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RESEARCH ARTICLE

CUMINUM CYMINUM L.: DEVELOPMENT OF PLANT REGENERATION PROTOCOL *IN VITRO*

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ABSTRACT

An efficient plant tissue culture methodology plays a significant role in genetic improvement associated with important regulatory genes. The present investigation was undertaken to develop a tissue culture system for cumin variety GC-2. Hypocotyl as an explant was inoculated into media supplemented with various concentrations of plant growth regulators for callus induction, proliferation, shooting and rooting. The MS (Murashige and Skoog) media with 0.1 mgL^{-1} BA (Benzylaminopurine) gave rise to callus induction, whereas upon increasing the concentration of BA (1 mgL^{-1}) callus proliferation was observed. The growth regulator free B5 basal media supported shoot and root induction simultaneously.

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INTRODUCTION

Cumin (*Cuminum cyminum* L.) diploid (2n = 14), traditionally well-known spice and medicinal plant belongs to family Apiaceae. It is widely grown in tropical regions, primarily in India, Iran, Egypt, China and Pakistan (Lodha and Mawar, 2014). India is prime producer as well as exporter to Japan, UAE, Brazil, UK, US, etc. The global demand of cumin is fulfilled by India with over 90% of total produce coming from Gujarat and Rajasthan states (Divakar and Anandraj, 2013). But the produce from crops is always influenced by various biotic and abiotic stresses during the developmental phase of plants. On the other hand, plants belonging to Umbeliferae family have slow; difficult and time-consuming breeding programs (Hunault *et al.* 1989) Seed dormancy is also one of the constraints for cultivation. The major drawbacks of the cumin plants are low genetic diversity and narrow genetic base, which cannot be developed by conventional breeding programs. Therefore, genetic improvement through transgenic plant production *via* tissue culture approach offers a great progression in obtaining superior plants which are stress tolerant (Aruna and Sivaramakrishnan, 1996). Numerous genetically modified crops containing desirable gene have been developed through transgenic approaches (Joshi *et al*., 2013, Tiwari *et al*., 2015, Pandey et al., 2016). The prerequisite for a transformation experiment is fully efficient tissue culture system (Hussain *et al*., 2011) which could develop rapid and pathogen free plants (Averre and Gooding, 2004). Somaclonal variation in *in vitro* raised plants may offer exceptional opportunities for growing new varieties of plants (Malik *et al*., 2004). In crop plants, tissue culture has been employed for creating useful variations such as: salt tolerance, drought tolerance, disease resistance, increased protein content in grains and enhanced nutrient biofortification (Khokhar *et al*., 2016). The plant regeneration system depends on numerous important aspects such as medium composition, use of the growth regulators, gelling agent, photoperiod quality and intensity, temperature and culture vessels (Reed, 1999; Praveen and Rama Swamy, 2011, Hussain *et al*., 2013; Khan *et al*., 2014; Abbassi *et al*., 2011). A proficient tissue culture system necessitates a suitable starting material (explant) and growth regulators. The optimal level of growth regulators (cytokinins and auxin ratio), in culture media plays a significant role in deciding the fate of further development from explant (whether triggering callus (indirect) formation or organ (direct) such as shoot and root development) (Jabeen *et al*.,2005; Sheeja *et al*., 2004; Gubis *et al*., 2004; Hussain *et al*., 2011; Hussain *et al*., 2013, Jan *et al*., 2015). The available *in vitro* regeneration in cumin reports the use of plant growth regulators.Beiki and his coworkers reported use of combinations of BAP-benzylaminopurine (0.0, 0.1 mgL^{-1}), NAA-naphthaleneacetic acid (0.2, 0.4, 0.6 mgL^{-1}) and IAA-indole-3-acetic acid (0.2, 0.4 mgL^{-1}) for three Iranian cumin landraces. Deepak *et al*., 2014 used BAP (1.0 mgL^{-1}) and NAA (4.0 mgL^{-1}) for callus development; kinetin (0.5 mgL^{-1}) and IAA (1.0 mgL^{-1}), BAP (0.1 mgL^{-1}), NAA (1.0 mgL^{-1}) with 25

