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Research Article

OPTIMIZATION OF *IN VITRO* REGENERATION PROTOCOL FOR *HELIANTHUS ANNUUS* CV. MORDEN

Nilesh Shirish Wagh[1], Rahul Lahu Chavhan[2] and [*]Gajanan Balasaheb Zore[1]
[1]*School of Life Sciences, SRTM University, Nanded (M.S.), India*
[2]*Department of Plant Biotechnology, Vilasrao Deshmukh College of Agricultural Biotechnology, Latur (M.S.), India*
**Author for Correspondence*

ABSTRACT
Present study was aimed at developing an efficient *in vitro* regeneration system for Sunflower cultivars susceptible to Sunflower Necrosis Virus (SNV) disease. SNV susceptible cultivars are recalcitrant in nature and thus development of efficient and rapid regeneration system to obtain SNV resistant varieties through genetic transformation are being optimized. Out of the four varieties tested in this study best response i.e. 100% germination was shown by *Helianthus annuus* cv. Morden within 48 h. Three different explants obtained from *in vitro* germinated seedling were used for organogenesis potential (shoot, root) and callus induction on twenty-five different combinations of PGR in MS media. Primordial meristem explants was found best for shoot and root regeneration i.e. organogenesis. It has shown best shoot (1 cm long and 0.27 cm thick) and root (10.11±0.60/explant) development in MS13 and MS11 media, respectively within 108 h. Hypocotyl segments could be induced in callus more efficiently compared to other explants as it has shown best callus (0.90 cm) in MS20 within 108 h. Significance of our study is that, we have optimized a protocol for successful, *in vitro* regeneration, organogenesis (shoot and root) and callus induction in recalcitrant and SNV susceptible variety of *H. annuus* cv Morden.

Keywords: *Sunflower Regeneration, SNV Susceptible, cv. Morden Regeneration, Shoot Regeneration, Root Regeneration*

INTRODUCTION
Helianthus annuus L. an annual sunflower is herbaceous C_3 plant (2n=34), member of Compositae family (Fiore *et al.*, 1997; Moghaddasi, 2011; Sujatha *et al.*, 2012). It is regarded as one of the most important oilseed crop grown worldwide (Fiore *et al*., 1997; Moghaddasi, 2011; Sujatha *et al*., 2012). Sunflower seeds are known for their high oil and protein content and thus used in confectionary and animal feed (Gürel and Kazan, 1998; Shin *et al.*, 2000). Sunflower litter has been reported to be allelopathic to weeds; having inhibitory activity on the germination response, density and percent ground cover of weeds (Leather, 1983). Sunflower, useful in all respects, accounts for nearly 5% of current oilseed production, and cultivated in an area of 1.48 million hectares with a production of 0.9 million tonnes (Sardaru *et al.*, 2013). However due to several factors like limited germplasm, disease susceptibility, seed dormancy, limited research resources, recalcitrance and other abiotic factors causes heavy economic losses due to decreasing yield (Wingender *et al.*, 1996; Dhaka and Kothari, 2002; Moghaddasi, 2011; Sujatha *et al.*, 2012).
Extensive efforts are in progress to develop high yielding varieties of sunflower with enhanced oil and protein content, disease and pest resistance, less recalcitrance and dormancy in recent years to meet the increasing demands (Moghaddasi, 2011; Müller *et al.*, 2001). Until recently attempts for sunflower improvement relied on the conventional breeding methods but genetic transformation methods became the method of choice (Moghaddasi, 2011; Sujatha *et al*., 2012). Due to limited use of wild species owing to the natural genetic barriers in the reproduction process, and the issues of self incompatibility, the conventional approach fails to achieve the perspective of sunflower improvement (Fiore *et al.*, 1997; Gürel and Kazan, 1998). Thus the application of genetic transformation tools for the improvement in all the prospects of nutritional, technological and agricultural qualities of sunflower varieties becomes the foremost and efficient alternative (Mohmand and Quraishi, 1994; Gürel and Kazan, 1998). However,

successful application of gene transfer technique relies solely on a reliable, reproducible *in vitro* regeneration protocol applicable on diverse sunflower genotypes (Hewezi *et al*., 2003; Moghaddasi, 2011; Sujatha *et al*., 2012).

