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Biochemical markers assisted screening of *Fusarium* wilt resistant *Musa paradisiaca* (L.) cv. Puttabale micropropagated clones

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An efficient protocol was standardized for screening of panama wilt resistant *Musa paradisiaca* cv. Puttabale clones, an endemic cultivar of Karnataka, India. The synergistic effect of 6-benzyleaminopurine (2 to 6 mg/L) and thidiazuron (0.1 to 0.5 mg/L) on MS medium provoked multiple shoot induction from the excised meristem. An average of 30.10 ± 5.95 shoots was produced per propagule at 4 mg/L 6-benzyleaminopurine and 0.3 mg/L thidiazuron concentrations. Elongation of shoots observed on 5 mg/L BAP augmented medium with a mean length of 8.38 ± 0.30 shoots per propagule. For screening of disease resistant clones, multiple shoot buds were mutated with 0.4% ethyl-methane-sulfonate and cultured on MS medium supplemented with *Fusarium oxysporum* f. sp. *cubense* (FOC) culture filtrate (5–15%). Two month old co-cultivated secondary hardened plants were used for screening of disease resistance against FOC by the determination of biochemical markers such as total phenol, phenylalanine ammonia lyase, oxidative enzymes like peroxidase, polyphenol oxidase, catalase and PR-proteins like chitinase, β-1-3 glucanase activities. The mutated clones of *M. paradisiaca* cv. Puttabale cultured on FOC culture filtrate showed significant increase in the levels of biochemical markers as an indicative of acquiring disease resistant characteristics to FOC wilt.

Keywords: Biochemical markers, Culture filtrate, Disease tolerances, Ethyl-methane-sulfonate, *Fusarium oxysporum* f. sp. *Cubense*

Musa paradisiaca cv. Puttabale (AB group) is an indigenous banana cultivar of Malnad region, Karnataka, India. Fruits are known for their delicious taste and flavour but highly prone to *Fusarium* wilt, caused by the pathogenic fungus *Fusarium oxysporum* f. sp. *cubense* (FOC) and the yield loss is estimated to 30-40%[1]. Few effective and economically safe methods have been developed to protect banana crop from *Fusarium* wilt disease by applying chemicals, fungicides and fumigants[2]. As a result, accumulation of non degradable chemicals in the soil leads to detrimental effects on environment. Alternately, plant breeding techniques acquire the center part for disease resistance lines[3]. However, success rate is very limited in plant breeding due to banana's polyploidy nature, heterozygosity, sterility, low fertility and limited genetic variability[4]. Mutation, somaclonal variation and *in vitro* selection technologies offer opportunities to enhance genetic variability for the improvement of agronomic traits such as disease resistance, than conventional plant breeding methods[5-7]. But, challenged by insertion and expression of a desired genes encoding antifungal activity[8].

Despite, its definite limitations, selection of disease resistance or tolerance regenerated clones by either mutagenic treatment[9,10] or co-cultivation with the fungal pathogen[11,12] are the important tool for the improvement of crop against the *Fusarium* wilt fungi. Use of chemical mutagens such as ethyl-methane-sulfonate (EMS), diethyl sulfate (DES) and sodium azide[13,14] and *Fusarium* culture filtrate or Fusaric acid[15,16] to produce *Fusarium* wilt tolerance has been reported in few varieties of banana[17]. Various biochemical enzymes[18-20] and gene markers[21-23] were reported as the major tools for the identification of disease resistant clones at the early stages. However, mass multiplication and induction of disease resistance Puttabale (AB group) clones against *Fusarium* wilt has not yet been reported. In the present investigation an attempt has been made to produce rapid propagation of plantlets and screening of disease resistance *M. paradisiaca cv.* Puttabale clones against *Fusarium* wilt using biochemical markers.

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Materials and Methods

Isolation of Fusarium—FOC was isolated from rhizomes region of infected *Musa paradisiaca* cv. Puttabale and cultured on potato dextrose agar (PDA) medium. A piece of sporulated mycelia was observed under the microscope for identification and the cultures were preserved at 4 °C.

Micropropagation—The *Musa paradisiaca* cv. Puttabale was regenerated *in vitro* through leaf calli[24]. In the present study banana clones were micropropagated using apical shoot-tip culture with some modification. Healthy and elite side-suckers of *Musa paradisiaca cv.* puttabale were collected from banana farmyards of Malnad region (Shimoga district), Karnataka, India. The suckers were thoroughly washed with tap water and shoot-tip explants prepared by removing the outer leafy sheaths of suckers with a clean knife. Tissue blocks containing shoot-tips and rhizomatous bases were surface sterilized for 5 min in 70% ethanol with two drops of Tween 20 and then with distilled water. The explants were then disinfested with 1% sodium hypochlorite for 10 min containing two drops of Tween 20, rinsed thrice in sterile double distilled water and excised a few outer leafy sheaths and part of corm tissue of disinfested explant. Aseptically, shoots-tips (~3-cm height) were cultured on MS medium[25] augmented with 160 mg/L Adenine sulfate (ADS) and 100 mg/L tyrosine with hormonal range of 2-5 mg/L 6-benzylaminopurine (BAP) and 0.1-0.4 mg/L indole-3-acetic acid (IAA) for initiation of culture. The regenerated shoots obtained from initiation, were cut into smaller pieces (usually 2-4) and transferred to MS medium composed of BAP (2-4 mg/L) and thidiazuron (TDZ) (0.1-0.5 mg/L) for induction of multiple shoot buds. Whereas, the proliferation medium (MS+5 mg/L BAP, 160 mg/L ADS and 100 mg/L tyrosine) was not incorporated with TDZ. When shoot attained a length of 4-5 cm, it is transferred to rooting media consisting of MS basal nutrients augmented with 0.2% activated charcoal without growth regulators for 15 days. The regenerated plantlets with roots were hardened primarily in cocopeats and maintained in polyhouse at 60-70% RH for two weeks. Plants were transferred to greenhouse for secondary hardening in polythene bags containing garden soil, sand and cattle dung manure (1:1:2). Plantlets were watered at 3-day intervals and fertilized twice a week.

