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Effect of plant growth harmones on the shoot regeneration system of groundnut (*Arachis hypogaea* L.) var. Kadiri 3

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Groundnut or peanut (*Arachis hypogaea* L.) is one of the principal economic crops of the world. The cotyledons, shoot apices, leaflets and embryo were selected as the explants to study the shoot regeneration system of groundnut from different explants of Kadiri 3. From the above set of experiments cotylydonary explants showed better response in shoot initiation medium (SIM), so that it was used in all the experiments on the optimization of regeneration system. The cotylydonary explants from mature seeds of peanut variety Kadiri 3 were cultured on different media formulations containing varying concentrations of BA and 2,4-D. Amongst the different media tested, MS containing 20 µM BA and 10 µM 2,4-D produced the highest frequency (73.3%). The explants bearing shoot buds were cut into two to four pieces and transferred on to shoot elongation medium, MS medium with 2 µM BA showed better response and the shoots were micropropagated on SEM through nodal explants for clonal multiplication and eventually rooted on MS medium containing 5 µM NAA where the other auxins didn't show any promising results when compared with the NAA.

Key words : Groundnut, Regeneration, Growth harmones

INTRODUCTION

The grain legumes are an important group of crops with major source of dietary protein and oil. Groundnut (*Arachis hypogaea* L.) is one of the world's most important oilseed crops. Besides income for the farmers, groundnut provides an inexpensive source of high quality nutrition. Groundnut seeds contain 44-56% oil and 22-30% protein on a dry seed basis. Due to lack of resistance to biotic and abiotic stresses in cultivated groundnut, the productivity remained low despite large acreage under cultivation (Savage and Keenan, 1994). The wild genotypes of *Arachis* are valuable sources of resistant genes against several pests, pathogens besides high oil and protein content (Cherry, 1977; Stalker and Simpson, 1995; Lynch and Mack, 1995; Holbrook and Stalker, 2003).

The multiplication and maintenance of wild *Arachis* germplasm is labour-intensive and involves specific protocols because many accessions are grown mostly under greenhouse/glasshouse conditions. For instance, the field-grown wild plants are uprooted and the soil has to be shifted to harvest the seeds (Stalker, 1997). Therefore, there is a limited supply of wild germplasm from the gene bank and it becomes difficult to maintain wild species of *Arachis* for its use in breeding programme.

Plant regeneration until the recent past in cultivated and in wild groundnut has been achieved either directly via organogenesis or indirectly through somatic embryogenesis (Kanyand *et al*., 1994; Li *et al*., 1994; Rani and Reddy, 1996a; Rani and Reddy, 1996b; Venkatachalam *et al*., 1999; Victor *et al*., 1999; Little *et al*., 2000). However, the reports on plant regeneration with intervening callus phase are few in cultivated genotypes and less in wild *Arachis* (Venkatachalam *et al*., 1996; Still *et al*., 1987; Vajranabhaiah *et al*., 1993; Li *et al*., 1993). The standardization of *in vitro* plant regeneration protocols with intervening callus phase would certainly help in the mass scale propagation of the wild species and also facilitate germplasm conservation *in vitro* (Gagliardi *et al*., 2002).

In addition, the protocol can also be exploited for generating new genetic variability in groundnut by somatic hybridization through protoplast fusion as has been demonstrated in other legumes (Arcioni *et al*., 2001).

Further, this is an attempt to study the effect of different plant growth regulators on morphogenetic response of shoot base-derived callus.

MATERIALS AND METHODS

Explant material and sterilization :

The cotyledons, shoot apices, leaflet and embryos were selected as the explants to study the shoot regeneration system. The different explants from groundnut variety Kadiri 3 were surface sterilized in 0.1% mercuric chloride with 1-2 drops of tween-20 for 7 min on the shaker. Explants were rinsed 3-4 times with sterile

distilled water and soaked in sterile water for 3 h before use.

After removal of the seed coat 5 to 8 seeds were implanted in each Petri plate (90 mm) containing MS basal medium (Murashige and Skoog, 1962). For the preparation of leaflet explants, the seed was split open and the leaflets were excised at the petiolar region of the embryo axis and cultured on shoot induction medium (SIM). The petiolar cut end of the explant was placed on culture medium with its abaxial surface in contact with the medium. The explants were cultured at a density of 8 per Petri-plate and sealed with parafilm.

