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DEVELOPMENT OF AN EFFICIENT AND REPRODUCIBLE *IN VITRO* REGENERATION SYSTEM IN CARROT (T-29)

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Abstract

Embryogenic calli were induced from stem segment of carrot (*Daucus carrota* L.) cultivar T-29 when cultured on callus induction (CI) medium containing MS salts, Myo-inositol, B5 vitamins, 3 mgL^{-1} 2,4-D, 1 mgL^{-1} Kinetin and 20 gL^{-1} sucrose. Somatic embryos were developed from calli upon transferring to MS medium, containing 30gL^{-1} sucrose without any additional plant growth regulators PGRs (MS_2 medium). To enhance the number of mature somatic embryos and plant regeneration, the induced calli were pretreated for two weeks on a modified CI medium that lacks 2,4-D but contains kinetin (0.6 mgL^{-1}). The production of somatic embryos and shoots increased 10-fold in pretreated calli when cultured on MS_2 medium compared with calli without pretreatment. The sucrose starvation treatment for two weeks, using modified CI medium that contains 20 gL^{-1} sucrose and reduced levels of kinetin, results in improved regeneration. Similarly, a greater number of mature somatic embryos and shoots primordia when transferred to a medium carrying 30 gL^{-1} sucrose were recovered. Thus, temporary sucrose and kinetin starvation remarkably enhanced plant regeneration that may be valuable in future experiments of genetic transformation.

Key words: Carrot, Embryogenesis, *In vitro*, Organogenesis, Nutrition stress.

Introduction

Highly efficient plant regeneration in elite cultivars is the essential requirement for plant transformation. Induction of efficient regeneration involves multiple factors including plant genotypes, use of appropriate gelling agent, choice of plant material (explant) for *In vitro* propagation, use of external physical factors like light and dark regime, optimal media; including the source of vitamins, auxin: cytokinin ratio and carbohydrate source. The type and quantity of carbohydrates also play a major role in somatic embryogenesis and shoot regeneration responses. Sucrose is commonly used as a carbon source for plant growth in tissue culture compared with glucose or fructose (Evans *et al*., 1981; Aslam *et al*., 2008). Addition or omission of sucrose in media has regulated plant regeneration with or without supplement of plant growth regulators (Nakagawa *et al*., 2001; Blanc *et al*., 2002). It has specific carbon effect and osmotic potential which helps in the modulation of plant regeneration responses (Yang *et al*., 2004).

Carrot (*Daucus carota* L.) is an excellent source of sugar, dietary fiber, vitamins A, C, K, and B6 and low in saturated fats and cholesterol. Dietary carotenoids act as cancer preventative agents. The richest source of α-carotene, the strongest cancer-protective agent, is carrot and its juice (Donaldson, 2004). Carrot is a biennial crop and economical to produce with higher biomass production and more consumption as raw food (Yan & Hunt, 1999). Carrot offers high regeneration potential and embryos can develop on most of the seedling parts in highly embryogenic genotypes (Neumann, 2000; Fujimura, 2014). Different explants including seed coat and hypocotyl (Ogata *et al*., 2005), stem segments (Kumar *et al*., 2004), petiole (Mashayeki & Neumann, 2006), cotyledons (Yang *et al*., 2004), apical tip segments (Kikuchi *et al*., 2005) and microspores (Li *et al*., 2013) were used to raise carrot plant tissue and cell cultures with different frequencies of embryo regeneration and conversion on different media. However, little is known about the role of gelling agents, sucrose, and growth hormones like kinetin in enhancing seedling growth and indirect regeneration responses in carrot. Hence elite indigenous cultivar T-29 was explored for the development of an efficient regeneration system that may help to establish transformation for the expression of economically important biomolecules in carrot plastome.

Materials and Methods

Seed sterilization and germination: Seeds of elite indigenous carrot cultivar T-29 were taken from Ayub Agriculture Research Institute (AARI), Jhang Road, Faisalabad for raising seedlings *In vitro*. Seeds were surface sterilized using 70% (v/v) ethanol plus 2-3 drops of Tween-20 for 2 minutes and 5% (v/v) sodium hypochlorite for 3-5 minutes followed by washings with sterile water. Seeds were cultured on medium in the Petri plates containing MS (Murashige and Skoog, 1962) salts, varying levels of sucrose with/without the addition of B5 vitamins (Table 1). Different solidifying agents viz. 2.66 $gL^{-1}$of phytagel (Sigma-Aldrich, USA) or 8-9 gL^{-1} of agar (PhytoTech Labs, USA) were also evaluated for their effects on plant growth and development, hyperhydricity and recovery of plants after transplantation. After 7-10 days of seed germination, the seedlings were transferred to their respective fresh medium in the culture jars for plant growth and development.

