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ORIGINAL ARTICLES

Screening of *In-vitro* Derived Mutants of Banana Against Nematodes Using Bio-chemical Parameters

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ABSTRACT

Investigations were carried out to screen the *in-vitro* derived mutants of banana cv. Robusta (Caveidish- AAA) and Rasthali (Silk- AAB) at Department of Fruit Crops, TNAU, Coimbatore, India by using certain bio-chemical parameters including some enzyme activities. The mutants tested were Ro Im V_4 6-1-1, Ro Im V_4 6-1-2, Ro Im V_4 6-2-1, Si Im V_4 10-5-3, Si Im V_4 6-2-5 along with respective susceptible checks (Robusta and Rasthali), tolerant check (Anaikomban- AA) and resistant check (Pisang Lilin- AA). Various bio-chemical assays used were total phenol, tannin content, lignin content, peroxidase, poly phenol oxidase, phenyl alanine ammonia lyase and ascorbic acid oxidase. The results revealed that the mutants namely Ro Im V_4 6-1-1 and Si Im V_4 10-5-3 were found to be resistant while the mutant Ro Im V_4 6-2-1 was moderately resistant. The rest of the mutants namely Ro Im V_4 6-1-2 and Si Im V_4 6-2-5 were found to be susceptible to nematodes. The resistant and moderately resistant mutants of banana could be further used in breeding programmes as well as be recognized as potential cultivars of commerce.

Key words: banana, nematode, resistance, biochemical parameters, enzymes, screening

Introduction

Musa production is threatened by pest and disease pressure, which has been increasing during the past 20 years. Most alarming has been the spread of more virulent types of nematodes like Burrowing nematode (*Radopholus similis*), Root lesion nematode (*Pratylenchus sp*), Root knot nematode (*Meloidogyne incognita*) apart from the serious threat by diseases like, Banana bunchy top disease, Sigatoka leaf spots (*Mycosphaerella sp)* and *Fusarium* wilt (*Fusarium oxysporum f. sp. cubense).* Crop losses caused by nematodes are very high, with 20% annual yield losses worldwide (15). The existing practice of chemical control of nematodes leaves lot of residues causing much threat to the environment. Hence, there is a need to develop commercially acceptable types of banana with resistance /tolerance to this biotic stresses. In response to these production constraints, efforts aimed at the genetic improvement of *Musa* have gained renewed interest to generate resistant cultivars. Classical breeding consisting of recombination and selection is difficult for banana. Polyploidy and sterility are both serious handicaps in the genetic improvement of *Musa* cultivars. An alternative procedure to synthesis nematode resistant cultivars would be to induce mutants under in-vitro conditions as vegetatively propagated crops like banana are usually heterozygous and the genetic nature of *Musa* is suitable for the application of mutation breeding.

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Materials and methods

The mutants derived from the *in-vitro* mutation studies from two commercial cultivars *viz*., Robusta (Cavendish group-AAA) and Rasthali (Silk group- AAB) were screened for nematode resistance along with Pisang Lilin (AA) (resistant check) and Anaikomban (AA) (tolerant check). Original Robusta and Rasthali were used as susceptible check.

Table 1: List of *in-vitro* derived mutants used for the study.

Sl. No	Name	Genome
1	Ro Im V_4 6-1-1 (Derived from Robusta)	Cavendish group-AAA
2	Ro Im V_4 6-1-2 (Derived from Robusta)	Cavendish group-AAA
3	Ro Im V_4 6-2-1 (Derived from Robusta)	Cavendish group-AAA
4	Robusta -Susceptible check	Cavendish group-AAA
5	Si Im V_4 10-5-3 (Derived from Rasthali)	Silk group- AAB
6	Si Im V_4 6-2-5 (Derived from Rasthali)	Silk group- AAB
7	Rasthali -Susceptible check	Silk group- AAB
8	Pisang Lilin - Resistant check	AA
9	Anaikomban - Tolerant check	AA

These mutants were selected based on their performance for yield and quality parameters during the preliminary screening trials.

