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Somatic embryogenesis in *Cicer arietinum* L: Influence of genotype and auxins

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Abstract

Plant regeneration through somatic embryogenesis was obtained in chickpea (*Cicer arietinum* L.) using immature cotyledons and immature embryonal axes as explants. 2,4-dichlorophenoxyacetic acid (2,4-D), 4-amino-3.5-6-trichloropicolinic acid (picloram) and 3.6-dichloro-0-anisic acid (dicamba) in concentrations 1, 2, 5, 10 mg dm^{-3} were found better than naphthaleneacetic acid (NAA) (10 - 20 mg dm^{-3}) for the induction of globular and heart-shaped somatic embryos. The embryos developed upto the dicotyledonary stage on medium supplemented with saccharose, mannitol and silver nitrate ($AgNO_3$) and developed further into plantlets on medium containing gibberellic acid (GA_3) and abscisic acid (ABA). The frequency of somatic embryogenesis was dependent on the genotype and auxins used.

Introduction

Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes of Asia, Africa and Latin America and is a rich source of dietary proteins. Grain legumes in general are not particularly amenable to *in vitro* culture and plant regeneration from callus is especially difficult (for review see Bajaj 1990). Previous reports on *C. arietinum* have dealt with the development of multiple buds from shoot apical meristems (Kartha *et al.* 1981) and regeneration from seedling explants (Sharma *et al.* 1979). Although globular somatic embryos were observed in leaflet derived cultures of *C. arietinum* (Rao and Chopra 1989), further differentiation into plantlets had not been recorded.

In the present communication, we report plant regeneration through somatic embryogenesis in *C. arietinum.*

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Materials and methods

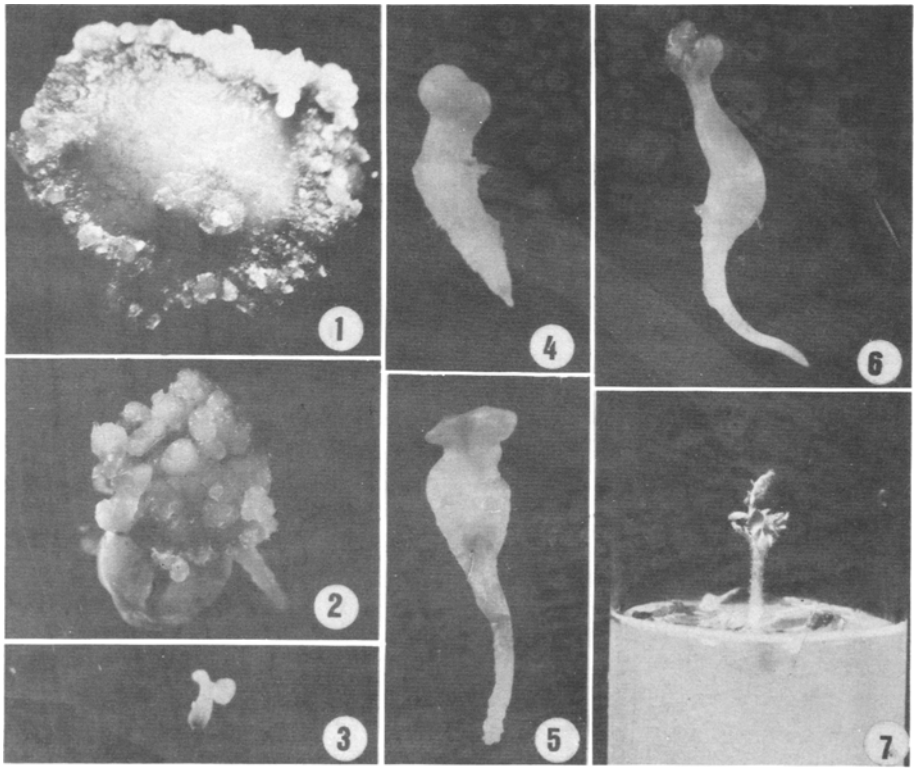
Chickpea (*Cicer arietinum* L.) cv. ICCC 32, ICCV-5 (Kabuli types) and ICCC-37 (Desi type) were grown in the Experimental Field Station at Trombay. Pods containing early mature stage embryos (18 - 21 d after flowering) were harvested. Staging was initially determined by observing the silhouette of immature seeds *in situ.* The pods were surface sterilized with 70 % ethanol (v/v) for 1 min. followed by sterilization with 0.1 % mercuric chloride (m/v) for 10 min. The pods were then rinsed five times with sterile distilled water, cut open and the seeds removed under aseptic conditions. The cotyledons measuring about 0.5 - 0.7 cm in length were carefully removed from the embryonal axis. Both cotyledons and embryonal axis were used for culture. The cotyledons were cultured with the abaxial side in contact with the medium.

