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BIOLIFE RESEARCH ARTICLE

Effect of Various Growth Hormone Concentration and Combination on Protocol Optimization for In vitro Rapid Propagation of *Withania somnifera*

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ABSTRACT

Withania somnifera(l.) Dunal is commonly known as ashwagandha and belongs to family Solanaceae. The plant is an erect undershrub with long tuberous roots. All the parts of the plants have shown remarkable significance in the field of pharmacology. The major chemical constituents of this plant is withanolide A and D. These are anti-inflammatory and enhance the bodies defence against infections and tumours. Generally leaves and roots are used as drug. Multiple uses of the plant have made it an endangered species. In view of these facts, the study was conducted for micropropagation of Withania somnifera. Murashige and Skoog (MS) media supplemented with different concentrations of 1-naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP) and kinetin (Kin) were used singly and in combinations. Different explants viz. young leaves, axillary shoots, hypocotyls, germinated seedling, root segments and crushed plantlets were used for establishing calli in M S media with varying phytohormonal combinations. Morphogenetic responses were observed only when there were auxins like NAA or 2, 4-D in combination with kinetin. In MS media + NAA (1mg/l) + kinetin (1mg/l to 3mg/l) the calli obtained from young leaves formed as much as 20 shoots. The hypocotyls formed brown callus in MS media+2, 4-D (1mg/l) + kinetin (0.5mg/l).The fresh and dry weight and moisture content data indicated good growth of callus, which is used in further studies of alkaloids production. Due to poor viability of stored seed and a lack of definite protocol for *in vitro* multiplication the present work was undertaken to examine the potential of different explants to respond under *in vitro* conditions with the possibility of developing a protocol for rapid propagation of *Withania somnifera.*

Keywords Groth hormone,Protocol optimization,*in vitro,*callus induction.*Withania somnifera*

INTRODUCTION

W*ithania somnifera* (L) Dunal commonly known as Ashwagandha or Indian ginseng, is an important medicinal plant belongs to family Solanaceae. It is mentioned as an important Ayurvedic drug of India. The plants is grayish in colour with long tuberous roots. All the parts of the plants have shown remarkable importance in the field of pharmacology (Shrivastava 2007). Ashwagandha contains withanolides A and D. Several chemotypes have been found differing in their withanolide content (Tyler et.al. 1981). Leaves and roots of the plant are used as drug and steroidal lactones occur in both. The total alkaloid content in the root of Indian types has been reported to vary between 0.13 and 0.31 percent (Singh and Kumar 1998).

Ashwagandha withanolides are anti-inflammatory and enhance the body's defense against infections and tumour (Jaffer et al. 1988, Devi and Sharada, 1992).Roots are prescribed as medicines for hiccups, several female disorders, bronchitis, rheumatism,

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dropsy, low energy, arthritis and skin diseases. The roots are also used as sedative for senile debility and for the prevention and inhibition of Alzheimer's disease.(Kirtikar and Basu, 1975). Collection of roots parts for pharmaceutical industries caused depletion of this plant from its natural habitat.(Shukla et al,2010). Tissue culture techniques can play an important role in conservation, clonal propagation and qualitative improvement of this medicinally important plant (Sivanesan 2007).

Propagation of ashwagandha is done mainly by seed but seed viability is limited to one year.Further there is lack of definite protocol for *in vitro* multiplication of the species concened, thus, present work was undertaken to examine the potential of different explants to respond under *in vitro* conditions Micropropagation of this plant allows the production of clones at a fast rate and in continuous manner. This work can lead to the development of an efficient protocol for callus induction and other tissues.

Materials and Methods

Collection of explants

Plants of *Withania somnifera* (Source-Sanjay Gandhi Biological Park, Patna) grown in the pot in the departmental garden of Botany, Patna University were used as an experimental material. Different explants viz, young leaves, axillary shoots, hypocotyls, germinated seedlings, root segments and crushed plantlets were used for establishing calli.

