Plant Physiology for Sustainable Agriculture

Chief Editor

Dr. G.C. Srivastava
Head, Division of Plant Physiology
Indian Agricultural Research Institute, New Delhi

Associate Editors

Dr. Karan Singh
University Professor and Head
Department of Plant Physiology
Rajasthan Agriculture University
SKNCOA, Jobner, India

Dr. Madan Pal
Scientist
Division of Plant Physiology
Indian Agricultural Research Institute
New Delhi

1999

Pointer Publishers
Vyas Building, S.M.S. Highway
Jaipur - 302 003 (India)
Phone : 568159, 518286

65

Multiple Shoot Induction in Groundnut (*Arachis hypogaea* L.)

T. Radhakrishnan, T. G. K. Murthy and A. Bandyopadhyay
National Research Centre for Groundnut, Junagadh, Gujarat

Summary

THE direct regeneration through multiple shoots from de-embryonated cotyledons (DC) of groundnut is a hitherto insufficiently exploited path. Regeneration through multiple shoots in *Arachis hypogaea* L. could be obtained from the DC (57.89% and >40 shoots per explant) and embryo-axes (up to 100% and 10-14 shoots/explant), when cultured on MS medium supplemented with 15 mg l^{-1} BA and above. Such regeneration was not possible when epicotyl (EC), hypocotyl (HC) and immature leaves (IL) were cultured under similar conditions. However, multiple shoots in a lesser frequency (a maximum of 13 shoots per explant) were obtained from mature whole seeds (MWS) cultured on MS medium with a very high level of BA (50 mg l^{-1}). Multiple shoot regeneration varied with the level of BA (20-80%). The response, number and type of shoots per explant showed cultivar dependent variation. Multiple shoots were confined to the proximal end (the region at which the cotyledon was attached to the embryo axis) of the DC, probably originated from the pre-existing meristematic initials at this region. Preliminary histological studies indicated the origin of multiple shoots from the vascular tissue in the cotyledons. Rooting could be induced on MS basal medium supplemented with 0.2 per cent activated charcoal and 200 mg l^{-1} casein hydrolysate.

Introduction

Seeds of groundnut (*Arachis hypogaea* L.) are a major source of edible oil and protein in India. Due to its wide adaptability and use as a source of diverse food products, the crop has a global economic significance. Despite consistent efforts in crop improvement, the yield of groundnut has remained static for the last few years. Groundnut being a self pollinated species, has a very limited reproductive efficiency. This comes in the way of generating large segregating populations, which is a prerequisite for breeding programmes and rapid multiplication of elite mutants. Micropropagation is one of the most exploited techniques in clonal multiplication, especially in horticultural crops (Auge and Boccon-Gibod, 1995). The potential application of this technique in groundnut would be for the rapid multiplication of elite breeding materials like hybrids and mutants. This technique has not been fully exploited in groundnut probably because of the lack, of suitable protocols for high frequency multiplication. A high frequency micropropagation system from de-embryonated cotyledons of mature seeds through multiple shoot regeneration and the factors influencing this method have been reported here.

Materials and Methods

Surface sterilised mature whole seeds (MWS), de-embryonated cotyledons (DC) and embryo axis dissected out of mature seeds (ZE) were used as explants in this study. Initial studies were done with the cultivar J 11 and to study the cultivar related differences in regeneration, GG 2, GAUG 10 and J 11 of Spanish type, and M 13 and Kadiri 3 of Virginia type were used. The MS medium (Murashige and Skoog, 1962) with the vitamins of B5 medium (Gamborg, *et al*, 1968), supplemented with one of the 10 levels of BA, viz. 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 mg l^{-1} was used for culture. Explants were cultured as two replicates with a minimum of 50 explants per replication and were incubated at 26+1°C and 16 h photoperiod. Data on frequency of the multiple shoot forming explants, the number of shoots per explant, the multiple shoot forming explants were classified into four categories, namely, A, B, C and D with <10, 10-25 and >40 shoots per explant, respectively, and the frequency in each category was recorded. The frequency of regenerating explants was converted into percentage and arc sine transformed for the statistical analysis.