Inoculation with nematodes

The suckers with rhizome weight of approximately 1.5 Kg were selected, pared and planted in earthen pots containing four kilograms of sterilized pot mixture (Red soil: Sand: FYM in the ratio 2:1:1 v/v) @ one sucker per pot. The experiment was conducted in a Completely Randomized Design (CRD) with two replications each. Banana mutants maintained in the pots were inoculated with mixed population of nematodes *viz*., infective juveniles of root-lesion nematode *Pratylenchus coffeae* (1000 no.s / pot) and burrowing nematode *Radopholus similis* (400 no.s / pot). The nematodes were extracted by modified Baermann funnel technique. The nematode suspension was then poured in the holes made around the rhizosphere of the plants after the emergence of the roots i.e at 45 days after planting. After inoculation the soil was lightly watered.

Biochemical Studies

Estimation of total phenols

The total phenol content was estimated by Folin Ciocalteau method. From the ethanol extract, one ml was taken in two different test tubes and the volume was made upto three ml with distilled water. One ml of Folin Ciocalteau reagent was added to each test tube followed by addition of 2ml of 20 per cent Sodium Carbonate solution. The tubes were kept in boiling water bath for a minute, cooled and diluted to 10 ml with distilled water. The intensity of deep blue colour developed was measured at 660nm wavelength in a Spectrophotometer (14). From the standard graph, amount of phenol present was calculated and expressed as mg/g of fresh weight.

Estimation of tannins

The tannin content in the leaves of banana was quantified by following Vanillin Hydrochloride method (10)

Estimation of lignin

The lignin content of banana leaf tissue was gravimetrically estimated following the method of Chesson (2). One gram of the banana leaf or root tissue was shaken in a mixture of 5ml of conc. H_2SO_4 and 50ml of HCl for 16 hr at 25°C in a shaker. The mixture was then transferred into a flask with 450 ml of distilled water. After boiling for 20 min, the content of the flask was filtered through a Geena G_3 glass filter. The acid residue was washed to neutrality with distilled water, dried at 105°C and weighed. The results were expressed in terms of per cent lignin content on dry weight basis of the tissues.

Assay of enzymes

Activity of the enzymes such as polyphenol oxidase, peroxidase, phenyl alanine ammonia lyase and ascorbic acid oxidase was estimated in leaves at 90 days after nematode inoculation.

The banana leaf samples were taken and homogenized at the rate of one gram per five ml of 0.1M phosphate buffer (pH 6.5). The homogenate was centrifuged for 20 minutes at 10,000 rpm at 4°C. Borate buffer 0.2M (pH 8.7) was used for the extraction of PAL. The supernatant was used as the enzyme extract and the activities were recorded as units min^{-1} g^{-1} fresh weight

Peroxidase

Peroxidase activity was analysed spectrophotometrically (5). The reaction mixture was prepared with 0.05ml of 20mM guiacol, 3ml of phosphate buffer, 0.1ml of enzyme extract and 0.03ml of H_2O_2. The changes in absorbance of the reaction mixture at 420nm were recorded at every 30 sec interval for 3 minutes.

Polyphenol oxidase

It was assayed using the modified method of Mayer *et al.* (7). Standard reaction mixture contained 1.5ml of 0.1M phosphate buffer (pH 6.5), 0.5ml of the enzyme extract and 0.5ml of 0.01N catechol. The changes in the absorbance were recorded at 495 nm and 30 sec interval for 3 minutes.

Phenyl alanine ammonia lyase (PAL)

The PAL assay was conducted as per the method described by Ross and Sederoff (11). The assay mixture containing 100 ml of enzyme, 500 ml of 50mM Tris HCl (pH 8.8) and 600ml of 1mM L-phenylalanine was incubated for 60 minutes. The reaction was arrested by adding 2N HCl. Later 1.5ml of toluene was added, vortexed for 30 sec, centrifuged (1000rpm, 5 min) and toluene fraction containing trans-cinnamic acid was separated. The toluene phase was measured at 290nm against the blank of toluene.

