The Bioscan 10(1): 167-171, 2015
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

EFFECTS OF PLANT GROWTH REGULATOR ON *IN VITRO* CALLOGENESIS OF GARDEN CRESS (*LEPIDIUM SATIVUM* L.)

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KEYWORDS
Garden cress
Callus initiation
Plant growth regulators
MS media.

Received on :
04.08.2014

Accepted on :
18.12.2015

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ABSTRACT

Three explants (seeds, shoot and root) of Garden cress (*Lepidium sativum* L.) were used for the standardization of previously reported protocol of callus initiation from seed and shoot explants under *in-vitro* conditions. The various combination of different concentration (0.0, 2.0, 4.0 and 6.0 mg/L) of plant growth regulators {á-naphthalene acetic acid (NAA), 2, 4-Dichlorophenoxyacetic acid (2, 4-D) and Kinetin (Kin)} were used in Murashige and Skoog (1962) medium (MS medium), to analyze the effect on explants of Garden cress for callus initiation. Plant growth regulators, 2, 4-D (4.0 and 6.0 mg/L) and Kin (2.0 mg/L) were found to be the most efficient combinations for calli formation (50 % and 20 % callus initiation from seeds and shoots inoculation, respectively). Globular and pale yellow callus was formed in the MS-medium containing 2, 4-D (4.00 to 6.00 mg/L). Similar trends were also obtained for callus formation from shoot explants using MS-medium containing 2, 4-D (4.00 to 6.00 mg/L) and Kinetin (2.0 mg/L). However, higher concentrations of Kinetin *i.e.*, more than 2.0 mg/L did not show any improvement on callusing and also reduced the callus induction frequency from both explants. Highest amount/ number of callus formation (50 % callus initiation) were observed in culturing of seed in MS medium than the culturing of shoot and root explants. This study may be useful for rapid micropropagation or callus regeneration of Garden cress in *in-vitro* condition in future *Lepidium* improvement programme.

INTRODUCTION

Garden cress (*Lepidium sativum* L.) is an edible with medicinal property and fast growing annual herb belonging to family Brassicaceae (Cruciferae). It is more popular in terms of consumers and producers because of its peppery taste and having health promoting substances such as glucosinolates and sterols (Tuncay *et al.*, 2011 and Wagh *et al.*, 2013). *Lepidium* seed is an important source of iron, folic acid, calcium and vitamins A, C and E. The seed also contains arachidic, linolic fatty acids and rich in protein (2.6 g/100 g), whereas the leaves are an excellent source of vitamin A, C and folate (Su and Arab, 2006; Juma, 2007 and Doke and Guha, 2014).

Now-a-days medicinal plants are important to the global economy, as approximately 80% of traditional medicine preparations involve the use of plants or plant extracts (Dhyani and Kala, 2005). Some of the most useful pharmaceutical compounds like aspirin, artemesinin, quinine, reserpine, digitoxin, diosgenin *etc.* were originally obtained from plants (Mali *et al.*, 2008, Seetharamulu *et al.*, 2012 and Wagh *et al.*, 2013). Pharmaceutical companies largely depend upon material procured from naturally occurring stands which are being depleted rapidly (Alyahya *et al.*, 1994). Pande *et al.* (2002) studied that the micropropagation regenerants showed maximum lepidine from the plantlets at vegetative stage.

Although, regeneration of plants by micro propagation can be achieved from auxiliary bud or shoot tips. The plants can be alternatively regenerated from unorganized callus tissues derived from different explants by dedifferentiation of callus induced by exogenous growth regulators (Aloni *et al.*, 2006 and George *et al.*, 2008). Plant regeneration from calli is possible by somatic embryogenesis or *de novo* organogenesis. Callus is a mass of cells and it is simply used for many cytological, protein and medicinal properties identification *in-vitro* conditions (George *et al.*, 2008 and Eltayb *et al.*, 2010). In spite of its great medicinal value, not much more literatures are available (Saba *et al.*, 2000; Pande *et al.*, 2002 and Eltayb *et al.*, 2010) on micropropagation or *in-vitro* propagation on Garden cress (*Lepidium sativum*). The *in-vitro* products could prove more fruitful than the continuous raw

material available for medicinal use as reported by Pande *et al.* (2002) and Eltayb *et al.* (2010). Thus, over exploitation concerns about possible extinction of the species, provide significant justification for the development of *in-vitro* propagation techniques for this crop.