Several successful reports are available describing sunflower regeneration protocols using different explants like cotyledons of mature seed (Greco *et al.*, 1984; Brar and Roberts, 2006; Sujatha *et al.*, 2012), shoot tips or embryonic axes (Elavazhagan *et al.*, 2009; Malone-Schoneberg *et al.*, 1994; Paterson, 1984), hypocotyls (Lupi *et al.*, 1987; Mohmand and Quraishi, 1994; Müller *et al.*, 2001; Sujatha *et al.*, 2012), somatic embryogenesis from immature embryos (Finer, 1987; Prado and Berville, 1990; Jeannin *et al.*, 1995; Lucas *et al.*, 2000; Dagustu *et al.*, 2010), leaves (Greco *et al*., 1984; Lupi *et al*., 1987; Paterson, 1984), protoplasts (Guilley and Hahne, 1989; Fischer *et al.*, 1992; Henn *et al.*, 1998), anthers (Mohmand and Quraishi, 1994; Thengane *et al.*, 1994), and unpollinated ovaries (Badea *et al.*, 1989).

Despite of these protocols, sunflower *in vitro* regeneration faces hindrances like recalcitrance, culture conditions, explants type, source and genotype, low rates of regeneration efficiency, difficulties in rooting, malformations in morphogenesis, premature flowering, vitrification of shoots, and hyperhydricity in cultures (Lupi *et al.*, 1987; Alibert *et al.*, 1994; Sarrafi *et al.*, 1996; Baker and Carter, 1999; Mayor *et al.*, 2003; Abdoli *et al.*, 2007).

Even the time interval required from the establishment of *in vitro* germination of seeds to the hardening of the regenerated plantlets in greenhouse is also an important factor adding to the problems in devising an efficient and rapid sunflower regeneration system. Moreover in the same genotype and similar laboratory conditions, difference may be observed in the division frequencies of the sunflower cultures (Guilley and Hahne, 1989; Santos and Caldeira, 1998).

Hence, optimizing a new regeneration protocol for any genotype, not responding on earlier protocols becomes a necessity.

Optimizations and changes in the cultural conditions, medium composition, careful observations of the cultures for response, and wise choice of explants like use of juvenile tissues such as embryonic meristem and primordial leaves have favored to achieve genotype independent regeneration of many recalcitrant species in sunflower (Sarrafi *et al*., 1996; Shin *et al*., 2000).

The present study aimed at developing an optimized, efficient and time saving *in vitro* regeneration system in Sunflower Necrosis Virus (SNV) susceptible cultivars of sunflower. The SNV susceptible cultivars are routinely used in field studies for comparative screening of SNV resistant varieties. However, these SNV susceptible recalcitrant cultivars were considered better for devising an efficient and rapid regeneration protocol that can help in genetic transformations to develop SNV resistant varieties. This could also help in optimizing Virus Induced Gene Silencing (VIGS) studies for functional genomics studies in SNV susceptible cultivars of sunflower. Moreover, this is an attempt to establish an efficient regeneration system in sunflower that can reduce the time for obtaining primary regenerants.

MATERIALS AND METHODS

Optimization of Rapid Germination and Shoot Regeneration Protocol

Seeds of sunflower cv. Mordern, CMS-17B, LSF-08, and LTR-07 susceptible to Sunflower Necrosis Virus (SNV) were obtained from the Oilseed Research Station, Latur. Seeds were rinsed thoroughly with distilled water for 10-15 min, washed with Labolene®-detergent for about 10 min, and rinsed several times with distilled water until all the foam was washed away. Seeds were then rinsed in Bavistin®-fungicide (1 gm L^{-1}), for 20 min and washed 3-4 times with distilled water.

Seeds then transferred to sterile distilled water, rinsed in 70% Ethanol for 2 min followed by surface sterilization in 2.5% Sodium hypochlorite solution (4% commercially available chlorine bleach) for 20 min with vigorous shaking.

The seeds were then treated with antibiotic solution of Streptocyclin®-antibiotic (50 µg ml^{-1}) for 1 min followed by 3-4 washings of sterile distilled water and finally kept for imbibition in sterile distilled water for a period of 48 h in dark at a temp of 4^{o}C. After 48 h, seeds were transferred to fresh sterile distilled water, rinsed again with 2.5% sodium hypochlorite for 5 min, followed by treatment of Streptocyclin®

for 30 sec. and finally rinsed 3-4 times in sterile distilled water. Seed coats were then removed before inoculating them in test tubes containing 5 ml half strength MS medium (Himedia Ltd, Mumbai, product code-PT051-25L and PL022-1×50ML) without any PGR (Plant Growth Regulator).
After 36 h inoculation at 25±2^{o}C in dark and further 12 h in light, germinated seeds were excised and cut in such a way that three types of explants were obtained; 0.5-1 cm hypocotyls, intact cotyledons, and embryonic meristem-primordial leaf.

