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Somatic embryogenesis in different root segments of *Punica granatum* L

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ABSTRACT

Three segments of roots i.e. root tip (the meristematic zone), root-middle (the cell elongation and differentiated zone), root base (near the root-hypocotyl junction) taken from in vitro germinated seedlings of Punica granatum L. var. Ganesh; showed differential response to various plant growth regulators when cultured in vitro on B5 medium. Addition of 2,4-D induced whitish grey globular callus from root tip, which on further subculture to 0.5 mg L^{-1} BA produced somatic embryos from the peripheral region of the callus. BA induced embryogenic callus in middle and base segments of root. Whereas direct somatic embryogenesis occurred in these two segments i.e. middle and base of the root when cultured on 2 mg L^{-1} Kinetin. NAA caused rhizogenesis in all the root segments. Differentiation of somatic embryos took place on B5 medium supplemented with 0.1 mg L^{-1} NAA + 0.5 mg L^{-1} BA + 2 mg L^{-1} Kinetin.

Key Words: *Punica granatum*, Somatic Embryogenesis, Morphogenesis.

Abbreviations: *BA = Benzyl Adenine, 2,4-D = 2,4-Dichlorophenoxyacetic acid, Kin. = Kinetin (6-furfuryl amino purine), NAA = α-Naphthalene Acetic Acid.*

INTRODUCTION

In vitro plant regeneration has been greatly helped by development of somatic embryogenesis technique, started more than five decades ago by Steward et al (1958).According to Sharp et al (1980) somatic embryogenesis in a culture can be initiated in two ways; (i) directly from the original explant tissues or (ii) indirectly through from callus or cell suspension culture. In present study induction and development of both direct and indirect somatic embryogenesis in pomegranate (*Punica granatum* L Var. Ganesh) is presented

The pomegranate is one of the oldest known edible fruits and an excellent tree for growing in arid zones for its resistance to drought conditions. The future of this fruit depends on the selection of high quality cultivars with soft seeds and fruits resistant to cracking and fruit borers . Breeding efforts are on for this purpose. Availability of somatic embryogenesis protocol would be immensely useful in this endeavour.

Ammirato (1983) has advocated advantages of somatic embryogenesis as an alternative technique for *in vitro* clonal propagation of plants. However, indirect somatic embryogenesis cannot be accepted as a method of clonal propagation as they show variation from the mother plant and can be used to produce somaclonal variants; thus offering advantages for genetic improvement and novel genotypes (Evans *et al* 1981).

Somatic embryos in pomegranate have been induced earlier through leaf explant explants (Omura 1987), cotyledonary tissues (Bhansali 1990) and petal cultures (Natraja and Neelambika 1996) and of pomegranate. They used either RBM II or MS medium.

In this paper we report induction of direct and indirect somatic embryogenesis in three segments of young roots of pomegranate using Gamborg et al's B 5 medium supplemented with various growth adjuvant individually or in different combinations. Explant types and plant growth regulators, both are known to influence somatic embryogenesis (Levi and Sink 1991). While studying the response of different parts of young root explants from seedlings of *Punica granatum* L. var. Ganesh, to various plant growth regulators, it was found that meristematic tissues like root apex did not directly produce somatic embryos. Whereas differentiated tissues from the base and middle part of root could produce somatic embryos. Here a study of morphogenetic response by root explants of *Punica granatum* to cytokinins (BA and Kin) and auxins (2, 4-D and NAA) is presented.

MATERIALS AND METHODS

Explant Source: Four weeks old seedlings of *Punica granatum* L. var. Ganesh; germinated on half MS medium; having 4 cm long roots without any lateral roots or lateral root primordia were taken as source of root explants.

To get the seedlings, seeds were drawn from the mature ripe fruits of *P.granatum*. The fleshy pulp surrounding the seeds was removed and then washed in running tap water. Seeds were sterilized by keeping them in 0.1% mercuric chloride for 2 min and then rinsing with autoclaved distilled water. Seeds were further treated with 0.5% sodium hypochorite containing few drops of Tween-20, for 10 min and rinsed twice with autoclaved distilled water. Seed coat was removed from the seeds under aseptic conditions and embryos along with cotyledons were inoculated on to half strength MS (Murashige and Skoog 1962) basal medium with 3% sucrose and 0.8% agar at pH 5.7 and incubated in dark for one week. After that it was transferred to 16 hrs photoperiod followed by 8hrs dark period. Four weeks after inoculation seedlings with 4 cm long main root without any lateral root were selected as source of root explants.

