J. Phytol. Res. **25**(2) : 183-191, 2012

REGENERATION POTENTIAL OF SEEDLING EXPLANTS OF CARROT *(DAUCUS CAROTA)*

SUHEL KHAN and RISHIKESH MEENA
Govt. J.D.B. Women PG College, Kota- 324005, India.
E-mail: skjpr2007@gmail.com

A study was conducted with hypocotyl, cotyledon, cotyledonary node and root segments of carrot as explants for regeneration on Murashige and Skoog (MS) medium supplemented with different concentrations and combinations of auxins and cytokinins. Seeds were surface sterilized with 4% (v/v) sodium hypochlorite and germinated in growth regulator free MS medium. Cotyledonary nodes and hypocotyls were excised from 10-15 days old seedlings and were cultured on MS medium supplemented with different concentrations of BAP, Kn singly or in combination with auxins. Cotyledonary nodes proved to be the better explant than hypocotyls for the shoot regeneration and proliferation. Use of BAP at 2.0 mgl^{-1} in combination with NAA (1.0 mgl^{-1}) induced the highest frequency (66%) of shoot induction as well as maximum number of shoots per explant (4.7 ± 0.8). For root induction, *in vitro* shoots were transferred to rooting media containing NAA or IBA. Regenerated plantlets were acclimatized successfully in the growth room for further development.

Keywords: Carrot; cotyledon; cotyledonary node; Hypocotyl; Root segments. Abbreviations : BAP : 6-bezylaminopurine, Kn : Kinetin, IAA : Indol-3-acetic acid, IBA : Indole-3-butyric acid, NAA : α-naphthaleneacetic acid, 2,4-D : 2,4-Dichloro phenoxy acetic acid, NaOCl : Sodium hypochlorite.

Introduction

Apiaceae (Umbelliferae) is a cosmopolitan family comprising of 455 genera and over 3500 species, which makes this family as one of the largest taxon among higher plants[1]. They can be distinguished by characteristic morphology of inflorescence, a compound umbel with flowers occurring in umbellets arranged radially.The most commonly cultivated members of the family are carrot *(Daucus carota),* Celery *(Apium graveolens)* etc. used as vegetables or feed crops grown on more than 1.2 million ha. Worldwide with annual production estimated at over 25 million tones. The importance of root vegetable carrot, which is the primary source of provitamin A in the human diet, is denoted by the fact that its production has increased over 40% during the last decade.

The Apiaceae taxa are also distinguishable by the appearance of Umbelliferose, Petroselinic acid and polyacetylenes, which are the characteristic compounds in this family. They also contain several specific phenols, phenyl propanoids, terpenes, saponins and coumarins in fruits, leaves or roots[2]. These bioactive compounds make Apiaceae species well recognized in traditional medicines. Also several Apiaceaeous condiments are desired for culinary purposes. Despite the large diversity of this family, the use of biotechnological methods for basic research is restricted to a small number of species only. Achievements in applied research, which would lead to the development of new technologies substantial for industry, are still in the shadow of top economically important crops. Nevertheless, substantial progress can be noticed as the number of studies empoying modern biotechnology in this family is increasing. Certainly carrot is the most potable Apiaceae species, to which biotechnology is widely utilized. So, Carrot *(Daucus carota* L.) is well-known as a model species for plant tissue culture systems.

Carrot is valued as food mainly because it is rich source of the fat-soluble hydrocarbon, carotene ($C_{40}H_{56}$) the β form of which is the precursor of Vitamin A. The modified root of carrot is the most widely used part as food but all parts of carrot plant are equally valuable. Carrot roots are used as vegetables for soups, stews, curries and pies, grated roots are used as salad, tender roots are pickled. Carrot juice is a rich source of carotene; it is used for coloring butter and other food articles. Besides the food value, different parts of carrot can be used for different medicinal purposes. Carrot roots are used as refrigerant and seeds as aromatic, stimulant and

carminative. They are useful in the kidney diseases, in dropsy, nervine tonic, aphrodisiac and given in uterine pain. An infusion of carrot has long been used as a folk remedy of threadworms. Carrot increases the quantity of urine and large amount of carrot to the diet has a favorable effect on the nitrogen balance. Normally carrot is propagated from seeds; however it has been a good experimental material for *in vitro* culture. Somatic embryogenesis in carrot has already been reported[3,4]. The present investigation was carried out to develop an efficient protocol for the *in vitro* multiplication of carrot through seedling explants.