Therefore, this investigation was emphasized to analyze the effects of plant growth regulators on callus initiation frequency and standardization of the previously reported protocol for callus initiation in Garden cress (*Lepidium sativum*) for rapid micropropagation or regeneration under *in-vitro* condition.

MATERIALS AND METHODS

Collection of explants, sterilization and cultivation conditions

The laboratory experiment was performed following the protocol of Eltayb *et al.,* 2010 with major modification. The experimental site is situated at 17.5ºN latitude, 78.27ºE longitude and altitude of 545 m above the mean sea level. The materials (seeds) were collected from the Department of Ayurveda, Institute of Medical Sciences, B.H.U., Varanasi. Explants (seeds) were washed by continuously running tap water for 3 to 5 minutes followed by washing (thoroughly) with sterile double distilled water thrice. Seeds were surface sterilized with 10 % sodium hypochlorite solution for 7 to 10 minutes under laminar air flow cabinet, then rinsed 3 to 5 times with sterile double distilled water and placed for germination in petri plates on germination paper. Some seeds were also inoculated in the basal MS medium (Murashige and Skoog, 1962) with 12 different combination treatments of plant growth regulators to standardize the combination of growth regulators on callus culture response.

Culture establishment

After 3 to 5 days germination of seeds were observed on germination paper in petri plates and then young seedlings were ready to use as explants for tissue culture works and followed the previous protocol of George *et al.* (2008) and Eltayb *et al.* (2010) with several necessary modifications. These aseptic seedlings were excised and their shoot and root parts were used as explants for callus induction in MS medium supplemented with different plant growth regulators combinations (Table 1 and Fig. 1 a and b). MS medium (Murashige and Skoog, 1962) was supplemented with 30.0 g L^{-1} sucrose (Hi-media, India) and pH of the medium was adjusted to 5.8 by using one or few drops of concentrated hydrochloric acid (HCl) or sodium chloride (NaOH). For solidification of the MS medium, 8.0 g L^{-1} purified agar (Hi-media, India) was used. Seeds and explants were incubated at 25 ± 02ºC temperature, under illumination of 50 µ mol m^{-1} s^{-1}, photoperiod 16/8 h (light/dark)/ light.

Assessment of the effect of auxins and cytokinin on callus induction

For assessing the effect of various concentration (0.0, 2.0, 4.0 and 6.0 mg/L) of auxins on callus initiation, explants (Seeds, shoots and roots part) were cultured in culture bottles containing MS basal media (Murashige and Skoog, 1962) supplemented with different levels of 2, 4-dichlorophenoxyacetic acid (2, 4-D), Naphthalene Acetic Acid (NAA) and Kinetin (Kin) as detail described (0, 2.0, 4.0 and 6.0 mg/ L of 2, 4-D, NAA and combinations of 2, 4-D and Kin) in Table 1.

After inoculation of the explants in the culture bottles, they were incubated in the dark room at room temperature. After 3 to 4 weeks, appearance of tiny creamy greenish and yellowish callus was observed. Callus was sub cultured on fresh MS medium (Murashige and Skoog, 1962) and kept in dark room for callus multiplication.