mgL^{-1}adenine sulfate showed efficient shoot bud and regeneration of plant from callus. Ebrahime *et al*., 2007 investigated that the combination 0.1 mgL^{-1}BA and 1 mgL^{-1}NAA was most effective combination for direct shoot proliferation from node explant. They also showed that low concentration of BAP (0.1 mgL^{-1}) had positive effect on shoot proliferation. Safarnejad showed highest callus induction inB5 medium with combination 0.2 mgL^{-1} NAA and 0.2 mg L^{-1} BAP. B5 media supplemented with 0.2 mgL^{-1} NAA, 0.2 mgl^{-1} BAP and free from any growth regulator were the best treatments for regeneration in *cuminum setifolium*. Twafik and Noga, in their studies, showed callus induction from hypocotyl and leaf portion with 4µM 2,4-D alone or with 2 or 4 µM kinetin. 0.5 or 1.0 µM kinetin showed development of plumules with and without roots. Also, half strength media with 1 µM indole-3-butyric acid (IBA) and 2% polyethylene glycol (PEG, 6000) were used for root induction. Soorni and coworkers proposed 1 mgL^{-1} 2, 4 D as best treatment for callus induction in leaf explant. With results from all aforementioned research, present investigation was designed for developing the *in vitro* regeneration protocol for cumin landrace GC-2 (variety susceptible to blight and wilt, suitable for late sowing season, with yield potential of 6.22q/ha and better grain quality) (Parsashar *et al*., 2014). The main aim in developing this protocol was to utilize it in gene transformation studies, which would help to develop resistance against biotic and abiotic stress and improve quality as well as yield parameters.

MATERIALS AND METHODS

For the objective, an experiment conducted in Plant tissue culture laboratory, Anand Agriculture University, Anand.

Preparation of culture medium:

Plant material: The seeds were collected from "Centre for Research on Seed Spices", Sardar Krushinagar Dantiwada Agricultural University, Jagudan, (Dist.: Mehsana) Gujarat. The seeds were surface sterilized using 0.1% mercuric chloride solution for 1 min and were rinsed three times with sterile distilled water. Seeds were then cultured on basal B5 media supplemented with half strength of macro- and micro-elements, vitamins and sucrose for 20 days at 4°C in dark for germination. The hypocotyls and cotyledons from the seedlings were cut into small segments of length 5 mm and were used as explants (Valizadeh and Tabar, 2009). The plant growth regulator, BAP (benzyl adenine purine) (0.05, 0.1, 0.2, 0.4, 0.8 and 1 mgL^{1}) with B5 (Gamborg medium, 2008, half strength) media and MS (Murashige and Skoog, 1962) was prepared and dispensed into test tubes (Borosil, India) using a Masterflexdigi-static auto dispenser. The media in test tubes were then sterilized by autoclaving at 121˚C at 15 psi pressure for 15 min. The pH of the medium was adjusted to 5.7 ± 0.1 prior to autoclave.

Callus induction and multiplication: The hypocotyl explants were inoculated on half strength B5 media supplemented with 0.05, 0.1, 0.2, 0.4, 0.8 and 1 mgL^{-1} BA, 2% Sucrose and 0.9% Agar (media composition for the initiation of cultures were adapted from Ebrahime *et al*., 2007). All the cultures were maintained at 25 ± 1 ^{0}C, relative humidity 60-70% and illumination intensity 3500 lux from 2.5 feet wide fluorescent tube (40 watt) placed at the level of cultures. The cultures were subjected to alternate 16-hour photo and 8-hour dark period.

Shoot initiation and Root induction: Callus proliferated cultures were transferred to half strength B5 media supplemented with BA (0, 0.1mgL^{-1}) and NAA (0, 0.1, 0.5, 1, 1.5 mgL^{-1}), 2 % Sucrose, pH 5.8 and 0.9% Agar (adapted from Safarnejad, 2011). All the cultures were transferred to fresh medium after every 21 days interval.

Statistical Analysis: The data obtained from the observations were recorded for callus induction, callus proliferation, shoot proliferation and root induction and were subjected to one - way analysis of variance (ANOVA) in the excel sheet using completely randomized design.

RESULTS

The objective of the study was to develop an indirect *in vitro* regeneration system for cumin plants efficient for transformation experiments in lab (Pandey *et al*., 2013, Pandey *et al.,* 2016), for production of disease-free plants and their use in various fundamental research programs (Ebrahimie *et al*., 2007).