Culture medium: The basal medium consisted of L-6 salts (Kumar *et al.* 1988), casein hydrolysate (200 mg dm^{-3}), 6 % saccharose (m/v), 1 % sorbitol (m/v) and 0.8 % agar (m/v) (*High Media,* Bombay). The medium was supplemented with 2,4-D, dicamba, picloram (1, 2, 5 or 10 mg dm^{-3}) or NAA (10 or 20 mg dm^{-3}). The cultures were incubated at 25 ± 2 °C and 14 h photoperiod (irradiance 121 µmol m^{-2} s^{-1}). The globular and heart-shaped embryos were transferred to MS medium (Murashige and Skoog 1962) containing 3 % saccharose (m/v), 3 % mannitol (m/v) and 10 mg dm^{-3} $AgNO_3$. After reaching the dicotyledonary stage the embryos were individually transferred to medium containing MS mineral elements, GA_3 (1 mg dm^{-3}) and ABA (0.26 mg dm^{-3}) for further growth. In average 20 embryonal axes or 40 cotyledons were used for each experiment and the experiments were repeated once.

Results

Cotyledons: Highly organized, round, creamish to white coloured protuberances differentiated from all over the surface of the cotyledons (Figs. 1 and 2) when they were cultured on L-6 medium supplemented with 2,4-D (1, 2, 5 or 10 mg dm^{-3}). In many instances, the epidermal cell layer of the cotyledon split up and organized structures emerged from the subepidermal layers. The number of organized structures was higher at the nodal region compared to the rest of the cotyledon. These organized structures closely resembled globular and heart shaped somatic embryos. When 2,4-D was tested at concentrations 1, 2, 5 and 10 mg dm^{-3}, 2,4-D at concentration 1 mg dm^{-3} produced the highest frequency of cultures showing somatic embryogenesis (100 %) (Table 1) and highest average number of somatic embryos per cotyledon (50, Table 2) in cv. ICCC-32. However, in cv. ICCV-5, 10 mg dm^{-3} of 2,4-D induced the highest frequency of responding cultures and 5 mg dm^{-3} of 2,4-D induced the highest number of somatic embryos (Tables 1 and 2). When picloram was used at 1, 2, 5 or 10 mg dm^{-3}, the frequency of responding cultures varied from 57 to 89 % in the different cultivars and average number of somatic embryos per

cotyledon ranged from 4.1 to 27.1 (Tables 1 and 2). However, in presence of 2 mg dm^{-3} picloram, cv. ICCC-37 produced somatic embryos which matured beyond the heart shaped stage into dicotyledonary stage. The embryos in general had typical "hour glass" shape (Fig. 5). In no other treatment did somatic embryos mature in a single step up to the "hour glass" shape.



Figs. 1 - 7. Development of plants through somatic embryogenesis in *C. arietinum* cv. ICCC-37.
Figs. 1, 2. Differentiation of highly organized structures from cotyledons on L 6 medium supplemented with 10 mg dm^{-3} 2,4-D at the end of 2 and 4 weeks, respectively.
Fig. 3. A dicotyledonary somatic embryo on MS medium supplemented with 3 % mannitol (m/v), 3 % saccharose (m/v) and silver nitrate (10 mg dm^{-3}) 6 weeks after initiation of the experiment.
Figs. 4, 5, 6. Well developed somatic embryos growing on MS medium supplemented with GA_3 (1 mg dm^{-3}) and ABA (0.26 mg dm^{-3}) after 8 - 10 weeks - Fig. 5 shows an "hour glass" shaped somatic embryo.
Fig. 7. A regenerated plant at the end of 3 months on MS medium supplemented with GA_3 (1 mg dm^{-3}) and ABA (0.26 mg dm^{-3}).

Dicamba was tested only in one cultivar (ICCC-37) and the response varied from 60 to 80 % and average number of somatic embryos ranged from 9 to 18 (Tables 1 and 2). When NAA (10 or 20 mg dm^{-3}) was used, callus formed at the cut end of the

cotyledon with profuse rooting in all the three cultivars. Although a few somatic embryos were observed initially, they later produced roots from root pole.

Table 1. Frequency of cotyledons of *C. arietinum* producing somatic embryos in presence of different auxins (percentage in parentheses).