Pathogenicity test[26]—(A) Root dipping method: The roots of *in vitro* derived plantlets were injured with a sterilized scalpel and then dipped in fungal spore suspension (10^6 spores/mL) for 30 min under laminar airflow hood in aseptic condition. The roots of the plantlets were blotted and aseptically transferred to the rooting medium fortified with 0.2% activated charcoal. Concomitantly, injured roots of the plantlets were also dipped in sterile water and considered as control.

(B) Pseudostem injecting method: Fungal suspension (100 µL; 10^6 spores/ mL) were injected into the middle part of pseudostem using sterile syringe under aseptic condition. The plantlets were then carefully transferred to the rooting medium and maintained at 26±2 °C with a 16 h photoperiod. Simultaneously, fungal spore suspensions (10^6 spores/mL) were directly inoculated onto the potted plant in *in vivo* condition.

Selection media—Pure culture of FOC was inoculated into potato dextrose broth and incubated on rotary shaker at 80–120 rpm for 15 days. The liquid culture was filtered through a four-layer gauze or cheesecloth and centrifuged at 5000 rpm for 20 min. The supernatant was passed through a membrane filter (0.22 µm pore size) to remove cell debris. The fungal filtrate at the concentration of range 5-15% was augmented aseptically to the autoclaved and cooled selection medium (MS+ 5 mg/L BAP + 5-15% (v/v) FOC culture filtrate) and used as a selection media.

Selection with mutagen treatment—The *in vitro* regenerated multiple shoot buds of banana cv. Puttabale were treated with aqueous solutions of 4% (v/v) dimethylsulphoxide (DMSO) and with a range of 0.1-0.5% (v/v) ethyl methane sulfonate (EMS) respectively. The cultures were agitated on a gyratory shaker (100 rpm) for two hours at 28±2 °C. After washing thrice with sterile double distilled water, the multiple shoot bud clumps were transferred to banana regeneration medium (MS+5 mg/L BAP+ 100 mg/L tyrosine) and cultured for 3 weeks. The well grown regenerants with shoots and roots were acclimatized in greenhouse condition. The plant height of 25-30 cm bearing 4-5 leaves were used for screening of disease resistance banana clones by biochemical markers against FOC fungus.

Selection with EMS and FOC fungal filtrate treatment—After mutagenic treatment (0.4% EMS), the proliferated shoot buds were transferred to the selection medium (MS+ 5 mg/L BAP + 5% (v/v)

FOC culture filtrate + 100 mg/L tyrosine). After four weeks of incubation of each, the growing shoot bud clumps were sequentially sub-cultured to the fresh selection media containing the increased concentrations of FOC culture filtrate 10 and 15 % respectively. After the successive rounds of selection, the clumps of multiple shoot buds, when attained a length of 4-5 cm with 3-4 leaf, were separated and transferred to rooting medium consists of MS basal salts and 0.2% activated charcoal.

For analysis of disease tolerance in *in vitro* condition, the mutagenic and FOC culture filtrate selection clones were aseptically isolated and subjected to *in vitro* pathogenicity test by root dip method and co-cultivated with rooting media. In *in vivo* experiment, the secondary hardened plantlets of selected clones were sporulated with FOC fungus. The pathogenic efficacies of FOC on these selected clones were evaluated by using biochemical markers.

Biochemical assays for screening of disease resistance banana clones—The enzymes activities and biochemical compounds were recorded after inoculation with FOC. Leaf samples were used for preparing the crude enzyme extract for the assessment of peroxidase (PO)[27], polyphenol oxidase (PPO)[28] and phenylalanine ammonia-lyase (PAL)[29] activities. Catalase activity[30] was expressed as µmol H_2O_2/min/g, phenol content[31] was expressed as catechol equivalents/g of protein. The crude enzyme was prepared with 0.05 M sodium acetate *p*H 5.2, 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and 2% polyvinylpolypyrrolidone (PVPP) for assay of chitinase[32] and β-1, 3 – glucanase[33] activities. The experiment was repeated thrice and all the data were analyzed using ezANOVA (0.98 Version) statistical software.

