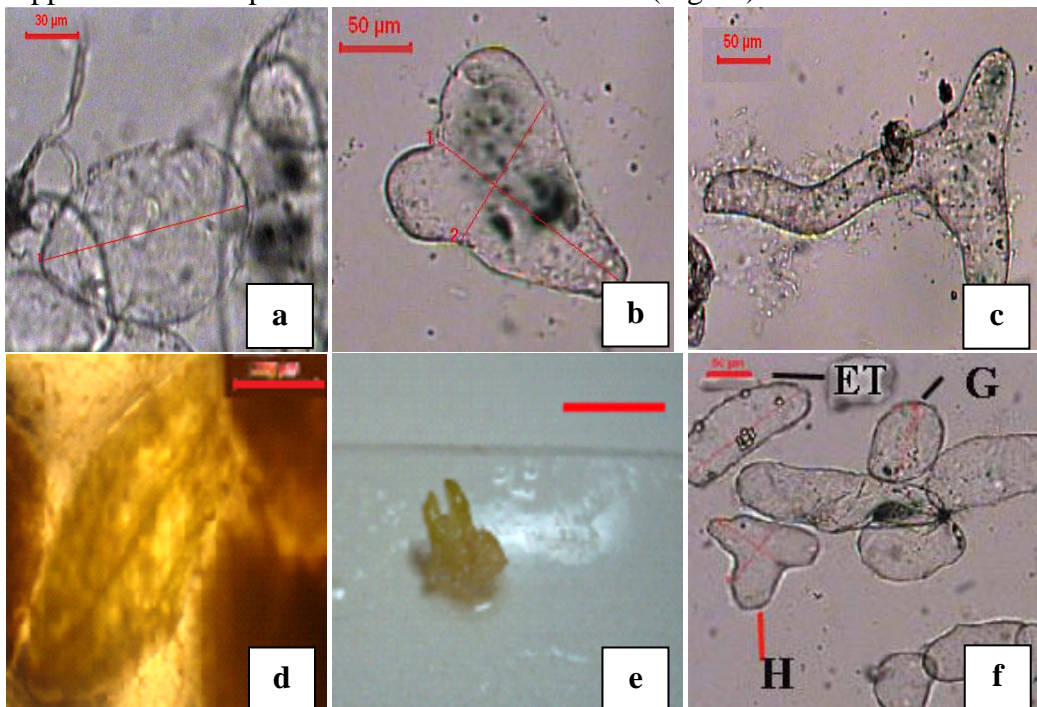


Figure 1: Stages of embryogenic callus of *I. mucronata*. **a** globular embryo (bar 30 µm). **b** heart shaped embryo (bar 50 µm). **c** torpedo shaped embryo (bar 50 µm). **d** cotyledonary embryo (bar 200 µm). **e** cotyledonary leaves (bar 1 cm). **f** embryos at different stages (bar 50 µm). (ET early torpedo)

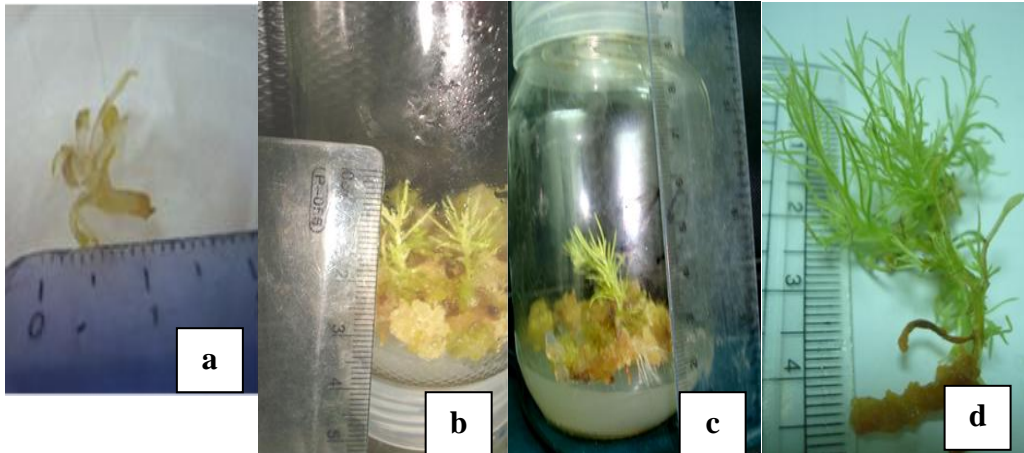


Figure 2: Plantlet regeneration of *I. mucronata* from embryonic callus. **a** & **b** shoot formation. **c** root formation. **d** regenerated plantlet.

