African Journal of Food Science and Technology ((ISSN: 2141-5455) Vol. 5(3) pp. 60-66, March, 2014
DOI: http:/dx.doi.org/10.14303/ajfst.2014.015
Available online @http://www.interesjournals.org/AJFST

Full Length Research Paper

Optimization of callus induction in *Lathyrus sativus* L

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ABSTRACT

Callus induction from internode explants of *Lathyrus sativus* L. cv. Nayagarh local was tested in different media (MS, LS, BM, B_5 and W) with different combination of auxins and cytokinins. Overall callus induction frequency and callus growth was very high in B_5 and BM medium. 2, 4-D was found to be effective for callus induction and growth, both in amount and rate, followed by NAA in all media tested. Maximum callusing response was revealed in B_5 medium with 2mg/l 2,4-D+0.5mg/l BAP fallowed by B5 + 2mg/l NAA + 0.5mg/l BAP, but the latter most frequently induced greenish white to dark green, loose, nodular and hard calli suitable for plant regeneration and genetic transformation.

Keywords: Callus induction, callus proliferation, growth regulators, *Lathyrus sativus* L.

INTRODUCTION

Grasspea (*Lathyrus sativus* L.) is an important short duration grain legume. It can thrive well in adverse abiotic stress (e.g., drought, salinity and flood) situations which paves the way for its preference by the growers despite its ban for cultivation in several countries owing to high neurotoxin (BOAA:β-N-oxalyl amino alanine) content in seed that causes lathyrism. Genotypes devoid of neurotoxin(zero BOAA content) is not available in *L. sativus*. Its closely related species also contain high neurotoxin (Liener, 1967). Preferential selection or cross breeding, would be of no use for substantial rectification of BOAA content. A number of researchers attempted mutagenesis to reduce the BOAA content but it could not be made stable in mutants. Biosynthetic pathways of BOAA synthesis have been established (Kuo and Lambein, 1991). Biotechnological approaches may be effective in turning off one or more gene(s) of metabolic pathways using antisense RNA (Whitaker and Feeney, 1973) or genetic transformation with bacterial gene capable of utilizing neurotoxins (Yadav *et al.,*1992).

Besides, *in vitro* culture techniques to generate somaclonal variation could be an alternative for elimination of nutritional stress factors (McHughen and Swartz, 1984). *In vitro* selection (Van Dorrestein *et al.,* 1998) and gene transfer may, therefore, provide grasspea to be fit for consumption. However, legumes and the grasspea, in particular, are recalcitrant to *in vitro* culture. The main bottleneck lies with low frequency of plant regeneration (Roy *et al.,* 1992, Van Dorrestein *et al.*,1998) and as such the report on callus induction and plant regeneration in this crop is limited. Therefore, the present pursuit aimed to establish a system of reproducible highly regenerative callus cultures in a suitable medium using internode explants in grasspea.

MATERIALS AND METHODS

A genetically pure line of grass pea (*Lathyrus sativus* L.) cv. Nayagarh local was used for *in vitro* culture at Sinha Molecular Breeding Laboratory, Department of Plant Breeding and Genetics, College of Agriculture, OUAT, Bhubaneswar-3 (India) during 2009-12. Fully matured, healthy, well dried seeds were pre-sterilized with 70% ethanol for 2min, washed thrice in double distilled water and surface sterilized with 0.1% $HgCl_2$ and with a mixture of sodium hypochlorite and teepol solution (7:5) for 10min each. Seeds were finally washed (5x) with double distilled water. The seeds were germinated in MS and B_5 basal media to raise seedlings. Internode explants from these aseptically grown seedlings, were tested for their callusing response in five media e.g., Linsmaier and

Table 1. Composition of different basal media used for *in vitro* culture in grasspea