Table 1. Compositions of plant growth and development media.

Ingredients	Plant development media				Callus induction (CI) medium
	MS_1	MS_2	MS_3	MS_4	
MS salts	4.33 g	4.33 g	4.33 g	4.33 g	4.33 g
B5 vitamins	-	-	1 mg	1 mg	1 mg
Myoinositol	-	-	100 mg	100 mg	100 mg
Kinetin	-	-	-	-	1 mg
2,4-D	-	-	-	-	3 mg
Sucrose	20 g	30 g	20 g	30 g	20 g
Agar	8 g	8 g	8 g	8 g	8 g

Explant source and culture procedures for callus induction (CI) and proliferation: After 4-6 weeks of seedling growth and development *In vitro*, the stem and petiole explants of 3-4 mm in size, were excised from these plants and placed in horizontal and vertical positions on CI medium containing 2,4-D and kinetin (Table 1) for callus induction under both long-day (LD) and dark (D) incubation conditions. After every 3-4 weeks, the calli were sub-cultured on the fresh medium for growth and proliferation.

Calli treatment, plant regeneration, and acclimatization: After 3-4 weeks of callus proliferation in stem segment explant, the calli were divided and were subjected to stress conditions to enhance the embryogenic potential of the calli by temporary placement for 2-3 weeks on pre-treatment media (T) containing full strength MS salts, 1 mgL^{-1} B5 vitamins, 100 mgL^{-1} myoinositol, 2% sucrose, 8% agar and different levels of kinetin (0, 0.2, 0.4, 0.6 and 1 mgL^{-1} referred as KT_o to KT_4, respectively). After pre-treatment for 2-3 weeks on T medium, the calli were placed on MS_2 medium for 3-4 weeks for embryogenesis and shoot regeneration. The germinating embryos were transferred to the culture jars containing plant growth and development medium (MS_3). The regenerated plants were transplanted in plastic pots (3" x 3.25") having sand and silt (2:1) as potting media for further growth and development of the regenerated plants and the plants were acclimatized following Khan *et al.*, (2006).

Incubation conditions: Agar (PhytoTech Labs, USA) was used (8-9%) for solidifying CI and regeneration media. Medium pH was adjusted to 5.7 ± 1 with 1M KOH. The medium was autoclaved for 20 minutes and dispensed in sterilized plastic plates and culture jars and sealed with parafilm. The cultures were incubated at 27±1°C temperature, 35µmole$m^{-2}s^{-1}$ light intensity under long days (LD; 16/8 h day and night cycle) and dark (D) conditions for callus induction.

Experimental layout and data analysis: The experiments were laid out following a Completely Randomized Design (CRD) in a factorial arrangement with at least three replications per treatment. Five to seven stem and petiole explants were cultured per Petri plate while seven to nine calli segments were cultured for proliferation and embryogenesis. The differences among means were determined using the LSD test and the new Duncan's Multiple Range Test (Damon & Harvey, 1987).

Results

Agar solidified MS medium enhanced plant growth, survival (%), and reduced hyperhydricity: Agar solidified MS_2 medium was creamy yellow to light brown color compared with phytagel medium having transparent white to off-white color. Fully expanded leaves were developed in 10-15 days old seedlings within 5-6 days on MS_2 medium. Plants developed from these seedlings were large and succulent with a higher growth rate on phytagel medium compared with plants raised on agar medium. Shoot length, root length, and plant height were markedly higher in seedlings raised on agar medium; however, leaf area was higher in seedlings raised on phytagel medium. No hyperhydricity was observed in the plants raised on the agar medium (Table 2).

Leaves appeared on shoots in phytagel medium were translucent, light green and glossy in color, elongated, succulent, and brittle. Desiccation and high plant mortality were observed within 2-5 days of the transfer of plants to *In vivo* conditions. Callus formation at the basal stem end of these plants raised on phytagel significantly reduced plant recovery (33.3%) compared to plants raised on agar medium (80%; Table 2). Contrastingly, in agar solidified medium, normal plant growth was observed with dark green leaves. Further, no callusing at basal stem end markedly enhanced post-acclimatization survival frequency (80%-90%) of plants.