Surface Sterilization

Explants were washed with running tap water and then with levolene detergent for 2-3 times in tap water (12 min). Then explants were surface sterilized with 0.1% (w/v) mercuric chloride for 2-3 min, washed 3-4 times with sterilized double distilled water and inoculated on agar-solidified MS media supplemented with different concentrations of NAA or 2, 4-D and Kinetin either alone or in combination. The pH of the medium was adjusted to 5.7-5.9 before sterilization. Cultures were maintained at 20^{o}-25^{o}C under 2000-4000 lux light intensity. Callus was sub-cultured after 25 days on same media

Media Preparation

Murashige and Skoog (MS) basic medium was prepared (Rao et. al. 2004).Standard procedure was followed for the preparation of media (Murashige and Skoog. 1962). The pH of the media was adjusted to 5.8 and heat resistant growth regulators (NAA, 2, 4-D, BAP and KIN) were added to the media prior to sterilization done at 15 lbs for 15 min. All media were solidified with 8g/l agar. After autoclaving further work was done under Laminar Air Flow.

Explant implantation & Culture Conditions

Stem, leaf (about 5 mm in length) and other explants were aseptically prepared and were implanted vertically on MS medium prepared with specific concentrations of BAP,NAA,2,4-D and Kin (0.5-10.0 mg/l) singly or in combination for callus induction. Stock culture, stem and leaf explants were incubated in dark in a culture chamber at 25°C.

For each treatment, 25 tubes were inoculated with desired explants and were incubated under optimal conditions as defined above. The experiments were terminated after an interval of 60 days. Fresh and dry weights of calli were determined from each tube. Mean and its standard error was calculated.

In another set of experiments, the shoot regeneration capacity was scored. The callus obtained from each explant was inoculated into the tubes containing MS medium supplemented with 2, 4-D and kinetin or BAP either alone or in combination. For each treatment at least 25 tubes were inoculated and incubated under optimal conditions of temperature, light and photoperiod as stated above. After an interval of 30 days the experiment was terminated and the data with respect to cultures producing shoots, number of shoots in each sub-culture, height of shoot, number of shoots rooted and root lengths were recorded.

Determination of Callus Fresh Weight

The callus was collected from tissue culture lab and its media were washed with sterile distilled water. They were placed under a fan (on a blotting paper) to remove water and weighed.

Determination of Callus Dry weight

After fresh weight determination, the materials were placed on Petri dishes and kept in an oven for 48 h at 65 °C for drying. Dry weight was weighed with an electronic balance.

Determination of Callus Moisture Content

The moisture content was determined using the fresh and dry weight of callus by the following ways:
A=weight of empty Petridish
B=weight of Petridish with fresh cell material
C=weight of Petridish with dried cell material
Moisture content percentage
=(B−A)−(C−A)/(B−A) x100

Observation and Discussion

Effect of Basal nutrient medium on Morphogenesis of *Withania somnifera*

The young leaves (Fig.01), axillary bud, hypocotyls and root segments, germinated seedlings, crushed plantlets, moistened seeds and seedlings cultured on MS Basal medium showed growth and differentiation as well as morphological changes. They formed calli.

Table-01. Morphological growth during establishment of culture

Sr. No.	Media	Explants	Response	Initiation period of callus	% of success	Nature of callus
1	MS+NAA 2 mg/l	Young leaves	Only rooting	10 days	80	Direct root formation
2	MS+2, 4-D (0.5mg/l)	Young leaves & hypocotyls	Black callus	30-40 days	15	Hard, black callus
3	MS+NAA 2 mg/l + Kinetin (0.5 mg/l)	Young leaves	Friable callus	12 days	70	Soft, oval shaped
4	MS+NAA 1.5 mg/l + Kinetin (1 mg/l)	Young leaves	Callus as well as shoots	15-17 days	40-50	Hard green callus forming single shoot
5	MS+NAA 1 mg/l + Kinetin (1.5 mg/l)	Young leaves	Callus as well as multiple shoots	15-25 days	30-45	Hard black callus formed single shoot
6	MS+NAA 0.5 mg/l + Kinetin (2 mg/l)	Young leaves	No response	-	-	-
7	MS+NAA 1 mg/l + Kinetin (3 mg/l)	Young leaves	Callus and Multiple shoots	20 days	85	1-2cm multiple shoots
8	MS+2, 4-D 0.5mg/l + Kinetin 0.2mg/l + BAP 0.5 mg/l)	Young leaves	Hard callus	15 days	90	Green hard callus with white edges
9	MS+2, 4-D 2mg/l + Kinetin 0.2 mg/l	Axillary bud	Friable callus	6-7 days	90	Soft, oval friable callus
10	MS+2, 4-D 2mg/l + Kinetin 0.2 mg/l	Leaves callus	Soft callus	7th days	80	Round shaped friable callus
11	MS+2, 4-D 2mg/l + Kinetin 0.2 mg/l	Root segments	Soft callus	30 days	100	Soft friable callus
12	MS+2, 4-D 2mg/l + Kinetin 0.2 mg/l	Seedlings	Callus	6-7 days	85	Callus with single shoot
13	MS+2, 4-D 1mg/l + Kinetin 0.5 mg/l	Hypocotyl	Hard callus	28 days	30	Brown colour
14	MS+2, 4-D 2mg/l + Kinetin 0.5 mg/l	Hypocotyl	No response	-	-	-
15	MS+2 BAP 4mg/l + Kinetin 2 mg/l	Crushed plantlets	Callus	12 days	70	Green hard callus
16	MS + NAA 1mg/l + Kinetin 1 mg/l	Seedlings	Friable callus	12-18 days	65	-
17	MS + NAA 1mg/l + Kinetin 2 mg/l	Callus	Shooting & rooting both	20-25 days	75	-
18	MS + BAP 2 mg/l	Seedlings	Friable callus	10-15 days	85	-
19	MS + BAP 2.5 mg/l	Callus	Green callus	11-15 days	70	-
20	MS+BAP 3 mg/l	Callus	Hard callus	30 days	20	-

