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Optimization of *Agrobacterium* Mediated Genetic Transformation in *Paspalum scrobiculatum* L. (Kodo Millet)

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Abstract: An efficient and reproducible protocol for *Agrobacterium tumefaciens* mediated genetic transformation was developed for kodo millet (*Paspalum scrobiculatum* L.) by optimizing various parameters. *Agrobacterium* strains EHA 105 and LBA 4404 harboring plasmids pCNL 56 and pCAMBIA 2300, respectively, provided the highest transformation efficiency. Addition of acetosyringone (AS) in infection medium (200 µM-EHA 105, 250 µM-LBA 4404) and co-cultivation medium (50 µM) increased the transformation efficiency. Transient and stable expression of *gus* gene was confirmed with histochemical assay of infected embryos and leaves of transformed plants, respectively. The best GUS response was obtained by pretreatment of callus with an antinecrotic mixture (10 mg/L Cys + 5 mg/L Ag + 2.5 mg/L As) at infection time of 20 min followed by co-cultivation for 3 days (EHA 105) and 5 days (LBA 4404) in dark. Regenerated transgenic plants were obtained after 8 to 10 weeks of selection on callus induction medium (NAA 0.5 mg/L, BAP 1 mg/L) containing 50 mg/L Kan + 250 mg/L Cef and were rooted for 2 weeks on MS medium containing PAA (1 mg/L) and phytagel. The plantlets established in greenhouse showed normal growth. Therefore, the protocol developed in the present study can be used for development of improved varieties of kodo millet.

Keywords: small millet; antinecrotic mixture; surfactants; GUS assay

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1. Introduction

Millet is a generic term used for unrelated small seeded forage grass cereals with coarse grains consisting of 10 genera, and are used for food, feed and forage worldwide [1,2]. They are staple food crops in developing nations of Africa and Asia and are known as a poor man's cereal or orphan crops [3].

Millets serve as excellent alternatives for cereals due to having an equivalent nutritional level, with the advantage of growing with minimum inputs [4,5]. They are often treated as nutri-cereals due to their rich protein and mineral profile along with high vitamin, calcium, non-starchy polysaccharide and fiber contents and a lower glycine index [6,7] and are considered as ideal components in different food formulations intended for specific target groups [8,9]. As per the Food and Agriculture Organization Corporate Statistical Database (FAOSTAT), the demand for cereals will increase to up to 2.89 billion tonnes by year 2030, while their production is expected to decline due to decreased irrigation, a lack of availability of large cultivable lands and other abiotic environmental threats.

Paspalum scrobiculatum L. (kodo millet) is one of the highly valued small millets. It is widely cultivated in South Asian countries such as India, the Philippines and Vietnam, with India being one of the largest producers [10,11]. The high dietary fiber, vitamin and essential amino acid content of the plant make it an excellent substitute for cereals [12]. The plant also possesses antidiabetic and high antioxidant activity among millets [6,13]. However,

susceptibility to the stress and the long maturity season of kodo millet have restricted its widespread use compared to other cereals. Considering the economic importance of the crop and the limitation associated with its incorporation into a balanced diet with other conventional cereals, it has become imperative to develop strategies for enhanced yield and increase abiotic stress tolerance in kodo millet [1,5].

Agrobacterium is commonly used for the transformation of monocotyledons due to its wide host range, stable T-DNA integration, relatively low transgene copy number and minimal equipment use [14]. Various factors including the type of explant, *Agrobacterium* strain, plasmid, promoter and selectable marker gene(s) along with time for infection and co-cultivation contribute to the development of successful transformation procedures [15,16]. Historically, monocots have been more difficult than dicots to transform using *Agrobacterium*, largely due to anatomical differences, limited wound response and production of *vir* gene inducing compounds [17]. Various attempts have been made to optimize regeneration and transformation of major millets including pearl millet, finger millet, foxtail millet and others [3,18]. The genus *Paspalum* is comprised of 350 different species and is one of the largest genera of family Poaceae, yet reports of successful genetic transformation only exist for *Paspalum notatum* [19,20] and *P. vaginatum* [21].

A successful and reproducible protocol for *in vitro* regeneration and multiplication of *Paspalum scrobiculatum* has already been reported in our previous study [3]. Therefore, in this current study, an efficient protocol for *Agrobacterium* mediated genetic transformation in kodo millet was developed. As per our knowledge, the present study is the first report of genetic transformation of such an important millet.

2. Materials and Methods

2.1. Plant Material and Preparation of Explants

Seeds of different varieties of *P. scrobiculatum* L. (RK 15, GPUK-3, RBK-155, RK-390-25, TNAU 86) were procured from All India Coordinated Millet Improvement Project (AICMIP), University of Agricultural Sciences, GKVK, Bangalore. The seeds were dehusked, rinsed with 20% (*v*/*v*) Extran (Merck, Mumbai, India) for 5 min and were surface sterilized with 0.1% (*w*/*v*) $HgCl_2$ (Merck, Mumbai, India) solution for 3 min followed by thorough rinsing (3 times) with sterile distilled water.