The explants were cultured on 25 different PGR combinations of 6-BAP, Benzyl Aminopurine or Benzyl adenine (0.0, 2.22, 4.44, 6.66 and 8.88 µM L^{-1}) and NAA, Naphthalene acetic acid (0.0, 1.34, 2.69, 4.03 and 5.37 µM L^{-1}).

Nine explants per treatment were used with 3 explants in each culture bottle of volume 250 ml containing 30 ml culture medium and the experiment was repeated thrice.

Cultures were incubated at 25±2^{o}C in a 16 h photoperiod provided by white fluorescent light (100 µE $m^{-2}s^{-1}$) for a period of maximum one week.

Results were recorded in terms of response of the explants to form shoot, root or callus after 0, 72 and 108 h of incubation.

The differences in the length and thickness of the explants at 0 h of inoculation to 108 h after inoculation were considered while evaluating the growth response.

Measurements were taken at 0 h, 72 h and 108 h after inoculation and the differences were calculated. Statistical analysis was carried out for further analysis.

In vitro Response of Different Genotypes for Germination

Seeds from four different cultivars susceptible for the Sunflower Necrosis virus i.e. cv. Morden, CMS-17B, LSF-08, and LTR-07 were analyzed for their *in vitro* germination response.

The seeds were surface sterilized as per the protocol optimized above and inoculated on half strength MS medium with 30 g L^{-1} sucrose, without any plant growth hormone, and gelled with 0.7% agar at pH 5.8. Seeds were allowed to germinate as per conditions optimized and the germination response of the seeds under *in vitro* conditions was recorded after 48 h of incubation.

Further the varieties showing 100% germination response after the end of 48 h and under same cultural conditions were only selected for further study.

In vitro Response of Different Explants for Shoot Regeneration Capacity
Three types of explants generated from the germinated seeds of sunflower were cultured on MS medium with 25 different treatment combinations of PGR under same cultural conditions to regenerate shoots. Observations were recorded at 0, 72 and 108 h after inoculation for the response in terms of increase in length and thickness or swelling of the explant.

Further steps to obtain complete shoots or regenerated plantlets were not done.

RESULTS AND DISCUSSION

In vitro Germination and Genotypic Response
Four different cultivars of Sunflower seeds showed variable response to *in vitro* germination on half MS media. A maximum germination response (100%) was recorded in a cv. Morden after 48 h (Figure 1 (A), 1 (B), 1 (C)). Thus it was selected for the further studies.