Vitamins (B5) enhanced stem and primary root growth in seedlings: Increase in sucrose level in media from 2% (MS_1) to 3% (MS_2) enhanced stem thickness and secondary root formation whereas the addition of B5 vitamins in combination with 2% of sucrose (MS_3 medium) enhanced stem elongation and the number of leaves (Fig. 1C). Besides, B5 vitamins enhanced primary root length and induced secondary root growth (Fig. 1C, D). Plant growth and development responses were found in following descending order on different media MS_3>MS_4>MS_2>MS_1 (Fig. 1A-D). Medium MS_3 was used for seedling growth and development since stem pieces of plants developed on this medium produced more embryogenic calli. Further, stem cuttings cultured on MS_2 and MS_3 media for micropropagation did not develop roots.

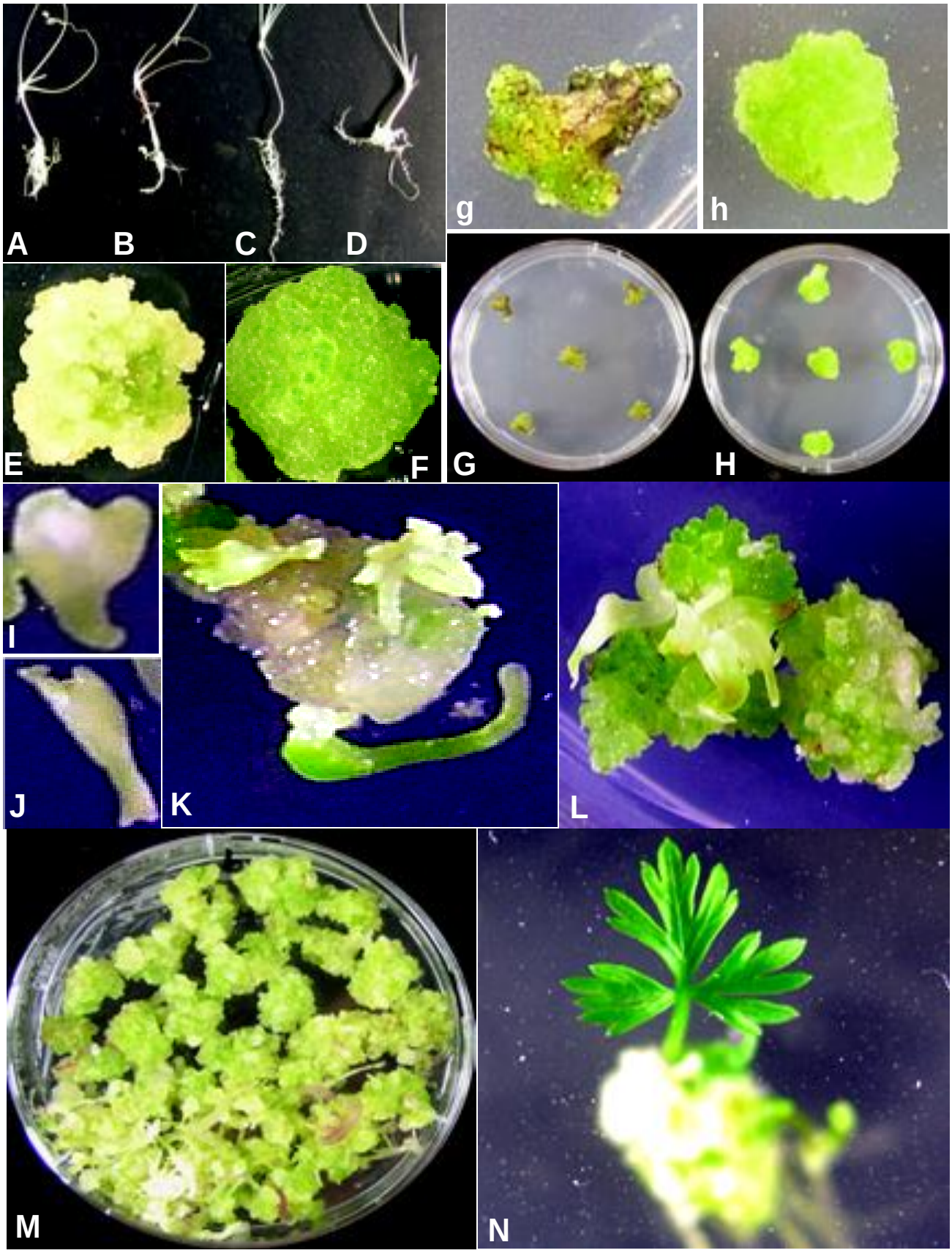

Fig. 1. *In vitro* plant development and regeneration responses from different explants under contrasting incubation conditions. Shown are the stem segment developments on different media in Fig. A-D (MS_1, MS_2, MS_3, MS_4 media, respectively), different types of calli developed from horizontal (E) and vertical (F) placement of stem segment explants on CI media; callus induction responses in stem segment (h, H) and petiole (g, G) explants under LD conditions on CI media; different types of embryos including globular to heart shaped (I), torpedo to cotyledonary stage (J) and embryogenesis (K,L) established on MS_3 media; shoot regeneration responses (M) in calli pretreated on kinetin media (KT) and developing plantlet (N) on MS_3 media.

Table 2. Characteristics of different gelling agents and their effect on plant.

Characteristics		Agar (8 gL-1)	Phytagel (2.7 gL-1)
Gel strength		Normal	High
Gel color		Creamy yellow to Light brown	Transparent white to Off-white
Growth response	Explant	Normal	High
	Plant	Normal	High
Stem length		2.78 ± 0.51	3.20 ± 0.34NS
Shoot (Leaf + Petiole) length		10.96 ± 1.2 *	8.98 ± 0.42
Root length		4.12 ± 0.91 *	3.18 ± 0.90
Plant height		18.34 ± 1.9 *	15.36 ± 0.71
No. of leaves/plant		4.20 ± 0.73	4.60 ± 0.40 NS
Leaf blade width		1.54 ± 0.24	1.64 ± 0.09 NS
Leaf blade length		1.12 ± 0.21	1.56 ± 0.15 *
Leaf area		5.32 ± 0.84	6.40 ± 0.19 *
Leaf color		Dark green	Light green & glossy