2.2. Screening of Potent Varieties

Sterilized seeds of all 5 varieties were cultured on callus induction medium composed of MS [22] basal salts supplemented with 3 mg/L (*w*/*v*) 2,4-dichlorophenoxyacetic acid (2,4-D) (Duchefa, Netherlands), 3% (*w*/*v*) sucrose (Loba chemicals, India) and 0.8% (*w*/*v*) agar (Bacteriological Grade, Merck, India) as gelling agent, with pH adjusted to 5.8 ± 0.05. The cultures were then maintained in culture chamber at 26 ± 1 °C and 24 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity with a photoperiod of 16/8 h for 6 weeks. Total 25 seeds with 5 seeds per flask were used for each treatment. Callus regeneration potential of all genotypes was recorded to identify the most responsive genotype, which was used for further experiments of gene transformation.

The calli of best responding variety were maintained through subsequent subcultures on callus maintenance medium (MS + 0.2 mg/L 2,4-D) every 4 weeks.

2.3. Antibiotic Sensitivity Test

Prior to optimization of the genetic transformation protocol using *Agrobacterium*, antibiotic tolerance capacity of the callus was estimated through a previously reported method [23]. All the antibiotics were procured from HiMedia, Mumbai, India. Callus regeneration media was augmented with varying concentrations of kanamycin (Kan) (0, 10, 20, 50 and 100 mg/L) and its effect on callus proliferation was recorded. The experiment was performed in triplicates for each concentration such that each plate contained 10 calli pieces (i.e., $n = 30$).

2.4. Bacterial Strain and Inoculum Preparation

Two *Agrobacterium tumefaciens* strains (EHA 105 and LBA 4404) harboring plasmid pCNL 56 (15.9 Kb), and pCAMBIA 2300 (8.7 Kb), respectively, were procured from IARI, New Delhi, India. Both plasmids consisted of a *gusA* reporter gene and *nptII* selectable marker gene controlled with a CaMV 35S promoter (Figure 1).

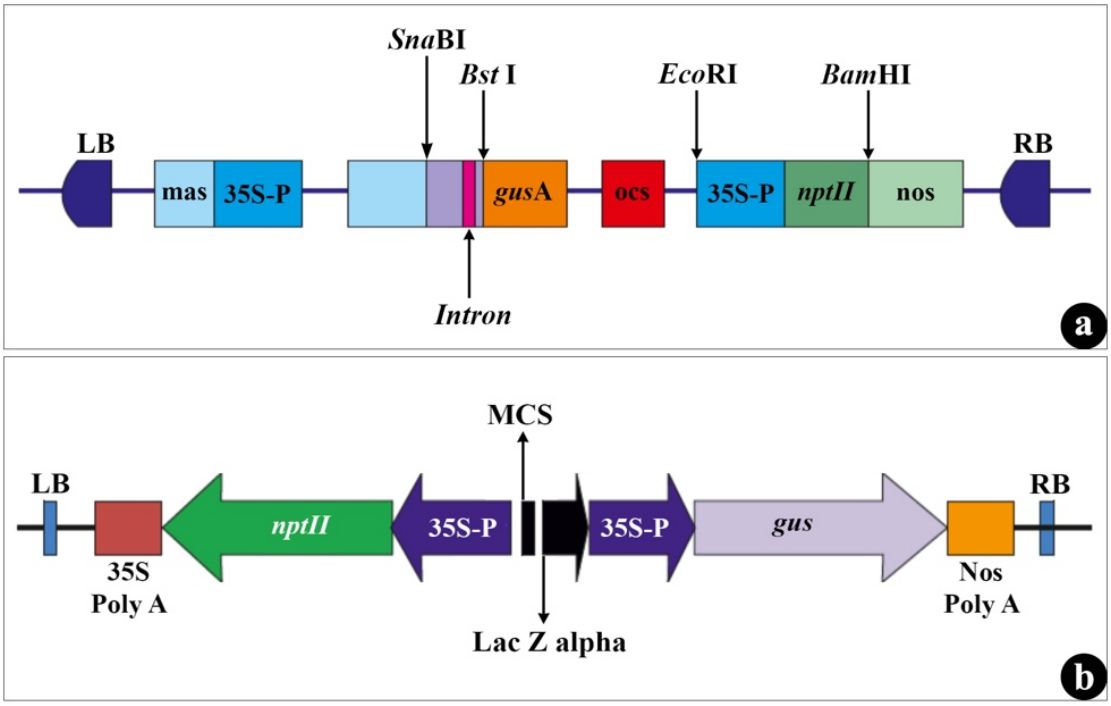

Figure 1. Genetic map of *Agrobacterium tumefaciens* strains (**a**) EHA 105 harboring plasmid pCNL 56, and (**b**) LBA 4404 harboring plasmid pCAMBIA 2300.

The culture medium types and antibiotics used are as follows (Table 1):

Table 1. Details of culture medium and antibiotics used for growth of different *Agrobacterium tumefaciens* strains.

Strain	Culture Medium	Antibiotics
EHA 105 (pCNL 56)	Luria-Bertini (LB) broth	50 mg/L Kan
LBA 4404 (pCAMBIA 2300)	Yeast extract broth (YEB)	50 mg/L Kan + 20 mg/L (Rifampicin) Rif + 50 mg/L Streptomycin (Strep)

The suspension cultures were incubated overnight in incubator shaker (Scigenics, Chennai, India) at 28 °C and 220 rpm.

