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Determination of Threshold Level of Kanamycin in Pigeon Pea Gene Transformation

Research article

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Abstract

Pigeon pea (Cajanaus cajan) is an important legume crop of Afro-asian countries cultivated as an important source of dietary protein. Annual pigeon pea losses due to biotic and abiotic stress have been increasing worldwide. Conventional plant breeding techniques have limitations to troubleshoot this above revealed difficulty in the course of developing resistance. This can be solved by enhancement of pigeon pea through genetic engineering in favor of transgenic plant development to build resistance against lepidopteron pest. This study was carried out to conclude the threshold concentration of antibiotics kanamycin in Pigeon pea crop towards transfer of unfamiliar gene in transgenic plant development. The effect of aminoglycoside antibiotic kanamycin was evaluated for its effects on mature embryo explant, for shoot development were evaluated through germination. On this basis, the practicable use of kanamycin as a selective agent in genetic transformation with neomycin phosphotransferase II gene as the selective marker was appraised. Kanamycin inhibited embryogenic growth, proliferation, as well as the initiation and development of embryonal axis sector.Percentage of germinated plants was recorded to study the effect of kanamycin on explant survival. Results analysis of variation in increase concentrations of kanamycin signify that there were significant differences between the concentrations at level of above 100 mg/lit were found to effectively inhibit the shoot development. The results of kanamycin effect reveals that the maximum germinated plants were seen in a treatments of control, 25 and 50 mg/lit. Kanamycin and the minimum regenerated plants were observed in concentrations of above 100 mg/lit kanamycin. The optimized parameters were comparable to the existing literature for Pigeon pea and other legume crops. Thus kanamycin concentration above 100 mg/lit could be used in transformation studies pertaining to Pigeon pea. Results demonstrated is compared to published cited literature in Pigeon pea and found that it was required high concentration of kanamycin for selection. Generally, result suggested that optimum lethal antibiotic kanamycin can be used for selection of transgenic tissues of Pigeon pea in genetic transformation programs.

Keywords: Pigeon pea; Embryo; Kanamycin; Gene transfer

Introduction

Pigeon pea (Cajanaus cajan) is a important legume crop in Leguminosae family, which is highly comprises of dietary proteins source, fiber and calories, growing under diverse climatic conditions of tropical to temperate zones[1]. Among the legume flowering plants pigeon pea is the largest cluster and acquire first position with diverse applications [2]. However, pigeon pea yields has turn down in last 25 years and the area of cultivation is instantly depleted due to abiotic and biotic stresses such as wide range of insect pests, water lodging, cold sensitivity during flowering, terminal drought during pod grain filling [3]. Fungal diseases such as Fusarium Leaf blight and wilt have been the most destroying pigeon pea diseases in India and oversea which are responsible for reduced yield and quality [4]. Conventional breeding techniques comprise of limited scope for improvement of quality characters in pigeon pea due to massive time consuming and laborious activities need to follow to plant breeders and require necessitate time for releasing new genotypes [5]. Compared to other classical breeding methods, gene transfer technique provides the prospect of integration of several foreign genes in plants for developing desired resistance against biotic and abiotic stresses [6].

Gene transformations methods consist of selectable marker gene, the aminoglycoside kanamycin, acting as a selective agent, which has been commonly applied in plant genetic transformation, this

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is because of kanamycin can kill plants of wild cells. The neomycin phosphotransferase II (npt II) gene is one of the most commonly used selective maker [7]. 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 drying of plant tissues [8]. 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 detoxifying the antibiotics in the selection medium [9].

Till date in Pigeon pea have been performed various experiments of genetic transformation which in them B- glucuronidase gene [10], hygromycin-phosphotransferase II [11] and neomycine phosphor transferase (NPT-II) gene use as selectable markers [12]. Kanamycin resistance is conferred by transgenic expression of neomycin phosphotransferase, the product of the NPT-II gene from the bacterial transposon Tn5. The enzyme neomycin phosphotransferase transfers a phosphate from ATP to the aminoglycoside and thereby inactivates it. The gene NPT-II was first established as a useful dominant selectable marker for plants [13].

Kanamycin has demonstrated to be the most widely appropriate selective agent [14] but the concentration is species specific. Species namely *Cicer arietinum*, *Lycopersicum esculentum* [15], *Brassica napus* [17]are selected at low concentration (15-100 mg/lit) whereas *Betal vulgaris* needs relatively high concentration of kanamycin (400 mg/lit).This reports demonstrated to determination of kanamycin threshold level for evaluating the potential of kanamycin for the selection of transformed pigeon pea cells at various developmental stage of transgenic putative plants through germination for gene transformation programs.

