Journal of Pharmacognosy and Phytochemistry

Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
www.phytojournal.com
JPP 2020; 9(5): 1500-1503
Received: 25-07-2020
Accepted: 27-08-2020

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Optimization of kanamycin concentration for screening of primary transformants in pigeonpea

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DOI: https://doi.org/10.22271/phyto.2020.v9.i5u.12551

Abstract
Pigeonpea is an important pulse crop which is widely cultivated throughout the tropical and sub-tropical regions. It is the major source of protein for the people with vegetarian diet. Major constraint in pigeonpea is the attack by pod borer. Conventional plant breeding techniques have limitations to develop resistance to the pest due to non-availability of resistance in the available germplasm. Therefore genetic engineering tools can be employed to transfer foreign genes into pigeonpea to impart pod borer resistance. Development of a reliable regeneration system coupled with efficient transformation has been a major limitation in developing transgenic pigeonpea with useful genes. *In planta* is an alternative approach to obtain transformed plants in short duration without involving tissue culture regeneration. However major limitations with this approach is screen more number of seeds to identify putative transformants which is time consuming and expensive. Therefore the present investigation was carried out to optimize the concentration of kanamycin for quick identification of putative transformants carrying *cry*1Ac and *npt* II using sand based assay. Seeds of Asha and BSMR736 were soaked in different concentrations of kanamycin (10-100, 200, 300, 400 and 500 ppm) for 5hrs and later sown in sand based medium. There was no effect of kanamycin on seedling growth up to 60 ppm and decrease in survival percentage in both the genotypes beyond this concentration. There was significant differences in growth of the seedlings in terms of shoot and root length at 100 ppm and beyond. Therefore this concentration was used to screen seeds of 32 primary transformants carrying *cry*1Ac and *npt* II and subjected to gene specific PCR analysis to confirm the usability of simple screening on kanamycin based sand medium for the identification of true transgenic plants. However some discrepancies between the growth inhibition and PCR results were observed.

Keywords: Kanamycin concentration, primary transformants, pigeonpea

Introduction
Pigeonpea is the important legume that belongs to the family *Fabaceae*. Pigeonpea is the excellent source of protein vitamins and minerals. A lepidopteron insect, the legume pod borer or *Helicoverpa armigera* is the most serious and widespread pest of pigeonpea. Since genes conferring resistance to pod borer is not available in the primary gene pool of pigeonpea therefore application of transgenic technology has been considered as one of the feasible options to overcome the problem. *In planta* method of plant transformation is quick, less expensive and good alternative to the tissue culture protocol but limitation is that screening of large number of plants to identify putative transformants. Most of the Genetic transformation methods consist of selectable marker genes for quick identification of putative transformants from non transformants. Most commonly transformation experiments in pigeonpea used neomycine phosphortransferase (*npt* II) gene as selectable markers (Krishna *et al.*, 2011) [7]. The neomycin phosphotransferase II (*npt* II) gene is one of the most commonly used selective maker (Flavell *et al.*, 1992) [4]. Kanamycin antibiotic binds to the active site of ribosomes to intracellular organelles and inhibit protein synthesis which leads to the degradation of chlorophyll and the inspection of browning and white spot on the plant morphologically leads to drying of plant tissues (Duan *et al.*, 2009) [2]. These antibiotics inhibit the growth of plant cells by binding to the 30S ribosomal subunit, there by inhibiting initiation of plastid translation. Plant cells transformed with the npt II gene detoxify the antibiotics in the selection medium (Kapaun *et al.*, 1999) [6]. Kanamycin was demonstrated to be the most appropriate selective agent (Curtis *et al.*, 1995) but the concentration is species specific. Species namely *Cicer arietinum*, *Lycopersicum esculentum* (Rajesh *et al.*, 2004) [8], *Brassica napus* (Malony *et al.*, 1989) were selected at low concentration (15-100 mg/lit) whereas *Beta vulgaris* needed relatively high concentration of kanamycin (400 mg/lit).