Results and Discussion

Micropropagation—The efficient protocol for mass multiplication of *Musa* species was standardized by many investigators[34-36] and commercialized. But, the optimal response was species and varietal specific[37]. *Musa paradisiaca* cv. Puttabale is an indigenous banana of Karnataka state, India known for its delicious taste but highly prone to *Fusarium* infection. High frequency regeneration of plantlets through leaf calli[24] and immature male flower calli[38] has been reported. In the present study micropropagated clones were derived from shoot tip culture by alterating the hormonal composition and growth adjuvants of the media. For initiation, MS basal nutrients supplemented with 160 mg/L ADS, 100 mg/L tyrosine and at the concentration of 4 mg/L BAP with 0.2 mg/L IAA. The frequency of initiation was noticed with 96.66% survival (Table 1), the explants remained green and shoot initiation was observed after 10 days of incubation (Fig. 1A). The effect of growth adjuvants decreased the oxidative stress of cultured explants as observed earlier[39,40] in sabri cultivar and Kanthali cultivars respectively. In the present study presence of adenine sulfate and tyrosine, exudation of phenolics was minimized. The presence of BAP at 5 mg/L (22.2 µM) was optimum concentration for efficient induction of shoot buds in banana cultivar Brasilian (AAA)[41]. Whereas, in cultivar Puttabale without interaction of the natural auxin IAA with BAP, shoot initiation was not observed and it was optimal at 0.2 mg/L.

The vertically cross excised propagule were subcultured on the multiplication medium fortified with 2-6 mg/L BAP and 0.1-0.5 mg/L TDZ. After 20 days of culture, photosynthetic bulb like structures were noticed and number of shoot bulb production increased upon subculture onto the same media. On an average, at the concentration of 4mg/L BAP and 0.3 mg/L TDZ was optimized with a mean number of 30.10 ± 5.95 shoot bulbs (Table 1) organized per propagule (Fig. 1B). However, further growth did not occur in the same medium and the growth was restricted to a single leaf primordium. The incorporation of BAP alone at the range of 4-6 mg/L in cultivar dwarf Cavendish 'Basrai'[42] was efficient for the induction of multiple shoots. But, in cultivar Puttabale synchronized effect of higher level of BAP and with lower level of TDZ was essential for the induction of multiple shoots from the propagule. The increased number of shoot bulbs was noticed upto 0.3 mg/L TDZ; beyond this concentration, shoots bulb production was decreased. Similar observation was noticed in the culture of banana cultivar Dwarf Cavendish[43]. The elongation of shoot bulb was achieved on proliferation medium augmented with 3-6 mg/L BAP and proliferation was highest at the concentration of 5 mg/L BAP with a mean length of shoots 8.38±0.30 cm/propagule. After 15 days of culture, the shoot bulb elongated with nodes and foliage leaves. Shoot proliferation in banana depends on cultivar. Arinaitwe *et al.*[44] observed that increased BAP above the optimal level did not significantly increase shoot proliferation in cultivar 'kibuzi'. In addition, multiple shoot induction was noticed in each

Table 1— Effects of growth regulators on shoot bud initiation and multiplication from the propagule of *Musa paradisiaca cv*. Puttabale

Plant growth regulators mg/L			Frequency of shoot initiation per explant %	Number of multiple shoot bud per propagule Mean ± S.D	Mean length Shoot per propagule (cm) Mean ± S.D
BA mg/L	IAA mg/L	TDZ mg/L			
2	0.0	-	10.00	-	-
2	0.2	-	20.00	-	-
2	0.4	-	13.33	-	-
3	00	-	23.33	-	-
3	0.2	-	30.00	-	-
3	0.4	-	36.66	-	-
4	0.0	-	56.66	-	-
4	**0.2**	-	**96.66**	-	-
4	0.4	-	66.66	-	-
5	0.0	-	53.33	-	-
5	0.2	-	76.66	-	-
5	0.4	-	60.00	-	-
6	0.0	-	73.33	-	-
6	0.2	-	83.33	-	-
6	0.4	-	56.66	-	-
2	-	0.1	-	04.40±2.88	-
2	-	0.3	-	22.80±2.35	-
2	-	0.5	-	05.70±1.49	-
3	-	0.1	-	07.80±2.35	-
3	-	0.3	-	28.50±6.33	-
3	-	0.5	-	07.10±1.45	-
4	-	0..1	-	10.90±2.13	-
4	-	**0.3**	-	**30.10±5.95**	-
4	-	0.5	-	12.80±2.97	-
5	-	0.1	-	12.10±1.45	-
5	-	0.3	-	26.40±4.25	-
5	-	0.5	-	14.10±2.28	-
6	-	0..1	-	13.50±1.65	--
6	-	0.3	-	23.80±2.78	-
6	-	0.5	-	15.50±3.60	-
2	-	-	-	-	3.01±0.43
3	-	-	-	-	3.68±0.27
4	-	-	-	-	6.58±0.41
5	-	-	-	-	**8.38±0.30**
6	-	-	-	-	7.28±0.34
F Value				**67.6**	433

The value of combination consisted of mean ± S.D. of 10 replicates.
The F-value is significantly different when $P< 0.05$.

shoot bulb (Fig 1C). The elongated shoots were transferred to rooting medium containing 0.2% activated charcoal. Similar to the work of Jarret *et al.,*[45] in the present study also roots were initiated from the base of small shoots after three weeks of culture (Fig. 1D). When shoots attained a length of 5-6 cm, they were transferred to poly trays and maintained in the green house for primary hardening (Fig. 1E). After one month, plantlets were transferred to poly bags and secondary hardened in the greenhouse (Fig. 1F) for two months. For the first time, an efficient protocol for mass multiplication of the banana cultivar Puttabale is reported here. With this protocol it is possible to produce 960 plantlets in a span of 8 months. The *in vitro* regenerated clones at the rooting stage and the two months old secondary hardened plants were used for pathogenicity test and biochemical markers assays.

