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Genetic fidelity in micro-propagated robusta (*Musa* AAA) plants based on quantitative and molecular analysis

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Abstract
The study was focused to obtain reliable estimate of genetic fidelity with respect to "number of subcultures" followed by comparing qualitative, quantitative and molecular level validation. Hardened "Robusta (*Musa* AAA)" plants derived from different multiplication stages namely; S6, S7, S8, S9, S10 and S11 were the experimental material used in the study. Stages of subculture were considered as different treatments as the study was mainly focused to limit the shoot multiplication stages to optimum subculture levels which ensures farmers demand of "clonal uniformity". Field analysis was performed using RBD with six treatments (S6, S7, S8, S9, S10 and S11) and four replication at Rice Research Station, Vyttila, Kerala and qualitative and quantitative comparisons were done. Molecular analysis was performed using DNA isolated from leaves of experimental plants. DNA isolated from sucker of mother plant used for initial culture establishment, represented as "S0" was the reference to confirm genetic uniformity. In order to compare variation pattern in field with molecular level, ten newly developed SSR markers having high homology with predicted genes were used and possible molecular interpretations were suggested.

Keywords: Robusta (Musa, AAA), genetic fidelity, somaclonal variation, SSR, ANOVA, divergent analysis, dendrogram

1. Introduction
The central concept of any plant micro propagation methodology is to provide uniform planting material and maintenance of true-to-type nature of a particular clone. The true-to-type nature can be assured by its genetic constitution which is referred to as genetic fidelity. Genetic fidelity and soma clonal variation are highly intervened, when soma clonal variation occurs, the genetic fidelity is lost and maintenance of genetic fidelity is the key to avoid soma clonal variation. Understanding the patterns of variations originating in tissue culture is vital to address the importance of genetic fidelity in micro propagation.

The variation occurring within cells can be used to understand biochemical or physiological process with relevance to transient expressions. The non-heritable variation expressed by primary regenerants can be exploited in asexually propagated plants but often some somatic instabilities are encountered. Epi-mutations can be exploited in seed propagated plants to induce targeted variations or exploit chance variations because of the lack or limitation of variation in agronomic traits which is needed for plant breeding programmes. But the scenario of micro propagation is different, it requires uniformity and the occurrence of variation which is important for crop improvement is a profanity here. Better understanding of soma clonal variation is needed to make it precise and directed to control the extent and type of variation which could be used in any tissue culture programme meant for creating agronomic uniformity.

The view of micro propagation is not just limited to the supply of sufficient planting material. It is fulfilled only when the quality of supplied planting material is strong enough to produce higher yield with provision to uniformity in harvest. Eight subcultures are permitted in banana for accredited labs before rooting initiation. However there is a difference in opinion between the production labs and researchers on the subcultural passages that could be considered as "safe" for shoot multiplication for different crops with respect to genetic uniformity of plants. In tissue culture it is well known that lesser the number of subcultures, lower will be the chance of soma clonal variation. However, it must also be realized that if the number of passages are far too small then the entire production process becomes economically unviable.

More than the economic concerns, the existing output from the labs are inadequate to meet the increased demand from farmer's side. Farmers demand cannot be compromised by providing unsafely multiplied clones. Hence confirming the clonal fidelity of tissue cultured plants using molecular markers in different passages is mandatory which is seldom followed in many labs.

The stability of a crop in the field is not only determined by the genome but also linked to the environment. Since uniformity is the major concern of a successful micro propagation programme, the chance for environmental effects imparted by sub culturing cannot be neglected which could influence qualitative and quantitative characters. So in order to solve the twin problems of meeting demands of farmers with no compromise to clonal uniformity both field analysis and marker analysis was conducted.

2. Materials and Methods

The experiments were conducted at Rice Research Station, Vyttila located 1.7 m above the mean sea level with 9˚58" N latitude and 76˚20" E longitude. Robusta (*Musa* AAA) plants derived from different multiplication stages (S6 to S11) were the experimental material. Stages of subculture were considered as different treatments. Hardened plants derived from different multiplication stages were used as planting material. Planting was conducted with a spacing of 2.4 m between rows and 1.8 m between plants. Quantitative characters were studied by recording biometric measurements from field grown plants raised in completely randomized design. Molecular screening using SSR markers in isolated plant genomic DNA from leaf sample was conducted with sucker of the mother plant from which initial culture establishment as the control. Data analysis based on 10 SSR markers was conducted to check clonal fidelity and genetic analysis of tissue culture raised banana (*Musa* spp.) plant.