Materials and Methods

Pigeonpea varieties *viz.*, ICPL 87119 (Asha), a moderately resistant variety for fusarium wilt, sterility mosaic disease and BSMR 736, a sterility mosaic disease resistant variety were used for the present investigation. Seeds of these cultivars were obtained from Agriculture Research Station (ARS), Gulburga. *Agrobacterium tumefaciens* strain LBA4404 carrying *cry*1Ac was used in the present study. The pBinBt3 carrying *cry*1Ac was procured from Dr. P. Anand Kumar National Research Centre (NRC) for plant Biotechnology, IARI, New Dehli as a part of ICAR-NFBSFARA funded pulse project. The *in planta* transformation method reported by Ramu *et al.* (2011) [9] for pigeonpea using embryonic axis as a source of explants with minor modifications were used to increase the transformation efficiency. Seeds of both Asha and BSMR736 were taken for this study. Initially seeds were washed thoroughly with double distilled water three times and soaked in different concentrations of kanamycin solution (10-500 ppm) for 5 hours. Along with this untreated seeds of respective genotypes were used as control. Seeds were sown in three replications in plastic cups (5 seeds/replications) containing sterile sand. Based on growth of seedlings 100 ppm of kanamycin was chosen to screen seeds from primary transformants. Progeny of 32 primary transformants were subjected for kanamycin screening. DNA was isolated from normal plants and plants showing stunted growth to confirm the presence of transgene through PCR. Genomic DNA was isolated from young leaves of progenies of primary transformants following the modified protocol of Edwards *et al.* (1991) [3] and Agbagwa *et al.* (2012) [1]. The quality and quantity of DNA checked by agarose gel electrophoresis and nanodrop spectrophotometer. The final concentration for PCR was made to 100 ng/µl. PCR components were nuclease free water: 13µl**,** taq buffer with Mg^{2+} (10X): 2 µl,dNTP's(2mM): 2µl**,** Primers (forwad and reverse: 5 pmol): 2 µl and DNA (100ng):1µl.The total volume prepared was 20 µl. PCR amplification consisted of pre-denaturation at 94 °C for 5 min; denaturation at 94 °C for 1min, annealing temp 55 °C for 30s; extension at 72 °C for 2 min, repetition for 32 cycles; and final extension at 72 °C for 10 min. The PCR products were separated on 1.2% agarose gel.

Results and Discussion

Generation of large number of primary transformants is very essential in order to obtain transgenic events with relatively high expression of transgenes. This necessitates the need for a high throughput yet stringent selectable marker-based screening for the initial identification of putative transformants. Significant differences were observed among the different concentrations (0 to 500 ppm) of kanamycin used. Plants were normal and did not show any death in control. Among the various concentrations of kanamycin used beyond 300 ppm seeds did not germinate at all. Both Asha and BSMR736 showed 100 per cent survival up to 60 ppm of kanamycin and the survivability decreased 70 ppm and above concentrations (Table 1). Also phenotypic differences in terms of reduction in root and shoot lengths were observed among the survived plants with the increasing concentration of kanamycin (Plate 1A, Fig.1). The differences were clear cut at 100 ppm concentration. Hence this concentration was used further to quickly screen the progeny of primary transformants (Table 2, Plate 1B). Of the progeny of 32 primary transformants screened total number of observed normal plants were 55 but only 3 plants were found positive in PCR screening (Plate 2). Interestingly out of 465 short plants 6 plants were found as PCR positive. Similarly Ganguly *et al.* (2018) [5] used 100 ppm kanamycin concentration to check segregation pattern of T_1 seeds and observed 12 Cry1Ac and 15 Cry2Aa T_1 events exhibit segregation of 3:1 ratio (KanR : KanS). Singh *et al.* (2018) [10] used 70 ppm kanamycin concentration for quartz sand-based screening of a large number of T_1 generation seedlings, resulted in the identification of 59 *cry*2Aa putative transformants.