To test the possibility of inducing multiple shoot regeneration from the seedling explants, epicotyl (EC), hypocotyl (HC) and immature leaves (IL) from *in vitro* germinated seedlings of the cultivar J 11 were used. To confirm the point of origin of multiple shoots, i.e. the proximal end of the cotyledons (the region at which the cotyledon was attached to the embryo axis), de-embryonated cotyledons without the primal end were also cultured under the same hormonal regimes described above.

For histological studies, multiple shoot forming explants of the cultivar J 11 were fixed in FAA at three day intervals from the date of culture. Paraffin embedding method

was used for preparation of microtome sections and double stained with safranine and fast green.

Results and Discussion

The embryo axes and de-embryonated cotyledons enlarged in size and turned green progressively in culture. The basal medium supported the full growth of the zygotic embryo into a seedling. In media supplemented with more than 25 mg l^{-1} BA, the multiple shoots arose from both shoot apices as well as axillary buds (Table 1). The primary root portion below the mesocotyl did not produce any shoots or buds, but produced slight callus in media containing lower concentrations of BA. In zygotic embryos, though cent per cent induction of multiple shoots was observed, the maximum number of shoots per explant was only 10-14. Multiple shoot formation from mature whole seeds had similar frequencies as that of zygotic embryos. On dissecting the multiples shoot forming seeds, it was observed that the shoots were originating from the embryo axis.

Table 1: Percentage of multiple shoot formation in the explants, de-embryonated cotyledons, zygotic embryos and mature whole seeds at 10 levels of BA.

BA mg.l^{-1}	% of multiple shoot forming explants		
	DC	ZE	MWS
5	25.00	-	-
10	33.00	-	-
15	57.00	-	-
20	27.62	-	20.00
25	22.00	18.00	22.00
30	14.00	100.00	45.00
35	11.40	100.00	60.00
40	09.27	100.00	74.00
45	12.00	100.00	76.00
50	06.10	100.00	82.00

DC = De-embryonated cotyledons, ZE = Zygotic embryo, MWS = Mature whole seed

In DC, multiple shoots were produced from the protein where the cotyledons were attached to the embryo. Very rarely, a cluster of tiny shoot buds arose from the tip of a small beak growing out from the proximal end (the portion where the cotyledons were attached to the embryo) of DC. Initiation of multiple shoots started at a very low concentration (of 5 mg l^{-1}) of BA in DC when compared to ZE (25 mg l^{-1}). Further, a maximum frequency (100%) of multiple shoot induction in ZE and 57% in DC was observed. However, the

number of shoots per explant were considerably high in de-embryonated cotyledons than in zygotic embryos (visual observation). Shoot bud regeneration from the de-embryonated cotyledons were first reported by Illingworth (1968) but the frequency of the induction was not worked out.

Table 2. Frequencies (in percentages of the responding explants) of the four different categories of multiple shoot formation in de-embryonated cotyledons of cultivar J 11.

	Category of explant based on multiple shoot formation				
	A	B	C	D	
BA mg.l^{-1}	(<10 shoots)	(10-25 shoots)	(25-40 shoots)	(>40 shoots)	Total
5	21.05	5.26	15.79	-	42.11
10	15.00	5.00	20.00	10.00	50.00
15	15.79	5.26	5.26	31.58	57.89
20	-	14.29	-	14.29	28.57
25	-	30.00	-	-	30.00
30	-	5.00	10.00	5.00	20.00
35	-	10.00	-	-	10.00
40	-	5.00	-	-	5.00
45	5.00	-	-	5.00	10.00
50	-	5.00	10.00	5.00	20.00

A=Explants with <10 shoots, B=Explants with 10-25 shoots, C=Explants with 25-40 shoots, D=Explants with >40 shoots.

