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AN EFFICIENT *IN VITRO* REGENERATION OF SHOOT FROM COTYLEDON DERIVED CALLUS OF PIGEON PEA (*CAJANUS CAJAN* (L.) MILLSP.)

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Abstract:-Pigeon pea (*Cajanus cajan* (L.) Millsp.) is an important grain legume of the semi-arid tropics. It provides protein rich food. Pigeon pea being recalcitrant species, regeneration of multiple shoots via callus induction and organogenesis was achieved from cotyledon explants of pigeon pea. Callus induction and shoot regeneration at various frequencies were observed using different concentration and combination of growth regulators. The highest callus formation was observed on MS medium + 1.0 mg l^{-1} IAA + 0.9 mg l^{-1} Kinetin. The Highest shoot formation was obtained on MS medium fortified with 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA and 0.4 mg l^{-1} GA, Regenerated plants were successfully established in the soil after acclimatization.

Keywords:*Cajanus cajan* (L.) Millsp.), *in vitro* regeneration, Cotyledon explants.

1. INTRODUCTION

Pigeon pea or red gram (*Cajanus cajan* L. Millsp.) is one of the major grain legumes of the tropics and subtropics. Pigeon pea is a high protein grain legume and caters the protein requirement of the majority of the population in the Indian subcontinent (George and Eapen, 1994). It is a source of protein for 80% vegetarian in India. In India, red gram is prone to more than 200 species of insects (Anonymous, 1978), among which pod borer (*Helicoverpa armigera*) causes enormous losses (46.6 - 63.6%). *Helicoverpa armigera* is a polyphagous lepidopteron insect, present throughout the year and can complete up to seven generations in a year under favorable conditions. The demand for Pigeon pea boosts the production of high yielding and disease resistant varieties in India and the world market. To meet the increasing demand for these crops *In vitro* tissue culture followed by gene transfer would be an efficient and economical method to obtain a large number of disease free plants within short span of time.

Legumes are one of the most important groups of crop plants and have been subjected of efforts to improve desirable traits including their *in vitro* culture response. Since legumes are notoriously recalcitrant to regenerate from tissue culture, much effort has been devoted to developing and optimizing efficiencies in vitro regeneration system to facilitate a variety of technologies (Murashige and Skoog 1962). In Pigeon pea attempts to regenerate plants from various explants have been attempted, these include leaves (Dayal and Lavanya, 2003; Eapen and George, 1993; Eapen *et.al*.,1998; Geetha *et.al.,*1999; Singh *et.al*.,2002) cotyledonary node (Frankalin *et.al*.,1998; Geetha *et.al*.,1999; Mohan & Krishnamurthy, 1999; Shiva Prakash *et.al*.,1994; Singh *et.al*.,2002), epicotyls (George and Eapen, 1994) and shoot apics (Singh *et.al.*,2002; Singh *et.al.*,2004).

The Pigeon pea is recalcitrant in its regeneration potential, which becomes difficult to apply genetic transformation techniques. Therefore in the present research study, an attempt has been made to develop an efficient regeneration protocol by using cotyledon explants of pigeon pea.

2. MATERIALS AND METHODS

Collection of Plant Material

Certified Seeds of (*Cajanus cajan* L. Millisp.) Co-6 cultivar were obtained from the Centre for Plant breeding & Genetics, Tamil Nadu Agriculture University, Coimbatore.

Explant Preparation

Mature surface sterilized seeds cultured directly on MS basal agar medium without any growth regulators. Cotyledon explants obtained from the plant after 7 days and are surface sterilized using the following procedure (Santombi & Sharma, 2007)

Soaked the explants in bavistin solution f or exactly 15 minutes

↓

Washed the explants with double distilled water twice

↓

Washed the explants with Tween 20 and Sodium hypochlorite for 4 minutes

↓

Washed the explants with autoclaved double distilled water 4 times

↓

Washed the explants with 70 % alcohol for exactly 30 seconds

↓

Immediately washed the explants with $Hgcl_2$ for exactly 2 and a half minutes

↓

Washed the explants with double distilled water 4 times to remove traces of ethanol and $HgCl_2$

Culture Media and Conditions

Cotyledon explants were transferred to regeneration media containing MS salts[10], B_5 vitamins[5] and 3% sucrose and the pH of the medium is adjusted to 5.6 (standard pH for MS medium). MS medium with 1.0 mg l^{-1} IAA + 0.9 mg l^{-1} Kinetin was used in the induction of callus and MS medium with 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA and 0.4 mg l^{-1} GA_3 was used in the shoot induction. The explants were transferred and sub cultured in fresh medium in every three weeks and regenerated callus were transferred to shooting media.