Callus induction and multiplication: Hypocotyls were excised from GC-2 genotype 10 days old, *in vitro* raised seedlings, as a source of explant. The explants were inoculated on MS basal media with different concentration of BAP (Table 1, fig 1). The data showed that 0.1% BA concentration was sufficient to induce callus induction in period of approximately two months (nearby 69 days) with highest callus frequency of 91.18%, and semi friable callus structure. Further, for callus multiplication among various concentration of BAP (0.05, 0.1, 0.2, 0.4, 0.8, 1.0 mgL^{-1}), 1.0 mgL^{-1} showed highest proliferation around 93 % (+++) with semifriable callus structure and yellow green white color (Table 2, fig 2). The results are in accordance with the study carried out by Ebrahimie and coworkers, who recorded the callus induction in cumin with 0.1 mgL^{-1} BA. It was postulated that BAP (cytokinin) remarkably induces callus formation *via* blocking direct regeneration pathways (Ebrahimie *et al*., 2003, 2006, 2007).

Shoot induction and root induction: With B5 medium, shoot induction from callus was observed in about 27 days after transferring it to growth regulator free medium. Also, our present study reports the simultaneous root induction on the same B5 media (Table 3, Fig 3). The data of shoot induction is supported by Safarnejad (2011) studies, who reported that MS media was good for callusing but failed to give further regeneration. Other previous studies on cumin regeneration also stated that callus proliferation and shoot elongation occur on transfer to basal media without growth regulators (Tawfik and Noga, 2002; Wakhlu *et al.,* 1990). The overview of the cumin regeneration protocol *in vitro* is shown in fig. 4. Although, the developed protocol was executed effortlessly, but few problems were encountered which are needed to be taken into consideration in future studies. Problems, such as, vitrification *i.e* glassy appearance of plants (fig 5 (i)), is caused due transfer of water from liquid phase into amorphous phase (Fahy *et al*., 1984). It poses a serious threat to shoot multiplication and culture vigour (Hammerschlag, 1986). Vitrified plants encounter chlorophyll a and b deficiency, hyperhydricity, hypertrophy, lack of cell wall lignifications and have leaves with large intercellular spaces in between spongy mesophyll and palaside cells (Pasqualetto, 1990).

Table 1. Effect of different concentration of benzyl adenine purine (BAP) on callus induction from hypocotyl in cumin

Sr. No.	Media Composition	Days to callus induction (Mean ± S.Em.)	Days to callus completion (Mean ± S.Em.)	Callus frequency (%) (Area of explant covered with callus)	Callus proliferation	Callus color	Callus Structure
1	MS + 2% Sucrose + 0.9% Agar	-	-	-	-	-	-
2	MS + 2% Sucrose + 0.05 mgl^{-1}BA + 0.9% Agar	-	-	-	-	-	-
3	MS + 2% Sucrose + 0.1 mgl^{-1}BA + 0.9% Agar	69.50 ± 0.66	90.33 ± 0.30^{c}	91.18	++	YGW	SF
4	MS + 2% Sucrose + 0.2 mgl^{-1}BA + 0.9% Agar	92.66 ± 0.18	138 ± 0.19^{a}	71.66	++	YG	NF
5	MS + 2% Sucrose + 0.4 mgl^{-1}BA + 0.9% Agar	93.33 ± 0.21	134 ± 0.11^{a}	64.89	+	YG	NF
6	MS + 2% Sucrose + 0.8 mgl^{-1}BA + 0.9% Agar	76.16 ± 0.47	110.12 ± 0.18^{b}	89.02	++	YG	SF
7	MS + 2% Sucrose + 1.0 mgl^{-1} BA + 0.9% Agar	86.00 ± 0.13	120.00 ± 0.45^{b}	52.11	++	YG	NF

G – Green, Y – Yellow, PY – Pale Yellow, W – White, NF – Non friable, F – friable, SF – Semi friable Data are in the form of mean ± SE '+'= 25% of culture vessel diameter, '++'= 50% of culture vessel diameter, '+++'= 75% of culture vessel diameter, '++++'= Full size of culture vessel diameter

Table 2. Effect of different concentration of benzyl adenine on callus multiplication in cumin

