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In vitro establishment of tolerant clones of banana against race-1 *Fusarium oxysporum* f. sp. *cubense*

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Abstract

A detailed study was conducted to develop gamma irradiation induced resistant clone of banana cultivar Rasthali against race-1 *Fusarium oxysporum* f. sp. *cubense.* Shoot buds of banana cultivar Rasthali irradiated by using Co^{60} in a gamma chamber at Indira Gandhi Center for Atomic Research, Kalpakkam, India were used for *in vitro* culture establishment and to develop resistant clones to race 1 of *Fusarium oxysporum* f. sp. *cubense.* The shoot buds were irradiated at doses *viz*., 20, 40, 60, 80 and 100 Gy Rad. In the treatment 40 Gy Rad treated shoot buds had the maximum shoots per culture and maximum per cent of culture establishment. Other doses *viz*., 60, 80 and 100 Gy Rad were found to inhibit the culture establishment when compared to untreated control. The shoots irradiated at 40 Gy Rad were used to develop resistant clones against toxins of race 1 of *F. oxysporum* f. sp. *cubense*. Firstly, to aim at standardizing an appropriate concentration of the culture filtrate of the pathogen for the tolerant clones selection, the multiple bud clumps were cultured on the Murashige and Skoog medium supplemented with 2 to 15 % crude culture filtrates. The growth on cultivars completely inhibited on the medium containing 10% culture filtrate. In the successive selection, the rate of survival of plantlets increased from 6.5 to 56.4% in the medium containing 10% culture filtrate of *F. oxysporum* f. sp. *cubense.* 50 per cent symptom less plantlets were obtained from 40 Gy Rad irradiated and survived plant lets under greenhouse conditions. The activity of peroxidase enzyme was more in the tolerant plantlets and four fold increased in their activity at 21 days after inoculation of *F. oxysporum* f. sp. *cubense.*

Key words: Fusarium wilt, banana, irradiation, tolerance, clones

Introduction

Fusarium wilt is a serious disease of banana in many cultivars. Chemical control of this disease is economically impracticable and its efficient control has been based on the use of tolerant varieties (Stover and Simmonds, 1987). Use of resistant variety is one of the methods to manage the disease problems in crop plants. Although there are varieties resistant to this disease (Ploetz *et al.*, 1990), transfer of the resistant genes into susceptible varieties by using traditional cross breeding is difficult because of triploidy and poor seed production (Novak, 1992). *In vitro* production of crop plants through tissue culture with toxin used selection is currently becoming popular. *In vitro* breeding combined with toxin selection is a very promising tool for induced mutation study. Mutation induced in the tissue culture have tolerance to the disease and was successfully exploited in other crops (Toyoda *et al.*, 1988; Wenzel and Wehr, 1990). On the other hand, it was shown that fusaric acid, a toxin responsible for the wilt symptom of the disease, was useful for the selection of resistant clones (Matsumoto *et al.*, 1995). Earlier, five per cent somaclonal variants by using an *in vitro* method for mass production of planting materials free of race 4 of *F. oxysporum* f. sp. *cubense* has been observed (Hwang *et al.*, 1984). Development of tolerant or resistant clones in toxin used method is now popular and useful to overcome problems prevailing in the conventional breeding methods. Study was undertaken to identify the effect of gamma irradiation on the shoot development and to develop toxin tolerant clones in banana against race 1 of *F. oxysporum* f. sp. *cubense*. In addition to this, the degree of tolerance mechanisms due to activity of peroxidase was assessed in the study.

Materials and methods

Extraction of culture filtrate of *F. oxysporum* f. sp. cubense: A nine mm disc of potato dextrose medium with a fungal colony of race 1 of *F. oxysporum* f. sp. *cubense* was inoculated in 100 ml Zapek Dox broth medium in a 250 ml Erlenmeyer flask. The cultures were incubated at room temperature (28 ± 2°C) for 21 days. The cultured liquid media were then filtered through four layers of cheese cloth and centrifuged at 10000 rpm for 20 min. The supernatant was filtered through a membrane filter (0.45mm pore size) to remove the fungal fragments completely. The filtrate used as toxins for the study.

