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Response of Exogenous Growth Regulators on Callus Proliferation, Chromosomal Instability and Morphogenesis in *Pisum sativum* Genotypes

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Abstract: Embryonic axis explants of four genotypes of pea (*Pisum sativum* L.) *viz*. IC 219023, IC 219006, EC 389374 and EC 385244 from 5 days old seedlings was cultured on MS medium with NAA (1.0 and 2.0 mg/l) and BAP (0.2 and 0.5 mg/l) in combinations along with control. Data were observed as days of explants swelling, callus induction and fresh and dry weight of obtained tissue. All the genotypes gave maximum fresh and dry weight of tissue when 2.0 mg/l NAA and 0.5 mg/l BAP was incorporated in the media while genotype EC 389374 recorded maximum callus growth at all hormonal combinations/treatments. Callus tissues from all culture types were also studied to detect chromosomal instability in terms of numerical and structural changes at 45 days of culture/age. The majority of cells studied were observed to be diploid (2n = 14) in nature. Among the numerical aberrations induced, tetraploid cells were observed to be most frequent. Bridges were also noticed to present among other structural changes. Effect of phytohormone was positive on chromosomal instability whereas genotypic effect was not observed with regard to chromosomal instability. Maximum regeneration via callus tissues into plantlets were achieved in genotypes IC 219006 and EC 389374 which indicates association of plant regeneration with chromosomal behaviour of the source, callus cultures.

Key words: Pea · Callus tissues · Chromosomal aberrations · Somaclonal variation · Organogenesis · Regeneration

INTRODUCTION

Pea (*Pisum sativum* L.) is an important pulse crop owing its importance due to high protein content. Moreover, protein quality of pea is superior to other legumes due to its high lysine content. In spite of intensive breeding efforts no major breakthrough in yield potential has been made in *Pisum spp.* This appears to be due to inadequate genetic variability for seed yield and its components. Hence, there is a need to generate more variability, which can be utilized for improving seed yield coupled with high nutritional value.

The discipline of plant tissue culture has been an area of intensive investigation during the last few decades and it has found applications in conventional breeding methods used for the improvement of crops. Genetic variability is the key factor in any breeding method which, created through conventional breeding techniques and dependent on recombination [1]. The developments in plant tissue culture have opened up new possibilities in creating genetic variability. Callus tissues have a unique potential for generating genetic variations. Although the chromosomal constitution of certain plants seems to be highly stable *in vitro* [2], much of the variability found in tissue culture can be directly or indirectly attributed to gross chromosomal changes and chromosomal abnormalities. Plant regeneration from relatively undifferentiated callus tissues possesses a vast array of genetic changes [3]. Eventually the plantlets regenerated from these callus tissues possess a vast array of genetic changes. This type of genomic instability is described as 'Somaclonal variation' [4]. The changes in chromosome structure and number induced in cultured cells may provide new variations for use in breeding programmes aimed at crop improvement. Variability through tissue culture techniques can be generated by using different starting tissues like cotyledon and embryonal axis which can be in turn induced to differentiate into new plantlets via either organogenesis or embryogenesis pathway.

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Somaclonal variation can be used to obtain desired ploidy levels and single gene mutants, which through conventional breeding methods are not possible [5]. On the other hand variation in chromosome structure and number disturbs the physiological and genetic balance of the callus leading to a loss in the capacity to regenerate plants [6, 7]. Thus, plant regeneration appears to be linked with chromosomal behaviour of the source, callus culture [8]. Therefore, there is a need to establish the nature and source of variation in culture cells and to regulate the degree of variation with a view to explore the possibility of regenerating plants with varying chromosome numbers [9, 10].

Development of reliable protocols applicable to evolve commercially desirable genotypes is required for successful utilization of *in vitro* techniques. Present investigation has been carried out to screen *in vitro* genotypes and a suitable nutrient medium to obtain maximum response.