Components	Final Concentration (mg/l)				
	LS	MS	BM	B_5	W
STOCK – A			**SALTS**		
NH4NO3	1,650.0	1,650.0	1.000	-	-
KNO3	1,900.0	1,900.0	1.000	2500	80
$Ca(NO_3)_2$, $4H_2O$	-	-	347.0	-	288
STOCK – B					
MgSO4, 7H2O	370.0	370.0	35.0	250	738
MnSO4,4H2O	22.3	22.3	4.4	10	6.65
ZnSO4, 7H2O	8.6	8.6	1.5	2	2.68
CuSO4, 5H2O	0.025	0.025	-	0.025	-
(NH4)2SO4	-	-	-	134	-
Na_2SO_4, 10 H_2O	-	-	-	-	454
STOCK-C					
CaCl2,2H2O	440.0	440.0	-	150	-
KCl	-	-	6.5	-	65
STOCK-D					
KI	0.83	0.83	0.80	0.75	0.75
CoCl2, 6H2O	0.025	0.025	-	0.025	-
STOCK –E					
KH2PO4, 7H2O	170.0	170.0	300	-	-
NaH2PO4,7H20	-	-	-	150	24
H3BO3	6.2	6.2	1.6	3.0	1.50
Na2MoO4,2H2O	0.25	0.25	-	0.25	-
STOCK –F					
FeSO4,7H2O	27.85	27.85	1.3	27.85	-
Na2EDTA,2H2O	37.25	37.25	19.5	37.25	-
Ferric citrate	-	-	-	-	2.0
	VITAMINS				
Myo-inositol	100	100	100	100	-
Nicotinic acid	-	0.5	0.5	1.0	0.5
Pyridoxine HCl	-	0.5	0.1	1.0	0.1
Thiamine-HCl	0.4	0.2	0.1	10.0	0.1
			AMINO ACIDS and ORGANIC ADDITIVE		
Glycine	2.0	2.0	2.0	-	3.0
Proline	100	100	100	100	100
Casein hydrolysate	500	500	500	500	300
	GELLING AGENT				
Agar	8,000.0	8,000.0	8,000.0	8,000	8,000
	CARBON AND ENERGY SOURCE				
Sucrose	40,000.0	30,000.0	30,000.0	20,000	20,000
pH	5.7	5.8	5.8	5.5	5.8

MS: Murashige T & Skoog F (1962), LS : Linsmaier, E.H. and Skoog, F. (1965), BM: Blaydes, DF (1966), and B5: Gamborg OL, Miller RA and Ojima K (1968), CIM: Callus Induction Medium, R: Regeneration medium .

Skoog (1965), Murashige and Skoog (1962), Blaydes (1966), Gamborg (1968) and White(1954) media, herein after mentioned as LS, MS, BM, B_5 and W(Table 1). Growth regulators (SIGMA, USA) used in this study, included four auxins e.g., 2,4-dichlorophenoxy acetic acid(2,4-D), α-naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA) and indole-3-butyric acid (IBA); and two cytokinins e.g., 6-benzyl amino purine (BAP) and Kinetin (β-furfuryl amino purine, Kn).

Following transfer to media, explants were cultured in inoculation room for callus induction and growth of callus. Cultures were incubated under light intensity of 2000 lux (Fluorescent tube, Philips, India) with a photo period of 12h /day at 25 ± 1^0 C and R.H. 68%.Totipotency of different explants for callus induction was evaluated as callus induction frequency (CIF %) from number of explants inducing callus out of total number of explants inoculated. Besides, colour, texture, and relative growth of calli were assessed for different media supplemented with varying concentrations of growth regulators.