Light

The light source is provided by fluorescent lamps and the photoperiod is controlled by an hourly timer. The fluorescent lamps produce better quality of light and which is distributed uniformly and generates less heat. The pigeon pea requires an illumination of around 2000 - 3000 Lux. The inoculated explants are maintained less than 16 hours photoperiod light for enhanced growth.

Acclimatization

Healthy plants with 5 - 7 cm long with different ages (15, 22 and 29 days) of rooted shoots were individually removed from the culture jars, and their roots washed carefully with tap water and were transferred to pots containing soil, soil with sand (1:1) and soil with compost (1:1) for observation on survivability of platelets under ex vitro condition.

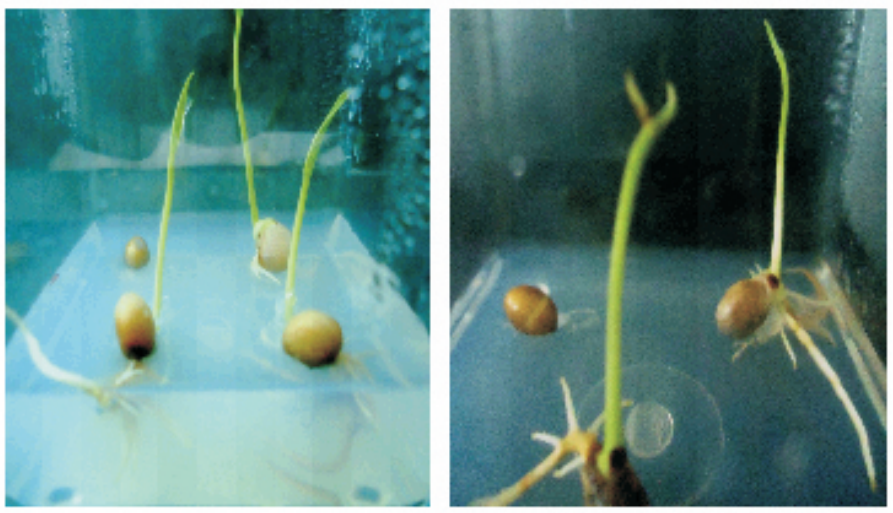
Statistical Analysis

The results obtained from the growth observation of the plants grown in different medium with cotyledon explants were statistically analyzed using a stat pack version - 4.01 (161209) copy written by Berty Edwin. The results are interpreted based on the T - Test representing the P - value along with the standard error of each sample. Through this analysis, P - value interprets the significant growth of the explants grown on a different medium.