Irradiation of plant material and culture conditions: Shoots of cultivar Rasthali (*Musa* spp.) which is susceptible to race-1 Fusarium wilt was used in this study. Rhizomes were collected from five month old plants, washed, roots and outer layer of tissues were removed and shoot tips measuring about 2 cm wide at the based were excised from the rhizomes and then they were air dried and packed in a polybag without any airspace under aseptic condition. The packed shoot tips were irradiated in 900 Gamma chamber at Indira Gandhi Center for Atomic Research, Kalpakkam by using Co^{60}. The irradiation was carried out at various doses *viz*., 20, 40, 60, 80 and 100 Gy Rad. Each bag containing 25 shoot buds was kept as replication. Four

replications were maintained. After irradiation, the shoot buds were surface sterilized with 0.1 % mercuric chloride for one min, washed with sterile water and again placed in 70 % ethanol for one min. The shoot buds washed with sterile water, were placed in Murashige and Skoog medium supplemented with 4 mg/l Benzyl Amino Purine (BAP) (the concentration of BAP already standardized in the study), 3% sucrose and incubated at 24 ± 2°C with 2000 lux light intensity under 14 h photoperiod by cool white fluorescent lights (56 m Mm^{-2} s^{-1}) (Matsumoto *et al.*, 1995). The unirradiated shoot tips grown as control. After 25 days of incubation, the responses of irradiated shoot tips were observed. The developed shoots from 40 Gy Rad doses were subcultured to fresh medium of same compositions. Second subculturing was done 10 days later and cluster of shoots were separated and used for identification of tolerant clones.

Standardization of culture filtrates of *F. oxysporum* f. sp. *cubense* against growth of MBCs: *In vitro* growth of multiple bud clumps (MBC) inhibition by culture filtrate of *F. oxysporum* f. sp. *cubense* in the proliferation medium was studied with a view to standardize an appropriate concentration for the selection of tolerant mutants. MBCs raised from dose of 40 Gy Rad were transferred to the above-referred medium supplemented with culture filtrate of *F. oxysporum* f. sp. *cubense* (2 to 15 %). The culture filtrate was added to the autoclaved medium, after filter sterilization through a membrane filter (0.45 mm pore size). The cultures were maintained in a controlled environment room at 24 ± 2°C with 2000 lux light intensity under 14 h photo-period by cool white fluorescent lights. After one month of culture, fresh weight of growing MBCs was measured.

Establishment of tolerant clones against the culture filtrates: The separated shoots were placed in MS medium supplemented with 4 mg/l BAP, 3% sucrose and amended with 10 % culture filtrate of *F. oxysporum* f. sp. *cubense* and incubated in the controlled condition (24 ± 2°C with 2000 lux light intensity under 14 h photoperiod by cool white fluorescent lights). After one month of the incubation, the buds were again subcultured with same concentration of the toxins in the MS medium. This selection process was repeated a further two times using the same concentration of toxins to reduce mutants and survivors by escape from the selection. After the successive selection, the growing mutants buds were subcultured another two times on the proliferation medium (MS medium + 4 mg L^{-1} BAP without toxin) for one month and transferred to regeneration medium composed of MS salts and 8 mg L^{-1} vitamins, 3 % sucrose, 0.25 % gellan gum and 1.55m mol α- naphthalene acetic acid (NAA). After one month of culture, regenerated plantlets were transferred to black polythene bags containing 2 L soil consisted of a mixture of loamy soil, sand and farm yard manure (2:1:1 v/v) in a greenhouse for further testing of growth. After 3 months, they were transferred into in a pot containing 5 kg of same type of soil with 5 % sand maize inoculum of pathogen *F. oxysporum* f. sp. *cubense* (10^6 cfu g^{-1} of inoculum). They were maintained for one month to select resistant plantlets and the disease symptoms were evaluated.

Assay of peroxidase activity: Banana plantlets were grown and rooted under axenic conditions as described by Novak *et al.* (1987). Roots were trimmed and plantlets were treated with microconidia suspension (5 x 10^5 conidia ml^{-1}) as described by Hwang and Ko (1987). Controls were treated with distilled water only. Treated and non treated plants were transplanted in sterile vermiculite and placed in a growth chamber at 29°C. At weekly intervals, two plantlets were removed and assayed for peroxidase activity. Root and leaf tissues were ground in mortar the tissue 0.1 M sodium phosphate buffer pH 6.8 (1.2w/v). The resulting homogenate was centrifuged at 14,000 rpm in an Eppendorf centrifuge and assayed for protein content according to Bradford (1976). In addition, the methods described by Garaway *et al.* (1989) were used to extract ionically bound peroxidase. Activity was measured in 1ml final volume reaction mixture. 5ml of tissue extracts were incubated in 0.3% guaiacol, 2mM hydrogen peroxide in 0.01M phosphate buffer, pH 6.0. After two minutes absorbance was read at a wavelength of 470nm and the protein content assayed.