Sr. No.	Media Compositions	Callus frequency (%) (Area of explant covered with callus)	Callus proliferation	Callus color	Callus Structure
1	MS + 2% Sucrose + 0.9% Agar	16.90^{d}	+	YG	NF
2	MS + 2% Sucrose + 0.05 mgl^{-1}BA + 0.9% Agar	54.86^{c}	+	YG	NF
3	MS + 2% Sucrose + 0.1 mgl^{-1}BA + 0.9% Agar	87.28^{a}	+++	YG	SF
4	MS + 2% Sucrose + 0.2 mgl^{-1}BA + 0.9% Agar	59.68^{c}	+++	PG	NF
5	MS + 2% Sucrose + 0.4 mgl^{-1}BA + 0.9% Agar	68.66^{c}	++	YG	NF
6	MS + 2% Sucrose + 0.8 mgl^{-1}BA + 0.9% Agar	78.28^{b}	+++	YG	NF
7	MS + 2% Sucrose + 1.0 mgl^{-1}BA + 0.9% Agar	93.33^{a}	+++	YGW	SF

G – Green, Y – Yellow, PY – Pale Yellow, W – White NF – Non friable, F – friable, SM – Semi friable '+'= 25% of culture vessel diameter, '++'= 50% of culture vessel diameter, '+++'= 75% of culture vessel diameter, '++++'= Full size of culture vessel diameter

Table 3. Effect of different media combinations on shoot regeneration from callus in cumin

Media Composition	Days to shoot initiation	Callus color	Callus intensity	No. of shoots	Length of shoots (cm)	No. of roots	Length of shoots (cm)	Remark
B_5 + 2 % Sucrose + pH 5.8 + 0.9% Agar	26.83 ± 0.17	GY	+	10.16 ± 0.46	4.66 ± 0.6	4 ± 0.15	3 ± 0.34	Many plants had vitrification problemin later stages
B_5 + 0.1 mgl^{-1} BA + 2 % Sucrose + pH 5.8 + 0.9% Agar	-	G	++	-	-	-	-	Callusing
B_5 + 0.1 mgl^{-1} NAA + 2 % Sucrose + pH 5.8 + 0.9% Agar	-	GW	++	-	-	-	-	Callusing
B_5 + 0.1 mgl^{-1} BA + 0.1 mgl^{-1} NAA + 2 % Sucrose + pH 5.8 + 0.9% Agar	-	WG	+++	-	-	-	-	Callusing
B_5 + 0.1 mgl^{-1} BA + 0.5 mgl^{-1} NAA + 2 % Sucrose + pH 5.8 + 0.9% Agar	-	GY	+++	-	-	-	-	Callusing
B_5 + 0.1 mgl^{-1} BA + 1.0 mgl^{-1} NAA + 2 % Sucrose + pH 5.8 + 0.9% Agar	-	YG	+++	-	-	-	-	Callusing
B_5 + 0.1 mgl^{-1} BA + 1.5 mgl^{-1} NAA + 2 % Sucrose + pH 5.8 + 0.9% Agar	-	Y	++	-	-	-	-	Callusing

G – Green, Y – Yellow, PY – Pale Yellow, W – White, data are in the form of mean ± SE '+'= 25% of the explant, '++'= 50% of the explant, '+++'= 75% of the explant, '++++'= Full size of the explants