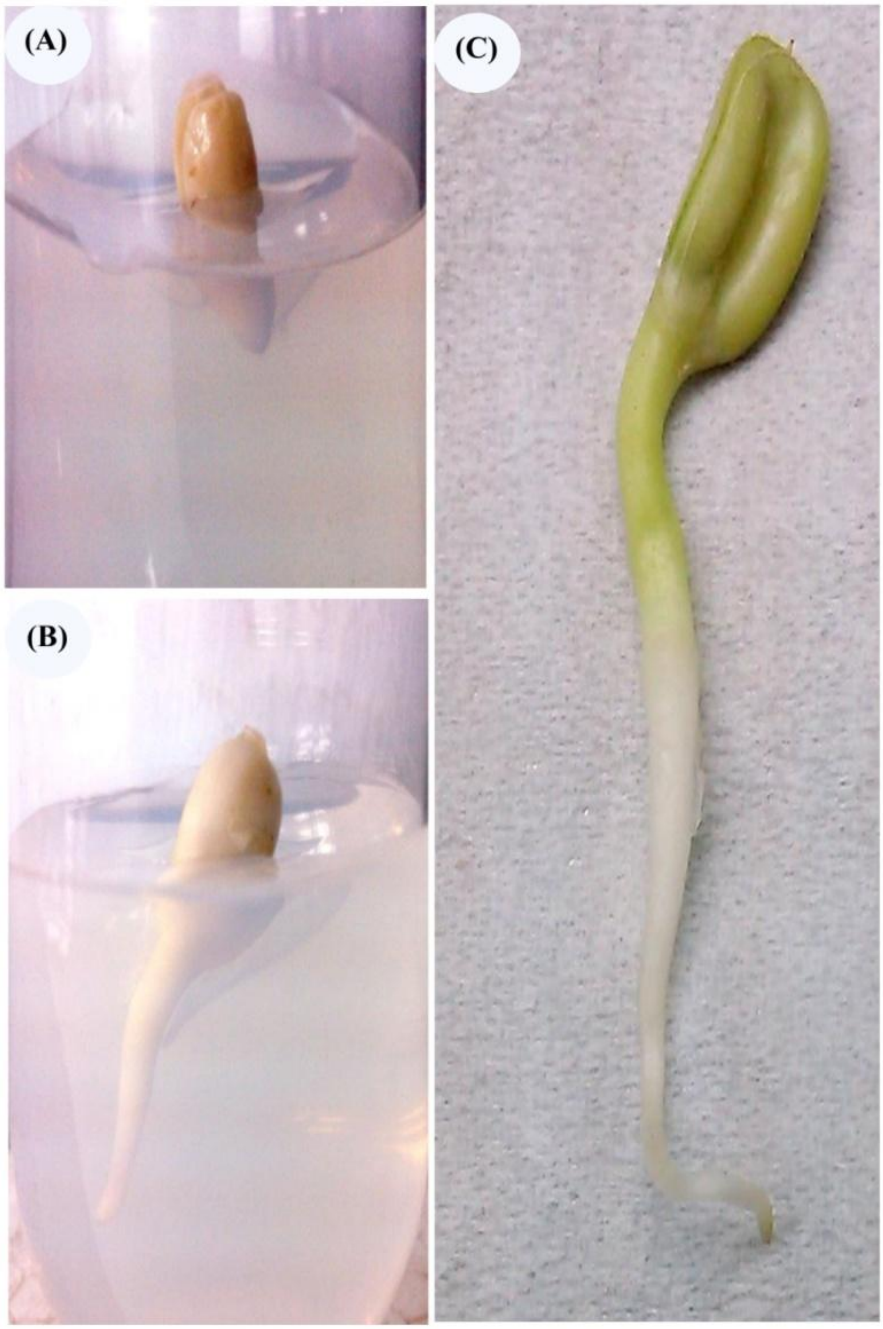

Figure 1: Developmental stages during *in vitro* germination in sunflower variety cv. Mordern. (A) Inoculated sunflower seed without seed coat on half MS media. (B) Inoculated sunflower seed after 36 h of incubation in dark condition. (C) Inoculated sunflower seed after further 12 h incubation in light condition

Shoot Regeneration Potential

Shoot regeneration potential of different explants (viz. primordial meristem, hypocotyl and cotyledons) obtained from *in vitro* germinated seedlings was tested using twenty five different combinations of PGR in MS media. *In vitro* regeneration potential of the explants i.e. development of shoot, root and callus was measured using the criteria like length and thickness of shoot and roots from 0 h to 108 h (Table 1, 2 and 3).