Culture conditions :

The MS medium and tubes were autoclaved at 15 psi pressure and 121^{0}C for 15 min. The culture medium was gelled with 0.8% (w/v) agar and pH of the medium was adjusted to 5.8 prior to autoclaving. All the cultures were maintained at 26 ± 2^{0}C under continuous light having 100 µEm-2S-1 irradiance provided by cool day light fluorescent lamps.

Induction of callus :

The cotyledonary explants were initially inoculated on the MS medium with different media formulations containing different concentrations of 2,4-D(1, 10, 20, 30, 40, 50 mgl^{-1}), Benzylaminopurine(1, 3, 5, 15, 15, 20 mgl^{-1}), and Thidi azuron (1, 3, 5, 15, 15, 20 mgl^{-1}) to study the effect of different hormonal concentration on callus formation.

Explants used for regeneration studies :

Mature seeds from Kadiri 3 peanut genotype were removed from mature pods and stored at 4^{0}C for testing the percentage of regeneration. The seed coat from the cotyledons was removed before culturing on the medium.

Regeneration of multiple shoots :

The cotyledonary explants were cultured on the MS medium which has various media formulations containing different concentrations of N6-benzyladenine (BA; 2.5, 5.0, 7.5, 10.0, 15.0, 20.0, 25.0 µM) and 2,4-dichlorophenoxy acetic acid (2,4-D; 1.0, 2.5, 5.0, 7.5, 10.0, 15.0, 20.0µM), to study the effect of the shoot hormones on the explant in shoot regeneration.

Elongation of multiple shoots :

The explants bearing multiple shoots were transferred into shoot elongation medium containing MS which has various media formulations containing different concentrations with GA3 and BA (1.0, 2.0, 3.0, 4.0, and 6.0). The subculturing was done for two to three passages of 28 days each for the development and elongation of adventitious shoot buds.

Transfer to rooting medium :

After the shoots were elongated on the shoot elongation medium they were transferred to the rooting medium which contains MS salts and 1.5, 2.5, 3.5, and 5.0 µM concentrations of different auxins (NAA, IBA, and IAA).

Transfer to glass house

Eventually after the formation of the roots, they were transferred to small pots which containing autoclaved sand only and then to glass house. After 10 days they were transferred to big pots containing soil, sand and manure in 3:2:1 ratio.

RESULTS AND DISCUSSION

In preliminary studies, different explants from groundnut genotype of Kadiri 3, were cultured on MS (Murashige and Skoog, 1962) media containing equal concentrations of BA and 2,4-D as per the usual method. Among all the explants, cotyledonary explants underwent regeneration at high frequencies (86.6%) followed by embryos (63.3%), leaflets (56.6%), and shoot apices (43.3%) (Table 1). However, only cotyledonary explants gave extended callus growth and better response in SIM. Similar result were reported *in vitro* callus culture and plant regeneration from different explants of groundnut (Venkatachalam *et al*., 1996) and in pigeonpea shoot regeneration (Sharma and Ortiz, 2000). Cotyledonary explants were proved to be the best explants for the callus formation in groundnut varieties (Fujimura and komamine, 1980; Maria Florencia Cucco, 2000). Among the 30 cotyledonary explants used in the present study, 26 have shown 86.6% of regeneration, which is a very similar frequency, reported by Sharma and Ortiz (2000). Therefore, the cotyledonary explants alone were used in all the experiments to optimize the hormonal influence on

Table 1: Effect of different explants on shoot regeneration of ground nut variety Kadiri 3.

Explant	Number of explants cultured	Number of explants regenerated	% Regeneration
Cotyledons	30	26	86.60
Shoot apices	30	13	43.30
Leaflets	30	17	56.60
Embryo	30	19	63.30

the regeneration process.