Table-02 Morphological changes on MS Medium supplemented with combinations of various phytohormones

Sr.No.	Combination of sub culture media	Origin of callus	No. of shoots	Length of shoot (in cm)	Initiation Period (Days)	No. of shoots rooted in%	Length of root
1	MS + NAA 1mg/l + Kinetin 3 mg/l	Young leaves	20	3.0	28	20	2.5-3cm
2	MS + NAA 1mg/l + Kinetin 2 mg/l	Callus	20	3.2 - 3.6	42	100	2.8-3cm
3	MS + NAA 1mg/l + Kinetin 1.5 mg/l	Young leaves	20	3.2 - 3.6	28	20	2.3-2.8cm
4	MS + NAA 1.5mg/l + Kinetin 1 mg/l	Young leaves	20	2.8 - 3.5	38	60	2.5-2.8cm

Effect of MS + Auxin on Morphogenesis of *Withania somnifera*

Young leaves and hypocotyls were placed in MS media containing NAA (2mg/l) showed only rooting, the initiation period was 10 days and success rate was 80%. The same explants inoculated on MS media + 2,4-D (0.5 mg/l), formed black Callus, the initiation period ranged from 30-40 days and success rate was 15%.

Effect of MS media +BAP on Morphogenesis of *Withania somnifera*

The seedling formed friable callus after 10-15 days with 85% success in MS and BAP (2mg/l).The calli were also used as explants and cultured in MS + BAP (2.5mg/l). They proliferated and formed green callus. The initiation period was 11-15 days and success rate was 70%. There was formation of hard callus after 30 days with 20% success in MS + BAP (3mg/l).

Effect of Auxin and Kinetin in combination on Morphogenesis of *Withania somnifera*

Young leaves, axillary buds, hypocotyls, callus obtained from leaves, root segments and seedlings and nodal explants were used as explants to observe the effect of MS media with Auxin and Kinetin in combinations (Table 1).

In MS media with NAA (2mg/l) and Kinetin (0.5mg/l), the young leaves formed friable callus after 12 days. The percentage of success was 70. Callus was soft and it was white in colour (Fig. 2).

In the MS media with NAA (1.5 mg/l) and kinetin (1mg/l), the young leaves formed calli as well as shoots after 15-17 days with 40 to 50%, success. Callus was green and hard. It formed single shoot only.

In the MS media with NAA (1mg/l) and kinetin (1.5mg/l), the young leaves formed callus as well as multiple shoots after 15-25 days but the success rate was 30-45%. The callus was hard and black and formed single shoot.

In the MS media with NAA (0.5mg/l) and kinetin (2mg/l), the young leaves showed no response.Young leaves in MS media with NAA (1mg/l) and kinetin (3mg/l) formed calli and multiple shoots after 20 days with 85% success. The multiple shoots were 1-2 cm.