Evaluation of pathogenesity — FOC isolated from rhizomes of infected *Musa paradisiaca* cv. Puttabale and pure culture on PDA medium form whitish cottony thread like colony (Fig. 2A) with multi-cellular spindle like spore under the microscope (Fig. 2B). Applying of spore suspension (10^6 spores/mL) to potted plant is an effective method

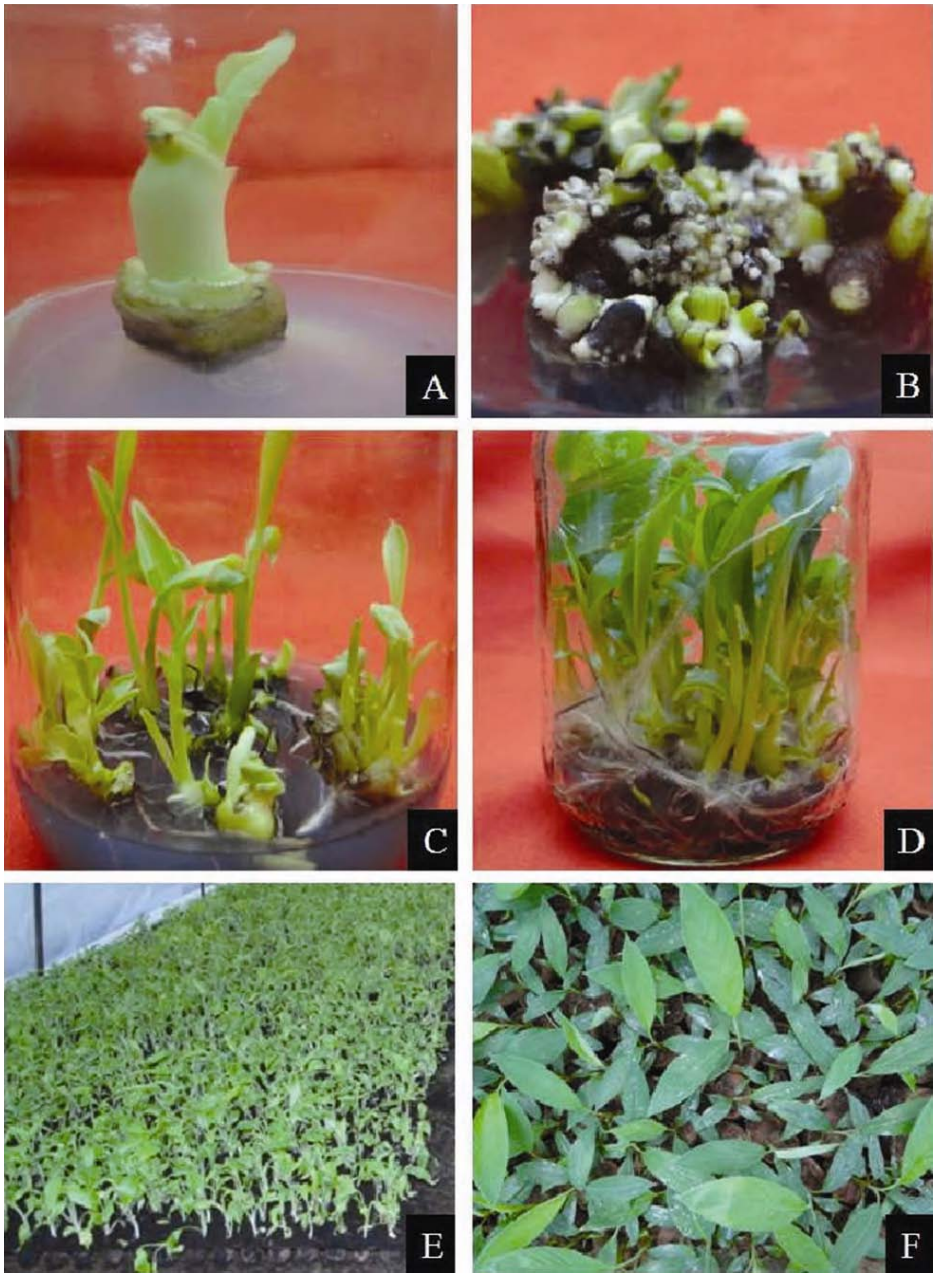

Fig. 1— A. sprouting of shoots from shoot tips culture, B: multiple shoot bulbs organized per propagule, C: elongation of shoot bulbs, D: rooting achieved in basal media with 0.2% activated charcoal, E: primary hardening of regenerants in cocopeat, F: secondary hardening of regenerants in Polythene bags.

for the induction pathogenicity[46]. The fungus grows much faster in culture medium than the plant tissues and quickly dominates the culture media leading to the obstruction of plant tissue growth by lack of nutrients or space, rather than susceptibility to the disease. The injured roots of the *in vitro* derived plantlets dipped in FOC spore suspension (10^6 spores/mL) showed symptoms of *Fusarium* wilt within fifth day of incubation. The leaf veins showed chlorosis gradually entire lamina turned yellow and resulted in the wilting of the leaves (Fig. 2C). Simultaneously, the fungal colony flourished on the medium in the form of whitish cottony thread like hyphae. In 26^{th} day old culture, the fungal colony completely spreads on the medium with reddish brown colour exudation into the medium, as a result complete wilting of the plantlet occurred (Fig. 2D).

