ARJLS

Advanced Research Journal of Life Sciences

Online Peer reviewed monthly Journal

ISSN 2455 – 3530

Volume 1/ Issue 1 / October 2015: 29 - 35

RESEARCH ARTICLE

In vitro Plant Regeneration from Cotyledonary Node Explants of Pigeon Pea *(Cajanus cajan* L.) Mill sp. cv. Vamban II *via* Direct Organogenesis

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Received 04 Jan 2015; Revised 13 Sep 2015; Accepted 03 Oct 2015

ABSTRACT

A simple and efficient protocol was developed for *in vitro* propagation of Pigeon pea [*Cajanus cajan (L.) Mill* sp.], Vamban II. The seeds were removed from the pods and the cotyledon along with embryonal axes were cultured on MS medium, with 3% sucrose and supplemented with various concentrations of BAP and KIN (1 mg/L, 2 mg/L, 5 mg/L and 10 mg/L). The cotyledonary node along with the mass of shoot - initials originating from 12 day old seedling were excised by removing the epicoyl and cotyledons within 1 - 2 mm of the node and used as explants. Direct shoot initials were observed from cotyledonary node explants after 2 weeks of culture initiation. The maximum number of multiple shoots was observed at the concentration of 1 mg/L of BAP. Addition of IAA (0.5 mg/L) to BAP (1 mg/L, 2 mg/L, 5 mg/L and 10 mg/L) enhanced the callus with multiple shoots. The optimum level of BAP (with 0.5 IAA) that promoted the highest number of multiple shoot (6.4 shoots/explants) was 1 mg/L. Addition of NAA (0.5 mg/L) to BAP (1, 2, 5, 10 mg/L) enhances shoot bud with callus. Multiple shoots (4) were observed in the medium supplemented with NAA (0.5 mg/L) and BAP (1 mg/L). Well developed elongated shoots (2 to 3 cm) were excised and cultured on ½ strength MS medium supplemented with different concentrations of IBA (0.1 - 0.5 mg/L) for root induction. Among these concentrations, 0.3 mg/L IBA produced the maximum number of roots. The highest percentage of plant regeneration *via* multiple shoots was noticed on MS medium supplemented with 1.0 mg/L. The mean number of shoots decreased with increasing in the concentration of BAP up to 1.0 mg/L. In addition cotyledonary node system has been efficiently used recently for *Agrobacterium*- mediated gene transfer technology in other legumes with high transformation frequencies.

Key words: Direct organogenesis, Vamban II and Micropropagation.

1. INTRODUCTION

Pigeon pea [*Cajanus cajan* (L.) Mill sp.] (Family: Fabaceae) also known as red gram is one of the major grain legume (protein rich pulse) crops grown in the semi-arid tropics and sub tropics and its importance in Indian agriculture has been described [22]. It is a diploid plant with 2*n* = 22 chromosomes. Among the leguminous crops, pigeon pea ranks fifth in area after soya bean, common bean, peanut, and chick pea [23]. India now constitutes the centre of diversity for this crop and among the pulses cultivated and produced in this region; pigeon pea ranks highest [20].

Its protein content is about 22.3 per cent and the seed contain globulins, cajanin and concojanin, accounting for 58 per cent and 8 per cent of the total nitrogen respectively, differing from each other in their sulfur and tryptophan content. The globulins, which form the chief proteins of the seed, appear to be characteristic of the genus. They are rich in tyrosine and moderately rich in cysteine, arginine and lysine. Fresh seeds contain vitamins, especially provitamin A and vitamin B complex. Per a 100 g edible portion, dry seeds contain 7 - 10.3 g water, 14 - 30 g protein, 1 - 9 g fat, 36 - 65.8 g carbohydrates, 5 - 9.4 g fibre and 3.8 g ash. The energy content averages 1450 kJ/100 g.

Several constraints that limit crop production or quality have been addressed by conventional breeding and enhanced management, but there are situations where the existing germplasm lacks the

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required traits [18]. Breeding incompatibility problems associated with wild species warrant the exploration of alternative approaches. Thus, genetic engineering technology plays a significant role as modern tool for the introduction of agronomically useful traits into high yielding cultivars/varieties.

However, the development of an efficient plant regeneration protocols is a prerequisite in recombinant technology to carry out genetic transformation. Pigeon pea has been reported to be recalcitrant to regeneration *via* tissue culture. Various constraints in the transformation include low percentage of plant regeneration, long - duration regeneration via callus phase, and influence of the genotypes on the regeneration system. An efficient plant regeneration method that avoids these problems is required [1].