2.5. Infection and Co-Cultivation

Preculture was prepared by transferring 2–3 week old calli (age after the initiation) on callus maintenance medium and incubating in culture chamber for 3–5 days prior to infection. For infection and co-cultivation, the bacterial cultures with varying cell densities were centrifuged, and the pellets were dissolved in MS liquid medium. Varied concentrations of acetosyringone (AS) (100 µM, 150 µM, 200 µM, 250 µM and 300 µM) with or without surfactants (Tween-20 and Pluronic acid F-68) were added and the medium was termed as the 'infection medium'. Calli with or without pretreatment with antinecrotic mixture (Table 2), were inoculated in the infection medium and were agitated gently for different time intervals (5–25 min) at varied temperatures. The calli pieces were then transferred to the 'co-cultivation medium' (MS + 0.2 mg/L 2,4-D) containing varied AS

concentrations (0–200 µM) and were incubated in the dark at lower temperatures (20 °C, 22 °C, 24 °C, 26 °C and 28 °C) for different time durations (0–5 days).

Table 2. Composition of different types of antinecrotic mixtures (AM) used for pretreatment of the calli.

Antinecrotic Mixture (AM)	L-Cysteine (mg/L)	Silver Nitrate (mg/L)	Ascorbic Acid (mg/L)
AM 1	10	5	1
AM 2	10	5	2.5
AM 3	10	3	2.5

2.6. Selection and Regeneration of Transformed Plantlets

After co-cultivation, calli pieces were washed with liquid MS medium containing 250 mg/L cefatoxime (Cef) and transferred onto 'callus maintenance medium' and incubated in a growth chamber. After a week of resting phase, the calli pieces were transferred onto the first selection medium (callus maintenance medium + 50 mg/L Kan + 250 mg/L Cef) for primary selection, followed by a subculture on second selection medium (callus maintenance medium) two weeks later, for secondary selection. In the third selection stage, the calli pieces were transferred on 'regeneration medium' composed of MS + 0.5 mg/L 1-naphthaleneacetic acid (NAA) + 1 mg/L 6-benzylaminopurine (BAP) (without antibiotics). The transgenic shoots developed within 3–4 weeks and were then transferred to a 'rooting medium' containing MS + 0.5 mg/L phenylacetic acid (PAA) (without antibiotics). The transgenic plantlets were then successfully acclimatized and hardened under greenhouse conditions.

2.7. GUS Histochemical Assay

For selection of transformants, GUS histochemical assay was performed as per Jefferson et al. [24] for both calli and regenerated plantlets, in which the presence of *gus* reporter gene was demonstrated through histochemical ß-glucuronidase assay in successfully transformed plantlets. GUS activity at each selection step was performed for calli and the regenerated plantlets. The plant segments were treated with 70% (*v*/*v*) ethanol for chlorophyll removal and were dipped in X-gluc staining solution (100 mg/mL X-gluc + 0.1 M sodium phosphate buffer + 20% Triton X) overnight at 28 °C.

The percentage of GUS expression was calculated as the ratio of number of GUS positive calli to the total number of calli infected, and the transformation efficiency was calculated as the ratio of number of kanamycin positive plants to the total number of transformed plants as reported by Sharma, et al. [25]. The assay was performed in triplicates and transformation efficiency for each was reported as mean of the three replicates ± Standard error (SE).

3. Result and Discussion

3.1. Selection of Variety

For establishment of a positive tissue culture experiment, the best responding variety of the plant must be used. Acknowledging this, 5 different varieties of *Paspalum scrobiculatum* L. were screened for their callus regeneration potential. All the varieties developed two types of calli: (i) nodular, creamish, imperforated, firm embryogenic and (ii) watery and yellowish non-embryogenic callus. Callus induction from immature seeds was observed on MS medium supplemented with 3 mg/L 2,4-D (Figure 2a). The 2 weeks old, watery and translucent callus was transferred on maintenance medium composed of MS + 0.2 mg/L 2,4-D (Figure 2b,c). After successful induction from the seeds, the callus showed very slow growth. This was in line with the previous report on embryogenic callus morphology, which suggested that both non-embryogenic and embryogenic callus are formed upon callus initiation. The non-embryogenic callus is sometimes the first to appear but lacks morphogenic ability and serves as nurse tissue [26]. Calli with different morphological and regeneration potential from same explant have been reported in barley as well [27].

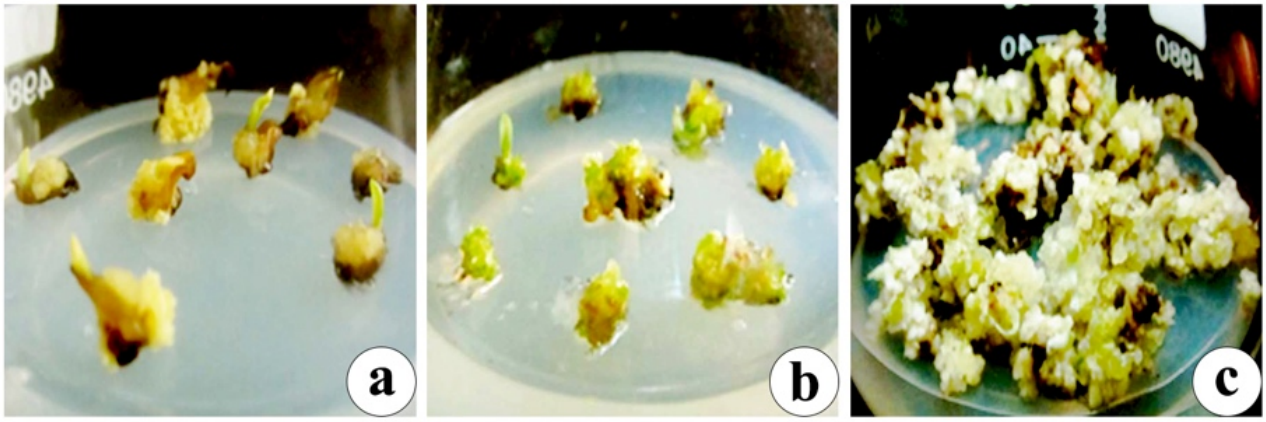

Figure 2. Response of GPUK-3 seeds on callus (**a**) initiation; (**b**) maintenance and (**c**) proliferation medium.

