The Bioscan 9(2): 599-604, 2014
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

HIGH FREQUENCY REGENERATION PROTOCOL FOR CALLUS CULTURES OF PEANUT LEAVES USING ETHYLENE MODULATORS AS CULTURE MEDIUM ADDITIVE

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KEYWORDS
Peanut
Ethylene modulators
Silver nitrate
Cobalt chloride
Putrescine,
Regeneration

Received on :
00.00.2013

Accepted on :
00.00.2014

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ABSTRACT

In order to develop high frequency regeneration protocols in peanut exploiting ethylene modulators, influence of four ethylene modulators *viz*. ethrel, silver nitrate, cobalt chloride and putrescine were studied on the regeneration behavior of immature leaves of peanut. Ethrel was found to inhibit the development *in vitro*. The addition of silver nitrate had enhanced the number of shoot buds regenerated per explant and hence could be used as a regular culture medium additive in peanut. Cobalt chloride was also found to increase the frequency of regeneration in peanut. The variation in the response induced by putrescine was also wider than the other additives. It was concluded that silver nitrate is the best ethylene modulator among the four additives studied and its use in culture medium at a level 0.01mM enhanced the regeneration frequency from the immature leaves of the peanut irrespective of the genotype used. In the new method of acclimatization developed by combining the rooting and hardening steps, prolonged growing of the plants on culture medium could be avoided. In this method no mortality was observed during the process of acclimatization. This method can be exploited in the nodal culture from field grown plants also without having the hazels of surface sterilizing the explants.

INTRODUCTION

Peanut is an important commercial crop throughout the world. The cultivated species of peanut (*Arachis hypogaea* L.) is vulnerable to an array of bacterial, fungal and viral diseases whereas, the wild species of peanut are resistant to most of the diseases. Hybridizations involving the wild species are often difficult due the breeding barriers like cross incompatibility and ploidy differences. Therefore, genetic transformation seems to be a viable approach for the production of elite varieties.

While an efficient tissue culture system for regeneration of plants from cultured cells and tissues is the key in success of plant genetic engineering (Pua *et al.,* 1996; Purnhauser *et al.,* 1987; Bharose *et al.,* 2014), enhancement in the regeneration frequency would be an added advantage in improving the genetic transformation protocols.

In majority of the regeneration protocols reported in peanut, cotyledons have been used as explant to get direct organogenesis. In callus mediated regeneration from leaf explants, the regeneration frequency reported has been very low (Radhakrishnan *et al.,* 2004). Differences in regeneration frequencies among different explants are due to their difference in the physiological state, endogenous level growth regulators and /or in their response towards growth regulators (Radhakrishnan, 1996; Pawar *et al.,* 2012). Supplementation of chemicals in culture medium has been found to modulate the level and availability of both exogenous and endogenous growth regulators to the tissues in culture. Thus, it was thought worthwhile to study the influence of ethylene-modulating chemicals as supplements to culture medium on the shoot bud regeneration from immature leaves of peanut *in vitro,* for developing a simple and high frequency regeneration protocol in peanut.

It is known that ethylene, a gaseous plant growth regulator, which is involved in the regulation of different plant physiological processes, is produced by tissues in culture, and is responsible for recalcitrance of mustard cell growth and development (Chi *et al.,* 1990; Pua and Chi, 1993) and other crucifer members including Chinese cabbage (Chi *et al.,* 1991), oil seed Brassica (Palmer, 1992) and *Arabidopsis thaliana* (Marton and Browse, 1991). Inhibition of ethylene biosynthesis or its action by the use of chemical inhibitors like silver nitrate, aminoethoxyvinylglycine (AVG) and putrescine greatly enhanced shoot regeneration in mustard cell (Pua *et al.,* 1996). However, from the experiment by Ozcan *et al.,*(1992), it is clear that the effect of these chemicals may not be the same in every type of explant.