For detailed observations on the frequency and type of shoots per explant, the experiment was repeated with DC only. The number of shoots produced per explant varied with the concentration of BA in the medium. The maximum percentage of multiple shoot forming explants (57.89%) were observed in the medium containing 15 mg l^{-1} of BA (Table 2). This was followed by 10 mg l^{-1} (50%) and 5 mg l^{-1} (42.11%). Levels higher than 30 mg l^{-1} BA had a negative effect reducing multiple shoot formation drastically to 10% at 50 mg l^{-1} BA. The reduction in the frequency of regeneration at very high doses of BA may be due to the alteration in the physiological status of the explant, by way of interference of BA in the other metabolic pathways of the explant. The role of the physiological status of the explant in the regeneration response is well established (Yeoman and Forche, 1980; Radhakrishnan, 1996). With 15 mg l^{-1} BA, nearly 32% of the explants could be grouped into category D (>40 shoots per explant). However, it is not very advantageous to have a very high number of shoots per explant, as it makes the subculturing of the tiny shoots difficult and thus affects the recovery of the shoots. The optimum concentration of BA for

multiple shoot induction in our study was found to be 15 mg l^{-1} as compared to 1 mg l^{-1} reported by Mhatre *et al.* (1985) and Bhatia *et al.* (1985). Although Mhatre *et al.* (1985) could induce high percentage of multiple shoots, the number of shoots per explant were only 10 to 12. Sastri *et al.* (1993), on the other hand reported that 25 mg l^{-1} BA + 2 mg l^{-1} NAA could induce maximum multiple shoots in de-embryonated cotyledons.

Distinct genotypic difference was observed in multiple shot formation in the cultivars studied (Table 3). In most of the cultivars, multiple shoots could be induced by 5 to 25 mg l^{-1} of BA in the culture medium. The most responsive cultures were J 11 and GG 2 giving nearly 60 per cent response, the former at 15 and the latter at 20 mg l^{-1} of BA. The poorest response was from GAUG 10 in the medium containing 10 mg l^{-1} of BA. In the cultivar GG 2, the shoots formed were mostly fused in nature (40%) and they were difficult to separate for further growth. In M 13 and GAUG 10, the maximum frequency (24%) of fused shoots were observed at 5 and 10 mg l^{-1} BA, respectively. Though 5 mg l^{-1} BA could induce multiple shoots in the maximum number of explants, in the cultivar M 13 the number of shoots per explant was not maximum at this level of BA. However, in GAUG 10, 10 mg l^{-1} was sufficient to induce the multiple shots in the maximum frequency and had the highest number of shots per explant. In other cultivars, the multiple shoots formed were distinctly separate and were amenable to further handling. Since the cultivars studied showed clear differences in their BA requirements for maximum response, standardisation of the cytokinin requirement will be essential prior to taking up a micropropagation programme involving any new cultivar. Mhatre *et al.* (1985) studied six cultivars and found that for five of them 1 mg l^{-1} BA was optimal for maximum frequency of multiple shoot production. In their study, the cultivars used were mutants and probably genotypically related and hence, their cytokinin requirements could not be identical. Differences in the regeneration potential within the same crop species, due to the varietal (genetic) difference were reported earlier in groundnut (Seitz *et al.*, 1987; Radhakrishnan, 1996) and other legumes (Oelck and Schieder, 1983).

Seedling explants did not produce any multiple shoots. Epicotyl as well as HC explants showed callusing in varying frequencies. Immature leaves remained green throughout and there was no callusing response. At higher concentrations of BA (40 mg l^{-1} and above) EC and HC explants turned brown and no callusing was observed. This response may be attributed to the release of phenols and their subsequent oxidisation in the medium.

From the de-embryonated cotyledons without their proximal end, no shoot initials were produced revealing their incapability to produce multiple shoots at any level of BA tried. Bhatia *et al.*, (1985) also reported that only proximal ends of cotyledons were capable of producing multiple shoots and development of a break from this region was essential for the production of shoot buds. Further, preliminary studies on the anatomy of the multiple shoot forming explants revealed that the shoots originate from the vascular region present at the proximal end of DC and this development was evident in 12-15 days

Table 3: Differential response of five groundnut cultivars in multiple shoot induction.

