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In vitro plant regeneration through immature seed culture of pigeon pea (*Cajanus cajan* [L.] Millsp.)

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Abstract — *An efficient protocol for multiple shoots induction and plantlet establishment was achieved through immature seed culture in Cajanus cajan cv ICP 26. Immature seeds of C. cajan were inoculated on MS/B_5 medium containing 10-30 gm/L of sucrose and also on MS medium supplemented with various concentrations of BAP (0.5-5.0 mg/L). Absolute percentage (100%) of seed germination was observed on MS medium supplemented with 25/30 gm/L sucrose. The maximum frequency of multiple shoos induction was observed on MS medium fortified with 2.0 mg/L BAP. The proliferated multiple shoots were elongated at 0.5 mg/L GA_3 in combination with 2.0 mg/L BAP. Profuse rhizogenesis was observed on MS medium fortified with 1.0 mg/L IBA. In vitro raised plantlets were acclimatized and successfully transplanted into the field with a survival rate of 70%. Regenerated plantlets are similar to donar plants. We have established the reproducible regeneration protocol in pigeon pea cv. ICP 26 through immature seed culture which can be used for in planta transformation to introduce gene(s) of interest in pigeon pea.*

Keyword — ***Cajanus cajan, Immature seed, Seed germination, Multiple shoos induction, Plantlet establishment.***

1. INTRODUCTION

Pigeon pea botanically known as *Cajanus cajan* (Linn.) Millsp is an important grain legume owing to its high protein content. The major centre of world production is undoubtedly India, where pigeon pea is the second most important pulse crop next to chickpea. In India, a vast majority of population depends on this grain legume for their protein requirement and in contrast, the world requirement is 20% (Sarangi *et al*., 1992). The world demand for grain legumes is increasing because of the increase in population in developing countries, for whom grain legumes cater to the protein requirement and also due to the growing trend towards plant based diets in developed countries. Genetic improvement of pigeon pea has not yet being achieved, even though various regeneration systems have been developed (George and Eapen 1994; Kumar *et al*., 1983; Mehta and Mohanram 1980; Prakash *et al*., 1984). This may be due to the incompatibility of existing regeneration protocols for transformation in *C. cajan* (Jacobsen 1993). Since conventional breeding has several constraints (Jaiwal and Gulati 1995), *in vitro* culture methods would serve as platform to produce *C. cajan* cultivars with desirable characters by using genetic engineering techniques. Legumes in general are recalcitrant to tissue culture and are highly genotype specific (Somers *et al*., 2003). Multiple shoos production and regeneration via organogenesis from different explants namely cotyledons, embryonic axes, cotyledonary nodes (Sarangi and Gleba 1991; Prakash *et al*., 1994; Franklin *et al*., 1998) and seedling petioles (Srinivasan *et al*., 2004) have been

reported. However, there is no report on *in vitro* regeneration, using the immature seed as an explant. Therefore in the present study, we report on the multiple shoots formation and plantlet establishment in *C. cajan* cv. ICP 26 for the first time through immature seed culture.

2. MATERIALS AND METHODS

Plant Material

The green immature pods of *C. cajan* cv ICP 26 were collected from the botanical garden, Department of Biotechnology, Kakatiya university, Warangal-T.S. These were surface sterilized with Tween-20 for 5 min, followed by 70% ethanol for 1 min, then sterilized with 0.1% (w/v) freshly prepared $HgCl_2$ for 4 min. Later they were rinsed with sterile double distilled water for 5-6 times. The sterilized immature pods were aseptically dissected with the help of forceps and scalpel to collect the immature seeds. These immature seeds were sterilized with 0.1% (w/v) $HgCl_2$ for 30 sec followed by rinsing with sterile double distilled water for 3-4 times and dried on a sterile tissue paper before placing on culture medium.