Though there are reports on rate of regeneration in peanut (*A. hypogaea*) cotyledon and leaf explants by the use of ethylene modulating chemical, silver nitrate (Pestana *et al.,* 1999; Ozudogru *et al.,* 2005), the effects of ethrel, cobalt chloride, and putrescine have not been reported in the regeneration of peanut. By supplementation culture media with a suitable ethylene modulator enhancing the regeneration frequency can lead to an improved protocol for peanut plant regeneration from immature leaves.

Hence, the present investigation was taken up to compare the efficiencies of the four ethylene modulators on enhancing the shoot regeneration from peanut leaves *in vitro* and to develop a simple rooting and hardening protocols for the regenerated shoots.

MATERIALS AND METHODS

In vitro culture and regeneration

The immature leaves from the dried and stored seeds of four peanut cultivars *viz*. GG 2, MH 2, Gangapuri and M 522 were used as explants. The seeds were sterilized and dissected to collect the explants as described by Radhakrishnan *et al.,* (2000). The immature leaves from the zygotic embryos were excised aseptically from the seeds and used as explants.

The culture medium and methods reported by Radhakrishnan *et al*. (1999) were followed. The callus cultures were initiated on MS medium with the growth regulators (3 mg/L BA and 1 mg/L NAA). After one week in culture, the explants with calli initiated were sub-cultured on MS medium with 3 mg/L BA and the NAA was replaced with the ethylene modulators *viz*. silver nitrate, cobalt chloride, putrescine and ethrel as additives. The filter sterilized additives were mixed with the media prior to the pouring of media to petri plates. The levels of additives were selected based on the information available from the work done in other crop species and the pilot experiments taken up in our laboratory (data not shown). Three levels of silver nitrate: 0.005, 0.01 and 0.02 mM; cobalt chloride: 0.0042, 0.0084, 0.0126 mM; putrescine: 10, 20 and 30 mM and ethrel (2-chloro-ethylphosphonic acid): 1.0, 2.0 and 3.0 mM were added to the culture medium as described earlier. Culture medium without additives was used as control.

Each treatment was replicated 4 times with 15 explants each. Cultures were sealed with two wraps of parafilm and were incubated at 26 ± 1 ºC and were exposed to 16 h photoperiod. The explants in culture were regularly observed for their physical changes and the regeneration behavior. The cultures, which produced shoot buds, were then subcultured on MS medium containing 3 mg/L BA and 1 mg/L GA_3 (without the ethylene modulator) for expansion of shoots as described by Radhakrishnan *et al*. (2000).

Rooting and hardening

Hardening is one of major bottlenecks in realizing the maximum output from micropropagation experiments. In most of the earlier works only 50-70% hardening could be obtained. A new approach which could save time and improve the efficiency by several folds was devised in the present study. A combined method for both rooting and subsequent hardening of the plantlets was devised by growing the shoots in open. The shoots of about 3-4 cm length were taken out from the culture and grown in Hoagland's solution containing 1 mg/L of NAA for rooting until sufficient number of well developed roots were induced and then in hormone free Hoagland solution the roots are sufficiently elongated and the plants have become sturdier enough to transplant to soil. The conventional method of supplementing 1mg/L of NAA in the culture medium *in vitro* for inducing roots was used as control (10 replicates with 25 shoots each).

RESULTS

Influence of different ethylene modulators on regeneration

All the ethylene modulators used except ethrel, induced varying degrees of changes in the frequency of the shoot bud formation in all the four cultivars used in this study. The explants showed callusing initially from the cut ends of the explants and slowly progress to cover the entire surface of the explants. The calli were compact in all cultures except in the medium with silver nitrate.

Shoot bud initials were first visible as small globular protuberances from the explants within two weeks after culture (Fig. 1.a). The shoot buds slowly opened up and were apparent on the explant (Fig. 1.b). The number of shoot buds varied in different cultures with the varying levels and the type of the ethylene modulators used.

