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Optimization of regeneration and transformation system in finger millet (*Eleucine coracana* L.)

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Abstract
A proficient *in vitro* regeneration protocol and transformation system is a requisite for genetic improvement of finger millet. Finger millet (*Eleucine coracana* L.) GN-4 was used for optimization of regeneration protocol. Using seed as a explants, highest frequency of callus induction was observed on MS supplemented with 2,4-D (1.0 mg.L^{-1}), Proline (500 mg.L^{-1}) and Casein enzyme hydrolysate (300 mg.L^{-1}). Among the different combinations used for shoot initiation, MS with kinetin (1.50 mg.L^{-1}) and BAP (0.50 mg.L^{-1}) produced maximum number of shoots (11.00) having highest shoot length (11.2 cm). Within six days, maximum numbers of roots (12.3) were obtained on half strength MS basal media fortified with NAA (1 mg.L^{-1}). In hardening process, maximum survival (44.51%) of plantlets with minimum days for new sprouting (4.4 days) was reported in vermicompost: sand: cocopeat (1:1:1 v.v^{-1}) mixture. *Agrobacterium tumefaciens* mediated genetic transformation system was optimized and putative transgenic plants were confirmed by PCR. When the effect of the age of the callus and co-cultivation duration were evaluated, forty five days old co-cultivated callus for five days yielded 1.33% frequency of transformation.

Keywords: *Agrobacterium* mediated transformation, finger millet, indirect regeneration.

Introduction
Finger millet is an allotetraploid (2n=4x=36), grown globally on more than 4 million hectares both in South East Asia and Africa, mainly India and Nepal [1]. Finger millet, commonly known as "Ragi" is an important minor cereal belongs to Poaceae family [2] and cultivated extensively in many regions of India [3].Total production of millet grains was 762,712 metric tons and the top producer was India with an annual output of 334,500 tons contributing 43.85% of total in year 2013. In India, finger millet production is on 6th after wheat, rice, maize, sorghum and bajra [4]. It constitutes about 81% of the minor millets produced in India, mainly grown in the states of Uttar Pradesh, Bihar, Tamil Nadu, Karnataka and Andhra Pradesh [2].

Finger millet is most important crop for people having low income because it is highly rich in calcium (0.38%), protein (6%–13%), dietary fiber (18%), carbohydrates (65%–75%), minerals (2.5%–3.5%), phytates (0.48%), tannins (0.61%), phenolic compounds (0.3–3%) and trypsin inhibitory factors, and hence could be used as nutraceuticals also [5]. Finger millet is also known for its other benefits to health like it is anti- diabetic, anti-tumerogenic, atherosclerogenic antioxidant and antimicrobial properties [5]. Finger millet is high in dietary fiber, also has medicinal attributes and used for making special foods for diabetics, gluten-free food for people suffering from celiac disease and weaning foods for infants [6]. As it is economically viable and also possess all these properties, it will have a immense future in the nutraceutical industry for reduction of chronic pathologies. As infants, age old females and sick person needs more calcium in their diet, it is used in preparations of foods for them [7]. Commercial production of finger millet products like cakes, biscuits, pudding, pasta, noodles, papad etc. are done by small scale industries, therefore it is considered as a industrially important crop. Utmost finger millet yield loss is due to fungal infection [8] and it is also susceptible to various abiotic stresses, particularly drought and salinity stress, in the field [9]. Genetic improvement of finger millet is a way to combat with this loss. Therefore it is indeed to have a *in vitro* regeneration pathway and a good transformation protocol for finger millet improvement.

Therefore here we attempted *in vitro* plant regeneration and transformation study in finger millet using *hpt II* as a marker gene to develop transgenic using *Agrobacterium* mediated gene transfer method.

Materials and method

Seed material of finger millet genotype GN-4 obtained from Hill Millet Research station, Waghai (Dang), NAU, Gujarat, India.

Surface sterilization of explants

Seeds were surface sterilized in 0.1% ($w.v^{-1}$) mercuric chloride for 6 minutes followed by 4-5 times rinses in sterile deionized water. Seven to eight seeds were placed in petriplates containing basal MS medium [10] and incubated in the dark condition for germination.