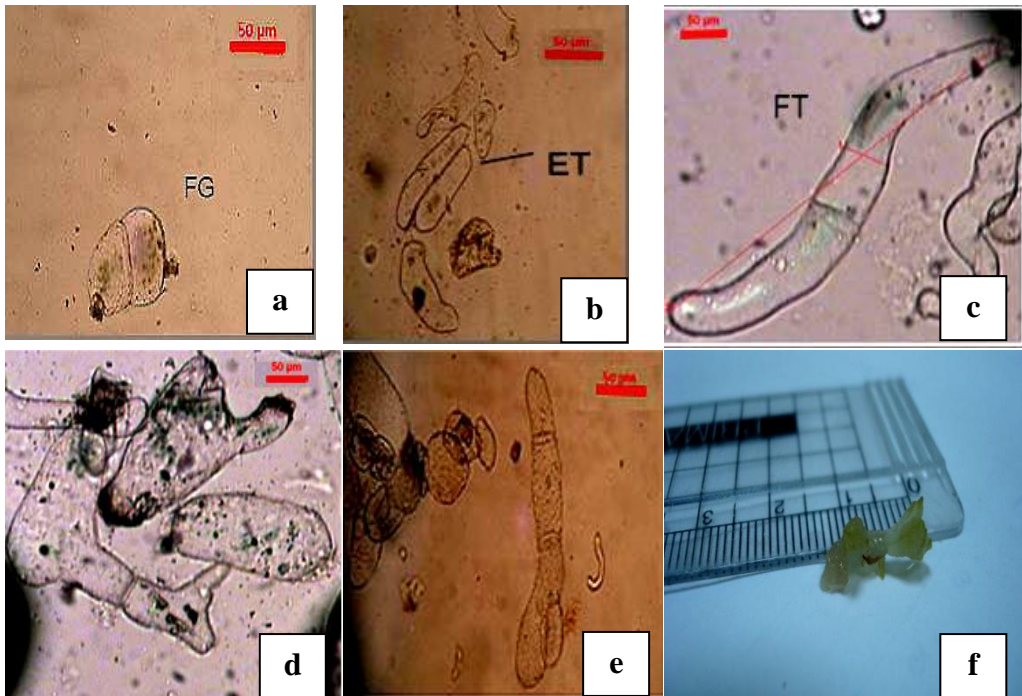


Figure 3: Anomalies in embryonic callus of *I. mucronata* (bar 50 μ m; a-e) **a** fused globular (FG). **b** early torpedo (ET). **c** fused torpedo (FT). **d** abnormal heart embryo. **e** anomalies in torpedo. **f** fused plantlet.

Embryogenic suspension culture

Biomass growing on suspension hormone free media (Fig. 4a) was separated and examined microscopically revealing different embryo stages, from globular to early cotyledonary stages (Fig. 4b-e). Globular embryos appeared in the first generation (Fig. 4b) while heart shaped embryos (Fig.

4c) appeared in the second generation, which then differentiated into torpedo stage (Fig. 4d). Moreover, heterogeneous embryos at different stages were also monitored (Fig. 4-e)

Plantlet regeneration in ESC

When the embryos were transferred to liquid media supplemented with 0.5 mg l^{-1} Kn, 5 mg l^{-1} ascorbic acid and 50 g l^{-1} sucrose in a trial for embryo germination, shoots (1 cm length) appeared after the 4th subculture. Unfortunately no further development appeared upon transfer of the developed shoot to semi solid media supplemented with 2 mg l^{-1} IAA (group A) (Fig. 4f). Abnormal embryos e.g. fused globular and torpedo shaped were noticed at the 4th generation of embryogenic suspension (Fig. 5).