Supplementation by the ethylene modulators in the culture medium was found to influence the regeneration significantly ($p = 0.01$). The variations due to the type of ethylene modulators used and their level in the medium were found to influence significantly the number of shoot buds produced per explant ($p = 0.01$). However, the genotypic difference in the response was not significant. The influence of the ethylene modulators and their level on the number of regenerating shoot buds from the immature leaf explants in four cultivars of peanut is summarized in the Table 1.

Ethrel

Ethrel was found to be totally inhibiting the development of the explant. The explants in this culture remained pale yellow without even enlargement.

Silver nitrate

The calli produced by cultures in media containing silver nitrate were friable and broke easily on handling. At a concentration of 0.01 mM in the medium had the maximum positive influence on the number of shoot buds produced per explant (ranging from 105 to 138% of increase over the control). Higher concentrations of silver nitrate in the culture medium did not produce more number of shoot buds. In fact, higher levels tended to reduce the number of shoot buds produced per explant in all the four varieties studied.

Cobalt chloride

The maximum increase in the number of shoot buds was observed in variety M 552 cultured in medium containing 0.0084mM cobalt chloride (87.2% over control). The percentage of enhancement varied from19% (at 0.0082mM in the cultivar MH 2) to 87.2% over the control. In the four varieties studied, the optimum concentration of cobalt chloride was 0.0084mM and in the cultivar MH2 higher concentration resulted in the decrease in the number of shoot buds per explant.

Putrescine

The explants cultured in medium containing putrescine remained pale green in colour despite their growth and development *in vitro* as compared to dark green explants growing in silver nitrate containing medium. Putrescine was

Table 1: Shoot bud regeneration in 4 varieties of peanut under the influence of the three ethylene modulators

Variety	Additive	mM	Shoot buds			
			Min.	Max.	Mean ± SD	% Increase
GG 2	Silver nitrate	0.0050	24.1	32.3	27.8 ± 3.4	51.1
		0.0100	38.6	47.9	43.8 ± 3.9	138.0
		0.0200	30.7	40.9	35.8 ± 4.7	94.6
	$CoCl_2$	0.0042	22.2	24.1	23.3 ± 0.8	26.6
		0.0084	24.1	28.9	26.6 ± 2.2	44.6
		0.0126	12.7	23.3	17.3 ± 4.4	-6.0
	Putrescine	10.000	22.2	25.5	23.5 ± 1.4	27.7
		20.000	23.0	28.4	25.1 ± 2.4	36.4
		30.000	13.2	20.4	18.3 ± 3.4	-0.5
	Control	-	14.2	20.8	18.4 ± 2.9	-
MH 2	Silver nitrate	0.0050	20.3	23.8	21.5 ± 1.6	10.3
		0.0100	32.0	49.3	38.7 ± 8.0	98.5
		0.0200	24.2	32.6	28.9 ± 3.6	48.2
	$CoCl_2$	0.0042	23.2	24.8	24.0 ± 0.8	23.1
		0.0084	22.5	24.1	23.2 ± 0.7	19.0
		0.0126	19.2	21.1	20.5 ± 0.9	5.1
	Putrescine	10.000	18.8	22.3	21.2 ± 1.6	8.7
		20.000	22.1	39.0	32.7 ± 7.4	67.7
		30.000	11.7	21.4	15.7 ± 4.4	-19.5
	Control	-	18.1	20.3	19.5 ± 1.0	-
Gangapuri	Silver nitrate	0.0050	27.3	30.5	29.0 ± 1.5	60.2
		0.0100	33.4	54.3	42.4 ± 8.9	134.3
		0.0200	27.1	31.7	29.4 ± 1.9	62.4
	$CoCl_2$	0.0042	26.0	29.0	27.1 ± 1.3	49.7
		0.0084	29.6	30.3	29.9 ± 0.3	65.2
		0.0126	21.3	25.8	24.2 ± 2.0	33.7
	Putrescine	10.000	21.7	27.6	23.5 ± 2.7	29.8
		20.000	23.8	26.4	25.3 ± 1.1	39.8
		30.000	16.8	25.7	21.7 ± 3.7	19.9
	Control	-	13.2	23.6	18.1 ± 5.0	-
M 522	Silver nitrate	0.0050	24.8	27.0	25.8 ± 1.0	65.4
		0.0100	29.8	34.0	32.0 ± 1.8	105.1
		0.0200	27.1	29.2	27.8 ± 1.0	78.2
	$CoCl_2$	0.0042	23.1	25.9	24.5 ± 1.4	57.1
		0.0084	28.3	30.2	29.2 ± 0.9	87.2
		0.0126	25.8	27.3	26.6 ± 0.7	70.5
	Putrescine	10.000	23.9	26.6	25.0 ± 1.2	60.3
		20.000	25.2	26.6	25.9 ± 0.6	66.0
		30.000	22.5	26.4	24.2 ± 1.8	55.1
	Control	-	14.2	17.0	15.6 ± 1.4	-

