EUROPEAN ACADEMIC RESEARCH
Vol. VI, Issue 5/ August 2018

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Design of Culture Medium and Leaf Clones are Determinant Factors in Callus Induction of *Calendula officinalis* L.

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Abstract:

In this present investigation, Leaf clones of Marigold, Calendula officinalis L., plant included four segments were used for callus stimulation. Three different types of MS induction media were tested. They were varied in KNO_3 and in sucrose concentrations. Both KNO_3 and sucrose were increased from 1900 to 2000 mgL-1 and from 30 to 32 gL-1 respectively. Data proved that this modifications in design of MS media enhanced callus induction. Also, the results expressed that the different clones exhibit different response for callus induction. C2, and C3 were the most responsive segments since they involved few days to begin callus stimulation compared with C1 and C4 clones.

Key words: MS Modification, Clones type, Callus induction, *Calendula officinalis* L..

INTRODUCTION

Marigold, *Calendula officinalis* L., is a winter annual plant species (Asteraseae), including 20 species of medicinal and ornamental plants of dual use of a high economically

importance (Dole and Wilkins, 2004). Generally callus establishment from explant pass into three development stages, induction, cell division and differentiation. Induction stage required the availability of endogenous hormones changes in cells of explants injured. There is a complex interaction between the plant material, composition of MS medium and environmental conditions during the incubation period which controlled callus induction (Dodds and Roberts, 1985). Many factors affect the frequency of callus formation and plant regeneration from *in vitro* system of callus induction, type of media, pretreatment of panicles, explant condition, genotype and growing conditions of the donor plants such as in rice (Shahnewaz and Bari, 2001) and *Trigonella foenum graecum* L. (Al-Mahdawe *et al*, 2013). Nitrogen is important in homeostasis of plants and therefore determines both of cells and tissues growth, development and yielding. Tissues can benefit nitrogen compounds as nitrate (NO_3^-) and other forms. It was reported that growth conditions determined the relative proportion of nitrogen forms depending on factors of oxygenation, acidity and humidity of growing environments as well (Taylor and Bloom, 1998). In callus induction media each of nitrogen and sucrose sources may significantly affect plant cell proliferation, roots growth and metabolite production (Cui *et al*., 2010, Zhang *et al*., 2011).

Sucrose has been used as a major carbohydrate source in the induction medium, for example, in rice, it was found that increasing the levels of sucrose from 3 to 4% was suitable to promote the induction and growth of callus. But the callus grown in a medium containing more than 5% of sucrose was differentiated into more of albino plants than those produced from low concentration of sucrose (Shahnewaz1 and Bari, 2004).

Type and nature of explants are critical principles in process of callus production, in other words number of cells and their genetic background influenced this process (Davey and Anthony, 2010). Additionally, in some cases explant might contain cells carrying special character which subsequently affect their physiological activities (Taiz and Zeiger, 2002). However, all the aforementioned effects of the culture medium modifications differ among the species, lines and type of cultures. This manuscript aimed to evaluate the response of each leaf clones of Marigold to callus stimulation with manipulation of KNO_3 and sucrose concentrations in induction medium.

MATERIALS AND METHODS

Leaf Clones Preparation:

Young Marigold plants were obtained from nurses in the main campus of university. Twelve weeks old plants grown in pots were transferred to room culture conditions (Fig.1-A). Healthy and fully-expended leaf was excised from plants (Fig.1-B), rinsed with tap water for 15 min., then soaked in solution consist of distilled water and bleaching solution (FAS, Babylon Comp. for detergents, Baghdad) 3:1 V:V ratio for 25 min. on orbital shaker, then washed by sterilized water for three times 3 min. for each (Długosz *et al*., 2018). Surface sterilized leaves were placed on autoclaved filter paper to remove the access of water, then transferred to sterilized 9.0 cm diam. Petri dish (Sterilin, UK) and cut into four parts (Fig.1-C).

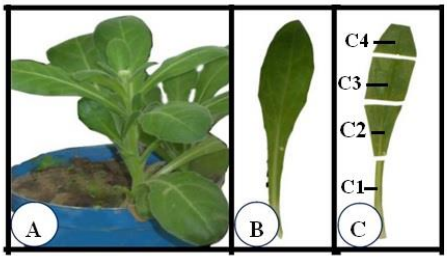

Fig. 1: Preparation of axenic leaf clones of Marigold, *Calendula officinalis.*

A: Intact plant of 12 weeks old grow in soil.

B: Single fully expanded leaf separated from plant in (A).

C: Clones produced from leaf in (B).

MS MODIFICATION:

Three forms of basal MS medium were prepared.