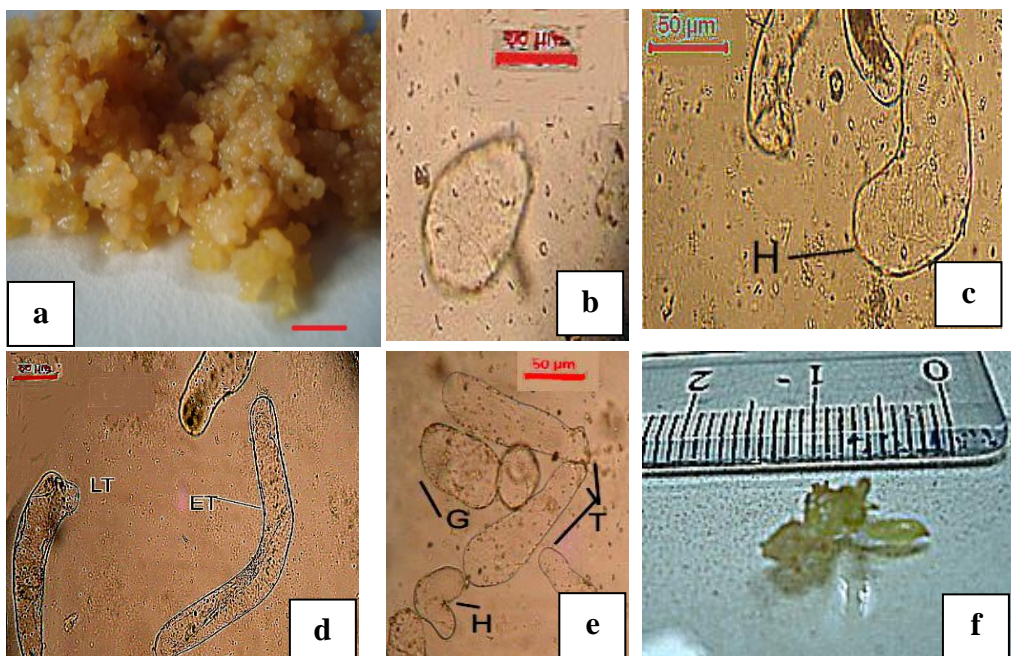


Figure 4: Somatic embryos of *I. mucronata* suspension culture. **a** Embryogenic suspension biomass (bar: 1 cm) **b** globular embryos. **c** heart shaped embryos . **d** torpedo shaped embryos. **e** different stages embryos (bar 50 µm, **b-e**). **f** undifferentiated plantlet.

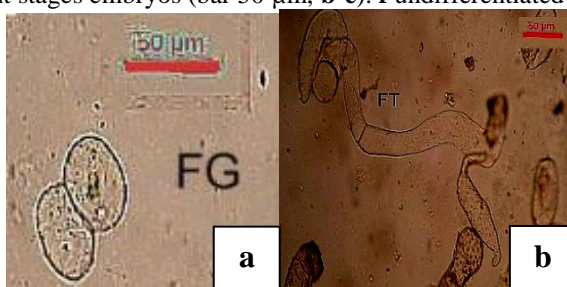


Figure 5: Fused embryos in suspension culture of *I. mucronata*(bar 50 µm). **a** fused globular. **b** fused torpedo.

Growth dynamics of ESC

Growth curve of ESC based upon dry weight measurement is represented in Fig. 6. It is noticed that the maximum dry weight was achieved after 9 days and continued a stationary phase after that. Growth parameters on dry weight basis were as following:

GI=1.381, RGR=0.04, $\mu=0.05$ and dt= 13.06d

Investigation of total phenolic and flavonoids contents

When phenolic contents were estimated (Fig. 7), EC showed the highest phenolic content as it represents 1.4 times the embryogenic suspension culture and 1.6 the regenerated plantlets. Moreover, It represents 2.9 times the parent plant itself. Follow up of the phenolic content through the whole generation of the 5th subculture of ESC on hormone free media, revealed the gradual decrease till reaching minimum value at day 6 followed by an increase at the 9th day reaching the highest level by day 13 then decreasing again (Fig. 8). On the other hand, the lack of flavonoid content was noticed in the regenerated plantlet and ESC while detected only in embryogenic callus (293 $\mu\text{g ml}^{-1}$) which represents about 16 % of the parent plant estimated previously (Al-Gendy *et al.* 2008).