Observation recorded and statistical analysis

Experiment was repeated thrice and data on callus induction

Table 1: MS media with different combinations of Plant Growth Regulators for callus induction

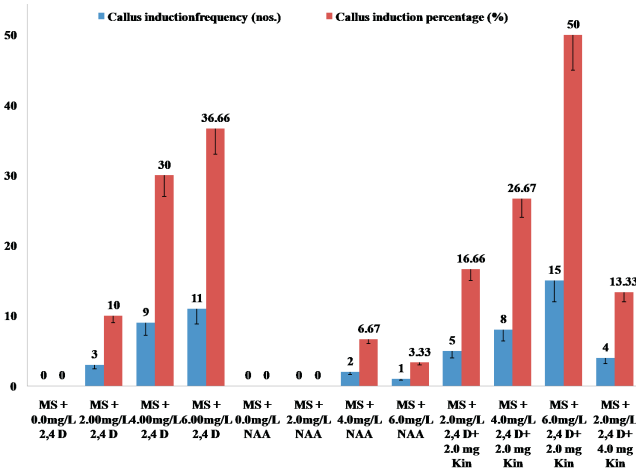
Treatment	Media Combination for callus induction	Treatment	Media Combination for callus induction
T_1	MS + 0.0mg/L 2,4 D	T_7	MS + 4.0mg/L NAA
T_2	MS + 2.00mg/L 2,4 D	T_8	MS + 6.0mg/L NAA
T_3	MS + 4.00mg/L 2,4 D	T_9	MS + 2.0mg/L 2,4 D+ 2.0 mg Kin
T_4	MS + 6.00mg/L 2,4 D	T_{10}	MS + 4.0mg/L 2,4 D+ 2.0 mg Kin
T_5	MS + 0.0mg/L NAA	T_{11}	MS + 6.0mg/L 2,4 D+ 2.0 mg Kin
T_6	MS + 2.0mg/L NAA	T_{12}	MS + 2.0mg/L 2,4 D+ 4.0 mg Kin

Table 2: Effect of plant growth regulators on Callus initiation frequency in Garden cress through seed inoculation.

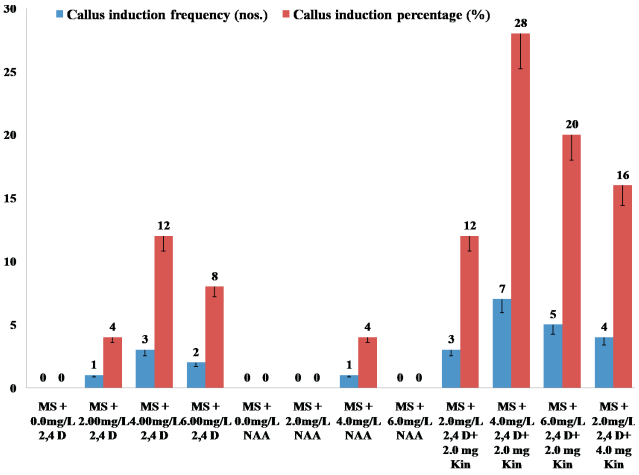
Media combination (Treatment)	Number of seeds placed in culture bottle	Callus induction frequency (nos.)	Callus induction percentage (%)
MS + 0.0mg/L 2,4 D	30	0.00	0.00
MS + 2.00mg/L 2,4 D	30	3.00	10.00
MS + 4.00mg/L 2,4 D	30	9.00	30.00
MS + 6.00mg/L 2,4 D	30	11.00	36.66
MS + 0.0mg/L NAA	30	0.00	0.00
MS + 2.0mg/L NAA	30	0.00	0.00
MS + 4.0mg/L NAA	30	2.00	6.67
MS + 6.0mg/L NAA	30	1.00	3.33
MS + 2.0mg/L 2,4 D+ 2.0 mg Kin	30	5.00	16.66
MS + 4.0mg/L 2,4 D+ 2.0 mg Kin	30	8.00	26.67
MS + 6.0mg/L 2,4 D+ 2.0 mg Kin	30	15.00	50.00
MS + 2.0mg/L 2,4 D+ 4.0 mg Kin	30	4.00	13.33

Table 3: Effect of plant growth regulators on Callus initiation frequency in Garden cress through shoot explants.