Material and Methods

Plant material and culture conditions-Daucus carota L., an important plant of family Apiaceae was taken for the present study. The seeds of *D. carota* were used for this experiment, which were bought from the local seed market of Kota. The seeds were first rinsed with 20% (v/v) Extran (Merck, India) followed by 3-4 washings with sterile distilled water. Seeds were then surface sterilized with 4 % (v/v) NaOCl (Qualigens, India) for 15 min. and rinsed with three changes of sterile distilled water to remove any traces of NaOCl. Seeds were then imbibed in sterile distilled water for two days, kept on rotary shaker. The seeds were then aseptically germinated on half strength MS[5] medium with 1% (w/v) sucrose and solidified with 0.8% agar, pH adjusted to 5.8. Hypocotyl, cotyledon, cotyledonary node and root explants were excised from 10-15 day old aseptically grown seedlings. These explants were inoculated on MS medium supplemented with various auxins *viz.* 2,4-D, IAA, IBA and NAA(0.5-2.0 mg/l) and cytokinins *viz.* BAP and Kn (0.5-2.0 mg/l) added singly or in various combinations. The MS medium supplemented with 3% (w/v) sucrose was used for all the experiments. The medium was solidified with 0.8% (w/v) agar (Qualigens, bacteriological grade), pH adjusted to 5.8 before the addition of agar and autoclaving at 121°C and 1.2-1.3 kg cm^2 pressure for 20 min. Three explants were kept in a single flask (100-ml 'Erlenmeyer' with 40 ml medium in each) for 4 weeks. All the cultures were incubated at 26±1°C with the 16-h light and 8-h dark cycle and the light intensity of 25 μM m^{-2} s^{-1} provided by cool white fluorescent tubes (Philips, India).

Sub-culturing procedure- The primary cultures were transferred to fresh medium after 4-5 weeks of culture incubation. The shoot buds induced from the explant were sectored into groups each having 3-4 of them. These clusters were then transferred on media containing various concentrations of auxins, cytokinins. Callus obtained during primary culture was transferred to fresh medium in order to analyse its morphogenic nature.

*Rooting of plantlets-*The regenerated, elongated shoots were transferred on medium containing various concentrations of different auxins (IAA, IBA, NAA) for development of proper root system.

*Field transfer of regenerated plantlets-*Regenerated plantlets with well-developed shoot and root systems were carefully taken out and washed with tap water to remove agar clinging to roots. These plantlets were then transferred to earthen pots containing garden soil and organic manure (1:1). To avoid rapid dehydration of *in vitro* regenerated plants, higher humidity level was maintained using poly bags. After one week of transfer, poly bags were perforated with the help of scissors every alternate day.

*Statistical analysis-*The observations recorded for the various experiments were subjected to following statistical analysis. Shoot buds longer than 0.5 mm with distinctly visible apical meristems only were counted in each treatment.

Average: The average (mean) was calculated by dividing the sum of values of observations for a particular treatment by the total number of observations for that treatment:

$$Av = \frac{\sum n}{N}$$

Where,

Av = Average

$\sum n$ = Summation of values of observations for a treatment

N = Total number of observations for that treatment

Standard Deviation - This is a measure of dispersion which is calculated by squaring the deviation of each observation from the mean, adding the squares, dividing by the number of observation and extracting the square root according to the following formula:

$$\sigma = \pm \sqrt{\frac{\sum d^2}{N}}$$

Where,

σ= Standard Deviation

$\sum d2$= Summation of squares of deviation of each observation from the mean

N = Number of observations

Resuls and Discussion

The seeds of D. carota were cultured on MS medium, which germinated after three days of inoculation. All the plantlets developed with normal roots and shoots. The plantlets measured about 7 cm height having 2.5 cm long root after two weeks of culture. The over all germination percentage of the seeds were 82%.

Cotyledonary Node

Effect of auxins-Cotyledonary nodes excised from 10-15 day old seedlings were cultured on MS medium supplemented with various concentrations of 2,4-D, IAA, IBA and NAA (0.5-2 mg/l). Yellow compact callus was developed all over the surface of explant on NAA (0.5-2.0 mg/l) supplemented medium. No response was observed on IAA/IBA/2,4-D (0.5-2 mg/l) supplemented medium

Effect of Cytokinins-The cotyledonary node explant cultured on MS supplemented with BAP (0.5 mg/l) initiated a shoot after one week of culture and started giving multiple shoots after four weeks of culture. The explant cultured on MS media supplemented with BAP (1 mg/l), shoots were induced after three weeks of culture while in BAP 2 mg/l, maximum number of shoots (4-5 shoots) were induced after one week of culture (Table 1). Various concentrations of Kn (0.5-2 mg/l) were tested to evaluate the morphogenic potential of the explants. Kn (5 mg/l) induced maximum callus from basal cut ends of the explant. Regular increase in mass of callus was observed with further increase in concentration of Kn (0.5-5.0 mg/l).