Callus induction and shoot initiation

MS basal medium in the presence of different plant growth regulators like 2,4-D (1.0 $mg.L^{-1}$), Proline (100-700 $mg.L^{-1}$) and Casein enzyme hydrolysate (CEH) (100-700 $mg.L^{-1}$) used separately and in combination. Plant growth regulators like BAP and Kinetin as well as in combinations of Kinetin (0.5-2.0 $mg.L^{-1}$), BAP (0.5 $mg.L^{-1}$) and NAA (0.5-2.0 $mg.L^{-1}$) were tried to obtain maximum shoot induction and multiplication. Murashige and Skoog (MS) medium containing 3% ($w.v^{-1}$) sucrose and 0.8% agar was used in all the experiments. Plant growth regulators at different concentrations were incorporated into the basal media and pH was adjusted to 5.8 with 1 N NaOH or 1 N HCl before autoclaving at 121^0C and 15 $lbs.Inch^{-2}$. All the cultures were incubated in a culture room at a temperature of 25 ± 2^0 C with relative humidity at 55 ± 5 per cent and plates were kept in dark condition for callus induction. For shoot initiation and multiplication, cultures were exposed to a photoperiod of 16 h light and 8 h dark with 55±5% of relative humidity (RH). From the different plant growth regulators and their combinations which showed highest response in terms of shoot induction were used to study effect of subculturing and multiple shooting.

***In vitro* rooting and acclimatization**

The trial on *in vitro* rooting was conducted on half MS medium solidified with 0.8 per cent agar. Each medium was supplemented with different concentrations of NAA (0.05-2.0 $mg.L^{-1}$) and IBA (0.05-2.0 $mg.L^{-1}$). After four weeks of incubation on rooting medium, the *in vitro* raised plantlets of finger millet with well-developed 10-15 roots were taken out from the culture tubes. The roots were washed thoroughly to remove adhering agar. The rooted plantlets of finger millet were then transplanted in plastic cups containing combination of sand, vermicompost and cocopeat after drenching it with 1.0 per cent bavistin and covered with cups. These plantlets were irrigated initially with one-third strength MS medium for five days. These were allowed to grow initially in culture room conditions (25 ± 2 ºC, 55 ± 5% RH, under 16h of photoperiod). Cups were removed after the new sprouting of leaves and these plantlets were hardened in soil for more growth.

Bacterial strain and plasmid

Rs-AFP2 gene was amplified from the plasmid pFAJ3105 [11]and cloned at BamHI site of pAHC17 under maize Ubiquitin (Ub1) promoter [12], to obtain pUb1-Rs. Plasmid pUb1-Rs was digested and cloned at HindIII site in pCAMBIA-1305.2 (Cambia, Australia) to obtain pUb1-Rs-T [13]. The binary vector (pUb1-Rs-T) was mobilized into *Agrobacterium tumefaciens* strain LBA4404 harbouring pSB1 [14].

Hygromycin sensitivity test

The test was performed in order to check the sensitivity of cultured tissues to hygromycin. Different concentration of hygromycin (0, 20, 40, 60, 80,100 $mg.L^{-1}$) were added to pre sterilized molten MS media by filter sterilizations through 0.22 µm pore size membrane filter. The treatment devoid of hygromycin was used as a control. Isolated callus were inoculated at different concentrations of hygromycin. Effect of the antibiotic concentration on callus was observed to determine its optimum concentration in selection media.