Replicated data derived mean values for subcultures and individual data of mother plant were subjected to analysis of variance. Mean value per replication of each observation as reported by Panse and Sukhatme in 1967 was used for tracing and comparing critical difference between subcultures. ANOVA was performed with the help of GRAPES (General R-shiny based Analysis platform Empowered by Statistics) version 1.0.0 (Gopinath *et al*., 2020) [2]. The magnitude of genetic divergence caused in *in vitro* generated plants because of sub culturing was studied by the technique of Mahalanobis D^2 statistics. Tocher's method (Rao, 1952) [3] was used to construct dendrogram based on the D^2 values. Sequentially agglomerated hierarchical non-overlapping (SAHN) clustering and dendrogram construction was performed using UPGMA (un-weighted pair group method with arithmetic average) based on simple matching co-efficient. Software NYSYSpc version 2.1 was used to perform molecular cluster analysis.

3. Result and Discussion

3.1 Analysis of variance

Significant difference was observed in subculture S11 from others for characters plant height (cm), pseudostem diameter (cm), leaf blade length(cm), leaf blade width (cm), male bud length (cm), male bud circumference (cm), fruit length(cm), number of hands/bunch, number of fingers/hand and bunch weight (kg). All the subcultures showed uniformity for characters number of leaves, fruit circumference (cm), fruit peel thickness (mm), brix (%) and number of suckers. Out of the 15 characters studied, significant deviation was observed for 10 characters in S11. Similar findings were reported by Kadam (2012) [4] in "Nendran" variety of banana. He revealed existence of high level of variation for quantitative characters among subcultures ranging from three to sixteen during early stages of comparison (2 months) which turned uniform at later stages (8 months) suggesting epi-genetic variations. The observed results of plant and yield characters were comparable among subcultures S6 to S10. The present investigation on robusta banana was found to have similar pattern for field stability of subcultures, true-to-type behavior was shown by subcultures S6 to S10. But there was a greater reduction in general performance and yield potential in subculture S11 of robusta plantlets indicating sub culturing beyond 10 should not be practiced for robusta. This can be due to the greater sensitivity of *Musa accuminata* genome to undergo mutation as suggested by study of Lakhwani *et al*. (2016) [5].

3.2 Divergence analysis

The comparison of subcultures S6, S7, S8, S9, S10 and S11 based on their divergence for quantitative characters were done using Mahalanobis D^2 statistics. The analysis clearly depicted the presence of two groups among subcultures. The intra-cluster distance observed in first group which included subcultures ranging from S6 to S10 was very small (18.41) on comparison with inter- cluster distance with the second group solely represented by subculture S11 (333.74). The inclusion of subcultures S6, S7, S8, S9 and S10 together in a group and S11 put alone to a group was reflecting the credibility of ANOVA. In order to genetically interpret the pattern of subculture induced variation dendrogram based on "Tocher method" was performed. dendrogram based on quantitative data with "Tocher" algorithm was conducted to serve twin purposes.

Dendrogram constructed based on divergence analysis was found representing two separate groups as observed in cluster distance categorization. The first group of cluster was represented by subcultures S6, S7, S8, S9 and S10. Subculture S11 was observed as an outlier that joined the first cluster with greater degree of divergence. The similarity of five quantitative characters for subculture S11 with other subcultures significantly influenced the cluster dendrogram. "Tocher" method produced agglomerated hierarchical cluster dendrogram, which at first consider all entries as separate clusters and later group them to similar clusters based on similarity of data observed. The "Tocher" cut-off value used in delimiting the clusters became high because of this similarity finally resulting in the formation of only one cluster. However the dendrogram was strong in addressing the greater degree of divergence found for subculture S11 on comparison with others.

