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Effective hygromycin concentration for selection of transformed embryogenic calli of cassava variety H-226

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Abstract

Cassava is a starchy root crop used as food, feed and for industrial purposes. Antibiotic resistance genes are used as a marker for the selection of transformed cells from non-transformed cells in genetic engineering. The optimum concentration of selective antibiotics is crucial for the effective transformation and regeneration of transformed plants. The current study aimed to determine the optimal cytotoxic concentration of hygromycin for screening both transformed and non-transformed calli. The embryogenic calli were subjected to particle bombardment using the binary vector pCAMBIA 1305.1, which included the GUS reporter gene and the *hptII* gene. Hygromycin was added to the callus induction and regeneration medium at 0, 25, 50, 75, and 100 mg L^{-1} concentrations to identify the optimal selective concentration. Results indicated that 50 mg L^{-1} of hygromycin inhibited non-transformed calli and maintained the health of transformed calli. This concentration provided a sufficient amount of selective pressure with minimal cytotoxic effects, thus serving as the optimal level for distinguishing transformed cells in the cassava genetic transformation system.

Key words: Cassava, callus induction, embryogenic calli, hygromycin, somatic embryo, leaf lobes, H-226

Introduction

Cassava (*Manihot esculenta* Crantz) is the most critical food source for over 800 million people in tropical regions. It is mainly used for food, animal feed, and starch production. It is the fourth-largest global calorie source and the second-largest starch source (Parmar *et al.*, 2017). Conventional breeding in cassava is limited due to high heterozygosity and poor seed setting. Hence, genetic transformation is crucial for the improvement of cassava through the incorporation of favourable traits. Recent research in plant biotechnology supports the development of transgenic cassava, and the priority areas for genetic improvement include pest and disease tolerance, improving starch quality, and reducing cyanogen content in cultivars. These molecular techniques are used to create cassava varieties that are more adaptable and resilient to satisfy future agricultural demands (Chavarriaga *et al.*, 2016). Generally, the friable embryogenic callus of cassava was used as an explant for genetic transformation. This tissue is compatible with large-scale production of transgenic events (Jayaseelan *et al.*, 2017).

A significant challenge in cassava transformation is the selective identification of genetically modified cells from non-modified ones, which is typically achieved by using antibiotic resistance genes such as kanamycin, hygromycin, geneticin, neomycin, gentamycin, and paromomycin for selecting transformed cassava. (Niklaus *et al.*, 2011). These antibiotics usually inhibit plant protein synthesis by binding to ribosomes (Davey *et al.*, 2010).

In certain studies, hygromycin has been shown to allow for more effective transgenic cell selection than kanamycin (Song *et al.*, 2012). Some species exhibit high sensitivity to hygromycin, and low concentrations of hygromycin have been utilized for the preliminary selection of transformed cells, such as 3.0 mgL^{-1} for rapeseed leaf petiole cells (Liu *et al.*, 2011). Meanwhile, higher amounts of hygromycin were used to select other species, such as 30 mg L^{-1} for Chinese rice cultivars (Zhao *et al.*, 2011).

The model cultivar lines TMS60444 are the only ones with information on the ideal concentration of different selectable markers for cassava. The data regarding the concentrations of the selective antibiotic hygromycin needed to be more precise for successful regeneration of transformants. However, such optimized results were lacking in many Indian cassava varieties, as there are only a few reports on stable protocols for transformation and regeneration. In the present study, a commercially available, high-yielding Indian cassava variety, H-226, was utilized to optimize the concentrations of the selective antibiotic hygromycin required for effective screening of transformed and non-transformed calli following biolistic genetic transformation. This work aims to close these gaps by balancing regeneration-related factors and selection efficiency to identify concentrations that optimize the transformation system's output.

Materials and methods

Nodal cuttings (5-8 nodes) of cassava cultivar H-226 were taken from mature greenhouse plants and sterilized by rinsing with tap water, followed by washes with distilled water, a 70% ethanol treatment, and a five-minute 2.5% sodium hypochlorite wash. The sterilized cuttings were then rinsed with sterile water, air-dried on Whatman filter paper, and placed individually in glass jars containing half MS basal medium (pH 5.7) and incubated at 25±2 °C for 25 days for the establishment of the *in-vitro* mother plant.

For embryogenic callus induction, immature cassava leaf lobes (0.5-1 cm) were taken from *in-vitro* mother plants cultured for 20-25 days and inoculated in CIM containing MS + 4 mg L^{-1} 2,4-D, and 0.01 mg L^{-1} NAA by placing the adaxial surface touching the

media. (Anuradha *et al.*, 2015). Plates were sealed with parafilm and kept in darkness at 26°C. Explants with callus growth were subcultured every 20 days under similar conditions to encourage the formation of embryogenic structures.