Auxins type	conc. [mg dm^{-3}]	Frequency of cultures showing somatic embryos ICCC-37	ICCC-32	ICCV-5
2,4 D	1	27/33 (81.8)	36/36 (100.0)	26/38 (68.4)
2,4 D	2	30/35 (85.7)	29/33 (87.8)	21/36 (58.4)
2,4 D	5	27/35 (77.1)	32/36 (88.9)	21/24 (87.5)
2,4 D	10	28/30 (93.3)	21/32 (65.6)	34/37 (91.9)
Picloram	1	33/39 (84.6)	32/37 (86.4)	32/36 (88.8)
Picloram	2	33/37 (89.2)	26.35 (74.2)	27/36 (75.0)
Picloram	5	29/33 (87.9)	33/39 (84.6)	25/31 (80.6)
Picloram	10	29/37 (78.4)	20/35 (57.1)	32/36 (88.9)
Dicamba	1	22/34 (64.7)		
Dicamba	2	20/33 (60.6)		
Dicamba	5	28/35 (80.0)		
Dicamba	10	14/28 (50.0)		

Table 2. Average number of somatic embryos (globular and heart shaped) produced from cotyledons and embryonal axis of *C. arietinum* on medium containing different auxins at the end of one month of cultivation. Mean ± S.E.

Auxins type	conc. [mg dm^{-3}]	Average number of somatic embryos per explant ICCC-37 cotyledons	ICCC-37 embryonal axis	ICCC-32 cotyledons	ICCC-32 embryonal axis	ICCV-5 cotyledons	ICCV-5 embryonal axis
2,4 D	1	9.3 ± 1.4	8.7 ± 1.2	50.0 ± 4.5	37.1 ± 3.4	12.7 ± 1.8	35.8 ± 5.0
2,4 D	2	13.4 ± 1.9	6.4 ± 2.3	11.6 ± 1.5	27.7 ± 6.0	8.4 ± 1.1	11.5 ± 2.6.
2,4 D	5	24.5 ± 3.8	5.5 ± 1.0	27.6 ± 3.3	48.2 ± 12.9	21.2 ± 3.3	18.9 ± 5.1
2,4 D	10	17.1 ± 2.6	3.8 ± 0.9	11.1 ± 2.1	4.3 ± 0.8	22.2 ± 3.2	11.0 ± 4.4
Picloram	1	15.0 ± 2.0	8.5 ± 2.7	15.1 ± 2.0	26.1 ± 4.7	10.8 ± 1.5	13.3 ± 2.3
Picloram	2	10.4 ± 1.7	7.5 ± 1.3	14.9 ± 2.9	8.3 ± 2.4	4.1 ± 0.5	5.6 ± 0.8
Picloram	5	16.9 ± 2.5	9.0 ± 2.5	27.1 ± 3.2	7.0 ± 1.0	14.9 ± 3.9	11.5 ± 3.3
Picloram	10	11.2 ± 1.9	6.7 ± 1.5	8.7 ± 2.3	8.0 ± 1.5	16.8 ± 2.7	9.7 ± 3.4
Dicamba	1	18.1 ± 3.7	26.6 ± 6.4				
Dicamba	2	13.0 ± 3.8	8.0 ± 4.0				
Dicamba	5	11.6 ± 1.9	6.9 ± 0.8				
Dicamba	10	8.9 ± 0.9	5.8 ± 0.6				

When NAA was added, no somatic embryo was found.

Embryonal axis: When tested at different concentrations, 2,4-D at 1 and 2 mg dm^{-3} produced 100 % response in cultivar ICCC-32, while it elicited the best response in cultivar ICCC-37 at 1 mg dm^{-3} (Table 3). In cv. ICCC-32, the highest number of

somatic embryos (48) was produced in presence of 5 mg dm^{-3} of 2,4-D, while in ICCV-5 1 mg dm^{-3} 2,4-D was more effective (Table 2). When 1 or 2 mg dm^{-3} picloram was used, 100 % of the embryonal axes produced somatic embryos in ICCC-37, whereas for ICCV-5 1 mg dm^{-3} picloram elicited the maximum response (Table 3). Out of four different concentrations tested, 1 mg dm^{-3} picloram produced the highest average number of somatic embryos in cv. ICCC-32 (Table 2). In presence of 2 mg dm^{-3} picloram, the embryonal axes gave rise to somatic embryos, that matured into the typical "hour glass" shaped embryos.

Table 3. Frequency of somatic embryos from embryonal axes of *C. arietinum* cultured on medium supplemented with different auxins (percentage in parentheses).

