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Highly Efficient Plant Regeneration of African Daisy (*Osteospermum hybrida*) Through Somatic Embryogenesis of Leaf Derived Callus

*Malabika Roy Pathak
Department Of Life Sciences, Agricultural Biotechnology Section, Arabian Gulf University, Manama, Kingdom Of Bahrain
*Corresponding Author: *Malabika Roy Pathak*

Abstract: *African daisy (Osteospermum hybrida) is a beautiful garden plant. The plant mainly propagate vegetatively by cutting while the propagation from seed is time consuming and difficult. To overcome this difficulty, tissue culture technique developed to regenerate a large number of plants rapidly. A highly efficient plant regenaration method has established from young leaf derived callus. Young leaves collected from 3-week old young seedlings developed in vitro on half strength Murashige and Skoog (MS) inorganic salt media. Leaf explant was cultured on inorganic MS salt media supplemented with Gamborg B5 vitamins in presence of different concentrations and combinations of N6-benzylamino purine (BAP), kinetin (KIN), indole acetic acid (IAA) and 2,4-dichlorophenoxy acetic acid (2,4-D). Compact, nodular and highly differentiating embryogenic calli developed from leaf explant culture in MSB5 media supplemented with 2.2 µM BAP, 2.3 µM KIN, and 1.2 µM IAA. The subcultured calli showed the highest plant regeneration capacity of 15 shoots /callus in presence 4.4 µM BAP, 2.3 µM KIN, and 0.5 µM IAA. The developed protocol established a highly efficient method of mass propagation of plantlets through callus derived culture of explant.*

Keywords: *African daisy, Embryogenic calli, Growth regulators, Plant Regeneration*

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I. Introduction

African daisy (*Osteospermum hybrida*) is lovely daisy-like flowering plant belongs to Asteraceae family. The flowers are available in a variety of shades and color viz., orange, cream, yellow, pink etc. Although the plants are native of Africa, they are growing throughout the Mediterranean region, Asia and Europe. The best way to propagate *Osteospermum hybrida* is to take cuttings from established plants, but the problem in serious infection of root rot diseases and several fungus diseases during cutting establishment for rooting. On the other hand, propagation from seed is time consuming and true to type plant is not a confirmed factor and representing variation in large way of propagation. To overcome this difficulty, plant tissue culture techniques applied to regenerate a large number of plants rapidly. Plant tissue culture technology has wide application in a large scale plant multiplication process in several purpose, apart from their use as a tool of plant science research (Hussain *et al*., 2012). Plant regeneration during *in vitro* culture may follow various pathways, of which somatic embryogeneis is one of the route for propagation of large number of plants (Arnold *et al*., 2002). Somatic embryogenesis has a great potential to improve plant production in a large-scale clonal propagation (Koh and Loh 2000; Punja *et al*., 2004; Sadeq *et al*., 2014a). This is the first report of application of tissue culture technique to regenerate African daisy plantlets by in vitro culture. The protocol developed here based on culture of young leaf explant in various culture media to develop highly efficient plant regenerating capacity.

II. Materials And Methods

2.1. Explant resource development:

In this experiment, young leaves of African daisy used as explant source for culture establishment. Young leaves collected from 21 days old seedlings of African daisy seedlings grown aseptically under *in vitro* culture method after germinating seeds on agar (0.8 %) solidified half strength inorganic MS (Murashige and Skoog, 1962) salt media. Seeds were surface sterilized using 50% (v/v) commercial bleach (Clorox, 2.67% w/v, Sodium hypochlorite) for 20 min, followed by 5 times rinsing with sterile distilled water before transfer to 70% alcohol for 1 min. Sterilized seeds allowed to imbibition for two hours before culture to develop young seedlings on half strength MS inorganic salt media at 21±2 °C and 14/10 h (light/dark) conditions for 4 weeks.

2.2. Explant culture

Young leaves of African daisy collected from four weeks old *in vitro* cultured seedlings as explant source for culture initiation and plant regeneration.