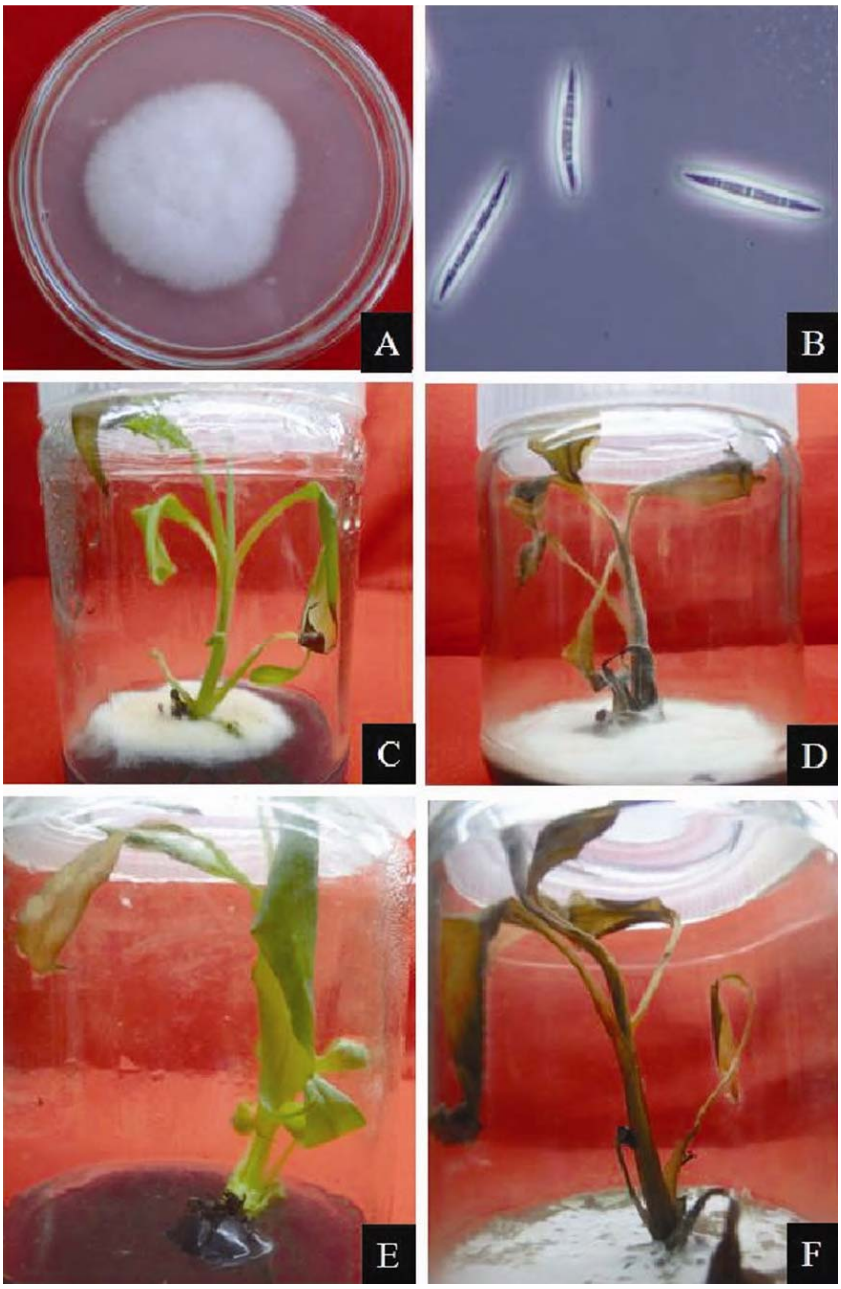

Fig. 2— A: colony of *Fusarium oxysporum* f. sp. *Cubense*, B: spores of FOC, C: initiation stage of FOC infection by root dip method, D: complete wilting of the leaves by wilting of the leaves, E: initiation stage of FOC infection by pseudostem injection method, F: later stage FOC infection by pseudostem injection method.

The *in vitro* regenerants directly injected with 100 µL of fungal suspension through the pseudostem exhibited the symptoms of *Fusarium* wilt after seven days of incubation. Initially, tender leaf and the apical meristem showed chlorosis and wilting (Fig. 2E), progressively it spreads to the other parts of the plant and finally the media also populated with the flourished colony of the fungi (Fig. 2F). Among these methods, efficient induction of *Fusarium* disease to banana plants occurred in root dip method. On the contrary, Steventon *et al.*[47] reported that the direct injection method was best for the induction *xanthomonas* wilt in banana. This variation is due to the nature of host and pathogen interaction.