Media combination	No. of shoot explants placed in culture bottle	Callus induction frequency (nos.)	Callus induction percentage (%)
MS + 0.0mg/L 2,4 D	25	0.00	0.00
MS + 2.00mg/L 2,4 D	25	1.00	4.00
MS + 4.00mg/L 2,4 D	25	3.00	12.00
MS + 6.00mg/L 2,4 D	25	2.00	8.00
MS + 0.0mg/L NAA	25	0.00	0.00
MS + 2.0mg/L NAA	25	0.00	0.00
MS + 4.0mg/L NAA	25	1.00	4.00
MS + 6.0mg/L NAA	25	0.00	0.00
MS + 2.0mg/L 2,4 D+ 2.0 mg Kin	25	3.00	12.00
MS + 4.0mg/L 2,4 D+ 2.0 mg Kin	25	7.00	28.00
MS + 6.0mg/L 2,4 D+ 2.0 mg Kin	25	5.00	20.00
MS + 2.0mg/L 2,4 D+ 4.0 mg Kin	25	4.00	16.00



Graph 1: Effect of different concentration of plant growth regulators in Garden cress on callus induction frequency from seed inoculation



Graph 2: Effect of different concentrations of plant growth regulators in Garden cress on callus induction frequency from shoot explants inoculation

were recorded after 4 weeks of inoculation. The frequency of callus induction (%) was measured using the formula given by Zaidi *et al.* (2006)

$$\text{Frequency of callus induction}(\%) = \frac{\text{No. of explants induced callus}}{\text{No. of cultured explants}} \times 100$$

RESULTS

Callus formation from seed explants

Sterilized seeds were used as explants for callus initiation of Garden cress in MS medium (Murashige and Skoog, 1962). These sterilized seeds were transferred on fresh media containing plant growth regulators and without plant growth regulators. After transfer of explant in the MS media containing different concentrations of growth regulators (2, 4-D and Kinetin), callus initiation begin from the explants after 4 weeks. The callus size increases with increase in incubation period. The higher concentration (6.00 mg/L) of 2, 4-D in the MS medium induced more callusing as compare to lower concentration (2.00 mg/L) of 2, 4-D where callus induction/ initiation frequency decreases, respectively (Table 1). Callus formation was observed maximum (50 %) on MS media containing 6.0 mg/L 2, 4-D supplemented with 2.0 mg/L Kinetin followed by MS medium containing 6.0 mg/L 2, 4-D alone and 4.0 mg/L supplemented with 2.0 mg/L produced 36.67 and 26.67 per cent callus formation, respectively. NAA alone at lower concentration (2.0 mg/L) did not respond very well to callus formation and at higher concentration, it is responsible for 3.33 per cent callus induction, only. Thus, MS medium containing 6.0 mg/L 2, 4-D supplemented with 2.0 mg/L Kinetin was found best combination for callus initiation from seed explants of Garden cress.

Callus formation from shoot explants

Shoot explants were dissected from germinated seedlings and transferred on MS medium (Murashige and Skoog, 1962) containing various concentration (0.0, 2.0, 4.0 and 6.0 mg/L) of auxins (2, 4-D and NAA) and cytokinin (kinetin) for callus induction. Callus initiation from shoot explants began after 3 to 4 weeks of incubation in same combinations of growth regulators; and seeds of Garden cress were also planted for callus induction to optimize the media combination. The higher frequency of callus initiation 28 % and 20 % callus induction were found in MS medium containing 4.0 mg/L 2, 4-D supplemented with 2.0 mg/L kinetin followed by medium containing 6.0 mg/L 2, 4-D supplemented with 2.0 mg/L kinetin,