The variety RK-390–25 was the least responding in terms of the percentage of callus produced, while GPUK-3 comparatively showed higher callus formation and shoot regeneration efficiency with maximum response on MS + 3 mg/L 2,4-D (Table 3). Hence, variety GPUK-3 was found to be best in terms of callus response (%) and was chosen for further investigations.

Table 3. Screening of morphogenetic competence among different varieties of *P. scrobiculatum* L. cultured on MS medium containing different concentrations of 2,4-D.

Variety	2,4-D Conc. (mg/L)	Callus Response (%)	Avg. No. of Regenerated Shoots/Explant (Mean ± SE)
TNAU 86	1	28	1.2 ± 0.2
	2	45	3.0 ± 0
	3	62	4.4 ± 1.1
	4	20	2.3 ± 0.8
RK-390-25	1	13	0
	2	17	0
	3	22	1.0 ± 0.2
	4	20	0
RK 15	1	20	0
	2	34	1.1 ± 0.8
	3	35	2.3 ± 0.5
	4	30	0
GPUK-3	1	34	2.1 ± 0.3
	2	57	3.2 ± 1.1
	3	78	6.8 ± 1.5
	4	32	2.5 ± 0
RBK-155	1	18	0
	2	33	1.5 ± 0.7
	3	28	1.3 ± 0.5
	4	25	1.2 ± 0.5

3.2. Antibiotic Sensitivity

The dose of antibiotic had a direct impact on the callus growth and proliferation. Callus growth began to wither, and the survival of callus slowed down with the introduction of even smaller concentrations of Kan (10 mg/L). Higher concentrations (50 mg/L) of Kan resulted in browning of the calli pieces within 10 days of inoculation (Figure 3). Callus turned brownish-black at a 100 mg/L concentration. Since extremely low doses of antibiotic in the medium might not be able to control the bacterial growth, 50 mg/L Kan was considered to be optimal for further experiments.

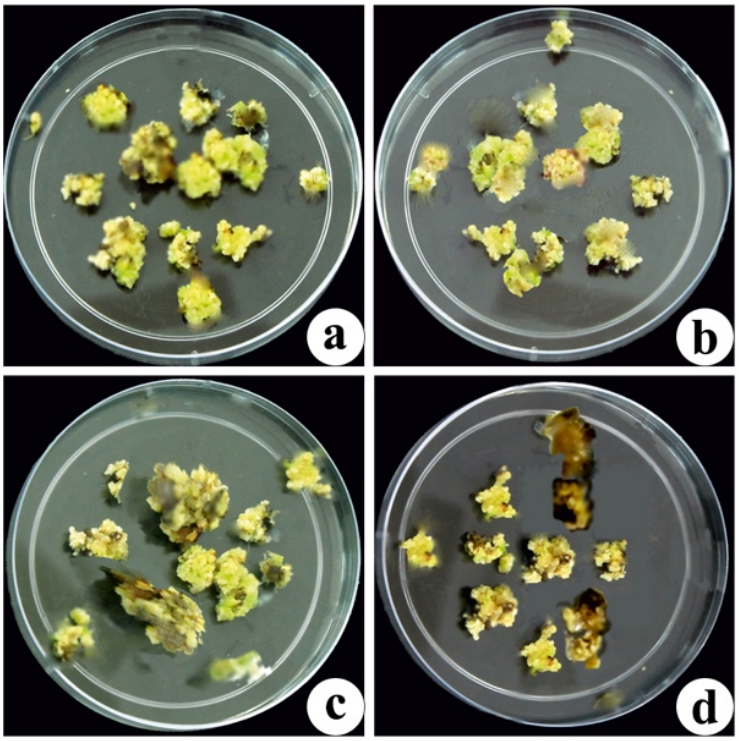

Figure 3. Antibiotic sensitivity of calli cultured on MS + 0.2 mg/L 2,4-D medium supplemented (**a**) without (0 mg/L) and with (**b**) 10 mg/L; (**c**) 20 mg/L and (**d**) 50 mg/L kanamycin.

We optimized the protocol for cultivar of barley and reported 50 mg/L Kan for profuse growth of the calli. Apart from Kan, hygromycin is also reportedly used in several transformation protocols, but the monocots were found to be sensitive against it [28].

3.3. Optimization of Different Parameters for Efficient A. tumefaciens Mediated Transformation

3.3.1. *Agrobacterium* Strain(s)

The experiment consisted of two *Agrobacterium* strains i.e., EHA 105 harboring pCNL 56 and LBA 4404 harboring pCAMBIA 2300. Plasmids pCNL 56 and pCAMBIA 2300 demonstrated maximum GUS expression percentages of 73% and 62%, respectively. The use of CaMV 35S promoter has been widely established for successful monocot transformation protocols [5,29].