0.5 cm long segments of roots from (a) tip having meristematic tissues (b) middle of root having cell elongation and differentiation zone and (c) root near base i.e. transition zone from root to hypocotyl having differentiated tissues were taken as explant.

Culture medium and conditions: Root segments were inoculated on Gamborg's B5 (Gamborg *et al* 1968) medium supplemented with different growth regulators either separately or in

combination. The growth regulators were 2, 4-D (2, 4-Dichlorophenoxyacetic acid); NAA (Naphthalene Acetic Acid), BA (Benzyl Adenine) and Kin (6-furfuryl amino purine). Table 1 contains the concentration range used.

Twenty combinations of NAA, BA and Kin (Table 2) were tried for plantlet differentiation from somatic embryos. Roots were incubated in dark for first ten days at 28^0C and then transferred to 16-h photoperiod at a light intensity of 50 µE m^{-2} s^{-1}; followed by 8 h dark period at 24^0C. A regular subculture at four weeks interval was maintained throughout the experiment.

Hardening of in vitro regenerated plants from somatic embryos- were tried on five different potting medium i.e. soil, vermiculite, 1:1 soil + vermiculite, 1:1 soil + vermiculite +1% cellrich, 1:1 soil + sand+1% cellrich.

RESULTS

The effect of different plant growth regulators are presented in table 1.

Culture Establishment: Within 5 days of inoculation, all the root segments turned black. However, a microscopic examination of the cross section of these roots revealed that only peripheral tissue was dark, but the central part of the root remained fresh and root segments continued to grow.

Morphogenetic Response: Three different root segments showed different response to the PGRs tried (Table 1).

Table 1.*In vitro* response by different segments of roots of *Punica granatum* L. to auxins (2, 4-D & NAA) and cytokinins (BA & Kin) supplemented to B5 medium

B5 + PGR (mg L^{-1})	Type Of Morphogenetic Response & (No. of explants responded)		
	Root Tip	Root Middle	Root base
2,4-D			
0.5	GC (24)	NR	NR
1.0	GC (18)	NR	NR
2.0	GC (7)	NR	NR
5.0	GC (2)	NR	NR
NAA			
0.01	NR	Rhi (2)	Rhi (3)
0.05	NR	Rhi (6)	Rhi (7)
0.1	NR	Rhi (20)	Rhi (18)
1.0	NR	Rhi (25)	Rhi (25)
Kin			
0.5	NR	DSE (4)	DSE (5)
1.0	NR	DSE (17)	DSE (15)
2.0	NR	DSE (24)	DSE (23)
5.0	NR	DSE (2)	DSE (3)
BA			
0.5	NR	ISE (23)	ISE (24)
1.0	NR	ISE (17)	ISE (17)
2.0	NR	ISE (5)	ISE (3)
5.0	NR	NR	NR

Results are mean of 100 replicas

(GC = Globular Callus, DSE = Direct Somatic Embryogenesis, ISE = Indirect Somatic Embryogenesis or embryogenic callus, Rhi = Rhizogenesis, NR = No Response).

2, 4-Dichlorophenoxyacetic acid - Within 4 weeks 2,4-D induced whitish gray, globular mass of callus in the *root tip segment*, which first appeared from the cut end of the root tip (Fig. 1a) and then from the whole surface. These globular callii did not grow further unless they were transferred to BA containing medium in the next sub-culture, where some of them continued to grow as callus. Transfer to hormone free medium was also not effective in continuing the growth of callus.

Root Middle and Root base did not respond to any of the tried concentration of 2, 4-D.

Naphthalene Acetic Acid - NAA could neither induce callusing nor embryos, but within fifteen days of inoculation rhizogenesis was observed in all the segments (Fig. 1d).

Benzyl Adenine - in presence of BA, after two subcultures i.e. in approximately 2 months, embryogenic callus appeared from the surface of all the explants taken from both the *middle and the base of the root segments* (Fig 1b & 1c). From these embryogenic calli, embryos developed within 15 days (Fig 1g). 0.5 mg L^{-1}BA was the most effective concentration; with increase in concentration the number of explants responded became fewer (Table 1). 5.0 mg L^{-1} did not elicit any response.