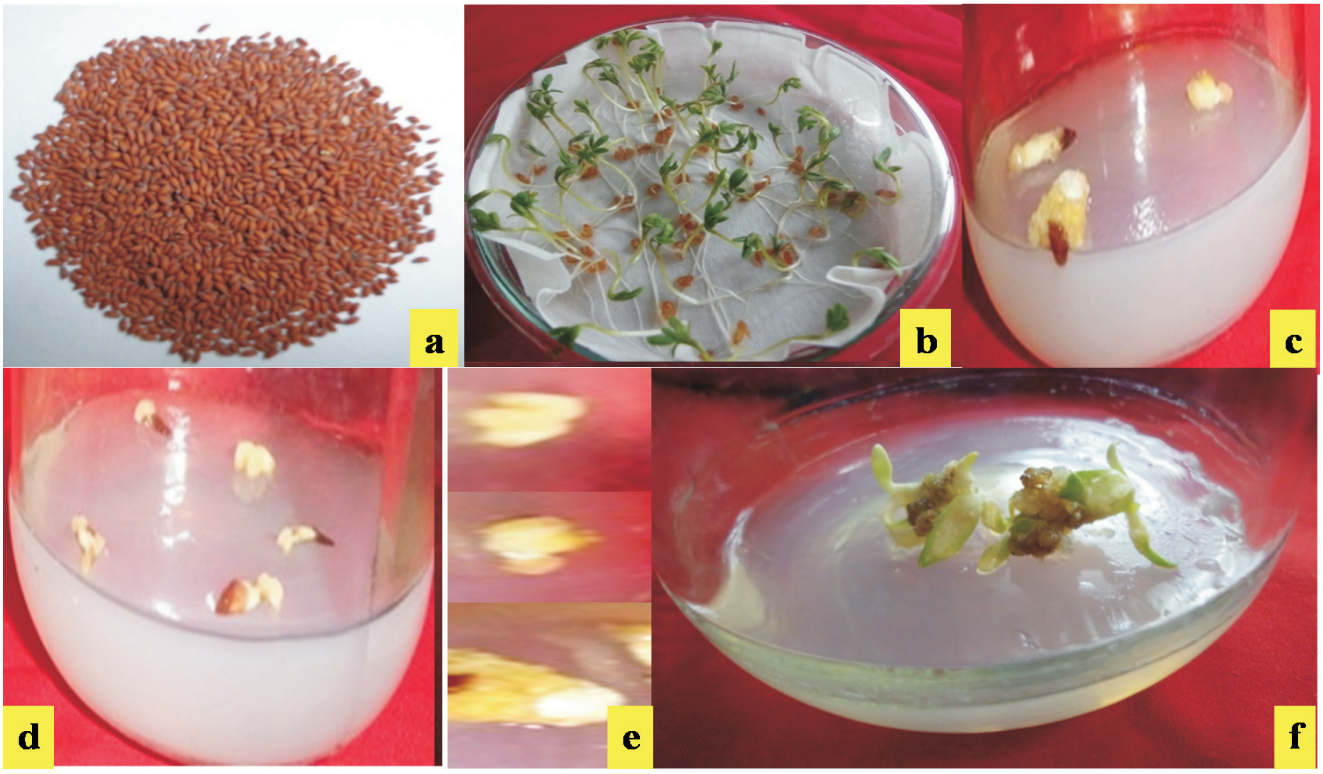


Figure 1: *In vitro* callogenesis in Garden cress (*Lepidium sativum* L.) through seed and shoot explant; 1a. Seeds of Garden cress used in *in vitro* condition for callogenesis; 1b. *In vitro* germination of Garden cress seeds in petri plates on germination paper to collect the shoot explants; 1c. Callus initiation from seeds of Garden cress cultured on MS medium with concentration of growth regulators *i.e.* 6.00 ml/L 2,4-D in Borosil culture bottles; 1d. Calli formation from seeds of Garden cress cultured on MS medium with concentration 6.00 ml/L 2,4-D + 2.00 mg/L kinetin of growth regulators in culture bottles; 1e. Proliferated callus from leave explant of Garden cress cultured on MS medium supplemented with 6.0 ml/L 2, 4-D growth regulator after 4 weeks of culture; and 1f. Calli formation from shoot explants of Garden cress cultured on same medium supplemented with 6.00 ml/L 2,4-D + 2.00 mg/L kinetin in Borosil culture bottle

respectively (Table 1 and Fig. 1d). Calli (12 %) were also induced from shoot explants in medium containing 4.00 mg/L 2, 4-D alone. NAA either at 2.0 mg/L or 6.0 mg/L did not induced callusing from shoot explants (Table 2). Higher concentration of kinetin (more than 2.0 mg/L) in combination with 2, 4-D, did not support more callusing and also reduced callus induction frequency in Garden cress.