Effect of cytokinins plus auxins-Various combinations of BAP/Kn (0.5-5 mg/l) + IAA/IBA/NAA/2,4-D (0.5-2 mg/l) were used to enhance the shoot bud production. BAP (2.0 mg/l) + NAA (1.0mg/l) induced highest number of shoots (5-7 shoots) (Table 2).Callus obtained on combinations of Kn (0.5-2 mg/l) + NAA (0.5-1.0 mg/l) was green, nodular, compact while callus formed on Kn (0.5-2 mg/l) + IAA (1-2 mg/l) was brown with some green spots and accompanied with wooly roots.

Hypocotyl

Effect of auxins- Hypocotyl segments were cultured on MS medium supplemented with 2,4-D, IAA, IBA and NAA(0.5-2.0 mg/l). Swelling accompanied with callusing and rooting was observed at the beginning of second week on MS medium supplemented with NAA (1.0-2.0 mg/l). Callus was light green to creamish and amorphous. No response was obtained on the medium containing lower concentrations of IAA or IBA (0.5-2.0 mg/l). Explants turned brown at higher concentrations of IAA, IBA or 2,4-D (2 mg/l).

Effect of cytokinins- The explants remained green, increased in size till 10 days of culture. Light brown friable callus alongwith few shoots were obtained from hypocotyl segments on medium supplemented with BAP (0.5-1.0 mg/l). Callusing was obtained on BAP (2.0-5.0 mg/l) supplemented medium (Table 1). 40% explants could produce shoots on 0.5 mg/l Kn supplemented medium. Further increase in the concentration of kinetin evoked callusing.

Effect of cytokinins plus auxins- Hypocotyls could induce production of shoots (4-5 shoots) on MS medium enriched with BAP (1.0 mg/l) in combination with NAA (0.5 mg/l) (Table 2). Increased concentration of NAA (2 mg/l) induced browning of the explant. BAP (0.5-2 mg/l) in combination with IBA/IAA/2,4-D (0.5 mg/l) evoke nodular callus formation Various combinations of Kn (0.5-2 mg/l) + IAA/IBA/2,4-D/NAA (0.5-2 mg/l) were less effective in inducing callus. Initial swelling followed by formation of little compact callus was observed on Kn. Light green and friable callus was formed on Kn (0.5mg/l) + 2,4-D (1.0 mg/l) combination.

Cotyledon

Effect of auxins - The cotyledons excised from 8-10 day old seedlings were cultured on MS medium supplemented with various concentrations of 2,4-D, IAA, IBA and NAA (0.5,1.0,2.0 mg/l). Creamish brown callus was evident on lower concentrations of 2,4-D (0.5-2.0 mg/l). In MS media supplemented with NAA 1.0 mg/l and 2.0 mg/l, small roots on the upper surface of the explant appeared after three weeks of culture which exhibited very slow growth and from the lower surface of the explant callus was developed which was yellow in colour.There was no response on IAA and IBA.

Effect of cytokinins - An experiment was conducted to compare the shoot regeneration response of the cotyledon. Swelling of explants was recorded within a week of inoculation irrespective of type or concentration of cytokinin. This was followed by callus induction at the cut ends of the explant which was more pronounced in the media containing BAP (0.5-2.0 mg/l). Moderate callus was also produced in media containing Kn.

Effect of cytokinins plus auxins - Callusing was obtained on all the combinations of auxins and cytokinins. The callus obtained on MS augmented with BAP (0.5 mg/l) + NAA (0.5 mg/l) was compact, nodular and morphogenic while it was yellow and non-morphogenic on MS supplemented with BAP + 2,4-D/IAA/IBA (0.5-2 mg/l). Callusing along with rhizogenesis was achieved on MS medium fortified with Kn (0.5-2 mg/l) in combination with NAA (0.5-2 mg/l). Explants remained quiescent on other combinations of Kn (0.5-2 mg/l) with IBA/IAA (0.5-2 mg/l). Little cream and friable callus was obtained on combination of Kn (0.5-2 mg/l) + 2,4-D (0.5-2.0 mg/l).