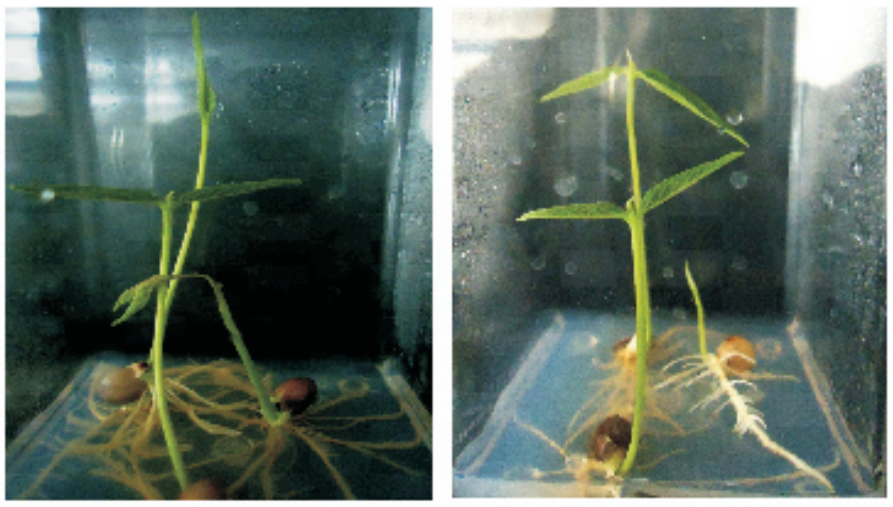
3. RESULTS AND DISCUSSION

Surface sterilized seeds, cultured directly on MS basal agar medium without any growth regulators in the phyta jar which showed 40 - 50% germination in 5 days; 95 - 100 % germination was observed after 7 days (Fig 1). This seedling plant cotyledon explants (excised from 7 days old seedlings), were used for morphogenic response. Callus induction was observed onto MS and B5 media containing different concentration and combination of IAA, Kinetin, 2, 4-D, NAA and BAP within 8 -15 days of incubation of cotyledon explants depending upon the concentration and combination of hormones. Callus induction noticed in all media formulations. But there was a wide range of variation in average fresh weight and color. The highest callus induction was observed on MS medium containing 1.0 mg l^{-1} IAA + 0.9 mg l^{-1} Kinetin (Fig. 2; Table 1, 1a). The highest callus growth in terms of fresh weight (0.802 g) was observed and color of calli was mostly whitish green and light green. It was observed only light green calli produce shoot buds. Proliferation of shoot buds was observed in MS + 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA and 0.4 mg l^{-1} GA_3(Fig. 2, Table 2, 2a). The shoot buds first appeared as nodular growth within 3-4 weeks of culture and at the end of 4 weeks this nodular growth increased in size and produced leaf primordial. The highest shoot induction in terms of height of shoots. Maximum number of shoot buds were obtained in MS + 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA and 0.4 mg l^{-1} GA_3, Root formation as recorded on MS + 1.0 mg l^{-1} IBA and 0.5 mg l^{-1} NAA (Fig. 2). Regenerated plants were successfully established in the soil.

The Pigeon pea *in vitro* regeneration depends on several factors like concentration of growth hormones, composition of media, media pH, types of explants, photoperiod time and light condition, In this study, cotyledons used as a explants, while this confirms previous reports



5 days old seedling plants



7 days old seedling plants

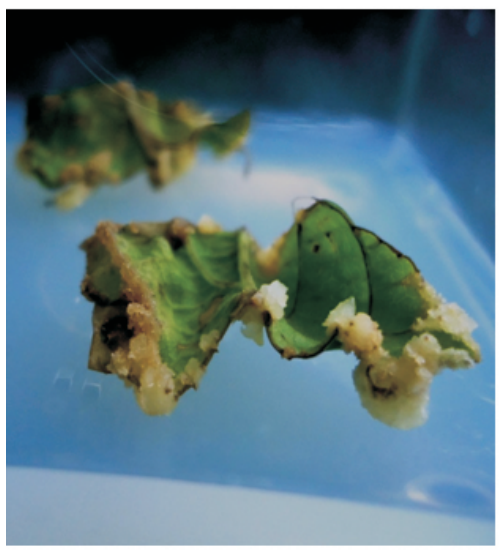


Fig A. 15 days' old regeneration

Fig B. 25 days' old regeneration

Fig C. 35 days' old regeneration

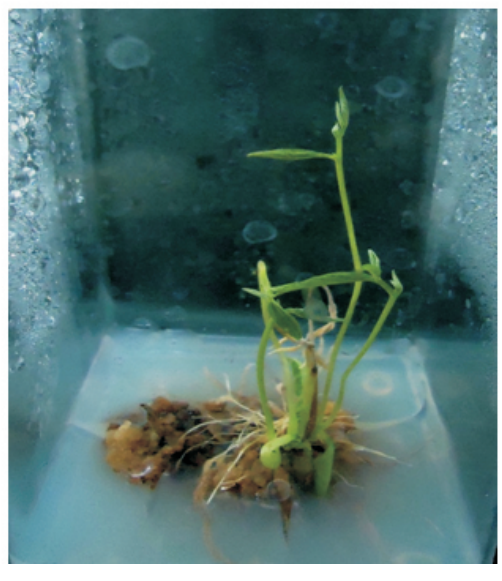


Fig D. 45 days' old regenerati

Fig 2. Cotyledon as explants on (A8) regeneration media, callus – 1.0 mg l^{-1} IAA + 0.9 mg l^{-1} Kinetin, shoot – 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 0.4 mg l^{-1} GA, root – 1.0 mg l^{-1} IBA + 0.5 mg l^{-1} NAA