MATERIALS AND METHODS

Plant Materials: The seeds of pea cultivars namely IC 219023, IC 219006, EC 389374 and EC 385244 were obtained from the Division of Germplasm Exchange, National Bureau of Plant Genetic Resources, New Delhi. The mature seeds were washed several times under running tap water and then surface sterilization was carried out with 0.1 per cent $HgCl_2$ for 5 minutes and washed three times repeatedly with sterile double distilled water. Aseptic seedlings were raised on growth regulator free MS medium [11] in 100ml conical flask.

Callus Studies: Five days old seedlings were taken for the preparation of embryonal axis explant. Explant was separated with the help of forceps and blade and transferred on MS medium containing supplemented with 5.37 μM NAA+ 0.88 μM BAP, 5.37 μM NAA +2.22 μM BAP, 10.74 μM NAA +0.88 μM BAP, 10.74 μM NAA +2.22 μM BAP. A control with no growth regular was also administered along with the different phytohormone treatment combinations. Each 25 ml volumetric flask with embryonal axis explant and plugged and capped with aluminum foil was incubated under complete darkness at 26±2°C in Growth chamber (Yorko, Sales Pvt. Ltd.) and B.O.D. incubator for subsequent recording of observations. Later the cultured flasks were subjected to 12 h photoperiod regime of 30 mmol m^{-2} s^{-1} luminance provided by white PAR lamps.

Chromosomal Studies: For the cytogenetic studies, samples of 45 days old callus were selected on the basis of their growth and fresh weight from various treatments. The selected calli were pre-treated with 3mM 8-hydroxy quinoline for 4-5 hrs at 16-18°C for arresting division at metaphase. The treated callus was washed thoroughly with distilled water and fixed in freshly prepared solution (3 parts of 95% ethanol and 1 part of glacial acetic acid) for 24 hours. Finally callus tissues were preserved in 70% ethanol and stored in refrigerator at 4°C for subsequent use. Temporary slides were prepared by squash technique using acetocarmine (2%) stain. Before squashing in 1% acetocarmine, the material was hydrolyzed in a mixture of 1N HCl. Microphotographs were taken with the help of Olympus Photomicrograph attachment with Olympus Occular Periplan O.K. 15x and Apocromatic objective 100 x NA 1.30 oil immersion.

Callus Mediated Organogenesis Studies: Callus tissues of all the four genotypes were transferred on shoot inducing/ shooting media (10.74 μM BAP + 0.88 μM NAA) for 25 days. The genotypes which showed shooting were transferred to rooting media with two concentrations of hormone viz. 10.74 μM NAA (rooting 1) and 26.85 μM NAA (rooting 2) for 25 days. The cultures were incubated at 16 hrs photoperiod at a light intensity of 55 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps (Philips, India) at 25±2°C. The cultures were examined after 4 weeks. The regenerated seedlings were removed from culture vessels, washed gently under slow running tap water and transferred to sterilized pots containing a mixture of sterilized sand, cow dung and soil in a ratio of 1:1:1 (v/v) . The plantlets were kept under controlled conditions. The percentage of survival was recorded after every 5 days.

Observations and Statistical Analysis: The experiment was laid out in Completely Randomized Design (CRD) with both equal and unequal number of replicates consisted of 400 - 500 embryonal axis. The cultures were examined every day to record observations on days to swelling, rating of callus growth, days to callusing, fresh and dry weight of obtained callus. The data were analysed statistically by the Duncan's multiple range test [12]. Mean figures followed by the same letter were not significantly different at $p<0.05$ level. Approximately 400-500 cells (dividing) were studied for each selected treatments to observe frequency of chromosomal abnormalities in terms of numerical changes, frequency

of chromosomal aberrations in terms of structural changes and effect of chromosomal abnormalities on regeneration frequency. The percentage values were calculated on the basis of total number of cells studied.

RESULTS AND DISCUSSION

The present investigation was initiated to study the effects of combination of some normally used phytohormone on callus growth and its chromosomal behaviour in embryonal axis explant of four different genotypes of pea (*Pisum sativum* L).