The horizontal distance present between individual subcultures represent the degree by which they are apart. The subcultures S6, S7, S8, S9 and S10 were found almost together in a single line representing the similarity between them for quantitative characters as the distance or level of divergence present among them were negligible. The Tocher cut-off value obtained from analysis was 102023.92. Dendrogram reveals that joining of horizontal line 6 representing subculture S11 with the other subcultures seen as almost together in a single line occurred before the cut-off

limit making only a single cluster. Even though only single cluster was formed, dendrogram clearly demonstrates that subculture S11 was observed as an outlier which joined the cluster with greater divergence on comparison to other subcultures with negligible divergence.

Table 1: Comparison of two groups (formed based on cluster distance)

Sl. No	Character	Group 1 mean	Group 2 mean	% decrease
1	Plant height	202.37	155.95	22.93
2	Pseudostem diameter	49.69	42.40	9.18
3	Leaf number	24.00	22.50	6.25
4	Leaf length	217.48	184.88	14.98
5	Leaf blade width	71.32	64.57	9.46
6	Male bud length	22.07	20.55	6.88
7	Male bud circumference	26.73	24.82	7.14
8	Fruit length	21.12	15.43	26.94
9	Fruit circumference	11.92	10.35	13.17
10	Fruit peel thickness	3.45	2.75	20.28
11	Brix value	22.74	21.38	5.98
12	Number of suckers	5.45	4.50	17.43
13	Hands per bunch	10.10	8.00	20.79
14	Fingers per hand	18.30	12.75	30.32
15	Bunch weight	20.99	13.81	34.20

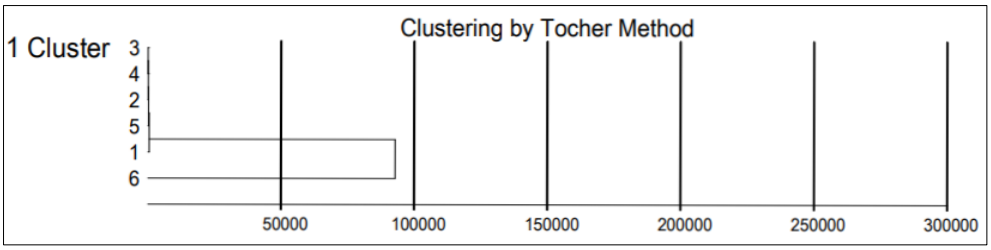

Fig 1: Clustering by "Tocher" method. 1- subculture S6, 2- subculture S7, 3- subculture S8,4- subculture S9, 5- subculture S10, 6- subculture S11

3.3 Electrophoresis and documentation in Gel Doc and polymorphic assay

A total of 91 bands were scored from seven set of DNA samples (mother and subcultures) with an average of 13 bands per sample. Average number of bands scored per primer was found to be 1.3 as 13 bands were observed from a sample using 10 SSR primers. 2 out of 13 bands scored in subculture 11 was found polymorphic. Polymorphic bands were produced from primers MaSSR 6.1 and MaSSR 11.1. The polymorphism associated with subculture S11 for two markers resulted dissimilarity in gel scoring for S11 on comparison with other subcultures which produced all the bands in monomorphic pattern.

Table 2: Scoring pattern for primers

Sl. No	Sample	Number of bands scored													
		MsSSR 1.1	MaSSR 2.1	MaSSR 3.1	MaSSR 5.1	MaSSR 6.1		MaSSR 7.1	MaSSR 8.1	MaSSR 9.1	MaSSR 10.1	MaSSR 11.1			Total
1	Mother	1	1	1	3	1	0	1	1	1	1	1	1	0	13
2	S6	1	1	1	3	1	0	1	1	1	1	1	1	0	13
3	S7	1	1	1	3	1	0	1	1	1	1	1	1	0	13
4	S8	1	1	1	3	1	0	1	1	1	1	1	1	0	13
5	S9	1	1	1	3	1	0	1	1	1	1	1	1	0	13
6	S10	1	1	1	3	1	0	1	1	1	1	1	1	0	13
7	S11	1	1	1	3	0	1	1	1	1	1	1	0	1	13
Total bands amplified by primer		7	7	7	21	7		7	7	7	7	14			91
Average no. of bands scored/primer		1	1	1	3	1		1	1	1	1	2			1.3
Location of band on comparison to 100bp ladder		350bp	350bp	Between 300 and 340bp	300bp to 500bp	300bp to 400bp		150bp to 200bp	300bp to 350bp	350bp to 400bp	300bp to 350bp	350bp to 450bp			300bp to 500bp

Two distinct bands were amplified with primer MaSSR 6.1 associated with marker found on chromosome 6 of banana. First band was scored above 350bp size which was observed monomorphically in mother and subcultures S6, S7, S8, S9, S10 but absent in S11. Second band scored just above 300bp was confined only to the subculture S11 hence revealing a polymorphism.

