International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.86

Agrobacterium mediated transformation of mature embryonl axes of three Pigeonpea genotypes *(Cajanus cajan* L.Millsp.)

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Abstract:

Pigeonpea is an important grain legume, however the genetic transformation of pigeonpea has been very challenging to date. In the present study, we describe a simple protocol for transformation of mature embryonal axis of three pigeon pea genotypes viz., LRG 30,ICPL 87, ICPL 85063.Agrobacterium mediated transformation was carried out using At 037 strain of LBA 4404 carrying Kanamycin antibiotic selection marker and GUS reporter gene. The percentage of transformants obtained were more in four days cocultivation than in two days cocultivationn. Four days co-cultivation exposed more number of meristematic cells to bacterial infection. The percentage of transformation was also more at cotyledonary node region than radical and plumule. Among three genotypes ICPL 87 showed highest frequency of transformation in both two days and four days cocultivation.

Keywords: Pigeonpea, mature embryonal axis, cotyledonary node, *Agrobacterium*, transformation.

Introduction:

Pigeonpea is one of the important protein yielding grain legumes of semi-arid tropics in India. Due to its importance as a pulse crop, pigeonpea improvement in terms of protein quality, stress tolerance and pest tolerance is necessary. Insect pest resistance has largely been introgressed in many crops, including pulses, by using the *cry* genes from *Bacillus thuringiensis.* However, many plant genes like lectins, protein inhibitors, etc. are also available that impart tolerance to insect pests and can be used for developing insect-tolerant plants (Rathore *et. al.* 2018). Developing resistant varieties is possible by conventional interspecific hybridization or introduction of resistant genes through genetic engineering. For production of transgenics, successful regeneration is important. In pigeonpea, there are several successful reports using different varieties and different explants. Regeneration was obtained via callus cultures (Kumar *et al.,* 1983), direct differentiation from leaf discs (Eapen and George, 1993; Geetha *et al.,* 1998), cotyledons (Geetha *et al.,* 1998, Mehta and Mohan Ram, 1980), cotyledonary nodes (Geetha *et al.,* 1998; Prakash *et al.,* 1994). somatic embryogenesis (Patel *et al.,* 1994; Kulkarni and Krishnamurthy, 1989) was also reported. Protoplast regeneration upto callus stage has also been reported (Sarangi *et al.,* 1992; Sagare *et al.*, 1997). Regeneration through multiple shoot induction (Geetha *et al.*, 1998; Franklin *et al.*, 1998, 2000; Niveditha *et al.*,2012)

Received: 8 October Revised: 15 October Accepted: 20 October
Index in Cosmos
November 2018 Volume 8 Number 11
UGC Approved Journal

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Agrobacterium is capable of infecting intact cells and introduces one to several copies of the transformed DNA into the plant genome. There are previous reports regarding transformation of pigeonpea by using different explants such as (Rathore & Lakshmi Chand,1997); callus from embryonal axes (Sagare *et al*.,1997); embryo axis (Geetha *et al*., 1999; Lawrence and Koundal, 2001; Satyavathi et al., 2003, Prasad *et. al*., 2004; Sawardekar *et. al,* 2012, Parekh *et. al.,* 2014, Ghosh *et. al.,* 2017), cotyledonary nodes (Geetha *et al.,* 1999; Prasad et al., 2004) and leaf disks (Arundhati, 1999), leaf segments (Yadav and Padmaja , 2003)., embryonal segments and cotyledons (Surekha *et al.,* 2005, 2007), three days old decapitated seedlings of pigeonpea cultivar ICPL 87119 were pricked at plumular meristem (Ganguly *et al.,* 2018.); differentiated and meristematic cells in pricked embryo axes (Kaur *et.al*., 2016).

Present study reported transformation of gus reporter gene in the mature embryonal axes of three pigeonpea genotypes.

Materials and methods:

Seeds of different pigeonpea cultivars viz LRG 30, ICPL 87 and ICPL 85063 were obtained from market yard, Guntur. At 037 strain of Agrobacterium LBA 4404 carrying the plasmid pBI121 carrying Kanamycin antibiotic selection marker and GUS reporter gene was used for transformation studies.

All the explants were inoculated on MSB2 medium (MS medium supplemented with 2mgL-^{1} BAP with 3% sucrose and 0.8% agar and maintained at a photoperiod of 16 h light and 8 h dark at 25±2°C for regeneration experiments [Murashige and Skoog,, 1962]. For transformation, the mature embryonal axes were excised from seeds that were surface sterilized with 0.1% mercuric chloride and soaked overnight. The bacterial cultures were grown overnight at 28°C in liquid LB medium (1% tryptone, 0.5% yeast extract and 1% sodium chloride at pH 7.0) containing 75 mg L^{-1} kanamycin and Rifampicin (10 mgL^{-1}).The Agrobacterium cells of 0.8 O.D was used for cocultivation.. The explants were inoculated for infection in 10 mL of bacterial suspension for 10 min. After 10 min incubation in bacterial suspension, the explants were blot-dried on sterile filter paper and cocultivated 3 days under dark at 25±2°C. After cocultivation for 2-4 days, explants were washed thoroughly and blot-dried on sterile filter paper and cultured for a week on MSB2 containing 75 mg L^{-1} kanamycin and 400 mg L^{-1} cefotaxime. Half of the explants were used for histochemical assay according to protocol (Hiei et al., 1994) and rest half were left for further growth.