Root Segment -The root explant obtained from *in vitro* culture of seeds, when cultured on MS + BAP (0.5 mg/l), formed green callus after four weeks of culture. On MS + NAA (0.5 mg/l), explants induced greenish callus after

Table 1. Morphogenic response of cotyledonary node and hypocotyl explants taken from seedlings of *Daucus carota* cultured on MS medium supplemented with BAP and Kn alone.
Culture period: 10 weeks

BAP (mgl^{-1})	Cotyledonary Node		Hypo-	
	% response	No. of Shoot buds/ explant Mean ± S.D.	% response cotyl	No. of Shoot buds/explant Mean ± S.D.
0.5	60	2.5 ± 0.5	20	1.6 ± 0.5
1.0	53	3.0 ± 0.7	40	1.8 ± 0.8
2.0	66	3.5 ± 0.8	46	C
3.0	46	3.1 ± 0.8	53	C
5.0	46	C	53	C
Kn (mgl^{-1})				
0.5	46	1.8 ± 0.5	40	1.6 ± 0.5
1.0	46	1.3 ± 0.5	20	C
2.0	33	C	26	C
3.0	26	C	33	C
5.0	20	C	20	C

Each experiment comprised of 5 replicates and were repeated twice
C - Callus formation

Table 2. Morphogenic response of cotyledonary node and hypocotyl explants taken from seedlings of *Daucus carota* cultured on MS medium supplemented with BAP in combination with NAA.
Culture period: 10 weeks

BAP/NAA (mgl^{-1})		Cotyledonary Node		Hypo-	
		% response	No. of Shoot buds/ explant Mean ± S.D.	% cotyl response	No. of Shoot buds/explant Mean ± S.D.
BAP	NAA				
0.5	0.5	46	3.1 ± 0.6	66	2.2 ± 0.6
	1	40	2.3 ± 0.5	53	2.4 ± 0.5
	2	26	1.5 ± 0.5	46	2.0 ± 0.5
1	0.5	53	3.2 ± 0.4	40	3.8 ± 0.7
	1	40	2.0 ± 0.7	40	2.5 ± 0.5
	2	60	C	46	C
2	0.5	53	3.0 ± 0.6	46	2.4 ± 0.5
	1	66	4.7 ± 0.8	46	1.7 ± 0.7
	2	53	C	53	C
3	0.5	46	2.1 ± 0.6	53	2.0 ± 0.7
	1	40	1.6 ± 0.5	26	1.7 ± 0.7
	2	66	C	53	C
5	0.5	46	2.4 ± 0.9	46	1.8 ± 0.6
	1	40	1.8 ± 0.7	40	1.6 ± 0.8
	2	73	C	60	C

Each experiment comprised of 5 replicates and were repeated twice
C - Callus formation

three weeks of culture. When the concentration of NAA was increased to 1 mg/l, roots were produced after three weeks of culture, which started to form callus after four weeks. Further increase in the concentration of NAA to 2 mg/l, roots appeared after two weeks of culture from which yellowish callus was induced after four weeks. On equal combination of NAA and BAP (0.5, 1 and 2 mg/l), the root segments swelled up and the cut ends gave callus, which showed no further responses.

D. carota is one of the important food-valued as well as medicinal plants of family Apiaceae. Many other economically important medicinal plants of family Apiaceaeae propagated *in vitro* by using different explant and different propagation methods[6,7]. Pant *et al.*[8], reported the formation of multiple shoot in *Cnidium officinale* by shoot tip culture. In *D. carota* though various parts were found to be totipotent to regenerate, the nodal explant and stem explant has been considered to be the appropriate explant for inducing the multiple shoots in the present investigation. Direct as well as indirect plant regeneration were obtained from the nodal and stem explants.

The root explant produced callus in most of the hormone concentration. At lower concentration of NAA and BAP, direct regeneration of callus was observed while at the higher concentration, rhizozenesis was formed followed by callus induction. The leaf segment of *D. carota* produced short roots on MS medium with NAA at both concentrations of 1 mg/l and 2 mg/l. The addition of NAA in the medium has favored the production of roots in the leaf explant. Similar results were observed by Bekhi and Lesley[9] and Kartha *et al.*[10] working on *Lycopersicum esculantum.* In the present study, in *D. carota* MS media alone was sufficient to regenerate the plantlet from the nodal explant. In MS medium supplemented with BAP (0.5 mg/l) multiple shoots were developed. In this condition highest number of multiple shoots were produced. When the concentration of BAP was increased to 1 and 2 mg/l, shoots were formed earlier. Hence, increase in concentration of BAP has positive effect on the earlier shoot formation, *i.e.* higher the concentration of BAP, less time taken for the formation of shoot. Sripichitt *et al.*[11] observed that BAP was more effective than kinetin in inducing shoot formation on MS medium. The nodal explant of *D. carota* gave shoots and callus on MS medium supplemented with 0.5 mg/l NAA and further increase in the concentration of NAA, the nodal explant differentiated into the whole plant. Plant differentiated in short time period in 2 mg/l of NAA as compared to 1 mg/l of NAA. Thus it was observed that higher concentration of NAA has favored the plant differentiation more rapidly. In the present investigation, in MS + NAA (0.5 mg/l) + BAP (0.5 mg/l), the nodal explant initiated the shoot formation followed by callus formation. Similar result was obtained when the concentration of BAP was increased to 1 and 2 mg/l.