The sensitivity of cassava embryogenic callus cultures to antibiotics was assessed through an experiment with four treatments and four replications to determine the optimal hygromycin concentration for selecting transformants. Embryogenic calli, initially produced on CIM, were transferred to Petri plate containing the same basal medium with varying concentrations of hygromycin (0, 25, 50, 75, and 100 mg L^{-1}) and incubated in dark condition for 30 days and subculturing was done in the same medium. Observations on growth and morphology of calli were recorded every 30 days. The calli were scored based on culture response, with pale yellow-coloured callus rated as (++++), dark yellow-coloured callus as (+++), callus beginning to brown as (++), and callus turning brown with inhibited growth as (+). The data on average weight of the callus in CIM with different concentrations of hygromycin were also taken after 30 days on callus induction medium.

Results

Single nodal cuttings from field-grown mother plants were inoculated in basal MS medium to produce *in vitro* mother plants. Fresh leaves developed 2-3 weeks after inoculation, with most cuttings exhibiting normal growth and well-developed leaves. Immature young leaf lobes were then excised for the induction of embryogenic calli. After 10 days, the leaf lobes exhibited swelling, and by 20 days, friable, unorganized calli formed at the cut ends of the leaf lobes. To encourage further proliferation, these calli were transferred to a fresh callus induction medium twice. After 40 days, some of the proliferating calli developed a smooth surface, which subsequently showed the formation of globular somatic embryos (Fig.1).

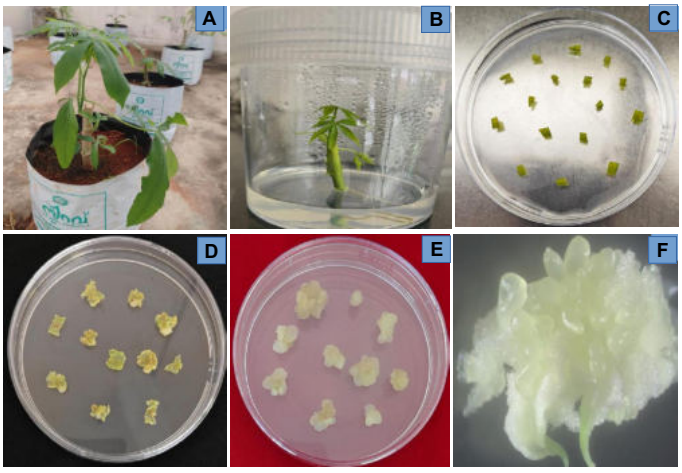

Fig.1. Somatic embryogenesis in cassava variety H-226 (A. Shoots containing 4-5 nodes; B. *In-vitro* mother plant after 30 days; C. Immature leaf lobes on SEIM; D. Leaf lobes after 10 days on SEIM; E. Appearance of globular stage embryo; F. Appearance of somatic embryo

Hygromycin concentration was tested in embryogenic calli of cassava variety H-226 to identify the optimal concentration for transgenic screening. The sensitivity assay showed that increasing hygromycin concentrations over four weeks progressively affected the color and viability of cassava embryogenic calli. At 25 mg L^{-1}, the calli maintained their ability to proliferate, remaining pale yellow with minimal stress. At 50 mg L^{-1}, the callus turned dark yellow, and growth was completely inhibited after four weeks, indicating that this concentration effectively

Table 1. Scoring of callus, viability percentage and average weight of embryogenic callus subjected to various concentration of hygromycin in callus induction medium after four weeks

Concentration of hygromycin (mg L^{-1})	Callus scoring	Viability percentage of callus (%)	Average weight of callus (mg)
Control (0 mg L^{-1})	_	100	338
T1 (25 mg L^{-1})	(++++)	20	264
T2 (50 mg L^{-1})	(+++)	0	192
T3 (75 mg L^{-1})	(++)	0	166
T4 (100 mg L^{-1})	(+)	0	158

suppressed non-transformed cells. At higher concentrations (75 mg L^{-1} and 100 mg L^{-1}), the calli exhibited discoloration to light and dark brown, respectively, revealing toxic effects that compromised callus health and viability (Fig. 2). Therefore, 50 mg L^{-1} hygromycin was determined to be the most effective concentration for selecting transformants, providing an optimal balance between selective pressure and cell viability over the four weeks.

This concentration effectively differentiates transformed from non-transformed tissues without inducing excessive toxicity. The average fresh weight of cassava embryogenic callus was 272 mg. In the control (0 mg L^{-1} of hygromycin), the average weight of embryogenic callus after 30 days increased to 338 mg due to calli proliferation. At 25 mg L^{-1} of hygromycin, the weight of the callus was 264 mg. At 50 mg L^{-1}, the callus weight was 192 mg (indicating complete growth inhibition). At 75 mg L^{-1}, the callus weight was 166 mg, and at 100 mg L^{-1}, the weight was 158 mg. The results on the average fresh weight of cassava embryogenic calli further support the conclusion that higher concentrations of hygromycin significantly inhibit growth.