Ascorbic acid oxidase

Ascorbic acid oxidase activity was analyzed spectrophotometrically by following procedure given by Drum *et al*., (3).

Observations

Root damage assessment

Root damage assessment was done at 90 days after inoculation. All the roots collected from each plant were divided into two categories *viz*., death and functional roots.

Root necrosis

The length of five selected roots was reduced to 10 cm and the roots were sliced lengthwise. Scoring was carried out at one half each of five roots for the per cent of root cortex showing necrosis. The maximum root necrosis per root half can be 20% giving a maximum root necrosis of 100% for the five halves together. Necrosis of the individual root was recorded. The sum is the total root necrosis of the sample (Carlier *et al*., 2002).

Root lesion index

Resistance rating for nematode screening under pot culture given by Sundararaju (1996) was followed.

S. No.	Number of lesions	Score
1	No infection	1
2	5-10 lesions	2
3	5-10 lesions with rooting	3
4	10-15 lesions	4
5	> 15 lesions with full rotting	5

Corm damage assessment

Corm damage assessment was worked out as per the procedure suggested by Carlier *et al*. (2002) and expressed as corm lesion index in a 1 to 5 scale.

S. No.	Lesion size and number	Score
1	No lesion	1
2	One small lesion	2
3	Several small lesion	3
4	One large lesion	4
5	Several large lesions	5

Host reaction

As per the terminology described by Bos and Parlevliet (1995), the host reaction was scored in banana which is described below:

- A susceptible plant allows nematode development and allows it to reproduce freely
- A resistant plant suppresses the nematode development and reproduction
- A sensitive plant shows much injury, even when relatively lightly infected with nematode
- A tolerant plant may suffer little injury, even when heavily infected with nematode

Results and discussion

Total phenols

Among the three mutants of Robusta, Ro Im V_4 6-1-1 registered the highest total phenol content (108.80 mg/100g) followed by Ro Im V_4 6-2-1 whereas the susceptible check Robusta recorded 65.75 mg /100g only. In Silk group, Si Im V_4 10-5-3 maintained superiority (106.9 mg/100g) than the susceptible check Rasthali (69.15 mg/100g). The resistant check, Pisang Lilin ranked top among all the tested mutants (118.75 mg/100g) while the tolerant check Anaikomban recorded the total phenol content of 103.4 mg/100g.

Tannin

The highest tannin in the leaves was recorded by Pisang Lilin (53.65 µg/g) which was the resistant check whereas the tolerant check Anaikomban recorded the tannin content of 48.63 µg/g. Among the mutants, Si Im V_4 10-5-3 and Ro Im V_4 6-1-1 recorded the higher tannin contents. The susceptible checks recorded very low values for tannin content.

Lignin

Lignin content in leaves at 90 DAI ranged from 0.185 to 0.765 mg/100g. The resistant check and the tolerant check registered very high values whereas the susceptible checks recorded very poor values. Among the mutants, Ro Im V_4 6-1-1 recorded the highest value for lignin content followed by Si Im V_4 10-5-3.

Enzymes

Among the five *in vitro* derived banana mutants 2 belonging to Cavendish group and one belonging to Silk group recorded higher values than their respective susceptible checks for the enzymes *viz*., peroxidase, polyphenol oxidase, Phenylalanine ammonia lyase and ascorbic acid oxidase. In Cavendish group, the highest value was recorded by Ro Im V_4 6-1-1 for peroxidase activity and in Silk group, the highest peroxidase activity was noticed in Si Im V_4 10-5-3. For poly phenol oxidase activity, the data were statistically significant among the mutants and their respective checks of banana. The activity ranged from 41.30 to 115.70 units min^{-1} g^{-1} fresh weight. The mutants Ro Im V_4 6-1-1 and Si Im V_4 10-5-3 registered higher enzyme activity which was comparable with resistant check (Pisang Lilin), however the other mutants namely Ro Im V_4 6-1-2 Si Im V_4 6-2-5 recorded comparatively lower enzyme activity which was almost equivalent to susceptible checks. The Phenyl alanine