3.3.2. Effect of Cell Density

The *Agrobacterium* strains (EHA 105 and LBA 4404) were grown to cell densities of 0.5, 0.8, 1.0 and 1.5 at 28 °C and 220 rpm. The bacterial suspension with cell densities of 0.8 (EHA 105) and 1.0 (LBA 4404) after 16 h and 18 h, respectively were found to be optimal for infection in terms of the highest percentage of embryogenic calli showing GUS activity (Figure 4a). Maximum transformation efficiency levels of 15.5% and 14.4% were obtained with cell densities of 0.8 (EHA 105) and 1.0 (LBA 4404) (Table A1). This was in agreement with findings on finger millet [25] and foxtail millet [30,31], while a cell density beyond 1.0 resulted in overgrowth of the bacterium, which in turn affected the viability of the infected calli. Hiei, et al. [32] reported that the overgrowth of *Agrobacterium* negates the flourishing of the callus culture and a particular density helps in adhering of bacteria to the explant surface, thereby allowing it to penetrate the plant cell.

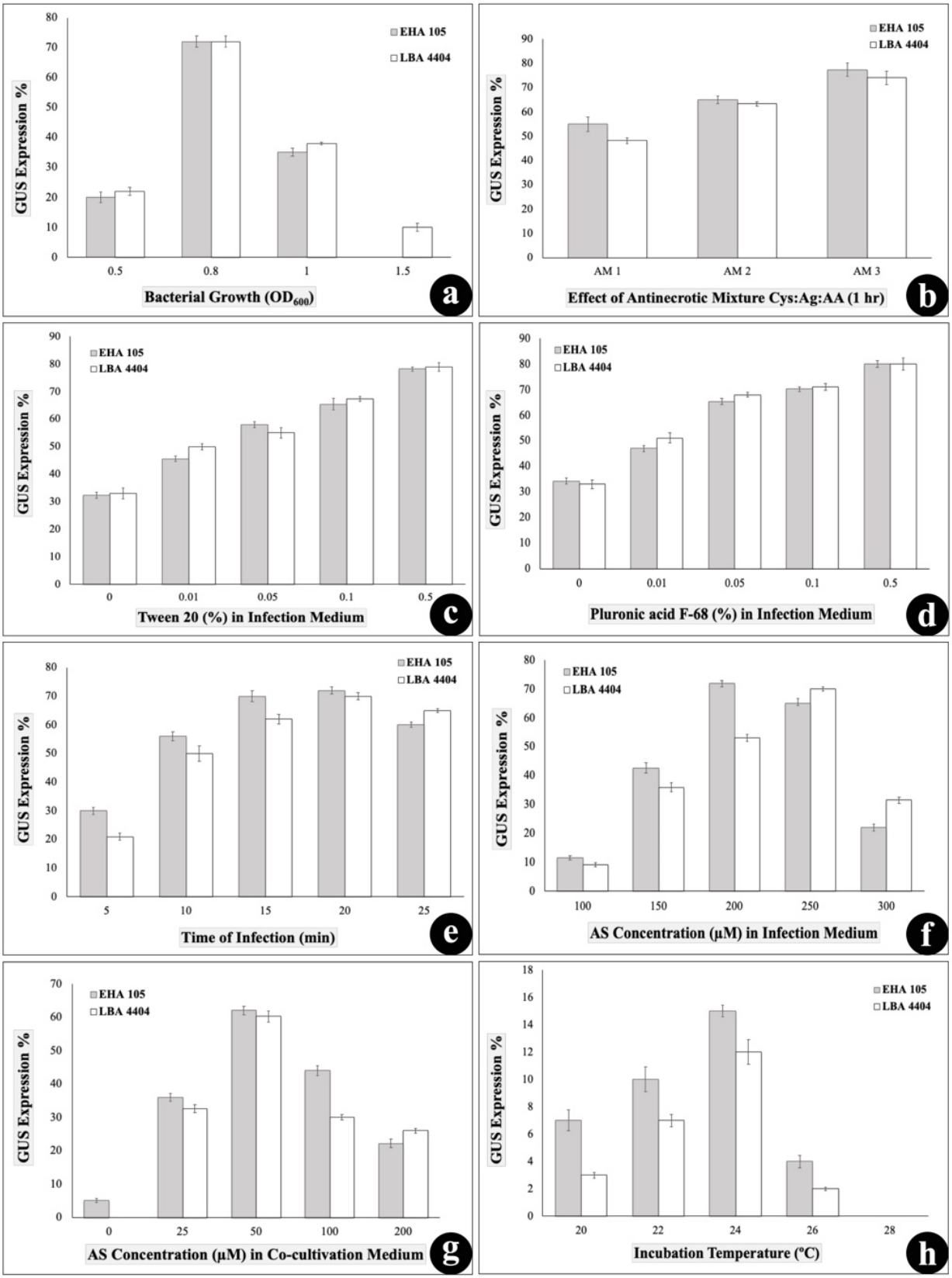

Figure 4. Effect of (**a**) Bacterial density; (**b**) Composition of antinecrotic mixture; (**c**) Concentration of Tween-20 in infection medium; (**d**) Concentration of Pluronic acid F-68 in infection medium; (**e**) Infection time; Acetosyringone concentration in (**f**) infection and (**g**) co-cultivation medium; and (**h**) Incubation temperature on GUS expression.