- ✓ Standard MS medium (Murashege and Skoog, 1962).
- ✓ MS medium with KNO_3 increased to 2000 mgL^{-1} (Mohamed *et al.,* 2015)
- ✓ MS medium with sucrose increased to 32 gL^{-1} Bari, (Shahnewazl and ALL, 2014)
- ✓ MS medium + KNO3 2000 mgL-1+ sucrose 32 gL-1.

They were prepared, pH adjusted to 5.8, distributed in conical flasks 250 ml capacity and autoclaved as usual at 121 ˚C for 20 min..

CALLUS INDUCTION:

Each of C1, C2, C3 and C4 were cultured on the above mentioned media, Five samples of each clone were placed on the surface of 20 ml of agar-solidified MS media in 100 ml glass jars. These jars were closed with Aluminum foil and kept in culture room conditions (25 $\pm$ 2 ˚C, 16 h. illumination 2000 Lux

of cool fluorescent tubes).The following media were tested for callus induction: MS0 (Hormone free medium).

MS + 0.1 mgL^{-1} 2, 4-D. ┐
MS + 0.5 mgL^{-1} 2, 4-D. ┘ (Al-Noah, 2013)

The following media were selected in this study:
MS + 0.1 mgL^{-1} BAP.
MS + 0.1 mgL^{-1} 2,4-D + 0.05 mgL-1 BAP.
MS + 0.5 mgL^{-1} 2,4-D + 0.05 mgL-1 BAP.

RESULTS:

The results revealed to the various responding existence of callus induction from C1, C2, C3 and C4 cultured on agar solidified-standard MS callus induction media. During the first week morphological changes were noted on these clones. Generally, C2 was more responsive for callus induction and recorded 100% in some cases (Table 1).

Table 1: Response of leaf clones of Calendula officinalis L. for callus induction on various MS induction media.

Design of media (mgL^{-1})	Callus Induction/ (Day)	Callus Induction (%)			
		Clones			
		C1	C2	C3	C4
MS0	NR*	0.0	0.0	0.0	0.0
MS + 0.1 2,4-D	13	53.8	85.7	76.9	50.0
MS + 0.5 2,4-D	13	66.6	**100**	33.0	50.0
MS + 0.1 BAP	12	80.0	**100**	80.0	83.3
MS + 0.1 2,4-D + 0.05 BAP	13	57.1	72.7	77.7	62.5
MS + 0.5 2,4-D + 0.05 BAP	13	60.0	66.6	63.6	46.1

No. of replicates 5 sample for each.
*N.R. explants not responded.

The time required for stimulation of calli from these clones varied according to the type of media provided with the plant growth regulators (PGR). Twelve days was needed for callus induction achieved, when a harmonious occurred between the medium supplemented with 0.1 mgL^{-1} of BAP and the clones tested. Variations in inducing callus were obvious when each of C1 (Fig. 2-A), C2 (Fig.2-B), C3 (Fig. 2-C) and C4 (Fig. 2-D) were cultured on solid MS medium containing 0.1 mgL^{-1} BAP.

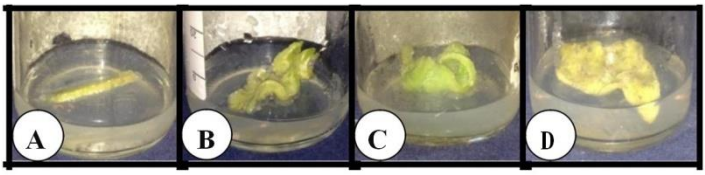

Fig. 2: Capability of clones derived from leaf of *Calendula officinalis* L.to induce callus on MS standard media.

A: C1 cultured on MS medium supplied with 0.1 mgL^{-1} BAP.
B: C2 cultured on MS medium supplied with 0.1 mgL^{-1} BAP.
C: C3 cultured on MS medium supplied with 0.1 mgL^{-1} BAP.
D: C4 cultured on MS medium supplied with 0.1 mgL^{-1} BAP.

The results pointed out the variations in capability of clones to induced callus when cultured on MS media with KNO_3 concentration increased to 2000 mgL^{-1} in the presence of auxin and cytokinin. Response of clones was increased compared to those cultured on MS0, and required fewer days to induce callus (Table 2).

Table 2: Response of leaf clones of *Calendula officinalis* L. for callus induction on MS media with KNO_3 increased to 2000 mgL^{-1}.

Design of media (mgL^{-1})	Callus Induction/ (Day)	Callus Induction (%)			
		Clones			
		C1	C2	C3	C4
MS0	NR*	0.0	0.0	0.0	0.0
MS + 0.1 2,4-D	6	85.7	75.0	87.5	81.8
MS + 0.5 2,4-D	8	50.0	75.0	75.0	**100**
MS + 0.1 BAP	6	75.0	**100**	77.7	75.0
MS + 0.1 2,4-D + 0.05 BAP	6	**100**	**100**	**100**	85.7
MS + 0.5 2,4-D + 0.05 BAP	5	33.3	80.0	75.0	83.3

No. of replicates 5 sample for each. *N.R. explants not responded.