BA ($mg.l^{-1}$)	Cultivars				
	GG2	GAUG 10	Kadiri-3	J 11	M 13
5	48.00	12.00	19.23	12.00	12.00
	(40.00)	(24.00)			(24.00)
10	40.00	28.00	32.00	16.00	24.00
	(32.00)	(24.00)			(12.00)
15	54.17	25.00	60.00	12.00	29.17
	(45.83)				(8.00)
20	60.00	21.05	26.92	12.50	20.00
	(40.00)				
25	11.54	8.00	20.69	12.00	36.00
30	26.92	4.00	13.04	13.04	21.74
35	-	8.33	9.09	16.67	33.33
40	-	12.50	0.00	33.33	12.50
45	-	12.50	0.00	32.00	14.29
50	-	4.17	8.00	31.82	36.36

Values in parentheses are the percentage of explants forming fused multiple shoots among the multiple shoot forming explants.

old explants in culture. Thus, it was apparent that the initials of multiple shoots were not produced *de novo*. The pre-existing meristematic initials were dormant and upon activation with cytokinin formed multiple shoot buds. For the activation of these meristematic initials, the level of cytokinin requirement varied from cultivar to cultivar and the concentration of the cytokinin also determined the nature of the shoots (fused or free) in many cases. However, the essentiality of the development of a beak prior to the multiple shoot formation reported earlier was ruled out in the present study as multiple shoots were induced directly from de-embryonated cotyledons without producing any break in most of the cases.

Rooting, Hardening and Field Establishment

The multiple shoots which were less than a centimetre in size were aseptically cut as a bunch and further subcultured on MS basal medium. When these shoots reached the size of about 5 cm, they were separated into individual shoots and each shoot was cultured on MS medium containing 0.2% charcoal and 200 mg l^{-1} casein hydrolysate. On continuously growing for about 4-6 weeks, the roots were produced from the basal part of the 10-13 cm long shoots. These plantlets were grown in a plant growth chamber at 3°C

and 80 per cent RH for about a week. Weak plantlets were supplemented with nutrient solution. Once the plantlets started growing normally in the growth chamber, they were transplanted to field and shaded with wheat straw. The shading was removed after the establishment of the plantlets in the field. When the transplantation was done in the rainy season, about 60 per cent of these plantlets were established in the field. However, transplantation during the summer season resulted in a higher rate of mortality, reducing the field establishment to 30-45% of the total plantlets transplanted.

REFERENCES

Auge, R. and Boccon-Gibod, J. (1995). Application in horticulture. In: *In vitro* culture and application in horticulture (ed.) H Vidalie, Oxford and IBH, New Delhi, pp. 65-90.

Bhatia, C. R., Murthy, G. S. S. and Mathews, V. H. (1985). Regeneration of plants from 'de-embryonated' peanut cotyledon cultures without nutrients and agar, *J. Plant Breeding,* 94(2): 149-155.

Gamborg O. L., Millar, R. A. and Ojiama, K. (1968). Nutrient requirements of suspension cultures of soybean root cells, *Exp. Cell Res.,* 50: 148-151.

Illingworth, J. E. (1968). Peanut plants from single de-embryonated cotyledons, *Hort. Sci.,* 3(4): 238, 175-176.

Mhatre, M., Bapat, V. A. and Rao, P. S. (1985). Micropropagation and protoplast culture of peanut, *Cur. Sci.,* 54: 1052-1056.

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

Oelck, M. M. and Schieder, O. (1983). Genotypic difference in some legume species affecting the redifferentiation ability from callus to plants, *Z. Pflazenzucht,* 91: 312-321.

Radhakrishnan, T. (1966). *In vitro* studies in the genus *Arachis.* Ph.D Thesis, University of Saurashtra, Rajkot.

Sastri, D. C., Nalini, M. S. and Moss, J. P. (1982). Tissue culture and prospects for improvement of *Arachis hypogaea* and other oil seed crops. In: Proc. COSTED symp. on tissue culture of economically important plants: National University of Singapore, Singapore, 28-30, April 1981, pp. 42-57.

Seitz, M. H., Stalker, H. T. and Green, C. C. (1987). Genetic variation for the regenerative response in immature leaflet cultures of the cultivated peanut, *Arachis hypogaea, Plant Breeding,* 98: 104-110.

Yeoman, M. M. and Forche, E. (1980). Cell proliferation and growth in callus culture, *International Review of Cytology*, IIA: 1-21.

—oo(O)oo—