RESULTS AND DISCUSSION

Lathyrus sativus seed has a hard seed coat. Pre-soaking

Table 2. Effect of different basal media for *in vitro* germination of seeds and growth of seedlings (after 10days of seed inoculation) in grasspea

Basal Medium	Germination %	Nature of germination
MS	69.2 ± 1.30	Fasciation of stem and leaf, stunted growth
½MS	96.8 ± 0.90	Normal growth
¼ MS	89.0 ± 1.52	Bit slow growth
1/10 MS	58.0 ± 2.12	Stunted growth and leaflets rarely differentiate
B_5	18.0 ± 2.58	Delay of seed germination and slow growth
½B_5	38.5 ± 2.78	Seeds just germinated and delayed growth

Figure1. *In vitro* germination of seeds to raise seedlings as a source of internode explants

in double distilled water for 1-2 hrs fallowed by surface sterilization resulted better germination. Seeds which imbibed water are generally assured of good germination provided the basal medium is potent enough to invigorate the zygotic embryos for germination. In the present investigation, four MS basal media and two B_5 basal media of different strength (Table 2) were compared for seed germination potential *in vitro.* Half- strength MS basal media excelled in germination over full strength and led to the highest germination percentage (96.8+ 0.90%) with normal growth of seedlings (Figure. 1).

Further reduction in strength of this medium resulted decrease in germination and slow growth of seedlings. Seedlings remained very stunted and even at 1/10 MS, leaflets rarely differentiated. Instead, full strength MS medium could lead to iso-diametric growth leading to fasciation of leaf and stem. However, Kysely and Jacobsen (1990) obtained good germination of pea seeds (*Pisum sativum*) on medium consisting of 1/10 MS macro salts, 1% sucrose and 7% phytagar. Gulati and Jaiwal (1990) reported *in vitro* germination of green gram (*Vigna radiata*) seeds on MS, B_5 and basal medium containing MS salts + B_5 vitamins without any comparisons.

Selection of appropriate plant growth regulators, auxin-cytokinin balance and their application schedule seems to be more critical than the basal medium for optimum growth of calli and morphogenetic potential in legumes (Ranjan *et al.,* 2003). Using internode as explant, different auxins were tested for their callusing ability with five different media, e.g., BM, B_5, LS, MS and W. In general, B_5 fallowed by BM medium were better for callus initiation and growth of calli (Figure. 2). MS and LS media induced average callusing, and both were equipotent for callus induction and growth (Table 3a), presumably due to similar composition and concentration of inorganic salts. Roy *et al.* (1991) reported MS media to be better than B_5 for callusing using leaf explant in *Lathyrus sativus*. In pigeon pea, however, BM was reported to be suitable among all the above five media for callus induction using leaflet and epicotyl explants (Suresh Kumar *et al.,* 1983). White(1954) media(W) with IAA and also IBA could not induce any callus and it elicited poor response when supplemented with 2, 4-D and NAA. The observed differences in callus induction and callus growth among media might be due to differences in concentration of inorganic nitrates and sulphates.

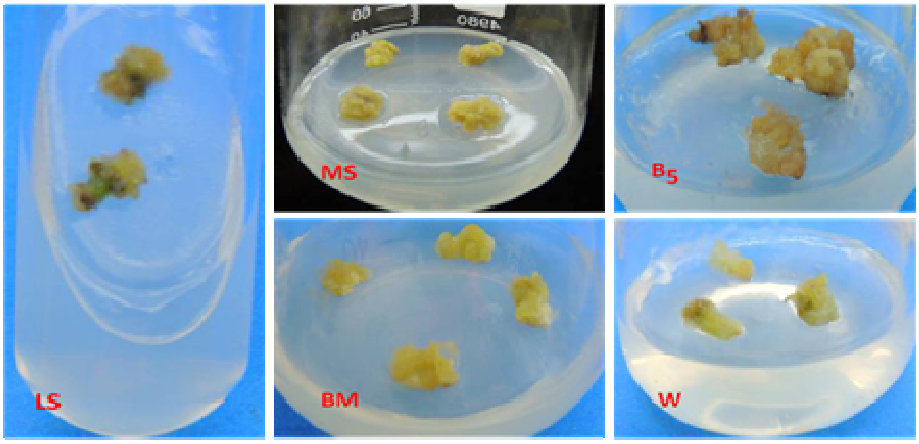

Figure 2. Callus induction and growth of calli in different basal media with 2.5mg/l 2, 4-D.