The fungal spore suspensions (10^6 spores/mL) applied to potted plant at *in vivo* condition showed symptoms of *Fusarium* wilt after 14 days of inoculation (Fig. 3A). Initially, appearance of slight

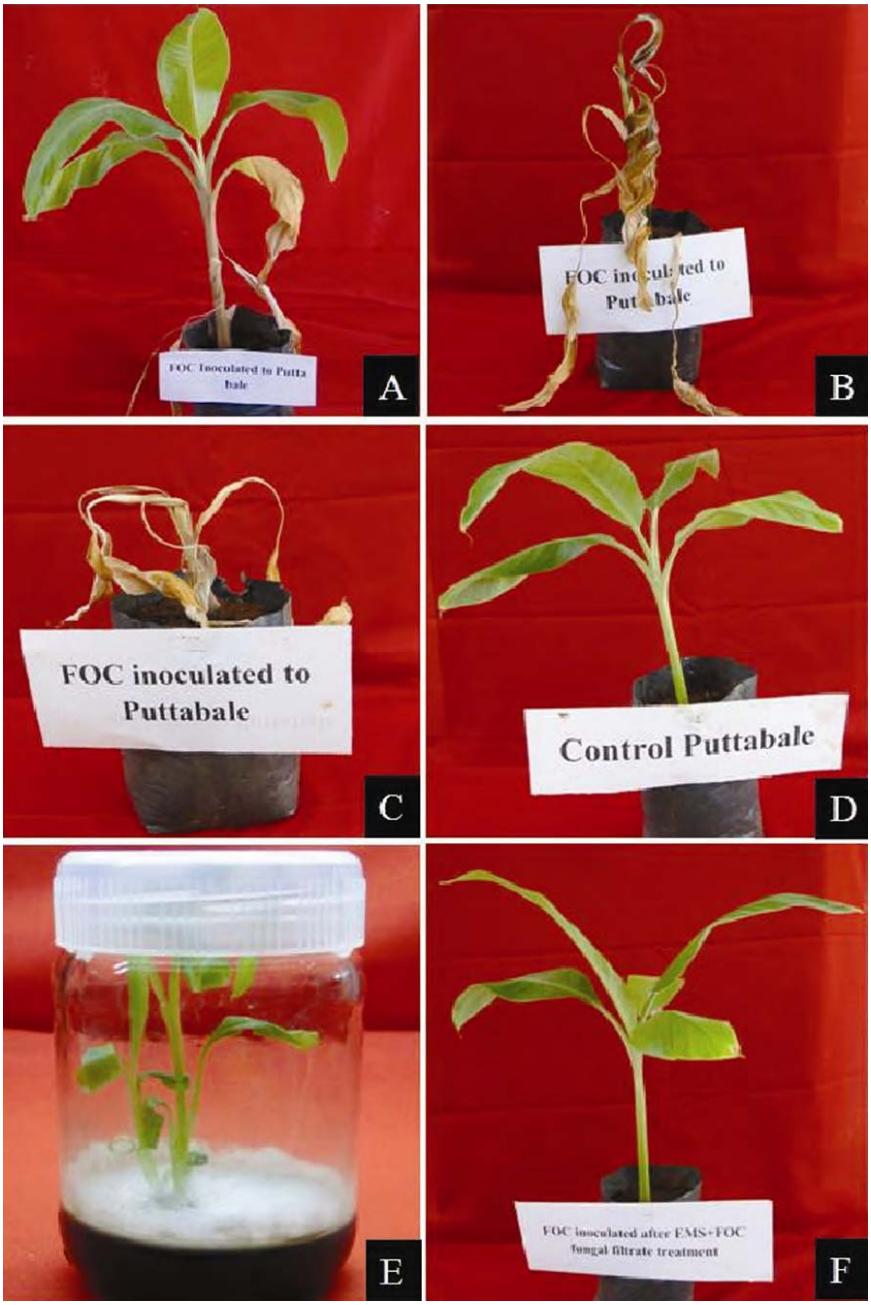

Fig. 3— A: *Fusarium* wilt symptoms after 14 days of FOC inoculation, B: after day 45th of FOC inoculation, C: complete death at 60th day, D: control plant with out FOC inoculation, E: no disease symptoms in EMS+FOC culture filtrate derived plantlets in *in vitro* condition, F: no disease symptoms in EMS+FOC culture filtrate derived plantlets even 60th day of FOC inoculation in potted plantlets.

vein clearing on the outer portion of the younger leaves, then epinasty (downward drooping) followed by stunting, chlorosis, yellowing of the lower leaves, wilting of leaves and young stems. Defoliation and necrosis of remaining leaves was observed after 50th day (Fig. 3B). Finally, whole canopy died at 60th day (Fig. 3C). Where as, the control plants inoculated with distilled water remained healthy (Fig 3D). FOC fungus was reisolated from infected plantlets for further experiments.