Among the phytohormone treatments studied, genotype EC 385244 showed minimum days for explant swelling (6.63 days) at phytohormone treatment 10.74 μM NAA +2.22 μM BAP whereas genotype EC 389374 exhibited maximum days (10.63 days) for explant swelling at 10.74 μM NAA +0.88 μM BAP phytohormone combination (Table 1). The control showed negligible swelling of explant with four genotypes under study. A visual rating of the callusing response by all the genotypes revealed that callus formation was observed at all the phytohormone combinations except for control. In all phytohormone combinations colour of the callus was creamy brownish (Figure 1 a-d) except for control, where it was dark brownish in colour. The genotypes EC 389374 and EC 385244 showed excellent callusing, whereas genotypes IC 219023 and IC 219006 showed very good callusing at phytohormone combination 10.74 μM NAA +2.22 μM BAP till the termination of the experiment (Table 2). Genotype EC 389374 also showed very good callus growth at 5.37 μM NAA + 2.22 μM. Fair callus growth was observed in genotypes IC 219023 and EC 385244 at 5.37 μM NAA+ 0.88 μM BAP treatment and in genotypes EC 389374 and EC 385244 at 10.74 μM NAA +0.88 μM BAP treatment. At control, all genotypes showed callus initiation till termination of experiment. All culture types exhibited a progressive tissue proliferation through the course of experiment till termination except for cultures at control which showed a stagnant performance. All genotypes exhibited early callus induction at all phytohormone treatments than control. Among the phytohormone treatments, all genotypes exhibited early callus induction at 10.74 μM NAA +2.22 μM BAP followed by 5.37 μM NAA +2.22 μM BAP.

Table 1: Mean values in Pisum sativum genotypes treated with various phytohormone combinations

Genotypes	Phytohormone combinations NAA+BAP (μM)	Days to swelling	Days to callus induction	Tissue fresh weight (gm)	Tissue dry weight (gm)
IC 219023	Control	-	15.000^{h}	0.0552^{g}	0.00574^{h}
	5.37+0.88	10.375^{e}	12.714^{de}	0.2134^{ef}	0.02478^{fg}
	5.37+2.22	8.750^{d}	12.286^{d}	0.7969^{b}	0.0854^{b}
	10.74+0.88	8.625^{d}	14.286^{gh}	0.2395^{def}	0.02902^{efg}
	10.74+2.22	7.375^{ac}	10.857^{bc}	0.8546^{ab}	0.09116^{b}
IC 219006	Control	-	14.714^{h}	0.0437^{g}	0.00484^{h}
	5.37+0.88	8.375^{cd}	11.429^{c}	0.3359^{cde}	0.03788^{def}
	5.37+2.22	8.375^{cd}	10.286^{b}	0.4443^{c}	0.04916^{cd}
	10.74+0.88	10.375^{e}	13.571^{fg}	0.2960^{def}	0.03108^{efg}
	10.74+2.22	7.250^{a}	9.429^{a}	0.8596^{ab}	0.09144^{b}
EC 389374	Control	-	14.571^{h}	0.0413^{g}	0.00410^{h}
	5.37+0.88	8.750^{d}	13.286^{ef}	0.4353^{c}	0.04988^{c}
	5.37+2.22	8.250^{cd}	12.143^{cd}	0.8114^{b}	0.08316^{b}
	10.74+0.88	10.625^{e}	14.571^{h}	0.2196^{ef}	0.02552^{efg}
	10.74+2.22	8.375^{cd}	10.571^{b}	0.9551^{a}	0.09536^{ab}
EC 385244	Control	-	14.429^{h}	0.0457^{g}	0.00494^{h}
	5.37+0.88	7.250^{a}	10.429^{b}	0.2066^{f}	0.02514^{fg}
	5.37+2.22	7.750^{bc}	11.429^{c}	0.3475^{cd}	0.03698^{def}
	10.74+0.88	8.125^{cd}	13.571^{fg}	0.2068^{f}	0.02124^{g}
	10.74+2.22	6.625^{a}	9.286^{a}	0.9732^{a}	0.10450^{a}
	SD	0.588	0.554	0.0648	0.0063