Leaf texture		Normal	Succulent
Hyperhydricity	Stem	Absent	Present
	Leaf	Absent	Present
	Plant	Absent	Present
Plant recovery after transplantation		80.0 ± 1.20 **	33.3 ± 0.88

Growth, development, Hyperhydricity and survival (%) after transplantation on MS3 media
* = Significant; ** = Highly significant; NS = Non-significant

Stem segment explant and long day (LD) conditions enhanced frequency of callus induction and proliferation: Explant placement on the CI media depicted a significant effect on the type and frequency of callus induction. Callus induction frequency was higher (75.67%) when the stem segment explant was placed vertically compared with its horizontal placement on the medium surface (Fig. 2). Calli obtained from vertically placed explants were rich in water content and had little embryogenic potential. In contrast, calli obtained from horizontally placed explants were embryogenic (Fig. 1E, F). Callus initiation responses were also highly affected by incubation conditions and explant types. Callus initiation was faster in the stem segment explant and under LD condition (7 and 8.7 days, respectively) compared with petiole explant and D conditions (Table 3). The frequency of callus induction (80% and 54%) and proliferation was higher in both stem and petiole explants under LD compared with D conditions (Fig. 1G, H; Table 3). Calli induced were green in color, smooth, compact and friable in both stem and petiole segments under LD conditions (Table 4, Fig. 1G, H). Conclusively, stem explant, and LD incubation conditions were better for callus induction responses compared with D conditions.

Somatic embryogenesis and plant regeneration in stem segments-derived calli: Embryogenesis and plant regeneration were observed upon transfer of small chunks (7-9 per plate) of ~40-45 days old proliferating calli on MS_2 medium (Table 5, Fig. 1K-N). Somatic embryo induction and development were more efficient in calli induced from stem segment under D (4-5 days; Table 5) with a higher number of embryos (3.74 and 2.38) and frequency of embryogenesis (21.89% and 33.67%) compared with LD conditions (Table 5). Calli induced from the petiole segment under both LD and D conditions did not regenerate after 30 days of culture.