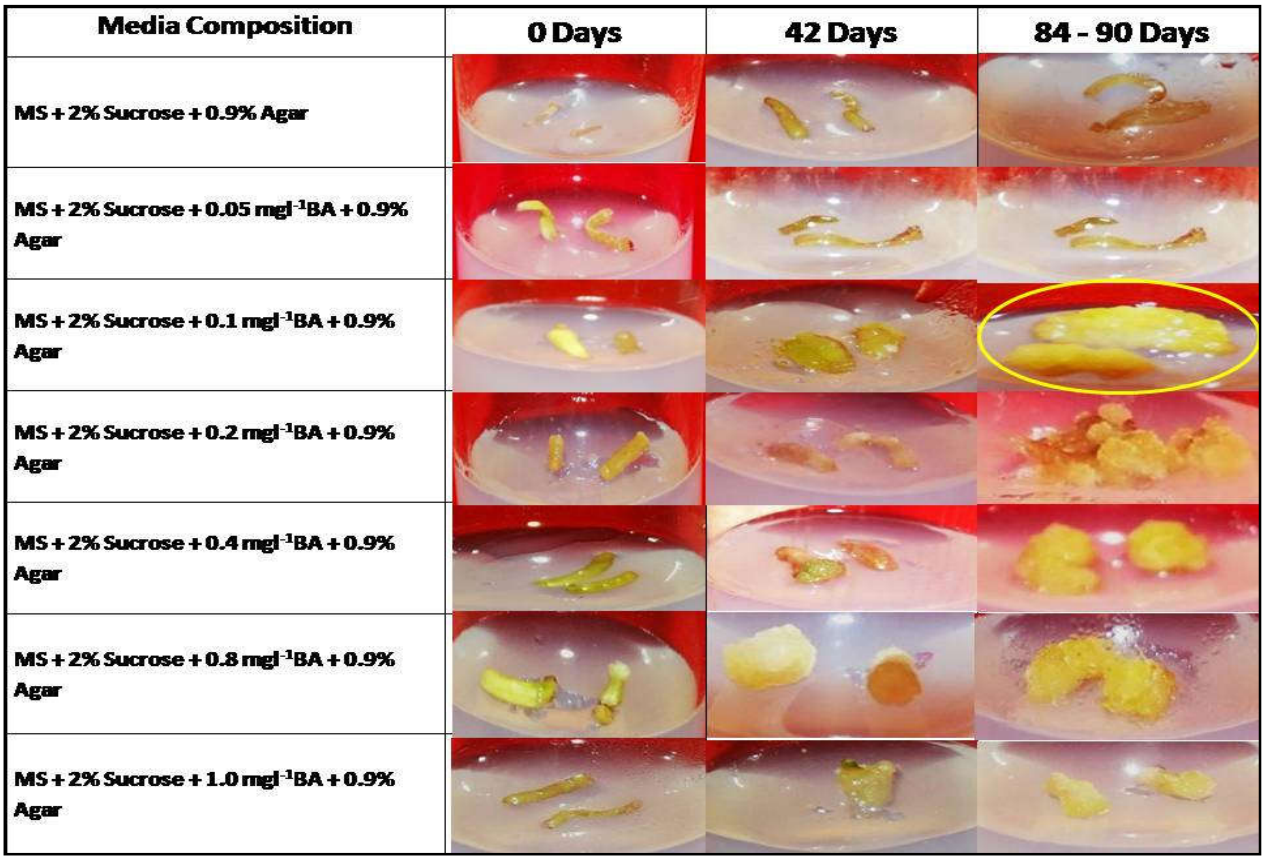

Fig 1 Callus induction in hypocotyl explants

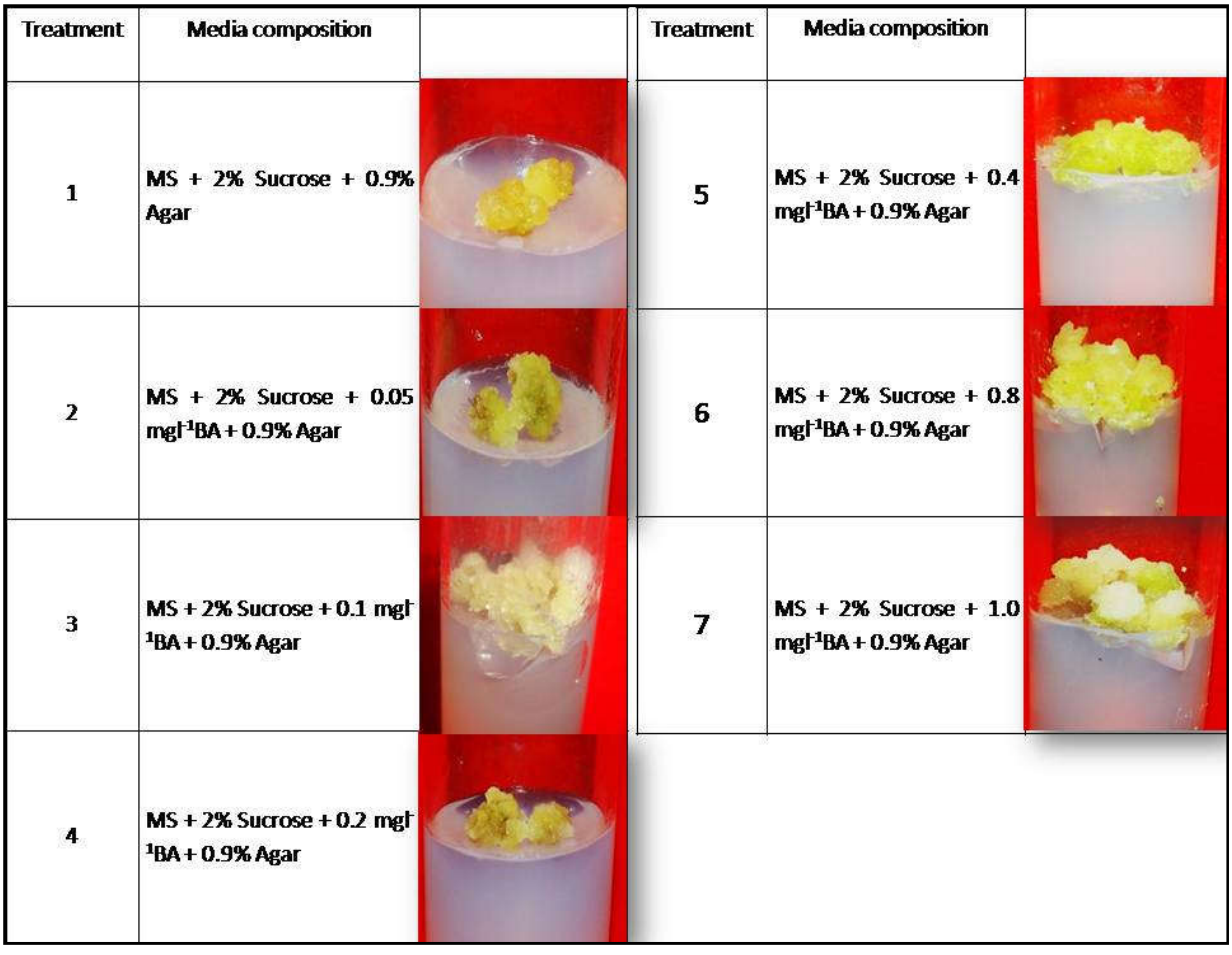

Fig 2. Callus multiplication in cumin Hypocotyl explants

Treatment	Media composition		Treatment	Media composition	
1	B_5 + 2 % Sucrose + pH 5.8 + 0.9% Agar		5	B_5 + 0.1 mgl^{-1} BA + 0.5 mgl^{-1} NAA + 2 % Sucrose + pH 5.8 + 0.9% Agar	
2	B_5 + 0.1 mgl^{-1} BA + 2 % Sucrose + pH 5.8 + 0.9% Agar		6	B_5 + 0.1 mgl^{-1} BA + 1.0 mgl^{-1} NAA + 2 % Sucrose + pH 5.8 + 0.9% Agar	
3	B_5 + 0.1 mgl^{-1} NAA + 2 % Sucrose + pH 5.8 + 0.9% Agar		7	B_5 + 0.1 mgl^{-1} BA + 1.5 mgl^{-1} NAA + 2 % Sucrose + pH 5.8 + 0.9% Agar	
4	B_5 + 0.1 mgl^{-1} BA + 0.1 mgl^{-1} NAA + 2 % Sucrose + pH 5.8 + 0.9% Agar				

Fig. 3. Shoot regeneration from callus in *Cumin cyminum* L.

