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Optimization of glufosinate-ammonium (Basta) dose for transgenics selection in banana cv. Grand Naine

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Abstract
Plant transformation techniques play an important role in modern agriculture. The dose rate of the selective agent is the primary determinant of the success rate in selecting putative transformants. The survival frequency of transformant and non-transformant cells is determined by selection pressure. In commercial banana cv. Grand Naine, for which we have already developed an effective *in-vitro* regeneration system, we examined the ideal dosage of a selective agent basta. Different concentrations of basta (1 mg/l, 2 mg/l, 3 mg/l, and 4 mg/l) were initially tested for their efficacy in regeneration media (RM) and germination media (GM). However, no optimal results were obtained within this range. Consequently, the concentration range was refined to lower levels, specifically 0.2 mg/l, 0.4 mg/l, 0.6 mg/l, 0.8 mg/l, and 1 mg/l, for further experiments. For both media, we found optimal results at 0.2 mg/l. Further, the affectivity of the optimal dosages of basta was examined for transgenic selection in banana cv. Grand Naine. After being transformed with the bar gene, the embryogenic cell suspension (ECS) cells were allowed to generate embryos and were transferred to RM and further to GM having 0.2 mg/l of basta. PCR analysis verified the presence of bar (*BlpR*) gene integration in putative transgenic banana plants.

Keywords: Banana, bar gene, basta, transgenic

Introduction
Crop genomes can be modified for different reasons, including higher yield, resistance to pests, herbicides and diseases as well as greater nutritional value. The following three steps are used to track the effectiveness of the stable genetic transformation process: evidence of DNA integration, protein expression, and transgene transmission into the next generation. When a foreign gene is introduced during genetic transformation, only a small proportion of the target tissue's cells, successfully integrate the transgene stably into their genome to become transgenic. It is crucial to use a selection agent to separate these transformed cells from the vast majority of non-transformed cells. A selectable marker gene should be present in the transformed cells to survive on a certain selection agent. According to reports, transgenic plant research is currently using or developing about 50 selectable marker genes (Miki and McHugh 2004; Tuteja *et al.* 2012) [1, 2]. The bar gene, identified from *Streptomyces hygroscopicus* (De Block *et al.* 1987) [3], provides resistance to phosphinothricin, an analogue of glutamate, a competitive inhibitor of the enzyme glutamine synthetase, and the active component of the herbicides bialaphos, liberty, and basta. The bar gene codes for phosphonothricin acetyltransferase, which acetylates phosphinothricin to render it inactive. This gene has been utilized extensively in genetic transformation research as a successful selectable marker in a variety of crop species such as pineapple (Sripaoraya *et al.* 2001; Espinosa *et al.* 2002) [4, 5], pear (Lebedev *et al.* 2001) [6], apple (Szankowski *et al.* 2003; Arcos *et al.* 2020) [7, 8], chick pea (Ali *et al.* 2009) [9], orange (Jardak *et al.* 2020) [10] and soybean (Nguyen *et al.* 2021) [11]. The concentration of the selection agent must be optimized to avoid being excessively high, which could exclude transformants with moderate resistance, or too low, which could permit the proliferation of undesired escapees. As a result, transgenic selection greatly depends on the optimization of the selective agent. Crop-to-crop variations exist in the selection of selective agents. In case of apricots, kanamycin was shown to be an effective selective agent since it also increased the proliferation rate of the transformed tissues (Petri *et al.* 2005) [12], however, hygromycin as a selective agent did not appear

advantageous on the development of mature turf grass embryos (Cao *et al.* 2006) [13]. In banana, hygromycin and basta are identified as the two most effective selectable markers (Sreeramanan *et al.* 2006) [14]. Further, basta is an effective selection agent for monocots such as sugarcane (Wang *et al.* 2017) [15], rice (Rachmawati *et al.* 2004); Yookongkaew *et al.* 2007) [16, 17], wheat (Nada 2016) [18], and maize (Fromm *et al.* 1990) [19]. Consequently, basta was chosen as the selective agent for our study's transgenic banana selection.