***Agrobacterium* mediated transformation and regeneration of transgenic plants**

Agrobacterium tumefaciens strain harbouring *hpt II* marker gene was used for transformation. Single, isolated colonies from YEB [15] medium plates were inoculated individually in 50ml YEP [15] medium containing 50$mg.L^{-1}$kanamycin and 10$mg.L^{-1}$ rifampicin and was grown at 28 ^{0}C with 200 rpm in an incubator shaker (Kuhner Germany). The culture was harvested at 0.4-0.6 O.D. at 600 nm and was utilized for transformation experiments. The culture was centrifuged at 5000 × g for 10 minutes. The pellet was resuspended in an induction medium (NH_4Cl 1.0 $g.L^{-1}$, $MgSO_4.7H_2O$ 0.3 $g.L^{-1}$, KCl 0.15 $g.L^{-1}$, $CaCl_2$ 0.01 $g.L^{-1}$, $FeSO_4.7H_2O$ 0.0025 $g.L^{-1}$, KH_2PO_4 0.272 $g.L^{-1}$, 2N morpholino ethane sulfonic acid [MES] 0.390 $g.L^{-1}$, Glucose 5.0 $g.L^{-1}$ and pH should be maintained 7.2) with 100µM acetosyringone and incubated for 4 hour in an incubator shaker at 175 rpm and 26 ^{0}C temperature. After this incubation period, the bacteria was centrifuged at 5000 rpm for 10 minutes and the pellet was resuspended in liquid co-cultivation media i.e liquid MS medium having MES 193 $mg.L^{-1}$, Glucose 2% and pH 5.65 incorporated with 100 µM acetosyringone and grown for 2 hours at 26^0C and 150 rpm in an incubator shaker. This culture was further used for infection of finger millet callus.

Infection and co-cultivation of the explants

For infection, 30, 45 and 60 days old callus on MS medium were taken as a explants. The latter were shacked with *Agrobacterium* culture and excess moisture was removed by blotting with sterilized Whatman No.1 filter paper. After infection, explants were put on solid co-cultivation media in dark for 3, 5 and 7 days respectively.

Selection of putative transgenic plant

After co-cultivation, explants were washed six times with sterile distilled water containing 250 $mg.L^{-1}$ cefotaxime. Washed calli were blotted dry using a sterile paper towel and cultured on the selection medium consisting of MS with 250$mg.L^{-1}$ cefotaxime and 40 $mg.L^{-1}$ hygromycin. Callus which was not inoculated with *Agrobacterium tumefaciens* were placed on the selection medium served as a negative control. The petridishes were incubated at a temperature of 28 ^{0}C under an 18 hours photoperiod and sub-cultured every 2 weeks for a period of 7 weeks. The resistant calli obtained after 7 weeks, were transferred to shoot and root regeneration media respectively and used for molecular analysis.

Confirmation of putative transformed plants through PCR

Genomic DNA isolated from leaves of plants by the CTAB method with minor modification [16]. Plasmid DNA was isolated by standard alkaline lysis method [16]. PCR amplification of a *hpt* II gene was carried out in Biometra thermal cycler using specific primers (Forward primer: 5'CCCTGATGGCATCCGAAGAGC3' and the reverse primer: 5'GAGGCAGCAGTGATGACATCC 3') to ensure integration of gene. The PCR reaction mixture consisted of 50 ng genomic DNA in a final volume of 20 µL containing 1x PCR buffer, 1.2 µL $MgCl_2$, 250 µM dNTPs, 1 µL each of primer and 0.3 units of Taq DNA polymerase. Amplification was carried out by Initial denaturation at 94 ^{0}C for 4 min followed by 30 amplification cycle at 94 ^{0}C for 50 sec, 53 ^{0}C for 1, 72 ^{0}C for 1 min. After completion of the 30 cycles, final extension was carried out at 72 ^{0}C for 5 min. PCR products were separated by electrophoresis with a 1.2% agarose gel and analysed.

Statistical analysis

Completely Randomized Design (CRD) was used for all the experiments. The data were subjected to analysis of variance (ANOVA) and treatment means were compared using the critical difference (CD) at a 1% level of significance [17].

Results

Increasing population demands higher production of a nutritive food like finger millet. In Indian scenario finger millet yield losses are mainly due to disease manifestation. Present investigation describes successful protocol of *in vitro* regeneration and *Agrobacterium* mediated gene transfer, through callus derived from seeds in finger millet plants.

***In vitro* callus induction from finger millet seeds**

Finger millet (genotype GN-4) seeds from the field are generally found with loads bacterial and fungal spores. Therefore it is mandatory to do surface sterilization. Mercuric chloride ($HgCl_2$) for 6 min which is found to be effective sterilant to reduce the risk of contamination (data not shown) and the successful establishment of the explants in the culture medium. As 2, 4-D is found to be most suitable for induction of callus in cereals [18], it was used in different concentration. Callus formation was induced by using 45 days old seeds (Figure 1a) and applying different concentration of 2, 4-D with combination of proline and casein enzyme hydrolysate (CEH). After 1 week of incubation in dark, soft watery white callus formation has been started from the seeds. Maximum 57.24% callus initiation was observed on MS media supplemented with 2, 4-D 100 mg.L-1, proline 500mg.L-1 and CEH 300mg.L^{-1} (Figure 1b)(Table 1). Proline and CEH were added in media to support and favour percent callus induction [19]. For callus maintenance and proliferation callus was put for 2 weeks on same media (Figure 1c).