The cotyledonary explants from mature seeds of peanut variety Kadiri 3 were cultured on different media formulations supplemented with varying concentrations of 2,4-D, BAP and TDZ. Among the three hormones, explants treated with BAP produced callus and cotyledon size increased for all the concentrations used. Cotyledons increased in weight and volume up to four times. The high concentrations of BAP produced a significant higher percentage of calluses than the lower concentrations (Table 2). When the explants were treated with different 2,4-D concentrations, they produced a high percentage of callus and cotyledonary size increased (Table 3). Explants which were treated with different TDZ concentrations, cotyledons size increased and they produced callus but not to that extent when explant were supplemented with 2,4-D and BAP (Table 4). Results obtained in the present investigation are similar to that reported by Maria Florencia Cucco, (2000), for *in vitro* regeneration of *Arachis hypogaea* L.

Among the different media tested, MS containing 20 µM BA and 10 µM 2,4-D (shoot induction medium; SIM) produced the highest frequency (73.3%) of multiple adventitious shoot buds (Table 5). However, better regeneration frequencies were observed when cotyledons were split into vertical halves following the method of Sharma and Ortiz (2000) which showed regeneration in 22 shoots out of the 30 explants subjected for the experimentation (Fig. 1). Each half of the split cotyledon responded with similar frequencies by producing greater number of adventitious shoot buds per explant. Results obtained by employing different concentrations of growth hormones were in agreement with the earlier studies of Sharma and Ortiz (2000) in groundnut shoot induction. Shoot regeneration from the cotyledonary explants in media supplemented with BA alone or in combination with 2,4-D were shown to yield better results in the earlier studies

Table 2 : Effect of different benzylaminopurine concentrations on cotyledonary explants of ground nut variety Kadiri 3

Explant	Concentration of BAP ($mg.l^{-1}$)	No. of explants	Callus percentage
Cotyledons	1	10	20
	3	10	20
	5	10	50
	10	10	50
	15	10	50
	20	10	60

Table 3 : Effect of different 2,4-dichlorofenoxiacetic acid concentrations on cotyledonary explants of ground nut variety Kadiri 3

Explant	Concentration of 2,4-D ($mg.l^{-1}$)	No. of explants	Callus percentage
Cotyledons	1	10	20
	10	10	40
	20	10	50
	30	10	50
	40	10	50
	50	10	60

Table 4 : Effect of different TDZ concentrations on cotyledonary explants of ground nut variety Kadiri 3

Explant	Concentration of TDZ (µM)	No. of explants	Callus percentage
Cotyledons	1	10	20
	3	10	20
	5	10	40
	10	10	50
	15	10	40
	20	10	40

Table 5 : Effect of BA and 2,4-D on shoot regeneration from ground nut cotyledon explants of Kadiri 3

Explant	Conc. of 2,4-D ($mg.l^{-1}$)	Conc. of BA ($mg.l^{-1}$)	Number of explants cultured	Number of multiple shoots regenerated
Cotyledons	1.5	2.5	30	5
	2.5	5	30	8
	3.5	7.5	30	12
	5.5	10	30	15
	7.5	15	30	16
	10	20	30	22
	15	25	30	18

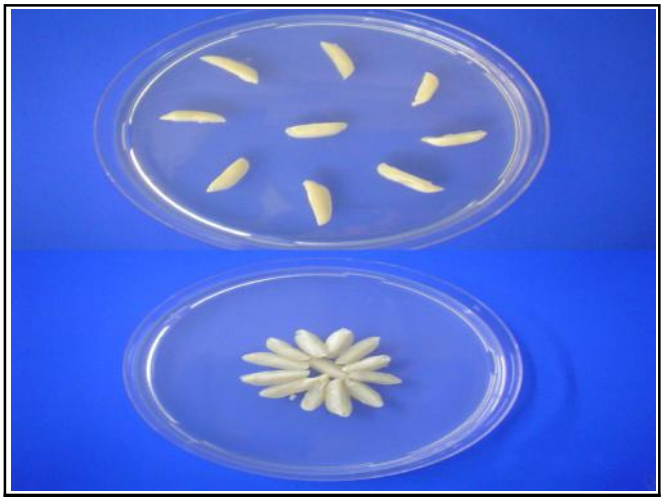

Fig. 1 : Inoculated cotyledonary explants for callusing

(Venkatachalam *et al*., 1999; Gagliardi *et al*., 2000).