Selection with mutagenic treatment—Induction of mutation in multiple shoot buds by EMS at the concentrations range 0.1-0.5% revealed the decrease in the meristem growth and decline in survival of the plants. Bidabadi *et al.*[48] also reported gradual reduction in survival, growth and regeneration

capacity of the treated shoot tips in banana cultivar 'Berangan Intan', 'Berangan' (AAA group) and 'Rastali' (AAB group), when concentration and duration of EMS treatment increased. In the present study, decline in survival of the mutated plants was observed beyond 0.5% treatment of EMS. On the contrary, the mutated plants acquired tolerance characteristics against the pathogenic fungi. In 0.4% EMS treated plants, symptoms of *Fusarium* wilt were seen after 45 days of sporulation (Table 2) .Whereas, at the lower concentration of EMS, disease symptoms were observed in early days and above 0.5% the plants became dead due to mutation. This clearly indicates that, EMS treated banana clones may or may not be tolerant to FOC. The results are in agreement with the work of Omar *et al.*[13].

Selection with EMS+FOC culture filtrate treatment—The regenerated clones were treated with 0.4% EMS, cultured on the selection media (5–15%) showed healthy growth even with flourished growth of pathogen (Fig. 3E). But, *Fusarium* wilt symptoms was observed after 45 days of co-cultivation with FOC. Where as, *in vivo* condition, symptoms of panama disease was not observed even after 60 days of sporulation (Fig. 3F). Bioactive constituents of culture filtrate may be responsible for disease tolerance and proven as an effective selective agent for screening of FOC resistance. Similar types of results have been reported by Thakur *et al.*[49] in variants of Carnation and Vanilla. Hence, selection using culture filtrate may be more effective for acquiring disease tolerant plant. Companioni *et al.*[11,12] induced disease resistant banana clones by the inoculation of concentrated culture-filtrate onto needle-mediated wounds of middle-aged banana leaves. This evaluation method is of great interest not only because it is non-destructive but also due to the very short time needed for evaluation. Rully *et al.*[50] reported that 0.6% EMS treated banana cv. Tangonon and Sangihe-1 cultured on medium without addition

Table 2— Determination of enzyme activities in leaves of *Musa paradisiaca* cv. Puttabale after EMS and FOC culture filtrate treatment challenged with *Fusarium oxysporum* f. sp. *cubense* (FOC).

Treatment	Total phenols	β-1,3 Glucanase	Chitinase	Phenylalanine ammonia-lyase	Polyphenol oxidase	Peroxidase	Catalase
Control	520.44±2.75	152.55±2.38	65.41±1.10	22.14±0.94	1.29±0.04	1.16±0.01	8.40±0.32
FOC inoculated	638.13±6.80 **	172.64±1.78 **	78.36±1.11 **	28.46±1.16 *	1.90±0.04 **	1.71±0.01 **	11.48±0.67**
FOC inoculated after 0.1% EMS treatment	649.93±0.66 **	166.86±1.06 **	63.34±0.63 Ns	24.86±0.86 Ns	1.85±0.02 **	1.62±0.01 **	9.82±0.20 **
FOC inoculated after 0.2% EMS treatment	641.25±0.56 **	166.38±1.05 **	66.95±0.71 **	24.75±1.24 Ns	1.79±0.02 **	1.64±0.02 **	10.46±0.36 **
FOC inoculated after 0.3% EMS treatment	635.30±0.60 **	162.89±1.03 *	69.42±0.58 *	25.87±0.40 *	1.77±0.02 **	1.66±0.02 **	11.04±0.36 **
FOC inoculated after 0.4% EMS treatment	695.64±2.37 **	202.08±2.11 **	87.11±1.24 **	32.16±0.81 **	2.05±0.05 **	1.91±0.04 **	14.45±0.46 **
FOC inoculated after 0.5% EMS treatment	656.05±1.79 **	169.37±0.44 **	72.83±0.28 **	28.99±0.37 **	1.94±0.01 **	1.91±0.02 **	12.50±0.15 **
FOC inoculated after 0.4% EMS + FOC culture filtrate treatment	839.74±1.50 **	307.87±1.17 **	168.20±0.44 **	50.52±1.18 **	3.11±0.08 **	3.72±0.08 **	27.22±0.79 **

Values of each treatment consisted of Mean ± S.E. of three replicates. Symbols represent statistical significance.* $p < 0.05$, ** $p < 0.01$ as compared to control. ns= not significant

The values of enzyme activities are expressed as: Total phenols – Catechol equivalents/g of fresh tissue; β -1,3 Glucanase- µg of glucose released/min/g of fresh tissue; Chitinase – nmol of N-acetylglucosamine equivalent/min/g of fresh tissue; Phenylalanine ammonia-lyase – µmol of Transcinnamic acid/min/g of fresh tissue; Polyphenol oxidase - nmol of catechol/min/g fresh weight, Peroxidase – nmol H_2O_2/min/g fresh weight & Catalase – µmol of H_2O_2/min/g of fresh weight.

of FOC culture filtrate showed inhibition of shoot proliferation. On medium containing 40% of FOC culture filtrate, the 0.6% EMS treated banana cultivars were able to generate shoots but most of the shoots were rotted and died. In this study, regeneration potency of shoot buds was not suppressed at 0.4% EMS and 5-15% FOC culture filtrate. Borras *et al.*[51] also reported that fungal filtrate could act as strong elicitors of plant defense reactions. Increase in biochemical markers is an indicative of disease tolerance. Hence, the level of tolerance was evaluated by biochemical markers against FOC (Table 2).