3.3.3. Effect of Pretreatment with Antinecrotic Mixture

The antinecrotic mixture AM 3 remarkably improved the GUS response (EHA 105—77.3%, LBA 4404—74%) and transformation efficiency (EHA 105–22.5%, LBA 4404—16.2%) against the untreated plants for both of the strains (Figure 4b, Table A2). Enríquez-Obregón, et al. [33] reported that the treatment with such formulations improves the cell survival and enhances the competence for foreign gene inclusion. The inclusion of L-Cysteine in antinecrotic mix or co-cultivation media enhances the transformation frequency and improves the viability of transformants by reducing the extent of cellular oxidation of explant tissues [34–37]. Similarly, ascorbic acid and silver nitrate has also been reported to reduce browning of transformed callus and *Agrobacterium* growth on the transformed tissues, respectively.

3.3.4. Effect of Surfactants

Augmentation of infection medium with surfactants helps with better attachment of bacteria, ensuring proper T-DNA delivery by reducing the surface tension. The GUS response enhanced when surfactants i.e., Tween-20 and Pluronic acid F-68 were added in the infection medium. Higher concentration of Tween-20 (0.5%) resulted in enhancement of GUS expression and transformation efficiency for both EHA 105 & LBA 4404 (Figure 4c, Table A6) Pluronic acid F-68 addition also increased the percentage of explants (80%) with blue coloration infected with either of both the strains (Figure 4d). The maximum transformation efficiency with Pluronic acid F-68 (0.5%) was observed to be 44% and 40% for EHA 105 and LBA 4404, respectively (Table A7). The study concorded with the findings of Ziemienowicz [38], who concluded that the addition of surfactants to suspensions of *Agrobacterium* increases the transformation efficiency. Pretreatment with such agents has been also been reported to increase the GUS expression efficiency in *Hordeum vulgare* [39].

3.3.5. Effect of Infection Time

The calli infected for 5 min did not display any overgrowth of the bacterial culture when transferred to co-cultivation medium, while those infected for 25 min proliferated the calli and resulted in enhanced GUS activity, but profuse *Agrobacterium* growth was observed around callus during co-cultivation. The calli pieces infected with *Agrobacterium* strains for 20 min resulted in maximum (EHA 105—72%; LBA 4404—74%) transient GUS expression (Figure 4e) and transformation efficiency (EHA 105—24.8%, LBA 4404—20%) (Table A3). Infecting the calli for more than 20 min led to a sharp decline in transformation efficiency, ultimately ceasing the calli growth, while a lower time interval did not allow the bacteria to attach to the explant surface sufficiently. Similarly, Ceasar and Ignacimuthu [40] reported that increasing infection time beyond a certain threshold resulted in sharp decline in the viability of the explant.

3.3.6. Effect of Acetosyringone

Millets are unable to produce phenolic compounds that facilitate *Agrobacterium* infection, hence supplementation of medium with phenolic compound(s) is a critical step in such transformation experiments. Addition of 200 µM and 250 µM AS in the infection medium proved to be efficient for EHA 105 and LBA 4404, respectively (Figure 4f). Similarly, transformation efficiency also increased significantly with the addition of 200 µM (27.7%) and 250 µM (23.3%) for EHA 105 and LBA 4404, respectively (Table A4). Moreover, the addition of 50 µM AS to co-cultivation medium of both strains further facilitated maximum GUS expression (Figure 4g) and transformation efficiency (EHA 105—30%, LBA 4404—23.3%) (Table A5). The transient GUS expression drastically dropped without AS addition in the medium and subsequent regeneration of the transformed plantlets could be achieved properly in many monocotyledonous species. Tyagi, et al. [41] and Ceasar and Ignacimuthu [40] also emphasized the use of AS in co-cultivation medium for induction of *vir* genes and extension of host range for the bacteria in rice cultivars and finger millet, respectively. Cheng, et al. [42] reported that higher concentrations of AS diminished the calli growth while a very low concentration administered in the medium did not allow

proper regeneration of the callus. Further, co-cultivation without AS has resulted in low *gfp* expression and no GUS expression in monocots, thereby affecting the transformation efficiency [23].

3.3.7. Effect of Co-Cultivation Time and Temperature

Co-cultivation of calli for 3 days and 5 days was optimum for EHA 105 and LBA 4404 mediated transformation, respectively. Increasing the duration of co-cultivation beyond 5 days led to decline in GUS expression of the explants and witnessed overgrowth of *Agrobacterium*, ultimately leading to callus necrosis and death. Almost two folds increase in callus growth was observed when infected calli were co-cultivated at lower temperature (24 °C) than the standard incubation temperature (26 ± 1 °C) for *in vitro* cultures (Figure 4h). Similarly, maximum transformation efficiency of 22.8% (EHA 105) and 16.1% (LBA 4404) was also noted at incubation temperature of 24 °C. A sharp decrease in both transient GUS activity and transformation efficiency was noted beyond this temperature and duration of co-cultivation (Figure 4h, Table A8). Lower temperature ranges are essential as the frequency of T-DNA transfer is affected by *virB* and *virD4* encoded proteins, whose optimal functioning is influenced by incubation temperatures [43]. The administration of lower temperature range in monocot *Agrobacterium* transformation has been reviewed extensively by Dillen, et al. [44], Cheng, Lowe, Spencer, Ye and Armstrong [42] and Hiei, et al. [45] and it corresponds with our present findings.

3.4. Co-Cultivation, Selection and Regeneration

The infected calli were transferred again to the callus maintenance medium, on which the non-transformed calli showed necrosis and poor growth. The selection medium ensured presence of antibiotics (Kan and Cef) at increasing concentrations at each step which was in agreement with studies by Karthikeyan, et al. [46], who reported improved efficiency of transformation by 15.33% in Indica rice (*Oryza sativa* L.) cultivar ADT 43. Calli that successfully endured the shock were transferred to the regeneration medium (Figure 5a,b) and successively to the rooting medium (Figure 5c). The regenerated plantlets were entrenched in greenhouse environment (Figure 5d).