Three distinct bands were observed on amplification with

primer MaSSR 11.1 associated with the marker present on chromosome 11 of banana. First band of size near 450bp was found monomorphic in all, second band of size near 350bp was found monomorphically in mother as well as subcultures S6, S7, S8, S9, S10 and third band of size near 300bp was amplified only in subculture S11 revealing polymorphism.

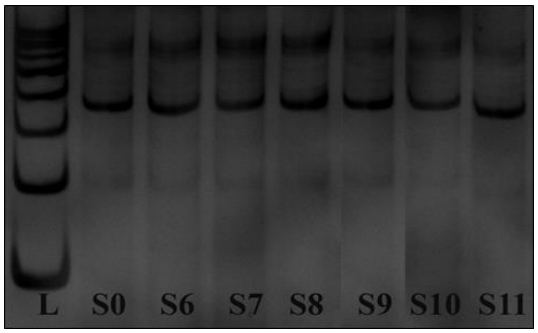

Plate 1: MaSSR 6.1

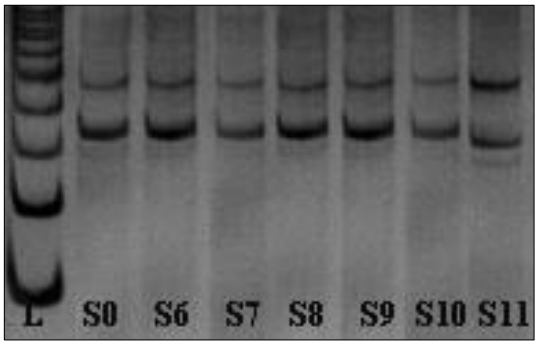

Plate 2: MaSSR 11.1

MaSSR 6.1 is associated with squamosa promoter-binding protein-like (SPL) genes encoding plant- specific transcription factors located on chromosome 6 of banana. MaSSR 11.1 is associated with the genes like AP2 (APETELA 2) / EREBP (ethylene responsive element binding protein family) which are like prototypes of family of transcription factors present in chromosome 11 of banana (Lestari *et al.*, 2019) [7]. The AP2/EREBP genes strongly influence fruit characters and abiotic stress response (Lakhwani *et al.*, 2016) [5]. SPL genes are key for miRNA-dependent temperature stress response in banana (Zhu *et al.*, 2019) [6]. Molecular analysis reflected the polymorphism encountered in subculture S11 for the markers MaSSR 6.1 and MaSSR 11.1 which indicates possibility for an error for the predicted genes. The genetic base for poor performance of subculture S11 expected due to reduced potential for field competence or susceptibility to higher environmental effects than others indicated by ANOVA was thus revealed. The relative delay in phase transition, caused because of error in SPL genes can be a reason for their poor performance of subculture S11 for all the quantitative characters compared. The AP2/EREBP genes associated with primer MaSSR 11.1, shown polymorphism in S11 had an important role in determining fruit characters. The highest percentage mean reduction occurred for fruit characters and yield revealed with ANOVA and divergence analysis can be possibly due to error caused to such genes.

4. Conclusion

The genetic fidelity analysis of micro propagated “Robusta” produced by direct organogenesis based on quantitative and molecular level validation revealed “true-to-type” behaviour of subcultures S6, S7, S8, S9 and S10 with the mother plant for the screened markers. The general yield reduction associated with subculture S11 was found to be in close agreement with the molecular interpretations. Even though true-to- type clones are produced up to ten subcultures, considering the random occurrence of mutation and verification of soma clonal variations associated with higher level of subcultures, the investigation suggests that the sub culturing can be safely carried up to subculture 9 for “Robusta” plantlets produced by direct organogenesis. Potential for S10 subculture can be further clarified with use of more molecular markers.

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