Culture medium and Culture conditions

The sterilized immature seeds were inoculated on half and full strength MS (Murashige and Skoog, 1962) and B_5 (Gamborg *et al.*, 1968) basal media with varying concentrations of sucrose (1-3%) and also on MS medium fortified with various concentrations (0.5-5.0 mg/L) of BAP/KIN. All the media were adjusted to P^H 5.8 with either 0.1N NaOH or 0.1N HCl before adding 0.8% (w/v) agar and autoclaved at 121^0C under 15ψ for 15-20 min. All the cultures were incubated at 25 ± 2^0C, 50 ± 5% humidity under 16h photoperiod provided by white-cool florescent tubes (3000 lux).

Multiple shoots proliferation and elongation

The explants with multiple shoots were subcultured on MS medium fortified with 2.0 mg/L BAP for further proliferation. For elongation, the micro-shoots were transferred onto MS medium supplemented with 0.5 mg/L GA_3+2.0 mg/L BAP.

In vitro rooting and plantlet establishment

The elongated micro-shoots were transferred onto MS medium augmented with various concentrations (0.5-2.5 mg/L) of auxins IAA/IBA alone for *in vitro* rooting. These *in vitro* rooted plantlets were taken out from culture vessels and washed with sterilized distilled water to remove remains of agar. Later these were shifted to plastic cups containing sterilized vermiculite: garden soil (1:1) and were covered with polythene bags for 3 weeks to maintain high relative humidity (80-85%). After 3 weeks they were shifted to plastic pots containing garden soil and kept in growth chamber for 3 weeks, for further acclimatization. These acclimatized plantlets were transferred into research field.

3. Statistical analysis

The data on days for germination, percentage of germination, average number of multiple shoots/explant, mean length of shoots, mean number of roots per micro-shoot and mean length of roots were recorded after 4 weeks of culture and the survival percentage of regenerates was also calculated. 20 replicates were maintained per each experiment and each experiment was repeated at least thrice. The data were statistically analyzed following the method of Pillai and Sinha (1968).

4. FIGURES AND TABLES

Table-1: Effect of type of medium and sucrose concentration on *in vitro* immature seed germination in *C. cajan* cv. ICP 26

Conc. Of sucrose (gm/L)	No. of days for seed germination	% of germination	Mean length of seedling (cms)±SE[a]
Full strength MS medium			

10	6	62	11.01±0.24
20	3	81	12.21±0.59
25	2	100	12.72±0.38
30	2	100	12.10±0.13
½ strength MS medium			
10	12	35	6.7±0.73
20	7	72	7.6±0.51
25	7	79	8.40±0.44
30	7	100	9.10±0.09
Full strength B_5 medium			
10	--	--	--
20	8	41	7.23±0.19
25	7	74	8.71±0.33
30	7	85	8.9±0.62
½ strength B_5 medium			
10	--	--	--
20	17	23	4.5±0.42
25	14	37	4.9±0.18
30	14	40	4.0±0.29

Table-2: Effect of BAP/KIN on *in vitro* multiple shoots induction from immature seeds in *C. cajan* cv. ICP 26

Conc. Of PGR (mg/L)	% of response	Mean number of shoots/explant (±SE[a])	Mean length of shoot (cms)±SE[a]
BAP			
0.5	50	2.0±0.28	3.5±0.17
1.0	68	2.6±0.22	2.7±0.21
2.0	95	6.2±0.45	2.1±0.44
3.0	78	4.1±0.19	2.3±0.01
4.0	65	2.2±0.32	1.7±0.36
5.0	43	1.2±0.10	1.2±0.53
KIN			
0.5	47	1.6±0.12	3.1±0.05
1.0	62	1.9±0.23	2.8±0.22
2.0	82	4.7±0.37	2.2±0.34
3.0	73	3.1±0.52	2.0±0.27
4.0	69	2.3±0.12	1.8±0.10
5.0	40	1.0±0.06	2.0±0.32