3.5. GUS Histochemical Assay, Transformation Efficiency and Field Transfer

After co-cultivation and regeneration, histochemical GUS assay activity was performed on infected callus and plantlets, respectively. Distinct blue coloration confirmed the activity of gus gene, indicating proper insertion of the T-DNA. A noticeable color difference between transformed and non-transformed calli could clearly be seen (Figure 5e). While the non-transformed calli and plantlets failed to obtain any color, the transformed calli and plantlets showed a striking blue color when incubated at 37 °C overnight, ratifying the transformation process (Figure 5e,f). Maximum transformation efficiency (44%) was obtained with strain EHA 105 when pretreated with surfactant (Pluronic acid F-68, 0.5%). These findings concorded with those reported for other millets [47].

The putative transformants generated were then successfully hardened under greenhouse conditions and their seeds were collected on maturity.

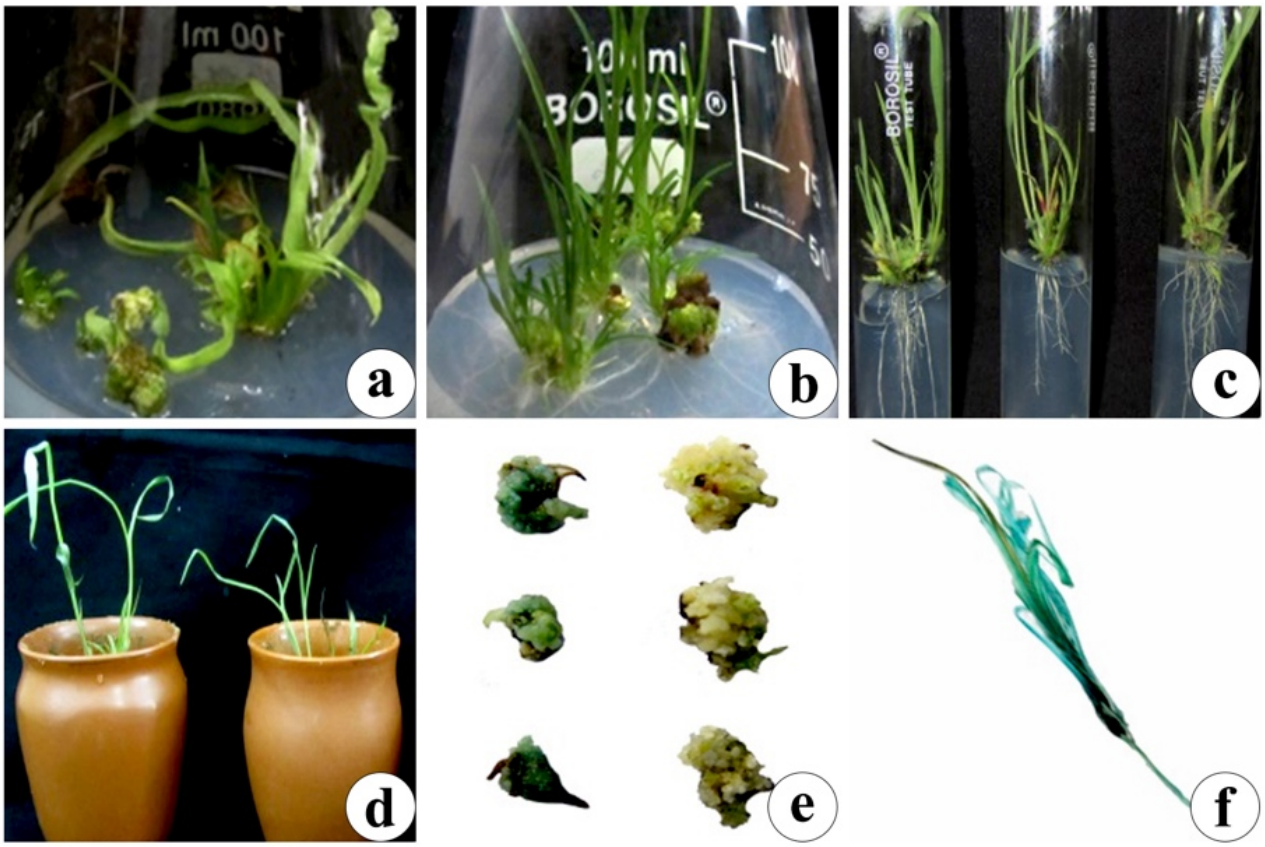

Figure 5. (**a**,**b**) Plantlet Regeneration on MS + 0.5 mg/l NAA + 2 mg/l BAP; (**c**) Rooting of regenerated shoots on MS + 0.5 mg/l PAA; (**d**) Regenerated plants transferred to pots and (**e**,**f**) GUS histochemical assay.