Materials and Methods

Explant preparation and culturing

Seeds of breeding pigeon pea line NTL-30 were collected from germplasm bank of Nirmal Seeds Pvt. Ltd Pachora, Jalgaon (M.S.) India. Seed were surface sterilized by washing with running tap water followed by treated with a 70% ethanol for 30 seconds. The seeds then treated with 0.1% $HgCl_2$ along with 0.1% of SDS for 3 minutes and thereafter wash 4 to 5 times with sterile distilled water. Seeds were incubated in conical flask filled with water for 24 hour on a rotary shaker at 50 rpm. Seeds were then transfer on sterile filter paper for dissecting seed coat and germinated on germination medium The medium was used MS half strength basal containing, 3% sucrose, 0.8% agar supplemented with 2 mg/L of BAP as plant growth regulator, The pH of the medium was adjusted to 5.8 and sterilized at 121^{0}C for 15 minute. The stock of kanamycin (100 mg/ml) was prepared by dissolving kanamycin (Himedia) in sterile distilled water and sterilized using sartorius 0.22 µm membrane. This stock solution was added to the cooled media (about 40 to 50 °C) after autoclaving, seeds were incubated for 10 days at room temperature (27 ± 1 °C) for 16 hours photoperiod.

Effect of Kanamycin on shoot Development

After the pre-culture stage, small germinated plants were placed on M.S. medium supplemented with 2 mg/lit BAP and different concentrations of kanamycin (0, 25, 50, 75,100,125,150,175 and 200 mg/lit) to study the effect of kanamycin on shoot development from embryo explants. Each experiment was repeated thrice maintaining three replications along with control in each experiment. Subculture explants on medium composed of different kanamycin after every ten day. The explants were incubated in a growth room at 16:8 h (light/dark) photoperiod at 24 ± 2 °C with cool white fluorescent light intensity of approximately 20,000 Lux. The percentage of mortality and germinated plants in each tissue cultured bottled were recorded to determine the effect of kanamycin .Plantlets which settled healthy were transferred to plastic bag containing soil and vermicompost were irrigated with water or half strength Hoagland solution .

Data collection and analysis

Embryo developmental growth and germination were recorded after five days of culture evaluation for observations of mottled green sectors on leaves, burnt symptoms and leaf survival. For each treatment 6 plants were recorded and averaged was calculated, after culture of 25 days total number of germinated embryo developmental stages was recorded. The experiment was arranged completely randomized design with three replications. Six explants were culture in each tissue culture glass bottle. The data were analyzed using

Figure 1: Effect of kanamycin higher dose.

Table 1: Effect of Kanamycin on % on germination in Pigeon pea.

Sr. no	Medium Name	BAP mg/lit	Kanamycin(mg/lit	% of germination
1	MSC	2	control	100
2	MSKA	2	25	100
3	MSKB	2	50	90
4	MSKC	2	75	80
5	MSKD	2	100	50
6	MSKE	2	125	30
7	MSKF	2	150	20
8	MSKG	2	175	10
9	MSKH	2	200	5

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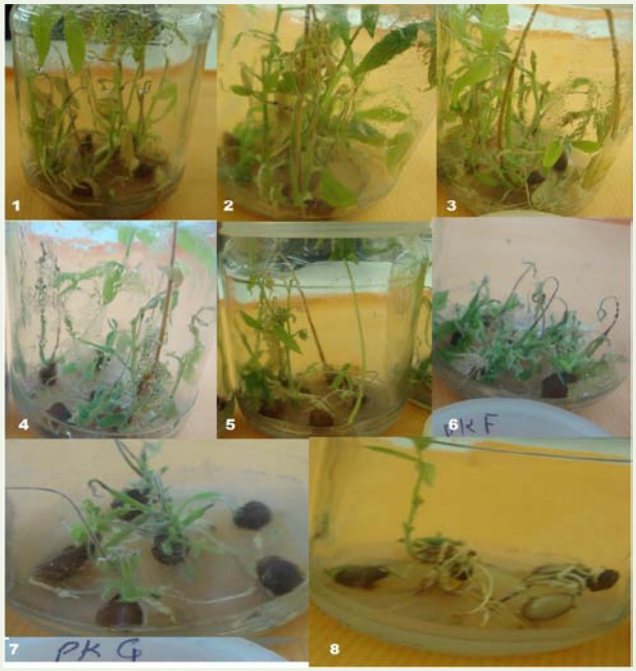

Figure 2: Significant shoot development on kanamycin gradiant 1(25 mg/lit), 2(50 mg/lit), 3(75 17mg/lit),4(100 mg/lit),5(125 mg/lit),6(150 mg/lit),7(25 mg/lit) and 8(200 mg/lit.).

statastical software and the least significant difference method was used to detect difference among the treatment.