Full response of callus was achieved when agreeable was happened between clones and MS medium supplemented with 0.1 mgL^{-1} 2,4-D interlaced with 0.05 mgL^{-1} BAP (Fig.3-A). Similar results were obtained from C2 (Fig.3-B) and C3 (Fig.3-C) cultured on the same medium, while, MS medium provided with 0.5 mgL^{-1} 2,4-D was convenient to C4 for achieved the complete induction of callus (Fig.3-D).

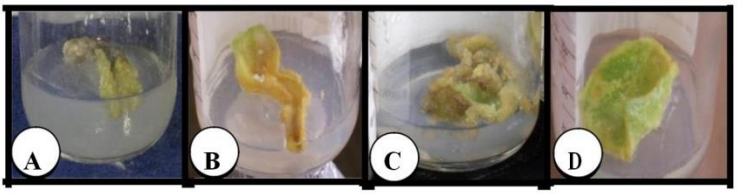

Fig. 3: Capability of clones derived from leaf of *Calendula officinalis* L.to induce callus on MS media with KNO3 increasing up to 2000 mgL^{-1}.

A: C1 cultured on MS medium supplied with 0.1 mgL^{-1} 2,4-D + 0.05 mgL^{-1} BAP.
B: C2 cultured on MS medium supplied with 0.1 mgL^{-1} 2,4-D + 0.05 mgL^{-1} BAP.
C: C3 cultured on MS medium supplied with 0.1 mgL^{-1} 2,4-D + 0.05 mgL^{-1} BAP.
D: C4 cultured on MS medium supplied with 0.5 mgL^{-1} 2,4-D.

Data exhibited high responses of the different leaf clones cultured on solid MS medium containing 32 gL^{-1} of sucrose. It showed that callus induction increased with this increasing of sucrose. Whereas clones cultured on standard MS0 failed to induce callus (Table 3).

Table 3: Response of leaf clones of *Calendula officinalis* L. for callus induction on various MS media containing 3.2 % of sucrose.

Design of media (mgL^{-1})	Callus Induction/ (Day)	Callus Induction (%)			
		Clones			
		C1	C2	C3	C4
MS0	N.R.*	0.0	0.0	0.0	0.0
MS + 0.1 2,4-D	11	40.0	**100**	**100**	**100**
MS + 0.5 2,4-D	11	80.0	**100**	**100**	**100**
MS + 0.1 BAP	7	**100**	**100**	**100**	**100**
MS + 0.1 2,4-D + 0.05 BAP	15	0.0	50.0	50.0	66.6
MS + 0.5 2,4-D + 0.05 BAP	15	**100**	66.6	66.6	33.3

No. of replicates 5 sample for each. *N.R. explants not responded.

Again all clones were achieved complete response when cultured on MS medium supplemented with 0.1 mgL^{-1} BAP only coupled with improvement of phenotypic characters of callus induced from all clones (Fig.4-A, B, C and D).

Additionally, they involved less time for callus induction. While, C2, C3 and C4 gave similar state of callus induction when cultured on MS medium supplemented either with 0.1 mgL^{-1} 2,4-D and the only difference was the number of days required. It was obvious that clones capability for callus induction decreased to 50% as they cultured on MS media provided with combination of 2,4-D and BAP.

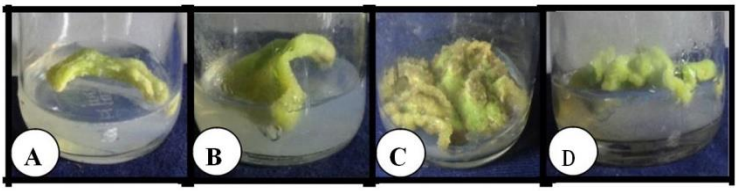

Fig. 4: Capability of clones derived from leaf of *Calendula officinalis* L.to induce callus on MS media with sucrose increasing to 3.2%.

A: C1 cultured on MS medium supplied with 0.1 mgL^{-1} BAP.
B: C2 cultured on MS medium supplied with 0.1 mgL^{-1} BAP.
C: C3 cultured on MS medium supplied with 0.1 mgL^{-1} 2,4-D.
D: C4 cultured on MS medium supplied with 0.1 mgL^{-1} BAP.