Table 3a. Effect of different basal media supplemented with common auxins (2.5mg/l) on callus induction and growth of calli in grasspea.

Auxin	LS	MS	BM	B_5	W
2,4-D					
CIF(%)	48.2±1.12	49.5±1.31[a]	58.7± 1.02	62.8±1.53	29.5±1.80
1st week	++++	+++	+++	++++	++
2nd week	+++	+++	++++	+++++	+
3rd week	++	++	++++	+++++	+
4th week	++	++	++++	+++++	+
IAA					
CIF(%)	15.3±1.81	13.2±2.21	10.5±2.32	18.5±2.10	0.0
1st week	0	++	++	++	-
2nd week	+	+	+	+	-
3rd week	-	+	+	+	-
4th week	-	-	-	-	-
IBA					
CIF(%)	16.9±2.52	20.5±2.13	19.5±1.80	17.2±2.01	0.0
1st week	0	+	0	+	-
2nd week	+	+	+	+	-
3rd week	-	-	+	+	-
4th week	-	-	-	-	-
NAA					
CIF(%)	33.7±2.13	35.1±2.11	45.8±1.32	50.6±1.31	17.0±1.98
1st week	++	++	+++	++++	+
2nd week	+	++	+++	+++	+
3rd week	+	+	+++	+++	-
4th week	-	+	+++	+++	-

CIF: Callus induction frequency(%), 0- No callus induction and growth, [a]values are mean ± S.E.
+, ++, +++, ++++, +++++: poor, moderate, good, fair and excellent callus growth respectively.

Overall callus growth was very high in B_5 and BM medium. While in MS and LS medium, calli turned brown within fortnight of its initiation and calli growth remarkably slowed down. Neelam *et al.* (1986) observed highly proliferated greenish to brownish calli in B_5 medium supplemented with various combinations of cytokinins and auxins in chickpea. In contrast, Rao and Chopra (1989) reported superiority of MS medium for callus induction against six other media in chickpea. The superiority was attributed to its high ammonium nitrate content. In the present investigation, explants as well as calli became necrotic in W media on two weeks of culture. Necrosis is *in vogue* associated with carbon source present in the medium in addition to other media recipes. Such calli lose their ability for proliferation and progressively die. This may be attributed to early production of phenolics and could be also due to higher Levels of sodium and magnesium sulphates (Suresh

Table 3b. Response of different hormonal concentrations in B_5 medium for induction and nature of callus in grasspea.