Axillary bud placed in MS + 2, 4-D (2 mg/l) and kinetin (0.2mg/l) showed friable calli. The initiation period was 6-7 days and success was 90%. The callus was soft, oval and friable.

Hypocotyls placed in MS media containing 2, 4-D (1mg/l) and kinetin (0.5mg/l), showed hard callus. The initiation period was 28 days with 30% success. The callus was brown in colour but hypocotyls placed in same media with changed concentration MS + 2,

Table-03 Fresh and dry weight of Callus

Sr. No.	Media	Explants	Cellus	Fresh weight after a month (in mg)	Dry weight (in mg)
1	MS+NAA 2 mg/l + Kinetin (0.5 mg/l)	Young leaves	Friable callus	988±8.8	130±2.8
2	MS+NAA 1.5 mg/l + Kinetin (1 mg/l)	Young leaves	Callus	884±4.5	104±4.1
3	MS+NAA 1 mg/l + Kinetin (1.5 mg/l)	Young leaves	Callus	892±6.8	110±3.0
4	MS+NAA 1 mg/l + Kinetin 1 mg/l	Seedlings	Friable callus	830±6.2	98±4.2
5	MS+NAA 2 mg/l + Kinetin 0.5 mg/l	Young leaves	Callus	875±2.8	115±2.8
6	MS+2, 4-D 0.5mg/l + Kinetin 0.2mg/l + BAP 0.5 mg/l)	Young leaves	Hard Callus	895±1.4	112±2.3
7	MS+2, 4-D 2mg/l + Kinetin 0.2 mg/l	Axillary bud	Friable callus	729±5.8	121±2.4
8	MS+2, 4-D 2mg/l + Kinetin 0.2 mg/l	Axillary bud	Callus	710±2.8	108±3.2
9	MS+BAP 4mg/l + Kinetin 2 mg/l	Crushed plantlets	Callus	680±4.8	100±3.6
10	MS+2, 4-D 1mg/l + Kinetin 0.5 mg/l	Hypocotyl	Hard Callus	669±5.6	92±2.6
11	MS+2, 4-D 2mg/l + Kinetin 0.2 mg/l	Leaves callus	Soft callus	580±4.1	89±2.4
12	MS+BAP 2mg/l	Seedlings	Friable callus	804±2.6	103±3.1
13	MS BAP 2.5mg/l	Callus	Green	732±1.8	115±2.2
14	MS + BAP 3mg/l	Callus	Hard	682±3.6	92±2.8

4-D (2mg/l) + kinetin (0.5mg/l) showed no response. Calli obtained after culturing young leaves were placed in MS + 2, 4-D (2mg/l) + kinetin (0.2 mg/l) showed soft callus. It was round and friable. The initiation period was 7 days with success rate 80%. Root segment as explants showed soft friable callus within 30 days and success rate was 100%.

The seedlings in same combination and concentrations showed calli with single shoot. The initiation period was 6-7 days and success rate was 85%. Seedlings in MS + NAA (1 mg/l) + kinetin (1mg/l) showed friable callus with 65% success. The initiation period was 12-18 days.

The calli as explants were placed in MS + NAA (1mg/l) and kinetin (2mg/l) showed shooting and rooting both with 75% success. The initiations period was 20-25 days.

Figure-1. Young juvenile leaves is inoculated on M S medium

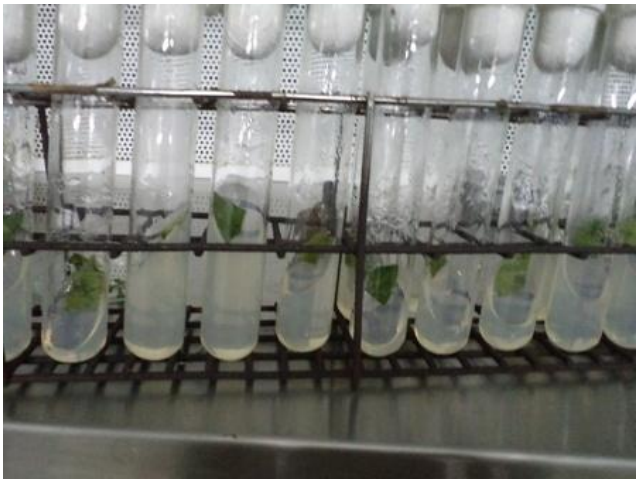

Figure-2. Callus obtained from one of the tubes

Effect of MS + BAP and kinetin on Morphogenesis of *Withania somnifera*

Crushed plantlets were used as explants in MS + BAP (4mg/l) and kinetin (2mg/l) showed morphogenetic response. It formed green hard calli. The initiation period was 12 days with 70% success.