The results indicate that response of leaf clones cultured on MS medium containing 2000 mgL^{-1} KNO_3 and 32 gL^{-1} sucrose were varied (Table 4). MS medium and the PGR 0.5 mgL^{-1} 2,4-D was perfectly appropriate for callus induction from C2 and C4. The results clearing that the lowest response was in C1 for all treatments compared to C2,C3 and C4. Except the non-influenced those clones cultured on MS0. All of clones were responded for callus induction, the time required to induced calli varied according to the PGR types and/or their concentrations used.

Table 4: Response of leaf clones of *Calendula officinalis* L. for callus induction on MS media containing of combination of increased concentrations of KNO_3 & sucrose to 2000 mgL^{-1} & 32 gL^{-1} respectively.

Design of media (mgL^{-1})	Callus Induction/ (Day)	Callus Induction (%)			
		Clones			
		C1	C2	C3	C4
MS0	N.R.*	0.0	0.0	0.0	0.0
MS + 0.1 2,4-D	8	78.5	87.5	87.5	80.0
MS + 0.5 2,4-D	8	67.5	**100**	90.0	**100**
MS + 0.1 BAP	5	54.3	92.5	73.2	83.3
MS + 0.1 2,4-D + 0.05 BAP	6	54.1	**100**	83.3	81.2
MS + 0.5 2,4-D + 0.05 BAP	5	76.3	92.8	92.8	**100**

No. of replicates 5 sample for each. *N.R. explants not responded.

High percentage achieved when C1 cultured on MS medium supplemented with 0.1 mgL^{-1} 2,4-D (Fig.5-A) compared to other C1 cultured on MS media supplemented with different PGR. Generally, C2 more responsive for callus induction (Fig.5-B), whereas C3 was not efficient (Fig.5-C). Finally C4 was similar in its response to C2 (Fig.5-D).

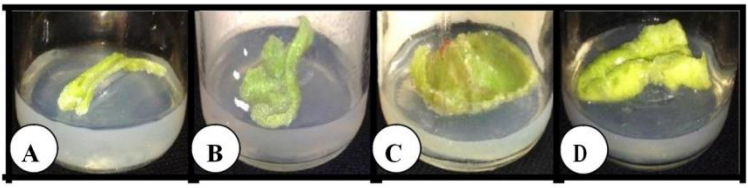

Fig. 5: Capability of leaf derived from clones of *Calendula officinalis* L. to induce callus on MS media containing of combination of KNO3 2000 mgL^{-1} and sucrose 32 gL^{-1}.

A: C1 cultured on MS medium supplied with 0.1 mgL^{-1} 2,4-D .

B: C2 cultured on MS medium supplied with 0.5 mgL^{-1} 2,4-D.

C: C3 cultured on MS medium supplied with 0.5 mgL^{-1} 2,4-D and 0.05 mgL^{-1} BAP.

D: C4 cultured on MS medium supplied with 0.5 mgL^{-1} 2,4-D.

DISCUSSION:

The variations among leaf clones ability to produced callus was expected, this is might due to the number and type of cells in

these clones. Moreover they probably differ in their endogenous hormones (Davey and Anthony, 2010). It seems likely that Calendula officinalis clones rolled a determinant factors in callus production when a compatibility occurs among clones, MS media structure and plant growth regulator (Nikam and Khan, 2014). The same authors pointed out that callusing capability from C. officinalis leaf clones cultured on modified MS medium supplemented with growth regulator affected callus induction process.

Interestingly, increasing of nitrogen as a nitrate (KNO3) and sucrose source sustained callus induction. Nitrate being the main source of mineral nitrogen known to have various influences on plant metabolite synthesis, transport, and distribution as well. Numerous reports indicate that nitrate is a preferred form leading to increase rate of synthesis of important bioactive substances (Długosz et al., 2018). The explanation that sucrose in culture medium functions both as a carbon source and osmotic regulator. Both the functions are critical for callus formation (Last and Brettell, 1990), and embryiod (Shahnewaz1 and Bari, 2004). Sucrose is rapidly hydrolyzed to glucose and fructose, nearly doubling the osmolality of the medium (Taiz and Zeiger, 2002). While nitrogen after the carbon and phosphorus constitutes the basics components needed for plant growth and metabolism.

Plant hormones are common among the most important physiological factors affecting the callus induction of plants *in vitro*. Several studies have been reported regarding the effects of plant growth regulators in callus induction of different plants (Neumman *et al.*, 2009). The major differences in the response of plant parts lie in the internal contents ratio of auxins to cytokinins (Skoog and Millar, 1967). A recent report (Nikam and Khan, 2014) indicated that callusing ability from *C. officinalis* L. leaves cultured on modified MS media

supplemented with plant growth regulator affected callus induction.

ACKNOWLEDGMENT:
Thanks are due to the department of biology, college of Education for Pure Sciences to carry out this work in biotechnology labs.

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