Means having different letters in a column differs significantly by Duncan's multiple comparison test ($p<0.05$)

Table 2. Rating of callusing response in embryonal axis of Pisum sativum genotypes at different days after inoculation

	Phytohormone combinations NAA+BAP (µM)															
	15 DAI					30 DAI					45 DAI					
Genotypes	Control	5.37+0.88	5.37+2.22	10.74+0.88	10.74+2.22	Control	5.37+0.88	5.37+2.22	10.74+0.88	10.74+2.22	Control	5.37+0.88	5.37+2.22	10.74+0.88	10.74+2.22
IC 219023	+	+	+	+	++	+	++	++	++	+++	+	++	+++	+++	++++
IC 219006	+	+	++	+	+++	+	++	+++	++	++++	+	+++	+++	+++	++++
EC 389374	+	+	++	+	+++	+	++	+++	++	++++	+	+++	++++	++	+++++
EC 385244	+	++	++	+	+++	+	++	++	++	++++	+	++	+++	++	+++++

+ callus initation, ++ fair, +++ Good, ++++ Very good, +++++ Excellent

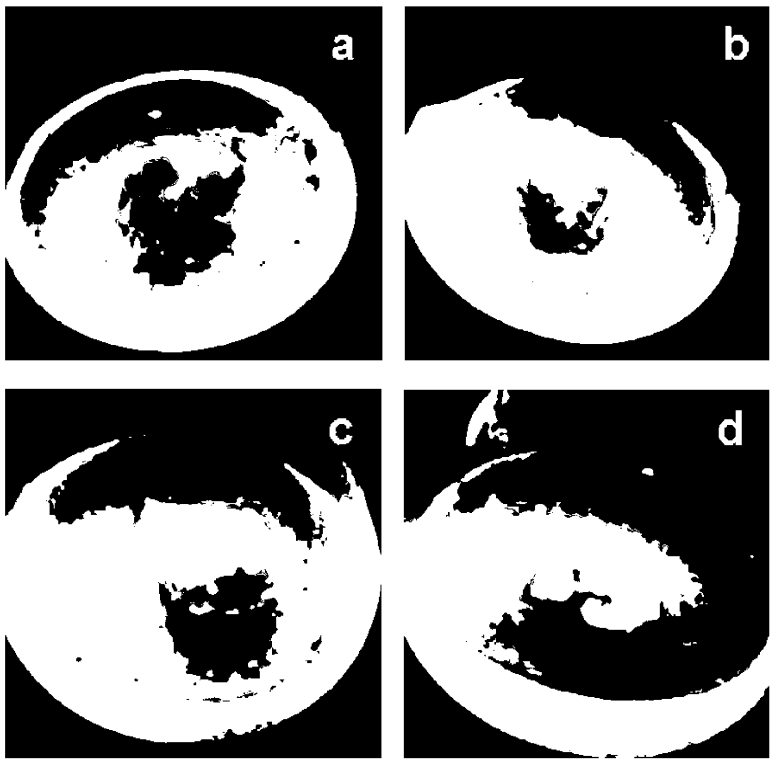

Fig. 1(a-d): Callus cultures derived from embryonal axis explant of *Pisum sativum* genotypes. a. IC 219023, b. IC 219006, c. EC 389374, d. EC 385244.

