In vitro Callogenesis and Plant Regeneration from Anther Culture in Groundnut (*Arachis hypogaea* L.)

SK Bera* and DM Bhatt

National Research Centre for Groundnut, Post Box-05, Ivnagar Road, Junagadh-362001, Gujarat, India

Anther or microspore culture is one of the efficient techniques for *in vitro* plant regeneration and has been exploited for haploid breeding. GG 2, a cultivated genotype of species *Arachis hypogaea* L. sub sp. *fastigiata* var. *vulgaris* was tested for *in vitro* callogenesis from anther and regeneration of anther derived calli. Callogenesis from anther starts four to five days after culturing. Callogenesis ranged from 31.5% to 91.2% in MS media with different combinations of growth regulators. Maximum callogenesis observed in medium containing MS salts with vitamins of B5+12.36 µM NAA+2.22 µM BAP+87.64 µM sucrose and 0.8 % agar (m/v). Somatic embryos were induced from anther callus in medium containing half strength MS salts with vitamins of B5+2.47 µM NAA+6.66 µM BAP+87.64 µM sucrose and 0.8 % agar. Regenerated plants were confirmed as diploid (2n=4x=40) by root tip analysis indicating that plants developed either from sporophytic tissue or diploidization.

Key Words: Anther culture, Callogenesis, Groundnut, *In vitro*, Plant regeneration

Introduction

Groundnut is the principal oil seed crop of India. It is annually grown in about 7.6 million ha with a total production of 7.8 million tonnes of nuts-in-shell. Although India is the largest producer of groundnut in the world, its share in the world is not proportionate as the crop is grown mainly as rainfed crop with high level of fluctuation in the production due to different abiotic and biotic stresses. Development of tolerant/resistant varieties at a regular pace is needed in the present breeding programmes involving both traditional and nontraditional methods.

Anther or microspore culture is one of the efficient techniques for *in vitro* plant regeneration and has been exploited in haploid breeding (Croughan *et al.*, 1987; Veilleux, 1994; Martinez *et al.*, 1996; Yang, 1997; Chen *et al.*, 1997). However, the success hampered by the difficulty of inducing callus from anther and inducing morphogenesis for different crops and cultivars (Keshari *et al.*, 1992;Yan *et al.*, 1996; Raina and Zapata, 1997). In groundnut, Bajaj *et al.* (1980) first reported induction of pollen embryos and pollen callus in anther cultures of *A. hypogaea* and *A. glabrata*. Bajaj *et al.* (1981) later reported regeneration of genetically variable plants from the anther derived callus of *A. hypogaea* and *A. villosa*. However, regeneration of plants from anther in groundnut remains unexplored due to low frequency of callogenesis and somatic embryogenesis. Several other workers (Martin and Rahechault, 1976; Sudhakar and Moss, 1990; Willcox *et al.*, 1991; Yeh and Tseng, 1999; Tseng and Yeh, 2000; Yeh and Liaw, 1998; Morginski and Fernandez, 1979; Morginski and Fernandez,1980; Still *et al.*,1987; Lee and Yeh, 2001; Pongsupasamit *et al.*, 2001; Sastri *et al.*, 1981; Pitman, 1981) though had reported anther culture, responsive stages of microspore, media for callus induction and differentiation but techniques failed to provide substantial progress in repeatability; high frequency callogenesis and plant regeneration from anther culture. The objective of this study was to develop an efficient protocol for induction of anther calli and regeneration of plants in high-frequency.

Materials and Methods

Healthy immature (2-3 mm in length) flower buds, suitable to bloom in next day morning were collected in tap water from field grown plants during 9-10 AM. Flower buds were then surface sterilized by agitating in 70% ethanol and freshly prepared 0.1% mercuric chloride solution for 10 minutes each and washed thoroughly in sterilized distilled water for three to four times. Sterilized flower buds then dissected longitudinally with sterilized needle inside the laminar flow and anthers of 0.5-1.0 mm in length were used as explants.