Table 2 Effect of various combinations of auxins and cytokinins supplemented to B5 medium; on plantlet differentiation from somatic embryos developed from root segments of *Punica granatum* L. Results are mean ± S.E. of 20 replicas.

B5 Medium Supplement With mg L^{-1}			Average Number Of Healthy Plantlets Generated/Segment
NAA	KIN	BA	
0.01	1.0	0.5	0
0.05	1.0	0.5	1.0 ± 0.006
0.01	1.0	1.0	1.5 ± 0.008
0.05	1.0	1.0	2.7 ± 0.011
0.01	2.0	0.5	8.3 ± 0.009
0.05	2.0	0.5	6.4 ± 0.007
0.01	2.0	1.0	5.1 ± 0.010
0.05	2.0	1.0	4.3 ± 0.004
0.01	-	0.5	0
0.01	-	1.0	0
0.05	-	0.5	0
0.05	-	1.0	0
0.1	-	1.0	0
-	1.0	0.5	1.3 ± 0.013
-	1.0	1.0	1.6 ± 0.010
0.01	1.0	-	0
0.01	2.0	-	0
0.05	1.0	-	0
0.05	2.0	-	0
1.0 2,4-D	0.5	0.2	0

6-Furfuryl Amino Purine - 2 mg L^{-1} Kin induced direct somatic embryogenesis in middle and the base of the root segments (Fig 1h, 1i, 1j). In root tip segment in only 5% explants, direct somatic embryos appeared only from the cut end, which was nearer to the cell elongation and

differentiation zone. It could be that responding tissues from the cut end of root tip were partly from the tissue-differentiated zone.

Maturation of Somatic Embryos: Development of globular embryos (Fig. 2a and 2b) into distinct bipolar structure or heart shaped (Fig. 2c) and torpedo shaped (Fig 2d) structure took place on the B5 medium for both directly (2 mg L^{-1}) and indirectly (0.5 mg $^{L-1}$) formed embryos From induction to maturation took 4 months. In indirectly formed somatic embryos, there were callus adjoining the embryos, which continued to produce more callus. From these calli regularly more somatic embryos were produced (Table –1).

Plantlet Differentiation from somatic Embryos,: Mature embryos from all the three root explants; on transfer to B5 medium supplemented with 0.01 mg L^{-1} NAA + 0.5 mg L^{-1} BAP + 2 mg L^{-1} kin (Table 2); differentiated into root and stem (Fig 3a,b,c).

Hardening and in field growth- As it can be seen in table – 3 and figure 3d both soil and vermiculite either separately or in combination can be used as potting substrate for hardening the plant. Fig 3e is field view of the 6 months old in vitro generated plants.

DISCUSSION

Although the first ever report of somatic embryogenesis was from the carrot roots (Steward et al 1958), the roots remained less preferred explant for production of somatic embryos (Jia et al 1989). However there are many report of somatic embryogenesis from the root explants (Fujimura and Kommamine 1979 and Vuorela et al 1992).

Thomas et al (1979), Ammirato (1983), Raghvan (1986) and Mathews et al (1993) found that auxins are most needed growth regulator for induction of somatic embryogenesis and its removal from the medium promoted further development of the embryos. But in the present work both the auxins were not found very effective. 2,4-D produced whitish-grey globular callus, which did not grow further even after the removal of 2,4-D. NAA could cause only rhizogenesis.

Right from the first report of somatic embryogenesis by Steward, who used coconut milk a well known source of cytokinin; many workers over a period of nearly 5 decades have reported importance of cytokinins in inducing and developing somatic embryos (Ranch et al 1963, Hiroaka and Tabata 1974, Bhojwani and Razdan 1983, Raghav and Nabor 1984, Kushalkar and Sharon 1996).

Whereas according to Fujimura and Kommamine (1979) BA and Kin have inhibitory effect on embryogenesis. Dodds and Roberts (1985) have also advocated that the role of cytokinins in embryogenesis is somewhat obscure; which was further supported by Pierik (1987) suggesting that cytokinins do not have a vital role in inducing embryogenesis.