Table 1: Response on Shoot Length and Thickness (Mean ± SD) cm

Explant	BAP (µM L^{-1})	0.0		2.22		4.44		6.66		8.88	
	NAA (µM L^{-1})	Thickness	Length	Thickness	Length	Thickness	Length	Thickness	Length	Thickness	Length
Primordial	0.0	0.05±0.03	0.82±0.13	0.11±0.05	0.93±0.07	0.13±0.03	0.67±0.09	0.21±0.06	1.08±0.10	0.38±0.06	0.76±0.07
Meristem/	1.34	0.06±0.02	0.64±0.05	0.08±0.05	0.50±0.07	0.21±0.05	0.60±0.09	0.30±0.13	0.41±0.06	0.32±0.02	0.34±0.07
Shoot tip	2.69	0.25±0.03	0.51±0.08	0.25±0.11	0.79±0.08	0.27±0.02	1.04±0.09	0.46±0.05	0.64±0.05	0.51±0.10	0.51±0.06
	4.03	0.13±0.06	0.44±0.05	0.18±0.07	0.42±0.07	0.31±0.05	0.41±0.08	0.50±0.02	0.58±0.07	0.63±0.03	0.34±0.05
	5.37	0.11±0.04	0.42±0.04	0.29±0.04	0.41±0.03	0.31±0.12	0.38±0.04	0.69±0.05	0.39±0.06	0.84±0.09	0.29±0.06
Hypocotyl	0.0	0.01±0.01	0.50±0.02	0.08±0.10	0.90±0.02	0.15±0.01	0.75±0.03	0.17±0.08	0.63±0.01	0.26±0.02	0.49±0.03
	1.34	0.10±0.03	0.16±0.04	0.19±0.04	0.21±0	0.17±0.07	0.10±0.03	0.34±0.03	0.05±0.03	0.32±0.07	0.03±0.01
	2.69	0.09±0.03	0.17±0.05	0.18±0.06	0.25±0.01	0.39±0.17	0.37±0.02	0.48±0.05	0.29±0.06	0.32±0.05	0.02±0.01
	4.03	0.24±0.10	0.04±0.02	0.30±0.03	0.15±0.01	0.48±0.04	0.12±0.01	0.32±0.03	0.13±0.05	0.90±0.06	0.49±0.03
	5.37	0.05±0.02	0.10±0.02	0.30±0.19	0.24±0.09	0.29±0.24	0.23±0.03	0.21±0.14	0.08±0.02	0.60±0.28	0.23±0.03
Intact	0.0	0.29±0.07	0.25±0.02	0.75±0.05	0.88±0.09	0.54±0.03	0.44±0.02	0.74±0.09	0.44±0.06	0.31±0.03	0.34±0.0
Cotyledon	1.34	0.34±0.06	0.84±0.11	0.96±0.01	0.23±0.02	1.03±0.04	0.96±0.14	0.50±0.04	0.74±0.03	0.87±0.02	0.30±0.06
	2.69	0.30±0.05	0.33±0.01	0.19±0.08	0.32±0.01	0.61±0.06	0.80±0.10	0.20±0.02	0.60±0.04	0.30±0.04	0.54±0.04
	4.03	0.12±0.03	0.18±0.06	0.20±0.03	0.30±0.04	0.09±0.03	0.08±0.05	0.39±0.01	0.96±0.15	1.38±0.04	0.69±0.09
	5.37	0.15±0.07	0.23±0.08	0.50±0.07	0.22±0.04	0.06±0.06	0.95±0.13	0.20±0.04	0.17±0.06	0.54±0.11	0.33±0.14

Table 2: Response on root formation using PM explant (Mean ± SD) cm

NAA (µM L^{-1})	BAP (µM L^{-1})				
	0.0	**2.22**	**4.44**	**6.66**	**8.88**
0.0	3.22±0.67	0	0	0	0
1.34	7.78±0.44	0	0	0	0
2.69	10.11±0.60	0	0	0	0
4.03	5.22±0.67	0	0	0	0
5.37	0	0	0	0	0