Anthers were inoculated in solidified medium consisting MS (Murashige and Skoog, 1962) basal salts, vitamins of B5 (Gamborg *et al.*, 1968), different doses of α-Naphthalene acetic acid (NAA) (2.47, 4.94, 7.41, 9.89, 12.36 and 14.83 µM), 6-Benzyl adenine (BAP) (2.22, 4.44 and 6.66 µM), 87.64 µM sucrose and 0.6 % agar (m/v). The medium was adjusted to pH 5.8 before autoclaving

*Corresponding author: Berask67@yahoo.co.in Fax:+91-285-2672555

at 121° C for 15 minutes and cooled before use. Single anther was inoculated on the surface of the solidified medium in 25 x 150 mm test tubes (Borosil, Mumbai, India) containing 10 cm^3 solidified media and then cultured at an irradiation of 30 $\mu molm^{-2}s^{-1}$ with 16-h photoperiod and 25±2°C temperature. All compounds and plant growth regulators used in the experiments were from Himedia laboratories Pvt. Ltd., Mumbai, India. Experiments were carried out with 3 replicates with 50 explants per replication and each treatment was repeated three times. Colour, physical properties of anther derived callus were recorded after 30 days of culture by visual observations. Callus was classified into green (G), brown (B), dark brown (D) and creamy (Cr). Physical nature of callus recorded based on compactness of callus and shape of individual growing cell of callus. Callus was grouped into compact (C) and friable (F) based on compactness of callus. Callus was further grouped into nodular (N) and normal/round (R) based on shape of growing cells. Data were statistically analyzed using Duncan's Multiple Range Test (DMRT) (Gomez and Gomez, 1976).

Results and Discussion

Callogenesis started four to five days after culturing in media with all combinations of NAA and BAP used in the experiment. However, maximum (91.2%) callogenesis recorded in hormonal combination of 12.36 μM NAA and 2.22 μM BAP (Table 1) and callus was green, nodular and compact. However, colour and structure of the callus varied with the combinations of growth hormones (Table 1). This indicates that combination of 12.36 μM NAA and 2.22 μM BAP is comparatively more effective with greater repeatability for induction of anther callus in groundnut. Lee and Yeh (2001) also reported 91.9% callogenesis. Occasional root development from callus after 25 to 30 days in same medium indicating that callus tended to be differentiated with times in the same medium. Tseng and Yeh (2000) reported similar results of root formation from anther-derived callus. Two weeks old anther calli were sub-cultured in medium consisting MS basal salts, vitamins of B5, 2.68 μM NAA, different doses of BAP (2.22, 4.44, 6.66, 8.88, 11.11, 13.33, 15.55, 17.77, 20.00, 22.22 μM), 2.89 μM GA_3, 87.64 μM sucrose and 0.6 % agar (m/v). The calli did not differentiate in any combinations of growth regulators till three weeks of culture. But cultured callus was comparatively more green and supportive in medium supplemented with 4.44 μM, 6.66 μM and 8.88 μM BAP. The calli cultured in medium with 6.66 μM BAP and 2.68 μM NAA and 2.89 μM GA_3

Table 1. Effect of NAA and BAP on anther callogenosis from field grown plants of *A. hypogaea* L. cv. GG2. Mean with different letters are significantly different according to DMRT (P<0.05)

NAA μM^{-1}	BAP μM^{-1}	Callus induction (%)	Type of Callus
2.47	2.22	56.4±0.45efg	GRF
2.47	4.44	31.5±0.82j	DRF
2.47	6.66	33.6±0.32j	CrRC
4.94	2.22	54.1±0.10g	GRF
4.94	4.44	64.8±0.37cd	GRC
4.94	6.66	43.1±0.78 hi	CrRF
7.41	2.22	48.4±0.54gh	GRF
7.41	4.44	54.5±0.48g	DRC
7.41	6.66	37.4±0.77ij	DRF
9.89	2.22	62.7±0.29def	BRF
9.89	4.44	62.9±0.20def	BRF
9.89	6.66	72.1±0.46bc	CrRF
12.36	2.22	91.2±0.17a	GNC
12.36	4.44	77.3±0.74b	DRC
12.36	6.66	68.3±0.64cd	GRC
14.83	2.22	55.5±0.17fg	BRF
14.83	4.44	64.0±0.92dc	GRC
14.83	6.66	64.4±0.73d	GRF