Table 1: Optimization of kanamycin concentration for identification of putative transformants using sand as a medium for growth

Kanamycin concentration (ppm)	Survival percentage (%)		Shoot length (cm)		Root length (cm)		Per cent reduction					
							Survival		Shoot length		Root length	
	Asha	BSMR 736	Asha	BSMR 736	Asha	BSMR 736	Asha	BSMR 736	Asha	BSMR 736	Asha	BSMR 736
Control	100.00	100.00	16.18	11.20	6.27	7.98	0.00	0.00	0.00	0.00	0.00	0.00
10	100.00	100.00	15.55	11.07	5.97	7.89	0.00	0.00	3.88	1.12	2.94	0.68
20	100.00	100.00	15.15	10.21	4.53	6.74	0.00	0.00	6.33	8.82	26.18	11.07
30	100.00	100.00	15.15	10.56	4.68	6.82	0.00	0.00	6.33	5.79	23.74	11.48
40	100.00	100.00	15.15	10.33	4.71	7.24	0.00	0.00	6.33	7.77	23.17	7.26
50	100.00	100.00	13.10	10.27	2.83	4.59	0.00	0.00	18.98	8.30	54.00	28.34
60	100.00	100.00	11.09	11.13	2.86	4.85	0.00	0.00	31.42	0.68	53.34	26.73
70	86.67	86.67	11.20	10.07	2.78	4.66	13.33	13.33	30.81	10.10	54.66	28.08
80	86.67	86.67	10.20	9.17	1.87	3.65	13.33	13.33	36.96	18.13	69.63	36.54
90	80.00	80.00	10.00	9.29	2.03	3.75	20.00	20.00	38.24	17.10	67.06	35.45
100	73.33	66.67	6.96	7.10	0.66	1.64	26.67	33.33	57.02	36.60	89.23	52.75
200	73.33	66.67	6.97	6.27	0.64	0.77	26.67	33.33	58.28	47.25	89.25	89.08
300	66.67	46.67	5.68	4.80	0.49	0.63	33.33	53.33	65.13	58.41	91.89	91.09
400	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
500	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
SED	6.41		0.32		0.18		1.43		1.12		1.46	
CD(5%)	12.85		0.64		0.37		2.85		2.23		2.92	
CD(1%)	17.13		0.85		0.49		3.78		2.96		3.88	
CV	9.05		3.90		6.50		8.12		4.35		4.11	

Table 2: Screening of progeny of primary transformants using 100ppm kanamycin

Sl. No.	Primary transformants	Number of seeds sown	Number of plant survived	Number of normal plants	Number of PCR positive normal plants	Number of short plants	Number of PCR positive short plants
1	1	45	24	1	1	23	0
2	2	45	19	1	0	18	0
3	3	45	13	1	0	12	0
4	4	45	10	2	0	8	0
5	5	45	12	1	0	11	0
6	7	45	18	0	0	18	0
7	8	45	20	0	0	20	0
8	10	45	17	2	1	15	0
9	12	45	16	0	0	16	0
10	13	45	11	3	0	8	1
11	15	45	18	0	0	18	0
12	16	45	18	2	0	16	0
13	18	45	21	5	1	16	0
14	20	45	18	0	0	18	0
15	21	45	17	0	0	17	2
16	24	45	18	0	0	18	0
17	27	45	20	1	0	19	0
18	29	45	22	0	0	22	0
19	30	45	12	4	0	8	1
20	31	45	19	0	0	19	0
21	34	30	7	1	0	6	0
22	35	45	18	0	0	18	0
23	42	45	16	0	0	16	0
24	44	45	23	3	0	20	0
25	47	45	14	1	0	13	0
26	50	45	20	9	0	11	0
27	52	45	23	10	0	13	0
28	61	45	17	5	0	12	0
29	64	30	7	0	0	7	0
30	68	30	2	2	0	0	0
31	69	45	15	0	0	15	0
32	70	45	16	2	0	14	2
Total	32	1395	521	56	3	465	6

Plate 1: Standardization of kanamycin concentration for the identification of putative transformants in pigeonpea.
A: Kanamycin concentrations, 1:100 ppm, 2:200 ppm, 3:300 ppm, 4: 400 ppm, 5: 500 ppm, C: control
B: Screening of progeny of primary transformants using kanamycin at 100 ppm.