Figure-3. Subculture of calli in flask for better proliferation and for better response

Effect of MS + 2, 4-D + kinetin and BAP on Morphogenesis of *Withania somnifera*

Young leaves were used as explants in MS+2,4-D (0.5 mg/l) + kinetin (0.2mg/l) + BAP (0.5mg/l) formed hard callus. The initiation period was 15 days with 90% success. Callus was green with white edges.

Figure-4 The callus with multiple shoots obtained from hypocotyle

Calli and their fate (Table 02)

Certain amount of calli (3 to 4 gms) were taken from the flask (Fig. 3) and were inoculated in shoot initiation medium and initiation of multiple shoots was

observed (Fig. 4). The shoots were kept for incubation to observe further response at 25°C for 3000 lux intensity.

Further shoots from the multiple shooting were transferred in fresh shooting medium for further proliferation. To increase rooting shoots derived from young leaves and calli as explants were excised and cultured on MS medium with different concentrations of auxins and kinetin. After 12-15 days, post transferred to rooting medium, the roots came out and by 30 days, many were found to be 2.3 – 3 cm long (Fig. 5,6 and 7).

Figure-5 Shoots obtained were transferred in rooting medium

Figure-6 The multiple shoots with callus were cultured separately in shoot multiplication medium

The best rooted plants were transferred to pots having 1:1 ratio of sand and soil, initially covered with beakers and kept at 25 ± 5°C. After 20 days it was transferred to glass house, the survival rate was 80%.

Figure-7. *in vitro* regenerated plants

Fresh and dry weight of calli

Young leaves, axillary shoots, hypocotyls, seedlings and crushed plantlets produced calli in MS medium supplemented with auxins or kinetin alone or in combination. The response was slow or fast. Calli obtained were soft or hard or friable. The calli were taken for fresh and dry weight in mg. The maximum fresh and dry weight of callus were recorded when it was obtained from MS+NAA (2mg/l) + kinetin (0.5mg/l). In this case the callus was friable. The second weight was recorded from hard calli obtained from young leaves cultured in MS+ 2,4 –D (0.5mg/l) + kinetin (0.2 mg/l) + BAP (0.5mg/l). The least fresh and dry weight was recorded in soft calli obtained by culturing calli from young leaves. The details of fresh and dry weight of calli in mg have been presented in Table-03.

The successful callusing from young leaves was also shown in *Physalis pubescens* and its flowerless mutant in MS medium containing BAP and NAA (Rani, Gita and Grover 1999).

Hypocotyls callusing in *Withania somnifera* was noticed between 15-20 days on MS media by Koch (1969) but in our case there was hard brown callus in colour and noticed between 27-28 days on MS + 2, 4-D (1mg/l) + kinetin (0.5 mg/l).

Regenerated shoots obtained from the axillary shoot base callus were rooted based on MS medium containing IAA (2 and 4 mg/l) or IBA (2 mg/l) and IAA (2mg/l). In our case, the rooting was the best with 100% success in multiple shoot obtained from callus as explants in MS media containing NAA (1mg/l) and kinetin (2mg/l).

Root segment was the best in callusing potential but no shoots could be regenerated from this source

of callus even by taking the help of different growth regulators.

To responsiveness of MS basal medium over others may be attributed to high salt concentration. Also the high energy level of MS medium may be responsible for conditioning of the sub-cellular components for yielding the best result by changing the pattern for gene expression (Shah et.al. 2003). The suitability of MS basal medium is due to higher concentration of Ammonium ion. This ion has been found to be essential for proper growth and differentiation of embryos (Benniamin et. al. 2004). The favourable effect of kinetin with auxin in formation of callus or multiple shoots might be due to enhancing the rate of cell division. There are many reports indicating BAP individually promoting organogenesis in plant tissue culture (Street 1996). In *W. somnifera,* however BAP induced callus formation either friable, green or hard.