differentiated after 35 days, which gradually produced multiple shoots after 55 days (Fig. 1). In general, delayed differentiation (between 35-45 days after culturing) of callus was noticed in groundnut and it requires one or 2 sub-culturing. The combination of 6.66 μM BAP and 2.68 μM NAA and 2.89 μM GA_3 was further repeated 7 times and confirmed delayed regeneration (between 35-45 days after culturing) and at least one sub-culturing after 15 days. Ninety four percent shoot induction observed in calli cultured in MS medium with 6.66 μM BAP, 2.68 μM NAA and 2.89 μM GA_3 (Table 2) and produced 1.5% shoots per callus (Table 3). The repeatability and high frequency regeneration are very much essential for further experimentation which is missing in earlier report (Yeh and Tseng, 1999; Tseng and Yeh, 2000; Yeh and Liaw, 1998; Lee and Yeh, 2001; Pongsupasamit *et al.*, 2001). Young generated shoot tips (1-2 cm) were shifted to growth medium (MS salts, vitamins of B5, 13.33 μM BAP, 5.37 μM NAA, 2.89 μM GA_3, 87.64 μM sucrose and 0.8 % agar (m/v) already standardized in the authors' laboratory. Later, growing shoots (4-5 cm) were shifted to rooting medium (MS salts, vitamins of B5, 5.37 μM NAA, 87.64 μM sucrose and 0.6% agar (m/v) already standardized in authors' laboratory. Hardening of plants carried out in test tubes containing liquid Hogland's solution under *in vitro* condition. Plants were kept initially covered with polythene sheet/packets to prevent excess transpiration and exposed to natural condition inside the culture room after 2 weeks. Healthy plants were shifted to earthen pots containing 50:50 mixtures (v/v) of soil