Table-3: Effect of GA_3+BAP on *in vitro* elongation of proliferated multiple shoots from immature seeds in *C. cajan* cv. ICP 26

Growth regulators (GA_3+BAP) (mg/L)	Average shoot length (cms)±SE[a]
0.1+2.0	1.7±0.43
0.2+2.0	2.3±0.11
0.3+2.0	2.9±0.32
0.4+2.0	3.2±0.19
0.5+2.0	4.5±0.25
0.6+2.0	3.6±0.09

Table-4: Effect of IBA/IAA on *in vitro* rooting of micro shoots developed through immature seed culture in *C. cajan* cv. ICP 26

Conc. of PGR (mg/L)	% of response	Average number of roots/ shoot (± SE[a])	Mean length of roots (cms) (± SE[a])
IBA			
MSO	Callus	Callus	Callus
0.5	69	3.1±0.11	2.11±0.04
1.0	100	6.08±0.9	4.14±0.31
1.5	93	5.1±0.07	3.11±0.17
2.0	89	4.1±0.18	3.08±0.34
2.5	85	4.0±0.15	2.29±0.19
IAA			
0.5	49	1.9±0.10	1.71±0.24
1.0	97	4.1±0.19	3.51±0.14
1.5	92	3.9±0.25	3.27±0.51
2.0	63	2.1±0.31	3.16±0.43
2.5	59	1.8±0.24	2.42±0.04

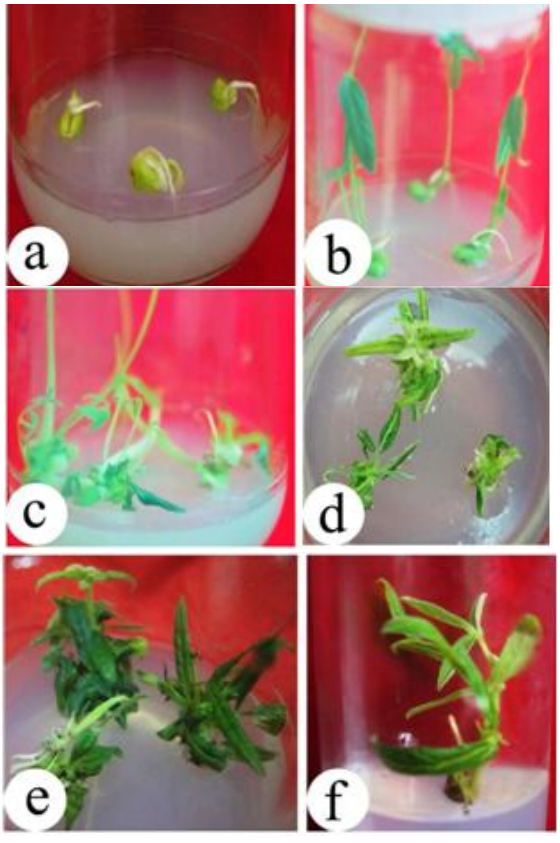

Fig.1 a-h: Effect of PGRs on *in vitro* multiple shoots induction from immature seeds in *C.cajan* cv. ICP 26. **a)** Initiation of germination from immature seed on MSO; **b)** Seed germination on MSO medium after 3 weeks of inoculation; **c)** Multiple shoots induction from immature seed on MS+2.0 mg/L BAP; **d-e)** Proliferation of multiple shoots on the same; **f)** Elongation of multiple shoots on MS+0.5 mg/L GA_3+2 mg/L BAP; **g)** Profuse rooting on MS + 1.0mg/L IBA; **h)** Acclimatization of regenerated plant.