2.3. Culture media and culture conditions

The basic media used throughout the culture of African daisy was modified MS salt media supplemented with B5 vitamins (Gamborg *et al*., 1968) supplemented with 3% sucrose, 0.01 % myo-inositol, 0.0001% Nicotinic acid, 0.0001 % Pyridoxine HCL, 0.001% Thiamine HCL. Media pH were adjusted to 5.8 before adding 1% agar and autoclaved at 121°C, 20 minutes at 15 psi. Modified MSB5 culture media supplemented with different combinations and concentrations of plant growth regulators (PGRs) according to culture steps. The cultures maintained at 16/8 h (light/dark) cycle with cool, white, fluorescent light intensity of 2000-2500 LUX, temperature of 21±2°C, 50-60% humidity.

2.4. Callus induction from young leaf culture

Young leaf explants collected from *in vitro* cultured plants, cut into small pieces around 0.5-1 cm and cultured for four weeks to see the responses in modified basic MSB5 (#1) Basic modified MSB5 media supplemented with different plant growth regulators and numbered viz.
(#2) 4.5 µM BAP; (#3) 4.6 µM KIN; (#4) 4.9 µM IAA; (#5) 4.5 µM 2,4-D; (#6) 2.2 µM BAP, 1.2 µM IAA; (#7) 2.3 µM KIN, 1.2 µM IAA; (#8) 2.2 µM BAP, 1 µM 2,4-D; (#9) 2.3 µM KIN, 1 µM 2,4-D; (#10) 2.2 µM BAP, 2.3 µM KIN; (#11) 2.2 µM BAP, 2.3 µM KIN, 1.2 µM IAA; (#12) 1 µM 2,4-D, 2.2 µM BAP, 2.3 µM KIN.

2.5. Shoot differentiation from calli and plantlet regeneration

Calli growing in media (#11) subcultured in the same media for 4 weeks and then transferred into various media for shoot differentiation using basic modified MSB5 media supplemented with different plant growth regulators and numbered viz.
(#1); (#11); (#13) 0.5 µM IAA, 2.2 µM BAP, 2.3 µM KIN; (#14) 0.5 µM IAA, 4.4 µM BAP, 2.3 µM KIN; (#15) 2.4 µM IAA, 2.2 µM BAP, 2.3 µM KIN; (#16) 2.4 IAA µM, 4.4 µM BAP, 2.3 µM KIN.
Differentiated shoots cultured for another 4 weeks for plantlet regeneration using basic modified MSB5 media supplemented with 2.2 BAP µM and 0.5 µM IAA (#17). Regenerated plantlets allowed for growth in media supplemented with 1.1 µM IAA for 6 weeks before transfer to soil.

2.6. Date analysis

The experiments carried out using completely randomized design (CRD) with three replications of each experiment and 5-6 explants per replication (depending on stages of experiments). Data collected in four weeks interval, which been mentioned in different steps of culture. Based on morphological response, such as leaf derived callus induction, callus differentiation to shoots and plantlet development data collected and statistically analyzed using SPSS Package. Means with slandered errors of 3 replicates per treatment were compared with control as well as multiple treatment groups. Mean comparisons were performed at $P \leq 0.05$ level of significance using one way analysis of variance (ANOVA) according to Duncan's multiple range test (DMRT) using JMP (Version 9) statistical software.

III. Results And Discussion

3.1. Effect of BAP, IAA, KIN and 2,4-D on callus initiation

The response of young leaf explant culture on Modified MSB5 media supplemented with BAP, KIN, IAA and 2,4-D has presented in Table 1. The presence of individual plant growth regulators (PGRs, BAP, KIN, IAA, 2,4-D) in culture media do not show any response in callus induction, their combinations showed differential responses. The leaf explants showed highly efficient callus induction in presence of 2.2 µM BAP with 1.2 µM IAA and 2.2 µM BAP with 1 µM 2,4-D. Similarly, callus was induced in media supplemented with 2.3 µM KIN with 1.2 µM IAA and 2.3 µM KIN with 1 µM 2,4-D. No callus developed in presence 2.2 µM BAP with 2.3 µM KIN while both callus induction as well as some shoot differentiation observed in media by addition of 1.2 µM IAA together with 2.2 µM BAP and 2.3 µM KIN.