Auxins		Frequency of somatic embryos per culture		
Type	conc. [mg dm^{-3}]	ICCC-37	ICCC-32	ICCV-5
2,4 D	1	12/13 (92.3)	15/15 (100.0)	16/19 (84.1)
2,4 D	2	8/11 (72.7)	15/15 (100.0)	10/12 (83.3)
2,4 D	5	11/17 (64.7)	11/13 (84.6)	11/13 (84.6)
2,4 D	10	5/11 (45.5)	6/15 (46.1)	6/11 (54.5)
Picloram	1	9/ 9 (100.0)	12/14 (85.7)	18/18 (100.0)
Picloram	2	15/15 (100.0)	9/15 (60.0)	14/19 (73.6)
Picloram	5	7/10 (70.0)	16/19 (84.2)	8/10 (80.0)
Picloram	10	12/15 (80.0)	32/36 (88.9)	7/11 (63.6)
Dicamba	1	10/12 (83.3)		
Dicamba	2	3/11 (27.5)		
Dicamba	5	10/16 (62.3)		
Dicamba	10	10/18 (55.5)		

Development of plants: For further development, globular and heart shaped somatic embryos obtained from cotyledons and embryonal axes were transferred to MS medium supplemented with 3 % saccharose, 3 % mannitol and 10 mg dm^{-3} $AgNO_3$. About 10 % of the globular and heart shaped embryos of all the three cultivars developed into torpedo and cotyledonary stages in this medium (Figs. 3, 4, 5, 6). Medium without $AgNO_3$ and mannitol did not promote the growth of the embryos. The majority of the cotyledonary embryos had fused cotyledons which did not open. The shoot meristems with primary leaves did not emerge in such cases. However, approximately 1 % of the cotyledonary type of somatic embryos showed the development of shoot meristems with the emergence of the first primary leaves when cultured on MS medium supplemented with GA_3 (1 mg dm^{-3}) and ABA (0.26 mg dm^{-3}). The developed somatic embryos were strikingly different from the zygotic embryos due to their small-sized cotyledons. Although all the three cultivars produced well developed somatic embryos, only 1 % of the somatic embryos of one cultivar (ICCC-37) developed into plantlets in this way. The plantlets developed upto 2 - 3 cm long with well developed leaves and roots (Fig. 7). Attempts are being made to transfer them to the field.

Discussion

The present studies have shown that it is possible to induce somatic embryogenesis and plant regeneration from immature cotyledon and immature embryonal axes of chickpea. Immature cotyledons (Lazzeri *et al.* 1987, Ozias-Akins 1989, Eapen *et al.* 1993), immature embryonal axes (Hazra *et al.* 1989) and immature embryos (Komatsuda and Ohyama 1988, Kysely *et al.* 1987) have been successfully used for somatic embryogenesis in other grain legumes as well.

In chickpea cultivars, 2,4-D was found to be the auxin of choice for the induction of somatic embryogenesis although picloram and dicamba were also effective. NAA was not effective in the induction of somatic embryogenesis at the concentrations tested. 2,4-D (Hazra *et al.* 1989, Lazzeri *et al.* 1987, Ranch *et al.* 1986, Griga 1993) and picloram (Kysely *et al.* 1987) have been widely used for somatic embryo induction in grain legumes. Addition of $AgNO_3$ and mannitol to the medium promoted the growth and development of somatic embryos in culture. Silver nitrate, an ethylene inhibitor, is known to enhance shoot morphogenesis in *Brassica campestris* (Chi and Pua 1989). Similarly, osmotic agents such as mannitol in the medium supported the induction of differentiation in long term cultures of rice (Kavikishore 1987). The well developed somatic embryos of chickpea were transferred to medium supplemented with GA_3 and ABA for further development into plants. ABA has been used in maturation of somatic embryos in *Carum carvi* (Ammirato 1973, 1974) and *Daucus carota* (Kamada and Harada 1981). GA_3 has been also found beneficial for development of somatic embryos in *C. carvi* (Ammirato 1977). Kavethekar *et al.* (1978) overcame somatic embryo dormancy with GA_3 in *Eschescholzia californea.* The frequency of somatic embryogenesis in the present study was found to be the result of interaction between the genotype, source material and the type and concentration of auxin used. Komatsuda and Ohyama (1988) observed that out of the 26 genotypes of soybean tested, different cultivars responded differently to hormones for somatic embryo induction. Parrot *et al.* (1989) found that the genotype had a significant effect on the ability of immature soybean cotyledons to undergo the auxin stimulated somatic embryogenesis.

Although we were successful in obtaining plantlets through somatic embryogenesis in chickpea, the frequency of plant development was low. Attempts are in progress to improve the efficiency of plantlet development from somatic embryos so that this technique could find application in transformation experiments.