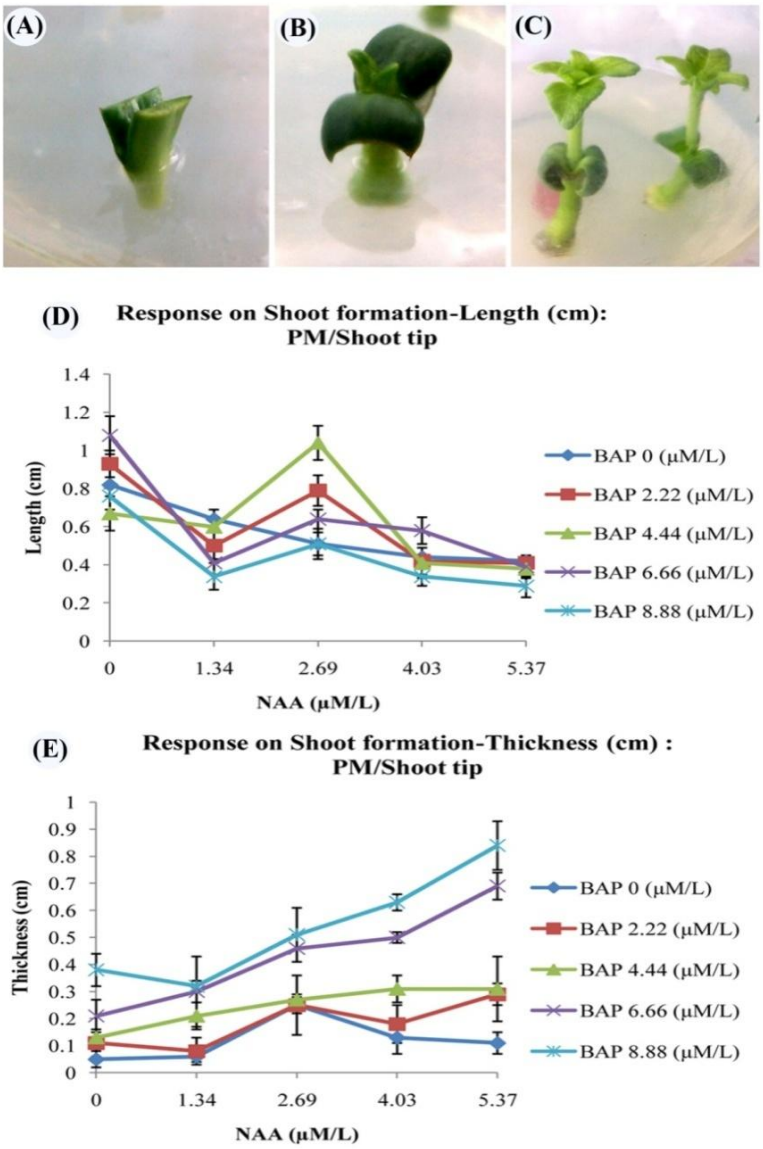

Figure 2: Developmental stages during *in vitro* shoot regeneration from Primordial Meristem (PM) / Shoot tip explant in sunflower variety cv. Mordern. (A) Inoculated Primordial Meristem (PM) / Shoot tip explant on MS13 medium. (B) Inoculated PM explant after 108 h of inoculation on MS13 medium. (C) Inoculated PM explants after incubation for 12 days from inoculation on MS13 medium. (D) And (E) Increase in the length and thickness of the Primordial Meristem/shoot tip explant in response to NAA and BAP combinations respectively. Each point is the Mean ± Standard deviation of the difference of the measurements before and after 108 h of incubation

Table 3: Response on Callus formation using PM and Hypocotyl explants: Thickness (Mean ± SD) cm

Explant	NAA (µM L^{-1})	BAP (µM L^{-1}) 0.0	2.22	4.44	6.66	8.88
Primordial	0.0	0	0	0	0	0.38±0.06
Meristem/	1.34	0	0	0	0	0.32±0.02
Shoot tip	2.69	0	0	0	0	0.51±0.10
	4.03	0	0	0	0.50±0.02	0.63±0.03
	5.37	0	0	0.31±0.12	0.69±0.05	0.84±0.09
Hypocotyl	0.0	0	0	0	0	0.26±0.02
	1.34	0	0	0	0	0.32±0.07
	2.69	0	0	0	0	0.32±0.05
	4.03	0	0	0.48±0.04	0.32±0.03	0.90±0.06
	5.37	0	0	0.29±0.24	0.21±0.14	0.60±0.28