Media	2,4-D µM	IAA µM	KI µM	BAP µM	Response
#1	-	-	-	-	-
#2	-	-	-	4.5	-
#3	-	-	4.6	-	-
#4	-	4.9	-	-	-
#5	4.5	-	-	-	-
#6	-	1.2	-	2.2	Calli
#7	-	1.2	2.3		Calli

#8	1	-	-	2.2	Calli
#9	1		2.3	-	Calli
#10	-	-	2.3	2.2	-
#11	-	1.2	2.3	2.2	Calli, Shoot
#12	1		2.3	2.2	Calli

Table 1. Effect of BAP, IAA, Ki, 2,4-D on *in vitro* response of leaf explant culture on basic MSB5 media after four weeks of culture.

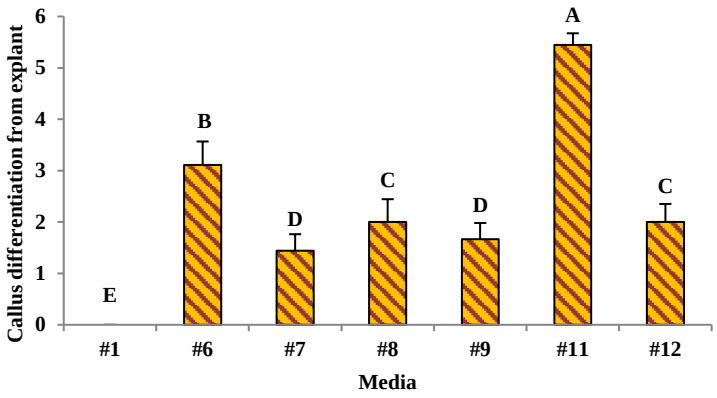

Figure 1. Effect of BAP, IAA, 2,4-D and KIN on *in vitro* explant response in terms of callus differentiation of African daisy after 4 weeks of culture. Results are the mean with standard error of number of young leaf segments responded to develop callus from explant of three sets individual experiments. Means followed by the same number are not significantly different at P≤0.05 according to DMRT.

The highest response of callus induction observed by 90% explants in presence of 2.2 µM BAP, 2.3 µM KIN and 1.2 µM IAA (Figure 1). Differential response of callus induction frequency in presence of different combinations and concentrations of PGRs statistically analyzed. The interactive effects of culture media based on analysis of variance of mean number of callus induction of explants were statistically significant ($P \leq 0.05$) as showed in (Figure 1). The analysis of variance revealed the combinations and concentrations of PGRs significantly affect callus induction frequency of explants. The highest callus induction frequency of young leaves was 5.4 in presence 2.2 µM BAP, 1.2 µM IAA, 2.3 µM KIN after four week of culture (Figure 1) and their interactive effect were statistically significant ($P \leq 0.50$). Different types of explant culture showed high frequency of callus induction and shoot differentiation in presence of auxin and cytokinin combinations in *Polianthes tuberosa* (Sangavia and Chellapandi, 2008), *Salvia Africana-lutea* (Makunga and Staden, 2008), *Phyllanthus amarus* (Sen *et al*., 2009). The shoot initiation as well as increased callus induction capacity observed in presence of BAP and IAA from the nodal segment culture of *Calligonum comosum* (Sadeq *et al*., 2014b) as well as *Leptadenia pyrotectina* (Sadeq *et al*., 2014a), young leaf explant of *Achyramthes aspera* (Sen *et al*., 2014) and *Stevia rebaudiana* (Roy Pathak, 2016).