Table 2: Rate of rooting and the establishment of plantlets in pots in the conventional method of rooting and acclimatization

Shoots cultured	Shoots Rooted	Establishment in pots
25	17 (68.0)	15 (88.2)
25	14 (56.0)	9 (64.3)
25	20 (80.0)	15 (75.0)
25	18 (72.0)	16 (88.9)
25	17 (68.0)	16 (94.1)
25	16 (64.0)	10 (62.5)
25	24 (96.0)	17 (70.8)
25	23 (92.0)	18 (78.3)
25	20 (80.0)	19 (95.0)
25	18 (72.0)	15 (83.3)
Mean	18.7 (74.8)	15 (80.04)

Values in parenthesis are % of response

found to enhance shoot bud formation to the maximum in the variety MH2 (67.7% over the control) followed by the variety M522 (60.3% over the control), both at the concentration of 20mM. A higher dose of putrescine was found to reduce the number of shoot buds per explant in all the varieties studied.

In all the explants with shoot buds induced after sub-culturing in the medium containing the ethylene modulator as additive were further transferred to the shoot elongation medium containing GA_3 had resulted in rapid elongation of the shoots from the buds (Fig 1.c).

Rooting and hardening

In conventional method the rate of rooting varied from 56 to 92% with a mean of 64.8% and their establishment ranged

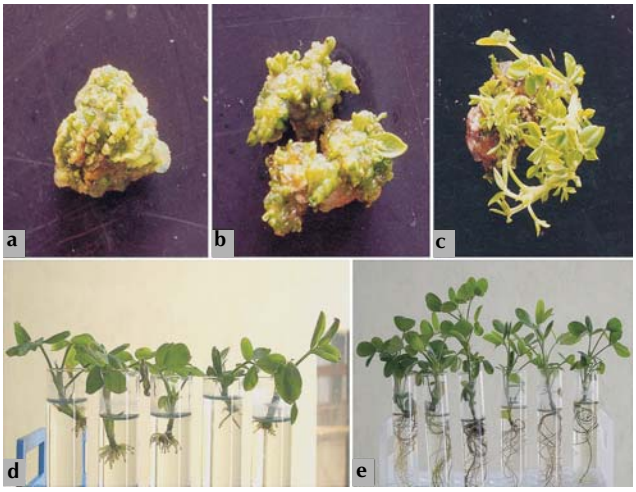

Figure 1: (a) Callus with shoot buds induced from the immature leaves of peanut in the culture initiation medium containing 3 mg/L BA and 1 mg/L NAA. (b) Shoot buds regenerating on callusing explants sub-cultured in medium containing the ethylene modulator silver nitrate as additive. (c) The shoot buds opened up and elongated in subcultures with medium containing GA_3 and ready for root induction. (d) The elongated shoots transferred to the Hoagland's solution containing 1 mg/L NAA for induction of roots. (e) The plantlets hardened in Hoagland's medium showing the development of secondary roots also.