Table 1— Regeneration of callus induction medium for Pigeon pea (*Cajanus cajan* L. Millsp.) *Co - 6*. Three combinations of medium responded well and A8 media responded maximum growth

Growth regulators (mg/l)	Explant type	Medium number	Growth observation (Cm) 8 days	12 days	15 days
A8 – 1.0 mg l^{-1} IAA + 0.9 mg l^{-1} KIN	cotyledon	A8	0.4	0.8	1.2
		B3	0.4	0.6	1.0
B3 –1.0 mg l^{-1} 2,4-D		C7	0.2	0.4	0.6
C7 – 1.0 mg l^{-1} KIN					

A8, B3, C7 – Different concentration and different combination of MS media
IAA – Indole Acetic Acid, KIN – Kinetin, 2, 4-D – 2,4 - Dichlorophenoxy acetic acid

Table 1a— Statistical growth observation analysis of cotyledon explants

OUTPUT / RESULT PANEL								
Treatments	n	Mean	SE	T-test	P-value	Result		
8 days	3	0.3333	0.0667	5.1962	0.0351	*		** - P < 0.01
15 days	3	0.9333	0.1764					* - P < 0.05
								NS - Not Significant

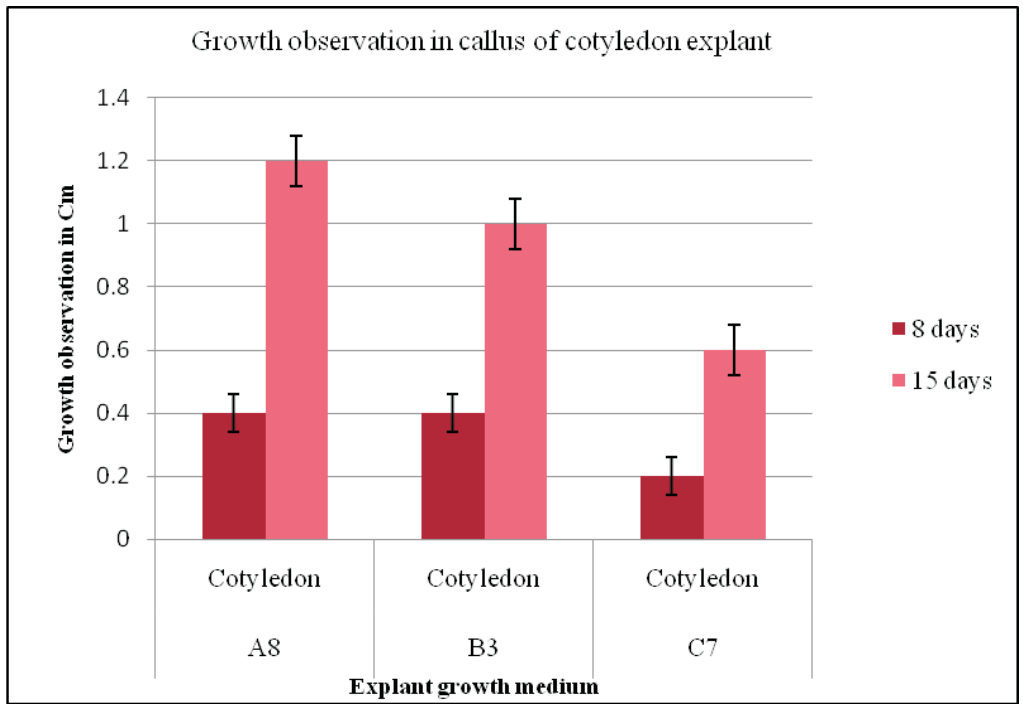


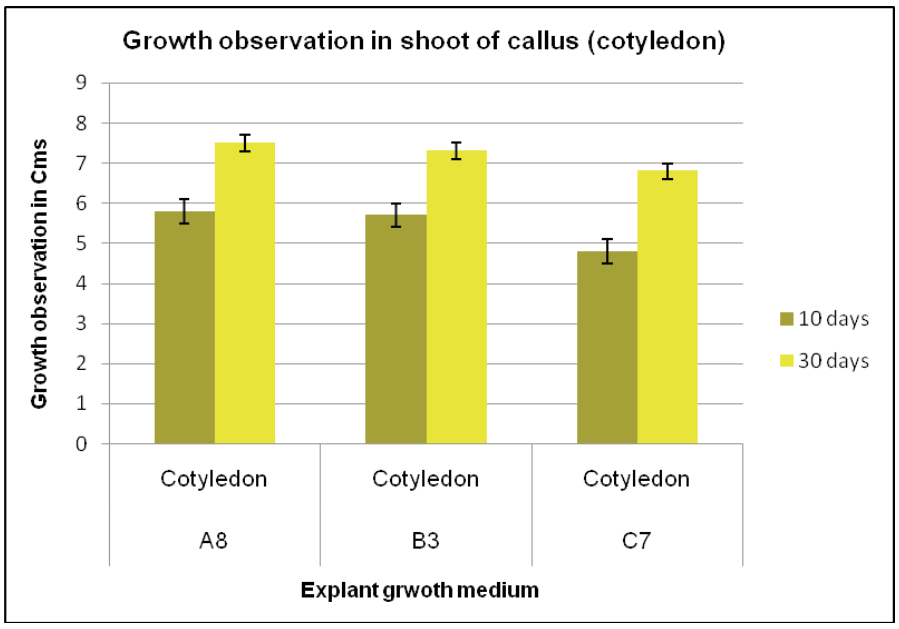
Table 2— Regeneration of shoot induction medium for Pigeon pea (*Cajanus cajan* L. Millsp.) *Co - 6*. Three combinations of medium responded well and A8 media responded maximum growth

Growth regulators (mg/l)	Explant type	Medium number	Growth observation (Cm) 10 days	20 days	30 days
A8 – 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 0.4 mg l^{-1} GA3	cotyledon	A8	5.8	6.9	7.5
		B3	5.7	6.7	7.3
		C7	4.8	5.9	6.8
B3 –2.0 mg l^{-1} BAP + 0.5 mg l^{-1} NAA					
C7 – 2.0 mg l^{-1} KIN+ 1.0 mg l^{-1} BAP					

A8, B3, C7 – Different concentration and different combination of MS media