3.2. Effect of BAP, IAA, KI on shoot differentiation and plant regeneration

The effect of different PGRs on shoots differentiation and plant regeneration compared on basic modified MSB5 media supplemented with different concentration of BAP, IAA and KIN. The flow diagram of Plant regeneration from callus differentiation to plantlet of African following somatic embryogenesis pathway in several steps presented in Figure 2. Leaf derived calli (A) were transferred to various media containing BAP, KIN, IAA for differentiation and showed plant differentiation (B). Development of large number of somatic embryos on the surface of calli observed in presence of 2.2 µM BAP, 2.3 µM KIN, 1.2 µM IAA (D). Differentiation of large number of plants from somatic embryos on the surface of highly compact calli (C, E) showed. Growth of fully regenerated plantlets observed in presence of 2.2 BAP µM and 0.5 µM IAA (F, G) and their further growth in soil (H). The ratio of auxin and cytokinins is an important factor to induce somatic embryogeneis, differentiation of somatic embryos in shoots through embryogenesis (Sharon *et al*. 2012).

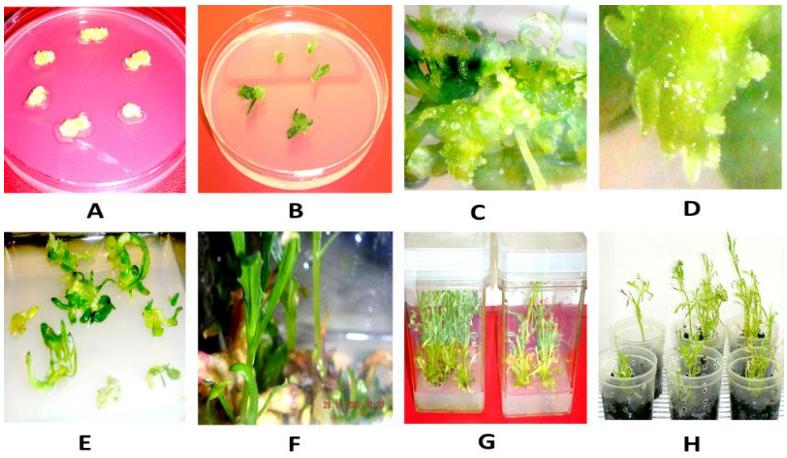

Figure 2. Stages of plant regeneration of African daisy from callus through somatic embryogenesis. A—Leaf derived calli; B—Leaf derived calli showing green spots and plant differentiation; C—Development of large number of somatic embryos and their differentiation into plantlets, D—Somatic embryos developed on the surface of newly developed calli; E— Plant differentiation; F-G—Differentiated plants growing in Magenta box; H—Regenerated plants growing in soil.

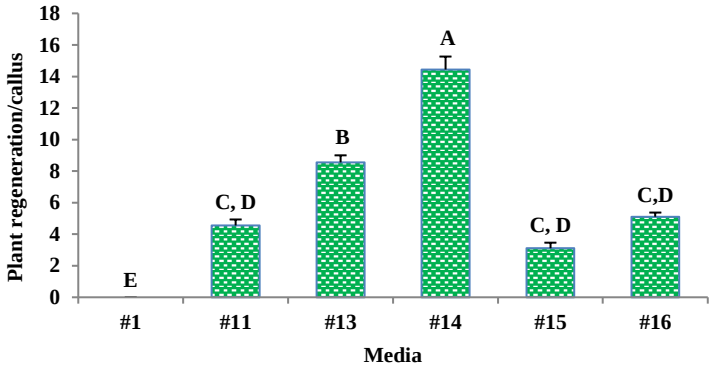

Figure 3. Effect of BAP, IAA, KIN in modified MSB5 culture media on callus differentiation to shoot initiation of African daisy after 4 weeks culture of African daisy. Results are the mean number of shoots regenerated per callus in different media of three set individual experiments. Means followed by the same number are not significantly different at P≤0.05 according to DMRT.