All though there are reports on organogenesis in pigeon pea from apical meristem [4], undifferentiated callus [10,12], differentiated non meristametic tissues like leaf [5, 6, 7, 9, 24], and various seedling explants such as hypocotyls [9], cotyledonary nodes [9, 12, 13, 14, 17, 19], epicotyls [9, 13, 17], and embryonal axes [8]. But only limited studies have been reported for Indian cultivars Vamban II, a popular cultivar from Tamil Nadu. Plant regeneration protocol is a pre-requisite for the genetic manipulation studies to enrich their nutritional value of plant variety. In this study, we report an efficient system for regeneration of pigeon pea (*Cajanus cajan*) Vamban II from cotyledonary node *via* multiple shoot formation.

2. MATERIALS AND METHODS

Plant material

Pigeon pea, *Cajanus cajan* L. (cv. Vamban II) were collected from the field grown plants. Pods (almost mature, 1 week before harvest) collected from field grown plants were agitated thoroughly in a dilute Tween 20 for 10 minutes. Seeds were then rinsed under running tap water and surface sterilized with 0.1% (W/V) mercuric chloride solution for 20 minutes, followed by 4 or 5 rinses of 2 minute duration in sterile distilled water. Under aseptic conditions, the seeds were removed from the pods. After removing the seed - coat, the cotyledon along with embryonal axes were cultured on MS medium, with 3% sucrose and supplemented with varying concentration of different cytokinins. The cotyledonary node along with the mass of shoot - initials originating from 12 day old seedling were excised by removing the epicoyl and cotyledons within 1 - 2 mm of the node and used as explants.

Culture conditions

The media used were based on MS basal (**Table 1**), containing 3% (w/v) sucrose (Hi-media, India) and 0.7% (w/v) agar (Hi-media, India). The pH of the medium was adjusted to 5.7 before autoclaving for 15 minutes at 121ºC. Cotyledon explants were placed on culture tubes (25 × 150 mm) containing 10 - 15 ml of medium with respective growth regulators. The cultures were incubated at 25±2ºC under 16/8 h (light/dark) photoperiod with white fluorescent light giving a photon flux density of 60 μ E m^{-2} $s^{-1.}$.

Table 1: Composition of a nutrient solution used for the culture of pigeon pea (*Cajanus cajan*) Vamban II

Chemicals	Concentration
Solution-1: Macronutrients	
$CaCl_2 2H_2O$	4.4 g/L
KH_2PO_4	1.7 g/ L
KNO_3	19 g/ L
$MgSO_4 .7H_2O$	3.7 g/ L
NH_4NO_3	16.5 g/ L
Solution-2: Micronutrients	
$COCl_2 .6H_2 O$	2.5 mg/ L
$CuSO_4.5H_2O$	2.5 mg/ L
H_3BO_3	620 mg/ L
$MnSO_4 .4H_2O$	2230 mg/ L
$Na_2MoO_4.2H_2O$	25 mg/ L
$ZnSO_4.5 H_2O$	860 mg/ L
Solution-3: Iron Source	
Fe.EDTA-Na salt	40 mg/ L
$FeSo_4$	27 mg/ L
Solution-4: Vitamins	
Glycine	400 mg/ L
Inositol	20000 mg/ L
Nicotinic acid	100 mg/ L
Pyridoxine HCl	100 mg/ L
Thymine HCl	10 mg/ L
Solution-5	
KI	83 mg/L
Others	
Sucrose	30 g/L
Agar	8 g/L

Effect of BAP and KIN for direct shoot bud regeneration

This experiment was designed to compare two cytokinins; BAP and KIN (1, 2, 5, 10), with respect to their effects on shoot bud regeneration. Cotyledonary node explants were cultured on MS medium supplemented with different concentrations of BAP and KIN (1.0 mg/L, 2.0 mg/L, 5.0 mg/L and 10.0 mg/L) for shoot bud differentiation. After 4 weeks of culture, the numbers of explants forming shoot buds were counted.

Effect of different PGRS for multiple shoot formation

Cotyledonary node explants were cultured on MS medium containing different combinations of BAP, NAA and IAA for multiple shoot formation. Three different treatments, BAP (1.0, 2.0, 5 .0 and 10.0 mg/L) alone, NAA (0.5 mg/L) + BAP (1.0, 2.0, 5 .0 and 10.0 mg/L), IAA (0.5 mg/L) + BAP (1.0, 2.0, 5 .0 and 10.0 mg/L) were employed. The cultures were maintained as mentioned previously and sub-cultured onto the same fresh media after 2 weeks. Shoots longer than 1.0 cm was counted and the data were recorded.