BAP – Benzyl amino purine, NAA – Napathalene acetic acid, GA3– Gibberellic acid, KIN – Kinetin,

Table 2a— Statistical growth observation analysis in the shoot of cotyledon explant

OUTPUT / RESULT PANEL								
Treatments	n	Mean	SE	T-test	P-value	Result		
10 days	3	5.4333	0.3180	14.6995	0.0046	**		** - P < 0.01
30 days	3	7.2000	0.2082					* - P < 0.05
								NS - Not Significant



Cotyledons (Frankalin *et.al*.,1998; Geetha *et.al.,* 1999; Mohan and Krishnamurthy, 1998; Shiva Prakash *et.al*.,1994; Singh *et.al.*, 2004), which can be induced to differentiate into adventious callus and shoots that can be used for efficient production of transgenic Pigeon pea.

The combination of BAP and NAA increased the number of multiple shoots, while this confirms previous results, regeneration of pigeon pea resulted best with MS medium supplemented with 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA + 0.4 mg l^{-1} GA_3 (Dayal *et.al*., 2003; Eapen and George 1993; Eapen *et.al*.,1998; Frankalin *et.al*.,1998; Geetha *et.al.*, 1999; George and Eapen 1994: Mohan and Krishnamurthy, 1998; Shiva Prakash *et.al*.,1994; Singh *et.al.*, 2004).

In the present investigation the dose of BAP and NAA is known to be produced critical multiple shoot organogenesis. Therefore we compared the response of various concentrations of BAP and NAA. The maximum number of multiple shoot buds were obtained in MS + 2.0 mg l^{-1} BAP + 0.2 mg l^{-1} NAA and 0.4 mg l^{-1} GA_3.

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