***In vitro* shoot induction, multiplication and rooting**

Isolation of five weeks old callus produced highest establishment percentage. Hence, for further investigations, five weeks old callus were used for study. In order to obtain shoot initiation from callus, different combinations of cytokinins were used. Effective shoot initiation (11 shoots per callus) (Figure 1 d,e) was obtained in $E_{10,}$ MS with BAP 0.50 mg.L^{-1}, Kinetin 1.50 mg.L^{-1} and NAA 1.0 mg.L^{-1} after 3 weeks of inoculation in shoot initiation media (Table 2). Rooting parameters were significantly influenced by the type and concentration of auxin. From the different auxin concentration used, half strength of MS medium supplemented with NAA 1mg.L^{-1} recorded significantly higher number of roots per plants (12.3) and minimum days (6) to initiate the root. While highest rooting frequency in terms of number of culture rooted was achieved on half MS with NAA 2.00 mg.L^{-1}. In our study NAA was more suitable for root formation than IBA.

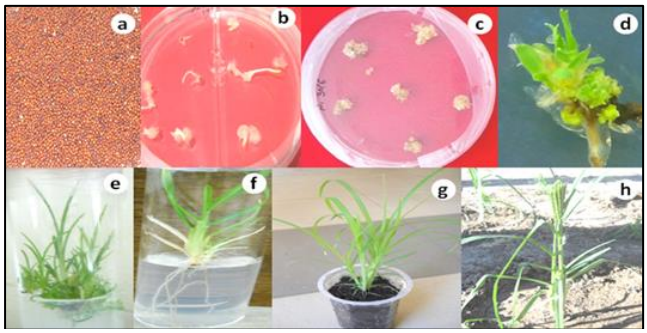

Fig 1: *In vitro* regeneration of finger millet a) Finger millet seeds b) Primary callus induction on MS+ 2,4 –D 100 mg/L, proline 500mg/L and CEH 300mg/L from seeds after 28 days of incubation in dark c) Callus maintenance d) Shoot induction and multiplication on MS + BAP (0.50 mg/l)+ Kinetin (1.50 mg/l)+ NAA(1.0 mg/l) d) Shoot elongation e) *In vitro* rooting f) Primary hardening of regenerated plants g) Growth of plant in soil

Table 1: Callus formation from seed of finger millet in presence of combinations of different growth regulators

Tr. No.	Plant growth regulators (mg.L^{-1}) with MS			(%) of callus formation
	2,4-D	Proline	CEH	
F_1	1.0	100	100	29.99
F_2	1.0	100	300	32.58
F_3	1.0	100	500	33.00
F_4	1.0	100	700	34.45
F_5	1.0	300	100	36.87
F_6	1.0	300	300	39.04
F_7	1.0	300	500	42.32
F_8	1.0	300	700	45.57
F_9	1.0	500	100	50.78
F_{10}	1.0	500	300	57.24
F_{11}	1.0	500	500	45.19
F_{12}	1.0	500	700	41.75
F_{13}	1.0	700	100	38.65
F_{14}	1.0	700	300	33.83
F_{15}	1.0	700	500	33.41
F_{16}	1.0	700	700	28.41
S.Em. ±				0.69
C.D. at 5%				2.01
C.V. %				3.08

Effect of serial sub culturing on multiple shoot regeneration through callus

To establish the exact number of subcultures to produce maximum number of superior quality plants, the best growth regulator combination of previous experiment were used for three cycles of subculture.

***In vitro* rooting and hardening**

Plants having well established roots were removed, washed and further were transplanted in poly bags having different potting mixture after drenching it with 1.0 per cent bavistin. Maximum survival (44.51%) of plantlets with minimum days

for new sprouting (4.4 days) was reported in Vermicompost : Sand : Coco peat (1:1:1 v/v) (Supplementary table 1).