The embryogenic calli of African daisy developed in presence of 2.2 µM BAP, 2.3 µM KIN, 1.2 µM IAA showed differential response in plant regeneration frequency in presence of different concentrations of BAP, KI and IAA (Figure 3).The highest plant regeneration of 15 per callus observed in presence of 0.5 µM IAA, 4.4 µM BAP, 2.3 µM KIN.). Differential response plant regeneration frequency in presence of different concentrations of BAP, KI, IAA were statistically analyzed. The interactive effects of culture media on differentiation of somatic embryos to plantlets performed based on analysis of variance of mean number of plant regeneration per calli with standard error. The PGRs treatments showed statistically significant ($P \leq 0.05$) results in plant differentaion of embryogenic calli (Figure 3). Plant regeneration capacity of African daisy calculated based on plant regeneration frequency of per calli and multiplication of frequency of initially developed callus per explant culture. The highest plant regeneration capacity of was observed when shoot initially developed in media containing 0.5 µM IAA in presence of 4.4 µM BAP and 2.3 µM KIN (Figure 4). Similar effect of BAP and KIN with several auxins in shoot induction reported in *Boerhavia diffusa* (Saini *et al*., 2011) and *Melissa officinalis* (Mohebalipour *et al*., 2012). The interactions of auxin and cytokinin play important role in embryogenic response such embryo differentiation and plantlet regeneration through embryogenesis (Sharon *et al*., 2012; Sadeq *et al*., 2014a).

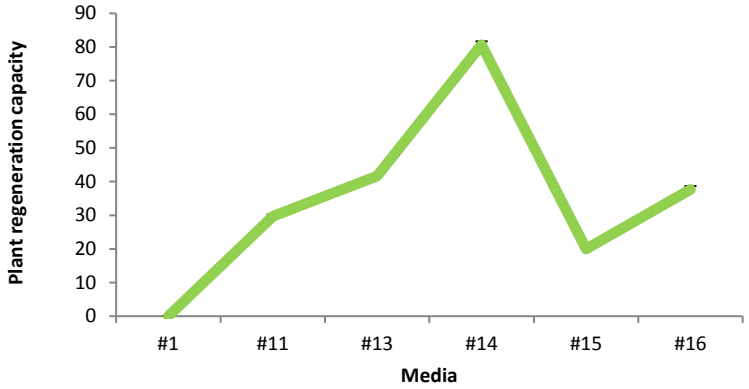

Figure 4. Effect of different plant growth regulators in plant regeneration capacity of African daisy in different culture media.

The proper concentration of KIN and BAP with proper ratio of auxin IAA induced the highest plant regeneration frequency (Figure 2), similarly the highest plant regeneration capacity (Figure 4). The calli developed in media fortified with 2.2 µM BAP, 2.3 µM KIN and 1.2 µM IAA showed dedifferentiation of nonembryogenic callus to embryogenic callus after subculture and developed large number of plantlets with time. Somatic embryogenesis under inductive condition largely influence the plant regeneration frequency where somatic cells develop somatic embryos through a series of morphological and biochemical changes in a rapid way (Komamine *et al*., 2005). Cellular totipotency of somatic embryos regenerated large number of plants in *Oryza sativa* (Roy *et al*., 1996), *Arabidopsis thaliana* (Ikeda-Iwai *et al*., 2002), *Caffea arabica* (Quiroz-Figueroa *et al*., 2002) and medicinal plant *Leptadenia pyrotectina* (Sadeq *et al*., 2014a). Their work also mentioned that the specific region of compact tissue mass showed differentiation of plants from embryogenic callus masses following somatic embryogenesis in presence of higher concentration of cytokines than auxins. In *Rauvolfia serpentine*, the embryogenic callus differentiated into plant development in presence of BAP and IAA (Singh *et al*., 2009). The phytohormones IAA, BAP, KIN are important plant growth regulators in controlling the developmental fate of pluripotent plant cells of explant culture through somatic embryogenesis in this study. The success of this protocol offers highly efficient method of the plant multiplication, which would be beneficial for plant tissue culturist, florist and nursery people where regular supply of plants is a large number required.

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