In the present work we found that both the cytokinins tried was able to induce somatic embryogenesis. Kin (2 mg L^{-1}) directly in all the three segments of the root whereas BA (0.5 mg L^{-1}) indirectly into tissue differentiated regions of the root but not in meristematic region i.e. root tip. The maturation and differentiation media for both types of embryogenesis (direct and indirect) was the same.

One important observation was that the cells from differentiated tissue zones responded better to cytokinins and produced somatic embryos; whereas meristematic zone of the root tip did not.

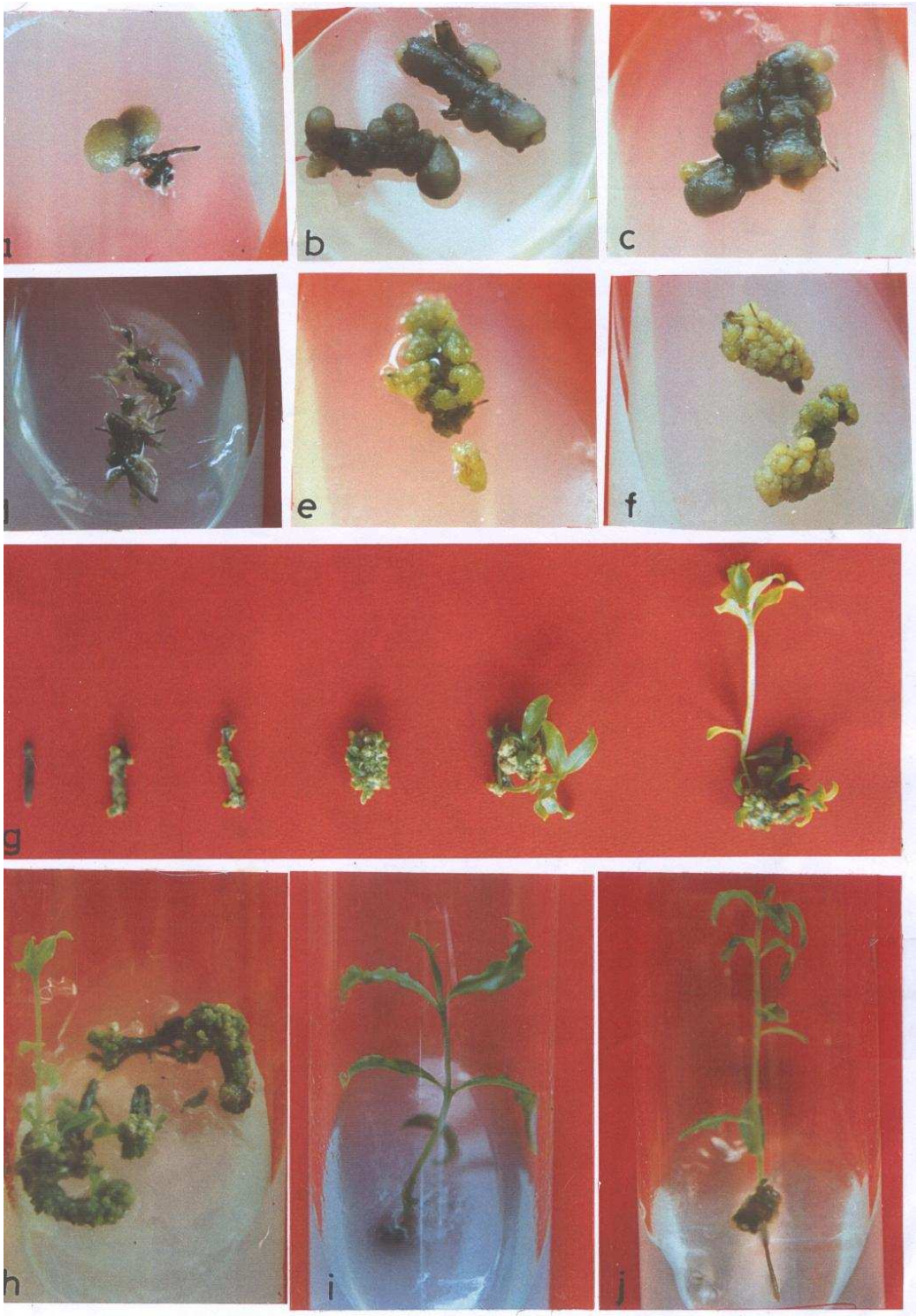

Figure – 1: In vitro response by different root segments of *Punica granatum* L cultured on B5 media to different pant growth regulators (a) root tip showing globular gray callus in presence of 2,4-D , (b & c) middle of root and root base respectively showing embryogenic callus in presence of BA (d) root segment showing rhizogenesis in presence of NAA (e & f) Kin induced direct somatic embryogenesis in middle & the base of the root segments (g) various stages of development of plantlets on medium containing 0.01 mg L^{-1} NAA + 0.5 mg L^{-1} BAP + 2 mg L^{-1} kin (h, i, j) direct somatic embryogenesis and plantlet development in roots on medium supplemented with2 mg L^{-1} Kin.