5. RESULTS AND DISCUSSION

Immature seeds of *C. cajan* cv. ICP 26 were cultured on MS/B_5 medium supplemented with various concentrations (10-30 gm/L) of sucrose (Table-1). Early germination of seeds was found on full strength MS medium containing 25/30 gm/L sucrose. Absolute percentage of germination was also recorded on the same medium (Fig. 1a-b), whereas on half-strength MS medium 100% germination was observed only with 30 gm/L sucrose. Less percentage of germination with more number of days for germination was observed on full strength B_5 medium. Very poor germination percentage, seedling growth and late germination were recorded on half-strength B_5 medium in pigeon pea cv. ICP 26.

Multiple shoot induction

Immature seeds were cultured on MS medium supplemented with varying concentrations (0.5-5.0 mg/L) of BAP/KIN (Table-2). Multiple shoots were induced from immature seeds in all the concentrations of BAP/KIN after 2-3 weeks of culture. Maximum number of multiple shoots per explant was observed at 2.0 mg/L BAP (6.2±0.45) (Fig. 1c-e) followed by 2.0 mg/L KIN (4.7±0.37). With the increase in the concentration of BAP, there is a gradual decrease in the number of shoots/explant. Of the two cytokinins used, BAP induced maximum number of multiple shoots compared to kinetin in pigeon pea cv. ICP 26. BAP is more effective than KIN to trigger the synthesis of endogenous cytokinin in the present study (Vankova *et al*., 1991). Direct multiple shoots were induced on MS medium supplemented with BAP from germinating seeds of Walnut (Roberto Rodriguez, 1982) and Hazelnut (Rodriguez *et al*., 1989). A similar result was also recorded on the multiple shoot induction and plant regeneration on BAP from seed explants of chick pea (Polisetty *et al*., 1997). Direct multiple shoots were induced on MS medium containing BAP from germinating seeds of *Solanum surattense* (Archana *et al*., 2012). *In vitro* raised multiple shoots of *C. cajan* were elongated on MS medium fortified with 0.5 mg/L GA_3 in combination with 2.0 mg/L BAP (Table 3; Fig. 1f).

Plantlet establishment

The elongated micro-shoots were transferred onto MS medium fortified with different concentrations of IAA/IBA (0.5-2.5 mg/L). Roots were induced from the micro-shoots cultured in all the concentrations of IAA/IBA used (Table 4). Profuse rhizogenesis (6.08±0.9) was recorded at 1.0 mg/L IBA (Fig. 1g), followed by 1.0 mg/L IAA. Our results were similar to that of Mohan and Krishnamurthy (1998) that 1.0 mg/l IBA induced maximum percentage of rooting in *C. cajan*. While, Eapen *et al.* (1998) have reported that 1 mg/l NAA induced maximum percentage (90%) of rooting in pigeon pea in contrast to our results. The *in vitro* rooted plantlets were shifted to plastic pots containing vermiculite: soil (1:1) and acclimatized in walk-in-chamber for 4 weeks (Fig. 1h). Later they were shifted to earthenware pots containing garden soil and maintained in the green house. After 4 weeks,

they were shifted in to research field. The survival percentage of the regenerated plants was found to be 70% and the plants were similar to parental plant by showing normal morphology, flowering and fruiting. In the present investigation, the protocol developed is useful for *in planta* transformation in *C. cajan* cv. ICP 26 similar to the protocol developed by Yasmeen *et al.* (2009) in Tomato and Keshamma *et al.* (2008) in Cotton by using *Agrobacterium tumefaecians* mediated genetic transformation.

6. CONCLUSION

In vitro immature seed germination was found maximum (100%) on MS medium with 25 and 30 gm/L sucrose. Maximum number of multiple shoots per explant was observed on MS medium fortified with 2.0 mg/L BAP. The present work demonstrates that the multiple shoots could be developed directly from immature seeds of *C. cajan* within a short period at higher frequency. Immature seeds cultured in the presence of 2.0 mg/L BAP for consecutive subcultures enhanced the regeneration frequency significantly. This protocol is simple, reliable and reproducible and would be more suitable to introduce gene (s) of interest into the genome of red gram via *in planta* transformation.

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