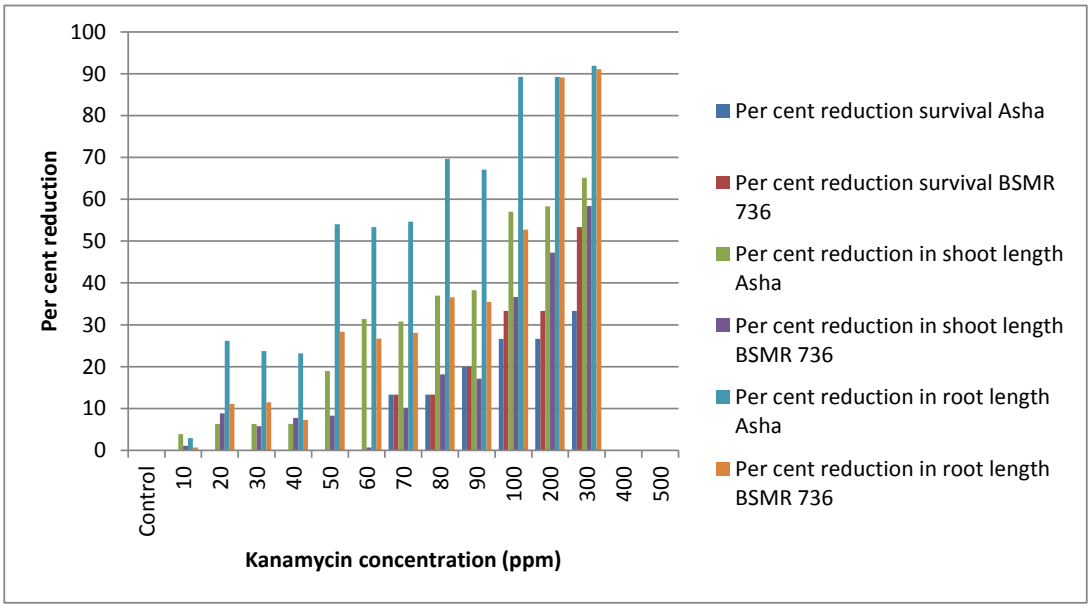

Fig 1: Effect of Kanamy on Plant Survival and growth

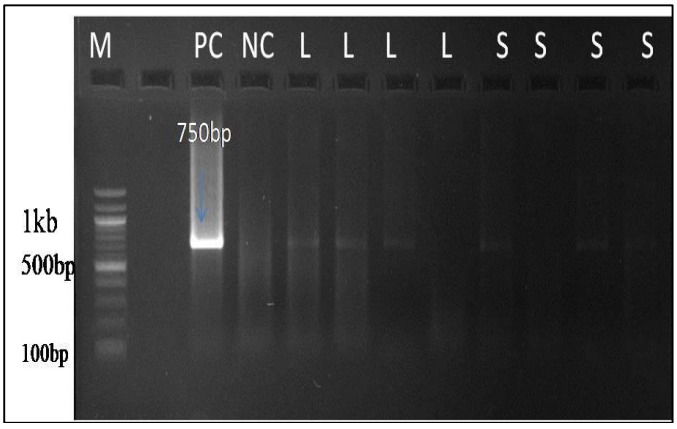

Plate 2: PCR analysis of the Kanamycin treated plants, PC: positive control, NC: negative control, L: long, S: short

Conclusion

For quick identification of transgenics in the T_2 generation, optimization of kanamycin concentration for sand based screening was attempted and kanamycin concentration of 100 ppm was found to give phenotypically differentiable progenies, however PCR confirmation test did not show the direct correlation between the presence of the transgene and the phenotype. So it needs to be further standardized.

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