Materials and Methods

Plant materials

The National Agri-Food Biotechnology Institute (NABI) at Mohali has a repository for banana germplasm. The immature male inflorescence of the banana cv. Grand Naine was utilized to generate banana ECS. Unless otherwise noted, Sigma Chemical Company (St. Louis, MO) and Merck (India) were the main suppliers of the molecular biology or cell culture grade reagents, compounds, and kits used.

Selection of basta dose for *in-vitro* regeneration and germination media

Immature male flower buds were used to generate ECS cells following the protocol established by Shivani and Tiwari (2019) [20]. The ECS was incubated on regeneration media without selection for up to two cycles, each lasting for 21 days. Subsequently, the regenerated cells were transferred to RM supplemented with varying concentrations of basta initially at 1 mg/l, 2 mg/l, 3 mg/l and 4 mg/l and further to 0.2 mg/l, 0.4 mg/l, 0.6 mg/l, 0.8 mg/l and 1 mg/l to determine the minimal inhibitory concentration (MIC).

Further, ECS were cultured on RM without a selection agent for up to four cycles. Mature embryos were allowed to germinate on GM without selection pressure. Subsequently, they were shifted to GM supplemented with varying concentrations of basta. Initially, concentrations ranged from 1 mg/l, 2 mg/l, 3 mg/l and 4 mg/l, which were later optimized to 0.2 mg/l, 0.4 mg/l, 0.6 mg/l, 0.8 mg/l and 1 mg/l. Both experiments were conducted in triplicates, and data was collected based on survival rate. Further optimization of basta concentrations was not performed for the subsequent step involving banana rooting media (BRM), as screening was effectively done at the germinated plantlet stage. Only survived plantlets were considered to the next stage.

Agrobacterium-mediated transformation of banana cv. Grand Naine ECS with the *bar* gene to evaluate the effectiveness of selected basta doses for transgenic selection

The banana ECS of cv. Grand Naine was used to perform *Agrobacterium*-mediated transformation of the vector pBUN411 (Addgene plasmid #50581). This vector carries basta resistance gene (*bilaphos resistance*, *BlpR*) (Figure 1) and was transformed in Agl1 strain of *Agrobacterium*. The *Agrobacterium*-mediated transformation protocol was performed by following the steps described by Shivani and Tiwari (2019) [20]. The putative transformed plantlets were selected at optimized range of basta on RM and GM to confirm the optimized MIC.

PCR analysis of transgenics

The leaf tissues (~100 mg) of putative transgenic plantlets were used for DNA isolation. Four random plantlets were selected and designated as T1, T2, T3 and T4. The DNaesy® plant mini kit (Qiagen, Germany) was used to isolate genomic DNA. The steps were followed as per manual provided in kit. The isolated DNA was quantified by Nanoquant (Thermo Fisher Scientific, USA). Primers for the *BlpR* gene were designed using the primer 3 tool (https://primer3.ut.ee/). These primers were used to confirm the presence of *BlpR* gene in the survived plantlets. The amplified product was ~ 250 bp. The forward and reverse primer sequence is given in table 1.

Results

Optimal dose selection of basta for regeneration and germination media

ECS was established from the immature male inflorescence of banana cv. Grand Naine. To determine the optimal dosage, ECS cultured on RM for two cycles was subsequently transferred to RM supplemented with concentrations of basta ranging from 1 mg/l to 4 mg/l. Observations were recorded and shown in figure 2. Early death signs were observed in all concentration range embryos on the first day (figure 2b-2e). To determine the optimal concentration, observations were recorded across a range of 0.2 mg/l to 1 mg/l. At concentrations of 0.8 mg/l and 1 mg/l, embryos died within the first day of transfer (Figures 2i-2j). At 0.6 mg/l, over 95% of the embryos died and showed blackening by the fifth day (Figure 2h). In contrast, at 0.4 mg/l, almost 40% embryo mortality was observed within a week (Figure 2g). At 0.2 mg/l (Figure 2f), the embryos exhibited characteristics comparable to the control group (Figure 2a), and this concentration was considered optimal for the regeneration medium.