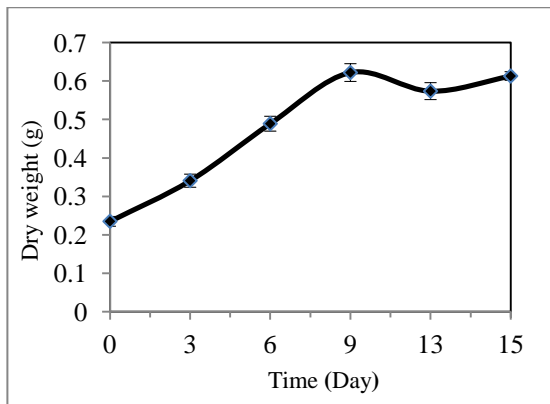


Figure 6: Growth curve of embryogenic suspension culture of *I. mucronata*. Mean \pm SD, n=3

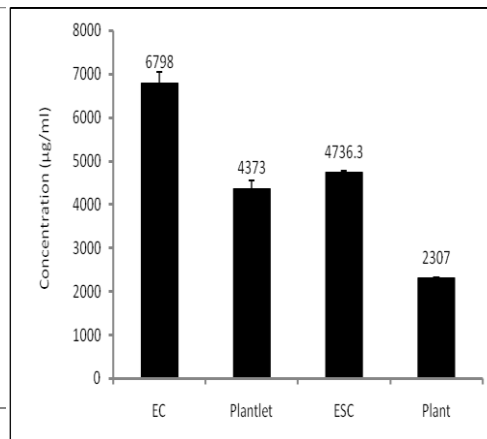


Figure 7: Phenolic contents of embryogenic callus and suspension cultures of *I. mucronata* compared with regenerated plantlet and parent plant. Mean \pm SD, n=3

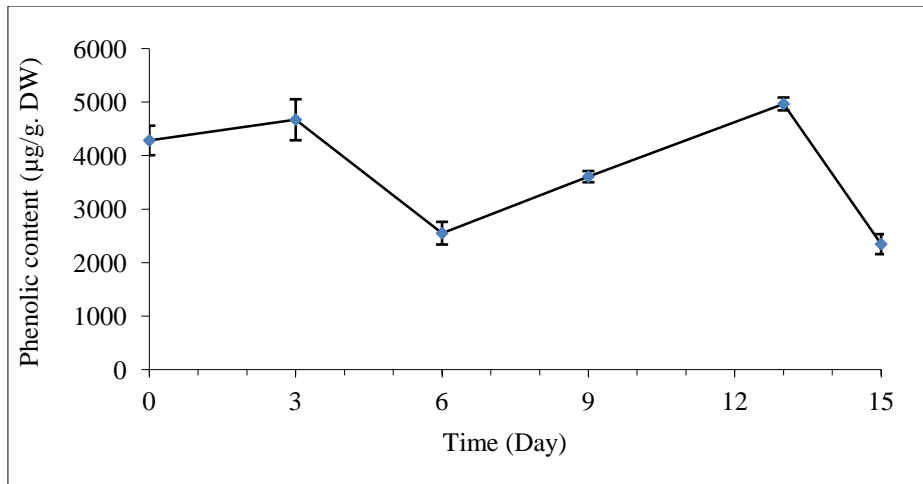


Figure 8: Follow up of phenolic content in *I. mucronata* embryogenic suspension culture (ESC). Mean \pm SD, n=3