Genotype EC 385244 showed maximum tissue fresh weight (0.973 gm) at 10.74 µM NAA +2.22 µM BAP treatment combination. Among the hormonal treatments maximum tissue fresh weight was recorded at 10.74 µM NAA + 2.22 µM BAP for all four genotypes. All the four genotypes showed minimum tissue fresh weight at control. Similarly, Cardi and Monti [13] reported increased density of callus with the presence of cytokinin in pea. Both tissue fresh weight and dry weight exhibited increasing trend with increase in the concentration of phytohormones. Genotype EC 385244 showed maximum tissue dry weight (0.1045 gm) at 10.74 µM NAA +2.22 µM BAP treatment combination followed by EC 389374 (0.0953 gm). De and Roy [14] reported that the callus showed best growth in NAA at 10.74 µM with either kinetin or BAP at 2.22 µM in pea. Raruqui *et al*. [15] observed best callusing response in media containing MS + NAA (5.37 µM) + BAP (2.22 µM) in *Pisum spp*.

The above results confirms the protocol of 10.74 µM NAA +2.22 µM BAP in MS medium given by De and Roy [14] in pea along with embryonal axis explant as it exhibited early explant swelling and callus induction and also gave maximum tissue fresh and dry weight in all the four genotypes. Genotype EC 389374 proved to be best as it exhibited earliest explant swelling and callus induction as well as excellent callus growth. It also showed maximum tissue fresh and dry weight.

A perusal of table 3 revealed that no genotypic differences were observed in terms of numerical changes induced at various phytohormone treatments.

Table 3: Numerical and Structural changes in chromosomes in embryonal axis callus cultures of Pisum sativum under different phytohormone treatments

Genotypes	Phytohormone combinations NAA+BAP (μM)	Diploid cells (%)	Triploid cells (%)	Tetraploid cells (%)	Aneuploid cells (%)	Fragments (%)	Bridges (%)	Rings (%)
	Control	89.03	0	8.99	0	0	0	0
IC 219023	5.37+0.88	73.89	0.86	17.74	3.81	1.06	2.13	0
	5.37+2.22	68.14	2.03	18.89	4.44	1.46	2.3	0.41
	10.74+0.88	65.67	2.9	19.12	4.89	1.96	2.39	1.16
	10.74+2.22	58.59	3.99	21.44	5.65	2.09	2.96	1.3
IC 219006	Control	83.07	0	12.13	0	0	0	0
	5.37+0.88	69.95	1.44	18.12	5.01	0.97	1.92	0
	5.37+2.22	63.69	2.13	20.06	5.9	1.23	2.09	0.2
	10.74+0.88	60.73	2.78	20.9	6.13	1.39	2.41	0
	10.74+2.22	54.71	3.87	22.12	6.68	1.99	3.16	0.61
EC 389374	Control	85.99	0	11.79	0	0	0	0
	5.37+0.88	72.63	1.21	17.98	3.22	1.17	2.04	0
	5.37+2.22	66.45	2.36	19.34	4.11	1.8	2.67	0.29
	10.74+0.88	63.16	2.9	19.99	4.82	2.01	2.9	0.38
	10.74+2.22	56.77	3.87	21.28	5.79	2.77	3.4	0.79
EC 385244	Control	84.75	0	12.01	0	0	0	0
	5.37+0.88	71.86	1.99	18.03	2.88	0.89	1.73	0
	5.37+2.22	67.68	2.91	18.92	3.45	1.34	2.07	0.33
	10.74+0.88	65.67	3.33	19.47	3.96	1.91	2.38	0.86
	10.74+2.22	58.91	4.69	20.94	5.09	2.14	2.91	1.41

The frequency of diploid cells was maximum in control. It ranged from 89.03% in genotype IC 219023 to 83.07% in IC 219006. The frequency of diploid cells was minimum at 10.74 μM NAA +2.22 μM BAP (54.71%) in IC 219006. In general it was observed that all the four genotypes exhibited decrease in the frequency of diploid cells with increase in concentration of growth hormones. Callus samples taken from all the treatment combinations revealed the predominance of diploid cell population and it was observed that the frequency of diploid cells decreased with the increase in concentrations of phytohormones. Genotypes did not show any specific trend in terms of variations induced at various phytohormone treatments. Gostimskii *et al.* [16] and Ezhova *et al.* [17] analyzed chromosomes of the callus cultures of *Pisum sativum* L. and reported that 85% of the callus cells under study were diploid cells.