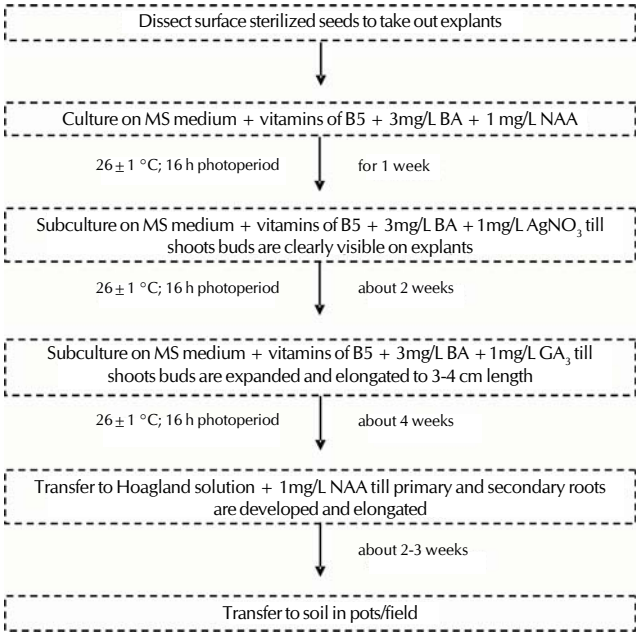

Figure 2. Diagrammatic representation of the protocol for high frequency regeneration of peanut immature leaf explants employing silver nitrate as ethylene modulator and the improved rooting and hardening procedure

from 62% to 95% with a mean value of 80.4% (Table 3). Despite the high percentage of establishment several shoots were weak and there was more than 20% mortality during acclimatization. However, all the plants in the new technique had rooted profusely (Fig. 1.d and 1.e) and were growing healthy outside the test tubes. All the regenerated plantlets established well in pots bringing down the rate of mortality in the acclimatization procedure to 0%.

DISCUSSION

Influence of different ethylene modulators on regeneration

Of the four ethylene modulators studied, ethrel totally inhibited the growth of the peanut leaf explants *in vitro*. Cultures *in vitro* produce high amounts of ethylene for the first 21 days (Pua *et al.,* 1996), and in this experiment the levels of the ethylene used as supplement in excess of the naturally produced ethylene might have caused total inhibition of the response of explants in culture. Earlier studies *in vitro* have reported the adverse effect of ethylene on callusing and regeneration (Songstad *et al.,* 1988; Roustan *et al.,* 1989, 1990; Biddington, 1992; Gisbert and Trujillo-Moya, 2012). We have used four levels of the ethrel and the initial level (10 mM) itself proved to be a higher dose inhibiting the growth. This is indicative of presence of a high level of naturally produced ethylene during *in vitro* growth of peanut leaf explants.

Silver nitrate was found to the most efficient ethylene modulator for enhancing regeneration frequency in peanut. The addition of silver nitrate enhanced the number of shoot buds per explant and hence, silver nitrate can be used as a regular culture medium additive to increase regeneration frequency in peanut. Silver nitrate has already been tried in other crop species also for enhancing the regeneration frequency (Chi and Pua, 1989; Pua *et al.,* 1996, Simonson *et al.,* 1997; Brar *et al.,* 1999; Bais *et al.,* 1999; 2000a, b; 2001a, b, c; Balkhande *et al.,* 2012; Yu *et al.,* 2012; Yasmin**,** *et al.,* 2013). Silver ions are also employed in the form of silver thiosulphate in several tissue culture studies (Eapen and George, 1997). Involvement of silver ion mediated responses in polyamines, ethylene- and calcium- mediated pathways, and their role in regulating physiological process including morphogenesis have been reported earlier (Roustan *et al.,* 1990; Nissen, 1994; Pua and Chi, 1993; Bais *et al.,* 1999; 2001b; Bais and Ravishankar, 2002; Kumar *et al.,* 2009). Although silver nitrate has proved to be a very potent inhibitor of ethylene action in plant tissue culture, the exact mechanism of its action is not fully known. However, the existing evidences suggest its interference in ethylene perception mechanism (Beyer, 1976a, b; Rodriguez *et al.,* 1999; Zhao *et al.,* 2002).