References

Ammirato, P.V.: Some effects of abscisic acid on the development of embryos from caraway cells in suspension culture. - Amer. J. Bot. **60** (Suppl): 22-23, 1973.

Ammirato, P.V.: The effects of abscisic acid on the development of somatic embryos from cells of caraway (*Carum carvi* L.). - Bot. Gaz. **135**: 328-337, 1974.

Ammirato, P.V.: Hormonal control of somatic embryo development from cultured cells of caraway: Intractions of abscisic acid, zeatin and gibberellic acid. - Plant Physiol **59**: 579-586, 1977.

Bajaj, Y.P.S.: Biotechnology in Agriculture and Forestry 10. Legumes and Oil Seed Crops I. - Springer-Verlag, Berlin - Heidelberg - New York 1990.

Chi, G.L., Pua, E.C.: Ethylene inhibitors enhanced *de novo* shoot regeneration from cotyledons of *Brassica campestris* spp. *in vitro*. - Plant Sci. **64**: 243-250, 1989.

Eapen, S., George, L., Rao, P.S: Plant regeneration through somatic embryogenesis in peanut (*Arachis hypogaea* L.) - Biol. Plant. **35**: 499-504, 1993.

Griga, M.: Some factors affecting somatic embryogenesis efficiency in soybean (*Glycine max* (L.) Merr.). - Biol. Plant. **35**: 179-189, 1993.

Hazra, S., Sathaye, S.S., Mascarenhas, A.F.: Direct somatic embryogenesis in peanut (*Arachis hypogaea*). - Biotechnology **7**: 949-951, 1989.

Kamada, H., Harada, H.: Changes in endogenous levels and effects of abscisic acid during somatic embryogenesis of *Daucus carota* L. - Plant Cell Physiol. **22**: 1423-1429, 1989.

Kartha, K.K., Pahl, K., Leung, N.L., Mroginwski, L.A.: Plant regeneration from meristems of grain legumes: soybean cowpea, peanut, chickpea and bean. - Can. J. Bot. **59**: 1671-1679, 1981.

Kavikishore, P.B.: Energy and osmotic requirement for high frequency regeneration of rice plants from long term cultures. - Plant Sci. **48**: 189-194, 1987.

Kavathekar, A.K., Johri, B.M.: *In vitro* response of embryoids of *Eschscholzia californica*. - Biol. Plant. **20**: 98-106, 1978.

Komatsuda, T., Ohyama, K.: Genotypes of high competence for somatic embryogenesis and plant regeneration in soybean *Glycine max*. - Theor. appl. Genet. **75**: 695-700, 1988.

Kumar, A.S.; Gamborg, O.L., Nabors, M.W.: Plant regeneration from cell suspension cultures of *Vigna aconitifolia*. - Plant Cell Rep. **7**: 138-141, 1988.

Kysely, W., Mayers, J.R., Lazzeri, P.E., Collins, G.B., Jacobsen, H.J.: Plant regeneration via somatic embryogenesis in pea (*Pisum sativum* L.). - Plant Cell Rep. **6**: 305-308, 1987.

Lazzeri, P.A., Hildebrandt, D.F., Collins, G.B.: Soybean somatic embryogenesis: effects of nutritional, physical and chemical factors. - Plant Cell Tissue Organ Cult. **10**: 209-220, 1987.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Ozias-Akins, P.: Plant regeneration from immature embryos of peanut. - Plant Cell Rep. **8**: 217-218, 1989.

Parrott, W.A., Williams, E.G., Hildebrandt, D.F., Collins, G.B.: Effects of genotype on somatic embryogenesis from immature cotyledons of soybean. - Plant Cell Tissue Organ Cult. **16**: 15-21, 1989.

Rao, B.G., Chopra, V.L.: Regeneration in chickpea (*Cicer arietinum* L) through somatic embryogenesis. - J. Plant Physiol. **134**: 637-638, 1989.

Ranch, J.P., Ogelsby, L., Zielinski, A.C.: Plant regeneration from tissue cultures of soybean by somatic embryogenesis. - In: Vasil, I.K. (ed.): Cell Culture and Somatic Cell Genetics of Plants. Vol. 3. Pp. 97-110. Academic Press, Orlando - San Diego - New York - Austin - Boston - London - Sydney - Tokyo - Toronto 1986.

Sharma, D.R., Kumari, R., Chowdhury, J.B.: Plant regeneration in *Cicer* species through tissue culture. - Indian J. exp. Biol. **17**: 607-609, 1979.