The results presented in this work showed that *D. carota* can be successfully propagated *in vitro.* Since the pioneering works of Reinert[12] and Steward *et al.*[13] on carrot somatic embryogenesis, these techniques have been applied to the propagation of a large number of Apiaceae species with particular incidence on somatic embryo formation and conversion into plantlets[14,15]. Although methods for *in vitro* propagation have been mainly used for crop species[16], there has been an increasing interest on their applicability to propagate rare or threatened species for purposes of plant conservation[17-19]. Propagation of endangered species is important not only for conservation purposes but also because wild taxa are a source of genetic diversity that can be used to improve or develop crops with new genetic characteristics through hybridization (sexual or somatic) or plant genetic transformation[20,21].

As part of a strategy for the conservation of endemic and threatened species of the Portuguese flora, we decided to apply *in vitro* culture techniques to the propagation of *D. carota subsp. halophilus*, an endemic Apiaceae located in one (Mediterranean basin) of the 25 world biodiversity hotspots[22,23]. This Portuguese endemism is also a salt-tolerant taxon that, as above stated, might be used to transfer this characteristic to domestic carrot cultivars. Moreover, it is an essential-oilproducing plant that is now being evaluated for potential medicinal purposes[24].

As far as we know, previous reports concerning *D. carota subsp. halophilus in vitro* culture were made by Imani *et al.*[25] and Thi and Pleschka[26]. In both cases, somatic embryogenesis was the technique used, but no data were presented about the rates of plant regeneration and acclimatization. Imani *et al.*[25] evaluated the potential of six *Daucus* species and six carrot subspecies for somatic embryogenesis induction, concluding that *D. carota* subsp. *halophilus* was one of the subspecies showing the capacity to form somatic embryos. These were obtained from petiolar explants through a two-step process in which embryogenic calluses were first induced in the presence of indol-3-acetic acid (1.13×10^{-5}M), whereas further embryo development occurred in an auxin-free medium. More recently, Thi and Pleschka[26] showed that the ability of several *D. carota* species and subspecies to undergo somatic embryogenesis was strongly correlated with the endogenous levels of ABA before culture of the petioles.

Explants showing the lowest endogenous ABA concentration were the most effective for somatic embryogenesis induction. Present studies confirmed the potential of *D. carota* subsp. *halophilus* to be propagated by somatic embryogenesis and showed that other explants, such as germinated cotyledons and root segments, could also be used for somatic embryogenesis induction. The conditions used in our experiments were also different since somatic embryos were induced and attained the cotyledonar stage of development in the original medium containing 2,4-D. This one-step process for somatic embryogenesis induction reduces the time for somatic embryo formation and plant regeneration. Attempts to optimize this protocol are now being carried out to reduce the number of precociously germinated somatic embryos observed in some cultures and to synchronize somatic embryo development, which is quite variable even in the same explant. Both goals are essential to improve the rates of plant regeneration and the effectiveness of the process. Previous work at our lab showed that the manipulation of the culture media can increase both the rate of somatic embryogenesis induction as well as the quality of the embryos[27]. Besides somatic embryogenesis induction and conversion, *D. carota* subsp. *halophilus* was also propagated by axillary shoot proliferation from shoot tips. This technique has been applied to *in vitro* propagation of a large number of crop and endangered species[16]. Through this method, high rates of multiplication can be obtained, and the regenerated plants are genetically uniform[28]. In our experiments, *D. carota* subsp. *halophilus* was, for the first time, propagated by this method. The explants used were shoot tips from germinated seedlings, which means that the obtained plantlets were seed clones and not clones of particular adult plants, thus assuring the genetic diversity of the propagated plantlets. Genetic variability is particularly important for plant conservation since the main objective is not to clone a particular genotype but to assure that genetic diversity is maintained or increased[29]. The results of our experiments showed that the concentration of BA slightly affects the number of shoots produced, with the highest concentration used giving the highest number of shoots. The role of cytokinins on shoot proliferation is well-known[30], and the type and concentrations most effective greatly vary among the different species. The data also indicated an increase in shoot proliferation in the second assay that can be explained by variations in the endogenous levels of cytokinins or other hormones. A habituation to the cytokinins[31] or an increase in the number of adventitious shoots formed from small organogenic calluses originated at the base of the explants might also have contributed to this increase.