Some of the physical properties of silver nitrate such as easy availability, solubility in water, specificity and stability make it very suitable for various applications in exploiting plant growth regulation and morphogenesis *in vitro*. Hence, from the results of the present study, 0.01mM of silver nitrate can be considered optimum for enhancing shoot bud regeneration in peanut and can be employed profitably in highly efficient regeneration protocols.

Cobalt chloride was reported to have a role in the shoot elongation of cowpea (Brar *et al.,* 1999) and in sunflower (Chraibi *et al.,* 1991). However, this is the first study on the influence of cobalt chloride on regeneration of peanut. Cobalt chloride also was found to increase the frequency of regeneration in peanut leaves *in vitro*. Cobaltous ions are known to inhibit ethylene synthesis (Lau and Yang, 1976).

Since this additive is already present in the culture medium as one of the constituents of the micronutrients used in the tissue culture medium, it may be easy to utilize its influence by just increasing the concentration of cobalt chloride in the medium. Moreover, this additive is co-autoclavable and more stable to light as compared to silver nitrate, thus making its exploitation easier. However, the effectiveness of cobalt chloride as compared to silver nitrate is much low.

Use of putrescine along with silver nitrate has enhanced regeneration in Chinese radish (Pua *et al.,* 1996). However, this is the first study on the influence of putrescine on regeneration of peanut, though putrescine could enhance shoot bud formation, the extent of increase was the lowest as compared the other three ethylene modulators studied. The variation in the response induced by putrescine was also wider than the other additives. The influence of putrescine on enhancing the regeneration may be by the competitive utilization of S- S-adenosylmethionine (SAM), a precursor of ethylene has already been reported by Miyazaki and Yang (1987) and Bais *et al.,* (2000b). Putrescine is considered to a highly corrosion inducing chemical and requires more of handling precautions. Hence, its use in regular media preparation is not as easy as cobalt chloride.

From the study it was concluded that silver nitrate is the best ethylene modulator among the four additives studied and it can be used in culture medium at a level 0.01mM for enhancing the regeneration frequency from the immature leaves of the peanut irrespective of the variety used.

Rooting and hardening

Though high frequency of root regeneration could be attained by conventional method of rooting on culture medium, the root induction in the new method reported here is cent per cent which makes this method superior to the available protocols. In the conventional approach, we have to pass the plantlets after the root induction through an acclimatization step which will lead to some mortality of the regenerated plantlets, bringing down the number of plants finally recovered. This is of a great concern in genetic manipulation studies where recovery of maximum number of putative transgenics are of prime importance. In the new method the acclimatization step has been done along with the rooting step. The advantage of the new method is that there was no mortality during the process of acclimatization. This will ensure the recovery of all regenerated plants and hence, is a highly superior approach. This method can have a wide spectrum of application which involves the nodal culture where we may have to grow nodal cuttings from field grown plants for multiplication purposes. This method can avoid the use of routine culture medium which contains sucrose as source of carbon which makes it prone to bacterial and fungal contamination. One of the major problems in nodal cultures from field grown plants is the high rate of contamination as disinfection of field grown plant explants is seldom effective. This new method of rooting and acclimatization avoids the growing of the plants on culture medium while providing the total requirement of nodal culture for producing plantlets.

Based on this experiment a protocol for regeneration was formulated and tested in several genotypes of peanut (data not presented). The sequences in the tissue culture procedure using the most effective type and dose of ethylene modulator as an additive in the culture medium and the various subcultures used to realize a high frequency of regeneration is illustrated in the diagram (Fig 2).

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