ammonia lyase activity differed significantly among the mutants and respective checks. Among the mutants of both Cavendish and Silk groups, the Phenyl alanine ammonia lyase activity was the highest (303.38 units min^{-1} g^{-1}) in Si Im V_4 10-5-3 which was on par with Ro Im V_4 6-1-1 (300.81 units min^{-1} g^{-1}). The lower Phenyl alanine ammonia lyase activity of 181.88 units min^{-1} g^{-1} and 186.12 units min^{-1} g^{-1} was recorded in Ro Im V_4 6-1-2 and Si Im V_4 6-2-5 respectively. Nematode infestation increased the activity of ascorbic acid oxidase in mutants in the order of Ro Im V_4 6-1-1, Si Im V_4 10-5-3, Ro Im V_4 6-2-1, Si Im V_4 6-2-5 and Ro Im V_4 6-1-2. The susceptible checks of both Robusta and Rasthali registered lower ascorbic acid activity.

Total number of roots and infected roots

The total number of roots at 90 DAI varied significantly among the mutants and their respective checks (Table 3). The total number of roots ranged from 52.4 to 72.0. The highest total number of roots was noticed in Si Im V_4 10-5-3 (72.0) which was significantly different from other mutants and it was followed by Pisang Lilin (70.6) which was a resistant check. The susceptible checks of both the groups recorded the lower values for total number of roots (54.8 in Silk and 52.4 in Cavendish). In the case of infected roots produced also statistically differed among the different mutants of banana. The number of infected roots ranged from 6.75 in Si Im V_4 10-5-3 to 29.17 in Rasthali -susceptible check. Among the mutants in Cavendish group, the least number of infected roots was recorded in Ro Im V_4 6-1-1 (6.99) while the highest number of infected roots was recorded in Ro Im V_4 6-1-2 (19.02). Similarly in Silk group, between the two mutants, the least value was registered by Si Im V_4 10-5-3, whereas the mutant Si Im V_4 6-2-5 registered higher number of infected roots. In both the groups the susceptible checks relatively recoded higher values for number of infected roots.

Root and corm damage assessment

The root and corm damage was assessed interms of per cent root necrosis, root lesion index and corm lesion index (Table 3). The total root necrosis per cent varied from 10 to 85. In Cavendish group the least per cent (15%) was recorded by Ro Im V_4 6-1-1 followed by Ro Im V_4 6-2-1 (20%). In Silk group, the least per cent (15%) was registered by Si Im V_4 10-5-3. in general the susceptible checks of both the groups comparatively recorded higher per cent of root necrosis. The root lesion index varied from 1 to 5 among the *in vitro* derived mutants of banana. The highest index (5) was recorded in susceptible checks while the lowest index (5) was recorded in Pisang Lilin which was a resistant check. Among the mutants of both the groups Ro Im V_4 6-1-1 and Si Im V_4 10-5-3 relatively recorded lower index (2). Similarly corm lesion index also ranged from 1 to 5. The least index (1) was recorded by Ro Im V_4 6-1-1 and Si Im V_4 10-5-3 which was on par with resistant check Pisang Lilin. The susceptible checks of both the groups recorded maximum index (5).

Based on the per cent root necrosis, root lesion index and corm lesion index, the level of resistance was assessed among the *in vitro* derived banana mutants. The mutants Ro Im V_4 6-1-1 and Si Im V_4 10-5-3 were found to be resistant while the mutant Ro Im V_4 6-2-1 was moderately resistant. The rest of the mutants namely Ro Im V_4 6-1-2 and Si Im V_4 6-2-5 were found to be susceptible to nematodes.