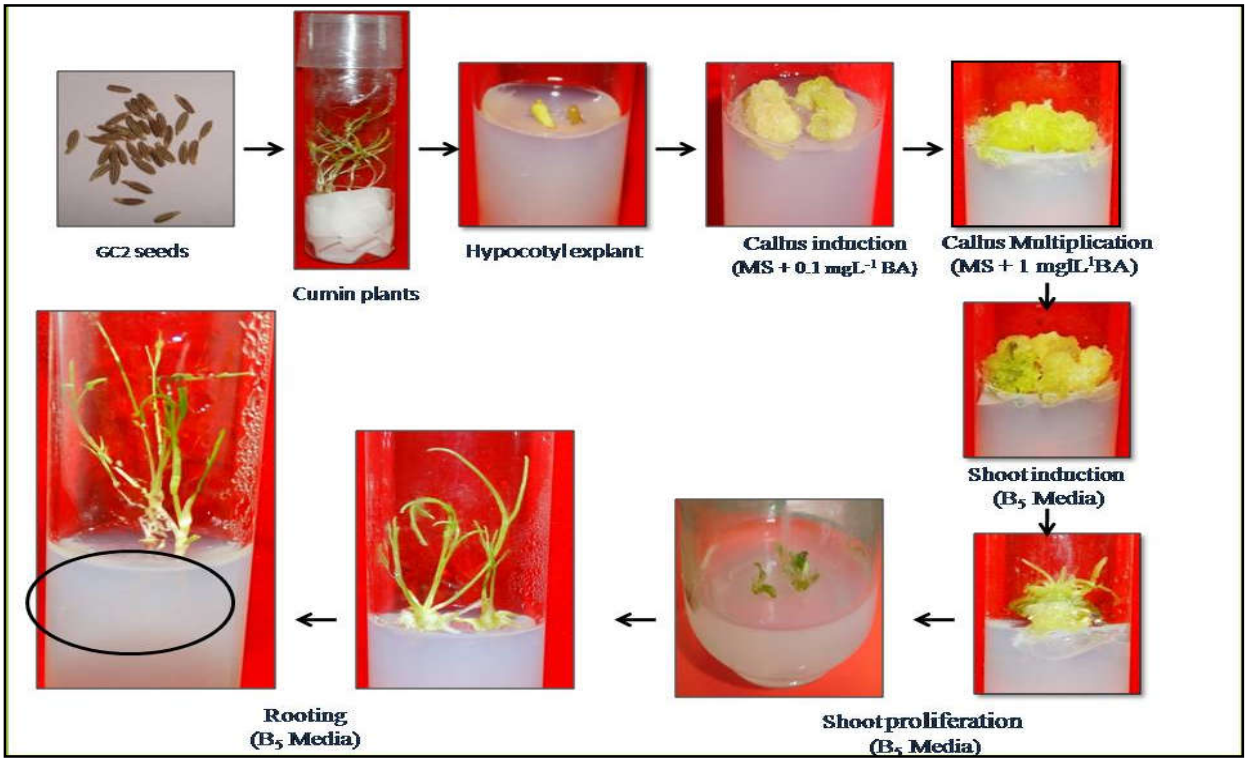

Fig. 4. Overview of cumin regeneration protocol

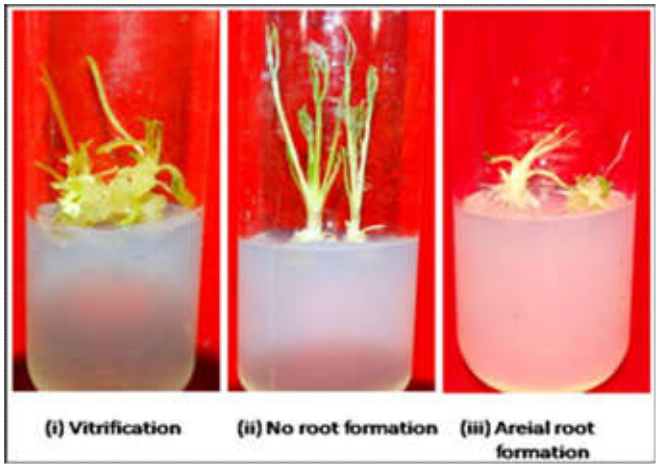

Fig 5. Troubles encountered in regeneration protocol of cumin (i) Vitrification (ii) No root formation (iii) Aerial root formation

Apart from vitrification, some cultures were completely lacking root formation, while some had aerial root formation (fig 5 (ii and iii). This may be due to auxin imbalance in plants with respect to media which could be solved *via* further optimizing media with various auxin types and concentrations.

Conclusion

In conclusion, present study reports a simple and efficient protocol for plant regeneration in cumin *via* callus development, callus multiplication, shoot development along with root development. It also describes the difficulties encountered during shoot and root development which can be addressed further. Overcoming these difficulties would help to develop more efficient protocol for plant regeneration.

Conflict of Interest: Authors state that there is no conflict of interest.

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