Pre-treatment of calli on medium with reduced Kinetin (KT) and sucrose enhanced its regeneration potential: A higher number of both globular to heart-shaped (G-H) and torpedo to cotyledonary stage (T-C) embryos (187 and 25) were developed after 2 weeks pre-treatment on MS_3 medium without kinetin (KT_o) compared with 3 weeks pre-treatment (Table 6). Among media treatments, KT_o proved better for the production of embryos at different developmental stages (Fig. 1I, J) compared with other treatments (KT_1-KT_4; Table 6). However, kinetin pre-treatment of the calli significantly enhanced the number of regenerated shoots and plantlets (101.67 and 34.67) in calli pretreated for 2 weeks on 0.6 mgL^{-1} of kinetin. Increase in time of treatment of calli from 2 weeks to 3 weeks sharply reduced number of regenerated shoots/plantlets. Estimation of total regenerants in calli treated at different levels of kinetin revealed a higher total number of embryos (228) in calli pretreated on control media (KT_o; Fig. 4). This embryogenesis response was ~10 times better than the embryogenesis obtained on MS_2 medium without any pretreatment (Fig. 3). Calli pre-treatment on KT_3 medium enhanced shoot regeneration response and up to 155 shoots were regenerated. Further addition of kinetin in medium showed a declined number of shoots (Fig. 4).

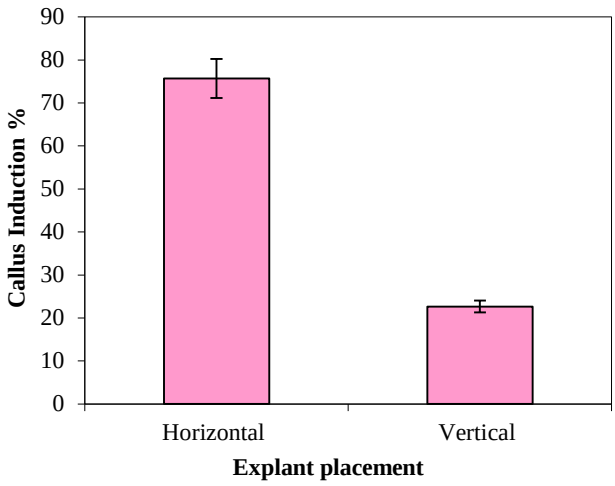

Fig 2. Callus induction (%age) in response to horizontal and vertical stem explant placement on medium containing MS salts, B5 vitamins, 20gL^{-1} sucrose, 3 and 1mgL^{-1} of 2,4-D and kinetin, respectively. Data are means ± SE of three Reps with 25 explants per rep.

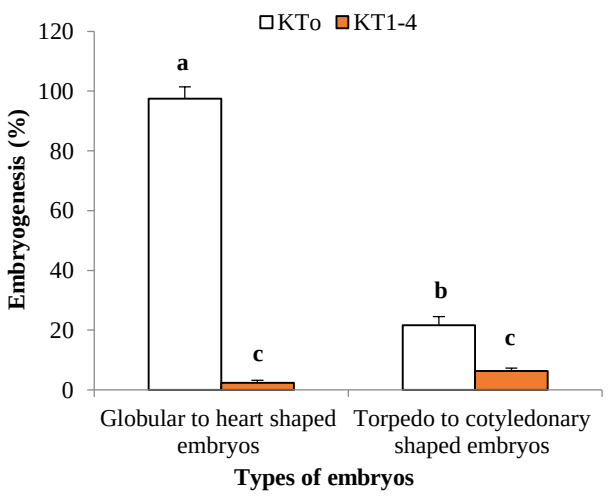

Fig. 3. Number and types of somatic embryos obtained on MS_2 medium after calli pre-treatment on medium containing kinetin (KT).