Discussion

The biochemical basis for resistance to nematode was studied in mutants. The mutants Ro Im V_4 6-1-1, Ro Im V_4 6-2-1 and Si Im V_4 10-5-3 possessed higher quantity of total phenol, tannin and lignin in the leaves (Table 2). The higher total phenol, tannin and lignin in the roots were attributed to polyphenol oxidase, phenylalanine ammonia lyase, ascorbic acid oxidase and peroxidase activities. Fogain (1996) and Valette *et al.* (1997) found higher amounts of phenolics in the resistant cultivar Yangambi km 5. Increased activity of peroxidase in tomato, phenylalanine ammonia lyase in brinjal was positively correlated with nematode resistance (Rajasekar *et al.*, 1997 and Sirohi and Dasgupta, 1993) respectively.

Several physiological processes in the host are stimulated due to the activation of certain enzymes. Enzymes like peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and ascorbic acid oxidase were found to be involved in the plant defense mechanism (Abbasttista and Matta, 1975). The mutants exhibited higher activities of peroxidase, polyphenol oxidase, phenylalanine ammonia lyase and ascorbic acid oxidase (Table 2). Enhanced peroxidase activity has been associated with resistant hybrids to nematodes (Fogain and Gowen, 1996; Valvette

et al., 1997). The PAL and ascorbic acid oxidase enzymes might have increased the chlorogenic acid and ascorbic acid content in the leaves, which act as toxic compounds against nematodes. Transcriptional activation of the genes encoding PAL has been observed within 5 minutes of elicitor treatment (Lam *et al*., 1986), confirming its role in defense mechanism. In susceptible mutants, the nematodes would have broken the defense barrier by producing certain offensive chemicals while in the case of resistant/ moderately resistant mutants, the plant would have synthesized certain toxic compounds known as phytoalexins. Usually these phytoalexins are low molecular weight antimicrobial compounds that are synthesized and accumulated in the cells. It has been well established that phenylalanine ammonia lyase is the prime enzyme involved in plant defense mechanism which is involved in the phenyl propanoid pathway in plant system Lignin and phenol are synthesized via phenyl propanoid pathway which impart resistance against nematode attack. The role of phytoalexins and other toxic compounds like phenols and lignin (which are otherwise called phytoanticipins and are synthesized as a part of the normal plant development) in resistance mechanism have been reported by earlier workers (Reuveni *et al*., 1992 and Sariah *et al*., 1999).

Table 2: Different bio-chemical contents in the leaves of tested mutants and respective checks at 90 DAI (Days After Inoculation)

Name	Genomic group	Total phenol (mg/100g)	Tannin (µg/g)	Lignin (%)	Peroxidase (Units min $^{-1}$ g^{-1} fresh wt)	Polyphenol oxidase (Units min $^{-1}$ g^{-1} fresh wt)	Phenyl alanine ammonia lyase (Units min^{-1} g^{-1} fresh wt)	Ascorbic acid Oxidase (Units min $^{-1}$ g^{-1} fresh wt)
Ro Im V_4 6-1-1 (Derived from Robusta)	Cavendish - AAA	108.80	48.57	0.751	48.65	110.11	300.81	52.64
Ro Im V_4 6-1-2 (Derived from Robusta)	Cavendish - AAA	70.23	25.53	0.238	22.25	55.56	181.88	57.14
Ro Im V_4 6-2-1 (Derived from Robusta)	Cavendish - AAA	100.28	39.87	0.695	43.82	90.28	260.72	53.64
Robusta (Susceptible check)	Cavendish - AAA	65.75	18.16	0.185	14.31	41.30	131.70	18.75
Si Im V_4 10-5-3 (Derived from Rasthali)	Silk- AAB	106.90	46.76	0.743	48.64	107.65	303.38	57.32
Si Im V_4 6-2-5 (Derived from Rasthali)	Silk- AAB	71.65	26.18	0.223	21.52	53.66	186.12	57.57
Rasthali (Susceptible check)	Silk- AAB	69.15	18.15	0.192	16.83	41.70	130.75	20.70
Pisang Lilin - Resistant check	AA	118.75	53.65	0.765	50.70	115.70	310.70	66.28
Anaikomban - Tolerant check	AA	103.40	40.06	0.705	44.80	91.15	265.70	50.64
SED		0.5615	0.5615	0.0071	0.6575	0.7071	2.2001	0.7122
CD 5%		1.2703	1.2703	0.0160	1.4872	1.5996	4.9772	1.6112