The explants turned green and underwent considerable enlargement within 4 days of culture initiation on shoot induction medium. Multiple shoot buds differentiated at the proximal cut end (Fig. 2) within 16 days among 80% of the explants. However, no further elongation occurred on the shoot buds even after 4 weeks on SIM (Fig. 3). Hence, the explants bearing shoot buds were cut into two to four pieces and transferred on to shoot elongation medium (MS medium with 3 µM BA) showed better response (Table 6 and 7; Fig. 4). Nevertheless, there was no effect of GA_3 on the shoot elongation and further it was also observed that GA_3 was responsible for the delayed rooting of the explant. The explants were subcultured for three passages of 3 weeks each and elongated shoots were rescued at the end of each passage (Fig. 5). Frequently, four to eight shoots

Fig. 2 : Developing callus in MS medium containing BAP

Fig. 3 : Developing callus with MS medium containing 2,4 D

Fig. 4 : Shoot induced cotyledonary explants

Fig. 5 : Shoot induced cotyledons after 4 weeks of culturing, showing shoot formation

Table 6 : Effect of different BA concentrations on shoot bud elongation of ground nut variety Kadiri 3

Explant	Concentration of BA (µM)	No. of shoot buds	Elongation percentage
Cotyledons	1	10	30
	2	10	40
	3	10	70
	4	10	50
	5	10	50
	6	10	40

Table 7 : Effect of different GA_3 concentrations on shoot bud elongation of ground nut variety Kadiri 3

Explant	Concentration of GA3 (µM)	No. of shoot buds	Elongation percentage
Cotyledons	1	10	10
	2	10	20
	3	10	20
	4	10	30
	5	10	30
	6	10	40

were recovered from each explant even though there was availability for ten shoots. To obtain high frequency of adventitious shoot bud regeneration the proximal cut end was placed embedded into the medium, so that it cpuld remain in contact with the medium during the first two weeks of culture. Results obtained in the present work are alike to the earlier reports of *in vitro* regeneration of *Arachis hypogaea.* L. (Maria Florencia Cucco, 2000).

The shoots were micropropagated on SEM through nodal explants for clonal multiplication and eventually rooted on MS medium containing different concentrations of auxins (NAA, IBA and IAA). Among the three auxins tested, NAA at 5uM concentration gave best results for root induction. Root induction was also observed with the other two auxins, however, the induction was poor compared to the NAA. Nevertheless, the IAA has shown better results compared to IBA (Table 8, 9 and 10; Fig. 6, 7 and 8). These results are agreement with those reported by Sharma and Ortiz (2000) during the production of transgenic peanut variety. Gagliardi *et al*. (2000) reported a similar result to the present findings in the induction of rooting (MS medium with 5.4 µM NAA) in *A. prostrata* and *A. aff*. Root induction in *A. villosa* on MS medium fortified with 5.37 to 26.8 µM NAA was reported by

Table 8 : Effect of different NAA concentrations on ground nut explants of Kadiri 3

Conc. of NAA (µM)	No. of plantlets cultured	No. of plantlets rooted
1.5	10	2
2.5	10	2
3.5	10	4
5.0	10	7

Table 9 : Effect of different IBA concentrations on ground nut explants of Kadiri 3

Conc. of IBA (µM)	No. of plantlets cultured	No. of plantlets rooted
1.5	10	0
2.5	10	1
3.5	10	1
5.0	10	2

Table 10 : Effect of different IAA concentrations on groundnut explants of Kadiri 3

Conc. of IAA (µM)	No. of plantlets cultured	No. of plantlets rooted
1.5	10	0
2.5	10	2
3.5	10	2
5.0	10	2

Fig. 6 : Developing multiple shoots in MS containing 10:20 ratio of 2,4-D and BA

Fig. 7 : Elongating multiple shoots in MS medium containing BA

Fig. 8 : Well developed roots on MS with NAA

Fig. 9 : Plantlets transplanted into pots

Vijayalakshmi and Giri, (2003).

The adventitious roots appeared within 2 weeks, developed in such a way that they were ready for transplantation to pots. After transplantation into the pots 60 per cent of the rooted shoots survived and appeared phenotypically normal (Fig. 9).

Conclusion :

In the present investigation, cotyledonary explants were found to be the best for callus induction form groundnut variety, Kadiri 3. Growth hormone concentration for the best root and shoot induction were optimized and the plantlets were successfully regenerated with great success rates. Hence, this protocol may be adopted for the commercial scale micropropagation of this groundnut variety.

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