In case of optimizing basta dosage for GM, we found almost similar results. On the next day of transfer of germinated embryos on germination media having even 1 mg/l basta, all were found dead (Figure 3b). The situation was also same for the higher dosage concentration (Figure 3c-3e). So, to optimize the ideal dosage, we further narrow down the basta range from 0.2 mg/l to 1 mg/l (Figure 3f-3j). In this experiment, we found blackening of all shoots on the next day in case of media having a dosage above 0.4 mg/l (Figure 3h-3j). Blackening was started after a week in the case of media with a dosage of 0.4 mg/l (Figure 3g). However, no death was observed up to 21 days in the case of media having 0.2 mg/l dosage (Figure 3f). So, this was considered optimal for regeneration media.

Agrobacterium-mediated transformation and selection of putative transgenic plants

After *Agrobacterium*-mediated transformation of ECS of cv. Grand Naine with vector having *BlpR* gene, the ECS were allowed to grow on RM without selection. After two cycles, the regenerated embryos were transferred to RM containing 0.2 mg/l basta (Figure 4b) and growth was comparable to the control (Figure 4a). The surviving mature embryos were subsequently transferred to GM supplemented with 0.2 mg/l basta (Figure 4d) and again growth was similar to the control (Figure 4c). Fully developed shoots were then transferred to banana rooting medium (BRM) for further growth and were labeled as putative transgenic plantlets.

PCR analysis of putative transgenics for *BlpR* gene

PCR analysis was performed on putative transgenic plantlets that survived selection pressure with basta using gene-specific primers. Genomic DNA was isolated from both wild-type control plants and four putative transgenics labeled as T1, T2, T3 and T4. PCR amplification was conducted using primers specific to the *BlpR* gene. The *BlpR* gene was absent in wild-type (non-transformed) plants, whereas the presence of the bar gene sequence was confirmed in putative transgenic plantlets subjected to basta selection pressure (Figure 5).

Table 1: Sequence details for the primer to check basta (*BlpR*) gene

Primer Name	Sequence (5' to 3')
BlpR_F	AGTCGACCGTGTACGTCTCC
BlpR_R	GAAGTCCAGCTGCCAGAAAC

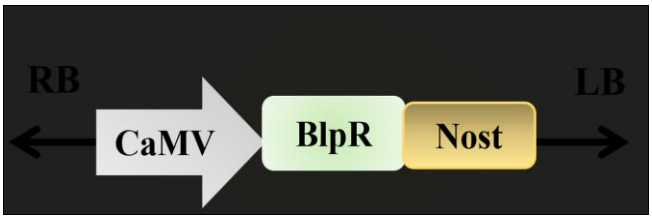

Fig 1: Schematic diagram of the vector carrying the basta (*BlpR*) gene whose expression is driven by CaMV35s promoter

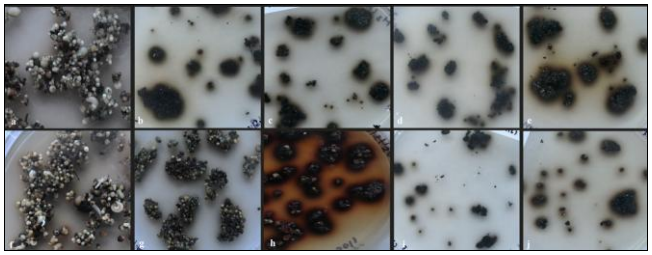

Fig 2: ECS response on regeneration medium (RM) at different levels of basta. (a) control (b) 1 mg/l (c) 2 mg/l (d) 3 mg/l (e) 4 mg/l (f) 0.2 mg/l (g) 0.4 mg/l (h) 0.6 mg/l (i) 0.8 mg/l (j) 1 mg/l

Fig 3: Shoot response on germination medium (GM) at different levels of basta. (a) control (b) 1 mg/l (c) 2 mg/l (d) 3 mg/l (e) 4 mg/l (f) 0.2 mg/l (g) 0.4 mg/l (h) 0.6 mg/l (i) 0.8 mg/l (j) 1 mg/l

Fig 4: Selection of transgenic plants on optimized level of basta. Control (a) and transgenics (b) on regeneration media having a selection level of 0.2 mg/l basta. Control (c) and transgenics (d) on germination media having a selection level of 0.2 mg/l of basta