Table 3: Root and corm parameters of tested mutants and respective checks at 90 DAI (Days After Inoculation)

Name	Genomic group	Total number of roots	Number of infested roots	% root necrosis	Root lesion index	Corm lesion index	Host response
Ro Im V_4 6-1-1 (Derived from Robusta)	Cavendish - AAA	68.4	6.99	15	2	1	R
Ro Im V_4 6-1-2 (Derived from Robusta)	Cavendish - AAA	56.6	19.02	40	4	4	S
Ro Im V_4 6-2-1 (Derived from Robusta)	Cavendish - AAA	67.8	12.67	20	3	3	MR
Robusta (Susceptible check)	Cavendish - AAA	52.4	26.13	85	5	5	HS
Si Im V_4 10-5-3 (Derived from Rasthali)	Silk- AAB	72.0	6.75	15	2	1	R
Si Im V_4 6-2-5 (Derived from Rasthali)	Silk- AAB	54.8	16.80	40	4	4	S
Rasthali (Susceptible check)	Silk- AAB	54.2	29.17	80	5	5	HS
Pisang Lilin - Resistant check	AA	70.6	7.47	10	1	1	R
Anaikomban - Tolerant check	AA	62.0	13.50	30	3	3	T
SED		0.544	0.915	-	-	-	-
CD 5%		1.23	2.07	-	-	-	-

R-Resistant;MR- Moderately Resistant;T- Tolerant;S- Susceptible;HS- Highly Susceptible

Good root development with healthy roots and corm favours resistance. In the present investigation, the resistant and moderately resistant mutants showed lower percentage of infected roots (Fig. 1)

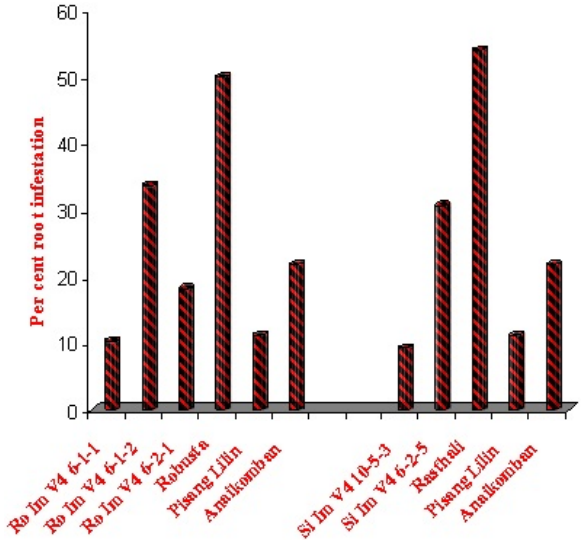

Fig. 1: Effect of ne matodes on % of infested root of in vitro derived mutants of banana.

Conclusion

The study vividly indicated the presence of resistance and tolerance in the in vitro derived mutants of banana. Further the role of different biochemical parameters and enzymes has also been well established in developing resistance mechanism against nematodes in banana. The resistant and moderately resistant mutants of banana could be further used in breeding programmes as well as be recognized as potential cultivars of commerce.

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