Rooting of the *in vitro* obtained shoots is an essential step for the success of plant regeneration[32]. In the current study, shoots were able to root without auxin treatment. Moreover, the presence of IBA did not increase the rate of root formation. Due to the high number of shoots formed, the rates of root formation observed are enough to assure that a considerable number of rooted plantlets could be obtained. However, attempts are being made to increase the rate of rooted shoots by treatments with other concentrations of IBA and other auxins and by the application of auxinic shocks, a procedure routinely used in many species. Our data also showed that root formation was not conditioned by the levels of BA used for shoot proliferation. However, other authors have pointed out that cytokinins seem to negatively interfere with further root formation[30]. It is possible that the concentrations used in the present study are not high enough to induce this inhibitory effect.

Shoots of *D. carota* subsp. *halophilus* were able to flower *in vitro* a kind of morphogenesis that has been observed in different species[33,34]. Flower formation was randomly observed, and the physical (light intensity and photoperiod) and chemical conditions *(e.g.,* plant growth regulators and sugars) that can control flowering were not evaluated. Preliminary observations showed that pollen grains of these flowers seem to be viable, since they germinated *in vitro* in a jellified medium. It would be interesting to optimize the conditions for *in vitro* flower production and determine if the life cycle of this species could be completed *in vitro* as occurs in other plants such as *Arabidopsis.*

Plants of *D. carota* obtained were successfully acclimatized and are now growing in their original habitat, thus showing the potential of this methodology for its conservation. This strategy should reduce pressure on wild populations. Further studies on this taxon will be focused on the evaluation of their genetic diversity through molecular markers and in the comparison between the essential oils produced *in vitro* and in the field.

References

1. Pimenov MG and Leonov MV 1993, *The genera of Umbelliferae.* A Nomenclator, Royal Botanical Gardens, Kew, UK, 156 pp
2. Hegnauer R 1990, Umbelliferae. In: Hegnauer R (ed.) *Chemotaxonomie der Pflanzan* (vol 9) Birkhauser Verlag, Berlin, Germany, pp 663-714.
3. Kamada H K, Kobayashi T, Kiyosue and Harada H 1989, Stress induced somatic embryogenesis in carrot