Discussion

Indole acetic acid was the most successful for plantlet regeneration. A previous study reported that IAA (2 mg l^{-1}) and Kn (2 mg l^{-1}) appeared to be a good combination for shoot regeneration in *Arachis hypogaea* (Narasimhulua and Reddy, 1983). Addition of 0.5 mg l^{-1} Kn showed a positive effect on regeneration when combined with 0.1 mg l^{-1} IAA and 0.5 mg l^{-1} 6-benzylaminopurine. When the concentration of Kn was decreased from 0.5 to 0.1 mg l^{-1} , the percentage of regeneration was also decreased from 80.60% and 62.2% to 6.0% and 14.6% in Pakistani wheat cultivars Kohsar and Khyber-87, respectively. These results may justify the increased embryogenesis when Kn was used at concentration 0.5 mg l^{-1} (Noor *et al.*, 2009). Another report for *in vitro* regeneration of *Citrus aurantifolia* (Rutaceae) revealed that IAA significantly influenced root proliferation and shoot growth (Al-Khayri and Al-Bahrany, 2001).

Appearance of abnormal embryos has been observed in many species. Reasons for their development are not well known. It has been suggested that this phenomenon is attributed to the developmental plasticity of somatic embryogenesis that is influenced by culture conditions. Possibly somatic embryos that failed to convert into plantlets were inclined to produce secondary embryos (Luo *et al.*, 1999; Carman, 1990). In this study, these abnormalities occurred in hormone free media, possibly due to failure to convert into plantlets.

The lack of flavonoid content in the regenerated plantlet may be due to the nature of flavonoids which are UV-B inducible (Cockell and Knowland, 1999) while the lamps used in the *in vitro* growth chamber did not provide wave lengths in the range of the UV radiation. An analogous

behavior was shown in callus cultures of *Passiflora* spp. where the UV-B irradiation was able to increase the production of flavonoids (Antognoni *et al.*, 2007; Lucchesini *et al.*, 2009).

Results seemed similar to that of thalamus-derived calluses of *Ranunculus asiaticus* L. where non differentiating callus was characterized by a high content of phenolic polymers and an elevated peroxidase and polyphenol oxidase activity in comparison with differentiating callus (Beruto *et al.*, 1996).

Upon studying phenolic content of *Echinacea. angustifolia*, the yields were the highest among the *in vitro* cultures and they were similar or higher than leaves of adult plants (Lucchesini *et al.* 2009). These results are matching with data obtained through this study (Fig. 7), where embryogenic callus culture has the highest phenolic content compared with ESC, regenerated plantlets and the parent plant.

Embryogenic suspension cultures have been established in only few crops, including sweet potato, cowpea and horsegram (Naik and Murthy, 2010). However, the quality of somatic embryos with regard to their germinability or conversion into plants has been generally very poor. This is due to the apparently normal looking somatic embryos are actually incomplete in development. (Bhojwani and Razdan, 1996; Amoo and Ayisire, 2005; Naik and Murthy, 2010; Pescador *et al.*, 2008; Yantcheva *et al.*, 1998). According to Canhoto *et al.* (1999), the most common abnormalities encountered are embryo fusion. Fused globular and torpedo shaped embryos were noticed at the 4th generation of *I. mucronata* embryogenic suspension culture (Fig. 5).

In a study to compare ESC and NESC for *Medicago sativa*, NESC gave a typical growth curve while in ESC the distinct phases were absent (Hrubcová *et al.*, 1994), this was the case in ESC of *I. mucronata*.

When estimating the growth parameters, GI of embryogenic suspension is relatively low (1.38) when compared with non embryogenic callus previously reported (Al-Gendy *et al.*, 2008). It needs longer time to reach double its initial weight (13.06 d) which is considered 1.6 the time needed for non embryogenic callus. So, embryogenic suspension culture is not a reliable method for obtaining biomass production.

Conclusion:

A protocol is established for the first time for somatic embryogenesis in callus and suspension culture of *I. mucronata* that can be used for micro propagation of this plant to save it from eradication, in addition to comparison of phenolic and flavonoid contents in embryogenic callus and suspension cultures with regenerated plantlets and parent plant.

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