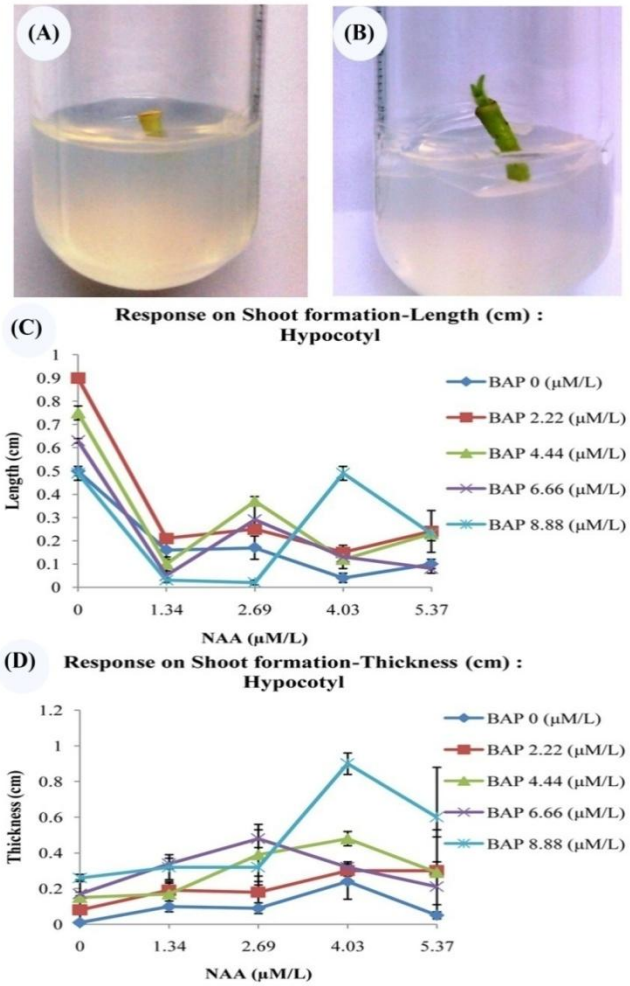

Figure 3: Developmental stages during *in vitro* regeneration from hypocotyl explant in sunflower variety cv. Morden. (A) Inoculated hypocotyl explant on MS2 medium. (B) Hypocotyl explant inoculated on MS2 medium after 108 h of incubation. (C) And (D) Increase in the length and thickness of the Hypocotyl in response to NAA and BAP combinations respectively. Each point is the Mean ± Standard deviation of the difference of the measurements before and after 108 h of incubation

Primordial meristem (PM) / Shoot tip explant showed best response towards the shoot regeneration as all the explants (100%) regenerated in to shoot within 108 h (Figure 2(A), 2(B), 2(C)). All the PM explants regenerated in single adventitious shoots with varying lengths (0.29 to 1.08 cm) and thickness (0.05 to 0.84 cm) (Figure 2(D), 2(E)).

Best response of PM explants towards the shoot regeneration was observed in MS13 (NAA: 2.69 µM L^{-1}, BAP: 4.44 µM L^{-1}). It showed development of 1 cm long and 0.27 cm thick, healthy shoot as response of other combinations was differential i.e. more length with thinner shoots or vice versa e.g. Increasing BAP concentrations lead to increase in length but decreased thickness forming a very thin and delicate shoot inappropriate for further culture manipulations. Response of hypocotyl explants towards the shoot regeneration using different media combinations was less compare to PM as increase in concentrations of BAP and NAA increased initiation of callus formation. Best response was observed in MS2 i.e. BAP (2.22 µM L^{-1}) alone which showed a shoot with 0.75 and 0.18 cm length and thickness respectively within 108 h (Figure 3(A), 3(B)). Hypocotyl segments showed very poor shoot regeneration response in MS12, MS13, MS14, MS17, MS18 and MS19 (Figure 3(C), 3(D)). Cotyledon explants did not show any shoot regeneration up to 108 h, however initiation of shoot formation was observed in MS12, MS13, and MS14 media indicating very poor and delayed response. Similar responses were reported in earlier studies by Greco (1984). In general, PM explant was found to be the best one for shoot regeneration compared to hypocotyl and cotyledons.

Root Regeneration

PM explants were found best for root regeneration as well, as it has shown very good root regeneration response on MS media with NAA alone (i.e. in MS1, MS6, MS11, and MS16). Media combinations inducing root formation inhibited regeneration of shoots. Results of NAA with BAP i.e. no root formation were consistent with earlier observations of Paterson (1984) and Gurel and Kazan (1998). MS11 medium induced maximum number of roots per explant (10.11±0.60) indicating MS11 is the best combination of root induction using PM explants. Hypocotyl and cotyledon explants could not regenerate in to roots in all the media combinations used in this study.

Callus Formation

Response of callus initiation was measured by measuring the thickness of explants i.e. increase in thickness is proportional to increase in response. Initiation of callus formation was observed in PM explants in the media combination MS5 with the maximum BAP (8.88 µM L^{-1}) without NAA. It showed increased response with increasing concentrations of NAA i.e. increase in NAA up to 4.03 µM L-1 and 5.37 µM L^{-1} reduced BAP requirement from 8.88 µM L^{-1} to 6.66 µM L^{-1} (callus of 0.5 cm) and 4.44 µM L^{-1} (callus of 0.3 cm) respectively. However best response was observed in the MS25 containing maximum concentrations of both the PGR (NAA: 5.37 µM L^{-1} and BAP: 8.88 µM L^{-1}). It showed callus of 0.84 cm thickness. Hypocotyl explants also showed similar response towards callus induction however best response was observed in the MS20 containing (NAA: 4.03 µM L^{-1} and BAP: 8.88 µM L^{-1}). Cotyledons could not be induced towards the callus initiation in all the media combinations used in this study.

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