Root induction

Elongated shoots were excised from all treatments and used for rooting. Shoots collected *in vitro* (> 3 cm long) were transferred to MS medium supplemented with different concentrations of IBA (0.1 mg/L, 0.2 mg/L, 0.3 mg/L, 0.4 mg/L and 0.5 mg/L) for rooting. Cultures were incubated as described previously.

Acclimatization

Rooted plantlets were gently washed with running tap water and followed by sterile distilled water and then successfully transferred to plastic cups containing sterile garden soil. The plastic cups were covered with porous polyethylene bags for maintaining high humidity. The hardened plantlets were maintained in the culture room for a week. The polyethylene bags were removed after 2 weeks and then hardened plantlets were successfully transferred to normal room temperature. These plants were lucratively transferred to net house for further growth and development. Observations were recorded on the percentage of response, number of roots per shoot and root length. Means and standard errors were carried out for each treatment.

Statistical Analysis

The experimental design was completely randomized block (CRD) and factorial with auxin and cytokinin as independent variables. The overall growth response of the shoot regeneration and root induction was recorded in terms of the percent of response, number of shoots per explants, number of roots per shoot and root length. The analysis of variance (ANOVA) was carried out using SAS programme. A significant level of 0.05 was used for all statistical tests.

3. RESULTS AND DISCUSSION

Pods (almost mature, 1 week before harvest) of Vamban II were collected from field grown plants. Under aseptic conditions, the seeds were removed from the pods. After removing the seed-coat, the cotyledon along with embryonal axes were cultured on MS medium, with 3% sucrose and supplemented with various concentrations of BAP and KIN (1, 2, 5, 10 mg/L). The cotyledonary node along with the mass of shoot-initials originating from 12-day-old seedling were excised by removing the epicoyl and cotyledons within 1 - 2 mm of the node and used as explants. The effect of various concentrations of cytokinins (BAP and KIN) on shoot regeneration from the cotyledonary node explants of Vamban II was studied. Direct shoot initials were observed from cotyledonary node explants after 2 weeks of culture initiation (**Fig 1**).

Green adventitious shoot buds was sub-cultured onto the same media for 2 to 3 weeks interval for shoot bud proliferation. The average number of shoots per explants was recorded after 3 weeks of culture (**Fig 2**). Highest regeneration frequency (100%) and number of shoots per explant (13.4 shoots/explant) were observed on MS media supplemented with 1.0 mg/L BAP (**Table 2**). The mean number of shoots decreased with increase in the concentrations of BAP. Sub-culture of regenerated shoots into same medium exhibited further shoot proliferation with minimal basal callus. In the present study, low regeneration frequency of shoot buds was observed in the medium supplemented with 10.0 mg/L KIN.

Callus induction was observed within ten days of explants cultured on MS medium fortified with different combination of plant growth regulators (BAP (1.0, 2.0, 5.0 and 10.0 mg/L) alone, NAA (0.5 mg/L) + BAP (1.0, 2.0, 5 .0 and 10.0 mg/L), IAA (0.5 mg/L) + BAP (1.0, 2.0, 5 .0 and 10.0 mg/L). The shoot clumps were started appearing on the calli after 2 weeks of culture. Shoot buds developed on the compact masses was sub cultured onto the same media within 2 to 3 weeks interval for shoot bud proliferation. Addition of IAA (0.5 mg/L) to BAP (1, 2, 5, 10 mg/L) enhanced the callus developing with multiple shoots. The optimum level of BAP (with 0.5 IAA) that promoted the highest number of multiple shoot (6.4 shoots/explants) was 1 mg/L (**Table 3**). Addition of NAA (0.5 mg/l) to BAP (1, 2, 5 and 10 mg/L) enhances shoot bud with callus. Multiple shoots (4) were observed in the medium supplemented with NAA (0.5 mg/L) and BAP (1 mg/L). The lowest regeneration frequency from cotyledonary node was observed in the medium

supplemented with BAP and NAA. Of the two cytokinin tested, BAP was found to be more effective than kinetin in inducing shoot development and multiple shoot induction. The shoots were sub cultured on the same medium and were allowed to grow till they attained 2 - 3 cm height (Fig 2).