Production of Putative Transgenic Plants

Hygromycin sensitivity was checked to determine necessary selection condition for recovery of transformants. After this study 40 mg.L^{-1} hygromycin was used to allow selected growth of transformed calli and killing non transformants. Under hygromycin selection pressure, most of the callus killed and some of the callus that was initially green, killed gradually, leaving only a few green callus (Figure 2 a, b). Rooting of the transformed shoot apices occurred when they were transferred from hygromycin selection medium to hygromycin free medium (Figure 2d). Co-cultivation duration for five days on an average gave maximum frequency of transformation (1.33%) while co-cultivation period for three days and seven days gave 0.33% and 1% frequency of transformation. No transformants obtained with thirty days seedling and three days of co-cultivation duration. The maximum frequency of transformation was obtained for 45 days old callus and five days of co-cultivation. From above described treatments, 30 and 60 days age of seedling and co-cultivation for three days gave low frequency of transformation.

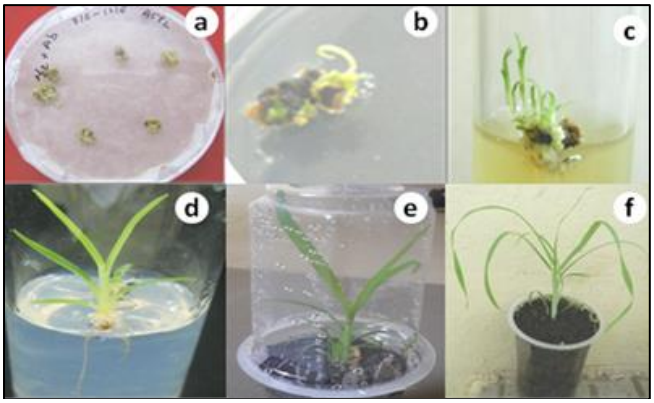

Figure 2: Selection of putative transgenic plant a) Finger millet calli on a selective media after *Agrobactrium* infection b) Putative transformed calli and shoot formation on shoot induction media c)Shoot multiplication d) Root initiation e, f) Primary and secondary hardening of putative transformed plant

Table 2: Effect of different combination of auxins and cytokinins for shoot and root formation from callus

Tr. No.	Cytokinin + Auxins (mg.L^{-1}) with MS			Multiplication (%)	No. of shoots	Shoot length (cm)
	BAP	Kin	NAA			
E_1	0.50	0.50	0.50	20.65	3.3	6.7
E_2	0.50	0.50	1.00	24.26	3.8	5.3
E_3	0.50	0.50	1.50	23.31	5.6	6.5
E_4	0.50	0.50	2.00	22.79	5.0	6.3
E_5	0.50	1.00	0.50	24.18	4.4	5.0
E_6	0.50	1.00	1.00	27.89	5.8	8.0
E_7	0.50	1.00	1.50	25.92	6.0	8.7
E_8	0.50	1.00	2.00	27.66	7.0	6.3
E_9	0.50	1.50	0.50	32.37	8.3	8.5
E_{10}	0.50	1.50	1.00	39.30	11.0	11.2
E_{11}	0.50	1.50	1.50	34.72	9.6	7.9
E_{12}	0.50	1.50	2.00	30.44	8.0	6.6
E_{13}	0.50	2.00	0.50	26.00	6.4	5.8
E_{14}	0.50	2.00	1.00	22.79	5.4	6.0
E_{15}	0.50	2.00	1.50	18.96	4.6	5.6
E_{16}	0.50	2.00	2.00	15.83	3.1	6.5
S.Em. ±				0.30	0.20	0.30
C.D. at 5%				0.87	0.58	0.88
C.V. %				1.99	2.43	3.47