The frequency of tetraploid cells was maximum in all genotypes cultured at different phytohormone combinations with regards to other polyploid cells. Mathur and Prakash [18] reported that frequency of tetraploid cells was higher as compared to cells with higher ploidy level. All the four genotypes under study showed maximum frequency of tetraploid cells in phytohormone combination 10.74 μM NAA +2.22 μM BAP *viz.* 22.12% in IC 219006, 21.44% in IC 219023, 21.28% in EC 389374 and 20.94% in EC 385244. The frequency of triploid cells was low as compared to diploid and tetraploid cells. At control triploid cells was absent in all four genotypes. Triploids also showed an increasing trend with increase in phytohormonal concentrations in all the four genotypes. Highest frequency of Triploid cells was observed in EC 385244 (4.29 %) at 10.74 μM NAA +2.22 μM BAP hormonal treatment. Gostimskii *et al.* [16]reported 11% tetraploid cells in callus cultures of pea whereas Tawakley *et al.* [19] reported 14% triploid cells and 67 % tetraploid cells in callus cultures of chickpea. The higher percentage of polyploidy cells indicates the chromosomal reduplication in the callus cells. Increase in the age of the callus enhanced the frequency of ploidy cells.

Aneuploids were observed in all treatment combinations except in control for all four genotypes studied (Table 3). The frequency of aneuploid cells also increased steadily with the increase in hormonal concentration. Maximum frequency of aneuploids was observed at 10.74 μM NAA +2.22 μM BAP phytohormone treatment viz. 6.68% in IC 219006, 5.79% in EC 389374, 5.65% in IC 219023 and 5.09% in EC 385244. Gostimskii *et al.* [16] reported 4% aneuploid cells in callus cultures of pea. The aneuploids were also observed in callus cultures by Ezhova *et al.* [17] in pea and Sekerka [20] in *Vicia sativa*. Mathur and Prakash [17] reported that frequency of aneuploid cells showed increase with increase in age of culture.

Fig. 2(a-c): Structural anomalies in callus cultures of *Pisum sativum*: a. bridge formation at anaphase, b. loop formation at metaphase, c. trinucleate callus cell.

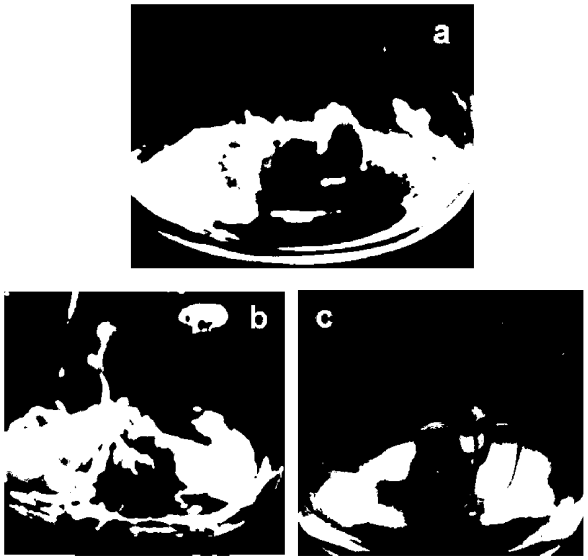

Fig. 3(a-c): Callus differentiation and organogenisis in embryonal axis cultures of *Pisum sativum*

Besides variation in chromosome number, the culture types also showed mitotic and interphase anomalies (Table 3) like anaphase bridges, fragments, chromosomal stickiness, ring chromosomes, cells with defected nuclear morphology and binucleated to multinucleated cells. In general it was observed that structural changes also increase with the increase in concentrations of phytohormones. The abnormalities were maximum at higher concentration of phytohormone *ie*. 10.74 µM NAA +2.22 µM BAP, for all four genotypes where as it was absent at control for all the four genotypes.