Growth regulator(mg/l)	CIF (%)	Callus growth	Colour	Nature of callus
2,4-D				
1.0	53.8 ± 1.23[a]	++[b]	white	Soft and powdery mass on surface
1.5	56.5 ± 1.52	++	-do-	-do-
2.0	66.1 ± 1.53	+++	Yellowish white	Very soft with powdery mass
2.5	62.5 ± 1.33	+++	-do-	Very soft without powdery mass
3.0	43.8 ± 2.01	++	Dirty white	-do- with occasional rooting
2,4-D +BAP				
1.0 + 0.10	58.5 ± 1.29	++	Bit yellowish	Soft and dying after 2-3 weeks
1.5 + 0.25	65.0 ± 0.89	++++	Greenish white	Soft, few powdery mass on surface
2.0 + 0.50	78.2 ± 0.98	+++++	White with green shades	Very soft, without powdery mass
2.5 + 0.75	68.1 ± 1.00	++++	-do-	-do-
3.0 + 1.00	57.3 ± 1.28	+++	Yellowish white	Very soft, even difficult to transfer
2,4-D + Kn				
1.0 + 0.10	43.2 ± 2.22	++	Yellowish white	Very soft
1.5 + 0.25	44.3 ± 1.98	+++	-do-	-do-
2.0 + 0.50	68.3 ± 1.21	++++	Whitish yellow	Soft
2.5 + 0.75	61.8 ± 1.32	++++	-do-	Soft
3.0 + 1.00	35.7 ± 2.13	++	Dull white	Soft
NAA				
1.0	40.2 ± 1.92	+	Greenish white	Hard and compact
1.5	48.0 ± 1.85	++	-do-	-do-
2.0	59.8 ± 1.50	+++	White with green shades	Less compact but hard
2.5	50.2 ± 1.38	+++	White	-do-
3.0	38.3 ± 2.13	+	Yellowish white	Less compact and bit soft
NAA + BAP				
1.0 + 0.10	45.3 ± 2.01	++	Whitish green	Bit loose and soft
1.5 + 0.25	62.8 ± 1.37	+++	Green with white shades	Loose, bit hard and friable
2.0 + 0.50	70.5 ± 0.88	++++	Dark green	Loose, hard and friable
2.5 + 0.75	58.2 ± 1.39	+++	Green	-do-
3.0 + 1.00	38.5 ± 2.11	++	Dull green	Bit compact, hard and less friable
NAA + Kn				
1.0 + 0.10	25.7 ± 2.81	+	Greenish white	Compact hard
1.5 + 0.25	31.3 ± 2.32	++	-do-	-do-
2.0 + 0.50	58.5 ± 1.80	+++	Yellowish green	Bit compact and less friable
2.5 + 0.75	52.2 ± 1.45	+	Greenish yellow	-do-
3.0 + 1.00	33.1 ± 2.91	+	Pale yellow	Bit soft and less compact

[a]Values are mean ± S.E. [b]Callus growth scored at 28th day of primary culture.

Kumar *et al.,* 1983).

Of the four auxins tested for callusing ability with seedling internode as explant, 2, 4-D was found to be effective for callus induction and growth, both in amount and rate, followed by NAA in all media tested. IAA and IBA induced callus in late (after 10-12 days) and calli became recalcitrant within one week of callus initiation. In *Dolichos lablab*, 2, 4-D (2gm/l) + Coconut milk (10%) was found to be optimum for callus initiation and its further growth, among all other auxins tested by Sounder Raj *et al.* (1991). Joyner et al. (2010) reported 100% callus induction from cotyledons in Soybean using MS medium with 3-21µM 2, 4-D. Among various media, B_5 containing 2mg/l 2, 4-D was reported to yield the best response in terms of callus formation (Ramulu and Rao 1989-91) in Bengal gram (*Cicer arietinum*) and guar (*Cyamopsis tetragonoloba*). In pigeon pea, among seven media tried by Kumar *et al*. (1996) including MS medium; Blaydes medium supplemented with auxins found better for callus induction.

A follow up experiment (primary culture) with the same explant (internode), was laid out to identify the most effective concentration of 2, 4-D and NAA both in single and combination with lower concentration of cytokinins, BAP and Kn. Results (Table 3b) indicated that 2, 4-D and NAA, each at 2mg/l, could induce good callusing in terms of both induction and growth. However, continuous culture in increased concentration of 2, 4-D (2.5-3mg/l) led to occasional rooting and that of NAA badly affected the nature of callus in terms of colour and

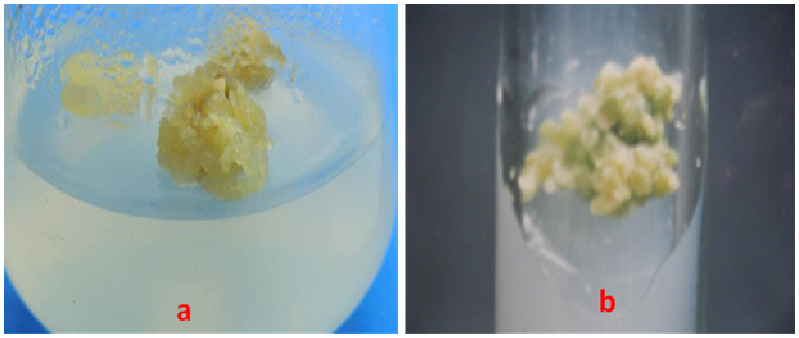

Figure 3. Callus induction and growth of calli in B_5 medium (**3a-** B_5 + 2mg/l 2, 4-D+0.5mg/l BAP, **3b-** B_5 + 2mg/l NAA+0.5mg/l BAP).