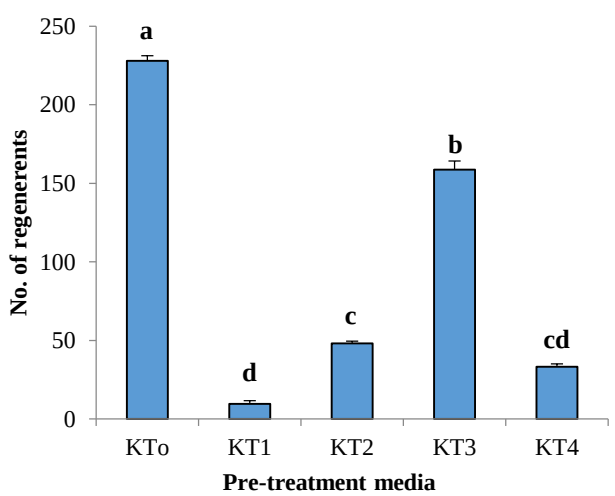

Fig. 4. Number of regenerants including embryos and shoots/plantlets obtained on MS2 medium after 2 weeks pre-treatment of calli on medium containing kientin (KT).

Discussion

Rate of plant growth, plant height, shoot formation, and leaf area were higher in plants raised on phytagel medium. However, these plants were characterized using the succulent stem, leaves, and callus formation at the basal stem end. Plants were more susceptible to desiccation and high mortality was observed upon transfer of plants to *In vivo* conditions. The succulent and vitrified morphology of carrot plants might have lowered down the photosynthetic capability of leaves leading to plant death as reported earlier (Majada *et al*., 2001). On the contrary, plants raised on agar solidified medium were better in growth with no hyperhydric response and higher survival rate. These differences in plant morphology and physiology on different media solidifying agents might be attributed to hyperhydricity developed due to variation in relative humidity and water potential (Roche, 1996), limited diffusion of medium components and water impurities (Nairn *et al*., 1995), reduced bioavailability of organic and inorganic salts at higher concentrations (Van Winkle *et al*., 2003; Aslam *et al*., 2008) and differences in gel strength (Winarto *et al*., 2004) of the two solidifying agents. Use of agar medium reduced hyperhydricity development in seedlings and regenerants with higher plant survival percentage suggesting limited use of phytagel in carrot plant regeneration. These findings are in agreement with similar reports in tomato (Karapanos *et al*., 2006) and French Tarragon (Mackay & Kitto, 1988). Phytagel produced hyperhydric shoots in other crops including pear (Kadota & Niimi, 2003), carnation (Winarto *et al*., 2004), mangosteen (Te-Chato *et al*., 2005) and tomato (Karapanos *et al*., 2006). Agar media developed plants produced quality explants that induced embryogenic calli on callus induction medium.

Better plant growth was obtained on MS_3 media containing 2% sucrose and B5 vitamins that produced a greater number of explants per plant (3-5) with higher embryogenic potential. In contrast, the germination rate of small citrus embryos was higher on B5 medium compared with their germination on MS and MT media (Viloria *et al*., 2005). Explants are sensitive to external physical factors like photoperiod. Effect of light on the growth of somatic embryos is known in certain crops however, little is reported about its effect on callus induction and proliferation responses. There was no influence of light on callus induction compared with dark conditions in quince (Morini *et al*., 2000); in contrast, LD conditions markedly reduced the time taken to induce calli in both explants. Similarly, callus induction and proliferation rate under LD was much higher compared with D conditions in carrot. Higher growth and proliferation were also reported in leaf disc derived calli in spinach and lettuce cultivars under LD on medium containing either 2,4-D or BAP (Usman *et al*., 2014). The exposure of tissues to light also increases tissue temperature which may trigger endogenous hormonal production, enhance the induction and proliferation of calli masses under LD compared with D.

Application of 2,4-D stimulates the accumulation of endogenous IAA to maintain callus proliferation, prevent the development of somatic embryos, and increase the level of auxin binding proteins (Feher *et al*., 2005). Callus induction

responses due to changes in auxin and cytokinin contents in CI media showed that both growth hormones are necessary for optimal callus initiation. The stem segment produced more embryogenic calli on medium containing 2,4-D and kinetin (CI medium) under both LD and D conditions. On the contrary, efficient callogenesis have been reported in carrot on MS medium supplemented with 2,4-D alone (Yang *et al*., 2004; Kikuchi *et al*., 2005). Use of a combination of high auxin: low cytokinin enhanced induction of embryogenic calli. Similar responses of inducing calli using kinetin in combination with 2,4-D have been reported in soybean that minimized the formation of 2,4-D amino acid conjugates and enhanced release of 2,4-D upon transfer to auxin free medium (Montague *et al*., 1981).