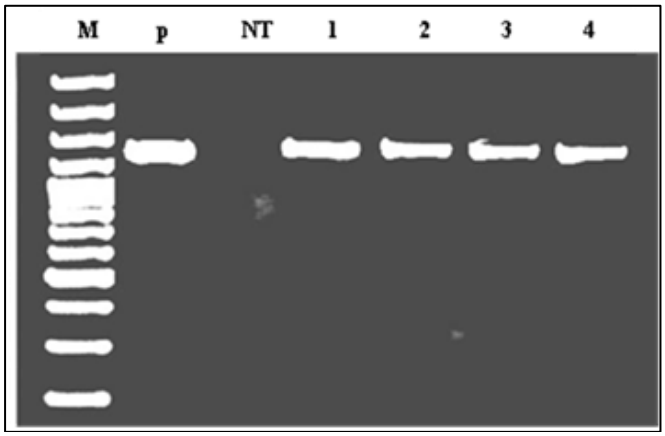

Fig 3: PCR analysis of genomic DNA of putative transgenic and non-transgenic plants M- DNA ruler, p- plasmid DNA, NT- Non transformed plant serving as a negative control, 1,2,3,4- Genomic DNA of putative transgenic plants in T_1.

Table 3: Effect of auxins and strength of the media on induction of *in vitro* rooting in finger millet

Tr. No.	Auxin (mg.L^{-1}) with ½ MS		Rooting (%)	Days taken for root initiation	No. of roots/plant
	NAA	IBA			
R_1	-	-	34.04	7.7	5.3
R_2	0.50	-	43.66	7.3	6.3
R_3	1.00	-	47.49	6.0	12.3
R_4	1.50	-	45.19	6.3	7.0
R_5	2.00	-	49.99	8.7	8.3
R_6	-	0.50	29.33	9.0	5.7
R_7	-	1.00	26.08	8.3	4.7
R_8	-	1.50	27.74	9.7	5.0
R_9		2.00	33.83	7.7	4.3
S.Em. ±			0.51	0.26	0.25
C.D. at 5%			1.47	0.77	0.72
C.V. %			2.34	3.76	2.94

Discussion

Finger millet variety GN-4 is having a greater yield but major breakthrough is fungal blast. Therefore for development of resistance to fungal pathogen Rs-AFP2 gene was successively transferred in finger millet. Here indirect organogenesis method was used for *in vitro* regeneration using seeds as explants. Less reports of callus induction was obtained from culturing of seeds, but many have reported induction of embryogenic callus from shoot tip as explants [20,21]. MS supplemented with 1.0 mg L^{-1} 2,4-D and other supplements give 57.24% of callus induction callus induction. Anju et al., 2016 [22] obtained 64.93% callus induction frequency for Co (Ra) 14 on MS supplemented with 2 mgL^{-1} 2,4-D and 0.5 mgL^{-1} BAP followed by CO 15 (62.02%) on MS medium supplemented with 5 mgL^{-1} 2,4-D and 0.5 mgL^{-1} Kinetin. In that, CEH is acting as a source of calcium, micronutrients and vitamins, while proline is used for osmotic adjustment and free radical scavenging [23]. BAP in the concentration of 0.50 mg.L^{-1} and Kinetin in 1.50 mg.L^{-1} were effective for shoot initiation and multiplication from soft callus. Further NAA (2.00 mg.L^{-1}) was found to best auxin for good root number and quality within 8.7 days. Satish *et al*. 2015[24] has reported best rooting in half-strength

MS medium containing 2.8 µM indole-3-acetic acid (IAA) in finger millet cultivar 'CO (Ra)-14', 'Piyur-2', and 'Try-1'. In present study, within six days 47.49% of root formation was there using half strength MS and NAA. Combinations of auxins and cytokinins are most often used in plant regeneration experiments, with the actual concentration of

these phyto-hormones varying from genotype to genotype [25]. Among the different subcultures, multiplication rate gradually increased till third subculture afterwards it showed declining trend and even the length of shoot after third subculture decreased gradually, it is similar to the findings of [26, 27]. Soil, sand and vermiculite in equal proportion for hardening and obtained 60% survivability in six genotypes of finger millet [28]. Since finger millet is a monocot plant it has low wound response for *Agrobacterium* infection, and the absence of associated activation of virulence genes in this plant causes a lower transformation efficiency compared with dicot plants [2]. In present investigation, it was found that several parameters like age of explants and days of co-cultivation also effect frequency of transformants.

Conclusion

A reproducible *in vitro* regeneration and transformation protocol has been developed using seed derived callus. PCR analysis of R_1 generation indicates stable inheritance of transgene in fingermillet. Till date very limited protocols are available, so this study proved the utility of development of transgenic plants with other agronomically important traits which enhances commercial cultivation of finger millet.

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