Well developed elongated shoots (2 to 3 cm) were excised and cultured on ½ strength MS medium supplemented with different concentrations of IBA (0.1 - 0.5 mg/L) for root induction. Among the concentrations tested, maximum number of root induction (8 roots/shoot) was observed on half strength MS medium containing 0.3 mg/L IBA (**Table 4**) followed by 4.5 roots/shoot found in the media having 0.2 mg/L IBA, whereas (4 roots/shoot) root induction was observed on MS medium containing 0.1 mg/L IBA (**Fig 3**).

When the plantlets were attained 6 - 8 cm long then they had developed a good root system subsequently they were transferred from growth room to the plastic cups and kept for fifteen days (Fig.4). Then, the plantlets were regularly sprayed with water, covered with polythene cover to maintain high humidity around juvenile plants. Plantlets were subsequently transferred into small pots and gradually acclimatized to the field condition. The survival rate of the transferred plantlets to soil was 60%.

Direct multiple shoot bud induction

In the present study, cotyledonary node of explants was treated with different concentrations of plant growth regulators for plant regeneration. Pods (almost mature, 1 week before harvest) of Vamban II were collected from field grown plants. Immature cotyledons and embryonal axes from 12-day-old seedling were excised by removing the epicoyl and cotyledons within 1-2 mm of the node and used as explants [10]. Cotyledonary nodes have been viewed as most successful for the induction of multiple shoots *via* organogenesis among grain legumes [3].

The cotyledonary node had better performance in direct shoot bud regeneration than hypocotyls, leaf, epicotyls and cotyledon explants. It indicates that, the cotyledonary node tissue of pigeon pea as a good explant source for plant regeneration. The cotyledonary node along with the mass of shoot-initials originating from 12 day old seedling were excised and cultured on MS medium supplemented with various concentrations of BAP and KIN (1 mg/L, 2 mg/L, 5 mg/L, 10 mg/L) and also with combination of IAA and NAA (0.5 mg/L) for multiple shoot induction. Direct shoot initials was noticed in all media formulations. A significant increase in shoot bud proliferation was observed over the culture period. Organogenesis has been the extensively used pathway compared to somatic embryogenesis, because of its wider adaptability among diverse genotypes [11, 15, 16, 21].

Shoot regeneration

The cotyledonary node along with the mass of shoot-initials originating from 12-day-old seedling were excised and cultured on MS medium supplemented with various concentrations of BAP and KIN (1 mg/L, 2 mg/L, 5 mg/L, 10 mg/L) also with combination of IAA and NAA (0.5 mg/L) for multiple shoot induction. The cytokinin BAP and KIN promotes cell division, shoot multiplication and auxillary bud formation. BAP alone was found to be suitable for both multiple shoot bud induction and proliferation. The morphogenetic response of tested explants differed depending on the concentrations of BAP and KIN in the medium. Differential response was noticed in cultivars and explants under the levels of BAP and KIN in the media.

Table 2: Effect of BAP and KIN on direct multiple shoot bud regeneration from cotyledonary node explants of pigeon pea after 3 weeks of culture

Cytokinin concentration (mg/l)	Percent of shoot bud regeneration	Mean No. of multiple shoots (Mean ±SE)
BAP		
1.0	100	13.4 ±2.01[a]
2.0	100	8.6 ±1.40[b]
5.0	100	6.4 ±1.32[c]
10	100	6.0 ±0.54[c]
KIN		
1	100	5.0 ±0.70[d]
2	100	5.6 ±0.50[d]
5	100	5.8 ±0.86[d]
10	60	2.22 ±0.91[e]

*Mean followed by the same letter within the column are not significantly different at P<0.0.5

Table 3: Effect of different PGRs on multiple shoot formation from cotyledonary node explants of pigeon pea after 4 weeks of culture

Hormones concentration (mg/l)	Percent of shoot bud regeneration	Shoot bud + callus	Mean No. of multiple shoots (Mean ±SE)
BAP + NAA			
1.0 + 0.5	60	++	4.0 ±1.15c
2.0 + 0.5	-	++	-
5.0 + 0.5	-	++	-
10 + 0.5	-	++	-
BAP + IAA			
1.0 + 0.5	100	++	6.4 ±0.67a
2.0 + 0.5	100	++	4.4 ±0.50b
5.0 + 0.5	100	++	4.0 ±0.31b
10 + 0.5	100	++	2 .0±1.09c

*Mean followed by the same letter within the column are not significantly different at P<0.0.5.