Statistical analysis: The experimental design was completely randomized. Percent data were transformed to arc sine transformation, used for analysis and back transformed. Data were subjected to statistical analysis using Duncan's Multiple Range Test, t- statistics and Fischer exact test.

Results and discussion

The results of the study revealed that the gamma irradiation significantly influences the culture establishment. Among the doses used in the study, 40 Gy Rad irradiation induced the maximum culture and number of shoots per culture which was followed by 20 Gy Rad (Table 1). The initial profusely proliferating culture was first noted from an explant irradiation at 40 Gy Rad. Doses *viz.*, 60, 80 and 100 Gy Rad levels were found to inhibit the shoot establishment when compared with unirradiated control. Earlier, De Guzman *et al.* (1980) reported that the gamma irradiation at low dosage could be stimulatory to bud formation and higher dose of irradiation created several morphological aberrations in shoots. So, 40 Gy Rad dose irradiated shoots was used for developing tolerant clones. After gamma irradiation, the shoots were grown in the medium containing various concentration of the culture filtrate of *F. oxysporum* f. sp. *cubense* to standardize the concentration. It was found that the growth of culture was completely inhibited on the medium containing 10% culture filtrate (Table 2). The multiple bud clumps tolerant to the culture filtrate were selected. The successive selection increased the surviving bud clumps from 6.5 to 56.4% in the medium containing 10% culture filtrate (Table 3). Similar results were observed in alfalfa (Arcioni *et al.,* 1987) and in banana (Matsumoto *et al.,* 1999). It was also observed that gamma irradiated buds regenerated the disease tolerant plants more frequently than treated control. After the gamma irradiation of 250 shoots, the 104 toxin resistant clumps were selected, 13.52 plants which showed resistant to the disease under green house conditions (Table 4). This indicated high level of somaclonal variation and mitotic instability (Reuveni and Israeli, 1990; Israeli *et al.*, 1991; Shepherd, 1996)

In the experiments, the plants regenerated from the multiple clumps resistant to the culture filtrates were also resistant to the disease in the green house. The disease resistance was further confirmed under green house conditions. The tests did not show clear but showed a level of tendency of the number of increased symptom less plants in the gamma-irradiated plantlets. Screening of large number of shoots against the wilt disease by using toxin may show little tolerance variation against the disease. Only five

variants showed tolerance to the wilt disease out of 20,000 in banana (Sita Hawa and Zakari, 1997).

Table 1. Effect of gamma irradiation on culture establishment in banana cv. Rasthali

Doses of gamma irradiation (Gy Rad)	*Percentage of culture establishment	*Number of shoots per culture
20	73.33(59.01) c	5.39 e
40	89.17(70.81) a	6.12 ef
60	41.48(40.09) d	2.79 c
80	19.27(26.04) e	1.06 ab
100	9.29(17.74) f	0.92 a
Control (unirradiated)	80.33(23.67) b	4.55 d
CD (p= 0.05)	3.46	0.66

*Mean of four replications

Values in parentheses are arcsine transformed values, mean followed by common letters are not significantly differ at p= 0.05 level as per Duncan's Multiple Range Test.

Table 2. Effect of culture filtrate of *Fusarium oxysporum* f. sp. *cubense* on the survival of multiple bud clumps

Concentration of culture filtrate (%)	Survival of multiple bud clumps (%)
2	64.89(54.06) b
4	43.56(41.29) c
6	15.57(23.75) d
8	11.11(19.45) de
10	0.00(0.91) f
12	0.00(0.91) f
14	0.00(0.91) f
15	0.00(0.91) f
Control	80.33(63.68) a

Values in parentheses are arcsine-transformed values,
Mean followed by common letters are not significantly different at p= 0.05 level as per Duncan's Multiple Range Test.

Table 3. *In vitro* selection of tolerant shoots to 10 % culture filtrates in a proliferation medium after one month of culture

Plantlets	Explants		
	Observed (number)	Survived (%)	Killed (%)
Gamma irradiated	85	56.4	44.6
Control (unirradiated)	17	6.5	83.5

Data showed significance at 1% level by Chi square test.