Screening of FOC wilt resistance clones by biochemical markers—Inducing the plant's own defense mechanisms by prior application of a biological inducer is thought to be a novel plant protection strategy and produces defense related compounds and enzyme activity such as total phenols, PAL and oxidative enzymes viz., PO, PPO, catalase, and PR-proteins like chitinase, β-1, 3 glucanase activity when challenged with FOC. In the present study, EMS+FOC culture filtrate treated *Musa paradisiaca* cv. Puttabale clones was found to be effective in reducing the incidence of *Fusarium* wilt under greenhouse condition (Table 2).

Total phenolics—Phenolics are involved in phytoalexins accumulation, biosynthesis of lignin and formation of structural barriers against pathogens. In *Musa paradisiaca* cv. Puttabale when FOC was inoculated, total phenol content was increased by 0.21 fold than the control. After 0.4 %EMS treatment, a slight increase in the accumulation of phenol to 0.31 fold was observed. The synergistic effect of EMS and FOC culture filtrate treatment increases as compared to control plants. Enhanced phenolic biosynthesis protects the crop plants against the pathogen infection as similar to the report of De Ascensao ARDCF *et al.*[52].

Phenylalanine ammonia-lyase (PAL) activity—PAL is the most important enzyme in phenylpropanoid metabolism for the synthesis of phenolics, phytoalexin and lignin. The PAL activity could be increased in plant pathogen interactions and fungal elicitor treatment[53]. Chen *et al.*[54] reported that high levels of PAL induced in cucumber roots when inoculated with *Pythium aphanidermatum.* Initially, PAL activity in puttabale clones was lower level and higher after challenging with FOC. The FOC inoculated clones showed 0.28 folds increased PAL activity than control clones. The PAL activity was increased to 1.3 folds after EMS and *Fusarium* culture filtrate treatment.

Peroxidase (PO) activity—PO catalyses H_2O_2-dependent condensation of phenolics into lignin, a structural barrier playing specific role to protect from pathogen[55]. In this study, PO activity in FOC inoculated clones increased to 0.2 fold. After EMS treatment, the level of PO increased to 0.69 fold than control. EMS+FOC culture filtrate treated clones further increases the level of PO to 3.21 fold. However, early induction of PO activity in susceptible variety conferred the stimulation of plant defense response and prevents the entry of pathogen[56].

Polyphenol oxidase (PPO) activity—PPO is copper containing enzyme, which oxidize phenolics to quinines and concerned to terminal oxidation of diseased plant tissue, which was accredited role in disease resistance. PPO activity increased to 0.51 fold in FOC inoculated clones. After EMS-treatment, clones showed elevated levels of PPO activity to 0.62 fold and 1.3 fold in EMS+FOC culture filtrate clones than control. Similarly, report was observed in banana roots treated with *F. oxysporum* f. sp. *cubense*-derived elicitors[57].

Catalase activity—Increased *catalase* activity provides a protection from oxidative damage by rapid removal of H_2O_2 in tolerant lines than resistant lines. The present study 3.25 fold catalase activities increased in EMS+cultured fungal filtrate clones than control, 0.75 fold in EMS treatment and 0.38 fold in FOC inoculated clones. These results are in agreement with antioxidant enzymes in tomato plant against *Fusarium wilt*[58].

β-1, 3-glucanase and chitinase activities—β-1,3-glucan and chitin are the major cell wall components of fungi. Since β -1, 3-glucanase and Chitinase have been shown to be capable of attacking cell wall of fungal pathogens, these enzymes have been considered as direct defense enzymes of the plants[18]. Only FOC inoculated clones showed slight increase in β-1, 3-*Glucanase* activity by 0.15 fold than the control**.** Where as, the clones treated with EMS+fungal culture filtrate showed increased β-1, 3-glucanase activity to 1.0 fold as compared to the control. The chitinase activity increased after EMS treatment to 0.35 folds. EMS+fungal culture filtrate clones showed increased chitinase activity to 1.58 fold than the control and 0.19 fold in FOC inoculated clones. The increase in β-1, 3-glucanase and chitinase activities are an indicative of inducing resistance and protecting to banana plants from *Fusarium* wilt. Similarly, reports of Mauch *et al.*[59]

also indicated that induction of chitinase and β-1,3-glucanase have been concerned in defense against further invasion of the pathogen in to banana plants.

Biochemical markers may or may not be part of the resistance mechanism but can be used to predict tolerance and resistance in banana to FOC. In the present study, a slight increase in defense enzyme activities were noticed in EMS treated clones as compared to control. But, combination of EMS+FOC culture filtrate clones showed significant increase in enzyme activities than the control and they did not showed symptoms of *Fusarium* wilt disease even after 60^{th} day of FOC inoculation. Hence, EMS+FOC culture filtrate treatment are the strong elicitor against *Fusarium* pathogen in banana cultivar puttabale.

Conclusion

Establishment of disease resistance plants depends on reproducible plant regeneration protocol, mutagenic action and efficient screening techniques. This study clearly describe the synergistic effect of EMS+FOC culture filtrate to induce disease resistance in *Musa paradisiaca* cv. Puttabale clones against *Fusarium oxysporum* f. sp. *Cubense* and the level of tolerance was evaluated by the estimation of defense enzyme activities.

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