texture. Once the callus turns to direct regeneration of rooting, there is remote possibility to reverse back to normal shoot regenerability. In the present investigation, inclusion of BAP (0.5 mg/l) exhibited more profuse callus growth and higher callus induction frequency (CIF) being highest at 2mg/l 2, 4-D + 0.5mg/l BAP (78.2± 0.98%)(Figure. 3a) fallowed by 2mg/l NAA + 0.5mg/l BAP(70.5 ± 0.88%)(Figure.3b). Thus, BAP was more effective than Kn. B5 supplemented with 2mg/l 2,4-D + 0.5mg/l Kn often produced whitish-yellow calli with white powdery mass on surface. Roy *et al.* (1991) also obtained very high frequency of nodular callus (94.0± 0.65%) in B_5 + NAA (2 mg/l) + BAP (0.5 mg/l) as compared to highest up to 34.0 ± 1.19% among kn combinations at 2mg/l NAA + 0.4 mg/l kn in *Lathyrus sativus.* Harisaranraj *et al.* (2008) obtained highest cell proliferation from explants cultivated in B_5 medium supplemented with13.3 µM BAP and 13.5 µM 2, 4-D using half-seed explant in blackgram. Similarly, Nazim *et al.* (2012) observed highest percentage of callus in MS+0.5mg/l 2,4-D, MS+0.5mg/l 2,4-D+0.5mg/l BAP and MS+1.0mg/l 2,4-D+0.5mg/l BAP using leaf and cotyledon explants of mungbean. However, a combination of picloram and BAP was reported to yield maximum response for callus induction in *Lathyrus sativus* (Sinha *et al.* 1982) and chickpea (Sawardekar 2007) using MS+10^{-8} M picloram + 10^{-6} M BAP and SS B-8 medium + 3.00 mg/l picloram + 0.5 mg/l BAP respectively.

Some workers reported use of cytokinins without any auxin for better callus induction in legumes. MS medium supplemented with B_5 vitamins + 4.0 mg/l BAP has been reported to be most effective to produce regenerative calli in blackgram (Varalaxmi *et al*., 2007). Similarly, Dadmal and Navhale (2011) reported higher response for callus induction using MS + 5.0mgl Kn in cowpea.

In the present investigation, induction and growth of callus in B_5 + 2, 4-D (2mg/l) + 0.5 mg/l BAP were excellent but, most of the calli were soft and non-chlorophyllous (Figure. 3a). In contrast, B_5 containing NAA(2 mg/l) and BAP (0.5 mg/l) most frequently induced light green to dark green, friable and nodular easily dissociated calli(Figure. 3b). Such calli could be most suitable for plant regeneration and genetic transformation in *Lathyrus sativus.*

CONCLUSION

The following are the conclusions from the research studies:

1 Half- strength MS basal media is suitable to grow seedlings *in vitro* from seeds.
2 B_5 and BM medium are better for callus initiation and growth of calli than either MS, LS or W growth media.
3 B_5 with 2mg/l NAA + 0.5 mg/l BAP is potent enough to produce green, hard, friable and nodular calli suitable for plant regeneration and genetic transformation.

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How to cite this article: Tripathy S.K., Swain D., Mishra P.K., Baisakh B. and Dash S. (2014). Optimization of callus induction in *Lathyrus sativus* L. Afr. J. Food Sci. Technol. 5(3):60-66