Results and Discussion

Antibiotic Kanamycin delayed the start of embryo growth in absence of kanamycin medium, the preliminary signs of initiation shown after third days of culture and germination on seventh day of culture. Medium supplemented with kanamycin commence delay and shown after seventh days and emerge rapidly at 10th days of culture. Germination ratio was not affected by kanamycin at 0 to 100 mg/lit, and was introverted totally above 100 mg/lit or higher (Figure 1).

However the germination was induced on medium increment with (25 to 200 mg/lit) of kanamycin, most of the embryo not develops further and turning brown and dead after 15 days of culture. Results of analysis and percentage of germinated plants under different concentrations of kanamycin indicated that there were significant differences between the concentrations 100 and 125 mg/lit. (Table 1).

The results of mean relationship in different concentrations of kanamycin designate significant illustrious with each set of kanamycin (Figure 2).

Two classes of antibiotics are use in genetic transformation method. One is a selectable agent (e.g. kanamycin, hygromycin) used to select transformants, and another (carbenicillin and cefotaxime) kills Agrobacterium. Whereas several published literature on transgenic plants development and the effect of antibiotics on plant tissue has infrequently been studied [18]. Kanamycin was widely use as selectable agent in plant transformation, even though kanamycin is widely used in Pigeon pea transformation, the optimal kanamycin selectable concentration and the activity of kanamycin at different stages of the pigeon pea regeneration process have been described by different user. In order to determine the concentration of the antibiotic kanamycin competent of tolerate the growth of pigeon pea, we tested different concentrations, and the lethal concentrations are described above.

The studies demonstrate that lesser amount of kanamycin concentrations (25, 50 to 75 mg/lit) are employed in selection procedure in various plants [19]. As the results of the study confirm lesser kanamycin concentrations have not been effectual for selecting pigeon pea plants and the tissue getaway will be occurred. Preferred concentration of kanamycin reported earlier for Tomato, Brinjal as a little more than the usual for other plants and nearly 100 mg/lit [20]. The studies showed that after the passage of two weeks symptoms such as yellowness and burnt were observed in the cultivated explants in higher kanamycin concentrations including 125 to 200 mg/lit. Results suggested that germinated plants obtained from embryo explants under 125 to 200 mg/lit kanamycin concentrations showed symptoms of yellowness, burnt, and growth discontinue.

The severity of the impact of the mentioned concentrations was in such a way that all the plants in them were completely turned yellow and get burnt. The results of the experiment showed that the maximum germinated plants were observed in the treatments of control, 25 and 50 mg/lit kanamycin and the minimum regenerated plants were observed in concentrations of 125 to 200 mg/lit kanamycin.

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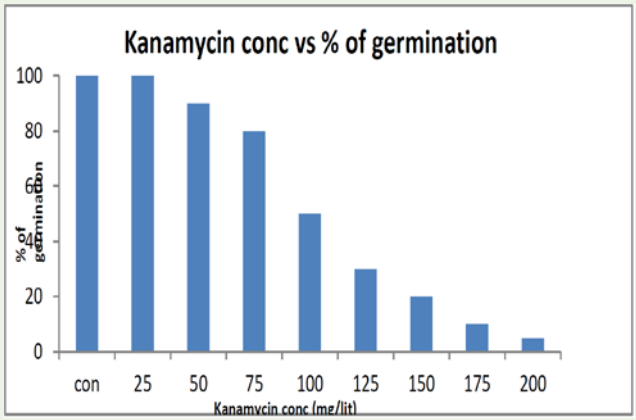

Figure 3: Effects of kanamycin on embryo development and germination of Pigeon pea.

Conclusion

The development of transgenic plants in Pigeon pea having an efficient germination and regeneration method necessitate the assortment of transgenic tissues by means of selection markers i.e. kanamycin. The outcome of the above study furthermore confirms that the question of escape and resistance arise in the explants. This trend can be recognized to secondary metabolite compositions in Pigeon pea. The biochemical compositions of Pigeon pea cause disorder in kanamycin destruction trend on plant cells so that symptoms of yellowness and burnt were not observed in explants under concentrations up to 100 mg/lit kanamycin as it was the case in the control treatment (absence of kanamycin). Results demonstrate that as kanamycin concentration increased number of germinated/ regenerated plants decreased so that the concentration of 200 mg/lit kanamycin caused symptoms of severe yellowness, burnt, and growth cessation in them. Results demonstrated which in compared to other plants in Pigeon pea was required high concentration of kanamycin for select transgenic tissues.

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