4. Conclusions

To summarize, the present report is the first attempt to develop an efficient protocol for modification of this ignored millet crop genetically. The protocol was designed by optimizing several parameters, which influence *Agrobacterium* infection. Profuse callus growth was found on MS medium enriched with 3.0 mg/L 2,4-D. EHA 105 and LBA 4404 strains were selected to obtain maximum transformation efficiency. Pretreatment with antinecrotic mixture, the addition of surfactants and lower infection temperature significantly improved transformation efficiency. The calli pieces co-cultivated at 24 °C for 3 days (EHA 105) and 5 days (LBA 4404), respectively, further improved transformation. The barrier for monocot transformation was overcome by the addition of phenolic supplement and acetosyringone in both infection and co-cultivation media. The transformation was confirmed with GUS histochemical assay. This protocol can be used eventually to transform this orphan crop with quality improving genes and traits that impart abiotic resistance to kodo millet, so it can thrive well in the era of nutritionally challenged soils and threatened environment conditions, becoming an excellent alternate for the counterpart cereals.

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Appendix A

Table A1. Effect of cell density on transformation efficiency of callus derived from seeds of *P. scrobiculatum* L.

Cell Density (OD_{600})	Callus Infected		Kan Resistant Calli		Kan Resistant Plants		Transformation Efficiency (%)	
	EHA	LBA	EHA	LBA	EHA	LBA	EHA	LBA
0.5	90	90	22	18	5	3	5.5	3.3
0.8	90	90	35	26	14	10	15.5	11.1
1	90	90	20	32	2	13	2.2	14.4
1.5	90	90	9	5	0	2	0	2.22

Table A2. Effect of pretreatment with Antinecrotic mix on transformation efficiency of callus derived from seeds of *P. scrobiculatum* L.

Antinecrotic Mixture (AM)	Callus Infected		Kan Resistant Calli		Kan Resistant Plants		Transformation Efficiency (%)	
	EHA	LBA	EHA	LBA	EHA	LBA	EHA	LBA
AM 1	80	80	16	9	6	5	7.5	6.2
AM 2	80	80	22	17	10	9	12.5	11.2
AM 3	80	80	36	32	18	13	22.5	16.2

Table A3. Effect of infection time (mins) on transformation efficiency of callus derived from seeds of *P. scrobiculatum* L.

Infection Time (min)	Callus Infected		Kan Resistant Calli		Kan Resistant Plants		Transformation Efficiency (%)	
	EHA	LBA	EHA	LBA	EHA	LBA	EHA	LBA
5	105	105	2	0	0	0	0	0
10	105	105	16	12	8	10	7.6	9.5
15	105	105	21	18	17	13	16.1	12.38
20	105	105	32	28	26	21	24.76	20
25	105	105	6	10	3	2	2.8	1.9

Table A4. Effect of AS concentration in infection medium on transformation efficiency of callus derived from seeds of *P. scrobiculatum* L.

AS concentration (µM)	Callus Infected		Kan Resistant Calli		Kan Resistant Plants		Transformation Efficiency (%)	
	EHA	LBA	EHA	LBA	EHA	LBA	EHA	LBA
100	90	90	27	19	8	2	8.8	2.2
150	90	90	36	25	17	9	18.8	10
200	90	90	42	32	25	18	27.7	20
250	90	90	25	38	14	21	15.5	23.3
300	90	90	6	11	0	0	0	0

Table A5. Effect of AS concentration in co-cultivation medium on transformation efficiency of callus derived from seeds of *P. scrobiculatum* L.

AS concentration (µM)	Callus Infected		Kan Resistant Calli		Kan Resistant Plants		Transformation Efficiency (%)	
	EHA	LBA	EHA	LBA	EHA	LBA	EHA	LBA
0	90	90	21	15	12	6	13.3	6.6
25	90	90	38	32	21	19	23.3	21.1
50	90	90	51	43	27	21	**30**	**23.3**
100	90	90	22	36	14	17	15.5	18.8
200	90	90	11	6	3	1	3.3	1.1

Table A6. Effect of Tween- 20 on transformation efficiency of callus derived from seeds of *P.scrobiculatum* L.

Tween-20 Conc. (%)	Callus Infected		Kan Resistant Calli		Kan Resistant Plants		Transformation Efficiency (%)	
	EHA	LBA	EHA	LBA	EHA	LBA	EHA	LBA
0 (Control)	75	75	12	6	4	2	5.3	2.6
0.01	75	75	26	15	16	9	21.3	12
0.05	75	75	33	27	22	18	29.3	24
0.1	75	75	38	31	24	21	32	28
0.5	75	75	42	39	32	29	42.6	38.6

Table A7. Effect of Pluronic acid F-68 on transformation efficiency of callus derived from seeds of *P. scrobiculatum* L.

Pluronic Acid F-68 Conc. (%)	Callus Infected		Kan Resistant Calli		Kan Resistant Plants		Transformation Efficiency (%)	
	EHA	LBA	EHA	LBA	EHA	LBA	EHA	LBA
0 (Control)	75	75	14	7	5	3	6.6	4
0.01	75	75	29	18	17	10	22.6	13.3
0.05	75	75	35	28	25	19	33.3	25.3
0.1	75	75	41	33	28	23	37.3	30.6
0.5	75	75	45	40	33	30	44	40

Table A8. Effect of incubation temperature on transformation efficiency of callus derived from seeds of *P. scrobiculatum* L.

Incubation Temp. (°C)	Callus Infected		Kan Resistant Calli		Kan Resistant Plants		Transformation Efficiency (%)	
	EHA	LBA	EHA	LBA	EHA	LBA	EHA	LBA
20	105	105	7	1	2	0	1.9	0
22	105	105	23	16	13	8	12.3	7.6
24	105	105	36	27	24	17	22.8	16.1
26	105	105	21	10	12	4	11.4	3.8
28	105	105	0	0	0	0	0	0

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