Results :

The embryonal axes of three genotypes in 2- 4 days co cultivation showed different frequencies of gus positives. In two day cocultivation the percentage of transformants were ranged from 37.5 to 45.4 (Table 1), where as in four days cocultivation the transformants ranged from 75 to 88.88 (Table 2). In two days of cocultivation none of them showed GUS positive reaction in plume region, but in four days of cocultivation two genotypes ICPL87, ICPL85063 showed GUS positive reaction in the plumule region also. In two days and four days of co-cultivation all the three

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genotypes showed GUS positive reaction at the cotyledonary node (Plate 2; b, c, d, e & Table 1). Only one genotype LRG 30 showed GUS positive reaction at radical in two days cocultivation. Controls showed negative response to GUS activity (Plate 1; a). Among the three genotypes highest frequency of transformation is observed in ICPL 87 in both two days and four days co-cultivation with 45.5% and 88.8% respectively, followed by ICPL 85063 with 38.8 and 78.57. LRG 30 genotype showed least frequency of transformation in both two days and four days co-cultivation with 37.5 and 75.

Table 1. Response of different merestimatic regions of the embryonal axes to Agrobacterium mediated transformation after two days of cocultivation.

Genotype	Number of Explants	Explants showing GUS positive Response		Part of embryonal axis showing GUS positive (%)		
		No	%	Plumule	Cotyledonary node	Radicle
LRG-30	16	6	37.5	Nil	31.25	6.25
ICPL-87	22	10	45.4	Nil	45.4	Nil
ICPL-85063	18	7	38.88	Nil	38.88	Nil

Table 2. Response of different merestimatic regions of the embryonal axes to Agrobacterium mediated transformation after four days of cocultivation.

Genotype	Number of Explants	Explants showing GUS positive Response		Part of embryonal axis showing GUS positive (%)		
		No	%	Plumule	Cotyledonary node	Radicle
LRG-30	12	9	75	Nil	75	42
ICPL-87	18	16	88.88	40	88.88	40
ICPL-85063	14	11	78.57	46	78.57	47

Discussion:

Genotypes, time of exposure to bacterial inoculums and other factors influenced the efficiency of transformation and GUS positive frequencies. The percentage of transformants obtained was more in four days cocultivation (Table.2) than in two days cocultivation (Table.1). Four

Received: 8 October Revised: 15 October Accepted: 20 October
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days cocultivation exposed more number of meristematic cells to bacterial infection. The number of cells in division at the time of transformation enhances the percentage of transformants. Development of an effective and reproducible in vitro regeneration protocol via multiple shoot induction was achieved using immature embryonal axes of pigeon pea genotype ICPL-87 (Niveditha *et. al*., 2012). Three pigeon pea genotypes showed highest frequency of transformation in four days co-cultivation than in two day co-cultivation. It is due to four days co-cultivation exposed more meristematic cells than two day co cultivation.

The present study reported the percentage of transformation at plumule, cotyledonary node and radical parts of mature embryonal axis in three genotypes. Transformation percentage was more at cotyledonary node region than radical and plumule. Higher percentage of cotyledonary node region to GUS positive reaction may be due to wounding. Among the three genotypes studied ICPL 87 showed highest frequency of transformation in both two days and four days co cultivation and also showed highest frequency from nodal region when compared to radical and plumule region. It is essential to develop and use insect-tolerant transgenics that have been developed by following the bio safety regulations, are high yielding, fit into popular cropping systems, and are expected to be remunerative to the stakeholders (Rathore *et. al*. 2018).

Conclusion:

Successful regeneration and transformation of pigeon pea is an important step in crop improvement. Previous reports of effective in vitro regeneration via multiple shoot induction from immature embryos protocol in ICPL 87 (Niveditha *et.al.,* 2012) and highest frequency of transformation at cotyledonary nodal region, it can be used to develop transgenic pigeon pea.

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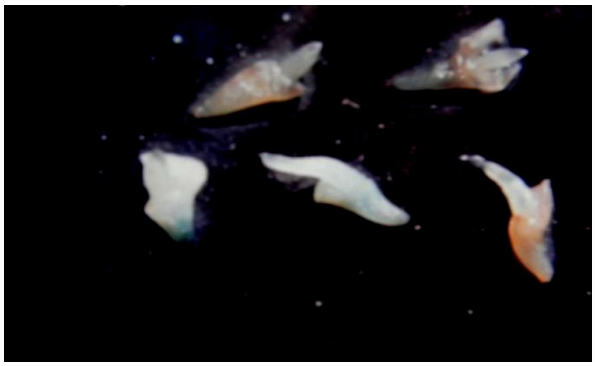
Received: 8 October Revised: 15 October Accepted: 20 October
Index in Cosmos
November 2018 Volume 8 Number 11
UGC Approved Journal

International journal of basic and applied research
www.pragatipublication.com
ISSN 2249-3352 (P) 2278-0505 (E)
Cosmos Impact Factor-5.86

Plate 1 a. control –embryonal axes of pigeon pea genotypes

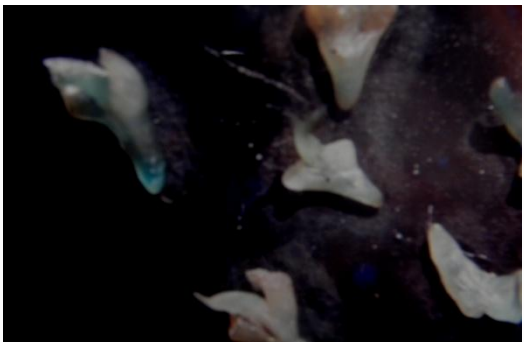
Plate 2 GUS positive embryonal axes of pigeonpea genotypes



b. LRG-30

c.ICPL-87

d. ICPL-85063



d.ICPL-87

Received: 8 October Revised: 15 October Accepted: 20 October
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November 2018 Volume 8 Number 11
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