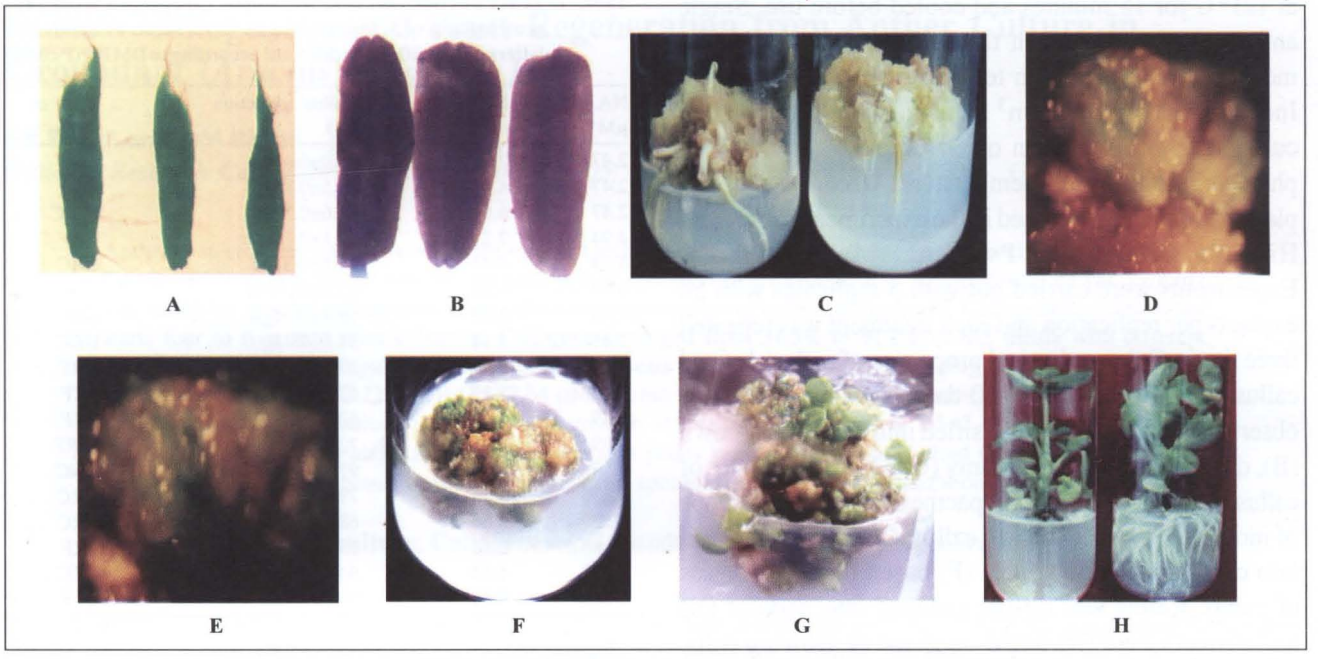

Fig. 1: Regeneration of plantlets from anther derived callus of *Arachis hypogaea* L.
A-Immature flower buds, B-Anthers used as explants, C-Induced callus with root formation, D & E-Somatic embryo, F & G-Organogenesis from somatic embryo, H-Growing shoots and plantlets

and sand. Root tip analysis of 26 generated plants confirmed 2n=40 chromosomes. It seems that plants have been regenerated from the anther tissue or from microspore mother cell. It is unlikely that regenerated plants could be spontaneous doubled haploids reported in other crops (Anderson, 2003) and callus takes more time (35-45 days) for regeneration. Further experiments may be required for confirmation of the origin and status of regenerated plants.

Table 2. Effect of NAA and BAP in regeneration of anther callus of *A. hypogaea* L. cv. GG2

NAA µM/l	BAP µM/l	Regeneration (%)	Type of callus
2.47	2.22	0	BNC
2.47	4.44	0	DRF
2.47	6.66	100 (50)	GRF
2.47	8.88	0	DRF
2.47	11.10	0	BRF
2.47	13.32	0	BRF
2.47	15.54	0	CrNC
2.47	17.76	0	BRF
2.47	19.98	0	GRC
2.47	22.20	0	GNC
4.94	2.22	0	BRF
4.94	4.44	0	GCF
4.94	6.66	0	DRF
4.94	8.88	0	DRC
4.94	11.10	0	BRF
4.94	13.32	0	GRC
4.94	15.54	0	CrRF
4.94	17.76	0	BRF
4.94	19.98	0	BRF
4.94	22.20	0	DRF

Table 3. Percent regeneration of anther callus of cv. GG 2 and number of shoot induction per callus in MS medium with hormonal combinations of 6.66 µM BAP, 2.68 µM NAA and 2.89 µM GA_3

No. of callus plated	Callus regenerated (%)	Number of shoot/callus
50	100	1.28
50	84.0	1.50
50	96.0	2.00
50	92.0	1.30
50	100.0	1.50
50	96.0	1.50
50	92.0	2.00
Mean	94.28	1.58

References

Anderson SB (2003) Doubled haploid induction in ryegrass and other grass, In: M Maluszynski, KJ Khasa, BP Forster, I Szarejko (eds.) *Doubled Haploid Production in Crop Plant*, A Manual, Kluwer Acad Publ Dordrecht, Boston, London, pp. 179-183.

Bajaj YPS, KS Labana and MS Dhanju (1980) Induction of pollen embryos and pollen-callus in anther cultures of *A. hypogaea* and *A. glabrata*. *Protoplasama* **103:** 397-399.

Bajaj YPS, KS Labana and H Singh (1981) Regeneration of genetically variable plants from the anther-derived callus of *A. hypogaea* and *A. villosa*. *Plant Sci. Lett.* **23:** 35-39.

Croughan TP, QR Chu, KS McKenzie, HJ Huang and MM Meche (1987) The use of anther culture to expedite the breeding and release of new varieties of rice. *Annu. Prog. Rep. la Agric. Exp. Stn.* **52:** 52.

Chen QF, R Afza, MV Duren and H Brunner (1997) Effects of gamma irradiation and other factors on the induction of calli and regeneration of green plants in rice anther culture. *Acta. Agric. Nucl. Sinica* **11:** 59-64.

Gamborg OL, RA Miller and K Ojima (1968) Nutrition requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* **50:** 151-158.