The cytokinins have been found to increase vascularization which enhances the frequency of shoot differentiation (Bimal 1985). It also helps on nucleic acid synthesis specially RNA which promotes protein synthesis (Kaushal 2004). In Ashwagandha, BAP as growth adjuvant work efficiently in inducing a callus but not the multiple shoot proliferation. The specific concentration is increased, the initiation period is extended with less success. Thus, BAP in 2mg or 2.5mg/l provided the critical level necessary for morphogenetically inducting effect in explants. The superiority of BAP over other cytokinins has been reported in many plants such as *Piper longum, Gymnema sylvestre,* strawberry and *Curila galivides* (Wickson and thimann 1960).

The auxins NAA (2mg/l) showed directly rooting in young leaves and hypocotyls. The initiation period was 10 days and the success rate was 80%. 2,4-D (0.5mg/l) in MS media induced hard black callus formation with initiation period as 30-40 days and with 15% success.

The combination of auxins and kinetin ratio plays a key role in differentiation and organogenesis in plants (Kumar et. al. 2011). It seems that the ratio is critical in inducing mitotic activity in the explants leading to callus formation. In MS media with NAA (2mg/l) nad kinetin (0.5mg/l), the young leaves formed friable callus after 12 days. Callus was soft, oval and white.

The best combination was MS media + NAA (1.5mg/l) + kinetin (1mg/l) where the young leaves formed calli as well as shoots after 15-17 days with 40-50% success. Callus was hard green.

In the MS media with NAA (1mg/l) and kinetin (1.5mg/l), the young leaves formed callus as well as multiple shoots. The callus was hard and black.

Young leaves in MS media with NAA (1mg/l) and kinetin (3mg/l) formed calli and multiple shoots after 20 days with 85% success. The multiple shoots were 1-2 cm.

Axiliary bud placed in MS + 2, 4-D (2mg/l) and kinetin (0.2mg/l) showed friable calli. The succss rate was 90% with 6-7 days initiation period. When the concentration of 2, 4-D is reduced (1mg/l) and kinetin increased (0.5mng/l), there was formation of hard callus by hypocotyls, Initiation period is extended and success rate is reduced. Root segments as explants showed soft friable callus within 30 days and success rate was 100%.

The calli as explants placed in MS + NAA (1mg/l) and kinetin (2mg/l) showed shooting and rooting with 75% success. The initiation period was 20-25 days.

BAP and kientin were tried with,2 4-D with young leaves as explants formed hard callus. The success rate was 90% with 15 days initiation period. Callus was green with white edges.

Thus, it is probable that for any morphogenetic events the auxins:cytokinins value must be specific for each experimental system and hold the key for reprogramming of cells or tissues for *in vitro* morphogenesis.

Rani and Grover (1999) noticed hypocotyls in callusing *Withania somnifera* in MS media with 15-20 days as initiation period but in our case, this callusing was noticed between 27-28 days. They reported the rooting from regenerated shoots from the axillary shoot base callus on MS medium containing IAA (2 and 4mg/l) or IBA (2mg/l) and IAA (2mg/l). In our case, the rooting was the best with 100% success in multiple shoots obtained from callus as explants in MS media containing NAA (1mg/l) and kinetin (2mg/l).

Root segment was the best in callusing potential but no shoots could be regenerated from this source of callus even by taking the help of various growth regulators.

In terms of number of shoots and culture steps, it is significant to note the multiple shoots as well as calli in MS medium with NAA (1.5mg/l) + kinetin (1mg/l) or NAA (1mg/l) + kinetin (1.5mg/l). Multiple shoots can also be obtained from young leaves in MS media + NAA (1mg/l) + kinetin (3mg/l).

MS + NAA (2mg/l) induced directly rooting in young leaves as explants. Most of the workers have reported rooting in presence of IAA or IBA. Nath Sangeeta and Burgohain Alak (2005). They reported maximum number of roots in MS media without auxin. These reports suggest that there is a sufficient level of endogenous auxins in the tissues (Shukla et.al. 2010) and Chaurasia Pratibha et al (2015).

The variation observed in the present investigation may be attributed due to the differences in culture conditions and the age of explants. The fresh and dry weight, and moisture content show good growth of callus.

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Compliance with Ethical Standards:

There is no involvement of ethical issues regarding the work.

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Conflict of Interests:

There is no conflict of interest among the authors. All authors have contributed in study and making the literature. authors declare that there is no conflict of interests regarding the publication of this paper.

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