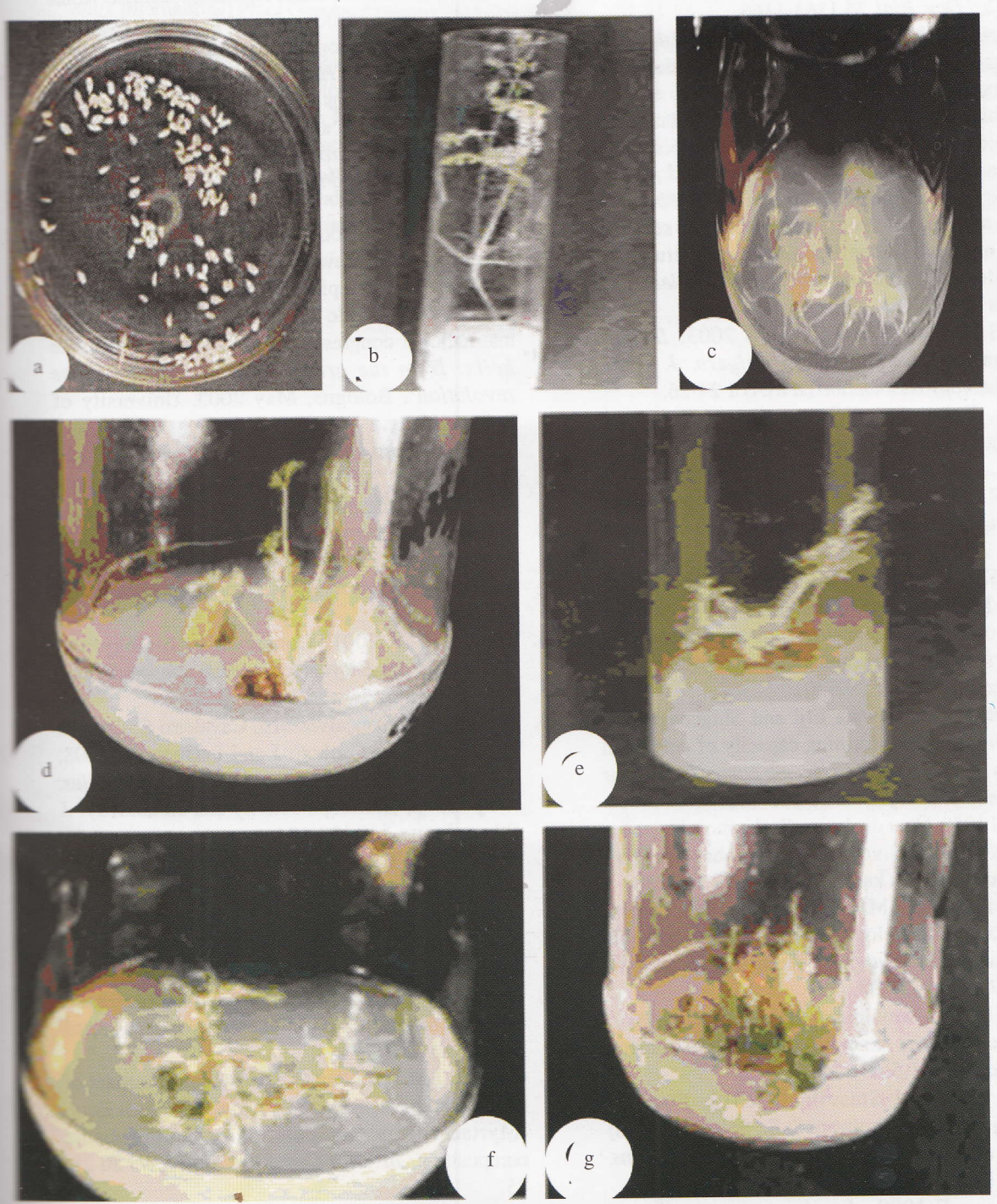

Fig. 1. Morphogenic response of seedling raised explants of *Daucus carota* on MS medium supplemented with various growth regulators.
a) Seeds of *Daucus carota;* b) Seedling of *D. carota* grown in MS medium (3 weeks old); c) 4 week old roots and callus formation from root explant of *D. carota* on MS + NAA (1 mg/l); d) 8 week old multiple shoots proliferated from cotyledonary node of *D. carota* on MS + BAP (0.5 mg/l); e) 9 week old multiple shoots proliferated from callus from cotyledonary node of *D. carota* on MS + BAP (2 mg/l) + NAA (1 mg/l); f) 9 week old root and callus from hypocotyl explant of *D. carota* on MS + NAA (1 mg/l); g) 10 week old callus proliferating multiple shoots from hypocotyl explant of *D. carota* on MS + BAP (1 mg/l) + NAA (0.5mg/l).