Table 4: Effect of IBA on *in vitro* rooting of elongated shoots after 3 weeks of culture

IBA concentration (mg/l)	Percent of rooting	Mean No. of roots (Mean ±SE)	Root length (Mean ±SE)
0.1	66.6	4.0 ±1.0[b]	4.13 ±0.670[b]
0.2	66.6	4.5 ±1.5[b]	3.81 ±0.315[c]
0.3	100	8.0 ±0.00[a]	5.04 ±0.290[a]
0.4	33.3	2.5 ±0.5[c]	3.37 ±0.775[c]
0.5	66.6	4.0 ±1.0[b]	2.89 ±0.230[d]

Fig 1: Direct shoot bud regeneration from Cotyledonary node explants of *Cajanus cajan* cv.Vamban II

Fig 2: Multiple shoot induction and Elongation of regenerated shoots

Fig 3: Rooting of elongated shoots

Fig 4: Establishment of *in vitro* regenerated plants in plastic cups

The medium supplemented with 1.0 mg/L BAP was most effective in induction of shoots from cotyledonary node explant. The average number of shoots per explants was recorded after 3 weeks of culture. Highest regeneration frequency and number of shoots per callus (13.4 shoots/explant) were observed on MS media supplemented with 1.0 mg/L BAP. Similar observations were reported [2, 19]. In the present work, it was observed that explants sub-cultured on media with different concentrations BAP or KIN (1 mg/L, 2 mg/L, 5 mg/L, 10 mg/L) alone to produce a shoots. But, the mean number of shoots decreased with increases the concentrations of BAP beyond 1.0 mg/L. Earlier studies showed that the presence of 5.0 mg/L BAP and 5.0 mg/L KIN combination produced maximum shoot bud development [19]. Addition of IAA (0.5 mg/L) to BAP (1 mg/L, 2 mg/L, 5 mg/L, 10 mg/L) enhanced the callus with multiple shoots. The optimum level of BAP (with 0.5 IAA) that promoted the highest number of multiple shoot (6.4 shoots/explants) was 1 mg/L. Addition of NAA (0.5 mg/L) to BAP (1, 2, 5, 10 mg/L) enhances shoot bud with callus. Multiple shoots (4) were observed in the medium supplemented with NAA (0.5 mg/L) and BAP (1 mg/L). The lowest regeneration frequency from cotyledonary node was observed in the medium supplemented with BAP (2 mg/L, 5 mg/L, 10 mg/L) and NAA (0.5 mg/L). The proliferation of multiple shoot initials from the explants was based on ratio of cytokinin and auxin [19]. In the present study, low regeneration frequency of shoot buds was observed in the medium supplemented with 10.0 mg/L KIN. BAP in general was found to be better than KIN for shoot bud regeneration.

Root formation

Well developed shoots (2 to 3 cm) were excised and cultured on half strength MS medium supplemented with different concentrations of IBA (0.1 - 0.5 mg/L) for root induction. The beneficial effects of using half-strength MS medium for rooting of *in vitro* induced shoots has already have been reported for pigeon pea [10, 17, 19].

Among the concentrations tested, maximum number of root induction (8 roots/shoot) was observed in half strength MS medium containing 0.3 mg/L IBA. However, it has been realized that the success of regeneration is highly influenced by the addition of BAP and KIN for direct organogenesis; and NAA for callus mediated shoot regeneration as well as in combination of dual cytokinins and auxin ratio.

Acclimatization

The regenerated plantlets were 6 - 8 cm long and had developed a good root system; they were gradually transferred from growth room to the plastic cups and kept fifteen days. For acclimatization plantlets were removed from rooting medium and transferred to plastic cups containing autoclaved soil and sand in the ratio of 1:1 and covered with polyethylene bags to maintain high humidity and was kept under culture room conditions for 15 days and then the plantlets were regularly sprayed with water once in two days interval. After two weeks, polyethylene bags were removed and were successfully established in the green house and placed under shade until growth was observed. The survival rate of the transferred plantlets to soil was 60%. The present plant regeneration protocol *via* direct shoot organogenesis can now be exploited for genetic transformation experiments.

In conclusion, the present study showed a development of an efficient tissue culture and plant regeneration protocol for local variety Vamban II cultivated in Tamil Nadu. Both callus induction and plant regeneration from cotyledonary node explants were noticed. Among different concentrations of cytokinins tested, direct shoot bud induction was observed in all the concentrations. The highest percentage of plant regeneration *via* multiple shoots was noticed on MS medium supplemented with 1.0 mg/l. The mean number of shoots decreased with increasing in the concentration of BAP up to 1.0 mg/l. In addition cotyledonary node system has been efficiently used recently for *Agrobacterium* - mediated gene transfer technology in other legumes with high transformation frequencies. Moreover this protocol might be potential system for the improvement of the genetic transformation efficiency.

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