Gomez KA and KA Gomez (1976) *Statistical procedure for Agricultural Research with emphasis of Rice.* International Rice Research Institute, Los Baños.

Keshari AK, RL Singh, SK Agarwal, GK Agarwal and VP Agarwal (1992) Studies on anther culture of rice cultivars of Nepal. *Biotech. Biodiv. Lett.* **2:** 1-10.

Lee JK and MS Yeh (2001) Studies on anther culture of peanut. IV. Pollen development, ksomatic embryogenesis and shoot regeneration from anther culture in *A. hypogaea* L. *J. Agriculture Forestry* **50:** 65-79.

Martin JP and H Rahechault (1976) *In vitro* culture of groundnut stamens (*Arachis hypogaea* L.). II. Establishing tissue cultures and organogenesis. *Oleagineux* **31:** 19-25.

Martinez CP, VF Correa, MC Amezquita and E Tula (1996) Comparison of rice lines derived through anther culture and the pedegree method in relation to blast (*Pyricularia grisea* Sacc.) resistance. *Theor. Appl. Genet.* **92:** 583-590.

Morginski LA and YA Fernandez (1979) Arachis anther culture. *Plant. Genet. Resource Newsl.* **39:** 28.

Morginski LA and YA Fernandez (1980) Production of plantlets by anther culture *in vitro* of wild species of *Arachis* (leguminosae) *Oleagineus* **35:** 89-92.

Murashige T and F Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiol. Plant.* **15:** 473-497.

Pitman RN (1981) *In Vitro* Culture of Peanut (*Arachis* spp.) Anthers, Cotyledons and Leaflets. PhD Oklahoma State University, Stillwater, USA.

Pongsupasamit S, K Kumpeerapang, C Pongsupasamit, S Ponsupasamit, K Kumpeerapang and C Pongsupasamit (2001) Shoot regeneration from anther culture of peanut *in vitro*. *Thai J. Agric. Sci.* **34:** 141-150.

Raina SK and FJ Zapata (1997) Enhanced anther culture efficiency of indica rice (*Oryza sativa* L.) through modification of the culture media. *Plant Breeding* **116:** 305-315.

Sastri DC, MS Nalini and JP Moss (1981) Tissue culture and prospect of for improvement of *Arachis hypogaea* and other oilseed crops. In: AN Rao (ed.) *Costed Symposium on Tissue Culture of Economically Important Plants,* Singapore.

Still PE, MI Plata, RJ Cambell, LC Bueno, EA Chichester and CL Niblett (1987) Regeneration of fertile *Arachis paraguariensis* plant from callus and suspension cultures. *Plant Cell. Tiss. Org. Cult.* **9:** 37-43.

Sudhakar Y and JP Moss (1990) Anther culture of Groundnut (*A. hypogaea* L): categorization and selection of flower buds. *Oleagineux* **45:** 487-490.

Tseng YW and MS Yeh (2000) Studies on anther culture of peanut III. Effects of ABA and starch on differentiation of anther-derived calli. *J. Agri. Forestry* **49**: 25-38.

Veilleux RE (1994) Development of new cultivars via anther culture. *Hort. Sci.* **29**: 1238-1241.

Willcox MC, SM Reed, JA Burns and JC Wynne (1991) Effect of microspore stage and media on anther culture of peanut (*A. hypogaea* L.). *Plant Cell Tiss. Org. Cult.* **24:** 25-28.

Yang Z (1997) Inheritance of photoperiod sensitive male sterility and breeding of photoperiod sensitive genic male sterile lines in rice (*Oryza sativa* L.) through anther culture. *Euphytica* **94:** 93-99.

Yan J, Q Xue and J Zhu (1996) Genetic studies in anther culture ability in rice (*O. sativa* L.). *Plant Cell Tiss. Org. Cult.* **45:** 253-258.

Yeh MS and YW Tseng (1999) Studies on the anther culture of Peanuts. II. Callus induction and development of microspores *Agric. Forest. Study Rep.* **48:** 27-37.

Yeh MS and JH Liaw (1998) Studies on the anther culture of peanut I. Callus induction and shoot differentiation from anther culture in *Arachis* species. *J. Agric. Forestry* **47:** 55-56.