DISCUSSION

A number of factors are affecting the callus induction frequency in plant tissue culture from shoot explants. In plant tissue culture, MS medium (Murashige and Skoog, 1962) containing high concentration of sucrose supported the growth of many micro-organisms, such as bacteria, fungi, *etc.* These microbes generally grow faster than the cultured tissue and finally kill it (Almeida *et al.*, 2003). Apart from that, the contaminants may also give out metabolic waste which is toxic to plant tissues for that, several precautionary steps were adopted, such as cleaning of hands, wrists, and forearms with spirit and should not pass the hand or arm directly over a sterile exposed surface, such as open agar plate (see, Saba *et al.*, 2000 and Almeida *et al.*, 2003).

To ensure the possibility of contamination, the sodium hypochlorite solution was used for surface sterilization of Garden cress seed as previously reported by several workers Pande *et al.* (2002); Hisashi and Francisco (2006) and Eltayb *et al.* (2010). The sodium hypochlorite solution upto 8 to 10 per cent were used and found 100 per cent seed disinfection by using 10 per cent concentration of sodium hypochlorite solution. Many other factors like explants, species, cultivar, plant growth regulators and light also affect callus induction, callus growth and callus quality for regeneration. Among these plant growth regulators, auxins and cytokinins are widely used for callus induction and directly influence the callus initiation (Singh *et al.*, 2003 and Eltayb *et al.*, 2010).

In most of the explants of Garden cress, 2, 4-D (auxin) promotes callus induction because it is potential source for plant cell division, cell elongation and vascular tissue differentiation. In our findings, MS medium containing 6.0 mg/L 2, 4-D supplemented with 2.0 mg/L Kinetin was found best combination for callus initiation from seed explants of Garden cress. It means auxin may be useful for the callusing, because it helps in cell division, elongation and many other processes that help to callus induction. Kinetin when present in combination of auxins also promotes callusing in Garden cress because it provides supplementary effects for callogenesis. Similar results were also reported by Eltayb *et al.* (2010).

Although, all normal plant tissue/ cells can produce itself from both the growth regulators auxin and cytokinins but generally young shoot organs are the major parts for auxin (IAA)

production and root tips are major parts for cytokinins synthesis in plants (Aloni *et al.,* 2006). Auxins and Cytokinins seem to be necessary for plant cell division. In plant tissue culture, auxins are broadly used for callus induction and involves in cell division, cell elongation, vascular tissue differentiation, rhizogenesis and root formation, embryogenesis and inhibition of axillary shoot growth (Pande *et al.*, 2002; George *et al.,* 2008; Chawla, 2009; Eltayb *et al.*, 2010 and Park *et al.*, 2010). It seems that auxin causes DNA to become more methylated than usual and this might be necessary for the reprogramming of differentiated cells and make them beginning division (George *et al.*, 2008). The auxin commonly used for callus induction is 2, 4-D, but NAA and IAA are also used by many workers (Pande *et al.*, 2002; Aloni *et al.,* 2006; Hisashi and Francisco, 2006 and Eltayb, *et al.*, 2010) for callus initiation in Garden cress and other plants while cytokinins like Kinetin (Kin), Benzyl adenine (BA) and Thidiazuron (TDZ).

Therefore, it can be concluded that the plant growth regulators, 2, 4-D at higher concentration supplemented with Kin (2.0 mg/L) was found to be the most efficient combinations in garden cress for calli formation from shoot and seed explants. It may be useful for rapid micropropagation or regeneration of Garden cress under *in-vitro* condition in future genetic improvement or transformation programme.

ACKNOWLEDGEMENT

Authors (SS) is highly grateful to the University Grant Commission, New Delhi, India for providing the fellowship and financial assistant during M. Sc. (Ag.) Plant Biotechnology programme at Banaras Hindu University, Varanasi and author (PS) also gratefully acknowledged to the Department of Science and Technology (DST) for receiving of DST-INSPIRE Senior Research fellowship (IF-120215).

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