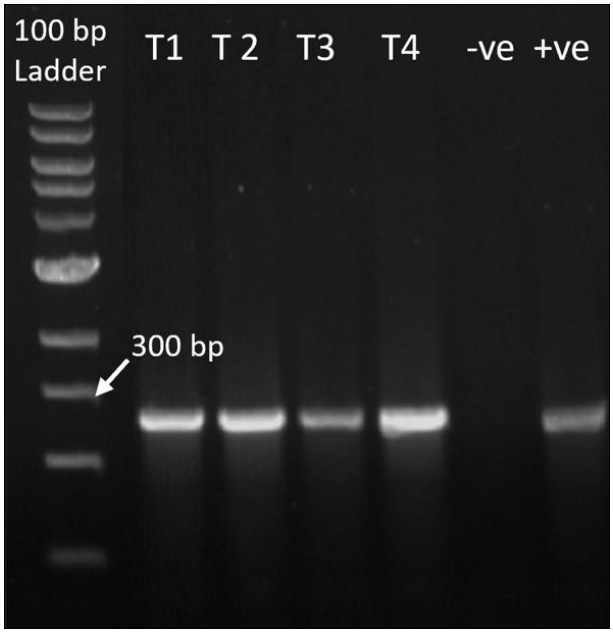

Fig 5: PCR analysis of putative transgenics for integration of bar gene. T1- transgenic plant 1, T2- transgenic plant 2, T3- transgenic plant 3, T4- transgenic plant 4, -ve- non-transformed control plant, +ve- positive control (Plasmid vector)

Discussion and Conclusion

One of the primary steps in creating transgenic plants is selecting transformed cells with a stably integrated gene. This can be done by determining the lowest concentration of a selective agent that can prevent the growth of non-transformed cells while enabling transformed cells to survive. This has made the transformation process more effective, which lowers the likelihood of chimeras. However, the complexity of *Agrobacterium*-mediated transformation increases in crops like banana, where the integrated copy of transgene remains in the genome, resulting in edited plants being classified as transgenic and subject to stringent biosafety regulations (Lakhani *et al.* 2022; Bansal *et al.* 2022) [21, 22]. Hence, the development of transgene-free editing is crucial for vegetatively propagated crops to fully explore the benefits of this technology (Lakhani *et al.* 2022) [21]. Nevertheless, until protocols for transgene-free genome editing in banana are optimized, stable transformation techniques remain the preferred approach for various studies. Sreeramanan *et al.* (2006) [14] emphasize the importance of optimizing the selective agent's dose rate and conclude that it is the most crucial stage in the selection of the modified plants since it simplifies and expedites the selection procedure. Parveez *et al.* (1996) [23] state that the selective agent's dose rate optimization is extremely species and tissue-specific. For example, the optimized basta concentration for banana plantains is reported to be 1 mg/ml (de GarcÃa and Villarroel 2005) [24], which differs significantly from the results of our study, where the optimal concentration was determined to be 0.2 mg/l. It could be the variation in endogenous resistance because monocots significantly depend on genotype.

In the current study, a range of basta levels were utilized for different stages of tissue culture to optimize MIC. A similar study has been conducted in sugarcane for transgenic events selection with the bar gene (Ijaz *et al.* 2012) [25]. For future applications of basta in transgenic or non-transgenic plants at the field level, this concentration range is safe for the soil microbial community and soil vegetation in banana plantation fields (Pattison *et al.* 2024) [26]. Therefore, it concluded that

optimization of optimal dose of selective agents should be done before the transformation of any crop plant to escape the non-transformant as well as to determine safe dosage for field application. Moreover, the vector used in the present study, pBUN411, together with other vectors such as pBUE411, pBUN421which also harbours basta as a selective agent has been used for CRISPR/Cas-based genome editing in monocots and dicots (Xing *et al.* 2014) [27]. So, for future endeavours, these vectors can be used for CRISPR/Cas-based genome editing in banana.

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Author Contributions
ST conceived the idea and designed the research. HL has performed various experiments. ST and HL contributed data compilation, analysis and writing of the manuscript.

Competing Interests
The authors declare that they have no competing interests.

Data Availability
All data supporting the findings of this study are available within the paper.

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