Table 4. Expression of symptom of vascular discolouration caused by race 1 of *Fusarium oxysporum* f. sp. *cubense* in plantlets under green house conditions

Explants	Explants observed	Plants showed symptoms	Survived plants
Gamma irradiated	26	12.48	13.52
Control (unirradiated)	30	23.68	4.32
Robusta cultivar	37	6.21	30.79

Robusta was tested as control for race of *Fusarium oxysporum* f. sp. *cubense* wilt resistance for comparing between control of gamma irradiated and irradiated plant lines. Data showed significance level of 0.412 for survived plants by Fischer's Exact Test.

In the experiments the plantlets which were regenerated from the multiple bud clumps tolerant to the culture filtrate were also resistant to the disease in the greenhouse conditions. However,

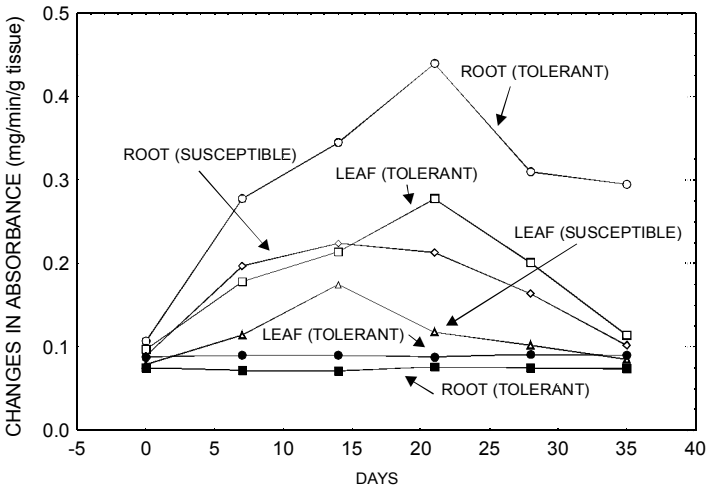

Fig. 1. Changes in peroxidase activity of susceptible and tolerant root and leaf

selection of toxin resistant ones was probably not the main response to pathogen but have acquired partial resistance to the disease delaying the pathogens growth and causes morphological changes like gel and/or tyloses production there it makes the host unfavourable to the pathogen. The survived tolerant and susceptible plantlets under green house conditions were used for assaying the peroxidase enzyme activity.

Root and leaf tissues of survived plants were analyzed for constitutive peroxidase activity. In tolerant plantlets, the highest peroxidase activity was found in root tissue. While the lowest activity was recorded in leaf tissue. However, the tolerant plantlets showed marked differences in peroxidase activity. In plantlets infected with *F. oxysporum* f. sp. *cubense* race1, both tolerant and susceptible plantlets showed a prompt increase in peroxidase activity with days after inoculation (Fig. 1). The general response of the irradiated tolerant and susceptible plantlets to race-1 was almost similar, with steady induction and a high level of activity over the whole experimental period.

Peroxidase is a multipurpose enzyme that catalyzes the condensation of phenolic compounds into lignin. The present strategy that involves peroxidases in defense mechanisms considers the condensation of phenolics derived from the phenyl propanoid pathway into insoluble polymers (Robb *et al.*, 1991). Peroxidase plays an important early and specific role in the hypersensitive containment of the pathogen (Peng and Kuc, 1992; Graham and Graham, 1991).

The results of this study demonstrated that the tolerant plantlets responded actively to infection of race-1 of *F. oxysporum* f.sp. *cubense*. The fact that in the compatible interaction, peroxidase activity increases rapidly suggests that in banana this enzyme might be involved in the defense response. These observations are in agreement with the model proposed by Morpurgo *et al.* (1994). The magnitude of induction of the defense mechanisms appears to be critical for the expression of resistance (Reuveni *et al.* (1992). Moreover, the activity of constitutive enzyme shows positive correlation between higher activity in tolerant and susceptible plantlets and the activity was rapidly elicited in response to pathogen infection. This finding is in agreement with the systemic defense mechanisms introduced by Hammerschmidt *et al.* (1982). However, this work was done using only irradiated tolerant plantlets and susceptible plantlets and

the actual role of peroxidase in the containment of *F. oxysporum* f. sp. *cubense* should be explained by a more elaborate screening by using different cultivars of *Musa*.

To conclude, this work shows that gamma irradiation at 40 Gy rad induced tolerance against the pathogen and degree of tolerance was explained by increased activity of peroxidase in the plantlets. But it needs more extensive research on the physiological and morphological mechanisms that narrates the resistance or susceptibility of banana to *F. oxysporum* f. sp. *cubense*.

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