Higher callus induction was obtained on optimum concentrations of 2,4-D, kinetin, and B5 vitamins with MS salts compared with media lacking B5 vitamins. It suggested that the use of B5 vitamins may also trigger callus induction and proliferation responses in carrot tissues if used in combination with MS salts and proper auxin: cytokinin ratio. Use of 2,4-D in combination with B5 vitamins and casein hydrolysate was better for callus induction compared to MS vitamins (Smith *et al*., 1997). The higher embryogenic potential has been reported from petiole explant derived calli using CN medium and B5 medium containing 2,4-D (Mashayekhi & Neumann, 2006). Callus induction and proliferation were obtained in carrot on medium containing Gamborg-B5 salts and vitamins, 1 mgL^{-1} 2,4-D, and 0.025 mgL^{-1} of kinetin (Klimek-Chodacka *et al*., 2018). Differences in callus induction responses might be attributed to different media formulations, PGRs, culture conditions, and cultivars used.

Table 3. Callus induction and proliferation responses on CI medium in different explants under contrasting incubation conditions.

Parameters	Incubation conditions	Types of explants		Means
		Stem segment	Petiole segment	
No. of days to induce calli	LD	6.2	11.2	8.7 B
	D	7.8	12.6	10.2 A
	Means	7.0 B	11.9 A	
Callus induction (%)	LD	80.60 a	54.06 b	67.33 A
	D	36.40 c	32.80 c	34.60 B
	Means	58.50 A	43.43 B	
Callus proliferation (%)	LD	96.60 a	90.60 b	93.60 A
	D	64.40 c	41.60 d	53.00 B
	Means	80.50 A	66.10 B	

Means sharing the same letters are statistically non-significant

Table 4. Morphological characterization of calli induced under contrasting incubation conditions.

Explant type	Incubation conditions	Callus induction	Callus proliferation	Calli color	Texture
Stem segment	LD	++++	++++	Light green to Green	Smooth, compact & friable
	D	++	+++	Cream to Yellow	Semi hard and watery
Petiole segment	LD	+++	++++	Green to Dark green	Smooth, compact & friable
	D	++	++	Cream to Yellow	Semi hard and watery

Data taken from three reps. ± SE from two independent experiments with 25-30 explants per rep
Excellent = +++++; V. Good = ++++; Good = +++; Normal = ++; Poor = +; - No growth

Table 5. Embryogenesis responses on MS_3 medium in calli under contrasting incubation conditions.

Embryogenesis	Incubation conditions	
	Long day	Dark
No. of days to embryogenesis	8.44 a	4.56 b
No. of embryo/calli segment	2.38	3.74
Embryogenesis (%)	21.89 b	33.67 a

Means sharing the same letters in a row are statistically non-significant
Data are means of three Reps. ± SE from two independent experiments with 25-30 explants per rep

Callus induction on auxinic medium and embryogenesis on auxin free medium has been used in a wide range of plant species (Samaj *et al*., 2003; Feher *et al*., 2003; Raghavan, 2004) and previously up taken 2,4-D was released leading to morphogenesis (Meijer *et al*., 1999). Somatic embryogenesis upon transfer of the carrot calli to hormone (2,4-D and Kinetin) free medium was noticed but the frequency of embryogenesis was low. The cell lines grew in an unorganized manner, with few embryos that appeared in calli after transfer to MS_2 medium. In contrast, no morphogenesis could be obtained on hormone-free medium in soybean (Montague *et al*., 1981) using kinetin and 2,4-D for callus induction. Similarly, no embryogenesis could be obtained in the carrot calli induced on medium containing 2,4-D, however, few morphological changes were observed (Michalczuk *et al*., 1992). Our results were better in the frequency of organogenesis and conversion of embryos into plantlets on hormone-free medium however, the frequency of occurrence was low. These findings also disagree with Kumar *et al.*, (2004)

who reported regeneration in carrot calli on MS medium containing B5 vitamins and 0.2 mgL^{-1} kinetin (MS_B). We obtained little regeneration upon transfer of calli to MS_B medium (data not shown) might be due to genotypic variation.