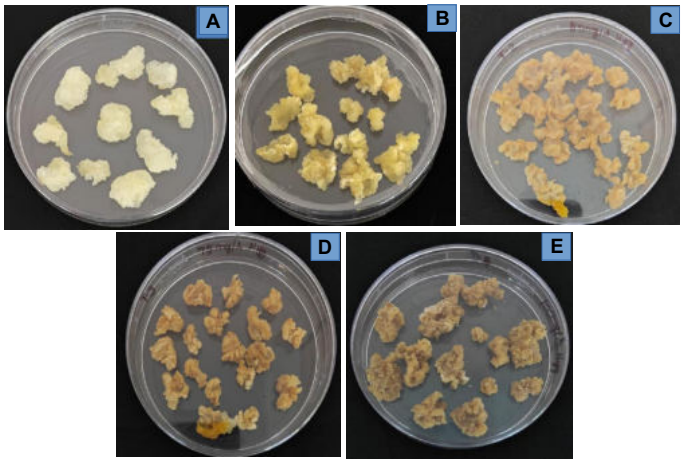

Fig. 2. Hygromycin sensitivity test for cassava embryogenic calli after 30 days (A. CIM+ Hygromycin 0 mg L^{-1} (Control), B. CIM+ Hygromycin 25 mg L^{-1}, C.CIM+ Hygromycin 50 mg L^{-1}, D. CIM+ Hygromycin 75 mg L^{-1}, E. CIM +Hygromycin 100 mg L^{-1})

Discussion

Establishing an optimal concentration of selectable marker genes is crucial for supporting the growth of transformed cells, and conducting antibiotic sensitivity tests is essential for developing a robust genetic transformation system. Kanamycin, spectinomycin, hygromycin are selection agents commonly used for genetic transformation in plants. The present study used a binary vector that includes the *hptII* gene as a selectable marker. The sensitivity test of embryogenic callus to various

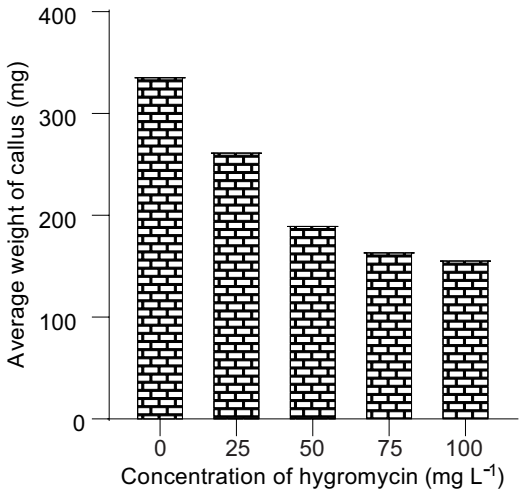

Fig. 3. Average weight of embryogenic callus subjected to various concentration of hygromycin in callus induction medium after four weeks

concentrations of hygromycin revealed that the optimum concentration for selecting transformed embryogenic callus of cassava was 50 mg L^{-1}. Similar findings were reported in previous research on rice, where the same concentration was effective for selecting transgenic plants from non-transgenic plants (Jayaraman *et al.*, 2018).

Pandey *et al.* (2023) reported that this same concentration (50 mg L^{-1}) was effectively used for selecting transgenic cvHD3090 wheat transformants, which were generated from the apical meristem tissues of germinating seeds. The use of hygromycin at 50 mg L^{-1} facilitated the selection of transformed plants and inhibited the growth of non-transgenic controls. So, the optimal antibiotic is essential for successful genetic transformation, which not only enhances transformation efficiency but also reduces the occurrence of false positives.

An increase in hygromycin concentration corresponded with a marked reduction in the average fresh weight of calli, reducing from 338 mg at 0 mg L^{-1} to 158 mg at 100 mg L^{-1}. This trend indicates that higher hygromycin levels inhibit callus growth significantly, ultimately halting calli proliferation. Similar findings were reported by Jhinjer *et al.* (2017), who also noted a substantial growth suppression in response to increased hygromycin levels.

The study identified 50 mg L^{-1} of hygromycin as the optimal concentration for selecting transformed embryogenic calli from immature leaf lobes of cassava variety H-226. This concentration can halt the growth of non-transformed calli and provide stable pressure to isolate transformed cells. Frequent selection is important in genetic transformation to ensure the proliferation of transformed cells. Lower concentrations of hygromycin may allow the growth of non-transformed cells and it may reduce the accuracy of selection. While a higher concentration of hygromycin becomes more toxic to transformed cells and reduces the regeneration efficiency.

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