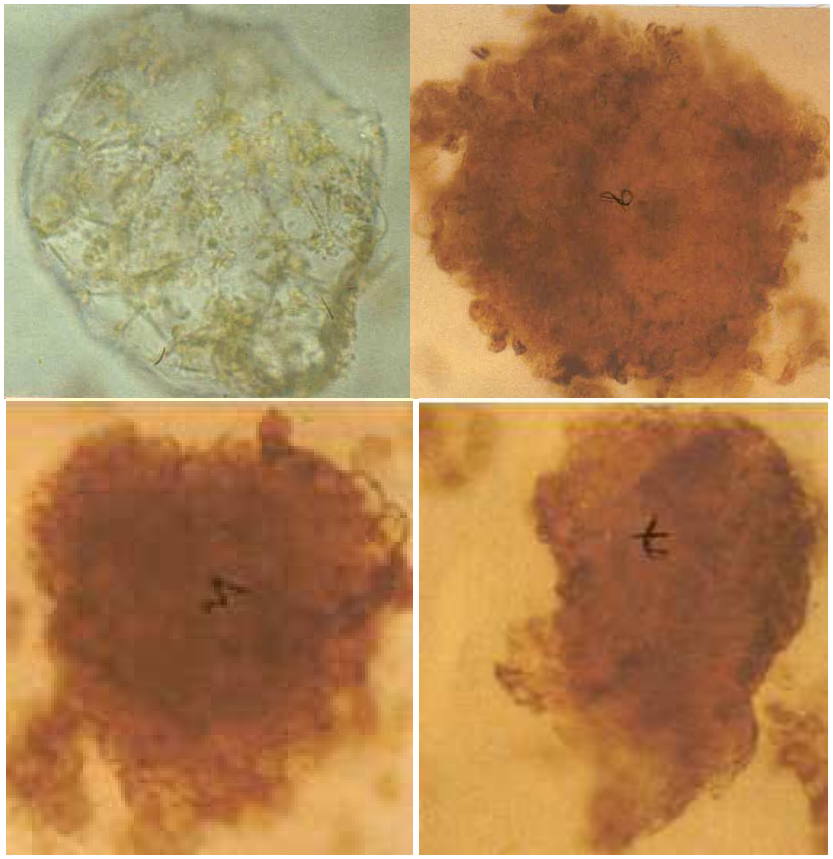

Figure – 2 . Microscopic observation of cultures of *Punica granatum L.* showing different stages of somatic embryo (a) initial stage of globular embryo (b) globular embryo (c) heart shaped embryo & (d) torpedo shaped embryo

This may be due to the consideration that somatic embryogenesis needs certain degree of maturation, which is perhaps not achieved by meristematic cells of the root tips. These observations suggest that explants as well as the cell type vary in their response to growth regulator, as expressed by their differential morphogenetic response to same growth regulator by three different segments of the root. However NAA was consistent in exerting its effect as rooting hormone in all the three segments.

Figure 3a – Plants regenerated through somatic embryo genesis are being hardened different potting mixtures (i) 1:1 sand and soil+ 1% cellrich (ii) 1:1 vermiculite and s + 1% cellrich (iii) 1:1 vermiculite and soil (iv) vermiculite (iv) soil

Figure 3b – 6 months old *Punica granatum* plants regenerated through somatic embryogenesis are growing in the field

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