Sucrose has been more effective in inducing embryogenesis compared with other carbohydrate sources due to rapid hydrolysis (Blanc *et al*., 2002). It is an essential source of energy in sub-cultured cells and is normally present at a 3% level in media (Mater, 1986). In contrast, we obtained a well proliferated embryogenic callus on CI medium containing reduced sucrose (2%). Reduction in sucrose i.e., alone and in combination with kinetin enhanced embryogenic potential of calli whereas reduction in MS salts and omission of sucrose did not affect embryogenesis. These findings are contrary to Lee *et al.*, (2001) who reported higher embryo induction after treatment on medium lacking sucrose. However, temporary reduction of sucrose content (2%) in the medium enhanced embryogenic potential in calli which responded profusely upon transfer to media with 3% sucrose. Similar enhanced embryogenesis associated with sucrose starvation stress has been reported in date palm (Veramendi & Navarro, 1996).

The omission of sucrose in the medium in earlier reports (Lee *et al*., 2001; Blanc *et al*., 2002) induced high-frequency embryogenesis in carrot with morphologically abnormal embryos and a lack of conversion to plantlets (Suhasini *et al*., 1996). Further, studies indicated inefficient micropropagation on solid medium (Saito & Nishimura, 1994) while suspension culture resulted in vitrification (Etienne *et al*., 1997). The above reports showed limited success in addressing these issues whereas present studies showed ~8-10 times higher rate of embryogenesis and organogenesis from stem segment derived calli using reduced sucrose and kinetin in the solid medium compared with conventional embryogenesis by transferring calli from auxinic to non-auxinic medium. A similar morphogenic variation is reported in carrot with varying levels of sucrose and IAA. Providing osmotic stress without hormonal treatment could also induce somatic embryogenesis in carrot. High concentrations of sucrose inducing a high frequency of somatic embryogenesis may be through specific carbon or osmotic potential effects (May & Trigiano, 1991), sucrose also acts as an osmoregulator**.** These findings suggested that temporary starvation of sucrose may trigger somatic embryogenesis at a higher rate. Shoot induction was directly proportional to the supplement of kinetin in MS_3 medium having kinetin (KT) and frequency of regeneration was better (3-5 shoots per calli segment) compared with no shoot regeneration obtained on MS_2 medium without pre-treatment of the calli.

Table 6. No. of somatic embryos and shoots/plantlets developed on MS_2 medium in response to the treatment time of calli on medium containing kientin (KT).

Media	Pre-treatment time (weeks)	Globular to heart embryos	Torpedo to cotyledonary embryos	Means
KT_o	2	187.00 a	25.00 b	106.00 A
	3	8.00 de	18.33 c	13.17 B
KT_1	2	3.33 efg	5.67 def	4.50 D
	3	0.67 fg	7.67 de	4.17 DE
KT_2	2	0.67 fg	5.67 def	3.17 DE
	3	7.67 de	2.33 fg	5.00 D
KT_3	2	0.67 fg	16.33 c	8.50 C
	3	4.00 efg	1.67 fg	2.83 DE
KT_4	2	1.67 fg	9.67 d	5.67 CD
	3	0.00 g	1.67 fg	0.83 E
		Shoots	Plantlets	Means
KT_o	2	14.33 de	2.33 g	8.33 DE
	3	27.67 c	27.67 c	27.67 B
KT_1	2	0.00 g	0.67 g	0.33 G
	3	9.67 ef	0.00 g	4.83 EF
KT_2	2	39.00 b	2.67 g	20.83 C
	3	11.00 e	5.00 fg	8.00 E
KT_3	2	34.67 b	101.67 a	68.17 A
	3	0.00 g	0.67 g	0.33 G
KT_4	2	3.33 g	18.67 d	11.00 D
	3	3.33 g	0.00 g	1.67 FG

Means sharing the same letters are statistically non-significant

Data are means of three Reps. ± SE from two independent experiments with 25-30 explants per rep.

Conclusion

Agar was more conducive to embryogenic callus induction and plant regeneration with no vitrification compared with phytagel. The medium containing MS salts, $20gL^{-1}$ sucrose in combination with B5 vitamins (MS_3 medium) showed better plant growth with more number of explants (3-5) per plant, and higher regeneration potential. Horizontal placement of stem explant induced higher frequency embryogenic calli under LD compared with D conditions. Calli pre-treatment for two weeks on media with reduced sucrose (2%) and kinetin ($0.6\ mgL^{-1}$) followed by transfer to MS_3 media with 3% sucrose enhanced embryogenesis and shoot regeneration up to ten times compared with 33% embryogenesis found in calli without sucrose starvation treatment.

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