and its application to synthetic seed production. *In vitro Cell Dev. Biol.* **25** 1163-1166.
4. Liu L, Earl G M and Eve S W 1994, Accumulation of petroselinic acid in developing somatic carrot embryos. *Phytochemistry* **37** 749-753.
5. Murashige T and Skoog F 1962, A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physol. Plant.* **15** 473-497.
6. Kim S W, Park M K and Liu J R 1996, High frequency plant regeneration *via* somatic embryogenesis in cell suspension cultures of coriander (*Coriandrum sativum* L.). *Plant Cell Reports* **15** 751-753.
7. Joshi S D, Pant B and Ranjit S 2003, *In vitro* propagation of *Foeniculum vulgare*. *J. Nepal Biotechnology Association* (JNBA) **1** 24-26.
8. Pant B, Kohda H and Namera A 1996, Clonal propagation of *Cnidium officinale* by shoot tip culture. *Plant Med.* **62** 281-282.
9. Bekhi R M and Lesley S M 1976, *In vitro* plant regeneration from leaf explants of tomato. *Can. J. Bot.* **54** 2409-2414.
10. Kartha K K, Gamborg O L, Shyluk J P and Contabel F 1976, Morphogenetic investigation on *in vitro* leaf culture of Tomato *(Lycopersicum esculentum* Mill. cv. *Starfire)* and high frequency plant regeneration. *Z. Pflazenphysiol.* **77** 292-301.
11. Sripichitt Prapa, Nawata Eiji and Shigenaga Soji 1987, *In vitro* shoot forming capacity of cotyledon explants in red pepper (*Capsicum annum* L. cultivar Yatsufusa). *JPN J. Breed.* **37** 133-142.
12. Reinert J 1958, Über die kontrolle der morphogenese und die induktion von adventivembryonene an gewebekulturen aus karotten. *Planta* **53** 318-333
13. Steward F C, Mapes M O and Mears K 1958, Growth and organized development of cultured cells. II. Organization in culture grown freely suspended cells. *Am. J. Bot.* **45** 705-708.
14. Ekiert H 2000, Medicinal plant biotechnology: Apiaceae family as example of rapid development. *Pharmazie* **55** 561-567.
15. Thorpe T A and Stasolla C 2011, Somatic embryogenesis. In: Bhojwani S. S.; Soh W. Y. (eds) *Current trends in the embryology of angiosperms.* Kluwer, Dordrecht, pp 279-336
16. Engelmann F 1999, *In vitro* conservation of horticultural genetic resources: review of the state of the art. *Acta Hortic.* **495** 245-250.
17. Jaramillo S and Baena M 2002, *Ex situ conservation of plant genetic resources: training module.* International Plant Genetic Resources Institute, Rome
18. Engels J M M 2003, Plant genetic resources management and conservation strategies: problems and progress. *Acta Hortic.* **623** 179-191.
19. Sarasan V A, Cripps R, Ramsay M M, Atherton C, McMichen P G and Rowntree J K 2006, Conservation *in vitro* of threatened plants- progress in the past decade. *Plant Cell Tissue Organ Cult.* **42** 206-214.
20. Pellegrineschi A 2005, The use of biotech for the introgression of new genetic variability in wheat varieties for developing countries. In: Tuberosa R.; Phillips R. L.; Gale M. (eds) Proceedings of the international congress. *"In the wake of the double helix: from the green revolution to the gene revolution"*, Bologna, May 2003. University of Bologna, Italy.
21. Maxted N, Ford-Lloyd B V, Jury S L, Kell S P and Scholten M A 2006, Towards a definition of a crop wild relative. *Biodivers. Conserv.* **15** 2673-2685.
22. Meyers N, Mittermeier A, Mittermeier C G, da Fonseca G A B and Kent J 2000, Biodiversity hotspots for conservation priorities. *Nature* **403** 853-858.
23. Ricketts T H 2001, Conservative biology and biodiversity. In: *Encyclopedia of life sciences.* Wiley, New York. http://www.els.net.
24. Tavares A C, Gonçalves M J, Cavaleiro C, Cruz M T, Lopes M C, Canhoto J and Salgueiro L 2008, Essential oil of *Daucus carota* subsp. *halophilus:* composition, antifungal activity and citotoxicity. *J. Ethnopharma.* **119** 129-134.
25. Imani J, Tran Thi L, Langen G, Arnholdt-Schmitt B, Roy S, Lein C, Kumar A and Neumann K H 2001, Somatic embryogenesis and DNA organization of genomes from selected *Daucus* species. *Plant Cell Rep.* **20** 537-541.
26. Thi L T and Pleschka E 2005, Somatic embryogenesis of some *Daucus* species influenced by ABA. *J. Appl. Bot. Food Qual.* **79** 1-4.
27. Canhoto J M and Cruz G S 1994, Improvement of somatic embryogenesis in *Feijoa sellowiana* Berg. (Myrtaceae) by manipulation of culture media composition. *In Vitro Cell. Dev. Biol. Plant* **30** 21-25.
28. George E F and Debergh P C 2008, Micropropagation: uses and methods. In: George E. F.; Hall M. A.; De Klerk G.-J. (eds) *Plant propagation by tissue culture.* 3rd ed. Springer, Dordrecht, pp 29-64.

29. Graudal L, Thomson L and Kjaer E 2001, Selection and management of *in situ* gene conservation areas for target species. In: FAO, DFS, IPGRI (ed) *Forest genetic resources conservation and management, vol 2. In managed natural forests and protected areas (in situ).* International Plant Genetic Resources Institute, Rome, pp 5-12.
30. Van Staden J, Zazimalova E and George E F 2008, Plant growth regulators II: cytokinins, their analogues and antagonists. In: George E. F.; Hall M. A.; De Klerk G.-J. (eds) *Plant propagation by tissue culture.* 3rd ed. Springer, Dordrecht, pp 205-226.
31. Geneve R L, Kester S T and Pomper K W 2007, Cytokinin habituation for autonomous shoot initiation in pawpaw. *Acta Hortic.* **738** 371- 374.
32. Bennett I J , McComb J A, Tonkin C M and McDavid D A J 1994, Alternating cytokinins in multiplication media stimulates *in vitro* shoot growth and rooting of *Eucalyptus globules* Labill. *Ann. Bot.* **74** 53-58.
33. Handro W and Floh E I S 2001, Neo-formation of flower buds and other morphogenetic responses in tissue cultures of *Melia azedarach. Plant Cell Tissue Organ Cult.* **64** 73-76.
34. Lin C S, Chen C T, Hsiao W W and Chang W C 2005, Effects of growth regulators on direct flowering of isolated ginseng buds *in vitro. Plant Cell Tissue Organ Cult.* **83** 241-244.