The analysis of cultured cells revealed that maximum frequency of bridges at anaphase (Figure 2a) was associated with maximum frequency of fragments. Maximum frequency of bridges and fragments were observed in genotype EC 389374 at 10.74 µM NAA +2.22 µM BAP hormonal treatment (3.40% and 2.77% respectively). Maximum frequency for ring chromosomes was also observed at 10.74 µM NAA +2.22 µM BAP. It ranged from 1.41% in EC 385244 to 0.61% in IC 219006. A fair frequency of interphase anomalies such as binucleated cells, multinucleated cells and cells with irregular shape of the nucleus was observed (Figure 2 b & c). It was observed that structurally changed cells were at a low advantage and were not able to divide and propagate in a manner similar to diploid cells. Zhang *et al*. [21] and Turkov *et al*. [22] reported a fairly high frequency of structural rearrangements in chromosomes of cultured cells and the most frequent were translocations/dicentrics and chromosome/chromatid exchanges. The above results revealed that combination treatment of phytohormones 10.74 µM NAA +2.22 µM BAP gave maximum frequency of both structural and numerical abnormalities, whereas genotypes did not show any specific trend for variations with regard to structure and number in chromosomes.

Regeneration via callus into plantlets was achieved in genotypes IC 219006 and EC 389374 (Figure 3a - c). Mathur and Prakash [17] reported plant regeneration from callus tissues of *Vigna mungo* L. after 30 days of inoculation. Both the genotypes exhibited maximum frequency of shooting at 0.88 µM NAA + 10.74 µM BAP while rest two genotypes viz. IC 219023 and EC 385244 have showed comparatively low frequency of shooting (Figure 4). Kosturkova *et al*. [23] reported that all screened genotypes were able to regenerate plants with a high efficiency (50-100%), although some differences in their organogenetic response were observed. The shoots proliferated where transferred to rooting medium with different concentrations of growth hormones viz. 10.74 µM NAA and 26.85 µM NAA for regeneration into complete plantlets. The shoots of genotype IC 219006 responded rooting at both the concentrations of NAA, whereas EC 389374 showed rooting only at 26.85 µM NAA. Natali and Cavallini [24] and Raruqui *et al*. [14]

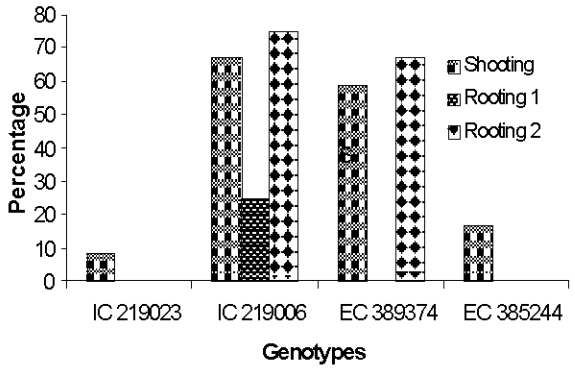

Fig. 4: Callus mediated organogenesis for embryonal axis explant of Pisum sativum genotypes

reported that genotype effect for regeneration was evident but the effect of media was prominent. The plantlets from both genotypes viz. IC 219006 and EC 389374 were transferred to pot with sterilized sand. The plantlets were of 8-10 cm in height having 4-6 leaves and they survived only for 15 days.

Overall it can be concluded that in the present study hormonal differences were observed in terms of callus growth as well as chromosomal aberrations induced. Genotypes IC 219023 and EC 385244 exhibited maximum frequency of various types of chromosomal aberration with very low frequency of plant regeneration. On the contrary, genotypes IC 219006 and EC 389374 exhibited comparatively low frequency of chromosomal aberrations with high frequency of plant regenerations. It clearly indicates that plant regeneration is associated with chromosomal behaviour of the source, callus cultures.

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